

Chemistry and Biology of Synthetic Retinoids

Edited by

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PREFACE

Retinoids continue to attract attention in two major areas — the fields of light energy transduction and vision and investigations into the processes of cell differentiation and proliferation — both of which are undergoing an exciting renaissance. It is the purpose of this monograph to present recent advances from a number of laboratories involved in retinoid research, with emphasis on analogs. Retinoids, including synthetic analogs and isotopically labeled substrates, are useful biochemical research tools and have therapeutic application in the treatment of proliferative diseases. Although several monographs on retinoids¹⁻⁴ have recently appeared, the growth in these areas of research clearly warrants a continuing update of even more recent and exciting advances. Accordingly, some of the leading researchers in retinoid chemistry and biology have graciously contributed to this volume. The authors were asked to focus primarily on their individual research areas, with inclusion of some background information as well as related research being conducted in other laboratories. The chemical syntheses of many of the analogs described in the chapters are included where appropriate. Research on retinoids requires dedicated and diversified work from scientists in a variety of disciplines. Cooperative ventures between chemists, biochemists, biologists, molecular biologists, biophysicists, physiologists, toxicologists, and clinicians, among others, have led to some of the most exciting advances in the field.

The retinoids are conveniently subdivided in terms of the three biological oxidation levels of the polar terminus on the retinoid side chain, namely, the retinols (alcohol end group), the retinals (aldehyde form), and the retinoic acids (carboxylic acid terminus). Various derivatives such as the esters of the retinols and the retinoic acids, as well as other condensation products, can be considered subgroups within these principal oxidation level subdivisions. Approximately the first half of this monograph is concerned with studies of retinals and their analogs in relation to the visual pigment and bacteriorhodopsin. The second half focuses primarily on the retinoic acids and their analogs and their effects on the processes of cell differentiation and proliferation, including cancer chemoprevention. The retinoids at the lowest oxidation level, namely, the retinols and their analogs, which are metabolic precursors to the aldehyde and carboxylic acid forms of retinoids, have not had the extensive synthetic manipulations that the other two oxidation levels have had for purposes of biological investigation, and therefore are not covered extensively here.

The monograph begins with four chapters devoted primarily to perhaps the most well-established area of retinoid research — the visual cycle. One of the most remarkable new discoveries in this field is the finding by Rando that all-*trans*-retinol (and its derivatives) is transformed to the corresponding 11-*cis*-isomer before oxidation to 11-*cis*-retinal (Chapter 1). Prior to this finding, it was thought that the 11-*trans* to 11-*cis*-transformation occurred at the aldehyde oxidation level. This transformation and other aspects of the visual cycle, including the roles of the retinol-binding proteins, are described in Chapter 2 by Bridges. Liu and Asato report in Chapter 3 the synthesis of various geometric isomers of the retinals and their analogs, including fluorinated and alkylated compounds. Their use of these compounds to probe and then refine the chromophore binding site of the visual pigment elegantly demonstrates the utility of analogs as biochemical research tools. In Chapter 4 Ito describes the syntheses of and studies on analogs to probe the active site of visual pigments, including the demonstration that the chromophoric group of the fly visual pigment is 3-hydroxy-11-*cis*-retinal.

The next three chapters by Sheves and Ottolenghi (Chapter 5), Crouch (Chapter 6), and Balogh-Nair and Nakanishi (Chapter 7) are concerned with related analogs and/or isotopically labeled compounds that probe both the visual pigments and bacteriorhodopsin. The final two chapters in the first section of this volume on the retinals (Chapter 8 by Hopf and Krause and Chapter 9 by de Lera, Chandraratna, and Okamura) focus on the use of acetylenes and allenes

in new synthetic approaches to novel analogs and on their chemical and spectroscopic properties.

Interest in retinoic acid chemistry and biology is undergoing a new resurgence with the recent exciting discoveries of the nuclear retinoic acid receptor proteins that are homologous to the receptors for steroid and thyroid hormones and vitamin D₃.⁵⁻¹⁰ Therefore, the second half of this monograph describes recent research on the effects that retinoids, particularly retinoic acid and its derivatives and analogs, have on the process of cell differentiation and how modifications of the retinoid skeletal affect activity in three different types of assay systems — cell culture, organ culture, and animal models. In addition to elucidating the fundamental role that retinoids have in mediating these processes, a major goal of these studies has been to develop new therapeutic agents that control these processes without having toxic side effects. Therefore, in this section the results of clinical trials on retinoids are also presented. In Chapters 10 to 13 are described the effects that retinoids have on the differentiation of four different cell lines — F9 embryonic carcinoma (Napoli), murine melanoma (Lotan and Lotan), HL-60 human promyelocytic leukemia (Shudo and Kagechika), and rabbit tracheal bronchial epithelia (Jetten). Emphasis has been placed on correlating retinoid structure with activity. These studies are extended to the hamster tracheal organ culture system in Chapter 14 (Schiff, Okamura, Dawson, and Hobbs), wherein retinoid activity in reversing keratinization is correlated to structural modification. Before studies on animal models are presented, Sani describes in Chapter 15 the possible roles that the retinoid-binding proteins play in mediating retinoid activity.

In Chapter 16 Dawson, Chao, Hobbs, and Delair present studies on the mouse model of tumor promotion. In addition to giving an overview of their own work in correlating retinoid structure with biological activity in inhibiting the induction of epidermal ornithine decarboxylase and in inhibiting skin tumor promotion, they review the research of Verma and Boutwell and their co-workers from which these studies derive and the tumor regression studies by the Hoffmann-La Roche group in Basel. Because much of this second section covers biological studies, in contrast to the first section in which many of the chapters focus on retinal chemistry, Chapter 16 also contains a summary of currently used retinoid synthetic methodology. In Chapter 17 Gensler and Meyskens discuss their research on the effects of retinoids in mouse skin models and in clinical studies on cutaneous cancer. Connor describes his research on retinoids in hairless mouse epidermis in Chapter 18. In Chapter 19 Moon and Mehta assess the effects that retinoids have in experimental carcinogenesis in the bladder and mammary gland. Coffey, Fielder-Nagy, Weisman, Woodward, and Welton discuss in Chapter 20 the antirheumatic and antiinflammatory effects of retinoids in animal models and in clinical studies. In Chapter 21, Willhite presents his research on the embryotoxicity of retinoids. Niculescu-Duvăz, Simon, and Voiculețz (Chapter 22) conclude this monograph with their minimal topological differences approach for analyzing retinoid structure-activity relationships.

We hope that you, the reader, will have as much enjoyment in reading this volume as we have had in editing it. We wish to express our thanks and appreciation to our fellow co-authors and our colleagues in the field for their contributions that made this volume possible.

Marcia I. Dawson
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TABLE OF CONTENTS

Chapter 1	
The Bioorganic Chemistry of Vision	1
Robert R. Rando	
Chapter 2	
The Molecular Basis of the Visual Cycle	27
C. D. B. Bridges	
Chapter 3	
The Binding Site of Opsin Based on Analog Studies with Isomeric, Fluorinated, Alkylated, and Other Modified Retinals	51
Robert S. H. Liu and Alfred E. Asato	
Chapter 4	
Synthesis and Application of Retinoids and Related Compounds to Vision Research and Cancer Studies	77
Masayoshi Ito	
Chapter 5	
Molecular Mechanism for the Function of Bacteriorhodopsin and Visual Pigments: Studies with Model Compounds and Artificial Pigments.....	99
Mordechai Sheves and Michael Ottolenghi	
Chapter 6	
Analog Pigments of Rhodopsin and Bacteriorhodopsin; Retinal Binding Site and Role of the Chromophore in the Function of These Pigments	125
Rosalie K. Crouch	
Chapter 7	
Visual Pigment and Bacteriorhodopsin Analogs	147
Valeria Balogh-Nair and Koji Nakanishi	
Chapter 8	
Acetylenic Intermediates in Retinoid Synthesis	177
Henning Hopf and Norbert Krause	
Chapter 9	
Synthesis and Studies of 12-s- <i>cis</i> -Conformationally Locked Retinoids	201
Angel R. de Lera, Rosshantha A. S. Chandraratna, and William H. Okamura	
Chapter 10	
The Biogenesis of Retinoic Acid: A Physiologically Significant Promoter of Differentiation	229
Joseph L. Napoli	
Chapter 11	
Inhibition of Melanoma Cell Growth by Retinoids: Structure-Activity Relationships	251
Reuben Lotan and Dafna Lotan	

Chapter 12	
Structure-Activity Relationships of a New Series of Synthetic Retinoids (Retinobenzoic Acids)	275
Koichi Shudo and Hiroyuki Kagechika	
Chapter 13	
Regulation of Squamous Differentiation in Tracheobronchial Epithelial Cells by Synthetic Retinoids	287
Anton M. Jetten	
Chapter 14	
Structure-Biological Activity Relationships of New Synthetic Retinoids on Epithelial Differentiation of Cultured Hamster Trachea	307
Leonard J. Schiff, William H. Okamura, Marcia I. Dawson, and Peter D. Hobbs	
Chapter 15	
Cellular Retinoic Acid-Binding Protein and the Action of Retinoic Acid	365
Brahma P. Sani	
Chapter 16	
The Inhibitory Effects of Retinoids on the Induction of Ornithine Decarboxylase and the Promotion of Tumors in Mouse Epidermis	385
Marcia I. Dawson, Wan-Ru Chao, Peter D. Hobbs, and Thierry Delair	
Chapter 17	
Role of Retinoids in Prevention of Cutaneous Cancer	467
Helen L. Gensler and Frank L. Meyskens, Jr.	
Chapter 18	
Epidermal Responses to Retinoids <i>In Vivo</i>	485
Michael J. Connor	
Chapter 19	
Retinoid Inhibition of Experimental Carcinogenesis	501
Richard C. Moon and Rajendra G. Mehta	
Chapter 20	
Retinoids as Potential Antirheumatic Agents	519
John W. Coffey, Christa Fiedler-Nagy, Stephen M. Weisman, Thasia G. Woodworth, and Ann F. Welton	
Chapter 21	
Molecular Correlates in Retinoid Pharmacology and Toxicology	539
Calvin C. Willhite	
Chapter 22	
Carcinogenesis Inhibitory Properties of Retinoids: A QSAR-MTD Analysis	575
I. Niculescu-Duvăz, Z. Simon, and N. Voiculeț	
Index	607



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Chapter 1

THE BIOORGANIC CHEMISTRY OF VISION**Robert R. Rando****TABLE OF CONTENTS**

I.	Introduction.....	2
II.	The Structure and Function of Rhodopsin.....	2
A.	Role of Schiff Base Charge and Charge Movement in the Formation of R*.....	3
B.	Nonactive-Site Lysine Modified Rhodopsins and Their Functions.....	8
III.	The Isomerization of all- <i>trans</i> -Retinoids to 11- <i>cis</i> -Retinoids in the Eye.....	10
A.	<i>In Vivo</i> Studies	12
1.	Studies on Drugs that Affect the Visual Cycle.....	12
2.	Analog Studies <i>In Vivo</i>	15
3.	Tritium Release Experiments <i>In Vivo</i>	16
B.	<i>In Vitro</i> Experiments	18
	Addendum.....	23
	Acknowledgments	24
	References.....	24

I. INTRODUCTION

Vision begins with the absorption of light by the membrane-bound chromoprotein rhodopsin. This absorption results in the *cis* to *trans* isomerization of its 11-*cis*-retinal Schiff base chromophore.¹ The isomerization leads to a series of conformational changes in the protein, which have been monitored by spectroscopic means.² One of the conformers, spectroscopically defined by metarhodopsin II and also referred to as "activated rhodopsin" (R*), catalyzes the exchange of GTP for GDP in a retinal G-protein often referred to as transducin.³ Upon binding GTP, the α subunit of the G-protein dissociates from the membrane and activates a c-GMP specific phosphodiesterase. This results in the rapid hydrolysis of the unbound c-GMP.⁴ Since c-GMP acts as an agonist for the plasma membrane sodium channels, maintaining them in the open state, the hydrolysis of c-GMP results in the hyperpolarization of the rod outer segments.⁵ This hyperpolarization of the rod cells initiates neural signaling in the retina. After further processing in the retina, this signal is transmitted to the brain where the visual image is constructed. The biochemical reactions described above occur in the rod outer segments. Some of the same interactions also occur in a variety of hormone-receptor systems, making the study of the visual cascade of some general interest.⁶

The photoisomerization of the 11-*cis* to all-*trans* chromophore of rhodopsin leads to the eventual hydrolysis of the retinoid to yield opsin and all-*trans*-retinal, in a process termed "bleaching".⁷ The liberated retinal is reduced in the retina and transported to the pigment epithelium, where it is esterified and stored as long-chain fatty acid esters.⁸ During dark adaptation, this process must be reversed to eventually form 11-*cis*-retinal, which can react with opsin to form the holoprotein rhodopsin.⁸ If the thermal regeneration of 11-*cis*-retinoids were not to occur, then vision would have the character of a once in a lifetime event.

The group of chemical reactions that are involved in the thermal regeneration of 11-*cis*-retinal comprise the so-called "visual cycle". None of the enzymes of this pathway have been purified, and until recently the isomerization process had never been demonstrated *in vitro*.⁹ This latter process is likely to be a key element in the control of dark adaptation. Under normal lighting conditions, the sensitivity of the human eye is largely determined by the relative amounts of rhodopsin to opsin.¹⁰ When the eye is dark adapted and maximally sensitive, virtually all of the opsin has been converted to rhodopsin.¹⁰ The sensitivity of the eye becomes markedly reduced when substantial light bleaching occurs.¹⁰ The extent of rhodopsin regeneration is, in turn, dependent on the amount of available 11-*cis*-retinal. Thus, the processing of all-*trans*-retinoids to 11-*cis*-retinoids in the eye is of enormous importance to human vision.

In this review article, two separate but related aspects of the bio-organic chemistry of vision will be considered. The first deals with the mechanism by which photolyzed rhodopsin achieves that conformational state, referred to as R*, which is capable of catalyzing the exchange of GTP for GDP in the retinal G-protein. The second aspect of this review is concerned with the beginnings of the identification and characterization of that hitherto elusive process responsible for the isomerization of an all-*trans*-retinoid to an 11-*cis*-retinoid.

II. THE STRUCTURE AND FUNCTION OF RHODOPSIN

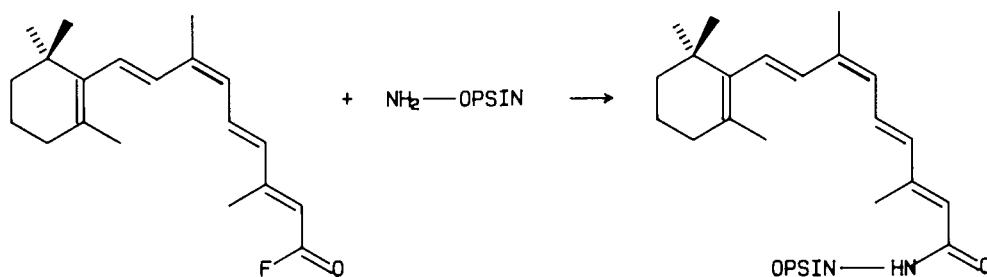
Rhodopsin (bovine) is a 40 kDa integral membrane protein found in the disks of the rod outer segments.¹¹ The primary sequence of this protein has been determined and a model for its structure in the native membrane has been proposed.¹¹ In this model there are seven transmembrane helices connecting the hydrophilic extra- and intradiskal sequences. Of the eleven lysine residues, only one is intermembranous (lys 296) and it is to this one that the Schiff base with 11-*cis*-retinal is formed.¹¹ Spectroscopic studies are in accord with the

belief that this Schiff base is protonated.^{12,13} Indeed, that the Schiff base is protonated is important for proposed models of energy storage and wavelength regulations.¹⁴ It is known that approximately two thirds of the energy of the absorbed light is stored in bathorhodopsin, one of the first intermediates detectable after light absorption by rhodopsin.¹⁵ This energy storage can be accounted for by the movement of the positively charged Schiff base away from its negatively charged counterion in a medium of low dielectric constant.¹⁴ This movement would, of course, be caused by the photoisomerization process. Wavelength control is related to the way in which opsins can drastically modify the ultraviolet-visible absorption characteristics of the retinal molecule. Retinal, and its unprotonated Schiff bases, absorb light at approximately 360 nm, whereas the mammalian rhodopsins maximally absorb light anywhere from approximately 440 to 580 nm.¹⁶ Substantial bathochromic shifts of the order of 60 nm are produced simply by protonating the Schiff base, and the remainder of the shift (the opsin shift) may be caused by the interaction of negatively charged amino acids of the protein with the positively charged retinal Schiff base^{16,17} The latter bathochromic shifts would be specific for the different opsins. This fact allows for an understanding of the observations that the various mammalian rod and cone pigments are all based on the 11-*cis*-retinal chromophore.^{14,16,17}

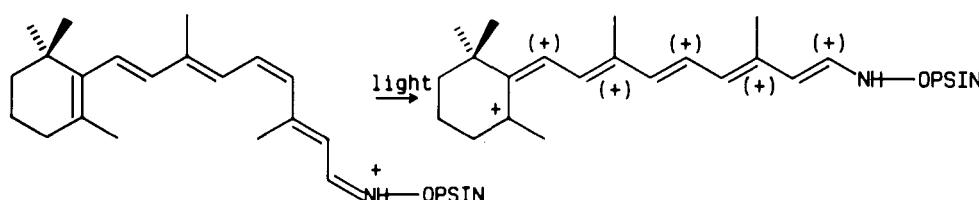
Spectroscopic evidence suggests that all but one of the observable intermediates that occur during bleaching contain a protonated Schiff base.^{2,12,13} The intermediate containing the deprotonated Schiff base, metarhodopsin II, is also presumed to be the spectroscopic signature of R*.¹⁸ Thus, judging primarily from spectroscopic evidence, it would appear that not only must rhodopsin be initially protonated at its Schiff base, but also that this proton must be lost to generate the biologically active form of rhodopsin R*. This deprotonation of the membrane-buried Schiff base must produce a substantial conformational change in the helical segments, which must then be transmitted to the G-protein either directly or indirectly. It has been suggested, based on limited proteolysis experiments, that the interaction with the G-protein is mediated through the extradiskal hydrophilic region of rhodopsin.¹⁹

A. ROLE OF SCHIFF BASE CHARGE AND CHARGE MOVEMENT IN THE FORMATION OF R*

The general question of interest in our laboratory is how the photochemical *cis*-to-*trans* isomerization in the chromophore of rhodopsin produces R*. We are further interested in determining those structural features of R* that enable it to interact with and activate G-proteins. We have employed retinal analogs and protein modification studies to approach these problems. A starting point is to probe the role of charge and charge movement in the functioning of rhodopsin. Initial studies focused on the use of retinal analogs to explore the question of the importance of Schiff base charge in the action of rhodopsin. The retinal analog 9-*cis*-retinoyl fluoride can be utilized to help answer this question. This analog, which is isosteric with 9-*cis*-retinal, would be expected to react with opsin to form an uncharged peptide rather than a charged Schiff base (Scheme 1).²⁰ In fact, the molecule reacts stoichiometrically with the active-site lysine of bovine opsin to form a new pigment, 9-*cis*-retinoyl opsin, which does not show an appreciable opsin shift.²⁰ That is, this pigment absorbs light with a λ_{max} very close to that of model retinoyl amides.²⁰ This new pigment photoisomerizes to the same photostationary state from both its all-*trans* and 9-*cis* forms without bleaching.²¹ Most important, photoisomerization does not produce R*, as determined by G-protein activation studies.²¹ G-protein activation was the assay used to determine if a structural state analogous to R* could be formed. These studies point to the importance of the protonated Schiff base in normal rhodopsin function. They eliminate the possibility that photoisomerization of the retinoid chromophore in and of itself will lead to the formation of R*. These studies also make it more likely that the mechanism of energy storage involves



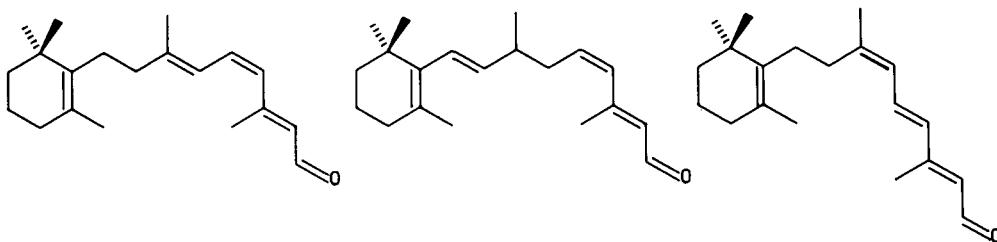
SCHEME 1. Reaction of 9-cis-retinoyl fluoride with opsin.



SCHEME 2. Light-induced charge migration into retinoid backbone.

charge separation and not the photochemical formation of some highly chemically strained retinoid intermediate. The fact that even the all-*trans*-retinoyl opsin adduct could also not activate the G-protein also makes it clear that more than a simple *cis*-to-*trans* isomerization is needed to produce R*.^{21,22} Other all-*trans*-retinoids, such as all-*trans*-retinal, its oxime, and the all-*trans*-retinylamine adduct, were also incapable of interacting with opsin to form a complex capable of activating the G-protein.²²

The results described above are entirely consistent with the idea that some sort of charge movement involving the protonated Schiff base is key in the mechanism of rhodopsin action. The next question is how the charge is redistributed as a consequence of the absorption of light. One possible mechanism has been previously described involving the movement of the positively charged Schiff base away from its counterion due to the double bond isomerization.¹⁴ Another possible mechanism would involve the light-induced redistribution of charge down the carbon backbone of the retinoid (Scheme 2). It is already clear that the excited state of protonated Schiff bases of retinal involve charge redistribution of the type.²³ One possibility is that a simple rotation about the C₁₃–C₁₄ bond of the excited state chromophore of rhodopsin could isolate the positive charge in the hydrophobic active-site pocket, thus causing the energization of the protein with a minimum of atomic motion. This hypothesis can be tested by studying the ability of 11- and 9-*cis*-dihydroretinoids to serve as the appropriate retinal surrogates (Scheme 3). The 11- and 9-*cis*-7,8-dihydro and 11-*cis*-9,10-dihydroretinals formed pigments with opsin.²⁴ Upon photolysis, all of these pigments proved capable of activating the G-protein about one-half as well as rhodopsin, itself (Table 1).^{22–25} Since positive charge could not migrate past the single bonds of these pigments, this tells us that charge migration past the 9,10-double bond of the retinoid in rhodopsin cannot be critical for the activation process. 11-*cis*-13,14-Dihydroretinal would have been exceedingly interesting here, but unfortunately the molecule does not form a stable Schiff base with opsin.²⁶ However, the studies with the available dihydroretinals are certainly more consistent with a mechanism of charge movement, if it indeed occurs, in which point charges are moved. In addition, they eliminate from consideration those mechanisms of activation that involve proton abstraction from a methyl group of the ionone ring followed by double-bond shifts.²⁷



SCHEME 3. Studies of 7,8-dihydro-11-cis-retinal, 9,10-Dihydro-11-cis-retinal, and 7,8-dihydro-9-cis-retinal.

TABLE 1
11(9)-*cis*-7,8-Dihydro- and 11-*cis*-9,10-Dihydro-Retinoid Chromophores and Rhodopsin-Mediated Activation of G-Protein.

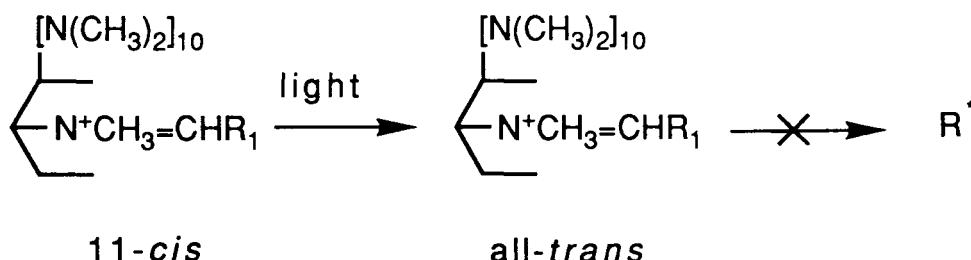
Modified Rhodopsin	G-Protein (GTPase) Activation ^a (%)
9- <i>cis</i> -7,8-dihydro-Rh	-4
9- <i>cis</i> -7,8-dihydro-Rh + light	43
11- <i>cis</i> -7,8-dihydro-Rh	8
11- <i>cis</i> -7,8-dihydro-Rh + light	52
11- <i>cis</i> -9,10-dihydro-Rh + light	38
Rh (reacted with NaBH ₄ in dark)	3
Rh (reacted with NaBH ₄ in dark) + light	98
11- <i>cis</i> -15,16-dihydro-Rh (retinylropsin)	-1
11- <i>cis</i> -15,16-dihydro-Rh + light	2

^a GTPase activation is presented relative to GTPase activation by native rhodopsin. All pigments were incorporated in egg phosphatidylcholine vesicles. The activity of GTPase was measured as production of free ³²P in the presence of approximately 0.75 μ M modified rhodopsin in the dark or under room lights as discussed under Materials and Methods of Reference 22. The relative GTPase activity was calculated as follows: [(GTPase activity in the presence of the modified rhodopsin) - (activity with rhodopsin in the dark)] - [(activity with rhodopsin in light) - (activity with rhodopsin in dark)] \times 100.

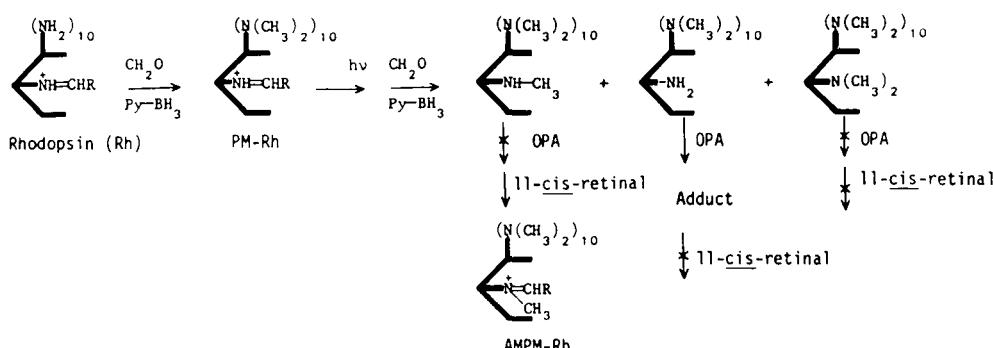
From Calhoon, R. D. and Rando, R. R., Biochemistry, 24, 6446, 1985. With permission.

The experiments described above are consistent with the hypothesis that the positive charge of the Schiff base of rhodopsin is important, at least at some stage of the bleaching cycle. Yet if the spectroscopic studies alluded to earlier are to be accepted at face value, metarhodopsin II, the spectroscopic signature of R*, contains a deprotonated Schiff base.^{2,12,13} It must be mentioned that others have argued that metarhodopsin II represents a hydrolyzed Schiff base rather than a deprotonated one, in which case the active-site lysine would still be protonated.²⁸ It was thus of interest to prepare a rhodopsin derivative in which the charge could not migrate from the Schiff base at all. This situation is, of course, opposite to that found with the retinoyl opsins. To these ends, active-site lysine monomethylated rhodopsin was prepared (Scheme 4).²⁹ We wished to test whether deprotonation is obligate in the formation of R* and in the formation of metarhodopsin II. Furthermore, we wished to know if the early intermediates that are produced prior to metarhodopsin II would be formed after light absorption. If they did form, then it would almost certainly mean that a full charge and not a partial one is on the Schiff base in the early intermediates during the bleaching of normal rhodopsin.

The actual sequence of chemical conversions used to prepare active-site methylated rhodopsin (AMPM-Rh) are shown in Scheme 5. The ten nonactive-site lysines of rhodopsin were first permethylated with formaldehyde/pyridine-borane, which incorporated 20 methyl



SCHEME 4. Active-site monomethylated rhodopsin (AMPM-Rh).



SCHEME 5. Preparations of AMPM-Rh.

groups to form permethylated rhodopsin (PM-Rh).²⁹ Fortunately, this material behaved similarly to native rhodopsin.²⁹ The absorption maximum of PM-Rh, its bleaching behavior at 4°C, and its ability to activate the G-protein were indistinguishable from that of rhodopsin itself.^{29,30} Bleaching of PM-Rh unmasked the active-site lysine residue, which could then be methylated to afford a mixture of the active-site lysine monomethylated, dimethylated, and unmethylated rhodopsins (Scheme 5).²⁹ The dimethylated derivative could not form a Schiff base, but the unmethylated could, making it important to remove this latter material. This was achieved with *o*-phthalaldehyde/mercaptoethanol (OPA), which are reagents capable of reacting with primary amines (lysine) but not secondary amines (methyllysine).²⁹ After this derivatization procedure was completed, 11-*cis*-retinal was added to regenerate the monomethyllysine opsin.²⁹ Purification of this mixture on hydroxyapatite led to the isolation of the nonactive-site permethylated, active-site monomethylated rhodopsin (AMPM-Rh).²⁹ Of immediate interest is the fact that AMPM-Rh showed a large bathochromic spectral shift when compared to PM-Rh or rhodopsin. AMPM-Rh had a maximal absorption at 523 nm — a 25-nm shift compared to PM-Rh and rhodopsin.^{29,30} This is not due to the methyl substitution per se because the protonated Schiff base formed between *n*-butylamine and retinal, and the Schiff base formed between *N*-methyl-*n*-butyl-amine and retinal had the same λ_{\max} .²⁹ Similar shifts were observed with 9-*cis* and 9,13-di-*cis*-retinals based AMPM-Rh pigments. The AMPM-Rh formed from 9-*cis*- had a λ_{\max} at 497 nm and that from 9,13-di-*cis*-retinal had a λ_{\max} at 495 nm.³¹ The rhodopsins formed from these retinals absorbed at 485 and 481 nm, respectively. The spectral shifts observed with the active-site methylated rhodopsin derivatives might have something to do with slight changes in the distances between the positively charged Schiff bases and their counterions. Possible mechanisms by which the negatively charged amino acids of opsin might shift the absorption spectra of the pigments have been considered by others.¹⁷

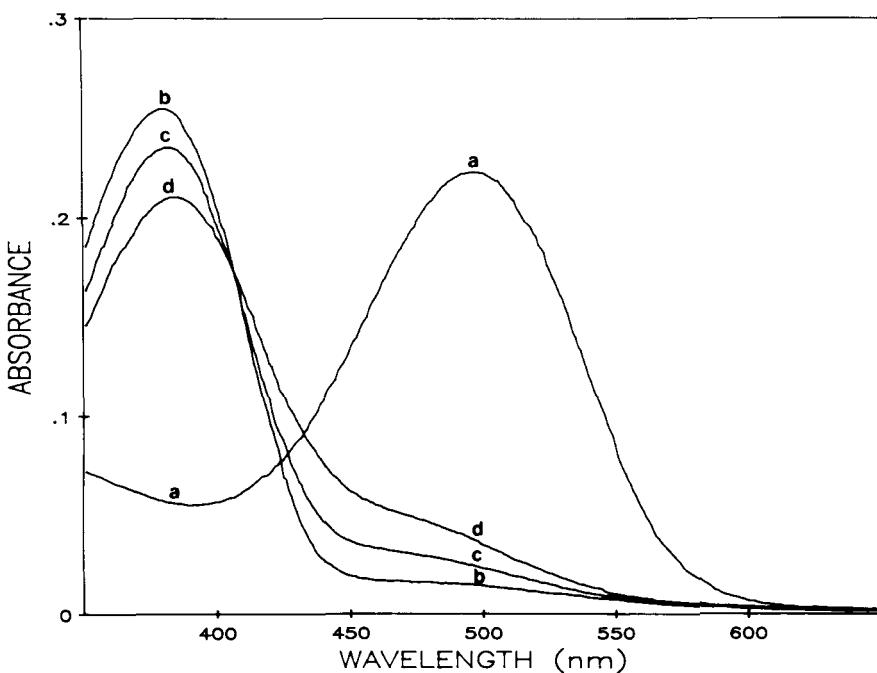


FIGURE 1. Bleaching of permethylated rhodopsin (PM-Rh) in dodecyl maltose buffer at 4°C. The spectra were recorded before (a) and 0.5 min (b), 1 h (c), and 4 h (d) after a 10-s exposure to orange light.

With the purified AMPM-Rh on hand, we were in a position to determine its bleaching behavior and to study its interaction with the G-protein. As noted before, the bleaching behavior of PM-Rh was similar to, if not identical, with that of rhodopsin when measured at 4°C.³⁰ Upon bleaching with orange light, a shift to metarhodopsin II was immediately observed that slowly decayed into metarhodopsin III and finally all-trans-retinal (Figure 1).³⁰ This behavior is to be contrasted with the results from bleaching of AMPM-Rh (Figure 2).³⁰ Here we did see an immediate shift to metarhodopsin II, which absorbed at 380 nm, but rather a shift to 485 nm was observed.³⁰ This we ascribe to a stable metarhodopsin I-like intermediate.³⁰ The same intermediate was observed when the AMPM-Rh formed from 9-cis-retinal was illuminated with orange light.³¹ This stable intermediate slowly decayed over a period of hours to all-trans-retinal and the methylated opsin (Figure 2).³⁰ There was absolutely no indication for the formation of either metarhodopsin II or III. We take this to mean that proton loss from the Schiff base is absolutely required for metarhodopsin II formation and that metarhodopsin III must arise sequentially from metarhodopsin II. Furthermore, these results indicate that metarhodopsin I can decay to afford all-trans-retinal directly without first proceeding through metarhodopsin II. The rate is so slow, however, that the direct connection would not normally be observed since it would be masked by the much faster metarhodopsin I to II conversion.²

If AMPM-Rh is really forming a stable metarhodopsin I-like intermediate that cannot proceed to a metarhodopsin II-like state, then it would be predicted that G-protein activation should not occur. This is exactly the case because the photolysis of AMPM-Rh, incorporated into phospholipid based vesicles, in the presence of the G-protein showed no inclination to activate the latter (Table 2).³⁰ These results indicate that Schiff base deprotonation is also obligate in the formation of R* and that there is a structural link between its formation and that of metarhodopsin II.

Since we now have a rhodopsin derivative that forms a stable metarhodopsin I-like state,

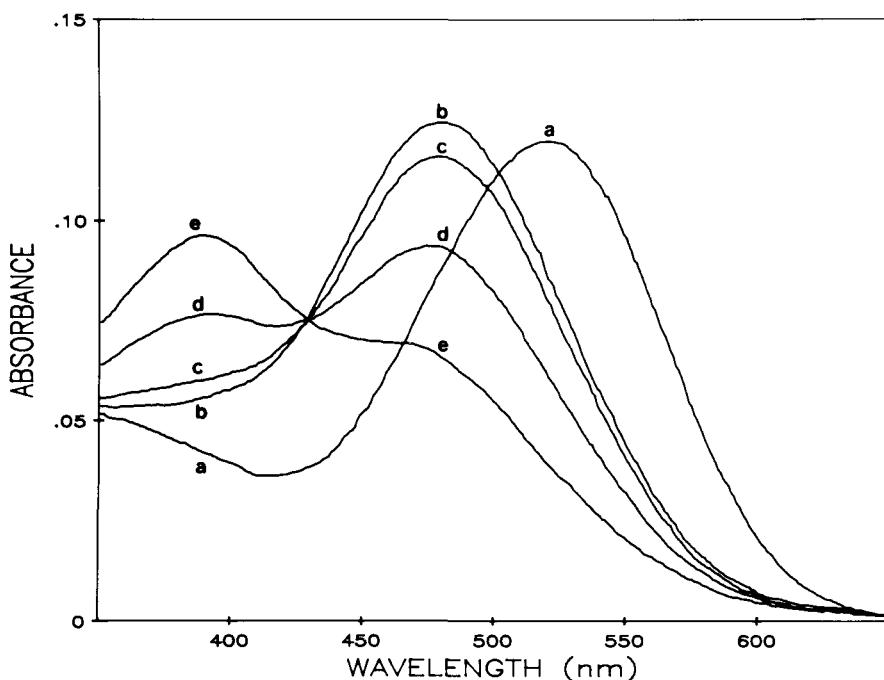


FIGURE 2. Bleaching of active-site methylated rhodopsin (AMPM-Rh) in dodecyl maltose buffer at 4°C. The spectra were recorded before (a) and 0.5 min (b), 5 min (c) 20 min (d), and 60 min (e), after a 10-s exposure to orange light.

we can ask questions that were hitherto unapproachable experimentally. For example, does rhodopsin kinase phosphorylate metarhodopsin I, II, or both, and will metarhodopsin I bind to the G-protein without activating it?

Recently, we have also carried out similar kinds of modification studies on bacteriorhodopsin (bR).³² This chromoprotein is not a sensory pigment like rhodopsin, but uses light energy to pump protons.³³ Bacteriorhodopsin contains all-trans-retinal or 13-cis-retinal attached to its active-site lysine residue by means of a protonated Schiff base.³³ Absorption of light causes a *trans*-to-13-cis isomerization in the retinoid, and as a consequence of this isomerization a proton(s) is pumped across the bacterial membrane. This process is coupled to ATP synthesis.³³ Several intermediates have been identified postlight absorption, with the most interesting one, for our purposes, being M 412.³⁴ This intermediate appears to be deprotonated at the Schiff base and has been implicated in the movement of the proton.³⁵ Is the Schiff base proton the one that is moved by light? To answer this question we prepared active-site methylated bacteriorhodopsin (AMbR) by procedures similar to those described for rhodopsin. AMbR proved to be blue, with a λ_{max} of 620 nm, and was in fact of the cyanopsin series.³² It was bathochromically shifted by 60 nm over bR and nonactive-site permethylated bR. This shift is even more striking than that observed with rhodopsin. When AMbR was incorporated into phospholipid based vesicles and illuminated, it underwent a reversible photoisomerization but was not able to pump protons.³² We conclude from these experiments that deprotonation of the Schiff base is obligate for the proton pumping ability of bR.

B. NONACTIVE-SITE LYSINE MODIFIED RHODOPSINS AND THEIR FUNCTIONS

Returning now to the rhodopsin modification studies, the fact that PM-Rh was capable of activating the G-protein as well as rhodopsin itself led to the question of the possible

TABLE 2
Activation of the G-protein by Active-Site Modified Rhodopsins

Modification of rhodopsin	% G-protein activation relative to native Rh	% Rh bleach required for equivalent G-protein activation*
Unmodified at the active-site		
Permethylated Rhodopsin (PM-Rh)	105	100
Active-site Modifications		
Preparation 1		
AMPM-Rh ^b	2	<0.5
AMPM-Rh ^c	2	<0.5
Preparation 2		
AMPM-Rh	17	1.5
10 min irradiation at 30°C	7	0.5
20 min irradiation at 30°C	4	<0.5
AMPM-Rh	14	1.2
20 min irradiation at 30°C	3	<0.5
OPA treated PM-opsin	10	0.8
Preparation 3		
AMPM-Rh ^d	3	<0.5
20 min irradiation at 30°C	4	<0.5
AMPM-Rh ^e	4	<0.5
OPA treated PM-opsin	6	0.5
Preparation 4		
AMPM-Rh	5	<0.5
OPA treated PM-opsin	2	<0.5
Combined Assays		
AMPM-Rh (16) ^f	3.0 ± 1.3	<0.5
OPA treated PM-opsin (6) ^f	4.8 ± 2.2	<0.5

Note: Each sample contained an excess of unregenerated protein over regenerated AMPM-Rh. The total protein was used to calculate the amount of lipid to be added in making vesicles. The values for each preparation are given as follows: micromolar concentration of AMPM-Rh (assuming $\epsilon_{280} = 40,000 M^{-1}cm^{-1}$), micromolar concentration of total protein (assuming $\epsilon_{280} = 64,000 M^{-1}cm^{-1}$), and volume of sample in ml. Preparation 1a: 3.7, 10.3, 1.0; preparation 1b: 2.3, 15.4, 3.0; preparation 2: 2.4, 15, 2.25; Preparation 3a: 3.3, 40.9, 1.5; preparation 3b: 2.2, 25.9, 1.5; preparation 4: 2.0, 16.1, 1.0.

* The curve relating the response of GTPase activation to partial rhodopsin bleach was generated by the standard protocol previously described.²¹ The curve for the G-protein preparation used in the above experiments was virtually identical to the dose-response curve already described.²¹

^b Protein purified on hydroxyapatite.

^c Protein purified on hydroxylalkoxypropylidextran.

^d Pool of concentrated fractions.

^e Pool of dilute fractions.

^f Number of assays.

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roles of the nonactive-site lysine residues in the interaction with the G-protein. Most of these lysine residues are found in the extradiskal connecting loops between helices 1 and 2, 3 and 4, and 5 and 6. These sites are potentially of interest in the interaction with the G-protein.³⁶ It has been speculated that the connecting loop between helices 1 and 2 may be important, because lysine-67 and leucine-72 are found in all vertebrate rod and cone pigments thus far sequenced.³⁶ The fact that dimethylation of this lysine has no apparent effect on

the ability of PM-Rh to activate the G-protein after photolysis weakens this notion. We have studied further lysine modification procedures to access the possible role of these residues in G-protein activation in greater detail (Table 3).³⁷ As can be seen here, none of the modifications save for the triphenylation and succinylation procedures had a significant effect on the abilities of the modified proteins to activate the G-protein after photolysis. Succinylation converts a positive charge to a negative charge and trinitrophenylation (picryl) removes the charge on the lysine and also adds a bulky hydrophobic residue. These drastic procedures would be expected to markedly modify the protein in a general way, or even to denature it. The acetylation result is most surprising, however (Table 3). This reagent neutralized the positive charge on many of the lysines, and yet the modified rhodopsin derivative still maintained its ability to activate the G-protein. This result is not understandable in terms of an important role for the bulk lysine residues in specific interactions with the G-protein, or any peripheral protein for that matter. In fact it is surprising that the protein maintains its tertiary structure after this treatment.

III. THE ISOMERIZATION OF ALL-*TRANS*-RETINOIDS TO 11-*CIS*-RETINOIDS IN THE EYE

Bleaching of vertebrate rhodopsin leads to the formation of all-*trans*-retinal and opsin. This physical detachment of the chromophore from vertebrate rhodopsin is important in dark adaptation after substantial bleaching for it enables the eye to control its sensitivity to light. Of substantial interest here is the fact that this control is exerted at the photoreceptor level. The greater the ambient light levels, the fewer the number of rhodopsin molecules there are to absorb light, and vice versa. The obscure logarithmic relationship found between rhodopsin levels and threshold sensitivities of the retina is of clear importance here.³⁸ By contrast, invertebrate rhodopsin does not bleach but rather undergoes a cyclical series of reactions, with one stage consisting of a long-lived metarhodopsin II.³⁹ That this occurs has been taken to mean that photochemical adaptation is not critical for these animals as their ambient light is maintained at fairly constant levels.

In order for vision to proceed after bleaching has occurred in vertebrates, the liberated all-*trans*-retinal must be converted into 11-*cis*-retinal by a thermal process. There have been several reports in the literature claiming the existence of a "retinal isomerase" but these have been incorrect due to a variety of experimental oversights.⁸

The investigation of this problem is complicated for several reasons. First of all, in the "visual cycle" the retinals are further processed to the retinols and their esters in the eye, so that the chemical isomerization need not occur at the retinal level (Scheme 6).⁸ Second, the isomerization could occur either in the retina or the pigment epithelium, an organ that is intimately associated with the retina. Indeed, the retinoids not associated with opsin are largely found in the pigment epithelium in their ester storage form.⁸ Third, the isomerization process is not a simple one chemically. This is because at chemical equilibrium 11-*cis*-retinoids account for only 0.1% of the mixture, whereas in a dark-adapted eye approximately 75% of the retinoids are 11-*cis*.^{8,40} Hence, one is far from equilibrium in the living eye, and energy must be put into the system in order to form appreciable amounts of 11-*cis*-retinoids. Since it is an intramolecular steric interaction within the retinoid backbone that renders the 11-*cis* isomer so unstable, one can expect that the equilibrium positions for the retinoids will be the same in a test tube as in the eye. It is distinctly possible that the isomerization process itself is energy requiring, making it an entirely new kind of enzymatic process. Finally, there are technical problems inherent in working with the retinoids that make analysis difficult. Foremost amongst these problems are the instability of the retinoids toward nonspecific reactions, including isomerization, and their lack of solubility in aqueous solutions. These difficulties notwithstanding, major strides have recently been made toward

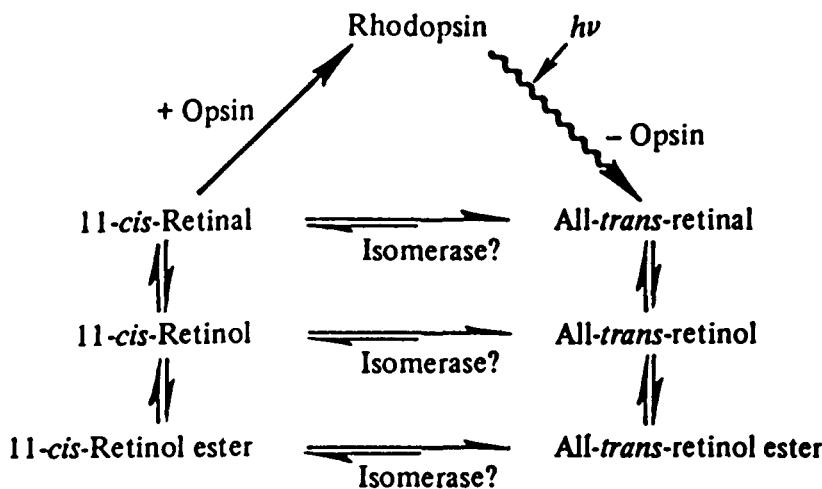
TABLE 3
Modification of Nonactive-Site Lysines and the Effect on G-Protein Activation

Modification to lysine	Lysines reacted (out of 10)	A_{278}/A_{500}		% regeneration of modified rhodopsin	% G-protein activation relative to native rhodopsin
		Before Reaction	After Reaction		
Dimethyl	>9 ^a	1.8—1.9	1.8—1.9	90—100	105
Ethyl	4.3	1.86	1.86	93	86
Acetimidyl	6.7	— ^b	1.85	85	69
Acetyl	8.8	1.89	1.89	53	95
Succinyl	8.2	1.88	1.98	62	3 ^d
Picryl	5.2	1.81	— ^c	0	2 ^d

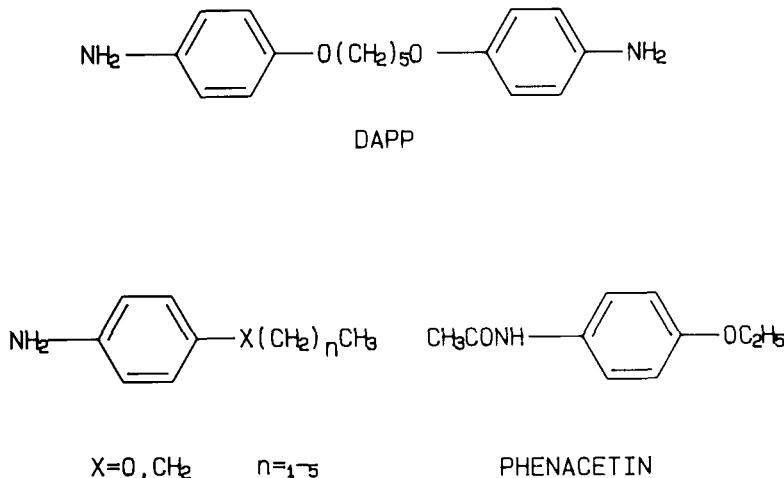
Note: Chemical modifications of nonactive-site lysines of rhodopsin and influence on ability to activate GTPase. Rhodopsin was reacted with a number of reagents (see Materials and Methods) to varying extents. All protein samples were able to withstand the reaction procedures with minimal denaturation or bleaching as shown by the ratio of the absorbance at 278 nm to the absorbance at 500 nm before and after reaction. Regeneration experiments and G protein-activation assays were carried out independently. Regeneration after bleaching with hydroxylamine (see Materials and Methods of Reference 37) is expressed as the percentage of initial regeneration observed before reaction. The GTPase activity of the G protein was assayed in the presence of 0.75 μM (modified) rhodopsin in the dark or under room lights. Concentrations of the modified rhodopsins were calculated from the absorption at 500 nm. The relative G protein activation was calculated as follows [(GTPase activity in the presence of the modified rhodopsin in light) — (activity with rhodopsin in dark)]/[(activity with native rhodopsin in light) — (activity with rhodopsin in dark)] \times 100.

- ^a Eight or nine with one round of methylation, >9 with two rounds.
- ^b The reaction was carried out with the rhodopsin in disk membranes.
- ^c Trinitrobenzene absorption interferes with this measurement.
- ^d Indistinguishable from zero by the assay used.

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SCHEME 6. The visual cycle.



SCHEME 7. Retinotoxic aromatic amines and the anti-inflammatory drug phenacetin.

a molecular understanding of the isomerization process. The remainder of this article will review some of these advances.

A. IN VIVO STUDIES

1. Studies on Drugs that Affect the Visual Cycle

Since very little is known about the visual cycle and its control, one way to initiate its study is to gain an understanding of the action of specific drugs that affect the function of the visual cycle. One class of drugs is represented by diaminophenoxypentane (DAPP) (Scheme 7). This drug, originally designed as an anti-Schistosomal agent, also causes night blindness and eventual total blindness in animals.^{41,42} On the physiological level, the drug was known to markedly decrease the rate of rhodopsin regeneration after a bleach.⁴² It was postulated that the drug functioned by inhibiting an ocular alcohol dehydrogenase — presumably an enzyme responsible for the conversion of 11-cis-retinol to 11-cis-retinal.⁴² However, the drug had never been shown to effectively inhibit any alcohol dehydrogenase, and was not structurally reminiscent of any known inhibitor of this class of enzymes. We

TABLE 4
Visual Cycle Effects of Phenetidine-Like Compounds with Hydrophobic Tail Modifications.

Aromatic amine	Dosage (mg/kg)	% rhodopsin regeneration at 2 h	% 11-cis-retinyl palmitate formation at 24 h
None	—	97 ± 2	24 ± 1
Aniline	100—200	90 ± 3 ^b	23 ± 2
p-Anisidine	40—100	74 ± 20	22 ± 4
p-Phenetidine	40—100	60 ± 8 ^c	7 ± 4 ^c
p-Ethylaniline	40—100	55 ± 15 ^c	9 ± 2 ^c
p-n-Butoxyaniline	50	37 ± 21 ^c	4 ± 2 ^c
p-n-Butylaniline	50—100	15 ± 2 ^c	3 ± 1 ^c
p-n-Hexyloxyaniline	60—100	10 ± 3 ^c	2 ± 0 ^c
p-n-Dodecylaniline	100 ^a	97 ± 2	20 ± 1
p-Aminophenol	100	98 ± 2	26 ± 2
p-Nitroaniline	50—100 ^a	95 ± 1	18 ± 1 ^b
<i>o</i> -Phenetidine	100	86 ± 1 ^c	19 ± 6
<i>m</i> -Phenetidine	100	59 ± 14 ^c	8 ± 2 ^c

^a These compounds were administered in ethanol rather than in acidified water.

^b Denotes significant reduction relative to control. ($0.01 \leq p < 0.05$ on a one-tailed unpaired *t*-test for $n = 2—8$). All values are \pm SEM.

^c Denotes highly significant reduction relative to control. ($p < 0.01$ on a one-tailed unpaired *t*-test for $n = 2—8$).

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reinvestigated this problem and found that the drug had physiological effects not interpretable by this kind of mechanism.⁴³ The drug inhibited rhodopsin regeneration because it depleted ocular stores of 11-cis-retinoids in the dark.⁴³ This depletion could, in principle, have arisen either by preventing the thermodynamically uphill biosynthesis of these retinoids or by enhancing their conversion to other retinoids.

Structure-activity studies on these drugs demonstrated that a bifunctional molecule was not important but that the monofunctional aromatic analogs of the type illustrated in Scheme 7 were also effective.^{44,45} In addition, molecules that could be transformed metabolically into these drugs, such as the anti-inflammatory drug phenacetin, were also active (Scheme 7).⁴⁴ Thus, any primary aromatic amine containing a suitable hydrophobic chain proved to be active (Scheme 7 and Table 4).⁴⁵ Aliphatic amines were not active.⁴⁵

Further studies showed that Schiff base formation between the drug and retinal was absolutely required for activity.⁴⁵ Furthermore, it could be shown that these Schiff bases were detected *in vivo* after the drugs were injected intraperitoneally.⁴⁵ The drugs did not work simply by trapping the retinals as Schiff bases because large stores of all-trans-retinoids were still found in the eye after drug treatment.⁴⁵ The drugs were also unlikely to affect the visual cycle by a specific receptor-drug mediated event, given the observed lack of structural specificity. Along these lines, it could be shown that the drugs did not affect the early burst of 11-cis-retinol formation that is observed in the amphibian.⁴⁵ This rules out the possibility that the drugs function by interfering with the biosynthesis of 11-cis-retinoids. This point will be returned to later in the section on *in vitro* studies.

The key to how these drugs functioned mechanistically was uncovered when it was found that they profoundly catalyzed the thermodynamically downhill isomerization of 11-cis-retinal to its all-trans congener when incorporated into membranes.^{44,46} These drugs were capable of catalyzing the isomerization of retinal by factors of approximately a thousand-fold over the uncatalyzed rate, compared at the same temperature, when incorporated into phospholipid based vesicles (Table 5).⁴⁶ Thus, the drugs functioned by short-circuiting the

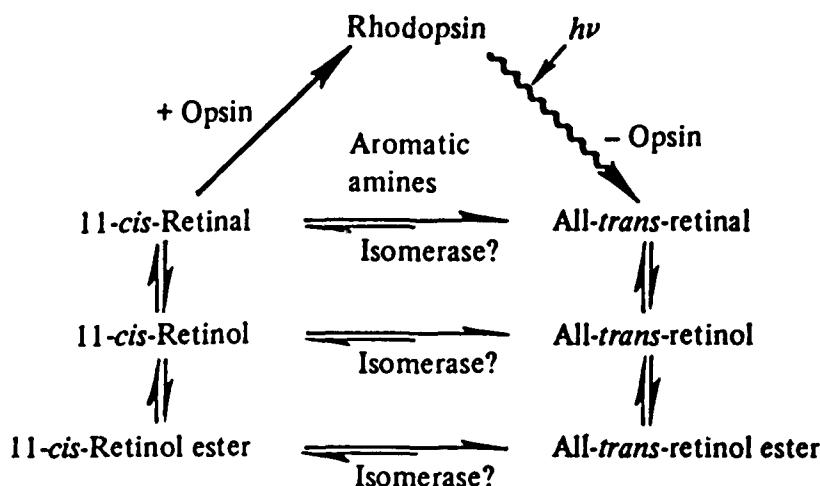
TABLE 5
Thermal Isomerization of 11-cis-Retinal and its *p*-n-Hexyloxyaniline Schiff Base (HSB) in Various Lipids

Retinoid	T (°C)	Lipid added	$k_1 (s^{-1}) \times 10^{-4}$
11-cis-retinal	37	<i>n</i> -hexane	<0.001
11-cis-retinal	37	PC	0.065
11-cis-retinal	37	PC (oxidized)	0.063
11-cis-retinal	37	Dimyristoyl PC	0.065
11-cis-retinal	37	Dipalmitoyl PC	0.093
11-cis-retinal	37	Dioleoyl PC	0.084
11-cis-retinal	37	Dilinoleoyl PC	0.077
11-cis-retinal	37	Triton X-100	0.034
11-cis-retinal	37	Sodium dodecyl sulfate	0.07
HSB	37	PC	1.6 ^a
HSB (1 eq. amine)	37	PC (oxidized)	0.2
HSB (5 eq. amine)	37	PC (oxidized)	2.0
HSB	37	Dimyristoyl PC	0.62
HSB	20	Dimyristoyl PC	0.31 ^b
HSB	37	Dipalmitoyl PC	0.56
HSB	43	Dipalmitoyl PC	0.99
HSB	37	Dioleoyl PC	1.6
HSB	37	Dilinoleoyl PC	2.5 ^b
HSB	37	Triton X-100	0.13
HSB	37	Sodium dodecyl sulfate	1.27

^a The isomerization followed first-order kinetics for at least four half-lives at which time a 92% decrease in initial percentage of 11-cis-retinoid had occurred.

^b Rate constants based on initial rate measurements.

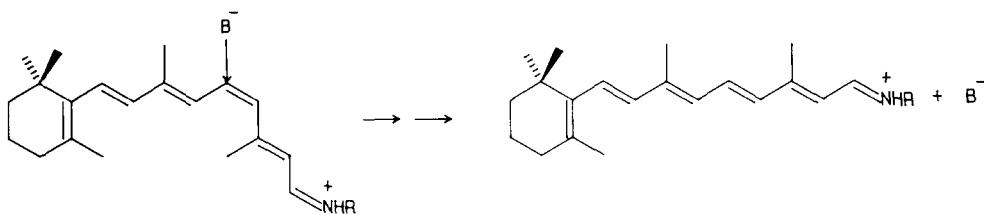
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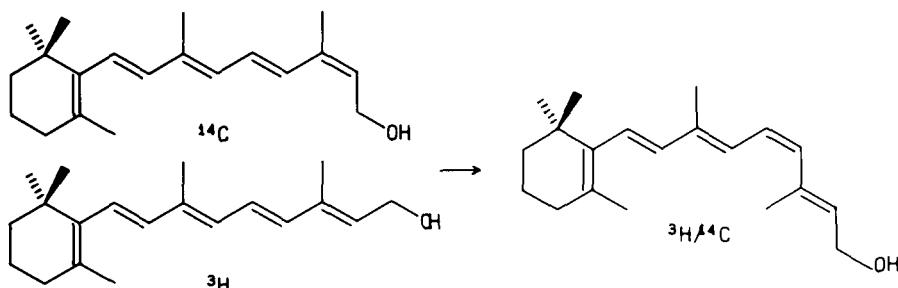
SCHEME 8. Short-circuiting the visual cycle with retinotoxic aromatic amines.

visual cycle as shown in Scheme 8.^{45,46} The mechanism of the catalysis of the isomerization process is depicted in an abbreviated form in Scheme 9.⁴⁶

Although the aromatic amine-containing drugs proved not to be capable of interrupting the biosynthesis of 11-cis-retinoids in the eye, their mechanism of action allowed the following valuable conclusions to be drawn about the operation of the visual cycle. The retinals



SCHEME 9. Acid-base catalyzed isomerization of retinotoxic aromatic amine Schiff bases of 11-cis-retinal.



SCHEME 10. Conversion of 13-cis-retinol and all-trans-retinol to 11-cis-retinol.

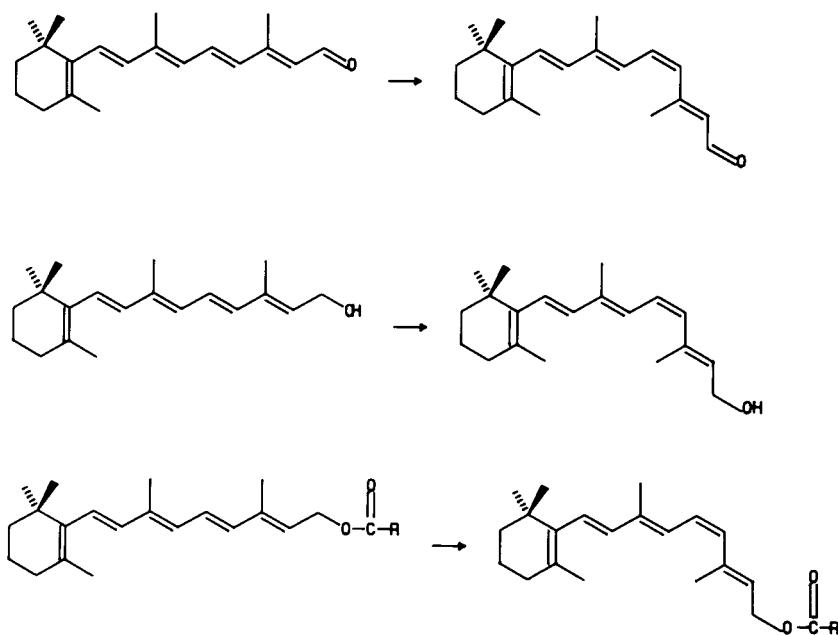
must exist, at least transiently, in a trappable form in the eye irrespective of the presence of retinoid-binding proteins. A productive nonenergy transducing "retinal isomerase" cannot exist in the eye because that is what the aromatic amines are. The enzymes of the visual cycle must process the retinoids in the dark, or else the drugs could not deplete the eye of 11-cis-retinyl esters since these esters are inert to the aromatic amines (as are the retinols). Therefore, whatever enzymatic regulation there is is not absolute. Finally, a new and general mechanism of ocular toxicity has been defined, and aromatic amines of the type described should be avoided as drugs and in the diet.

2. Analog Studies *In Vivo*

In order to study the specificity of the isomerization process, *in vivo* studies with retinoid analogs were performed. Initial studies were concerned with probing the stereospecificity of the process.⁴⁷ Since the retinoids do not contain an asymmetric center, diastereomeric probes had to be utilized. Specifically, we wished to know if 13-cis-retinols could be processed to 11-cis-retinoids *in vivo*.

Intraocularly injected all-trans-retinol is processed to 11-cis-retinoids in the frog.^{8,47} During time-course studies, it was noticed that 13-cis-retinoids were formed early on, only to disappear at later time points.⁴⁷ It was inferred from this that 13-cis-retinoids could be formed in the eye but were later converted into 11-cis-retinal in the dark. To test this hypothesis directly, double-label mixtures of [³H]-all-trans-retinol and [¹⁴C]-13-cis-retinol (Scheme 10) were injected into the animals, and the relative extents of conversion of these two molecules into 11-cis-retinal were determined.⁴⁷ Interestingly, both molecules entered the 11-cis pool with almost equal facility, thus proving that the isomerization process could be nonstereospecific *in vivo*.⁴⁷ Three mechanistically different possibilities could explain this result. The isomerization process could be nonenzymatically mediated and chemical in nature. It could be enzymatically mediated by a nonstereospecific isomerase. Finally, there could be multiple isomerizing systems present. Evidence will be described later that points toward the latter possibility.

To further probe the specificity of the isomerization process, the isomeric all-trans-

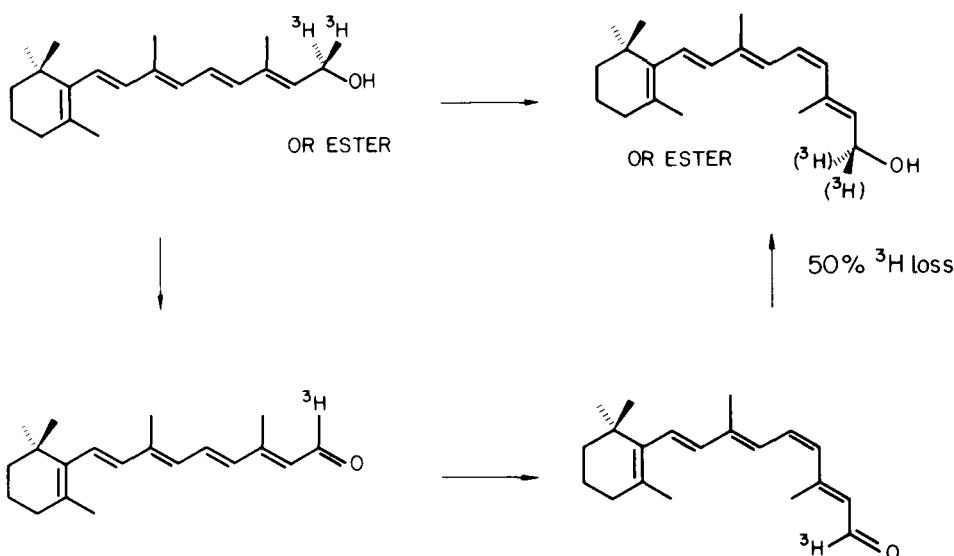
SCHEME 11. 15-Methyl-all-*trans*-retinol and all-*trans*-retinol methyl ether.SCHEME 12. *Trans*-to-*cis* isomerization of aldehyde, alcohol, and ester forms of vitamin A.

retinol analogs shown in Scheme 11 were studied. First of all, the *O*-methyl ether of all-*trans*-retinol was not processed at all in the living eye.⁴⁸ The 15-methyl-all-*trans*-retinol was oxidized to the analogous ketone, but not isomerized.⁴⁸ These experiments, taken together, indicate a specific isomerization process.

3. Tritium-Release Experiments *In Vivo*

Before proceeding with *in vitro* experiments, it was important to know which form of retinoid is isomerized. Three possibilities are depicted in Scheme 12. All three retinoid forms have been speculated to be the actual substrates, but on the basis of incomplete if not incorrect information.⁸ An experimental approach to at least determining the relevant substrate oxidation state involved in the isomerization is shown in Scheme 13. If [15-³H]all-*trans*-retinol can be converted either directly or through its ester form into 11-*cis*-retinol(ester), theoretically, no tritium loss should occur. Some loss might occur, however, due to a nonproductive exchange catalyzed by the ocular alcohol dehydrogenases. The tritium in the 15-position of the retinol is stable to chemical exchange. It has been known for many years that alcohol dehydrogenases can distinguish between the prochiral hydrogen atoms in the reduced substrate.⁴⁹ Thus, up to one half of the tritium could be removed by alcohol dehydrogenase mediated oxidation. However, less than half should be removed at early times, unless oxidation to the retinal is obligate in the isomerization process. If the oxidation is obligate, then one half of the tritium should be lost in the initially formed 11-*cis*-retinol(ester).

Double-label experiments using mixtures of [¹⁴C]all-*trans*-retinol and [³H]all-*trans*-



SCHEME 13. Tritium washout and the substrate for isomerization.

TABLE 6
Double-Label Experiments in Rats — 4 h

Drug	% retention of ³ H label (relative to ¹⁴ C label)*			
	11-cis-retinyl esters ^c	All-trans-retinyl esters ^c	11-cis-retinal oximes	All-trans-retinol
None	67 ± 6	81 ± 13	50 ± 2	48 ± 12
4-MP ^b (500 mg/kg)	81 ± 4	92 ± 4	58 ± 4	44 ± 12
DAPP ^b (100 mg/kg)	73 ± 8	85 ± 4	48 ± 4	52 ± 4

* All values are mean ± S.D. for n = 3. The starting ³H:¹⁴C ratio was 5.2:1.

^b Abbreviations: 4-MP, 4-methylpyrazole; DAPP, 1,5-di-(p-aminophenoxy)pentane.

^c Retinyl esters in the rat are predominantly a mixture of retinyl palmitate and retinyl stearate.

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retinol were performed *in vivo* both on the rat and on the amphibian.⁵⁰ In both cases, the labels were in the 15-position of the retinoid. The results from the rat experiments are shown in Table 6.⁵⁰ It can clearly be seen that in the rat, significantly more than 50% of the starting tritium was retained in the initially formed 11-cis-retinyl ester. This retention could be enhanced in the presence of 4-methylpyrazole, a well studied inhibitor of alcohol dehydrogenases that blocks dark adaptation in the rat.⁵¹ DAPP, another inhibitor of dark regeneration in the rat that does not involve alcohol dehydrogenase inhibition, does not significantly affect tritium loss. These experiments mean that isomerization must occur at the alcohol level of oxidation. Of course, they do not tell us anything about the nature of the putative enzyme-bound intermediates that occur during the isomerization process.

Two further deductions about the visual cycle can be gleaned from these experiments. First, after 24 h in the dark, 50% washout of tritium was observed in all of the retinoids.⁵⁰ This confirms one of the conclusions from the DAPP experiments, namely that the enzymes of the visual cycle are operative in the dark. Secondly, the reisolated all-trans-retinol from these experiments had lost 50% of its tritium at all times measured during these experiments. It might be inferred from this result that it is not retinol itself that is a substrate for the

TABLE 7
***In Vitro* Regeneration Experiments in Eye Cups from Light-Adapted
Frogs**

Medium Used	<i>In vitro</i> lighting conditions (h) ^a	% rhodopsin regeneration (mean \pm S.D.) ^b
Bleached eye	—	<10 ^c
Amphibian tissue-culture medium (ATM)	2 D	90 \pm 9 (n = 9)
ATM	2 D/0.5L	23 \pm 4 (n = 3)
ATM	2 D/0.5 L/2 D	83 \pm 4 (n = 6)
ATM	17 D/0.5 L/2 D	69 \pm 16 (n = 2)
ATM + 0.1% NaN ₃	2 D	21 \pm 9 (n = 2)
ATM + Argon	2 D	40 \pm 13 (n = 2)
ATM + 2,4-Dinitrophenol	2 D	13 \pm 4 (n = 2)
2 mM 2,4-Dinitrophenol ^d	2 D	

^a D = incubation in the dark; L = incubation in the light; (i.e., 2 D/0.5 L corresponds to a 2-h dark incubation followed by a 0.5-h light incubation).

isomerization process, but rather a diversion product from it, such as retinyl ester. This conclusion cannot be regarded as unambiguous since the kinetics of the reactions of the visual cycle are unknown at this time. When the experiments described above were conducted on the frog, uninterpretable results were obtained because of the rapid, uninhibitable tritium washout catalyzed by the frog ocular alcohol dehydrogenases.⁵⁰

B. *IN VITRO* EXPERIMENTS

Before further analysis of the isomerization process could occur, an *in vitro* system capable of the biosynthesis of 11-*cis*-retinoids was required. Examination of the literature was not helpful in this regard because even excised eye cups have never been clearly shown to be able to synthesize 11-*cis*-retinoids *de novo* after a thorough bleach. Typically, around 50% pigment regeneration was observed in the dark after a single bleach.⁵² There were probably enough endogenous 11-*cis*-retinoids in these eye cups to account for this.³ Since possible 11-*cis*-retinyl ester synthesis was not measured in the dark, it is impossible to determine if net 11-*cis*-retinoid biosynthesis had occurred. Based on these kinds of experiments, one could not eliminate the possibility that the *de novo* biosynthesis of 11-*cis*-retinoids was somehow controlled by the central nervous system and would not occur outside the living organism.

The possibility that an afferent neural signal from the brain affected regeneration could be ruled out by leasoning experiments.⁴⁸ Leaving the ocular blood supply intact and cutting the optic nerve had little or no effect on pigment regeneration in light-adapted living frogs and rats after placing them in the dark, as compared to the appropriate controls.⁴⁸ Furthermore, when freshly dissected eye cups from frogs were placed in Ringers solution containing added amphibian tissue culture medium (ATM), evidence for clear *de novo* pigment regeneration was obtained after bleaching (Table 7).⁴⁸ Multiple bleaching regeneration cycles were possible, and this regeneration could be inhibited by energy uncouplers (Table 7). In the absence of added ATM, the results were more scattered and less clear. Furthermore, analysis of the esters from the ATM incubated eye cups also showed that new 11-*cis*-retinyl ester synthesis had occurred (Table 8).⁴⁸ These results unequivocally demonstrate that 11-*cis*-retinoid biosynthesis can occur outside the living organism. These results prompted us to determine if the amphibian eye cup system could be further simplified on the way to the eventual purification of the isomerase system. To this end, frog retina-pigment epithelium preparations were thoroughly sonicated and centrifuged at 600 \times g to remove any unbroken

TABLE 8
In Vitro Production of 11-cis-Retinyl Palmitate in
Eye Cups from Light-Adapted Frogs Incubated in
ATM

<i>In vitro</i> lighting conditions (h)*	% 11-cis-Retinyl palmitate in total retinyl palmitate pool (mean \pm S.D.)	
2 D	6 \pm 4	(n = 2)
17 D	28 \pm 8	(n = 6)

* D = incubation in the dark; L = incubation in the light; (i.e., 2 D/0.5 L corresponds to a 2-h dark incubation followed by a 0.5-h light incubation).

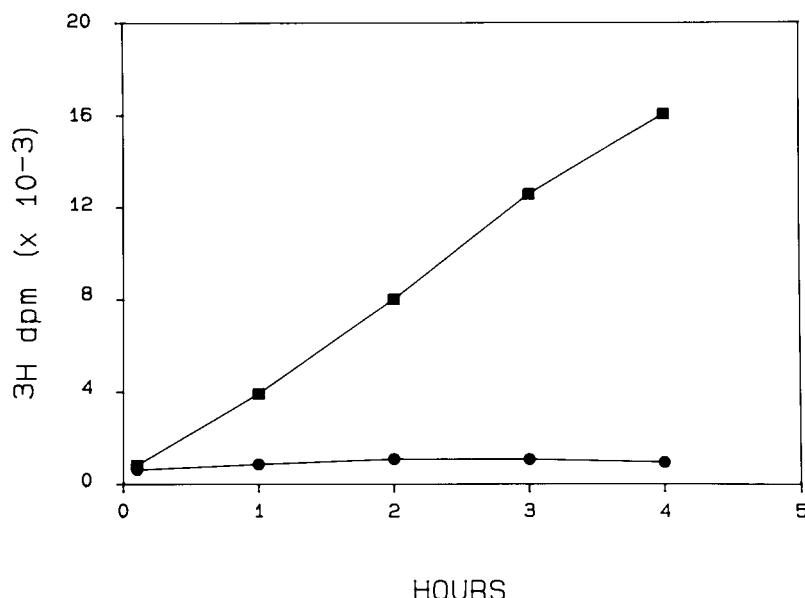


FIGURE 3. Formation of [³H]11-cis-retinol *in vitro*. A 1.0-ml aliquot of 600 \times g supernatant from light-adapted frog retina/pigment epithelium (two eyes) was incubated with 4 μ Ci of [11,12-³H]all-trans-retinol, and [³H]11-cis-retinol content was assayed hourly from 200- μ l portions. ■, [³H]11-cis-retinol content in retina/pigment epithelium supernatant. ●, [³H]11-cis-retinol in a control incubation with no eye tissue present in the buffer.

cells and nuclear material. This supernatant was treated with [11,12-³H]-all-trans-retinol, and the synthesis of radioactive 11-cis-retinol was followed as a function of time (Figure 3).⁹ As can be seen here, a time-dependent increase of 11-cis-retinol was observed.⁹ Since 11-cis-retinoids account for only 0.1% of an equilibrium mixture, the substantial synthesis observed here (30 to 50%) signals an energy-requiring, biologically significant process. In Figure 4 is shown the synthesis of 11-cis-retinol as a function of time compared to that of the nonphysiologic and spontaneously forming 13-cis isomer, which does not increase with time.⁹ It should be mentioned that we have also detected a soluble, possibly nonspecific, isomerization activity from the retina/pigment epithelium that interconverts all-trans- and 13-cis-retinoids.⁵³ Whether this is of biological significance remains to be determined, but it might explain our earlier double-label experiments with 13-cis- and all-trans-retinols (Scheme 10).⁴⁷ Also shown in Figure 4 is the decline in all-trans-retinol as a function of time.⁹ That 11-cis-retinol was being formed, and not some other isomer or retinoid degradation product, was shown in the following ways.⁹ First, the labeled putative 11-cis-retinol

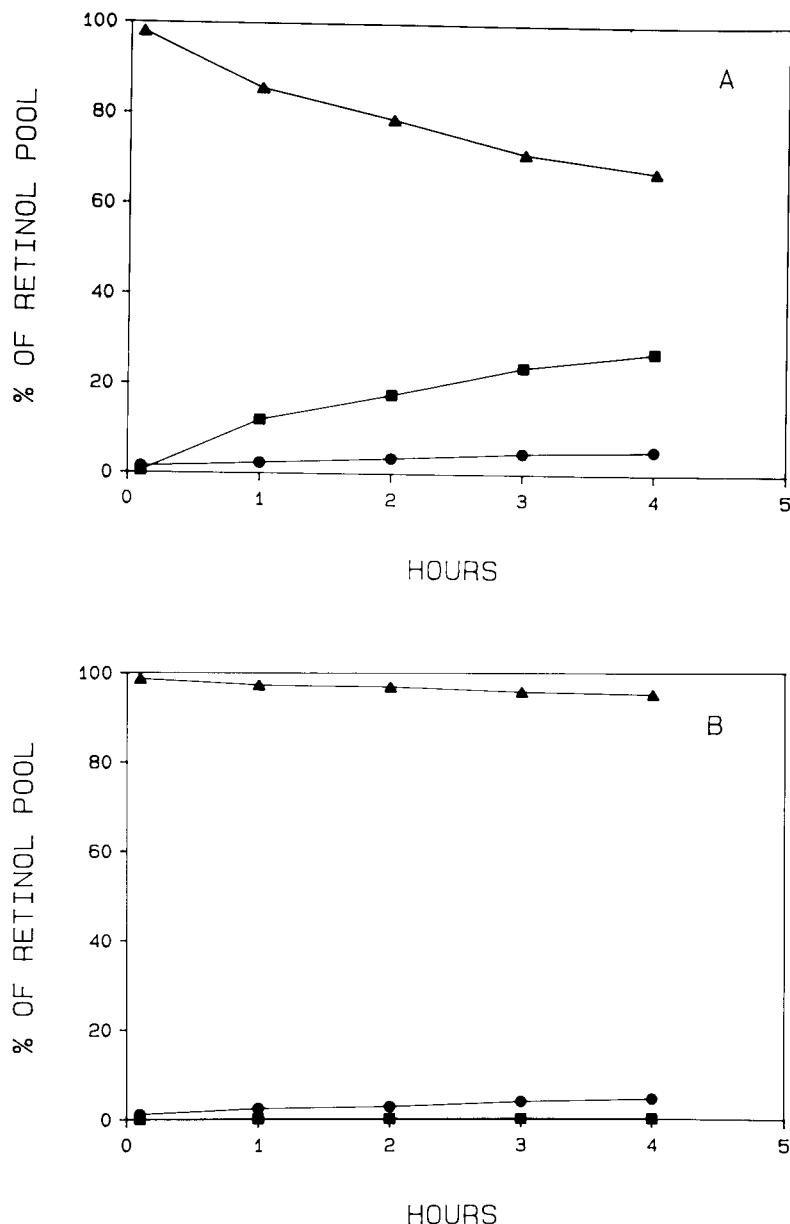


FIGURE 4. Isomeric distribution of [³H]retinol during *in vitro* incubation. A light-adapted frog retina/pigment epithelium $600 \times g$ supernatant (A) and a control tube containing no eye tissue (B) were incubated with [$11,12\text{-}^3\text{H}$]all-*trans*-retinol as described in Materials and Methods. HPLC analysis of [³H]retinol isomers was performed hourly. ■—■ is [$15\text{-}^3\text{H}$]11-*cis*-retinol, ●—● is [³H]13-*cis*-retinol, and ▲—▲ is [³H]all-*trans*-retinol.

co-chromatographed identically by HPLC with authentic 11-*cis*-retinol. Second, the putative 11-*cis*-retinol was esterified with palmitoyl chloride and shown to produce 11-*cis*-retinyl palmitate. Third, the putative 11-*cis*-retinol was isomerized by iodine and shown to produce the same isomeric mixture of retinols as obtained from authentic 11-*cis*-retinol. Thus, without a doubt 11-*cis*-retinol biosynthesis has been shown to occur in a cell-free system. It should be mentioned that these membranes also make substantial quantities of 11-*cis*-retinyl palmitate and 11-*cis*-retinal.⁹

TABLE 9
***In Vitro* Production of Isomeric Retinols from [³H] all-*trans*-Retinol in 600 × g
Supernatants of Eye Homogenates Incubated 3 h^a**

Experiments	%11-cis-Retinol	%13-cis-Retinol	%All-trans-retinol	%Total recovery ^b	Protein (mg/ml)
No eye (n = 9)	0.2 ± 0.1	2.9 ± 1.1	96.9 ± 1.1	100	—
Light-adapted eye extract (n = 7)	25.6 ± 3.8	7.6 ± 2.4	66.9 ± 6.0	13.9 ± 3.1	4.2 ± 0.6
Light-adapted eye extract boiled 5 min (n = 3)	0.4 ± 0.1	5.9 ± 0.5	93.7 ± 0.5	105.6 ± 3.9	5.4 ± 1.6
Dark-adapted eye extract (n = 3)	12.3 ± 2.8	9.4 ± 4.8	78.5 ± 7.5	17.8 ± 4.1	4.2 ± 0.8
Dark-adapted retinal extract (n = 3)	2.7 ± 1.5	7.0 ± 1.4	90.3 ± 2.0	43.0 ± 13.2	3.2 ± 1.4
Dark-adapted pigment epithelium extract (n = 3)	10.6 ± 5.0	9.3 ± 4.1	80.1 ± 8.7	10.2 ± 1.8	1.0 ± 0.6
Light-adapted eye 150,000 × g pellet (n = 5)	32.8 ± 14.1	16.8 ± 4.9	50.5 ± 18.1	7.4 ± 1.7	2.0 ^c
Light-adapted eye 150,000 × g supernatant (n = 5)	1.6 ± 1.1	1.7 ± 0.9	96.7 ± 1.6	58.1 ± 2.8	2.8 ^c

^a Incubations and analyses were as described in Reference 9. All values are mean ± S.D.

^b Total recovery of all isomeric retinols relative to concurrent control incubations with no eye tissue present.

^c Protein content was measured a single time.

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mentioned that these membranes also make substantial quantities of 11-cis-retinyl palmitate and 11-cis-retinal.⁹

Further experiments were performed to characterize this 11-cis-retinoid biosynthetic activity.⁹ As shown in Table 9, the activity is heat sensitive, more active when isolated from a light-adapted eye, and confined largely to the pigment epithelium.⁹ The biosynthetic activity proved not to be sensitive to the aromatic amine retinotoxic agents described above, further confirmation of their proposed mechanism of action.^{48,53} The isomerase activity also proved to be protease and phospholipase C sensitive.⁵³ The latter sensitivity suggests that the protein(s) is membrane bound, which could be directly shown by centrifugation experiments.⁹ Increasing the speed of centrifugation decreased the amount of activity in the supernatant and increased it in the pellet.⁹

The tritium-release experiments performed *in vivo* showed that isomerization takes place at the alcohol level of oxidation. It was of interest to repeat these experiments on the *in vitro* preparation. That the isomerization process occurs at the alcohol state of oxidation also was shown by the experiments exhibited in Table 10 and by double-label tritium-washout experiments of the kind described above in the *in vivo* experiments.⁵³ In the experiments shown here, washed membranes depleted of nicotinamide dinucleotide cofactors have substantially lost their ability to convert all-*trans*-retinal but not all-*trans*-retinol to 11-cis-retinoids. Double-label experiments show that there is no loss of tritium from the 15-position of all-*trans*-retinol when it is processed to 11-cis-retinol by these membranes.⁵³ Also shown in Table 10 are results employing added all-*trans*-retinyl palmitate as a possible substrate. Its apparent lack of conversion to any 11-cis-retinoid might suggest that the ester is not the substrate for isomerization. However, given the extreme hydrophobicity of this compound and the fact that it does not undergo intermembranous transfer at an appreciable rate, a conclusion of this type cannot be made at this time.⁵⁴ It should be noted that the frog is not the only vertebrate species where we have found an isomerase activity. We have also

TABLE 10
***In Vitro* Formation of 11-cis-Retinoids from [³H] all-*trans*-Retinoids in Washed and Unwashed 600 × g Supernatants of Eye Homogenates (3 h)**

[³ H]-all- <i>trans</i> -retinoid supplied	Retinol % total		Retinal % total		Retinyl palmitate % total	
	% 11-cis	recovery ^b	% 11-cis	recovery ^b	% 11-cis	recovery ^b
Retinol (no eye, n = 6)	<1	>99	—	<1	—	<1
Retinol (unwashed membranes, n = 3—5)	36.9 ± 5.9	15.6 ± 2.6	45.8 ± 15.4	11.6 ± 1.4	12.7 ± 5.2	72.8 ^c
Retinol (washed membranes, n = 4—6)	40.3 ± 9.9	12.0 ± 3.3	14.5 ± 14.1	8.5 ± 3.4	14.4 ± 5.9	79.5 ^c
Retinal (no eye, n = 5)	—	<1	1.8 ± 1.2	>98	—	<1
Retinal (unwashed membranes, n = 2)	33.7 ± 8.4	18.4 ± 1.6	31.8 ± 9.4	14.0 ± 3.1	14.7 ± 2.8	67.6 ^c
Retinal (washed membranes, n = 2)	16.6 ± 2.2	7.9 ± 4.1	1.5 ± 0.7	60.2 ± 5.1	12.0 ± 1.1	31.9 ^c
Retinyl palmitate (no eye, n = 2)	—	<1	—	<1 ^c	<1	>99
Retinyl palmitate (unwashed membranes, n = 2)	—	<1	—	<1 ^c	<1	>99

^a Incubations and analyses were as described in Reference 9. All experiments in this table were performed with no ethanol present, while those of Tables 2 and 3 had 0.4% ethanol in the incubation mixture. All values are mean ± S.D.

^b Combined recovery of all isomers of the particular retinoid relative to total recovery of all retinoids in concurrent control incubations with no eye tissue.

^c Total recovery was not determined in these instances, but the experiments of Tables 2 and 3 indicate that the sum of recoveries for all three major retinoids is always approximately 100%.

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found the activity to reside in bovine pigment epithelium membranes.⁵⁵ In washed membranes from this system, only all-*trans*-retinol served as substrate.⁵⁵ This should allow for an approach to the isolation and eventual purification of this important isomerase system.

The fact that 11-*cis*-retinoid biosynthesis occurred only in the pigment epithelium of the mammal, and largely if not exclusively in the same organ in the amphibian, allows us to understand why *de novo* pigment regeneration had never been observed in excised retinas.^{56,57} Interestingly, published attempts to demonstrate 11-*cis*-retinoid biosynthesis in pigment epithelium cultures from added all-*trans*-retinol have also met with failure.⁵⁸ However, we now know that this failure was not due to anything significant, but was due to experimental design. In the reported cases, 2 to 4% ethanol was used as a carrier for the added retinoids. This concentration of ethanol completely abolished isomerase activity both in the amphibian and mammalian systems.^{48,55}

The experiments reported above allow us for the first time to entertain the possibility of fully understanding the missing reaction in the visual cycle, that is, the one that produces 11-*cis*-retinoids and hence completes this cycle. On a physiological level this will clearly be of great interest, for it will enable us to understand the nature of the visual cycle and how the biosynthesis of 11-*cis*-retinoids is controlled. What relationship this might bear to diseases of the eye, such as Retinitis Pigmentosa, will deserve careful scrutiny. On a biochemical level, the isomerization process is also of great interest because of its endergonic nature. What is the energy source for the biosynthesis of 11-*cis*-retinoids, and how is it coupled to the isomerization process? These and other intriguing questions await the purification and characterization of this unique system.

ADDENDUM

A major issue in the investigations on the isomerase mechanism involved the nature of the energy source which can drive the thermodynamically uphill isomerization of an all-*trans*-retinoid to an 11-*cis*-retinoid. It turns out that the issue of energy source is also inextricably linked with the nature of the putative substrate for the isomerase. As already mentioned, membranes from the pigment epithelium process added all-*trans*-retinol to produce 11-*cis*-retinol, 11-*cis*-retinyl palmitate, and in certain cases 11-*cis*-retinal. Moreover, the all-*trans*-retinol is rapidly esterified by the membranes to produce retinyl esters, chiefly of the palmitate series.

In early studies we were able to show diverse chemical agents, such as ethanol, hydroxylamine, and *p*-hydroxymercuribenzoate, inhibited the processing of all-*trans*-retinol to esters and 11-*cis*-retinol in a roughly parallel manner. This suggested the possibility of a link between the formation of esters and the eventual isomerization reaction. One possible hypothesis concerning this mechanism involves a sequence of events whereby the all-*trans*-retinol is initially esterified by the membranes to form a retinyl ester in an obligate fashion before isomerization can ensue. A mechanism of this type would have the intrinsic advantage of identifying the energy source necessary to drive the isomerization reaction. This is because the process of a *trans*-retinyl ester directly to 11-*cis*-retinol would provide a mechanism by which the energy of hydrolysis of the retinyl ester could be used to drive the isomerization reaction. A mechanism of this type would involve the elimination of the ester group followed by isomerization around the C₁₁-C₁₂ single bond followed by the readdition of water to yield the final product.⁵⁹⁻⁶¹ A mechanism of this type makes two predictions. First, since the C₁₅ prochiral center becomes symmetrical at the intermediate state, it is possible that inversion of stereochemistry at C₁₅ accompanies isomerization. Second, the mechanism predicts the cleavage of the carbon-oxygen bond of vitamin A in the isomerization event. Both predictions were realized.^{60, 62, 63} Furthermore, this type of mechanism suggests that the polyene system of the vitamin should remain intact in order for isomerization to ensue, and in fact, this has been found.⁶⁴ Further, experiments also bear on this mechanism. We

have been able to solubilize the isomerase and ester synthetases in detergent and partially purify them, and have found that the two activities appear to copurify.⁶⁵ Finally, we have found that if the pigment epithelial membranes are specially treated by pre-irradiation and thorough washing to remove the endogenous retinoids, then added all-trans-retinyl esters can be processed to 11-cis-retinol.⁶⁶

The studies alluded to above are entirely consistent with the mechanism in which there is an obligate formation of a retinyl ester prior to the formation 11-cis-retinol, and that the energy source for this reaction is found in the "high energy" acyl ester bonds of the retinyl esters. It should be noted that retinyl esters are formed here by a transesterification of vitamin A with an endogenous lecithin (phosphatidylcholine) to produce a retinyl ester along with the 2-acyl-lysophospholipid lipid.^{65,66} Thus, the energy initially comes from the membranes in the form of the chemical energy of the acyl bonds of the phospholipid constituents. This suggests that under certain conditions, membrane phospholipids can be used to drive otherwise unfavorable reactions by group-transfer mechanisms such as those found in the biochemistry of ATP.

ACKNOWLEDGMENTS

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Chapter 2

THE MOLECULAR BASIS OF THE VISUAL CYCLE**C. D. B. Bridges****TABLE OF CONTENTS**

I.	Introduction	28
II.	The Visual Pigments	28
A.	Rhodopsins and Porphyrins	28
B.	Bleaching and Regeneration of Rhodopsin	30
III.	Retinol Isomerase	31
A.	Localization in Pigment Epithelium	32
B.	Occurrence in Different Vertebrate Species, Appearance during Development	34
IV.	Extracellular Transport of Retinoids Through the Interphotoreceptor Matrix During the Visual Cycle — Interstitial Retinol-Binding Protein (IRBP)	34
A.	Immunocytochemical Localization of IRBP	35
B.	Properties of Purified IRBP	36
C.	Primary Structure of Human IRBP	36
D.	IRBP mRNA, Chromosomal Localization and Restriction Fragment Length Polymorphism of the Human IRBP Gene	36
E.	Distribution in Different Vertebrate Species	39
F.	Biosynthesis of IRBP	39
G.	Expression of IRBP during Retinal Differentiation and in the Neoplastic Cells of Retinoblastoma.....	40
H.	IRBP in Mammalian Pineal	40
I.	Function of IRBP in the Visual Cycle	41
V.	Intracellular Retinoid Binding Proteins, IRBP, and the Visual Cycle	41
VI.	Conclusion	44
	References	44

I. INTRODUCTION

The initial event in vision is the absorption of light by visual pigments. These pigments are integral transmembrane proteins, where the protein (opsin) is coupled to an 11-*cis*-retinal prosthetic group by a protonated aldimine (Schiff base) bond. The reaction associated with light absorption always involves isomerization from 11-*cis* to all-*trans*, a process leading to "bleaching". In order to maintain visual sensitivity in the light (or to recover it subsequently in the dark), bleached visual pigment molecules are continuously regenerated by providing them with fresh supplies of 11-*cis*-retinal. This cyclic process of bleaching and regeneration, isomerization to all-*trans* and re-isomerization to 11-*cis*, operates through the interplay of a number of enzymes working in conjunction with intracellular and extracellular retinoid transport proteins. The present chapter will discuss recent developments that have advanced our understanding of this process.

II. THE VISUAL PIGMENTS

A. RHODOPSINS AND PORPHYROPSINS

Visual pigments are divisible into two large classes. The rhodopsins are based on 11-*cis*-retinal and the porphyropsins on 11-*cis*-3-dehydroretinal. The evolution of these two systems has been discussed at length.¹⁻¹¹ Porphyropsins typically have absorption spectra displaced to longer wavelengths, hence they are prevalent in organisms that spend their lives in freshwater environments where the ambient light is often reddish in color. One example is goldfish porphyropsin, which has its λ_{max} shifted to 522 nm when compared with frog and bovine rhodopsins, which have their λ_{max} near 500 nm. The retinas of many vertebrates, however, have mixtures of rhodopsin and porphyropsin, the balance between these two pigments depending on a variety of factors.

One factor appears to be stage in the organism's life cycle. In general, adult terrestrial amphibians have rhodopsins, but their larval forms possess porphyropsin.¹²⁻¹⁷ The switch from porphyropsin to rhodopsin often occurs abruptly, and coincides with emergence of the forelimbs at metamorphosis and the invasion of dry land. Apparently, the visual pigment change has adaptive significance, because adult amphibians that remain in the freshwater habitat where they were hatched, retain porphyropsin. The situation is by no means clear cut, however, because adult bullfrogs have porphyropsin in the dorsal one third of their retinas¹⁴ and toad tadpoles have rhodopsin.⁶

Other organisms that switch between rhodopsin and porphyropsin during their life cycle include lampreys, eels, and salmonid fishes.^{15,18-23} Ocean-caught Pacific salmon have retinas dominated by rhodopsin. As the fish move into tidewater there is a gradual change towards porphyropsin, until at the spawning site after the upstream migration is over porphyropsin often accounts for more than 90% of the visual pigment.¹⁹

A second set of factors is related to the environment, notably illumination. Ranid tadpoles kept in darkness slowly develop rhodopsin-dominated retinas but return rapidly to porphyropsin-dominated retinas when transferred to the light.²⁴⁻²⁶ Figure 1 illustrates an experiment in which tadpoles were kept in darkness for 38 d, at which time the proportion of porphyropsin was declining. Groups of these tadpoles were then exposed to two levels of yellow-green light. After a further 6 d, the porphyropsin in the dark tadpoles had declined still further, but it had increased dramatically in the brightly illuminated animals.

Many purely freshwater fishes also have mixtures of rhodopsin and porphyropsin, their proportions being highly variable (Figure 2).²⁷⁻³² As in tadpoles, the intensity and duration of light exposure is an important environmental factor and is probably the causative agent in the seasonal visual pigment changes observed in these fishes. Unlike tadpoles, darkness usually favors porphyropsin and light favors rhodopsin.

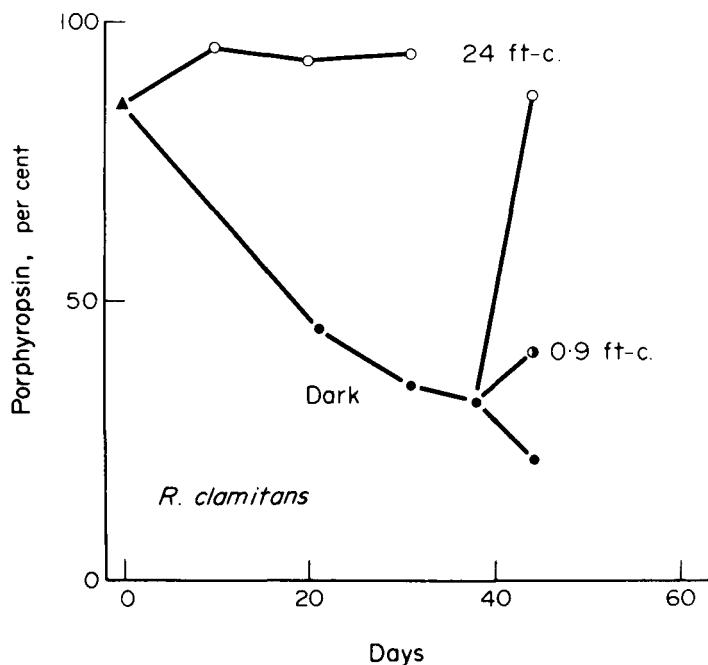


FIGURE 1. Effects of light and darkness on the visual pigments of tadpoles. *R. clamitans* tadpoles were kept in the dark (●), then exposed to two levels of yellow-green light (○, ◉); ▲, composition initially.²⁶

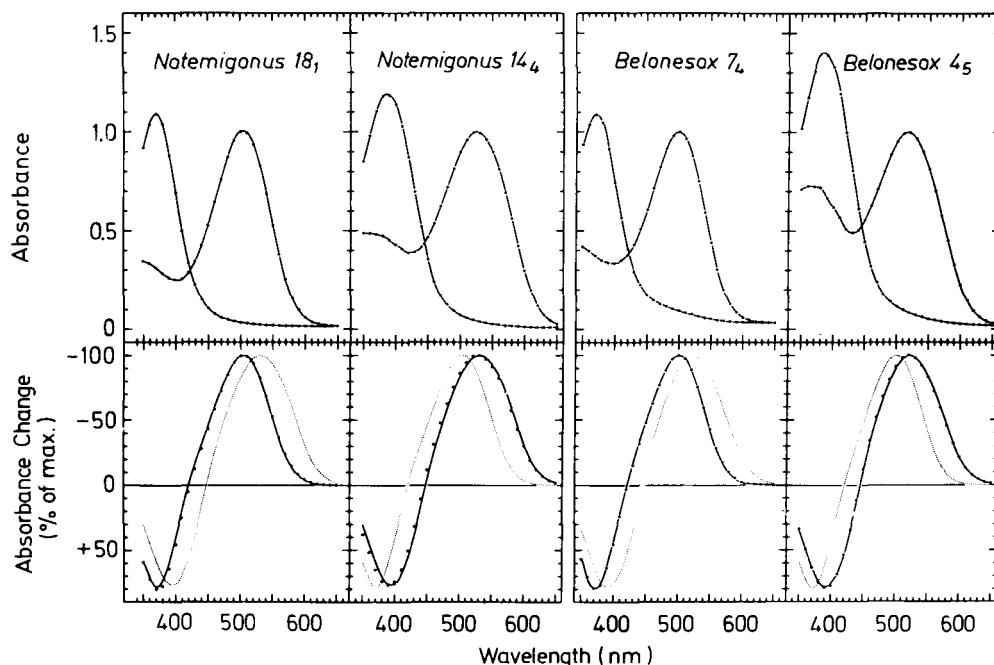


FIGURE 2. Extreme variations in the rhodopsin-porphyrins composition of retinas from individual fish — the freshwater cyprinid *Notemigonus* and the brackish-water poeciliid *Belonesox*. The difference spectra are plotted as points in the lower panels, the continuous curves representing the pure difference spectrum of the preponderating visual pigment and the dotted curves that of the pigment in lesser amount.³

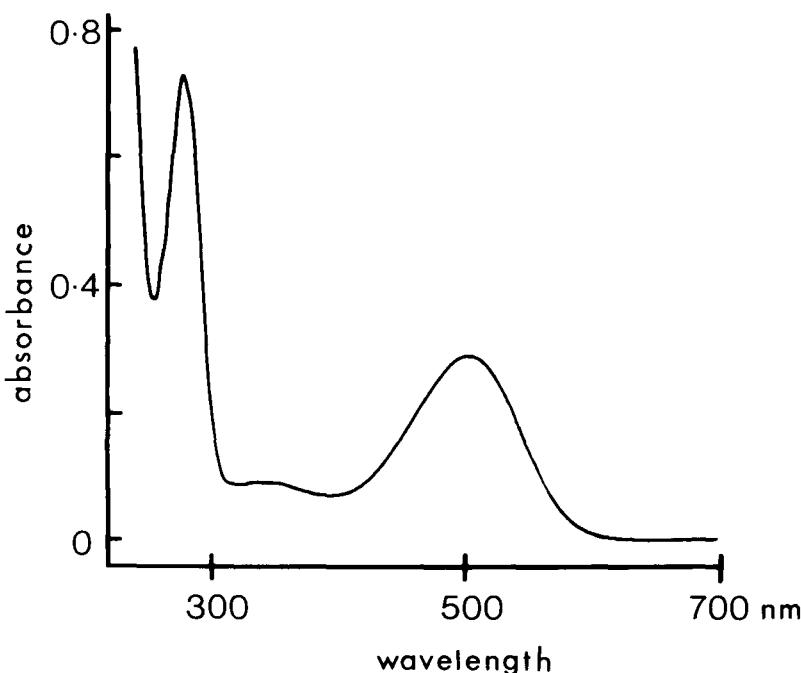


FIGURE 3. Absorption spectrum of frog rhodopsin. (*Rana pipiens*; solvent, 1% octyl glucoside in 50 mM sodium phosphate, pH 7.0; visual pigment concentration, 7.1 μ M).³⁵

Using opaque caps fitted over one eye, Bridges and Yoshikami²⁸ showed that the two eyes of individual fishes could be induced to change their visual pigments independently of one another, demonstrating that the system was not under central control. Subsequently, Bridges⁵ made similar observations with tadpoles that had one eye occluded. In the case of tadpoles, the effect appears to be mediated by light absorbed in the rod photoreceptors.²⁶

B. BLEACHING AND REGENERATION OF RHODOPSIN

Rhodopsin (usually from frog or bovine retinas) is better studied than porphyropsin, and will be the subject of the remainder of this chapter. The structure and function of rhodopsin has been recently reviewed.^{33,34} The absorption spectrum of frog rhodopsin³⁵ is shown in Figure 3. Even when prepared from individual animals, it is heterogeneous and can be resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) into two closely-spaced species with molecular masses of 34,700 and 37,000. Both are phosphorylated if the retinas are light-adapted. As shown in Figure 4, the low M_r band focused in two bands at pH 8.8 (band I) and 8.1 (band IIa). The high M_r band focused at pH 8.0 (band IIb; see also References 37, 38). In contrast, cattle rhodopsin is homogeneous from the standpoint of molecular weight and isoelectric point.³⁹

Rhodopsin bleaches with a quantum efficiency of about 0.67⁴⁰⁻⁴² and the 11-cis-retinal prosthetic group isomerizes to all-trans.⁴³ At physiological temperatures and pH, the bleached pigment is hydrolyzed to opsin and all-trans-retinal. Picosecond and low-temperature spectroscopy have identified a variety of intermediates that appear between the initial absorption of light and the appearance of free all-trans-retinal.⁴⁴⁻⁴⁶ In the human retina at 36°C, free all-trans-retinal has a half-life of 23 s⁴⁷ because it is reduced in the rod outer segments to all-trans-retinol by a membrane-bound alcohol dehydrogenase. Frog and bovine retinol dehydrogenases require NADPH as their cofactor⁴⁸⁻⁵⁰ and have specificity for the all-trans isomer.⁵⁰⁻⁵²

The all-trans-retinol formed in the rod outer segments then flows into the contiguous

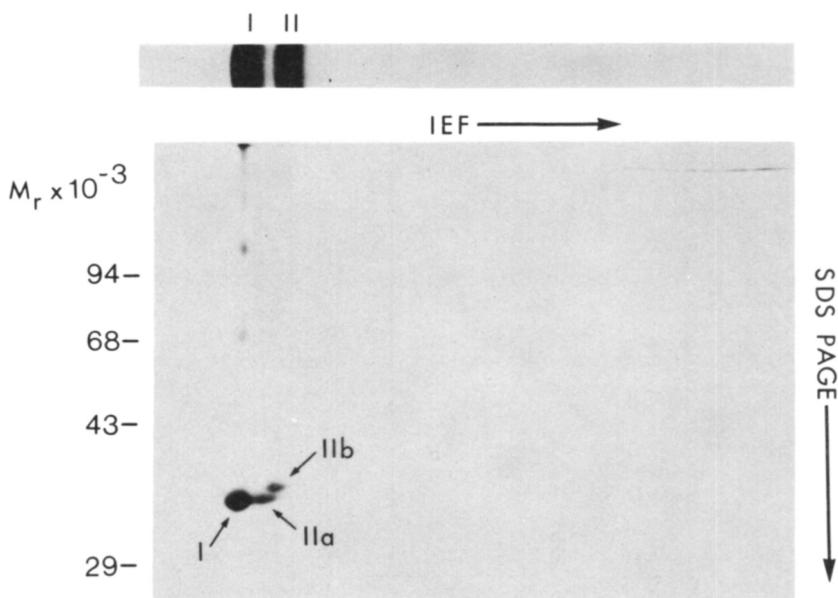


FIGURE 4. Two-dimensional gel electrophoresis demonstrating 3 forms of frog rhodopsin. An octyl glucoside extract containing 50 μ g of frog rhodopsin was subjected to isoelectric focusing (IEF) followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).³⁶

layer of cells known as the pigment epithelium,⁵³⁻⁵⁶ where most of it is esterified.^{54,57-60} The major esters are palmitate and stearate, the former heavily predominant in most animals investigated⁶¹⁻⁶³ (Figure 5). In order to support rhodopsin regeneration, the retinoid lost to the pigment epithelium must be restored to the photoreceptor cells. This process is manifested by recovery of rhodopsin in the dark, or maintenance of a steady-state level in the light. A number of steps involving the interplay of retinoid-binding proteins and enzymes, many with well-defined stereochemically restricted requirements, have been identified. One step entails mobilization of retinyl esters. While there have been many studies on the synthesis by pigment epithelium of retinyl esters, information on their hydrolysis has been lacking until recently. Blaner et al.⁶⁴ found that homogenates of human pigment epithelium cells hydrolyzed 11-cis-retinyl ester at a rate that was approximately 20 times greater than that for the all-trans. These results clearly indicate that there are two distinct activities for the hydrolysis of 11-cis- and all-trans-retinyl esters.

The pigment epithelium hydrolase with specificity for 11-cis-retinyl esters may act in concert with an 11-cis isomer-specific oxidoreductase in bovine pigment epithelium.^{51,52} Together, these two enzymes have the potential to convert stored 11-cis-retinyl ester first to 11-cis-retinol and then to 11-cis-retinal. The question of how the 11-cis isomer is generated from all-trans will be addressed in the next section.

III. RETINOL ISOMERASE

Although 30 years have elapsed since it was shown that 11-cis-retinal isomerized to all-trans when rhodopsin was bleached,⁴³ until recently virtually nothing was understood about the reverse process that generates 11-cis-retinal for rhodopsin regeneration. A "retinal isomerase" originally reported by Hubbard⁶⁵ appears to be a protonated Schiff base composed of all-trans-retinal and phosphatidylethanolamine.⁶⁶ On exposure to light, this compound isomerized to a mixture of isomers that included 11-cis. However, unlike the system in many invertebrates, the vertebrate visual cycle does not rely on photoisomerization to generate 11-cis isomer. This is evident from experiments showing that exposure to potentially

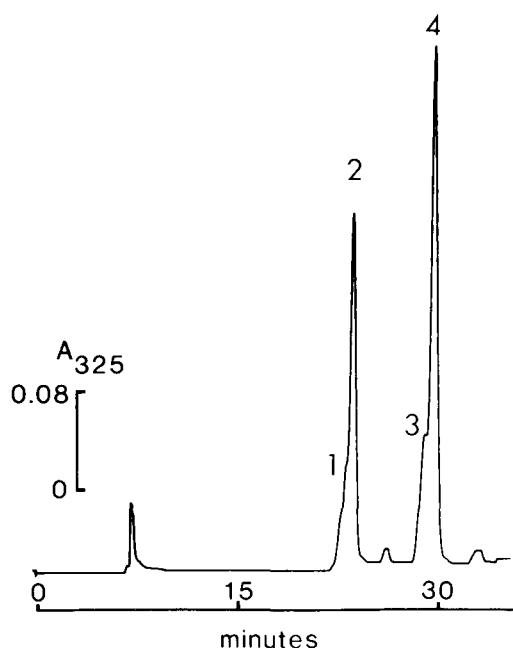


FIGURE 5. High-performance liquid chromatography of retinyl esters stored in the pigment epithelium of human eyes. Peaks 2,4 — 11-cis- and all-trans-retinyl palmitates: the shoulders (1,3) correspond to the stearates. The unlabeled peak following all-trans-retinyl palmitate is all-trans retinyl oleate.⁶²

isomerizing light does not influence the subsequent course of dark adaptation.⁶⁷⁻⁶⁹ Recent studies^{69a,70,71} have now demonstrated that vertebrates possess an eye-specific, membrane-bound enzyme that converts all-trans- to 11-cis-retinol in the dark, and therefore fulfills the primary requirement for an isomerase that plays a central role in dark-adaptation.

A. LOCALIZATION IN PIGMENT EPITHELIUM

Although a large body of negative evidence had suggested otherwise, there is now basic agreement that retinol isomerase is concentrated in the pigment epithelium. Experiments from our own laboratory on which this conclusion is based entailed incubating tissue homogenates with radiolabeled all-trans-retinol, then extracting the mixture with ethanol/hexane and analyzing the various retinoids and their isomers by high-performance liquid chromatography. A typical set of data from the combined retina and pigmented layers of light-adapted frogs is shown in Figure 6. The radioactivity profile in panel C shows a prominent peak corresponding to 11-cis-retinol and a smaller peak due to the 13-cis-isomer. The latter is observed in medium that does not contain tissue homogenate, and appears to be generated thermally. The truncated peak corresponds to the unchanged all-trans-retinol substrate. The isomerizing reaction that generates 11-cis-retinol is enzymatic because it is abolished by preheating the enzyme source (panel D), by phenylmethylsulfonyl fluoride and by digestion with trypsin. The enzyme appears to be membrane-bound. It is not detectable in liver (panel B) or brain homogenates. When retinas from dark-adapted frogs are carefully separated from the underlying pigmented layers, virtually all of the retinol isomerase activity is found in the pigment epithelium (see Table 1; no significant difference between light- and dark-adapted tissues can be detected). At present, the activity observed is not high, and better conditions need to be found before an *in vitro* system capable of regenerating rhodopsin is developed.

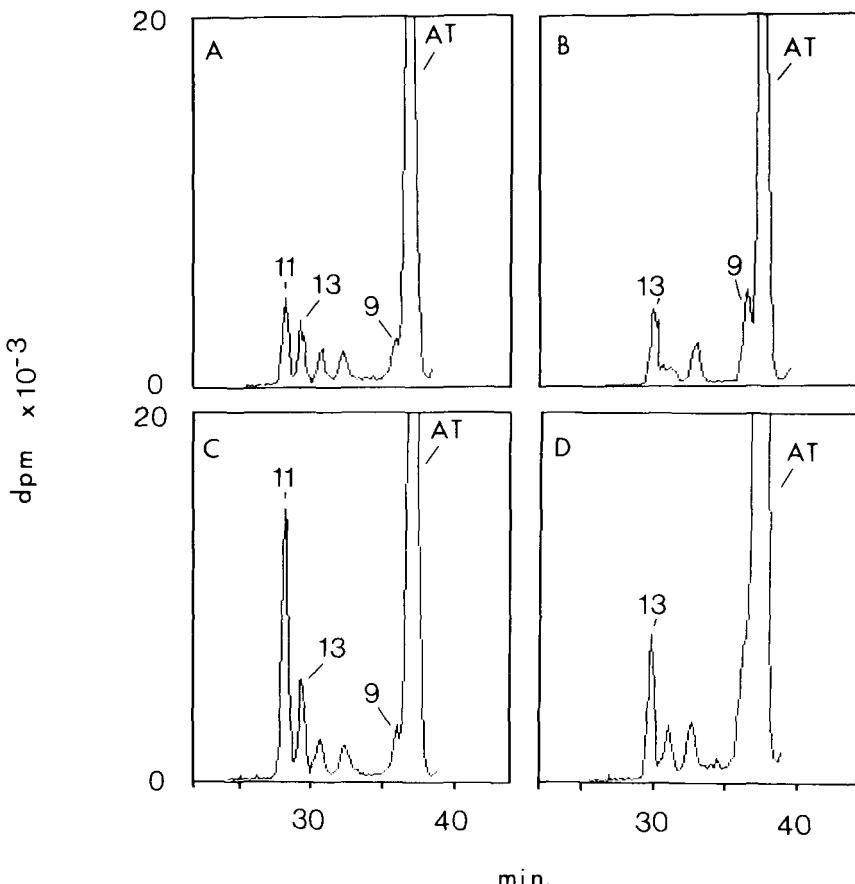


FIGURE 6. Abolition of retinol isomerase activity by heat, absence of retinol isomerase in liver. 700 g supernatants (about 0.8 mg of protein per milliliter) were incubated with 27 pmol all-trans-[11,12-³H]retinol. Except for panel A, which was for 1.5 h, all incubations were for 3 h. A,C,D — combined retina, pigment epithelium and choroid from dark-adapted frogs (in D, the 700 g supernatant had been heated at 100°C for 5 min and cooled to room temperature before addition of substrate); B — frog liver. No 11-cis-retinol peak is evident in B and D. Note that the 9-cis-retinol peak is more prominent in the liver preparation.⁷⁰

TABLE 1
Distribution of Retinol Isomerase
Activity Between Pigment
Epithelium and Retina in
Vertebrates

Species	% Retinol isomerase activity in pigment epithelium
Human	93.8
Cattle	96.4
Goldfish	81.8
Turtle	87.9
Chicken	94.3
Frog	95.3

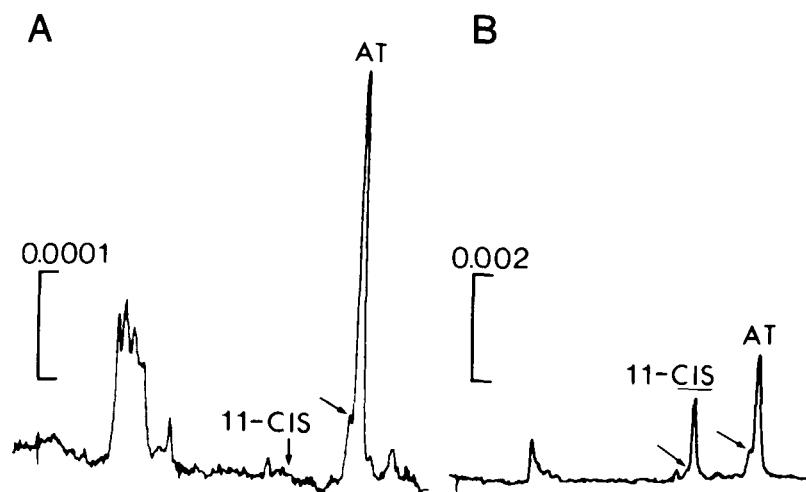


FIGURE 7. Appearance of 11-cis isomer during development in the mouse. Retinyl esters extracted from the eyes of dark-adapted mice were subjected to high-performance liquid chromatography on normal-phase columns. Note absence of 11-cis-retinyl ester in A (P4, 0.9 pmol/eye of all-trans-retinyl palmitate). A substantial amount of 11-cis-retinyl palmitate is present in B (P16, 0.6 nmol/eye of all-trans- and 11-cis-retinyl palmitates). AT, all-trans-isomer (major peak = palmitate); slanted arrows indicate the position of all-trans- and 11-cis-retinyl stearates.⁷²

B. OCCURRENCE IN DIFFERENT VERTEBRATE SPECIES, APPEARANCE DURING DEVELOPMENT

In addition to one amphibian (frog), we have demonstrated retinol isomerase activity in the ocular tissues of representatives of the mammals, birds, fish, and reptiles, showing that it is probably universally distributed throughout the vertebrates. Regarding the invertebrates, we have been unable to detect the enzyme in cephalopods. As Table 1 summarizes, retinol isomerase is concentrated in the pigment epithelium in every case, suggesting that the same basic visual cycle is present in each species.

Although the activity of retinol isomerase has not been directly determined during development, spectroscopically identifiable rhodopsin is first detected in mice at postnatal day 6-7 (P6-7), when rod outer segment discs are being formed.⁷² Although all-trans-retinyl esters are present at P4, the appearance of 11-cis-retinyl esters in the pigment epithelium coincides with the appearance of rhodopsin in the retina. Their proportion rises to almost 50% of the total at P16 (Figure 7).

Whether the variable isomerase activity observed in isolated retinas is due to contamination by pigment epithelium or whether it results from an endogenous enzyme has not been established. The localization of retinol isomerase in the pigment epithelium, however, would explain the essential role of this layer in rhodopsin regeneration^{73,74} and would account for the need to transfer all-trans-retinol from bleached rhodopsin in the photoreceptors to the pigment epithelium during light-adaptation.⁵⁴⁻⁵⁶ In order to pass between these two layers, retinol must traverse the interphotoreceptor matrix. In the next section, we shall discuss the properties of the protein that transports vitamin A compounds through this medium.

IV. EXTRACELLULAR TRANSPORT OF RETINOIDS THROUGH THE INTERPHOTORECEPTOR MATRIX DURING THE VISUAL CYCLE — INTERSTITIAL RETINOL-BINDING PROTEIN (IRBP)

Retinoids are insoluble and often susceptible to oxidative degradation. When they are not stored as retinyl esters, they usually occur attached to a variety of binding proteins (see

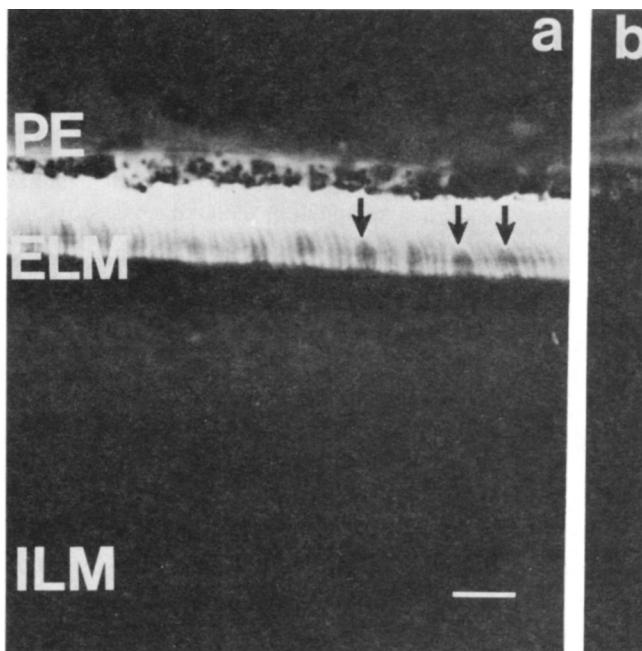


FIGURE 8. Immunocytochemical localization of IRBP in the human retina using an FITC-labeled second antibody. IRBP immunoreactivity is clearly localized in the area between the pigment epithelium (RPE) and external limiting membrane (ELM). The apparent staining of the RPE seems to be attributable mainly to nonspecific or to autofluorescence, because it is also present in the control section (b), which had been treated with pre-immune serum. The specific staining between the RPE and ELM appears to be outside of, rather than within, the photoreceptor cells. It is especially obvious that the cones themselves are not stained (some are indicated by the arrows). Although less evident, the rods, which are thinner, are also not stained. ILM, internal limiting membrane; bar = 30 μ M.⁹⁵

also next section).^{46,57,75-79} Serum retinol-binding protein (RBP) is one of the first known examples. It transports retinol from its storage depots in the liver to a variety of target tissues that include the pigment epithelium. Pigment epithelium cells have been shown to have receptors for RBP on their basal plasma membranes.^{80,81}

Until recently, RBP was the only example of a protein that transported retinoids extracellularly. In 1982, there appeared several reports demonstrating the presence in interphotoreceptor matrix of a high-molecular weight glycoprotein that carried retinoids of endogenous origin.⁸²⁻⁸⁴ This protein is known as IRBP, an acronym for interphotoreceptor matrix or interstitial retinol-binding protein.^{83,84} Some workers now refer to IRBP as interphotoreceptor retinoid-binding protein.⁸⁵⁻⁸⁸ Although it sediments at 5.7 to 5.8S,^{83,85} IRBP is probably identical with a 7-8S retinol-binding entity observed on sucrose gradients by Wiggert et al.⁸⁹ IRBP is the most abundant protein constituent of the interphotoreceptor matrix, and its discovery has prompted an enormous surge of interest in this area (e.g., Reference 90). Although it will not be discussed further, it should be noted that purpurin is also of particular interest in this connection. Purpurin is a neural retina adhesion and survival molecule that is also an extracellular retinol-binding protein with amino acid sequence homology to RBP.⁹¹

A. IMMUNOCYTOCHEMICAL LOCALIZATION OF IRBP

Using antibodies directed against the purified bovine, frog, and monkey proteins, IRBP has been immunohistochemically demonstrated at the light-microscope and electron microscope level in the interphotoreceptor matrix of cattle, human (Figure 8), frog, rat, mouse,

ground squirrel and monkey.^{57,92-106} No study has compellingly demonstrated the presence of IRBP intracellularly, except in cases where it appears that the protein has gained entry into damaged cells.^{102,103}

B. PROPERTIES OF PURIFIED IRBP

IRBP has been purified and characterized from human, bovine, monkey, rat, and frog eyes.^{83-85,88,94,95,107-109} Bovine IRBP has an apparent molecular mass of 240,000 to 280,000 on gel-filtration columns compared with 140,000 to 144,000 on SDS-polyacrylamide gels (Figure 9). Sedimentation equilibrium analysis suggests a true M_r of 132,000 to 133,000.^{85,88} These data are reconciled by the conclusion that bovine IRBP (and probably human, rat, monkey, and frog IRBPs) is an elongated molecule. It consists of a single polypeptide chain that binds two molecules of 11-cis- or all-trans-retinol with a K_D of about $10^{-6}M$ and an $E_{1cm}^{1\%}$ (all-trans-retinol) of 7.88 at 330 nm.^{88,108} In addition to retinol, IRBP binds exogenous retinal, cholesterol, alpha-tocopherol, and all-trans-retinoic acid, all of which can be displaced by all-trans-retinol.¹⁰⁸ The affinity of alpha-tocopherol for IRBP is at least several orders of magnitude less than that of all-trans-retinol. Recent studies by Alvarez et al.¹¹⁰ have been unable to detect any traces of alpha-tocopherol bound to IRBP in bovine interphotoreceptor matrix, suggesting that it does not transport this compound under physiological conditions. There is evidence, however, that IRBP may function in fatty acid transport.¹¹¹ The carbohydrate content of bovine IRBP is 8.4%.¹⁰⁸ Approximately 40% of the N-linked oligosaccharide chains are monosialylated hybrid-type; the remainder are di-, tri-, and tetrasialylated complex biantennary structures.^{108,112,113} Different degrees of sialylation may be responsible for four isoelectric forms with isoelectric points ranging from 4.4 to 4.8. The function of the carbohydrate (if any) has not been elucidated. It has no detectable influence on the shape of the molecule, its secretion, or its retinol-binding capacity.^{108,112,114,115} It may influence its lifetime in the interphotoreceptor matrix,¹¹² but this has not been investigated.

C. PRIMARY STRUCTURE OF HUMAN IRBP

We have obtained a 3.77 kb cDNA sequence from three overlapping clones and used it to deduce the complete amino acid sequence of human IRBP. Most of this sequence can be aligned with the amino acid sequences of a series of peptides purified from tryptic digests of bovine IRBP. The degree of homology between human and bovine IRBP is 86 to 88%. The deduced amino acid sequence suggests that human IRBP, like the majority of secreted proteins, has a hydrophobic signal peptide. This peptide consists of 16 amino acids, and is cleaved between an alanine and a glycine residue. Subsequently, an additional two or five amino acids are excised from some IRBP molecules, yielding mature proteins with three N-terminal sequences.¹¹⁶ Rabbit and rhesus IRBPs each display two N-terminal sequences, presumably the result of similar processing.^{116,117} In contrast, the IRBPs from cattle, sheep, pig, guinea pig, bush baby, hamster, and frog appear to be homogeneous, although some have an extra N-terminal three to six amino acid segment.¹¹⁶

The full amino acid sequence of human IRBP suggests that the molecule arose by a series of internal gene duplications that yielded four homologous domains.^{118,119} It is possible that these domains fold and interact to form two retinol-binding sites. An analogous situation is observed in serum retinol-binding protein, which contains two homologous regions that contribute to its one retinol-binding site and which may also have arisen by partial gene duplication.¹²⁰⁻¹²²

D. IRBP mRNA, CHROMOSOMAL LOCALIZATION AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM OF THE HUMAN IRBP GENE

Although the predicted size of an RNA coding for IRBP is about 3.6 kb, a cloned 2184 bp cDNA (H-4 IRBP) for human IRBP was found to hybridize to a 5.2 kb polyA RNA from

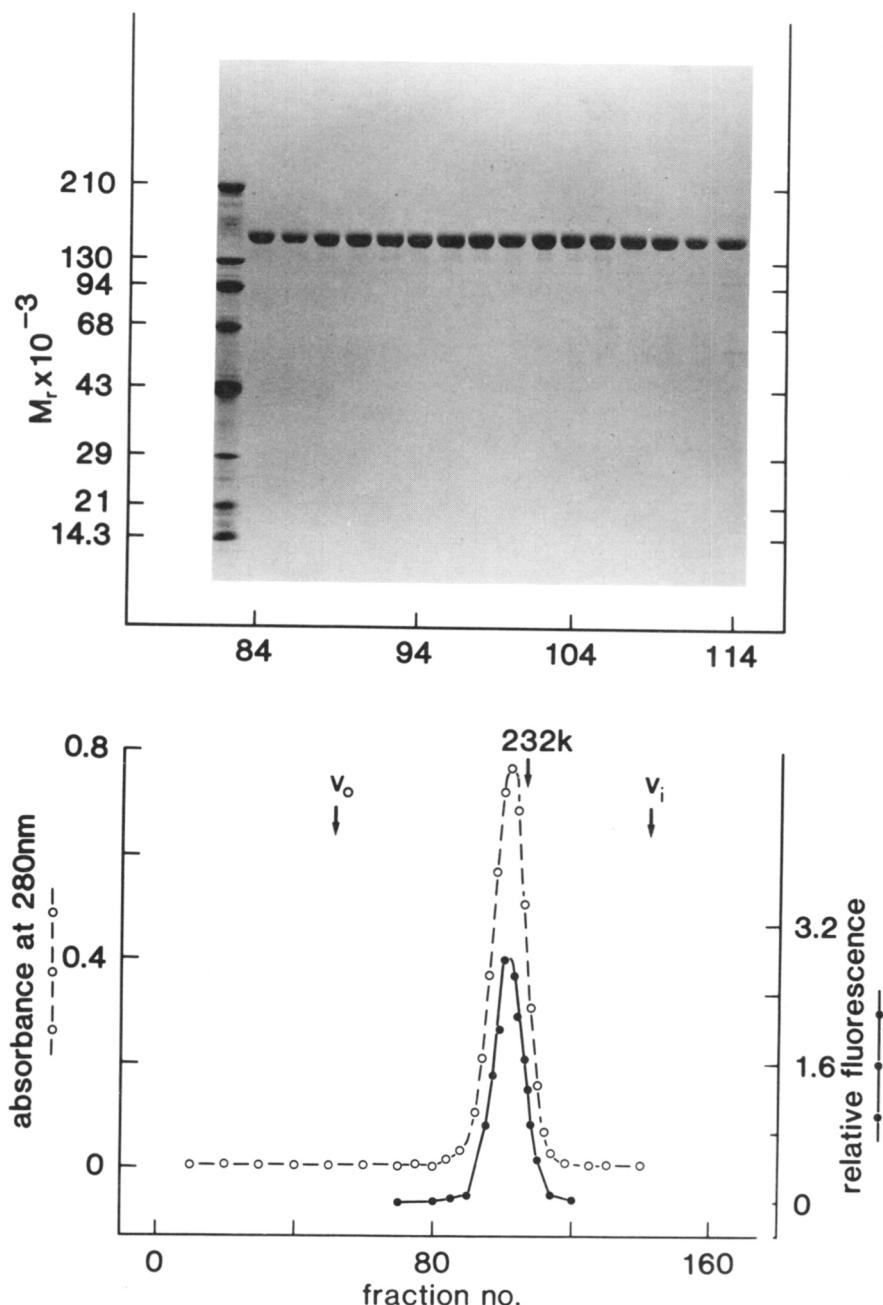


FIGURE 9. Gel-filtration chromatography (lower panel) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (upper panel) of bovine IRPB. Fluorescence (●) and A_{280} (○) are plotted in the lower panel. The marker at 232K (arrow) corresponds to the elution position of catalase. See Fong et al.¹⁰⁸ for further details.

human retina and a 6.5 kb polyA RNA from bovine retina (Figure 10 top; Reference 119). In cattle, it has been demonstrated that IRBP is not derived from a correspondingly larger precursor molecule (Figure 10 bottom), and it appears that the large size of IRBP mRNA is due to an extensive 3'-untranslated segment.¹²³

The human chromosome containing the IRBP sequences was identified by probing

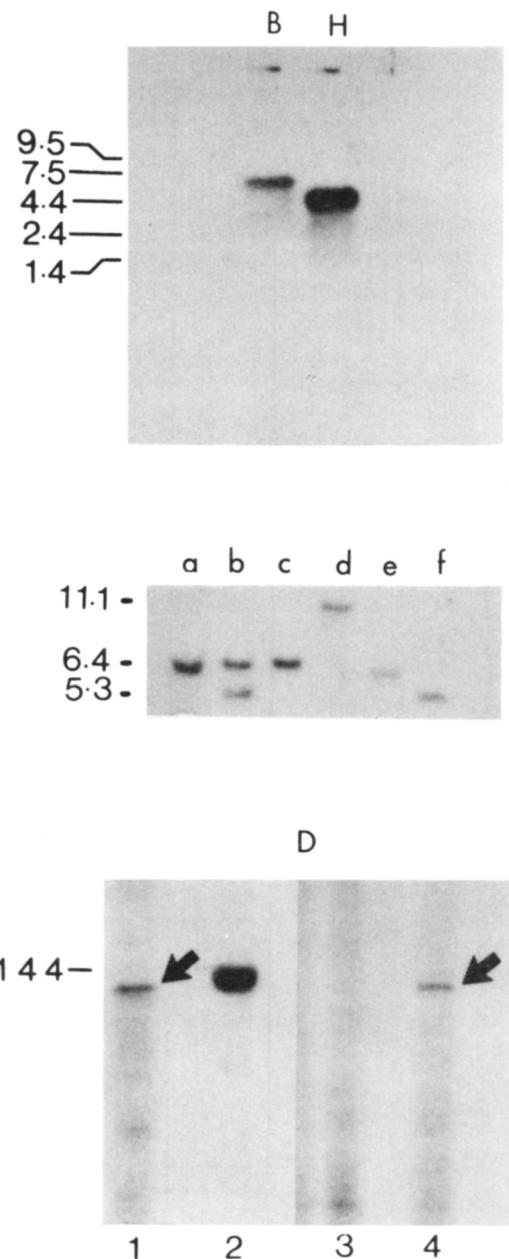


FIGURE 10. Northern blots of human and bovine retina polyadenylated RNA probed with a human IRBP cDNA probe, H-4 IRBP (upper panel); Southern blots of Bgl I-digested DNA from rodent, human and hybrid cells probed with H-4 IRBP (center panel); *in vitro* translation of bovine retina polyadenylated RNA (lower panel). Top panel: B — bovine retina polyA RNA; H — human retina polyA RNA. RNA size markers in kilobases. Center panel: a,b,c — DNA from hamster/human somatic cell hybrids containing different human chromosomes; d — mouse DNA; e — hamster DNA; f — human DNA. Note that a diagnostic human fragment is present only in lanes b and f. Lower panel: lanes 1,4 — immunoabsorption of the rabbit reticulocyte lysate mixture with rabbit antibovine IRBP IgG and protein A-Sepharose; lane 3 — IgG from preimmune serum; lane 2 — metabolically-labeled bovine IRBP. The immunoprecipitated protein formed by the *in vitro* translation system is indicated by the arrows in lanes 1 and 4. The size of this protein is actually slightly less than mature IRBP, the difference being due to lack of glycosylation by the rabbit reticulocyte system.^{119,123}

Southern blots of restriction enzyme-digested DNA from 29 rodent-human somatic cell hybrids.¹¹⁹ Digestion with Bgl I yielded diagnostic fragments at 11.1, 6.4, and 5.3 kb for mouse, Chinese hamster, and human DNAs, respectively, as shown in the center panel of Figure 10 in lanes d, e, and f. The results from three hybrid cell lines are shown in lanes a, b, and c. The 5.3-kb human signal is evident only in lane b. The frequency of discordancy between this signal and each of the 24 human chromosomes in the panel of hybrids enabled us to map the structural gene for IRBP to chromosome 10. *In situ* hybridization suggested a regional localization near the centromere.¹¹⁹ The possible linkage of the IRBP gene to autosomal dominant retinitis pigmentosa (which has not been assigned to a particular chromosome) and to various diseases with known loci on chromosome 10 has recently been made possible by the discovery of a 2-allele Bgl II RFLP recognized by H-4 IRBP.¹²⁴ A recent study has already reported tight linkage between the multiple endocrine neoplasia type 2a locus and the IRBP gene.^{124a}

E. DISTRIBUTION OF IRBP IN DIFFERENT VERTEBRATE SPECIES

As might be expected from the localization of retinol isomerase in the pigment epithelium of all vertebrate species examined — hence necessitating the transfer of all-*trans*-retinol to this tissue during the visual cycle — IRBP occurs in the interphotoreceptor matrix of animals from all the major vertebrate classes. These include representatives of the mammals, birds, reptiles, bony fishes, cartilaginous fishes, and amphibia.^{94,125,126} The method entails electrophoretically transferring the interphotoreceptor matrix proteins from SDS-polyacrylamide gels to nitrocellulose sheets, then probing the sheets with rabbit antiovine or antifrog IRBP immunoglobulins followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. An immunoreactive protein is observed in all cases. In most animals, immunoreactivity is observed in a protein with molecular mass of $134,200 \pm 8,600$ ($n = 17$). In the bony fish, however, the molecular mass is about half this value, i.e., $67,600 \pm 2,700$ ($n = 8$). This observation led Fong et al.¹²⁶ to suggest that there had been at least one gene duplication in the evolution of IRBP. Although it is now likely that this is the result of post-translational processing, the idea has been vindicated by the observation that there are four homologous segments in the full amino acid sequence of human IRBP.

F. BIOSYNTHESIS OF IRBP

As demonstrated in experiments where isolated retinas from cattle, rats, mice, humans, monkeys, and frogs have been incubated with labeled sugar (fucose, glucosamine) or amino acid precursors, IRBP is synthesized and secreted by the retina.^{72,93-95,108,109,112,114} In each case, labeled protein with an electrophoretic mobility identical with that of IRBP and immunoprecipitable with rabbit antibodies against bovine or frog IRBP appeared in the incubation medium. Secretion was not prevented by tunicamycin,^{108,112,114} an antibiotic that inhibits the assembly of oligosaccharides linked by *N*-glycosidic bonds to proteins. Secretion is also unaffected by agents such as castanospermine and swainsonine, which interfere with oligosaccharide processing.¹¹² In similar experiments, no biosynthesis or secretion of IRBP by the pigment epithelium cells could be detected.¹⁰⁸

The first evidence that the photoreceptors were the source of IRBP was provided by Gonzalez-Fernandez et al.,^{93,127} and is illustrated in Figure 11. Royal College of Surgeons retinal-dystrophic rats progressively lose their photoreceptors during postnatal development, without any abnormalities being evident in other retinal cells. Retinas from 50-day-old dystrophic rats and their age-matched congenic controls were incubated with [³H]leucine. After 4 h, media and retinas were collected separately. The retinas were homogenized, and the cytosol collected after centrifuging. Media and cytosol samples were subjected to SDS-PAGE fluorography.⁹³ In Figure 11, it is clear that labeled IRBP has been secreted into the medium by the control retinas (lane 4) but not by the retinas that had lost their photoreceptors

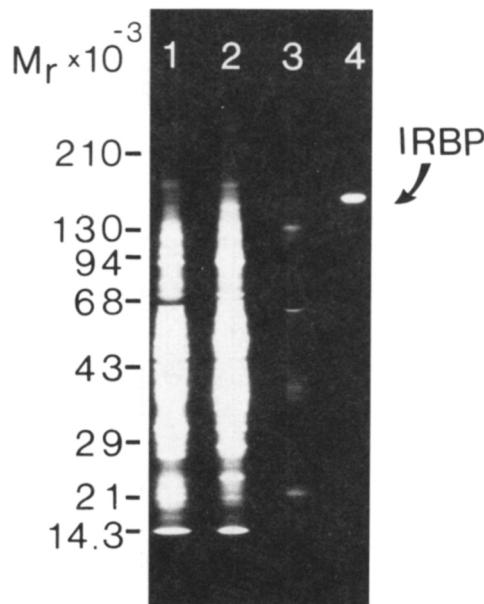


FIGURE 11. Evidence that retinal photoreceptors synthesize and secrete IRBP. Lanes 1,2 — fluorograms of cytosol fractions from RCS rat dystrophic and control retinas, respectively. Lanes 3,4 — fluorograms of the corresponding media. Note that IRBP is only secreted into the medium by retinas that possess photoreceptors (lane 4), and is absent from the medium in which retinas lacking photoreceptors had been incubated (lane 3).^{93,142}

(lane 3). Similar results have been obtained with the retinas of *rd* mice.⁷² The conclusion that the photoreceptors synthesize IRBP has been confirmed by *in vitro* incubation experiments⁹⁸ and by *in situ* hybridization, where mRNA transcripts for IRBP have been demonstrated in bovine retinal photoreceptors.¹²⁸ Thus these cells are involved in the biosynthesis of two glycoproteins that play a central role in the biochemistry of the visual process, namely rhodopsin and IRBP.

G. EXPRESSION OF IRBP DURING RETINAL DIFFERENTIATION AND IN THE NEOPLASTIC CELLS OF RETINOBLASTOMA

In comprehensive studies carried out with mice, it has been demonstrated that expression of IRBP by the photoreceptor cells coincides with inner segment differentiation.⁷² Thus IRBP was only detectable at embryonic day 17, which corresponds to a stage at which rudimentary inner segments can be observed.

In retinoblastoma, however, the situation is different. Retinoblastoma is a malignant tumor of the infant retina. With the exception of the Y-79 cultured cell line, IRBP mRNA transcripts have been demonstrated in the undifferentiated transformed cells of primary tumors and of all other retinoblastoma cultured cell lines examined.¹²⁹ These cells are also capable of synthesizing and secreting IRBP, even though they do not display evidence of morphological differentiation.¹³⁰⁻¹³²

H. IRBP IN MAMMALIAN PINEAL

Unlike the situation in submammalian vertebrates¹³³ the presence of functional photoreceptors in the adult mammalian pineal has not been demonstrated although in young rats

the pineals may be capable of photoreception.¹³⁴ However, it is now known that the vertebrate pineal contains a range of proteins previously found only in retinal photoreceptors. In mammals, for example, the pineal contains rhodopsin kinase, arrestin (S-antigen, 48k protein), and opsin,¹³⁵⁻¹⁴¹ implying that the organ shares a common photochemistry with the retina. Recently, it has been demonstrated that IRBP and cellular retinal-binding protein (CRAIBP) occur in the mammalian pineal organ,¹⁴²⁻¹⁴⁴ their presence raising the possibility that they are associated with photochemical events dependent on vitamin A, including its 11-cis isomer, which is specifically bound to CRAIBP (see Section V).

While IRBP provides yet another biochemical link between pinealocytes and retinal photoreceptors, the significance of CRAIBP, which is not a photoreceptor protein, is an interesting question (see also Section V). In the eye, it is found in the Müller (glial) and pigment epithelium cells.⁹² Consequently, pineal CRAIBP may originate from a subpopulation of pinealocytes derived from these cells.

I. FUNCTION OF IRBP IN THE VISUAL CYCLE

Cattle interphotoreceptor matrix contains IRBP at a concentration of 30 to 100 μm . Each eye contains 3.0 to 3.3 nmol of IRBP ($0.12 \text{ to } 0.13 \text{ nmol/cm}^2 = 17.2 \text{ to } 19.0 \mu\text{g/cm}^2$), an amount that can bind 6 to 7 nmol of retinol, or about 40% of the rhodopsin present.¹⁰⁸ The first evidence that IRBP was important in the visual cycle was the finding that in bovine eyes it carried retinoids of endogenous origin.⁸²⁻⁸⁴ The three major retinoids reported by different laboratories are 11-cis-retinal, 11-cis-retinol and all-trans-retinol.^{83,88,108,115,125,145} There is general agreement that when bovine eyes are bleached the amount of all-trans-retinol bound to IRBP increases by a factor of 5 to 7 times. The degree of binding site saturation can be as high as 30%. Bunt-Milam et al.¹⁴⁵ were unable to demonstrate delivery of retinol to the pigment epithelium, however. Adler and Evans¹¹⁵ found that the amount of 11-cis-retinal was less in bleached eyes.

Unfortunately, bovine eyes obtained from the slaughterhouse are poorly suited for physiological experiments on light- and dark-adaptation. On the other hand, frogs have a well-studied visual cycle.^{53,56} In terms of unit area, the amount of IRBP in frog eyes is similar to cattle, and averages $0.16 \pm 0.08 \text{ nmol/cm}^2$ ($n = 14$). However, because there is relatively much more rhodopsin in frog eyes, IRBP averages only 3.2% of the rhodopsin present in frogs, while IRBP averages 18.6% of the rhodopsin present in cattle. In the eyes of living dark-adapted frogs, the predominant retinoids bound to IRBP are 11-cis-retinal and 11-cis-retinol (Figure 12, DA).¹⁴⁶ A minor proportion of all-trans-retinol is also present. Between 0.5 h and 1 h of light adaptation, which bleached nearly all the rhodopsin, there is a sharp drop in the amount of 11-cis-retinal. The amount of all-trans-retinol increases during the initial 0.5 to 1 h and drops thereafter, presumably because it has passed into the pigment epithelium. The amount of 11-cis-retinol declines slightly. These findings support the suggestion that IRBP transports retinol from the rod outer segments to the pigment epithelium. Further, they provide evidence that IRBP delivers 11-cis-retinal to the rod outer segments to support rhodopsin regeneration. At present, we cannot easily account for the occurrence of 11-cis-retinol as an endogenous ligand. It remains to be determined whether it is in transit from the pigment epithelium to the retina.

V. INTRACELLULAR RETINOID BINDING PROTEINS, IRBP, AND THE VISUAL CYCLE

In the eye, retinoids of importance to the visual cycle are bound to two cytosolic proteins, i.e., CRBP, which carries all-trans-retinol, and CRAIBP, which carries 11-cis-retinal and 11-cis-retinol.⁷⁸ Both CRAIBP and CRBP have been demonstrated in the pigment epithelium and in the Muller cells of the retina.^{57,77,92,147} CRAIBP occurs only in the eye and the pineal

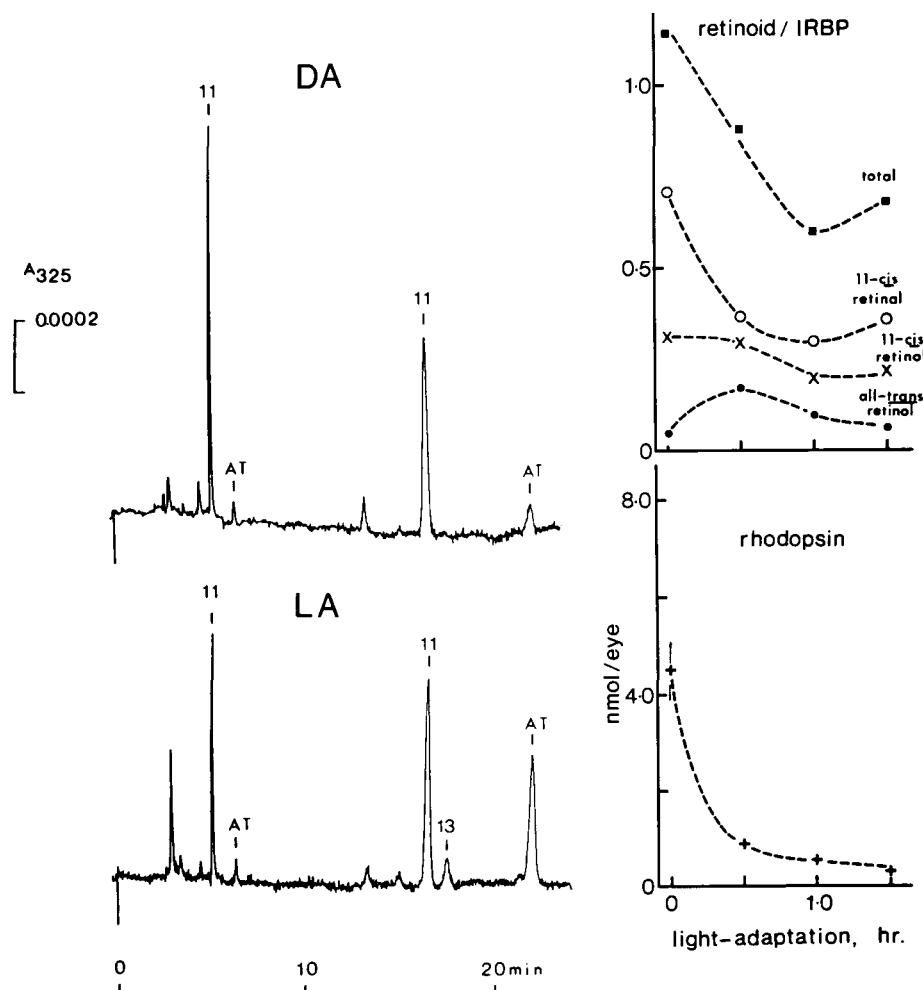


FIGURE 12. Endogenous retinoids bound to IRBP in light- and dark-adapted frogs. High-performance liquid chromatography profiles are shown on the left. The early-eluting peaks labeled "11" and "AT" are 11-cis- and all-trans-retinal, the later-eluting peaks are 11-cis- and all-trans-retinol. DA — dark-adapted frogs; LA — frogs light-adapted for 1 h.¹⁴⁶

organ (Section IV.H), but CRBP occurs in most tissues of the body.⁷⁵ It has been shown that all-trans-retinol bound to CRBP and 11-cis-retinol bound to CRAIBP serve as excellent substrates for the membrane-bound ester synthase in the pigment epithelium. The enzyme removes these ligands from their corresponding binding proteins.⁵⁷ Additionally, 11-cis-retinal bound to CRAIBP is reduced easily to 11-cis-retinol by a dehydrogenase in bovine pigment epithelium; in this instance the ligand remains attached to the protein.¹⁴⁸ The reverse reaction is also feasible.

In the pigment epithelium, CRAIBP contains almost exclusively 11-cis-retinal. CRBP saturated with all-trans-retinol is also present.⁷⁸ Although the isomerase system is functional in the absence of CRAIBP and CRBP,⁷⁰ it seems unlikely that under physiological conditions these proteins are important carriers of its substrate and product. A possible scheme in which CRBP and CRAIBP interact with established enzyme systems in the pigment epithelium is shown in Figure 13.

Many questions remain. The binding constants favor retinol transfer from IRBP to CRBP or CRAIBP, but not the reverse process. The corresponding binding constant for 11-cis-

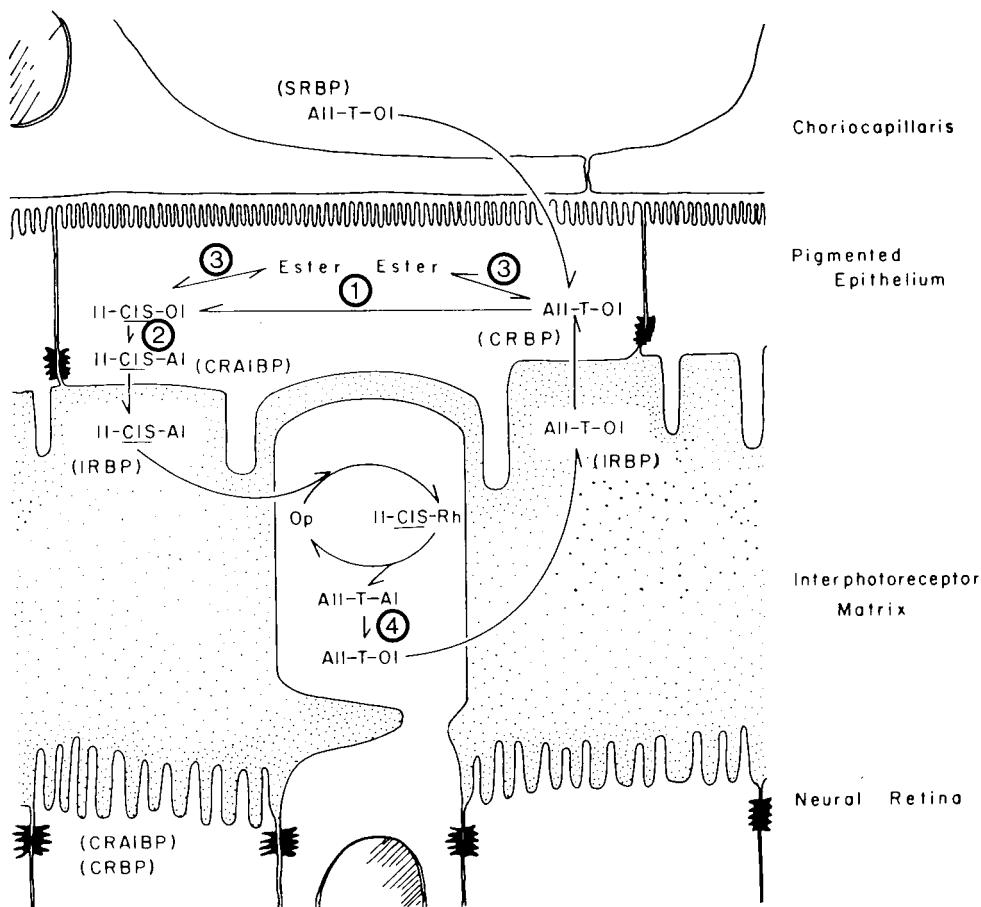


FIGURE 13. Interactions of retinoid-binding proteins and retinoid-processing enzymes in the vertebrate visual cycle. (A) Retinoid-binding proteins: SRBP — serum-retinol binding protein; CRBP — cellular retinol-binding protein; CRAIBP — cellular retinal-binding protein (specific for 11-cis isomers of retinal and retinol); IRBP — interstitial retinol-binding protein. The cells in the neural retina that contain CRBP and CRAIBP are the Müller cells. Their processes extend into the interphotoreceptor matrix, and their tight junctions with the photoreceptor cells constitute the external limiting membrane (see Figure 8). (B) Enzymes: 1 — retinol isomerase; 2 — 11-cis-retinol oxidoreductase; 3 — retinol ester synthase, hydrolases (there appear to be two hydrolase activities, one for 11-cis- and the other for all-trans); 4 — all-trans-retinol oxidoreductase (in rod outer segment). (C) Op — opsin; 11-cis Rh — rhodopsin; Ol — retinol; Al — retinal. See text for references.

retinal bound to IRBP has not been determined, so the efficacy of transfer from CRAIBP to IRBP cannot be judged at present. One of the most important questions concerns the presence of 11-cis-retinol bound to mammalian IRBP in the interphotoreceptor matrix. Is it in transit from the isomerase in the retinal pigment epithelium to CRAIBP in the Müller cells? In addition to being bound to CRAIBP in the retina, a substantial quantity of 11-cis-retinol is also present in membrane fractions from bovine retina homogenates,¹²⁵ raising the question of why 11-cis-retinol is present in tissue that cannot oxidize it to 11-cis-retinal and hence cannot use it for rhodopsin regeneration. There appears to be a species difference here, because although bovine retina does not contain an 11-cis-retinol oxidoreductase,^{51,52} there is evidence for the presence of this enzyme in frog retina.⁵⁰ Consequently bleached bovine and other mammalian retinas do not regenerate rhodopsin when provided with 11-cis-retinol, while frog retinas do.¹⁴⁹⁻¹⁵² Observations by Defoe and Bok¹⁵³ are also puzzling. These authors suggest that even in darkness there is an exchange of prosthetic group between

opsin molecules, a finding reminiscent of Bridges and Yoshikami,¹⁵⁴ who concluded that "in darkness, therefore, there is a mechanism for abstracting retinaldehyde from rhodopsin in the photoreceptors and replacing it with labeled material from the circulation".

VI. CONCLUSION

The present chapter has attempted to synthesize the current status of our knowledge of the visual cycle. The identification and localization of retinol isomerase has clarified the role of the pigment epithelium in rhodopsin regeneration as well as the need to transport retinol between this tissue and the retina. The transport vehicle, IRBP, has been purified, its primary structure determined and its endogenous retinoid ligands characterized during the physiological processes of light- and dark-adaptation. These findings, coupled with other recent observations on retinoid-binding proteins and retinoid-processing enzymes are beginning to provide important new insights into the molecular basis of the visual cycle.

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Chapter 3

**THE BINDING SITE OF OPSIN BASED ON ANALOG STUDIES
WITH ISOMERIC, FLUORINATED, ALKYLATED, AND OTHER
MODIFIED RETINALS****Robert S. H. Liu and Alfred E. Asato****TABLE OF CONTENTS**

I.	Introduction	52
II.	Synthesis of Retinal Analogs	52
A.	Stereoisomers of Retinal	52
1.	7- <i>cis</i> Isomers	52
2.	9- <i>cis</i> Isomers	53
3.	11- <i>cis</i> Isomers	54
4.	13- <i>cis</i> Isomers	54
B.	Fluorinated Retinals	55
C.	Alkylated Retinals	57
D.	Alpha-Retinals	57
E.	Miscellaneous Retinal Analogs	57
III.	Visual Pigment Analogs	58
A.	Binding Interaction with Opsin	58
B.	Stereoselectivity of the Binding Site of Opsin	58
C.	The Shape of the Binding Site of Opsin	63
D.	Refining the Binding Site	66
E.	Other Limitations within the Binding Site of Opsin	67
F.	Fluorinated Rhodopsins. Specific Protein-Substrate Interaction and ^{19}F NMR	69
	Addendum	71
	Acknowledgments	71
	References	71

I. INTRODUCTION

Visual pigment analog studies began with the classical study of Wald, Hubbard, and co-workers where six geometric isomers of retinal (*all-trans*, 13-*cis*, 11-*cis*, 9-*cis*, 9,13-di-*cis*, and 11-13-di-*cis*) were employed to react with the apoprotein opsin.¹ This was followed by the use of 3-dehydroretinal isomers by the same group² and other modified retinals by Blatz³ and by Kropf.⁴

Beginning in the mid-1970s, with the involvement of several organic research groups, visual pigment analog studies reached a higher level of activity. Most notable is the Columbia group under the direction of Nakanishi. The initial study of examining the effect of methyl substituents at C₁₃ and C₁₄ on conformational properties of the chromophore and on protein-substrate interactions⁵ was followed by a steady stream of structurally diverse analogs in the last decade. These works have been summarized elsewhere.⁶

In the early 1970s the research group at Hawaii established a photochemical entry to the previously unknown sterically crowded 7-*cis* geometry in compounds in the vitamin A series.⁷ This finding paved the way for the synthesis of other missing retinal geometric isomers. This effort culminated in the successful synthesis of the last two hindered isomers (*all-cis* and 7,9,11-tri-*cis*) of vitamin A in 1983.^{8,9} The unexpected finding of stable pigment formation of 7-*cis* isomers of retinal¹⁰ not only revived the interest in reexamining the stereospecificity of the binding site of opsin but also launched an independent program of visual pigment analog studies at Hawaii. In this review, a summary of this synthetic and bioorganic endeavor is presented. The effect of modification of the retinyl chromophore on photochemical properties of pigment analogs has recently been reviewed¹¹ and will not be repeated here.

II. SYNTHESIS OF RETINAL ANALOGS

A. STEREOISOMERS OF RETINAL

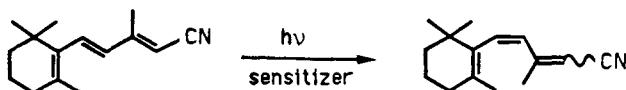
All sixteen possible geometric isomers of retinal (**1**) are now known (Figure 1). The synthetic pathways leading to these isomers are as plentiful as they are diverse and imaginative. Comprehensive surveys that examine these preparative methodologies include those of Mayer and Isler,¹² Frickel,¹³ and Liu and Asato.⁹ The latter two reviews canvas the chemical literature through 1982 while the Mayer and Isler text remains as prerequisite reading for researchers in this area.

Despite rapid advances in HPLC separation technology that permit the facile obtainment of milligram quantities of pure retinoids from complex synthetic mixtures, the need for stereoselective preparative protocols still exists. This is evident in the case of mixtures that defy HPLC separation attempts and in the synthesis of labile radiolabeled retinoids.

Several proven methodologies that afford gram quantities of isomers of known configuration in relatively high stereochemical purity are summarized later.

1. 7-*cis* Isomers¹⁴

The most practical route to retinoids containing the highly hindered 7-*cis* configuration is selective sensitized photoisomerization of appropriate triene precursors such as β-ionylideneacetonitrile:



Separation of the 7-*cis* and 7,9-di-*cis* isomers is readily achieved by straightforward column chromatography.¹⁴

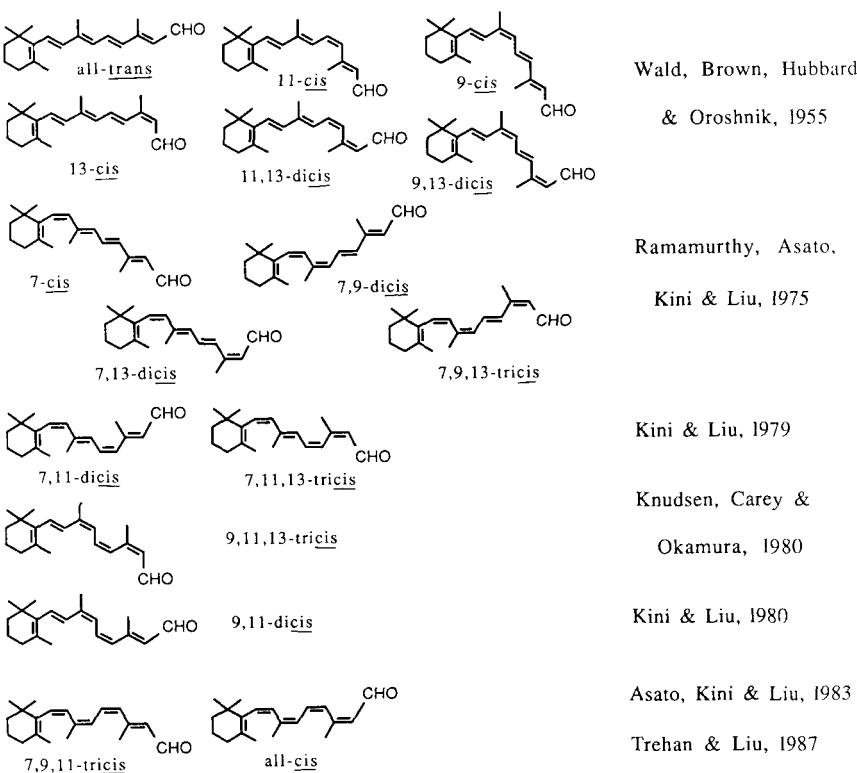
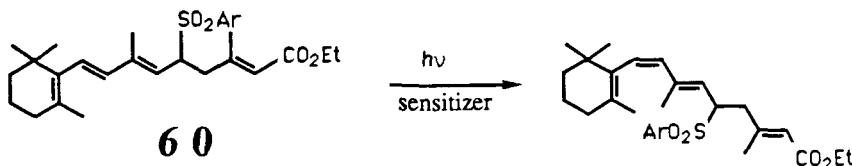
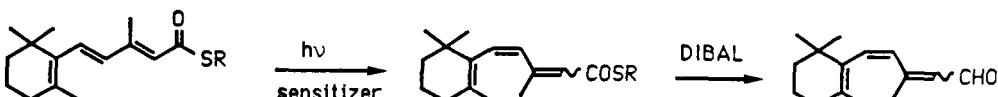


FIGURE 1. All 16 known geometric isomers of retinal arranged in chronological order of their preparation.

In favorable cases the *7-cis* isomer is formed almost exclusively as for C₂₀-sulfone **60**¹⁵ and the precursor to *7-cis,9-cis*- β -ionylideneacetaldehyde.¹⁶

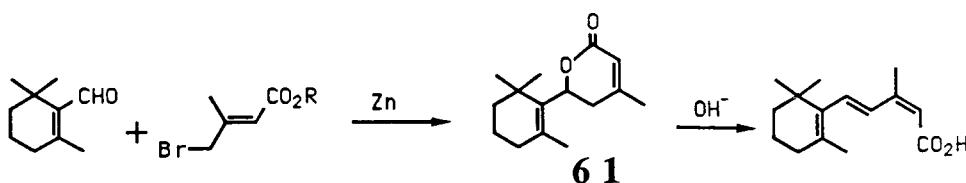


The latter aldehyde is also obtained in excellent yield by the photosensitized isomerization of C₁₅-thiol esters followed by DIBAL reduction.¹⁷



2. *9-cis* Isomers

The base-induced ring opening of C₁₅-lactone **61** is one of the more practical synthetic approaches to large quantities of configurationally pure *9-cis* isomers that has been routinely employed in our laboratories. This lactone is, in turn, prepared by the Matsui or Reformatsky condensation of β -cyclocitral with senecioic acid or its γ -bromoester, respectively:^{18a,b}

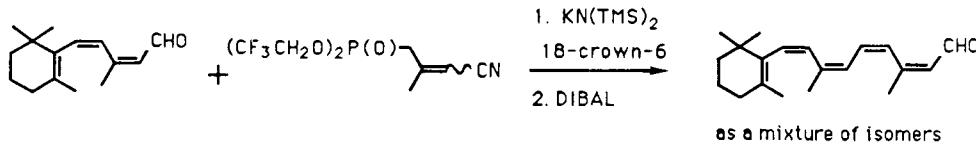


3. 11-cis Isomers

Isolation of gram quantities of the visually important 11-*cis*-retinal from photo-mixtures by fractional recrystallization was successfully demonstrated by Brown and Wald.¹⁹ However, now the isolation of this geometric isomer is usually carried out by HPLC separation.^{14,20} Synthetically, the catalytic semihydrogenation of 11-dehydroretinoids has been, and continues to be, the method of choice for the preparation of the important 11-*cis* isomers of retinal.²¹

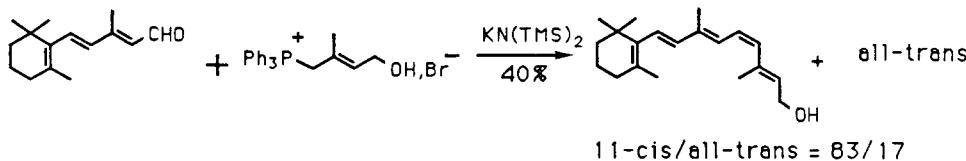
Two recent modifications of traditional olefination reactions hold some promise as synthetically useful methodologies for gaining access to the 11-*cis* manifold.

Enhanced Z-selectivity in double-bond-forming Horner reactions was recently reported by Still.²² The method has recently been incorporated by D. Mead and A. Trehan in our laboratories in routes for the nonstereoselective synthesis of the unstable trifluorinated 11-*cis*-retinal²³ and the labile all-*cis*-retinal.²⁴

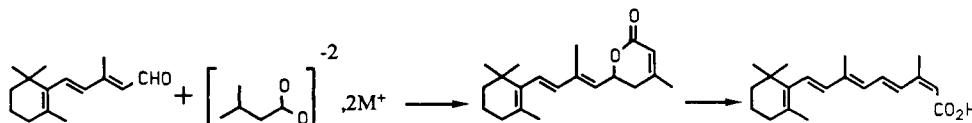


It should be noted that because of its ready conversion to 7-*cis*,9-*cis*,13-*cis*-retinal, presumably via consecutive 6e-electrocyclization,²⁵ spectra of pure all-*cis*-retinal are difficult to obtain. Its *UV-VIS* spectrum was successfully recorded only after the employment of a diode-array detector. The absence of the 360-nm peak (Figure 2), characteristic of other retinal isomers, reveals the highly twisted conformation of the all-*cis* chromophore.

An equally viable alternative to the above method has been recently reported by Kobayashi. In his “oxido-ylid” method, the double bond newly formed in a modified Wittig reaction had very high *cis*-stereoselectivity in acceptable overall yields.²⁶



It is anticipated that both procedures will find increasing usage for the preparation of *cis*-double bond-containing polyolefins.



4. 13-cis Isomers

As in the case of the stereoselective preparation of 9-*cis* isomers, the 13-double bond

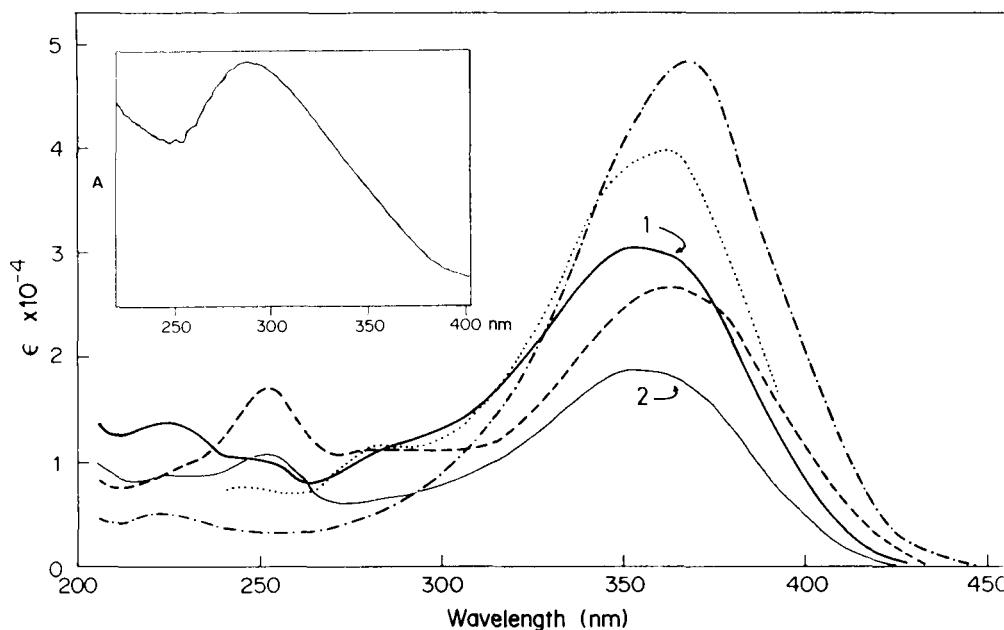
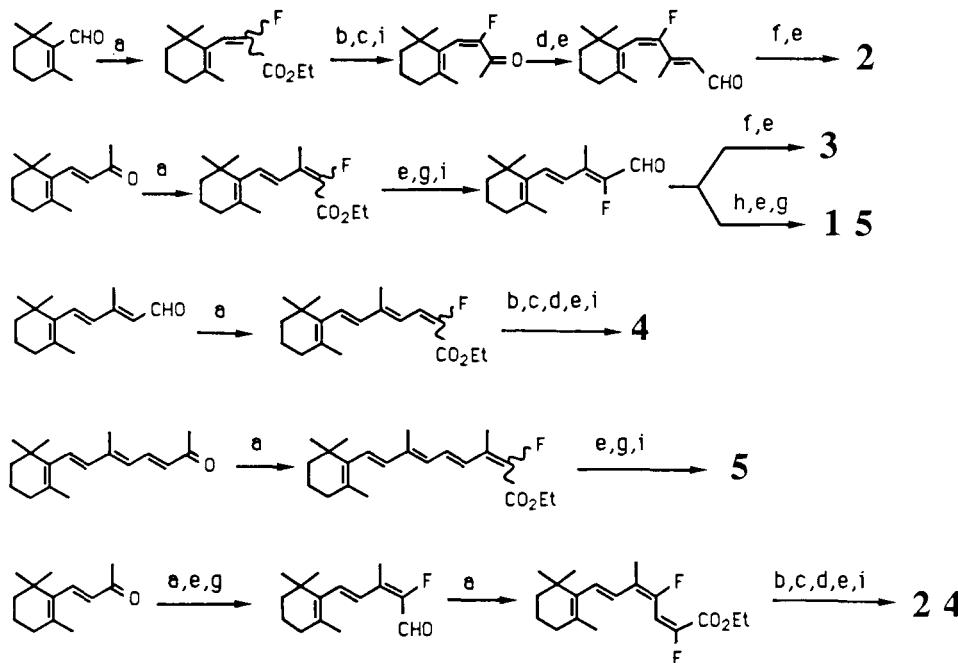


FIGURE 2. UV-VIS absorption spectra of selected isomers of retinal in hexane (—·—, all-*trans*; - - - - , 9-*cis*; ···, 11-*cis*; solid line 1, 9-*cis*,11-*cis*; solid line 2, 7-*cis*,11-*cis*). Insert: spectrum of all-*cis*-retinal recorded on a Hewlett-Packard diode array detector (solvent: 5% ether in hexane) extinction coefficient not determined.²⁴

can be configurationally directed to the *cis*-form by a C₁₅ + C₅ Matsui-type condensation:^{18b,27}

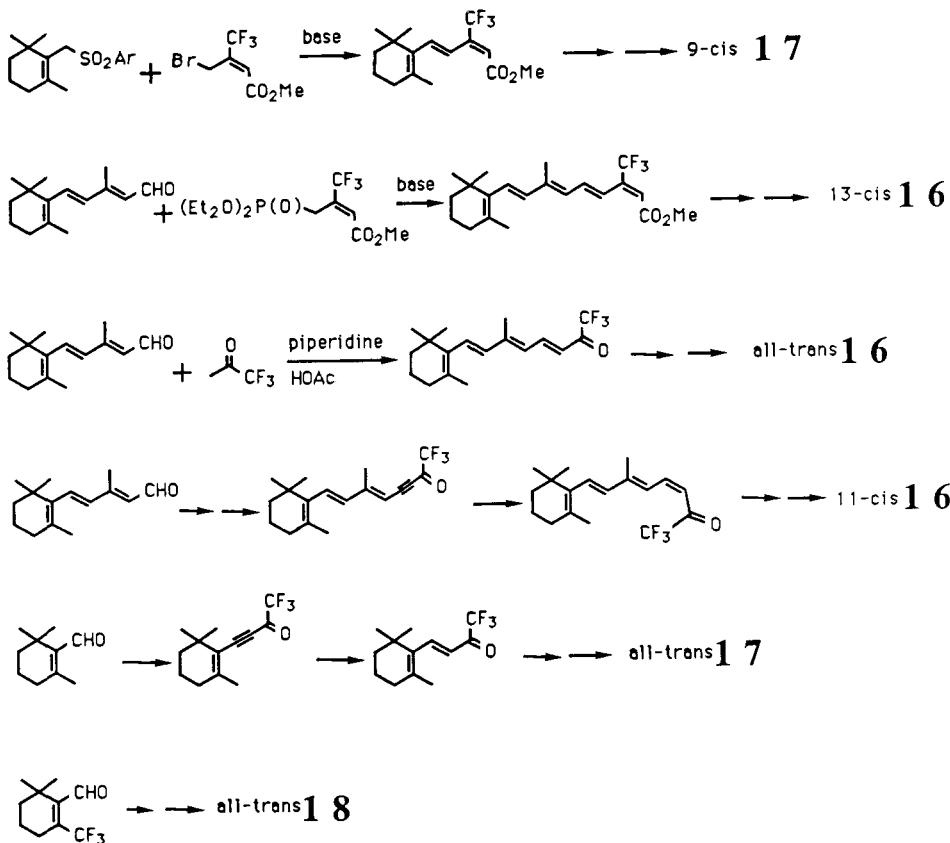
B. FLUORINATED RETINALS

Experimental procedures for the preparation of fluorinated derivatives of vitamin A were first reported by Machleidt.²⁸ We extended the methodologies he devised to the preparation of fluorinated retinal analogs for vision research,²⁹ as illustrated by the following examples:

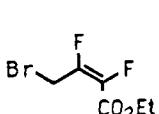


- a). $(EtO)_2P(O)CHFCO_2Et$, LDA; (b) aq. KOH/MeOH; H_3O^+ , Cl^- ; (c) MeLi, Et₂O; (d) $(EtO)_2P(O)CH_2CN$, LDA; (e) DIBAL, hexanes; wet silica gel; (f) $(EtO)_2P(O)CH_2C(CH_3)=CHCN$, LDA; (g) MnO_2 ; (h) $(EtO)_2P(O)CH_2C(CH_3)=CFCO_2Et$; (i) FOC or HPLC separation.

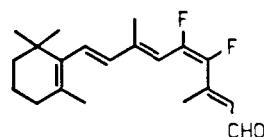
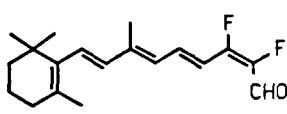
Several procedures have been independently devised in our laboratory and by the Tokyo-based research group of Kobayashi for the preparation of retinal analogs **16** to **18**, wherein the methyl groups have been selectively replaced by the highly electron-deficient trifluoromethyl moiety:^{23,30-34}



Difluorolefins of the type RCF=CFR' exhibit *cis-trans* isomerism, wherein the three bond F–F coupling constant in the ¹⁹F NMR spectrum is of the order of ~0 to 20 Hz and ~120 to 140 Hz for the *cis* (*Z*)- and *trans* (*E*)-forms, respectively. This dramatically large difference could, in principle, be exploited to establish the configuration of difluorolefin-containing macromolecules. Accordingly we set about to incorporate this novel fluorinated moiety into the retinal skeleton. The development of a simple method for the stereoselective preparation of the highly functionalized C₄-synthon, ethyl (2*Z*)-4-bromo-2,3-difluoro-2-butenoate, permitted the successful synthesis of fluorinated retinal analogs **25** and **62**.³⁵



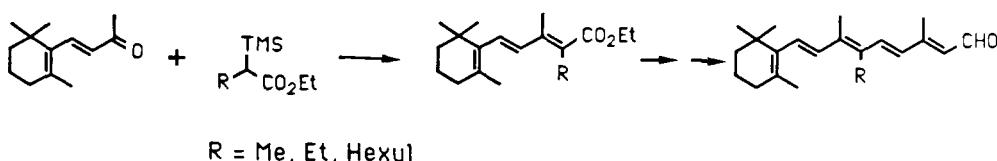
2 5



6 2

C. ALKYLATED RETINALS

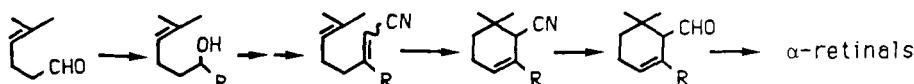
Since the report on 14-methylretinal, which provided direct evidence for the 12-*s-trans* conformation in native rhodopsin,⁵ numerous alkylated retinal analogs have been prepared, including 13-demethylretinal,³⁶ 19-methylretinal ("9-ethyl retinal"),⁴ 20-methyl- and 20-ethylretinal,³⁷ 10- and 11-methylretinal.³⁷⁻³⁹ In our laboratory, the introduction of C₁₀-alkyl substituents was readily effected by the Petersen reaction of β-ionone with a series of α-trimethylsilylalkanoates, followed by straightforward elaboration to the requisite retinal analogs.^{17,39}



$R = Me, Et, Hexyl$

D. ALPHA-RETINALS

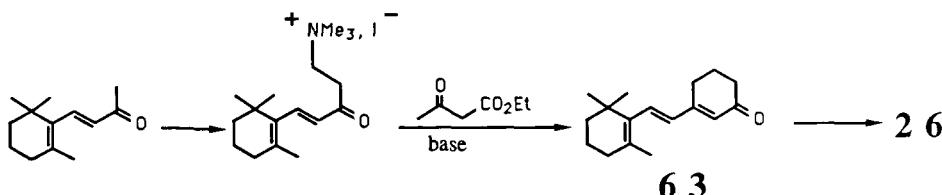
A series of α-retinal analogs modified at the 5-position were recently prepared for the purpose of investigating the binding site requirements of the hydrophobic pocket in rhodopsin. The general method of synthesis for these α-retinal analogs (**34** to **36**, **50**, **51**) is outlined below:



In this sequence the predominant α-isomer was accompanied by lesser amounts of the corresponding β- and γ-forms.

E. MISCELLANEOUS RETINAL ANALOGS

Other analogs of retinal containing the 10-chloro-,³⁸ 12-chloro-,⁵⁸ and 11,19-ethano substituents, bicyclic, aryl, and naphthyl end groups as well as acyclic analogs have also been prepared in our laboratories.¹⁷ The key step in the synthesis of "9-*trans*-locked" retinal analog **26** (11,19-ethanoretinal) was the construction of the cyclohexenone derivative **63**.⁴⁰ This important intermediate was assembled by standard methodologies originally developed by Karrer in 1952.⁴¹



This retinal analog was independently synthesized by Sheves and co-workers in Israel^{42,43} by a shorter reaction sequence, which featured the general synthesis originally developed by Schlessinger for β-formylcycloalkenones.⁴⁴

The preparation of aryl retinoids was effected in a conventional manner starting from the appropriately substituted benzaldehyde and two repetitive chain-extension reactions using a C₅-phosphononitrile.⁴⁵

III. VISUAL PIGMENT ANALOGS

A. BINDING INTERACTION WITH OPSIN

Because procedures for the regeneration of visual pigment (interaction of opsin with 11-*cis*-retinal) and formation of new pigment analogs (opsin with retinal analogs) are in the literature,^{46,47} they will not be repeated here. Digitonin has been the most commonly used detergent to solubilize opsin. Recently many new detergents (alkyl glucosides, Triton, CHAPS, and CHAPSO) have also been employed. ROS (rod outer segment) suspensions have also been used.

This variety of conditions unfortunately occasionally provides seemingly disparate data in yields and rates of pigment formation, making quantitative comparison of data difficult. The problem is further complicated by the fact that the purity of a given detergent (digitonin, in particular) could have a significant effect on pigment formation. Hence, in the absence of a standard condition for pigment formation, pigment yield data are usually only qualitatively meaningful. Therefore, we categorize all pigment analogs prepared at Hawaii (Table 1) into three groups: those of high (+ + +, >70%), moderate (+ +, 30 to 70%) or low yield (+, 3 to <30%). Anything less than 3% is listed as inactive (−). Structures for the corresponding retinal analogs are shown in Figure 3. Those retinal analogs and their isomers that failed to form pigment analogs are shown in Figure 4.

B. STEREOSELECTIVITY OF THE BINDING SITE OF OPSIN

The observation that in addition to 11-*cis*-retinal only the structurally similar 9-*cis* isomer formed a pigment analog, while the remaining four isomers (all-*trans*, 13-*cis*, 9,13-di-*cis*, and 11,13-di-*cis*) either failed to give pigment or did not yield conclusive results led Wald and co-workers to conclude that the binding site of opsin is highly specific.¹ However, two independent observations in the mid 1970s prompted a reevaluation of this postulate. First, Nakanishi and co-workers⁴⁸ showed by extraction of chromophore from the pigment analog derived from 9-*cis*,13-*cis*-retinal that the pigment analog retained the original geometrical integrity. Second, four new isomers of retinal,⁴⁹ all containing the hindered 7-*cis* geometry, were shown to form new pigment analogs with absorption properties substantially blue shifted (λ_{max} 440 to 455 nm) from those of other known isomeric rhodopsins (480 to 500 nm).¹⁰ Subsequently a complete study with all stable isomers of retinals (Table 1)⁵⁰ revealed that opsin displayed little selectivity in accepting nearly all singly, doubly, and even triply bent isomers, the only exception being the all-*trans* and the 13-*cis* isomers.^{50,51} A similar trend of lack of stereoselectivity extended into other structurally modified retinal analogs (Table 1) although the overall pigment yields were generally lower.

Limited data on rates of pigment formation for different isomeric retinals are available in the literature.⁵⁰ The rates of reaction are slower in detergents than with ROS suspension (probably due to a higher effective local retinal concentration in the latter cases). But within each set, the general trend of faster rate of reaction for 11-*cis* and the structurally similar 9-*cis* isomer is evident. Hence, based on the criteria of rates of reaction, the early statement of a highly stereoselective binding site of opsin is still valid. The protein-binding site, unlike a rigid lock as envisioned in the key-and-lock analogy for protein-substrate interaction, is capable of conformational rearrangement to accommodate isomers different from the native shape of the binding site. Hence, the initial structural incompatibility is reflected in reduced rates of reaction but not necessarily in yields of pigments.⁵⁰

TABLE 1
Isomers of Retinal Analogs and Visual Pigments from Interaction with Bovine Opsin

Retinal Analog		Pigment Analog	
Compound	Isomer	λ_{\max} (nm)	Yield ^a (detg) ^b
Retinal, 1^c	all- <i>trans</i> ^d	—	— (S, D)
	13- <i>cis</i> ^d	(465) ^e	— (D)
	11- <i>cis</i> ^d	498	+ + + (S, D)
	9- <i>cis</i> ^d	483	+ + + (S, D)
	7- <i>cis</i>	450	+ + (S)
	11,13-di- <i>cis</i> ^d	?	? (D)
	9,13-di- <i>cis</i> ^f	481	+ + + (D)
	7,13-di- <i>cis</i>	455	+ (D)
	9,11-di- <i>cis</i>	480	+ (D)
	7,11-di- <i>cis</i>	455	+ + (D)
	7,9-di- <i>cis</i>	460	+ + (S)
	9,11,13-tri- <i>cis</i> ^g	?	
	7,11,13-tri- <i>cis</i>	?	
	7,9,13-tri- <i>cis</i>	455	+ + (D)
	7,9,11-tri- <i>cis</i>	462	+ + (D)
	all- <i>cis</i>	?	
8-Fluoro-, 2^{h,i}	all- <i>trans</i>	—	— (C)
	13- <i>cis</i>	—	— (C)
	11- <i>cis</i>	463	+ + + (C)
	9- <i>cis</i>	460	+ + + (C)
	7- <i>cis</i>	453	+ (C)
	7,13-di- <i>cis</i>	440	+ (C)
10-Fluoro-, 3^{j,k}	all- <i>trans</i>	—	— (C)
	13- <i>cis</i>	—	— (C)
	11- <i>cis</i>	489	+ + + (C)
	9- <i>cis</i>	486	+ + + (C)
	7- <i>cis</i>	484	+ (C)
	11,13-di- <i>cis</i>	461	+ + (C)
	9,13-di- <i>cis</i>		+ (C)
	7,9-di- <i>cis</i>	464	(D)
	7,9,13-tri- <i>cis</i>	460	(D)
12-Fluoro-, 4^l	all- <i>trans</i>	—	—
	13- <i>cis</i>	—	—
	11- <i>cis</i>	507	+ + + (D)
	9- <i>cis</i>	493	+ + + (D)
	7- <i>cis</i>	—	—
	7,11-di- <i>cis</i>	500	(D)
	7,9,11-tri- <i>cis</i>	—	(D)
14-Fluoro-, 5^m	all- <i>trans</i>	—	—
	13- <i>cis</i>	—	—
	11- <i>cis</i>	527	(D)
	9- <i>cis</i>	511	(D)
	7- <i>cis</i>	—	—
10-Chloro-, 6ⁿ	all- <i>trans</i>	—	—
	13- <i>cis</i>	—	—
	11- <i>cis</i>	480	+ + (D)
	9- <i>cis</i>	474	+ (D)
	7- <i>cis</i>	—	—
12-Chloro-, 7^m	11- <i>cis</i>	?	— (D)
	9- <i>cis</i>	488	+ + (D)
14-Chloro-, 8ⁿ	all- <i>trans</i>	—	—
	13- <i>cis</i>	—	
	11- <i>cis</i>	544	+ + (D)
	9- <i>cis</i>	524	+ (D)

TABLE 1 (continued)
Isomers of Retinal Analogs and Visual Pigments from Interaction with Bovine Opsin

Retinal Analog		Pigment Analog	
Compound	Isomer	λ_{\max} (nm)	Yield ^a (detg) ^b
10-Methyl-, 9^k	all-trans	—	—
	13-cis	—	—
	11-cis	508	+
	9-cis	497	+++ (D)
	7-cis	506	+(D)
11-Methyl-, 10^b	all-trans	—	—
	11-cis	498	+(D)
	9-cis	479	+(D)
12-Methyl-, 11^m	all-trans	—	—
	13-cis	—	—
	11-cis	489	+(C)
	9-cis	487	+++ (C)
	11,13-di-cis	486	+(C)
14-Methyl-, 12^{b,n}	all-trans	—	—
	13-cis	—	—
	11-cis	508	(T)
	9-cis	493	(T)
	9,13-di-cis	494	(T)
10-Ethyl-, 13^b	all-trans	—	—
	13-cis	—	—
	11-cis	480	— (D)
	9-cis	494	+(D)
	7-cis	—	— (D)
5,6,7,8-Tetrahydro-, 14^o	9,13-di-cis	493	+(D)
	11-cis	424	++ (D)
	9-cis	426	++ (D)
	9,13-di-cis	426	+(D)
10,14-F ₂ -, 15^p	all-trans	—	—
	13-cis	—	—
	9,13-di-cis	510	(D)
20,20,20-F ₃ -, (13-CF ₃), 16^q	all-trans	—	—
	13-cis	—	—
	11-cis	542	(D)
	9-cis	516	(C)
19,19,19-F ₃ -, (9-CF ₃), 17^q	all-trans	—	—
	13-cis	—	—
	11-cis	456	(D)
	9-cis	447	(D)
	9,13-di-cis	453	(C)
18,18,18-F ₃ -, (5-CF ₃), 18^q	all-trans	—	—
	13-cis	—	—
	11-cis	457	(D)
	9-cis	454	(D)
18-Ethyl-, (5-n-propyl), 19^b	all-trans	—	—
	13-cis	—	—
	11-cis	475	++ (D)
	9-cis	482	+(D)
	7-cis	470	+(D)
18-Propyl-, (5-n-butyl), 20^r	11,13-di-cis	480	+(D)
	11-cis	—	— (D)
	9-cis	458	+(D)
19,19-Dimethyl-, (9-isopropyl), 21^b	13-cis	—	—
	11-cis	494	+(D)
	9-cis	490	+(D)

TABLE 1 (continued)
Isomers of Retinal Analogs and Visual Pigments from Interaction with Bovine Opsin

Retinal Analog	Compound	Isomer	Pigment Analog	
			λ_{max} (nm)	Yield ^a (detg) ^b
19-Butyl-, (9-pentyl), 22 ^h		all-trans		
		13-cis		
		11-cis	480	+
		9-cis	—	—
		7-cis	462	+
13-Demethyl-, 23 ⁱ		all-trans	—	(D)
		13-cis	—	
		11-cis ^x	498	++
		9-cis ^x	483	+++
		11,13-di-cis	500	++
		9,13-di-cis	484	++
		9,11-di-cis	477	+
		9,11,13-tri-cis	485	++
10,12-F ₂ -13-demethyl-, 24 ^b		all-trans	—	
		13-cis	—	
		9,11-di-cis	492	+
13,14-F ₂ -13-demethyl-, 25 ^{b,i}		all-trans	—	
		13-cis	—	
		9-cis	520	+
11,19-Ethano-, (9,11-ring-locked), 26 ^u		all-trans	—	
		13-cis	—	
		11-cis	483	+
		11,13-di-cis	471	+
11,20-Ethano-, (11,13-ring-locked), 27 ^h		all-trans		
		13-cis		
		9-cis	489	+
3,7,11-Trimethyldodeca-2,4,6,8,10-pentaenal, 28 ^v		all-trans	—	
		13-cis	—	
		11-cis	507	+
		9-cis	508	++
10-Isopropyl-3,7,11-trimethyl-2,4,6,8,10-dodeca-pentaenal, 29 ^h		all-trans		
		11-cis	496	+++
		9-cis	481	+++
		7-cis	473	+
3-Dehydro-, 30 ^w		all-trans	—	
		13-cis	—	(D)
		11-cis ^x	505	(D)
		9-cis ^x	498	(D)
		7-cis	464	
12-Fluoro-3-dehydro-, 31 ^h		all-trans	—	
		11-cis	520	(D)
α -Retinal, 32 ^r		all-trans	—	
		13-cis	—	
		11-cis ^y	468	++
		9-cis ^y	462	++
		9,13-di-cis		
		9,11-di-cis	476	+
10-Fluoro- α -, 33 ^r		all-trans	—	
		13-cis	—	
		11-cis	463	++
		9-cis	460	++
		9,13-di-cis	458	+++
18,18-Dimethyl- α -, (5-isopropyl), 34 ^r		13-cis	—	
		9-cis	462	++

TABLE 1 (continued)
Isomers of Retinal Analogs and Visual Pigments from Interaction with Bovine Opsin

Retinal Analog			
Compound	Isomer	λ_{\max} (nm)	Yield ^a (detg) ^b
18-Propyl- α -, (5-butyl), 35^c	all- <i>trans</i>	—	
	11- <i>cis</i>	462	++ (D)
	9- <i>cis</i>	—	
18-Hexyl- α -, (5-heptyl), 36^d	9- <i>cis</i>	460	+++ (D)
	all- <i>trans</i>		
	13- <i>cis</i>	—	
3,7-Dimethyl-9-phenyl-2,4,6,8-nonatetraenal (Phenylretinal), 37^e	11- <i>cis</i>	494	+(D)
	9- <i>cis</i>	480 ^{aa}	-(D)
	all- <i>trans</i>	—	
<i>o</i> -Tolylretinal, 38^f	13- <i>cis</i>	—	
	11- <i>cis</i>	496	+(D)
	9- <i>cis</i>	482	+(D)
Mesitylretinal, 39^g	all- <i>trans</i>	—	
	13- <i>cis</i>	—	
	11- <i>cis</i>	480	++ (D)
10-Fluoromesitylretinal, 40^h	9- <i>cis</i>	470	+(D)
	11- <i>cis</i>	472	++ (D)
	9,13-di- <i>cis</i>	462	+++ (D)
2'-Chloro-6'-fluoro-phenylretinal, 41ⁱ	9,13-di- <i>cis</i>	458	++ (D)
	all- <i>trans</i>	—	
	13- <i>cis</i>	—	
41^j	11- <i>cis</i>	460	+(D)
	9- <i>cis</i>	470	+(D)

^a ++ = >70% yield; + + = 30—70% yield; + = 3—30% yield; — = 0—<3% yield.

^b Detergent used: D = digitonin, C = CHAPS, T = Triton 100, S = ROS suspension; Blank, yield not determined.

^c Reference 50.

^d Reference 1.

^e Reference 52.

^f Reference 48.

^g Knudsen, C. G., Carey, S. C., and Okamura, W. H., [1,5]-Sigmatropic rearrangement of vinylallenes: a novel route to geometric isomers of the retinoids possessing 11-*cis* linkages including 9-*cis*,11-*cis*,13-*cis*-retinal, *J. Am. Chem. Soc.*, 102, 6355, 1980.

^h Reference 70.

ⁱ Reference 81.

^j Reference 68.

^k Reference 38.

^l Reference 82.

^m Reference 58.

ⁿ Reference 5.

^o Fenstemacher, R., Matsumoto, H., and Liu, R. S. H., Visual pigment analogs from isomers of 5,6,7,8-tetrahydroretinal. The importance of the trimethylcyclohexyl ring, *Bioorg. Chem.*, 11, 404, 1982.

^p Reference 51.

^q Reference 23.

^r Reference 66.

^s Reference 36.

^t Reference 35.

^u Reference 40.

^v Reference 72.

^w Matsumoto, H., Asato, A. E., and Liu, R. S. H., 7-*cis*-Porphyropsin from 7-*cis*-3-dehydroretinal and cattle opsin, *Photochem. Photobiol.*, 29, 695, 1979.

^x Reference 2.

^y Reference 67.

TABLE 1 (continued)
Isomers of Retinal Analogs and Visual Pigments from Interaction with Bovine Opsin

^a Reference 45.

^{aa} Motto, M. G., Sheves, M., Tsujimoto, K., Balogh-Nair, V., and Nakanishi, K., Opsin shift in bovine rhodopsin and bacteriorhodopsin. Comparison of two external point-charge models, *J. Am. Chem. Soc.*, 102, 7947, 1980.

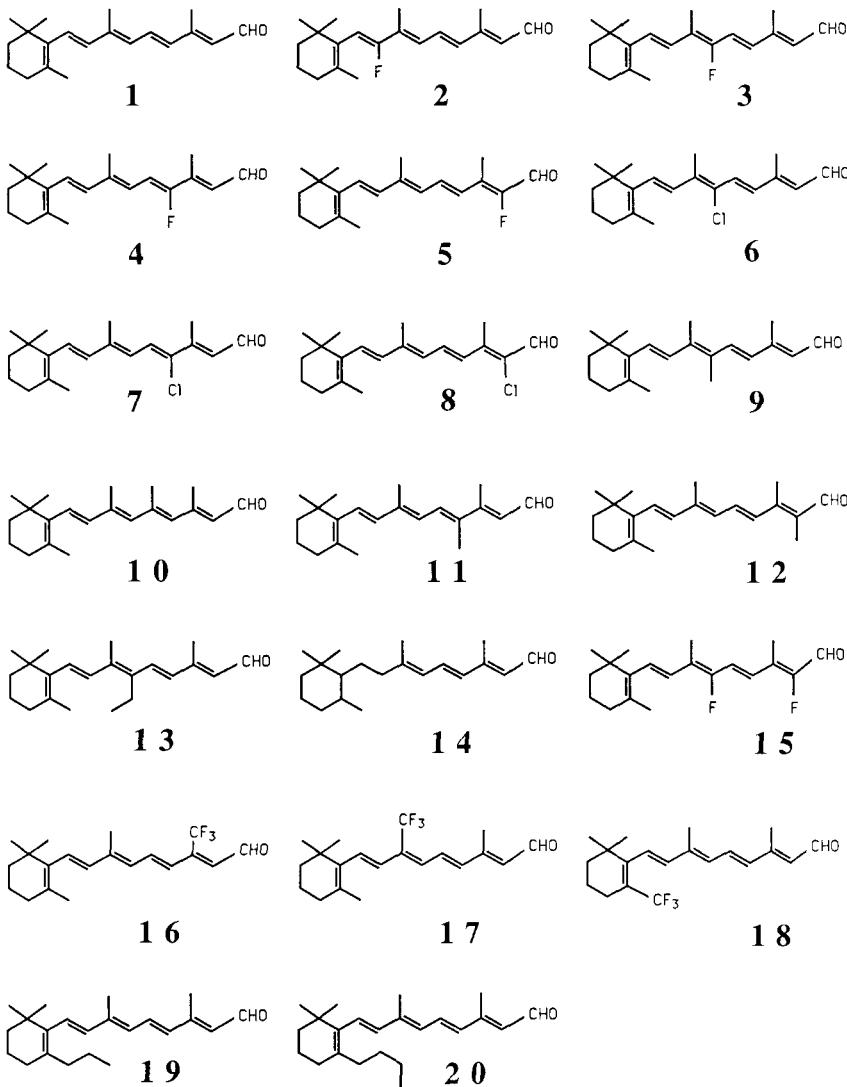


FIGURE 3A. Structures of retinal and analogs with isomers active toward bovine opsin (Table 1).

C. THE SHAPE OF THE BINDING SITE OF OPSIN

What stands out from the current data is the failure of only the all-trans and the 13-cis isomers of retinal to form stable pigment analogs. (The pigment absorbing at 465 nm listed for the 13-cis isomer was the transient photo-meta-rhodopsin that was postulated to have the 13-cis geometry.⁵² Several attempts have been made to account for the exceptional behavior of these two isomers.

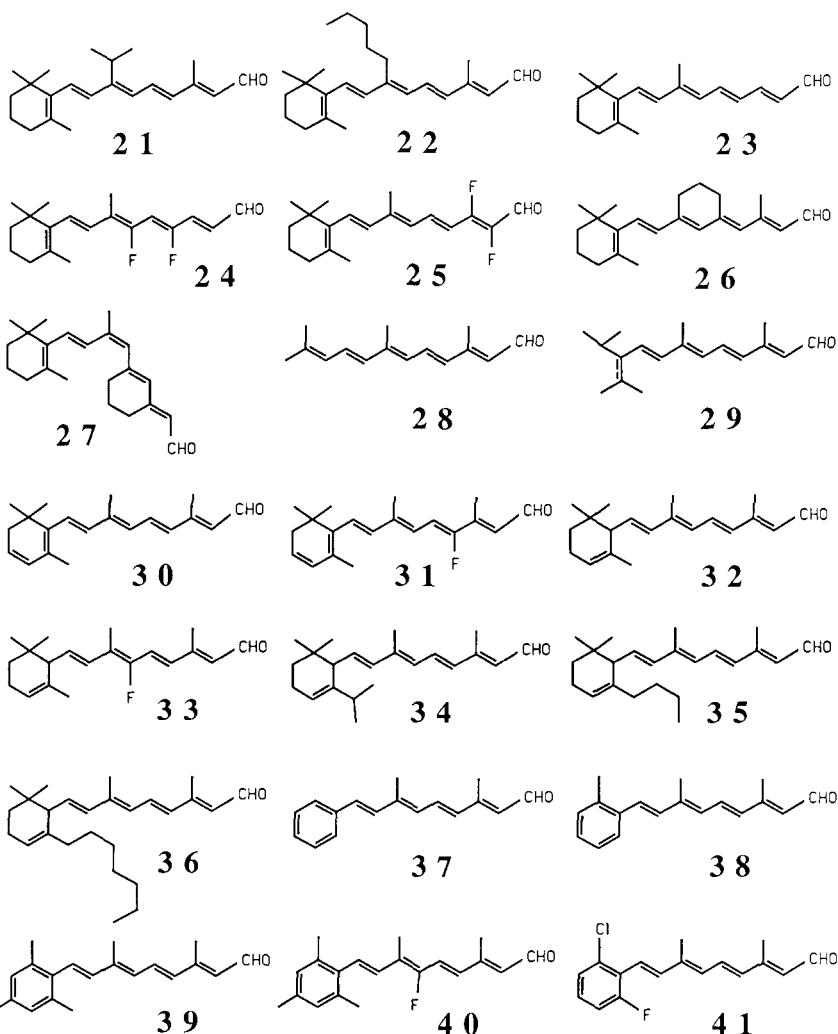


FIGURE 3B. Structures of retinal analogs (Table 1).

The first attempt was that of Matsumoto and Yoshizawa,⁵³ who first demonstrated the presence of a hydrophobic pocket in the binding site through rhodopsin inhibition experiments⁵⁴ and then introduced the concept of the existence of a longitudinal restriction within the binding site of opsin. Because the all-*trans* isomer has the longest critical distance between the center of the cyclohexenyl ring and the carbonyl carbon, they reasoned that the failure of binding interaction of this isomer with opsin was due to its excessive length. Because 13-*cis*-retinal has the next longest critical distance, they invoked the same explanation for the negative result. However, this explanation failed to account for the reported pigment formation of 7,9-di-*cis*-rhodopsin¹⁰ and, subsequently, of 9,11-di-*cis*-rhodopsin,⁵⁵ the chromophore of both isomers having the same critical distance as that of the 13-*cis* isomer.⁵³ Resolution of the crystal structure of 13-*cis*-retinal⁵⁶ and methyl 7-*cis*,9-*cis*-retinoate⁵⁷ provided to the rationale that steric crowding in the latter led to a nonplanar conformation and thus a shortened critical distance.⁵⁷

A different approach to the critical distance was introduced in 1984.⁵⁸ This model continued to recognize the existence of a hydrophobic pocket for the nonpolar trimethylcyclohexenyl ring⁵⁴ by retaining the center of the ring as one of the two anchors for the

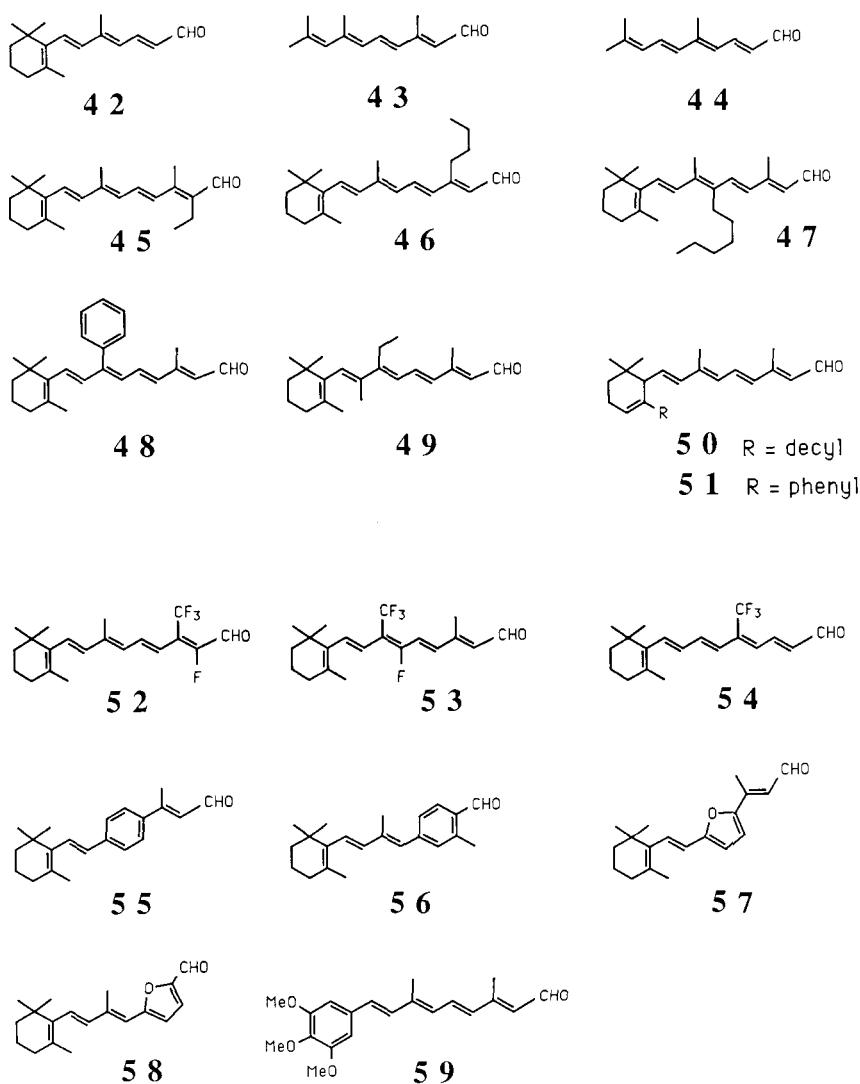


FIGURE 4. Structures of retinal analogs without any active isomers toward bovine opsin.

chromophore. To mimic the nonbonding nature of the hydrophobic interaction, the ring was allowed to pivot freely. Retinal, however, is known to be attached to opsin through a protonated Schiff base linkage involving the ϵ -amino group of Lys-296.⁵⁹ Hence, the second point of attachment for the chromophore should be the α -carbon of Lys-296. The butyl group of lysine added in this model serves to accommodate those retinal isomers (or even photobleaching intermediates)⁶⁰ of slightly different lengths through conformation reorganization.

A model for the rhodopsin chromophore (*11-cis*) was constructed with the two anchors affixed in place.⁵⁸ The restricted chromophore was then converted to all other isomers of the chromophore. Positions of the carbon atoms in the two-dimensional projections of all isomeric chromophores were marked. Figure 5 shows the result of this exercise. Summation of the carbon atoms for all active isomers (solid circles) should approximate the shape of the binding site of opsin and the van der Waals radii of the outer carbons should define the perimeter of the binding site (Figure 5).

Since the all-*trans* and the 13-*cis*-retinyl chromophores (marked in triangles and circles)

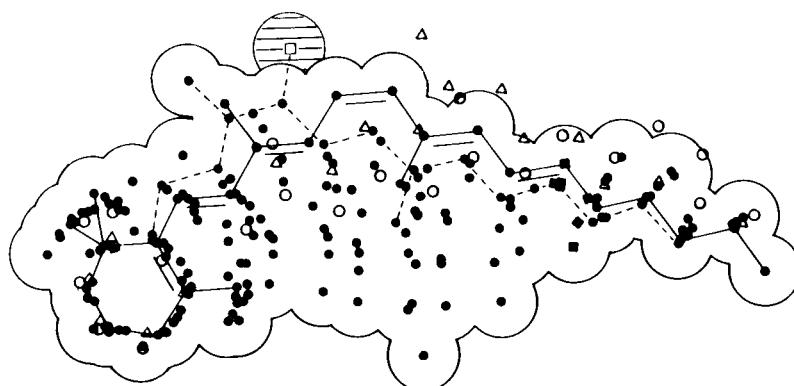


FIGURE 5. A two-dimensional projection map for the binding site of opsin constructed according to Liu et al.⁵⁸ The solid dots are projected positions of carbon atoms of all isomeric rhodopsin chromophores. The perimeter is the summation of van der Waal radii of outermost carbon atoms. The triangles and circles are those of the 13-cis and all-trans isomers. The shaded area is the proposed expanded binding site due to results of 10-substituted analogs (see text).

can also be constructed within the restriction of the lengthened critical distance, their failure to interact with opsin cannot be due to the length of the tethered chromophore. However, their two-dimensional projections did reveal the existence of a region uniquely occupied only by atoms in these two nonbinding isomers. This region, corresponding to positions occupied by C₁₂-C₁₅ atoms of the two isomers, clearly protrudes beyond the perimeter of the binding site. Hence, it was suggested that their failure to interact with opsin is due to a blocking group(s) from the protein already occupying this region. Being proximal to the protonated imino nitrogen, this region is likely to be occupied by a counterion. Indeed, its approximate area (that of three second row atoms) implicates a carboxylate ion.⁵⁸

This analysis agrees with the current notion that Asp-83 is likely to be the counterion to Lys-296.⁶¹ Furthermore, the bidentate nature of the carboxylate ion makes it not only a candidate for a counterion to the imino nitrogen, but, also possibly, the origin of the second point charge nearby C₁₂,⁶² as postulated by the Columbia group.⁶³ This conclusion that the counterion and the second point charge could be one and the same has since been reached independently by two other groups.^{64,65}

This two-dimensional binding-site map also successfully accounted for the unusual observation that the 11-cis isomer of 12-methylretinal (**11**) gave a negligible amount of a pigment analog, while the 9-cis isomer gave a high yield. The map clearly shows that only for the 11-cis isomer would an alkyl substituent at C₁₂ protrude into the forbidden zone.⁵⁸ The active region in the map also readily accounts for pigment formation for isomers of the structurally related α -retinals^{66,67} and aromatic retinals⁴⁵ (Table 1).

D. REFINING THE BINDING SITE

It is clear that the perimeter of the binding site as determined by the stereoisomers of retinal is limited by the atoms present in these isomers. Conceivably the perimeter at several places could be larger than what is indicated. Indeed, in a recent study of 10-substituted retinals and their pigment analogs, such a situation was shown to exist.³⁸

9-cis-10-Fluororetinal was found to give a high yield of a pigment analog.^{38,68} This is not unexpected because the fluorine substituent is only slightly (~20%)⁶⁹ larger than a hydrogen. But the 10-methyl- and 10-chloro-9-cis-retinals with the large substituents protruding beyond the perimeter in Figure 5 also gave moderately high yields of pigment analogs.³⁸ Hence, the perimeter near C₁₀ must protrude beyond the region prescribed by

atoms in retinal isomers. The observation that the 9-*cis* isomer for 19,19-dimethylretinal (9-isopropyl) also yielded a pigment analog⁷⁰ appears to be consistent with this suggestion. Hence, it was proposed that the binding site of opsin should be enlarged near this region as indicated in Figure 5.³⁸ Parallel studies should be extended to other regions to determine whether further expansion of the binding site is necessary.

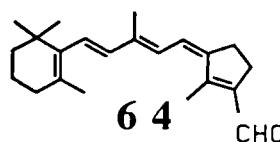
The 11-*cis* isomers of 10-methyl- and 10-chlororetinal also behave in an unexpected manner, giving pigment yields lower than the 9-*cis* isomer (Table 1). This is not predicted based on the proposed shape of the binding site. A possible answer to this anomaly was provided by results of molecular mechanics calculations of the hindered retinal isomers.³⁸ It was found that increased steric crowding between the 10-substituent and 13-methyl group caused an increased nonplanarity of the polyene chromophore by a simultaneous twist of the 10,11- and the 12,13-single bonds. The two-dimensional projection maps in Figure 5 do not reflect such a change of molecular shape. We suspect the twisted chromophore has resulted in an overlap of a portion of the molecule with the protein side chains above and below the chromophore (i.e., restrictions of the binding site in the third dimension). It is hoped that with accumulated data on binding interactions with different retinal analogs, especially those with defined molecular shape, such restrictions can be better identified.

More recently, we have attempted to apply the technique of molecular modelling to map the binding site of opsin in all three dimensions. A preliminary attempt to depict the cavity as occupied by four active isomers of retinals (11-*cis*, 9-*cis*, 7-*cis*, and 7,9-di-*cis*) is shown in Figure 6.⁷¹

E. OTHER LIMITATIONS WITHIN THE BINDING SITE OF OPSIN

The ever increasing data base of the retinal analogs is likely to lead eventually to a complete understanding of factors determining the selectivity of the binding site of opsin. While at this stage it is premature to make such an analysis, it will nevertheless be of interest to identify other restrictions within the binding site, as revealed with data derived from currently available analogs.

The longitudinal restriction of the binding site of opsin alluded to in a previous section has a lower as well as an upper limit.⁵⁷ Hence, the C₁₇-aldehyde **42**⁵⁷ and the related linear polyenals **43** and **44**⁷² (Figure 4) did not form stable pigments with opsin. Along the polyene chain, the accumulated data suggest that the region nearby the protonated Schiff base is sterically congested. Hence, while 11-*cis*-14-methylretinal was reported to form a stable pigment,⁵ 11-*cis*-14-ethylretinal did not.⁷⁰ Similarly 20-propylretinal (13-butylretinal, **46**) was found to be inactive. Interestingly, the slightly more constrained ring-fused analog **64** was reported to be active.⁷³



The binding site surrounding the middle portion of the polyene chain is also sensitive to steric and electronic perturbation. The effect of substitution at C₁₀ has been discussed in an earlier section. Substituent variations at the C₉ position has afforded some interesting results. In his pioneering work with retinal analogs, Kropf⁴ reported the successful regeneration of rhodopsin analogs using 19-methyl ("9-ethyl")- and 19-propyl ("9-butyl")-retinals. Nakanishi and co-workers have also prepared a visual pigment analog wherein a potential photoaffinity label, the diazoacetoxy reporter group, is appended to the C₉ position.⁷⁴ We expanded the C₉ manifold of analogs to include the 19,19-dimethyl ("9-isopropyl")- and 19-demethyl-9-phenylretinals, **21** and **48**, respectively. While the former readily

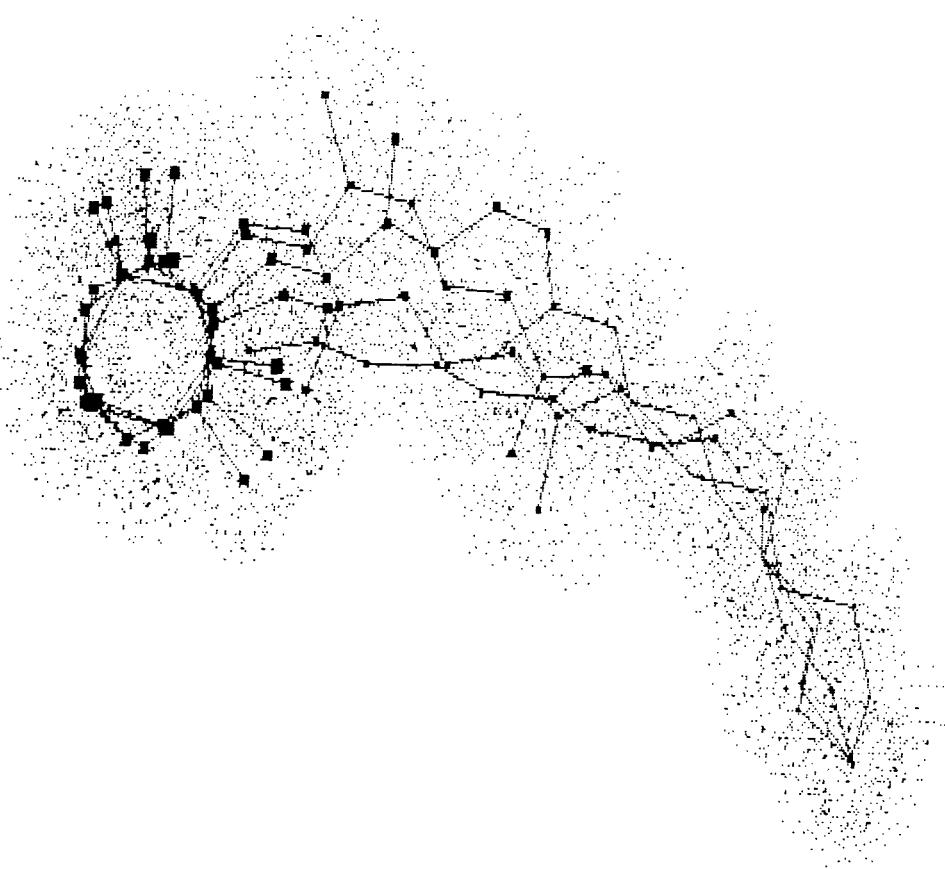


FIGURE 6. A molecular modelling (CHEMGRAF) representation of the binding site of opsin as revealed by rhodopsin, 9-*cis*-rhodopsin, 7-*cis*-rhodopsin and 7,9-di-*cis*-rhodopsin. The minimized structure of 11-*cis*-retinyl Schiff base was constructed using a minimized structure of 11-*cis*-retinal (MM-II-85), followed by the attachment of a minimized pentyl group. The structures for the other isomeric chromophores were created in a similar manner but adding the restriction of maximum possible overlap of the last atom of the pentyl group (the α -carbon of lysine) and the six ring carbons. The resultant structures have energies within 2.0 kcal/mol of the minimum energy of each isomeric chromophore.⁷¹

formed a visual pigment analog, the latter was unreactive to the apoprotein.⁷⁰ This result is a probable manifestation of conformational effects (i.e., deviations from planarity) brought about by the substantial resonance interaction of the aromatic ring with the polyene side chain as well as possible steric interaction with the protein itself. Additional work must be done to clarify the "space available" in this particular region of the binding site. The negative results with the fluorinated analogs **52** to **54**⁷⁵ indicate the sensitivity of this region of the binding site to electronic perturbation. Several ring-fused compounds (**55** to **58**) were prepared in conjunction with the predicted 10-*s-cis*- or 12-*s-cis*, all-*trans* geometries for photobleaching intermediates of rhodopsin,^{60,76} which understandably were shown to be incompatible with the binding site.^{41,70}

Lastly, a series of α - and β -retinal analogs modified at the C₅ position have been prepared and, in some cases (**19**, **20**, and **33** to **36**), successfully incorporated into the apoprotein. The results clearly indicate that there is substantial space available for a hydrocarbon reporter group in this region of the binding site of rhodopsin. There is, as to be expected, a finite limit to the size of the C₅-alkyl substituent that the apoprotein will recognize and accept. Thus, when a *n*-decyl group is attached to the C₅ position, there is no artificial visual pigment

TABLE 2
Quantum Yields of Photobleaching of
Bovine Rhodopsin Analogs⁸¹

Rhodopsin Analog	Quantum Yield ^a	
	11-cis	9-cis
Rhodopsin	0.67 ^b	0.2, ^c 0.24, 0.22 ^d
10-F-Rdp	0.65	0.09
10-Cl-Rdp	0.33	0.07
10-CH ₃ -Rdp	0.32	0.08
8-F-Rdp	0.6	0.22
14-F-Rdp	0.51	0.40

^a ± 20%.

^b Data from Dartnall, H.J.A., *Handbook for Sensory Physiology*, Vol. 7. Part 1, Springer, Berlin, 1972, 122.

^c Data from Hubbard, R. and Kropf, A., *Proc. Natl. Acad. Sci. U.S.A.*, 44, 130, 1958.

formation.⁷⁰ A phenyl substituent in the C₅ position of α-5-demethyl-5-phenylretinal **51**, as at C₉ led to an unacceptable substrate for the apoprotein.⁷⁰ Conformational flexibility of the hydrophobic pocket surrounding the trimethylcyclohexenyl ring is also indicated by its ability to accommodate analogs containing the adamantyl end group⁷⁷ and a diazoacetoxy photoaffinity-labeling group at C₃.⁷⁴ However, the negative result⁴⁵ with the trimethoxyphenyl derivative (**59**) reveals limitations of this flexibility.

For several of the systems that failed to form stable pigments we have detected the formation of a blue-shifted pigment (λ_{\max} 405 to 415 nm), which is only moderately stable in hydroxylamine. Such a pigment probably accounts for the inhibiting effect of many retinal analogs toward rhodopsin formation.⁴⁵ Recently, for other systems (e.g., 9-trans-locked **24** and 5-substituted α-retinals) we have detected formation of a prepigment with a λ_{\max} in the same region, which subsequently converts to the more stable red-shifted pigment analog.⁷⁸ (The same observation has been made by the Columbia group in their reinvestigation of 9,10-dihydrorhodopsin.)⁷⁹ It will be of interest to determine whether the prepigment is the unprotonated Schiff base or an unperturbed protonated Schiff base.

F. FLUORINATED RHODOPSINS. SPECIFIC PROTEIN-SUBSTRATE INTERACTION AND ¹⁹F NMR

The use of the ¹⁹F label for NMR studies of protein-substrate interactions is a well established technique.⁸⁰ The advantages of such an NMR label (small size, high sensitivity, and the absence of background noise) are, however, occasionally counter-balanced by altered protein-substrate interactions due to the high electronegativity of fluorine atoms. Hence, to examine the possible application of such techniques to studies of visual pigment analogs the existence of fluorinated analogs with unaltered properties must first be demonstrated.

Recent success in the synthesis of vinylic^{33,36} and allylic (trifluoromethylated)^{23,28} fluorine-labeled retinals (see above) allows the preparation of such labeled pigment analogs. Their photochemical behavior has been examined in an attempt to detect possible altered properties in fluorinated rhodopsin. While many analogs have similar photochemical and bleaching characteristics, we have also come upon a new form of specific protein-substrate interaction that could provide useful information concerning the orientation of the chromophore in the binding site.

The quantum yields of photobleaching of several vinylic labeled fluororhodopsins were measured at room temperature.⁸¹ The data (Table 2) were compared with isomers of the parent rhodopsin and other related analogs.⁸¹ The following trends were noted.

First, the reactivities of 8-, 10-, and 14-fluorinated 11-*cis*-rhodopsins appear to be unaffected, all approaching that of the parent rhodopsin. In addition, in a separate study⁸² it was shown that the low-temperature photobleaching characteristics of 12-fluororhodopsin are virtually identical to those of rhodopsin. Therefore, a fluorine substituent appears to have little effect on isomerization at the 11,12-bond. However, the quantum yield of isomerization of 9-*cis*-10-fluororhodopsin is noticeably lower than that of 9-*cis*-rhodopsin, while those of 8-fluoro- and 14-fluoro-9-*cis*-rhodopsins remain as high or higher than that of 9-*cis*-rhodopsin. Therefore, the reduced quantum yield of reaction of 9-*cis*-10-fluororhodopsin is a regiospecific effect, observable only for a fluorine atom at the 10-position for isomerization of the 9,10-bond.

Recent studies on intermolecular or intramolecular H,F-hydrogen bonding in small molecules in solution⁸³ and in crystal⁸⁴ indicate the definitive presence of such, albeit weak, interactions. Hence, the regiospecific interaction in 9-*cis*-10-fluororhodopsin was probably due to interaction between the 10-fluoro substituent and an acidic protein residue of opsin.⁸¹ This suggestion is consistent with Hargrave's tertiary structure of opsin⁶¹ which shows the presence of only three carboxyl-bearing amino acid residues in the seven helices comprising the binding site. Because Asp-83 in helix-2 is generally considered the counterion to the imino nitrogen, only the two glutamic acid units (122 and 134) in helix-3 are possible acidic proton sources. Indeed, helix-3 lies adjacent to the 9,10-bond,⁶¹ making one of these amino acidic residues ideally located for interaction with F₁₀.

It should be noted that H,F-interactions of this type are estimated to be small, of the order of 2 to 3 kcal/mol.⁸³ Therefore, it was not surprising that previous NMR studies did not lead to conclusive evidence for such interactions in other fluorine-labeled proteins.⁸⁰ However, photobleaching of rhodopsin analogs involves short-lived excited singlet-state species. Its efficiency could easily be perturbed by a weak environmental effect.⁸⁵ Hence, in this situation we believe photochemistry has become a sensitive handle for detection of a weak specific protein-substrate interaction. While independent low-temperature spectroscopic studies have confirmed the unusually low reactivity of 9-*cis*-10-fluororhodopsin,⁸⁶ it will be of great interest to obtain other corroborative evidence (such as FT-IR)⁸⁷ on the postulated H,F-interaction.

While Glu-122 and 134 are likely sources of the acidic proton, it should be recognized that these units may exist as part of an ion-pair by interacting with the nearby basic units of Trp-126 and Arg-135, which, in turn, may act as the proton donor. Such ion-pairs have been discussed in other studies.⁸⁸

¹⁹F NMR spectra of labeled visual pigments are complicated, however, by the broad peaks associated with detergent-solubilized samples. For example, for 12-fluororhodopsin, its peak width (recorded at 141 MHz) was in excess of 120 Hz.⁸² This line width unfortunately obscures meaningful conformational and configurational structural information of the protein. However, the chemical shift data, of which ¹⁹F nuclei exhibit an unusually wide range (hence, sensitivity to environmental effects), are unaffected by the line width problem. For example, the ¹⁹F signal for 12-fluororhodopsin appears at 25-ppm lower field than that of the photobleached product.⁸² Because ¹⁹F chemical shifts of these polyenes are rather insensitive to the nature of the end group (aldehyde, Schiff base, or protonated Schiff base),⁸⁹ the large Δδ must be due to local protein perturbation.⁹⁰ The direction of the shift suggests decreased shielding around F₁₂, which is in agreement with the postulate of the presence of a second point charge nearby C₁₂.⁶⁸ Such a negatively charged species should lead to polarization of the C-F bond with a decrease of electron density around F₁₂.

For 9-*cis*-10-fluororhodopsin it was found that the pigment peak showed an upfield shift of 14 ppm from that of the oxime of the photobleached product.⁹⁰ A shift in the direction opposite to that of 12-fluororhodopsin is consistent with the notion of the presence of a nearby positively charged species. This observation coupled with the photochemical prop-

erties of 9-*cis*-10-fluororhodopsin⁸¹ led to the suggestion of a protonated basic amino acid residue nearby F₁₀ such as in an ion-pair.⁹⁰

These preliminary ¹⁹F NMR data show that despite the difficulty of excessive line width, the method could provide useful information related to protein substrate interactions in visual pigments. With many pigments already available, it is anticipated that more useful information will be forthcoming in the near future. Also, it will be of interest to see whether the magic angle spinning technique, successfully employed in studies of ¹³C-labeled visual pigments,⁹² can be applied to fluororhodopsins. The closeness of ¹⁹F resonance frequency with ¹H, however, is expected to make this technique less routine for ¹⁹F than for ¹³C.

ADDENDUM

Several relevant references have since appeared after the closing date of the manuscript. A reexamination of the chromophore configuration of pigments formed during reconstitution of 9,13-di-*cis*-retinal with opsin by the chromophore extraction/HPLC method revealed an extensive isomerization (from 70 to 100%) of the di-*cis* geometry to the more stable 9-*cis* pigment.⁹³ This observation contradicts those reported by Crouch et al.⁴⁸ but is in agreement with the earlier observation by Wald et al.¹ By the same procedure, the extent of retention of chromophore configuration for three other di-*cis*-retinals has been determined, ranging from complete retention of configuration for the 7,9-di-*cis* isomer (0% isomerization) to partial isomerization for the 9,11-di-*cis* isomer (60%) and nearly complete isomerization for the 7,13-di-*cis* isomer (>97%).⁹⁴

The approach of mapping the two-dimensional shape of the binding site of rhodopsin has since been extended to all three dimensions using computer assisted molecular modeling.⁹⁵ The flexibility of the binding site is also reflected in successful preparation of several isomers of a highly branched ring opened rhodopsin analog,⁹⁶ a C-19 all-*trans*-rhodopsin analog⁹⁷ and the 11-*cis* isomer of naphthalene analog (437 nm, +)¹⁰⁰ of which its retinochrome analogs are in the literature.⁹⁸

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Chapter 4

SYNTHESIS AND APPLICATION OF RETINOIDS AND RELATED COMPOUNDS TO VISION RESEARCH AND CANCER STUDIES**Masayoshi Ito****TABLE OF CONTENTS**

I.	Introduction	78
II.	Synthesis of Retro- γ -Retinals	78
III.	Synthesis and Photochemical Reaction of 9- <i>cis</i> -Retro- γ -Rhodopsin	78
IV.	Synthesis of Retinal Analogs Having the 11- <i>cis</i> -Locked-Cyclopentatrienylidene Structure	79
V.	Synthesis and Photochemical Reaction of 11- <i>cis</i> -Locked-Cyclopentatrienylidene Rhodopsin	81
VI.	Circular Dichroism (CD) Spectrum of Rhodopsin	83
VII.	The Chromophore of the Fly Visual Pigment	86
VIII.	Synthesis of Retinoidal Butenolides.....	89
	Acknowledgments	93
	References.....	94

I. INTRODUCTION

It is generally accepted that vitamin A is particularly important for the promotion of general growth, the maintenance of visual function, the regulation of proliferation and differentiation of epithelial tissues, and reproduction. The mode of action of retinol (vitamin A) and retinal (vitamin A aldehyde) in the visual cycle was elucidated by Wald.¹ The visual pigment rhodopsin (**1**) (Scheme 1) has been shown to contain 11-*cis*-retinal (**2**) which is bound via a protonated Schiff-base linkage to the ϵ -amino group of a specific lysyl residue of the apoprotein opsin.^{2,3}

The photobleaching process of rhodopsin has been well investigated so far. In the first bleaching steps, rhodopsin is converted to bathorhodopsin at liquid-nitrogen temperature by absorbing a photon.⁴ A number of previous studies on the primary step of the bleaching process proposed mainly two kinds of hypotheses to explain the process:

1. A proton translocation mechanism⁵ (bathorhodopsin formed from rhodopsin by translocation of a proton at the C₄-position to the Schiff-base nitrogen without any *cis-trans* isomerization).
2. Isomerization mechanism⁶ (an 11-*cis* to 11-*trans* isomerization resulting in the formation of bathorhodopsin from rhodopsin).

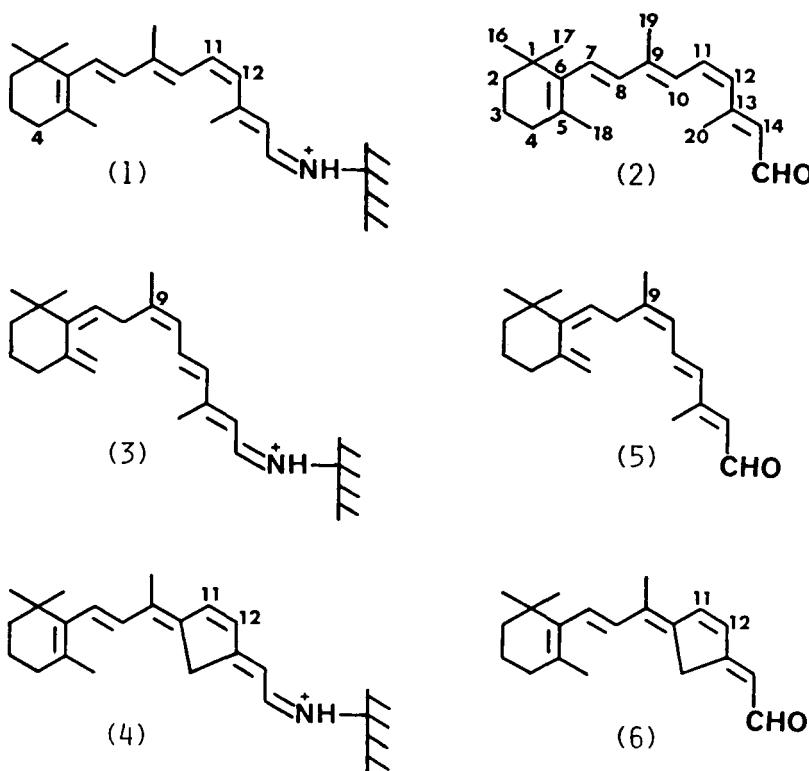
In order to clarify the primary photochemical event by chemical methods, two types of rhodopsin analogs (**3** and **4**) were synthesized. The former (**3**) was derived from 9-*cis*-retro- γ -retinal (**5**) having two dissected chromophoric systems, namely, one diene and one trienal chromophore. The latter (**4**) was prepared from an 11-*cis*-locked-cyclopentatrienylidene retinal analog (**6**). If the proton translocation mechanism is correct, retro- γ -rhodopsin (**3**) should not form the corresponding batho-product on irradiation at liquid-nitrogen temperature. If the *cis-trans* isomerization hypothesis is correct, retro- γ -rhodopsin (**3**) might produce the corresponding batho-product and 11-*cis*-locked-cyclopentatrienylidenerhodopsin (**4**) should yield no batho-intermediate.

II. SYNTHESIS OF RETRO- γ -RETINALS

Five isomers [all-*trans*- (**7**), 11-*cis*- (**8**), 9-*cis*- (**5**), 13-*cis*- (**9**), and 9,13-di-*cis*- (**10**)] of retro- γ -retinal were synthesized according to the route shown in Scheme 2.^{7,8} Irradiation of methyl β -ionylideneacetate (**11**) with a high pressure (500W) Hg lamp using a Pyrex filter gave a mixture of 9-*trans*- (**12**) and 9-*cis*- (**13**) isomers of methyl retro- γ -ionylideneacetate.^{9,10} The separated 9-*trans* isomer (**12**) was converted by reduction with Red-Al followed by treatment with triphenylphosphonium bromide ($\text{Ph}_3\text{P}^+\text{HBr}$) into the Wittig salt **14**. Wittig condensation (KOH) of this salt (**14**) with *n*-butyl γ -oxoseneioate¹¹ gave *n*-butyl retro- γ -retinoate (**15**), which was transformed by reduction (LAH or Red-Al) and oxidation (MnO_2) to a mixture of retro- γ -retinal isomers, the chromatographic purification of which led to the all-*trans*- (**7**) and the 11-*cis*- (**8**) isomers. Similarly, 9-*cis*-retro- γ -retinal (**5**) was prepared from the corresponding isomer (**13**). Photoisomerization of **7** and **5** by a fluorescent lamp (30 W) produced the 13-*cis* isomer (**9**) and the 9,13-di-*cis* isomer (**10**), respectively. Characteristic spectral data for the retro- γ -retinals are summarized in Table 1.

III. SYNTHESIS AND PHOTOCHEMICAL REACTION OF 9-CIS-RETRO- γ -RHODOPSIN

An artificial visual pigment, 9-*cis*-retro- γ -rhodopsin (**3**, 420 nm) was prepared from 9-*cis*-retro- γ -retinal (**5**, 339 nm) and cattle opsin (0.7% digitonin solution).^{7,12} The photo-



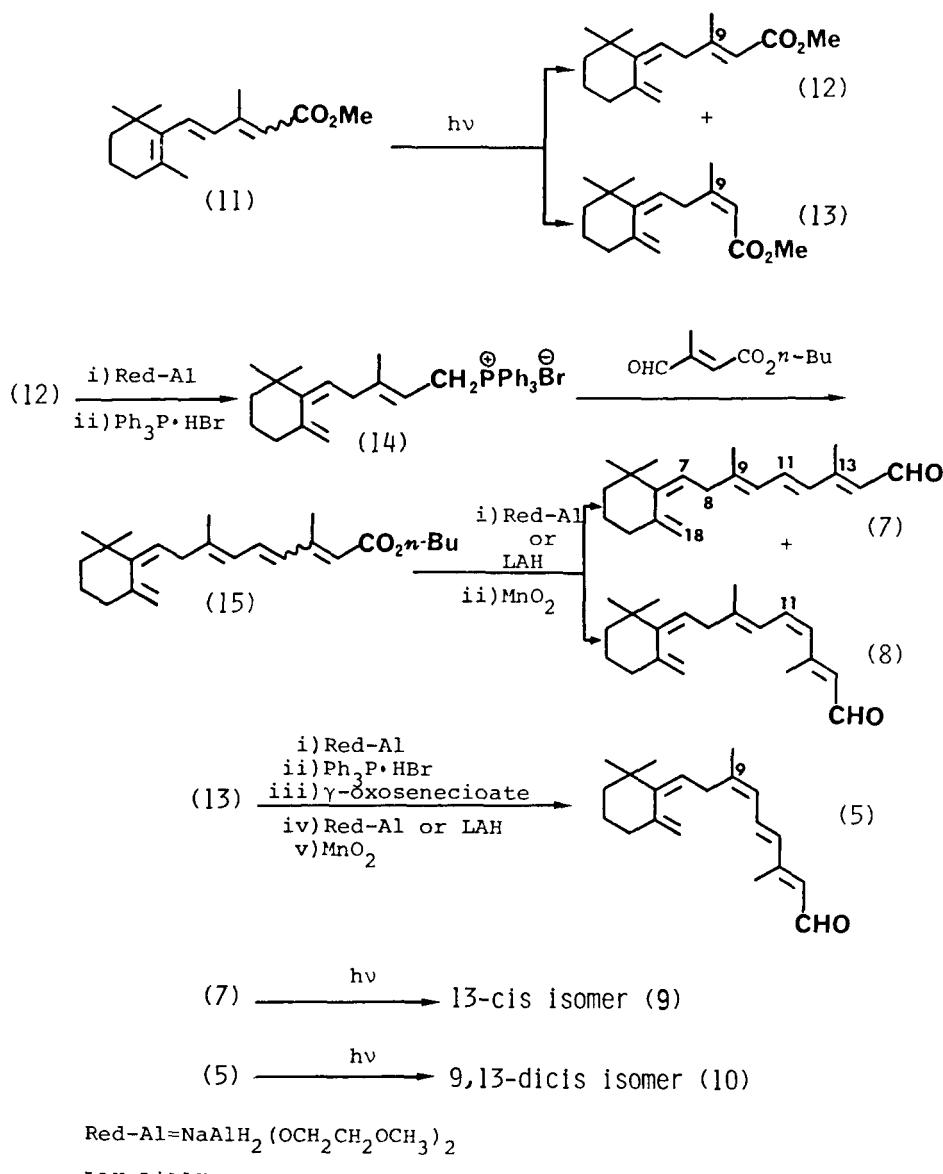
SCHEME 1

chemical reaction of this rhodopsin analog at -185°C showed the formation of the corresponding batho-product. This was the first example that the artificial visual pigment possessing the dissected chromophore underwent the photochemical reaction. From its photochemical behavior, it was inferred that the conversion of rhodopsin to bathorhodopsin did not involve the proton translocation mechanism but was caused by the isomerization of the 11,12-*cis*-double bond to *trans* geometry. In the same manner as retro- γ -rhodopsin, the 5,6-epoxy- and 5,8-epoxy-rhodopsin analogs **16a,b**¹³ and **17**¹⁴ (Scheme 3) were also prepared from the corresponding retinals^{15,16} with cattle opsin.

IV. SYNTHESIS OF RETINAL ANALOGS HAVING THE 11-CIS-LOCKED-CYCLOPENTATRIENYLIDENE STRUCTURE

In order to obtain direct evidence for the isomerization mechanism by chemical means, the 11-*cis*-locked rhodopsin analog **4** was prepared from cattle opsin (2% digitonin solution) and the 11-*cis*-locked-cyclopentatrienylidene retinal analog **6** having an 11-*cis*- and 12s-*trans*-fixed retinylidene chromophore in the five-membered ring.^{17,18} The 11-*cis*-locked-retinals **6**, **18**, **19**, **20**, and **21** were obtained by a short-path synthesis from the β -ionyl sulfone **22**,¹⁹ as shown in Scheme 4.

Reaction of β -ionyl sulfone **22** with 4-acetoxycyclopentenone **23**²⁰ using LDA gave two β -ionylidenecyclopentenone isomers **24** and **25**, which were cleanly separated by low-pressure liquid chromatography. Horner-Emmons reaction (*n*-BuLi) of the 9-*trans* isomer (**24**) with diethyl cyanomethylphosphonate (**26**) and subsequent reduction (DIBAH) of the resulting nitrile at -70°C led to a mixture of the 11-mono-*cis*-aldehyde **6** and its 11,13-di-*cis* isomer **18**. Each unstable isomer was purified by preparative TLC (pTLC) followed by

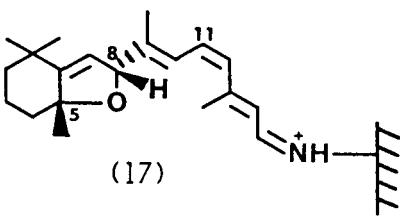
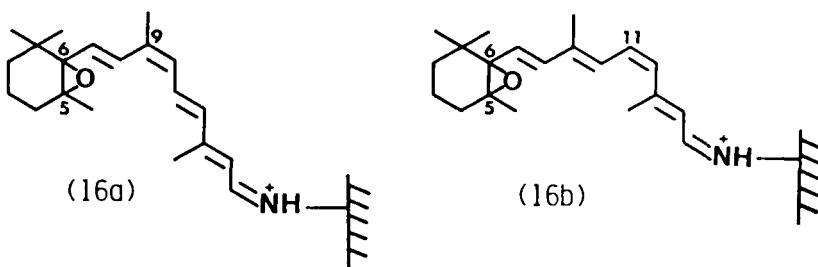


SCHEME 2

HPLC in the dark. Using a similar procedure, the 9,11-di-*cis*-aldehyde **19** was produced as the main product from the corresponding 9-*cis* isomer **25**. The structures of the three products were confirmed on the basis of their UV and ¹H NMR spectral data (Table 2). The three isomers have absorption maxima at longer wavelengths (ca. 20 nm) than that (380 nm) of all-*trans*-retinal. This suggests that the former have greater chromophoric coplanarity. This behavior is particularly notable for the 11-mono-*cis* isomer **6**, the UV maximum of which is at 30 nm longer wavelength than that (375 nm) of 11-*cis*-retinal (**2**). This is the first example of a retinal analog having the same chromophore as **2**, and indicates that **6** has a considerable degree of coplanarity in its conjugated structure. In addition, ¹H NMR spectral data (Table 2) confirm that the 11-*cis*-locked-retinal **6** has a high degree of coplanarity, particularly in the C₉-C₁₄ triene region. This is further supported by MO calculations.²¹

TABLE 1
Characteristic Spectral Data For Retro- γ -Retinals 5, 7, 8, 9, and 10

	7	8	5	9	10	
UV (EtOH)		339 (13900)				
λ_{max} nm(ϵ)	340 (24300)	228 (8900)	339 (23400)	337 (24000)	335 (18100)	
¹ H-NMR (90 MHz) (δ , CDCl ₃) (<i>J</i> in Hz)	13-Me 18-H ₂ (d, <i>J</i> 3) 4.99 (m) 14-H (d, <i>J</i> 8) 12-H (d, <i>J</i> 15) 11-H (dd, <i>J</i> 15,11) 8-H ₂ (d, <i>J</i> 8) CHO (d, <i>J</i> 8)	2.31 (s) 4.58 (d, <i>J</i> 3) 5.01 (m) 5.99 (d, <i>J</i> 8) 6.28 (d, <i>J</i> 15) 7.04 (dd, <i>J</i> 15,11) 2.97 (d, <i>J</i> 8) 10.14 (d, <i>J</i> 8)	2.38 (s) 4.62 (d, <i>J</i> 3) 5.01 (m) 6.09 (d, <i>J</i> 8) 6.41 (d, <i>J</i> 12) 6.63 (dd, <i>J</i> 12,10) 2.99 (d, <i>J</i> 8) 10.12 (d, <i>J</i> 8)	2.30 (s) 4.64 (d, <i>J</i> 3) 5.09 (m) 5.97 (d, <i>J</i> 8) 6.25 (d, <i>J</i> 15) 6.94 (dd, <i>J</i> 15,11) 3.14 (d, <i>J</i> 7) 10.13 (d, <i>J</i> 8)	2.11 (s) 4.56 (d, <i>J</i> 2.7) 4.97 (m) 5.82 (d, <i>J</i> 8) 7.12 (d, <i>J</i> 15) 6.89 (dd, <i>J</i> 15.5,11) 2.96 (d, <i>J</i> 7) 10.19 (d, <i>J</i> 8)	2.10 (s) 4.62 (d, <i>J</i> 2.8) 5.06 (m) 5.81 (d, <i>J</i> 8.1) 7.15 (d, <i>J</i> 15) 6.91 (dd, <i>J</i> 15,11) 3.12 (d, <i>J</i> 7.3) 10.18 (d, <i>J</i> 8.1)

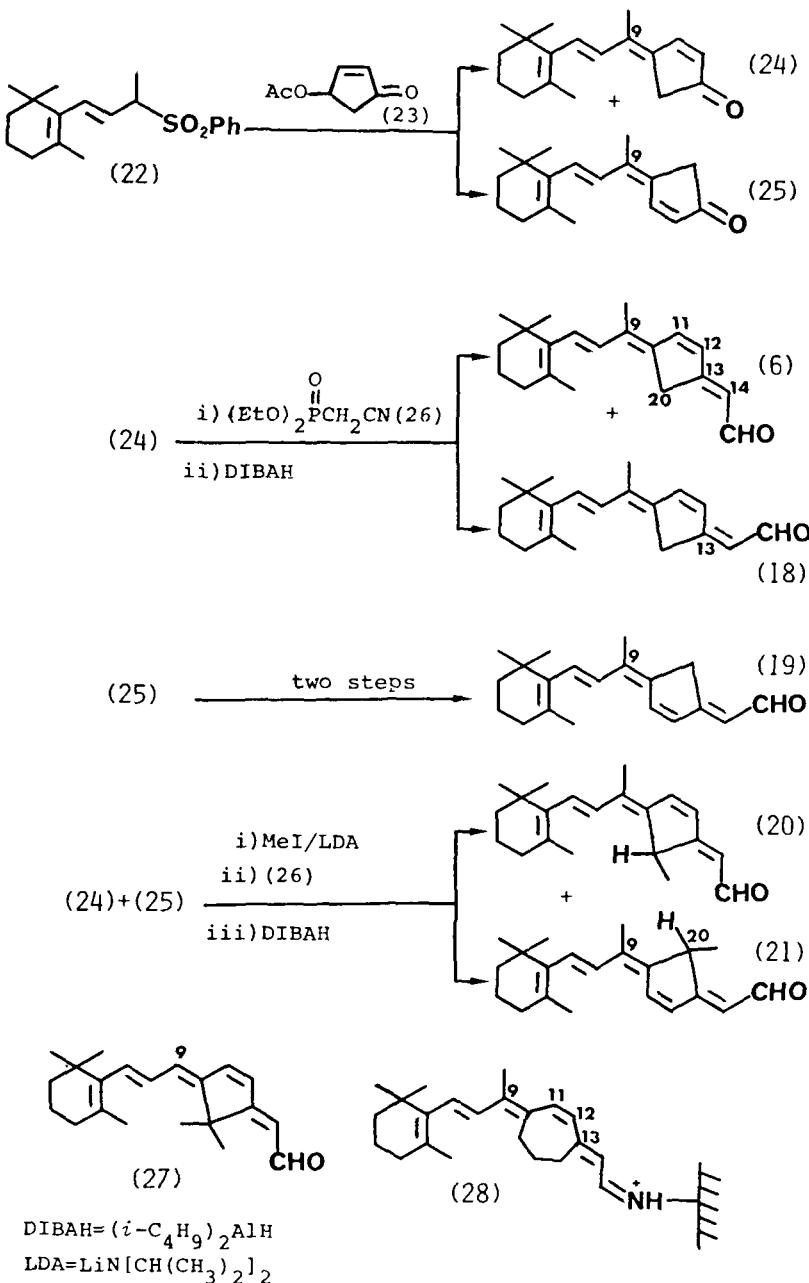


SCHEME 3

The 20-mono-methylated pentaene aldehyde isomers **20** and **21** were derived from a mixture of **24** and **25** as shown in Scheme 4. The 20,20-dimethylated 9-desmethyl analog **27** was synthesized by another route.²²

V. SYNTHESIS AND PHOTOCHEMICAL REACTION OF 11-CIS-LOCKED-CYCLOPENTATRIENYLIDENE RHODOPSIN

In a binding experiment using bovine opsin, **20** and **27** gave no pigment while **6** produced the artificial rhodopsin analog **4** (495 nm) at a rate that was slow compared with that of the regeneration of rhodopsin.²³ This finding suggests that a selective binding of opsin to a



SCHEME 4

twisted 11-*cis*-retinal (**2**) in a 12*s*-transoid form and a spatial restriction in an opsin cavity are strict requirements for the regeneration of rhodopsin.

Irradiation of **4** at -196°C with either blue light or orange light caused no spectral change supporting the *cis-trans* isomerization hypothesis for formation of bathorhodopsin. The same confirmation was also obtained by Prof. Nakanishi's group employing a cycloheptatrienylidene rhodopsin (**28**)^{24,25} (Scheme 4), which was forced into a noncoplanar 9,11,13-triene conformation by virtue of the seven-membered ring.

TABLE 2
**Characteristic Spectral Data For 11-cis-Locked-Cyclopentatrienylidene Retinals 6,
 18, 19, 20, and 21**

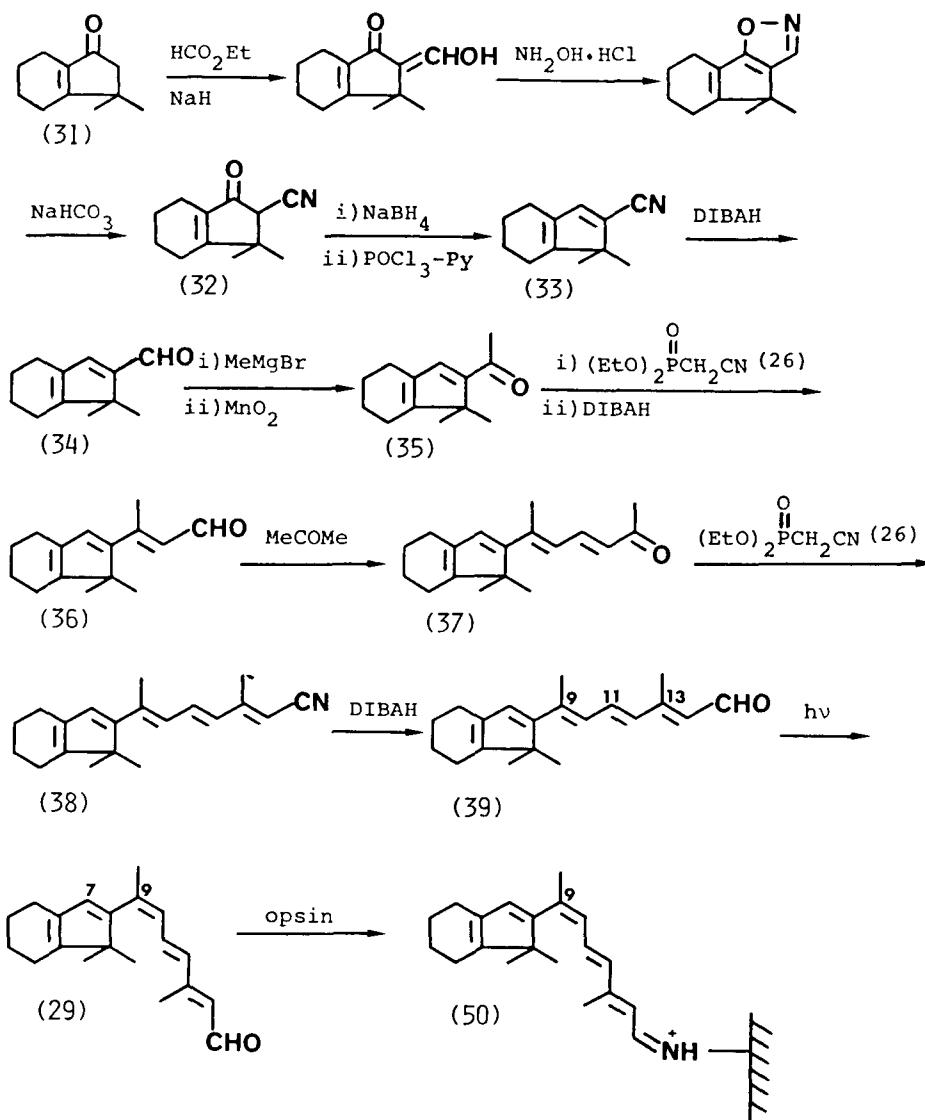
		6	20	19	21	18
UV (EtOH) λ_{\max} nm (ϵ)		405 (18500) 263 (13300)	400 260	402 (11500)	395	392 (10000)
¹ H-NMR (200 MHz) (δ , CDCl ₃) (<i>J</i> in Hz)	9-Me	2.05 (s)	2.05 (s)	2.02 (s)	2.06 (s)	2.05 (s)
	20-Me	—	1.35 (d, <i>J</i> 7)	—	1.38 (d, <i>J</i> 7)	—
	20-H	3.73 (d, <i>J</i> 1.5)	4.04 (q, <i>J</i> 7)	3.70 (d, <i>J</i> 2)	3.98 (q, <i>J</i> 7)	3.48 (s)
	14-H	5.97 (td, <i>J</i> 7,1.5)	5.89 (d, <i>J</i> 8)	5.98 (td, <i>J</i> 7,2)	5.90 (d, <i>J</i> 8)	5.88 (dd, <i>J</i> 8,1.5)
	12-H	6.61 (d, <i>J</i> 5)	6.49 (d, <i>J</i> 5)	6.59 (d <i>J</i> 5.5)	6.48 (d, <i>J</i> 5)	7.28 (d, <i>J</i> 5.5)
	11-H	7.27 (d, <i>J</i> 5)	7.16 (d, <i>J</i> 5)	7.36 (d, <i>J</i> 5.5)	7.25 (d, <i>J</i> 5)	7.36 (dd, <i>J</i> 5.5,1.5)
	8-H	6.42 (d, <i>J</i> 16)	6.37 (s-like)	6.62 (d, <i>J</i> 16)	6.60 (d, <i>J</i> 16)	6.33 (s-like)
	7-H	6.32 (d, <i>J</i> 16)	6.27 (d, <i>J</i> 16)	6.28 (d, <i>J</i> 16)	6.28 (d, <i>J</i> 16)	6.28 (s-like)
	CHO	9.88 (d, <i>J</i> 7)	9.90 (d, <i>J</i> 8)	9.89 (d, <i>J</i> 7)	9.91 (d, <i>J</i> 8)	9.99 (d, <i>J</i> 8)

VI. CIRCULAR DICHROISM (CD) SPECTRUM OF RHODOPSIN

The clarification of the origin of the induced CD of rhodopsin would be of particular interest for the conformational analysis of the photobleaching intermediates of rhodopsin. So far, two general mechanisms for the induced chiral activity of rhodopsin have been proposed, namely, a twisted chromophore,²⁶⁻²⁸ and a dipole-dipole interaction in the rhodopsin molecule.²⁹⁻³¹

The CD spectrum of **4**, which has a nontwisted conformation around a 12s-*trans* bond, showed a negligible α -band.²³ A comparison of the CD data for rhodopsin and the cycloheptatrienylidene rhodopsin (**28**)²⁴ with our results showed that the presence of the α -band in the induced CD spectrum of rhodopsin is due to the twisted 12s-bond in the chromophore. Subsequently, in order to investigate the origin of β -CD band of rhodopsin, two kinds of 9-*cis*-bicyclic retinal analogs-I (**29**)³² and -II (**30**),³³ possessing a conformationally 6s-*cis*-fixed retinal chromophore, were synthesized (Schemes 5 and 6). The bicyclic retinal analog-II (**30**) has a C₈-C₁₈ bonded structure bearing a *gem*-dimethyl group at the C₄-position instead of at the C₁-position compared to 9-*cis*-retinal, whereas the bicyclic retinal analog-I (**29**) has a *gem*-dimethyl group at the C₁₈-position to retain a conjugated double bond in the cyclopentadiene ring.

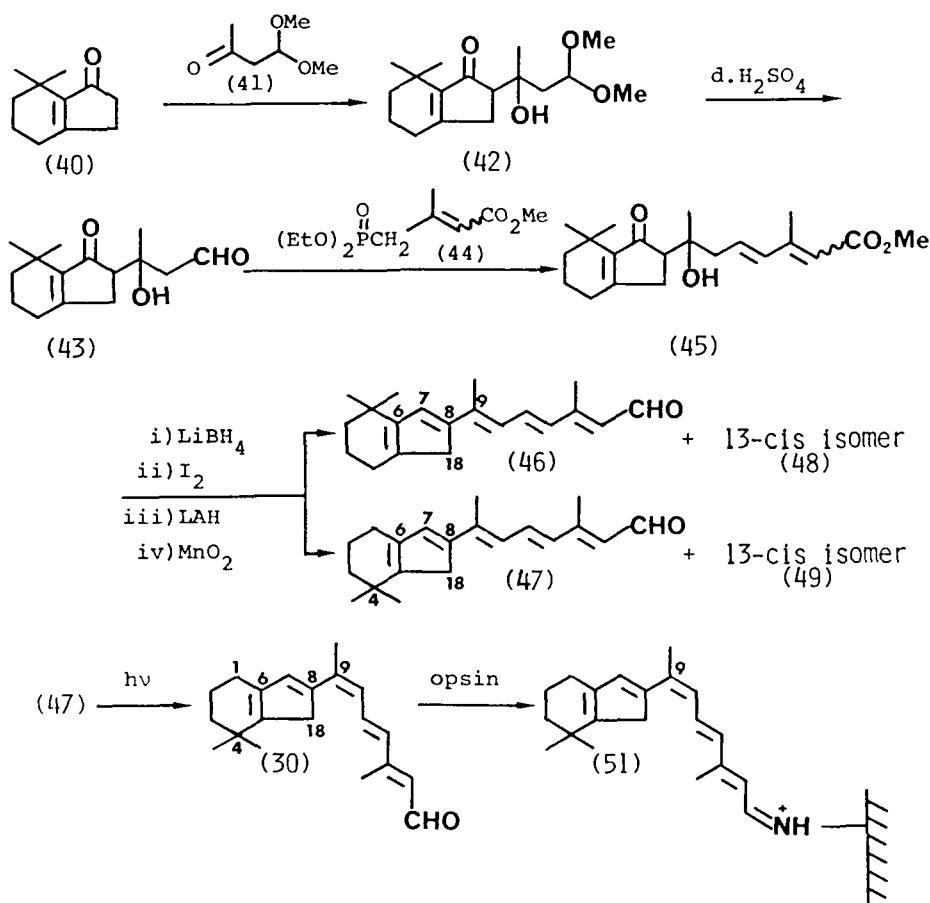
The keto-nitrile **32** was prepared from tetrahydroindanone derivatives **31** via the isoxazole intermediate (Scheme 5). NaBH₄ reduction of **32** followed by dehydration with POCl₃-pyridine led to the diene-nitrile **33**, which, in turn, was converted to the diene-aldehyde **34**^{34,35} by DIBAH reduction. Grignard reaction of **34** with methylmagnesium bromide and a subsequent MnO₂ oxidation provided the diene-ketone **35**, which was transformed into the *trans*-triene aldehyde **36**³⁶ by condensation (*n*-BuLi) of **35** with cyanomethyl phosphonate **26** followed by DIBAH reduction. Aldol condensation (NaOH) of 9-*trans*-aldehyde **36** with excess acetone gave the all-*trans*-ketone **37**, which was converted by a Horner-Emmons reaction (*n*-BuLi) with **26** into the pentaene-nitrile **38**. Reduction of the nitrile group (DIBAH), followed by chromatographic purification (pTLC and HPLC) in the dark, afforded



SCHEME 5

the all-*trans*-bicyclic retinal analog-I (**39**)³² the stereochemistry of which was determined from the spectral data (Table 3). The electronic absorption maximum of **39** is at a much longer wavelength than that (380 nm) of all-*trans*-retinal, showing that the bicyclic retinal analog-I (**39**) has a highly coplanar conjugated system. Irradiation of the all-*trans* aldehyde **39** in MeOH using a high-pressure (300 W) Hg lamp with a Pyrex filter produced an isomeric mixture (all-*trans*:9-*cis*:9,13-di-*cis* = 7:20:3), pTLC and HPLC of which gave the new 9-*cis* (**29**) and 9,13-di-*cis* isomers in pure form. The structure of 9-*cis* isomer **29** was characterized spectroscopically (Table 3). The upfield shift of the ^1H NMR resonances in **29** relative to those of **39** and the hypsochromic shift of the UV absorption maximum (370, 335 nm) in **29** suggest that the single bonds in its chromophore are strongly twisted.

The 9-*cis*-bicyclic retinal analog-II (**30**)³³ was prepared according to the route in Scheme 6. Aldol condensation (LDA) of the bicyclic pentenone **40**³⁷ with the methyl ketone-acetal **41** gave the hydroxy-acetal **42** as a mixture of diastereoisomers, which, without separation,



SCHEME 6

TABLE 3
Characteristic Spectral Data For Bicyclic Retinal Analogs-I 39 and 29 and Bicyclic Retinal Analogs-II 46, 47, and 30

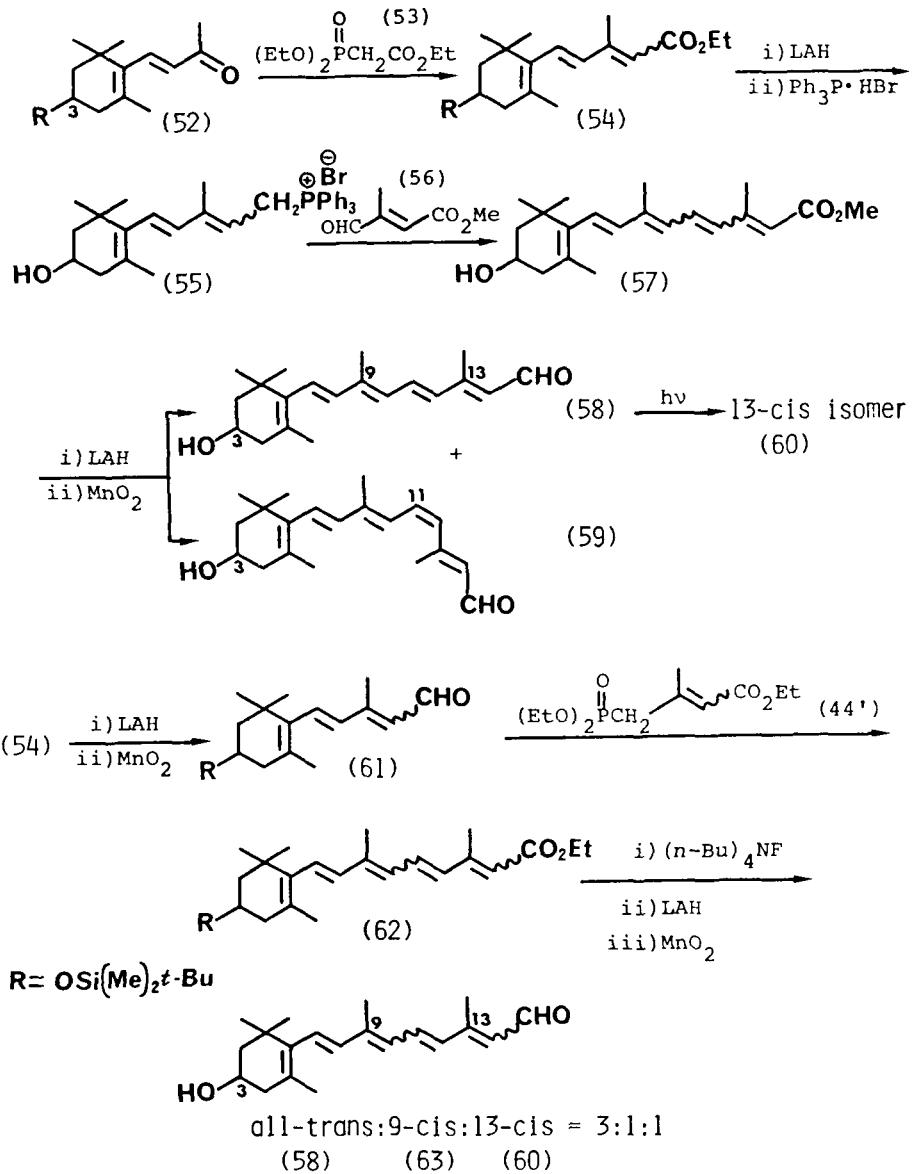
	Bicyclic retinal-I		Bicyclic retinal-II			
	39	29	46	47	30	
UV (EtOH)	425 (33100)	370 (13100)	425 (29000)	425 (32000)	417 (25000)	
λ_{\max} nm (ϵ)	292 (8300)	335 (14800)	295 (11000)	295 (10000)	295 (16000)	
$^1\text{H-NMR}$ (200 MHz) (δ , CDCl_3) (J in Hz)	13-Me 9-Me 12-H (d, J 15) 11-H (dd, J 15,11) 10-H (d, J 11) 7-H CHO 18-H	2.34 (s) 2.11 (s) 6.42 6.43 7.21 6.47 (s) 10.10 —	2.20 (s) 2.07 (s) 6.26 6.20 6.92 5.99 (s) 10.05 —	2.33 (s) 2.09 (s) 6.36 6.36 7.15 6.63 (s) 10.10 3.12(sharp s)	2.33 (s) 2.07 (s) 6.39 6.40 7.15 6.45 (s) 10.10 3.16(br s)	2.32 (s) 2.06 (s) 6.32 6.07 7.37 6.45 (s) 10.10 3.20(br s)

was deprotected with dilute H₂SO₄ to afford hydroxyaldehyde **43**. Condensation (*n*-BuLi) of aldehyde **43** with the C₅-phosphonate **44** yielded the diene-ester **45**, which was reduced by LiBH₄. The reduction product was dehydrated with iodine to give isomeric mixtures of the bicyclic retinoates. The mixture of esters was converted to the bicyclic retinals by LAH reduction and MnO₂ oxidation; preparative HPLC (pHPLC) in the dark, gave four bicyclic retinal isomers **46**, **47**, **48**, and **49** in pure form. The structures of the four isomers were determined by comparison of the ¹H NMR data including nuclear Overhauser experiments (data for **46** and **47** are compiled in Table 3) with those of **39**. Irradiation of **47** using a fluorescent lamp (30 W) in MeOH yielded predominantly 9-*cis* isomer **30**, which has an absorption maximum at 417 nm. Its stereostructure was determined from the ¹H NMR spectral data (Table 3). Comparison of the UV and ¹H NMR data of **30** with those of 9-*cis*-bicyclic retinal analog-I (**29**) and 9-*cis*-retinal³⁸ suggests that the 9-*cis*-bicyclic retinal analog-II (**30**) has a coplanar structure in the C₆-C₁₀ region of the molecule. In a binding experiment using bovine opsin, 9-*cis*-bicyclic retinals **29** and **30** gave two new 9-*cis*-bicyclic rhodopsin analogs **50** (Scheme 5) and **51** (Scheme 6), respectively. Unfortunately, the 9-*cis* isomer derived from **46** gave no rhodopsin analog. Rhodopsin analog **51** showed an absorption maximum at 519 nm and a CD spectrum [α-band: 512 nm (+ 13.6), β-band: 326 nm (- 2.1)],³³ whereas the other analog **50** exhibited an absorption maximum at 539 nm and a different CD spectrum [α-band: 527 nm (+ 16), β-band: 328 nm (- 8.2)].³² A very weak β-CD band in the 9-*cis*-bicyclic rhodopsin analog-II (**51**) suggests that the β-band in the induced CD of rhodopsin originated from the twist of a C₆-C₇ single bond. Negative signs for the β-CD band in **50** and **51** have not been reported so far for artificially prepared visual pigments. It is likely that a chirally twisted chromophore having a partially constrained planar structure plays an important role in the CD spectrum.

VII. THE CHROMOPHORE OF THE FLY VISUAL PIGMENT

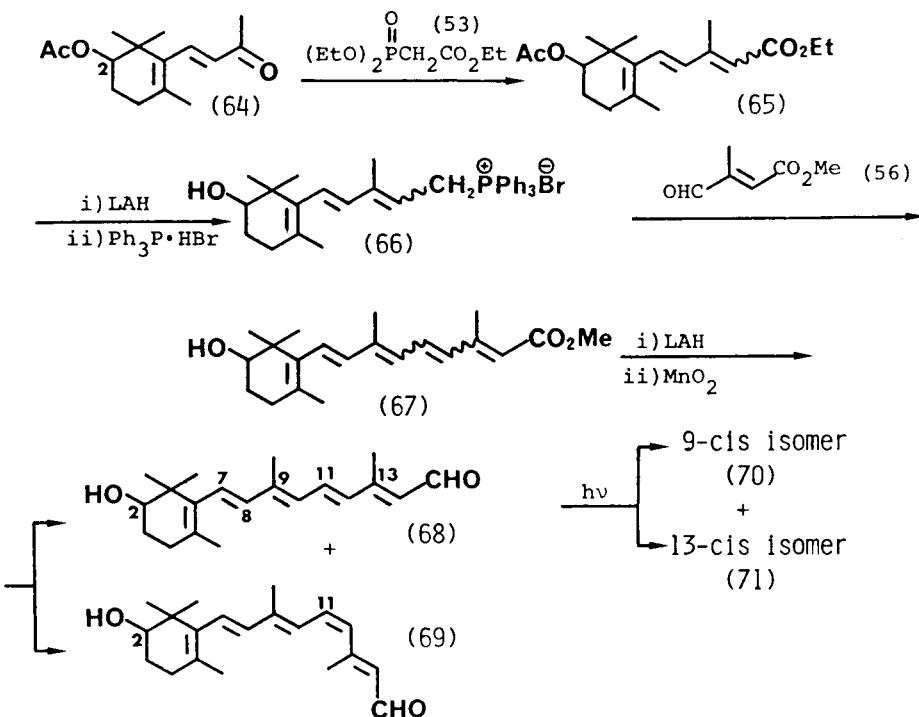
In 1984, Vogt and Kirschfeld³⁹ proposed that the chromophore of the fly visual pigment was not retinal but 3-hydroxyretinal on the basis of some chemical and UV spectral evidence. However, based on chemical evidence only, the possibility that 2-hydroxyretinal could also be the chromophore remained unresolved. In addition, there was no confirmation of the geometry around the conjugated double bonds. In order to confirm the structure of the chromophore, the geometrical isomers (all-*trans*, 13-*cis*, 11-*cis*, and 9-*cis*) of 2- and 3-hydroxyretinals were synthesized by minor modifications of typical methods (Schemes 7 and 8).^{40,41}

Horner-Emmons reaction (*n*-BuLi) of the *t*-butyldimethylsilyl ether **52**⁴² of 3-hydroxy-β-ionone^{43,44} with ester-phosphonate (C₂-phosphonate) **53** gave in a high yield 3-hydroxy-β-ionylideneacetate derivative **54**, which was treated with LAH and Ph₃P-HBr to afford Wittig salt **55**. Condensation (NaOEt) of **55** with formyl-ester **56** yielded 3-hydroxyretinoate **57**, which, on LAH reduction of the ester group and subsequent MnO₂ oxidation, furnished a mixture of 3-hydroxyretinal isomers. The chromatographic separation [column chromatography (CC) and pHPLC] gave the pure all-*trans* and 11-*cis* isomers **58**,^{45,46} and **59**, respectively. The 13-*cis* isomer **60** was isolated in moderate yield from the irradiation products of the all-*trans* isomer **58** in benzene. Horner-Emmons reaction of 3-hydroxy-β-ionylideneacetaldehyde derivative **61**, derived from **54**, with the C₅-phosphonate **44'** gave a mixture of 3-hydroxyretinoate derivatives **62**. After deprotection, the retinoates were converted by the usual method (LAH and MnO₂) to a mixture of 3-hydroxyretinal isomers [all-*trans* (**58**):9-*cis* (**63**)⁴²:13-*cis* (**60**) = 3:1:1], which was carefully separated in the dark into the respective isomers by CC followed by pHPLC. The structures of four 3-hydroxyretinal isomers were confirmed mainly on the basis of comparison of ¹H NMR signals (Table 4) with the data reported³⁸ for retinal isomers.



SCHEME 7

In analogy with 3-hydroxyretinals, the synthesis of 2-hydroxyretinals was accomplished (Scheme 8) via 2-acetoxy- β -ionone (**64**), which was prepared from β -ionone by us^{47,48} and other groups.^{49,50} Condensation (*n*-BuLi) of **64** with the C₂-phosphonate **53** gave a mixture of 9-*cis*- and 9-*trans*-2-hydroxy- β -ionylideneacetate derivatives (**65**), which on reduction with LAH and treatment of the resulting alcohol with $\text{Ph}_3\text{P} \cdot \text{HBr}$ afforded the phosphonium salt **66**. Wittig condensation (NaOEt) of **66** with formyl-ester **56** resulted in the formation of a mixture of 2-hydroxyretinoate isomers **67**. This ester mixture was converted by the usual method into a mixture of 2-hydroxyretinal isomers, from which the pure all-*trans* and the 11-*cis* isomers (**68** and **69**), respectively, were isolated by a combination of CC and pHPLC. Both the 9-*cis* (**70**) and the 13-*cis* (**71**) isomers were isolated in pure form from irradiation products of the all-*trans* isomer **68** in methanol, using pHPLC. The spectral data



SCHEME 8

TABLE 4
Characteristic Spectral Data For 3-Hydroxyretinal Isomers

		all-trans (58)	13-cis (60)	11-cis (59)	9-cis (63)
UV (EtOH)		379 (44000)	372 (31000)	375 (21000) ca.282(sh) 254 (14000)	372 (32000)
λ_{\max} nm (ϵ)			257 (6500)		
$^1\text{H-NMR}$ (200 MHz)	13-Me	2.34 (s)	2.15 (s)	2.36 (s)	2.32 (s)
(δ , CDCl_3) (J in Hz)	12-H	6.39 (d, J 16)	7.31 (d, J 15)	5.95 (d, J 12)	6.32 (d, J 15)
	11-H	7.14 (dd, J 16,11)	7.04 (dd, J 15,11.5)	6.69 (t-like, J 12)	7.21 (dd, J 15,12)
	10-H	6.20 (d, J 11)	6.23 (d, J 11.5)	6.54 (d, J 12)	6.12 (d, J 12)
	8-H	6.15 (d, J 16)	6.16 (d, J 16.5)	6.13 (d, J 15)	6.67 (d, J 16)
	7-H	6.29 (d, J 16)	6.38 (d, J 16.5)	6.28 (d, J 15)	6.27 (d, J 16)
	3-H	ca.4.02 (br m)	ca.4.02 (br m)	ca.4.03 (br m)	ca.4.03 (br m)
	CHO	10.12 (d, J 8)	10.21 (d, J 8)	10.09 (d, J 8)	10.12 (d, J 8)
	OH	1.60 (br s)	1.57 (br s)	1.62 (br s)	1.60 (br s)

TABLE 5
Characteristic Spectral Data For 2-Hydroxyretinal Isomers

		all-trans (68)	13-cis (71)	11-cis (69)	9-cis (70)
UV (EtOH)		381 (42000)	375 (30000)	376 (23000)	372 (32000)
λ_{\max} nm (ϵ)			259 (8500)	254 (15000)	
¹ H-NMR (200 MHz) (δ , CDCl ₃) (<i>J</i> in Hz)	13-Me	2.34 (s)	2.15 (s)	2.36 (s)	2.32 (s)
	12-H	6.39	7.31	5.95	6.31
		(d, <i>J</i> 15)	(d, <i>J</i> 15)	(d, <i>J</i> 11.5)	(d, <i>J</i> 15)
	11-H	7.14	7.04	6.70	7.22
		(dd, <i>J</i> 15,11)	(dd, <i>J</i> 15,11.5)	(t-like, <i>J</i> 12.5,11.5)	(dd, <i>J</i> 15,11.5)
	10-H	6.20	6.23	6.54	6.11
		(d, <i>J</i> 11)	(d, <i>J</i> 11.5)	(d, <i>J</i> 12.5)	(d, <i>J</i> 11.5)
	8-H	6.18	6.16	6.13	6.67
		(d, <i>J</i> 16.5)	(d, <i>J</i> 16.5)	(d, <i>J</i> 16)	(d, <i>J</i> 15.5)
	7-H	6.30	6.38	6.28	6.28
		(d, <i>J</i> 16.5)	(d, <i>J</i> 16.5)	(d, <i>J</i> 16)	(d, <i>J</i> 15.5)
	2-H	3.57	3.57	3.55	3.58
		(dd, <i>J</i> 9,3.5)	(dd, <i>J</i> 9,3.5)	(dd, <i>J</i> 9,3.5)	(dd, <i>J</i> 9,3.5)
	CHO	10.12	10.21	10.10	10.11
		(d, <i>J</i> 8)	(d, <i>J</i> 8)	(d, <i>J</i> 8)	(d, <i>J</i> 8)
	OH	1.54	1.57	1.52	1.57
		(br s)	(br s)	(br s)	(br s)

(Table 5) of the four 2-hydroxyretinal isomers gave satisfactory results for the assignment of the respective isomeric structures on comparison with the spectra of the analogous 3-hydroxyretinals.

Simultaneous separation of a mixture of eight isomers of 2- and 3-hydroxyretinals was achieved completely by HPLC under the analytical condition shown in Figure 1. An extract, (prepared by the formaldehyde method⁵¹) from heads of *Drosophila melanogaster*, was examined using this analytical procedure. The chromophore of the visual pigment of *D. melanogaster* was found to be not 2-hydroxyretinal but 3-hydroxyretinal. In the formaldehyde method, isomerization during the extraction was a possibility. Therefore, in order to determine the geometry about the conjugated double bond, we adopted the oxime method⁵²⁻⁵⁴ in the extraction of fly heads. Before the extract was subjected to the HPLC analysis, the simultaneous separation of the eight pure isomers of the 3-hydroxyretinal oxime was accomplished by HPLC using the solvent system shown in Figure 2. A HPLC chromatogram of an untreated sample from *D. melanogaster* showed the existence of the two main *syn* peaks of the 11-cis and all-trans isomers. Considering the ϵ values of both *syn* isomers at the wavelength of detection, the 11-cis isomer was found to be a main component of the extract. Because the all-trans isomer did not form the pigment of the fly head, the chromophore of the fly visual pigment was determined to be 11-cis-3-hydroxyretinal.

VIII. SYNTHESIS OF RETINOIDAL BUTENOLIDES

In the course of synthetic work to develop a new antitumor retinoidal compound, we became interested in the preparation of retinoidal lactones. First, retinoidal 3(2H)-furanones **72a,b** and **73a,b** were synthesized,⁵⁵ using a Claisen-type condensation between polyene ester **74** and a α -hydroxy methyl ketone **75** in the presence of freshly prepared LiNH₂ followed by treatment of the resulting hydroxy polyconjugated enolic β -diketones **76** and **77** with dilute H₂SO₄ (Scheme 9). Unfortunately, no activity of those conjugated vinyllogous lactones against B-16 melanoma was observed *in vivo*.⁵⁶

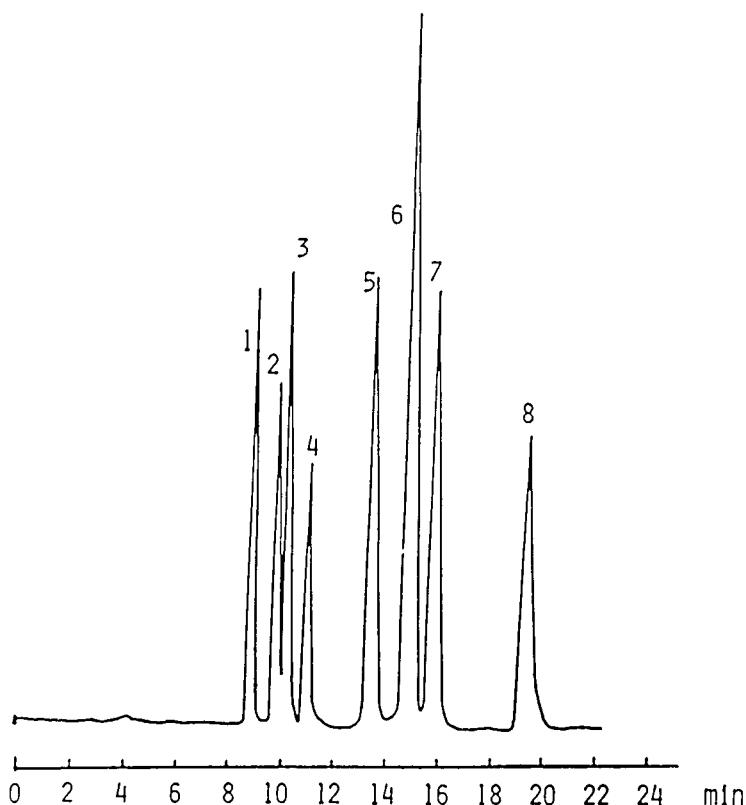


FIGURE 1. HPLC elution pattern of a mixture of 2- and 3-hydroxyretinal isomers. Column, LiChrosorb Si-60-5 (30 × 0.75 cm I.D.); eluant, 0.7% methanol-17% THF in *n*-hexane; flow rate, 4 ml/min; UV detection, 350 nm; 1 = 13-*cis*-2-hydroxyretinal (OH-R), 2 = 11-*cis*-2-OH-R, 3 = all-*trans*-2-OH-R, 4 = 9-*cis*-2-OH-R, 5 = 13-*cis*-3-OH-R, 6 = all-*trans*-3-OH-R, 7 = 11-*cis*-3-OH-R, 8 = 9-*cis*-3-OH-R.

Retinoidal 4-ylidenebutenolides **78a,b** and **79a,b** were synthesized (Schemes 10 and 11).^{57,58} Aldol condensation (piperidine) of β -cyclocitral (**80a**) with pyruvic aldehyde dimethylacetal (**81**) gave, in 60% yield the (*E*)-acetal dienone **82**, which was treated with C₂-phosphonate **53'** using *n*-BuLi as the base to yield a mixture of the (*9E*)- and (*9Z*)-acetal esters **83** in 94% yield. This mixture, without separation, was refluxed in methanol with an acid catalyst to afford in 68% yield methoxylactone **84**, which was converted to the hydroxylactone **85** by an acid hydrolysis. Hydroxylactone **85** was also directly obtained in 74% yield from **83** under the same acidic conditions. PCC oxidation of **85** led to the unstable conjugated anhydride **86**, which was condensed with acetyl phosphorane **87** to afford a mixture of three ketolactones **88**, **89**, and **90**, which were cleanly separated by pTLC. Wittig condensation of **88** or **89** with ester-phosphorane **91** produced a mixture of retinoidal 4-ylidenebutenolides **78a** and **79a**. The corresponding 3-methoxy derivatives (**78b** and **79b**) were synthesized starting from 3-methoxy- β -cyclocitral (**80b**) using the same pathway.⁵⁸ The *E* and *Z* isomers of additional related 4-ylidene compounds (**92**, **93**, **94**, **95**, **96**, and **97**) having the common structure **98** (Scheme 11) have been prepared to compare the chemical shifts of 3-H with those of retinoidal 4-ylidenebutenolides. Consequently, in compounds of this type, the NMR signal for 3-H in the 4*Z* isomer was observed at δ 7.00 to 7.20, whereas the corresponding signal in the 4*E* isomer was found downfield below δ 7.40.^{58,59} These values were used to determine the stereochemistry of the ylidene part in the double bond system.

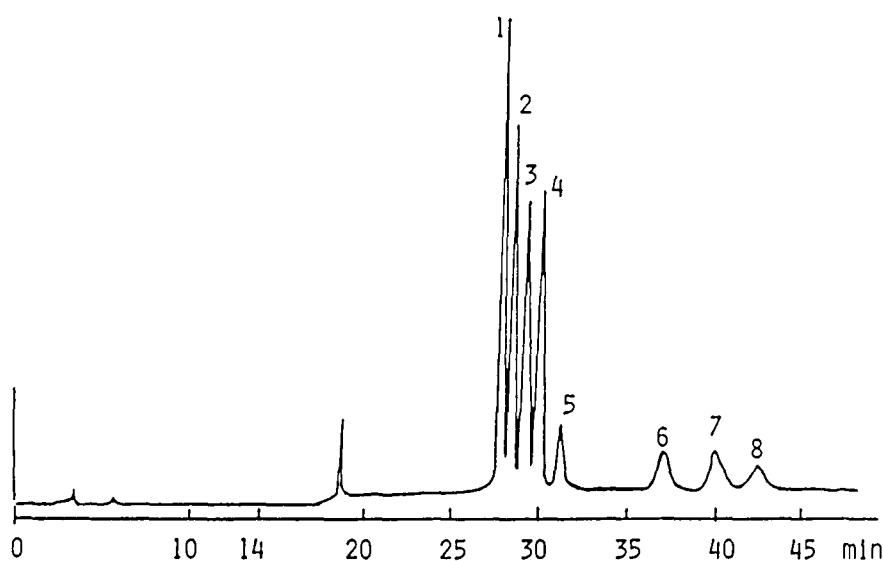
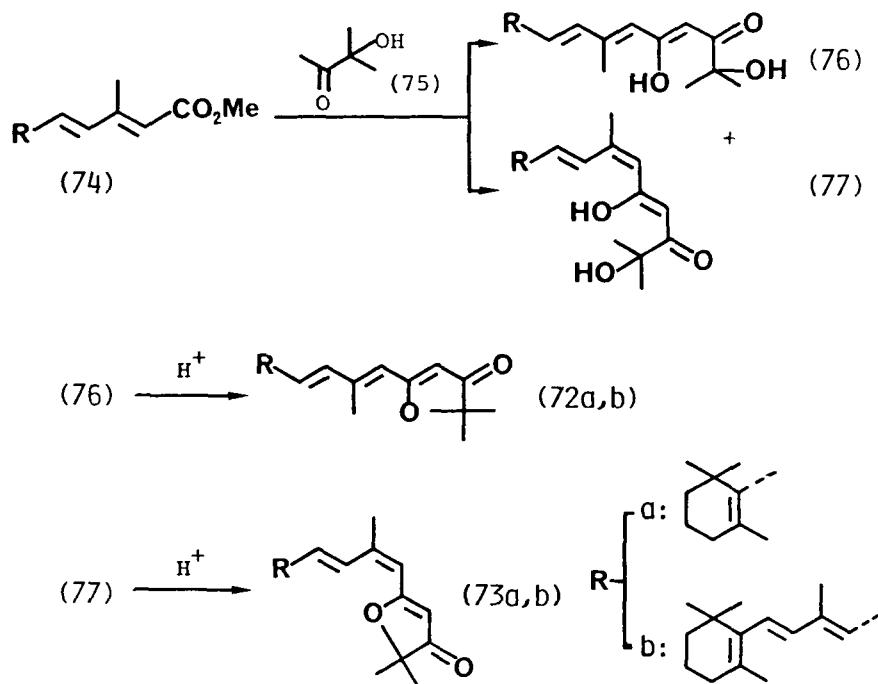
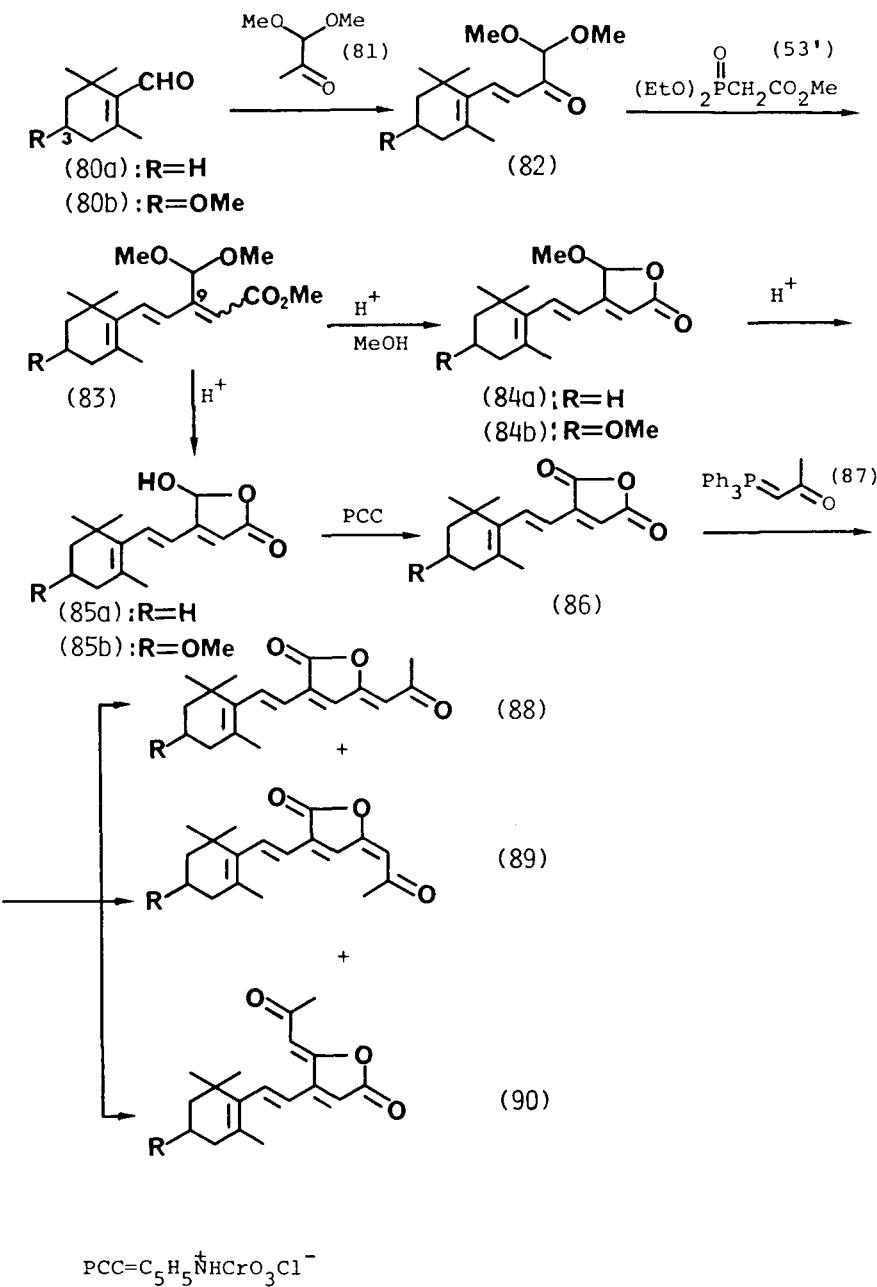


FIGURE 2. HPLC elution pattern of a mixture of eight geometrical isomers of 3-hydroxyretinol. Column, YMC-Pack A-012-3(S-3 SIL) (15×0.6 cm I.D.); eluant, 0.1% methanol-1.2% *t*-butyl methyl ether in benzene, after 14 min, the eluant was changed to 1% methanol-6% *t*-butyl methyl ether in benzene; flow rate, 1.7 ml/min; UV detection, 350 nm; 1 = 9-*cis*-syn, 2 = 13-*cis*-syn, 3 = 11-*cis*-syn, 4 = all-*trans*-syn, 5 = all-*trans*-anti, 6 = 13-*cis*-anti, 7 = 9-*cis*-anti, 8 = 11-*cis*-anti.



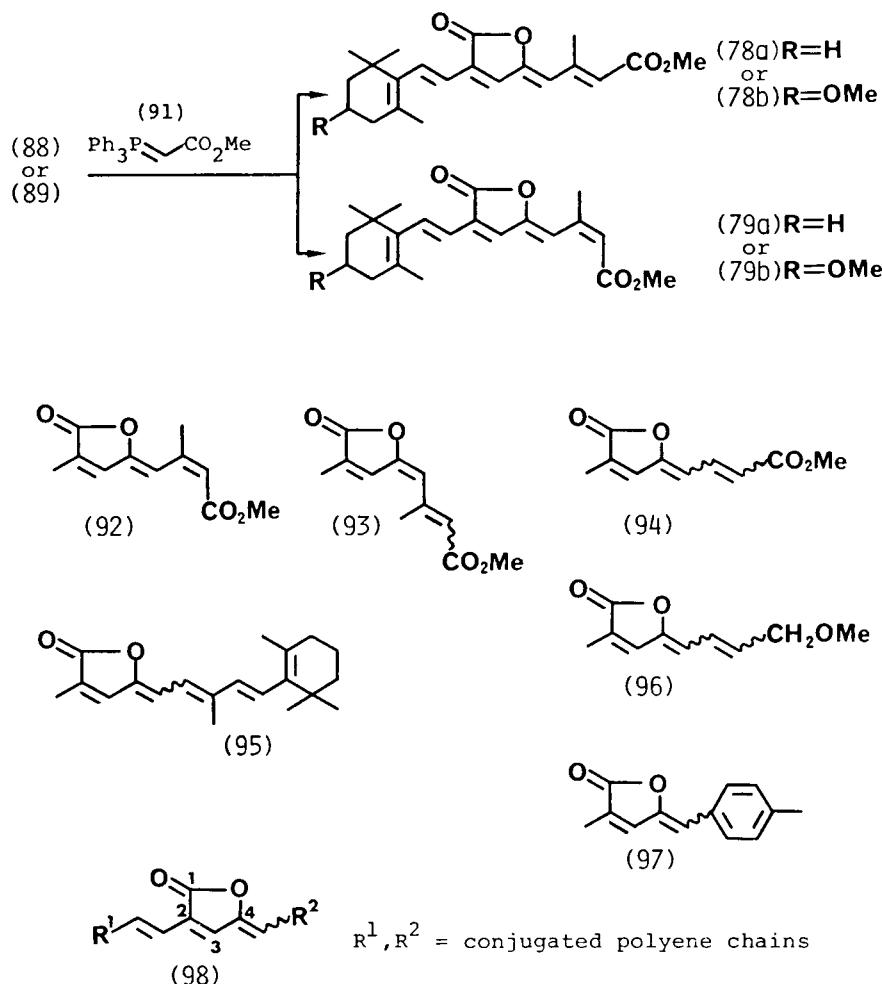
SCHEME 9



SCHEME 10

The cytotoxic activity of retinoidal butenolides **78a**, **84a,b**, and **85a,b** on mouse neuroblastoma and rat glioma cells was examined.⁶⁰ Butenolide **85a** was found to be the most potent compound in the series and was about 90 times more potent than retinoic acid. Therefore, the higher homologue **99** and aromatic analogs (**100a,b,c,d,e,f,g,h**) (Scheme 12) were synthesized⁶¹ in order to find more active compounds. Some of these analogs had activities on the *in vitro* mouse neuroblastoma system.

In the connection with our work on hydroxylactone **85a**, a new synthetic route to another hydroxylactone (**101**) was developed (Scheme 12).⁶² Treatment of sulfone **102**, derived from

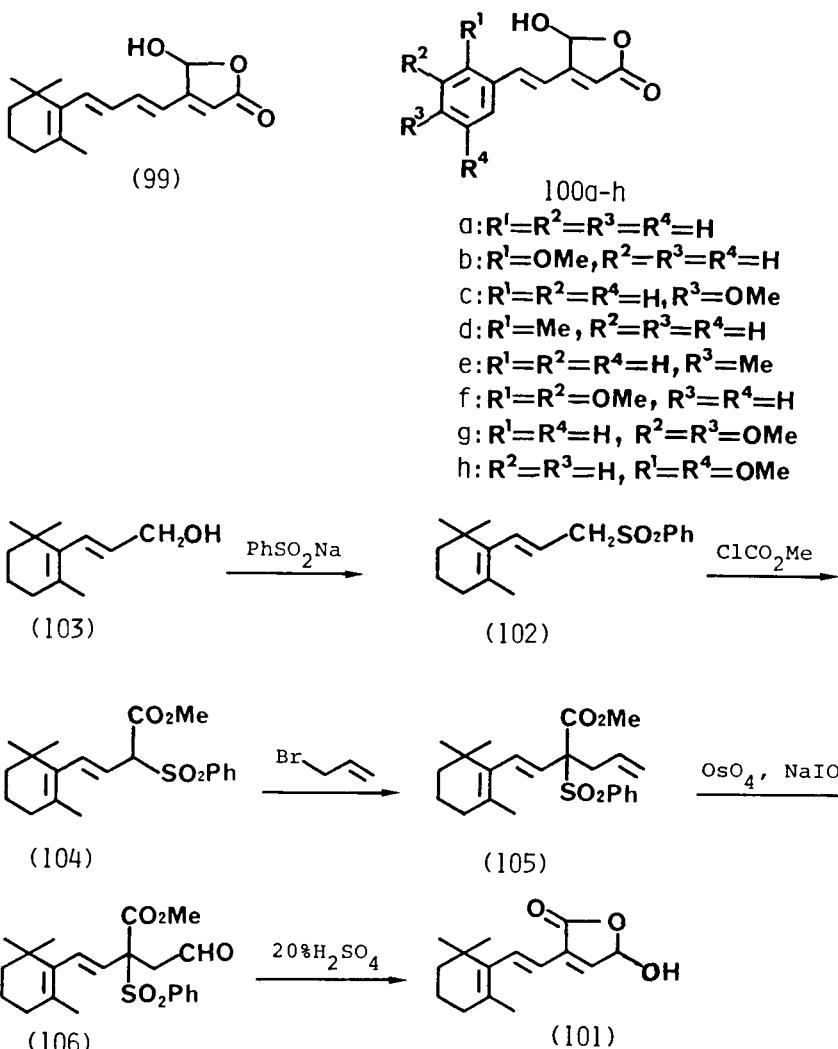


SCHEME 11

dienol **103**,⁶³ with methyl chlorocarbonate in the presence of *n*-BuLi gave carbomethoxylated-sulfone **104** in 97% yield, based on recovered starting sulfone. Sulfone **104** was converted to **105** in 94% yield by treatment with allyl bromide and NaH. Oxidation of **105** with sodium periodate and a catalytic amount of osmium tetroxide afforded in 50% yield the formyl-sulfone **106**, which was cyclized with 20% H₂SO₄ in refluxing THF to hydroxy-lactone **101** in 72% yield. The antitumor activity of **101** is under examination.

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SCHEME 12

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Chapter 5

**MOLECULAR MECHANISM FOR THE FUNCTION OF
BACTERIORHODOPSIN AND VISUAL PIGMENTS: STUDIES
WITH MODEL COMPOUNDS AND ARTIFICIAL PIGMENTS****Mordechai Sheves and Michael Ottolenghi****TABLE OF CONTENTS**

I.	Introduction	100
II.	The Opsin Shift in Bacteriorhodopsin.....	100
A.	Model Systems	100
B.	Artificial bR Pigments.....	103
III.	Vibrational Spectroscopic Criteria for Chromophore-Opsin Interactions in the Vicinity of the Schiff Base Linkage.....	105
IV.	The pK _a of the Protonated Schiff Base of bR and Its Alteration during the Photocycle	108
V.	The Primary Photochemical Event in bR and in Bovine Rhodopsin.....	111
A.	Bond Isomerizations and Rotations	112
B.	Retinal-Opsin Interactions	113
VI.	Subsequent Stages in the Photocycle of bR	114
VII.	Thermal Isomerization of Retinal Isomers	115
VIII.	Summary and Conclusions	117
	Note Added in Proof	118
	Acknowledgment	119
	References.....	119

I. INTRODUCTION

The visual process is initiated via light absorption by rhodopsin (Rh) pigments consisting of a retinyl polyene chromophore (11-cis) covalently bound to a membrane apo-protein (opsin) through a protonated Schiff base linkage with a lysine residue.¹⁻⁴ Excitation leads to changes in the electrical potential of the membrane, which are transmitted to the brain through appropriate synaptic processes.

Bacteriorhodopsin (bR), a retinyl-protein closely related to visual pigments, is responsible for the photosynthetic activity of the purple membrane of the halophilic microorganism *Halobacterium halobium*.⁵ The role of bR is to convert light energy into a proton gradient across the membrane, which is subsequently used to synthesize ATP via a chemiosmotic mechanism. It was found⁵ that the photobiological activity of bacteriorhodopsin is due to an all-trans-retinal-protein complex (bR₅₇₀) of the kind previously found only in visual pigments.

The absorption maxima of bR and of visual pigments are considerably red-shifted relative to the corresponding protonated retinal Schiff base (RSBH⁺) in (methanol) solution. This energy difference (in cm⁻¹), which contributes to the high response of both pigments in the visible range of the spectrum, was termed the "Opsin Shift"⁶ (OS).

The biological activity of retinal pigments is based on photocycles (see Figure 1) during which both retinal and the opsin moieties undergo a series of structural changes. In spite of its basic (all-trans) structure, the photocycle of bR₅₇₀ shows basic analogies to those of the visual pigments in which the retinal moiety is in the 11-cis configuration.^{3,7} Accumulated evidence⁸ suggests that the primary event, which leads to a red-shifted primary photo-products (bathorhodopsin in the visual photoprocess and K₆₁₀ in that of bR₅₇₀), is associated with cis-trans isomerizations around the C₁₁=C₁₂ and C₁₃=C₁₄ bonds, respectively.

In this chapter we review our work to clarify the molecular aspects of the function of bR and visual pigments using two main approaches: (1) constructing synthetic retinal analogs bearing nonconjugated charges, as well as altering the retinal Schiff base environment, in order to evaluate the effects of electrostatic and solvation interactions on the retinal chromophore, and (2) constructing artificial pigments based on synthetic retinals to provide insights into the chromophore-opsin interactions and their relevance to the absorption spectra of the pigments and their photoprocesses.

II. THE OPSIN SHIFT IN BACTERIORHODOPSIN

One of the most perplexing problems in the investigation of the molecular basis of vision is the nature of the red-shifted absorption of all pigments relative to the respective protonated retinal Schiff bases in solution (e.g., in methanol). A similar phenomenon is found in bacteriorhodopsin. It has been proposed^{6,9,10} that red shifts in retinal systems can be induced by electrostatic interactions with (negative) protein charges or dipoles in the vicinity of the retinal molecule. Work with model RSBH⁺ systems has provided important information in this respect.

A. MODEL SYSTEMS

The capability of nonconjugated negative charges to influence the absorption maximum of protonated polyene Schiff bases was directly demonstrated¹¹ by examining chromophores **1** and **2**, which exhibited red shifts of 2500 and 300 cm⁻¹, respectively, upon conversion of the carboxyl groups to carboxylates. To further investigate the influence of nonconjugated charges on the absorption maximum of RSBH⁺ we synthesized a series of modified retinals bearing nonconjugated positive charges at various locations along the retinal skeleton and measured the absorption maxima of their protonated retinal Schiff bases¹²⁻¹⁴ (Table 1). It

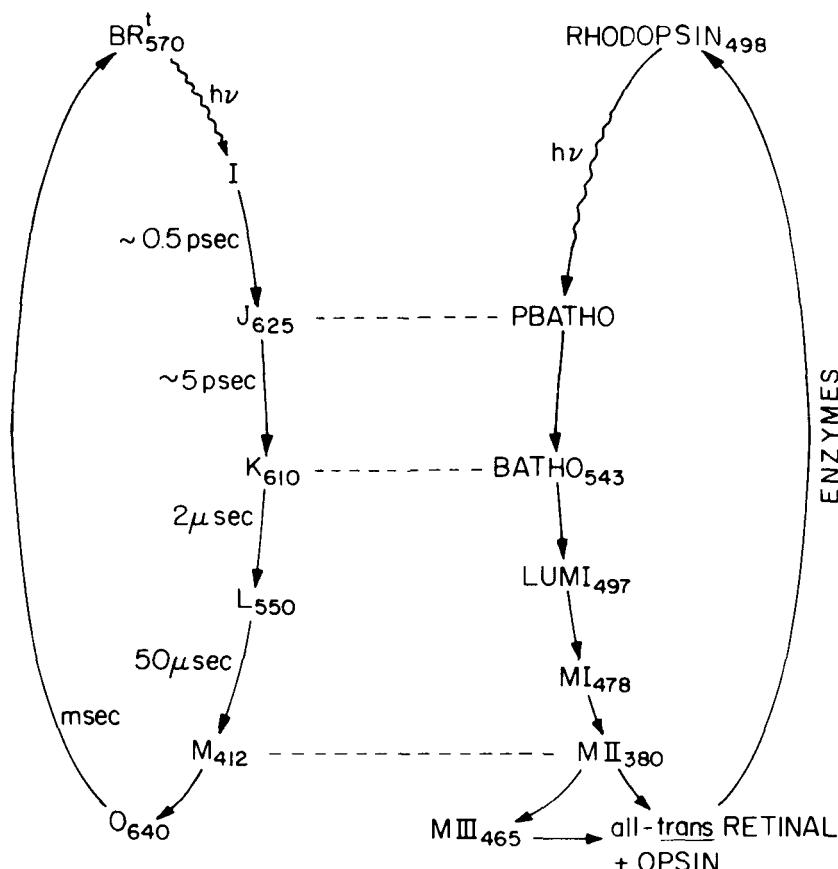
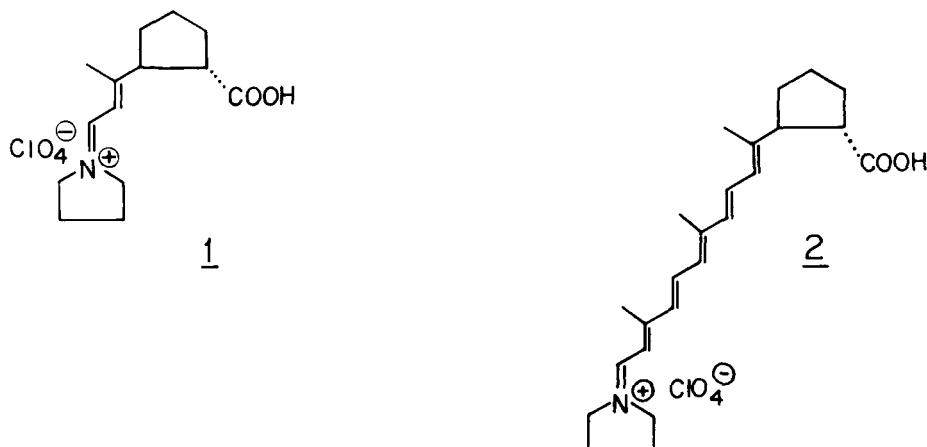
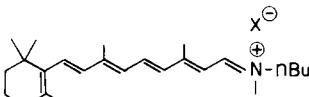
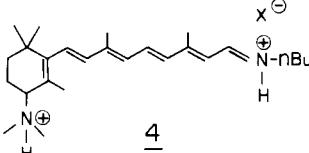
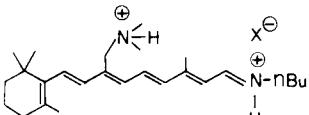
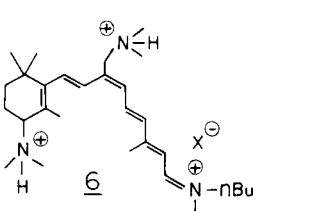


FIGURE 1. The photocycles of bacteriorhodopsin (all-trans) and bovine rhodopsin (11-cis). Subscripts refer to wavelengths of maximum absorption. Time notations are approximate room-temperature values. Horizontal dotted lines indicate analogous intermediates (see text).



was found that the absorption maximum (in CH_2Cl_2) is blue-shifted due to the presence of the positive charge in the vicinity of C_4 (chromophore **4**) and C_9 (chromophore **5**) by 1150 and 1320 cm^{-1} , respectively. However, the maximum is not significantly affected by a charge located in the neighborhood of C_{12} (chromophore **7**) and is red-shifted by 950 cm^{-1} due to the presence of a positive charge in the vicinity of the Schiff base linkage (in chromophore **8**). We concluded that the location of the external (positive) charge is crucial in determining the spectrum of the Schiff base. The observed blue and red shifts are most

TABLE 1
Absorption Maxima Values of Various Protonated Schiff Bases^a

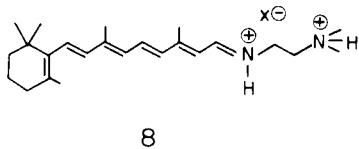
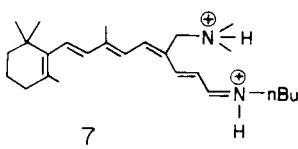
Chromophore	λ_{\max} (nm)				
	EtOH ^b	TFE ^b	HFIP ^b	CH ₂ Cl ₂ (1 eq. TFA) ^c	CH ₂ Cl ₂ (1 M TFA) ^c
	440	467	492	448	513
<u>3</u>					
	423 (810)	431 (1800)	442 (2300)	426 (1150)	461 (2200)
<u>4</u>					
	419 (1035)	419 (2450)	428 (3050)	423 (1320)	455 (2500)
<u>5</u>					
	396 (2525)	412 (2850)	—	405 (2370)	406 (5140)
<u>6</u>					

^a Values in parenthesis indicate difference in energy (cm^{-1}) between the corresponding chromophore and its parent compound (without the nonconjugated charge).

^b Protonation was carried out using HCl(g) in cases in which the chromophore was not protonated by the solvent itself.

^c Protonation was carried out using 1 eq. (or 1 M concentration) of TFA; Schiff base concentration was $0.5 \times 10^{-5} M$.

probably due to destabilization of the excited state and of the ground state, respectively. Accordingly, the different influence of the nonconjugated charges reflects different π -electron delocalization along the various polyene carbons. It should be noted that shifts of opposite direction, but similar magnitudes, are expected in the case of nonconjugated negative charges.



We also found that the magnitude of the above shifts largely depends on the degree of ion-pairing between the nonconjugated positive charge and its counteranion. Diminishing ion-pairing by increasing counteranion solvation causes a more effective interaction of the nonconjugated positive charge with the retinal skeleton, enhancing the spectral shift. This effect was achieved either by using excess trifluoroacetic acid (TFA),^{13,15,16} which introduces a homoconjugated effect in nonprotic solvents (such as methylene chloride) or by applying fluorinated alcohols.¹⁴ The latter strongly solvate anions but relatively only weakly solvate cations.¹⁷ This behavior differs from that of other hydrogen-bonding solvents, such as ethanol, in which solvation of cations and anions occurs with equal efficiency. Therefore, by using fluorinated alcohols we have achieved strong anion solvation with only a weak solvation of the nonconjugated positive charge, resulting in a remarkable shift of the absorption maxima (Table 1).

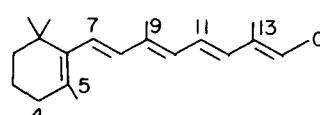
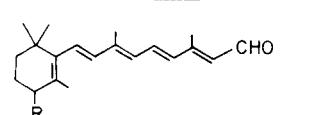
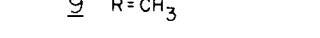
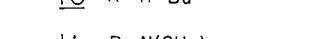
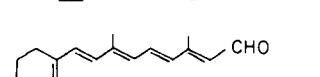
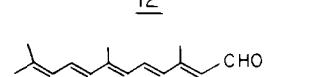
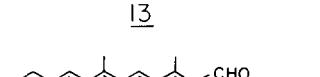
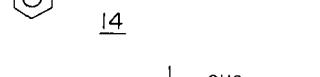
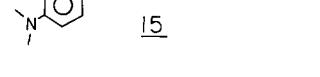
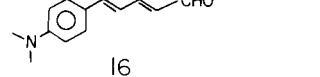
The absorption maximum of RSBH⁺ is also affected by the mode of interaction between the positively charged Schiff base linkage and its counteranion. Increasing the size of the counteranion induces a red shift in the absorption maximum. Thus, Baltz et al.¹⁸ demonstrated that Cl⁻ caused a red shift of approximately 1150 cm⁻¹ in RSBH⁺ relative to that of Cl⁻ in chloroform. Excess TFA in methylene chloride induced a more significant shift (about 2500 cm⁻¹),^{13,15} probably because hydrogen bonding diminished ion-pairing between the anion and the positively charged nitrogen. The effect is associated with destabilization of the ground state, leading to a red shift.

Experiments with fluorinated alcohols demonstrated another interesting possibility of introducing a red shift in the spectrum of RSBH⁺, which is directly associated with solvation of the Schiff base. Despite the similar (and relatively high) dielectric constants of EtOH and trifluoroethanol (TFE), the positively charged Schiff base nitrogen is much more effectively solvated in EtOH. In the absence of ion-pairing with anions in such polar ("leveling") solvents, this solvation leads to a red shift in TFE due to a decreased ground state stabilization relative to EtOH. Thus, RSBH⁺ absorbs at 467 nm in TFE, at 492 nm in hexafluoro-2-propanol (HFIP), and at 440 nm in EtOH (a shift of approximately 2440 cm⁻¹ relative to HFIP).¹⁴

B. ARTIFICIAL bR PIGMENTS

Evidence that chromophore-opsin interactions affect the absorption maxima of bR has emerged from studies with artificial pigments derived from synthetic retinal analogs. Retinals **9** to **11** (substituted at the C₄ position),¹⁹ **12** (lacking all ring methyl substituents),²⁰ and the linear chromophore²¹ **13a** (Table 2) may be expected to affect sterically the chromophore-opsin interactions without altering the basic all-*trans* polyene system. We found that the pigments **9** and **10** derived from C₄-substituted analogs are unique in exhibiting two distinct absorption bands in the visible range: a main (blue) band at 465 nm, characterized by a negligible OS_{bR} value — analogous to that of the 4-dimethylamino derivative **11** — and a less pronounced shoulder around 550 nm, reminiscent of the red band of bR (normal OS_{bR}). A second class of artificial pigments, in which a relatively small OS_{bR} value is observed, is derived from analogs in which the basic polyene structure is perturbed by introducing aromatic rings (pigments **14** to **17**) with or without shortening the polyene chain (Table 2). The major conclusion from all such observations is that a substantial drop in the value of OS_{bR} is observed whenever the ring region is seriously perturbed either by C₄ substitution (the above "blue" pigments) or by replacing the cyclohexane ring with an aromatic one. A similar drop in OS_{bR} is observed in the linear chromophore **13a**. Therefore, large steric effects appear to be induced by bulky substituents at the C₄ position that drastically alter the conformation of the ring region of the molecule. The decrease in OS_{bR} in the case of the linear molecule, the ring-desmethyl derivative, as well as the aromatic chromophore derivatives²² may be attributed to a release of steric constraints that result in a ring-chain

TABLE 2
Spectral Data For Synthetic Retinal Schiff Bases (RSB) And Their Artificial Pigments (9 — 17)

Pigment	Absorption maxima (nm)			Spectral Shifts (cm^{-1})		
	RSB (MeOH)	RSBH ⁺ (MeOH)	Pigment	OS _{BR}	$\Delta\nu$ (bR/K)	OS _M ^a
	360	440	570	5180	2440	3500
all- <i>trans</i> -Retinal						
	360	440	$\frac{465^b}{550^c}$	$\frac{1220^b}{4500^c}$	$\frac{2640^b}{2310^c}$	$\frac{1500^b}{3100^c}$
9 R = CH ₃						
	360	440	$\frac{465^b}{550^c}$	$\frac{1220^b}{4550^c}$	$\frac{2640^b}{2310^c}$	$\frac{1500^b}{3100^c}$
10 R = n - Bu						
	360	440	455	750	2560	2140
11 R = N(CH ₃) ₂						
	368	457	535	3200	2125	2480
12						
	373	468	527	2400	2300	1800
13						
	367	448	512	2600	3000	2250
14						
	398	524	590	2135	2240	0
15						
	384	511	576	2210	1490	
16						
	352	460	510	2130	1430	
17						

^a Whenever OS_M is not specified, it implies that an M-intermediate was not observed.

^b Main absorption band (the "blue" band).

^c Less pronounced band (the "red" band).

conformation similar to that of a free protonated retinal Schiff base in solution. It is also possible that the above steric effects may change the value of OS_{BR} by affecting the distance between a protein (negative or dipolar) charge and the ring region of the polyene moiety.

Of special relevance is the observation that, regardless of the drastic perturbation of the ring region in the aromatic, linear, and ring-desmethyl derivatives, a “residual” opsin shift of approximately 2500 cm^{-1} is observed in all cases. Opsin shifts of a similar magnitude were found in artificial bR pigments based on shortened linear polyenes²³ and on dihydroretinals.^{24,25} This “residual” value is attributed to specific interactions of the Schiff base linkage with the opsin and differs from those prevailing between the Schiff base moiety and its environment in the case of model RSBH⁺ in methanol solution. The effect may be interpreted on the basis of the sensitivity of RSBH⁺ spectra to environmental factors of the Schiff base. As discussed above, red shifts of about 2500 cm^{-1} in the spectrum of a model RSBH⁺ may be achieved either by diminishing ion-pairing between the positively charged nitrogen and its counteranion or by stabilizing the nitrogen moiety via hydrogen bonding (e.g., with fluorinated alcohols compared with methanol). The very small value of OS_{br} observed in the cases of C₄-substituted retinals, which is below the above “residual” value, was attributed¹⁹ to specific, yet undefined, ring-opsin interactions causing a blue shift, which counterbalance the red shift induced by interactions of the opsin with the Schiff base nitrogen.

The results described above led to the conclusion that the opsin shift in native bR ($\sim 5,000\text{ cm}^{-1}$) is due to comparable contributions of opsin-chromophore interactions in the vicinity of the ring and to interactions in the region of the Schiff base nitrogen. This conclusion is qualitatively in agreement with experimental data,^{24,25} derived mainly from synthetic dihydroretinal pigments and from ¹³C NMR measurements, and suggests that the opsin shift in bR is due to: (1) a planar *s-trans* ring-chain conformation induced by the opsin²⁶ (contribution of $\sim 1300\text{ cm}^{-1}$), (2) an additional contribution of $\sim 1,000\text{ cm}^{-1}$, possibly introduced by electrostatic interactions of the chromophore with a protein dipole in the vicinity of the ring,²⁴⁻²⁶ the feasibility of which was confirmed by studies with model compounds,¹³ and (3) a main shift of $\sim 3,000\text{ cm}^{-1}$ introduced by interactions in the Schiff base vicinity²²⁻²⁶ which as discussed above, is in keeping with our work with artificial pigments.

The opsin shift of bovine rhodopsin will be discussed in the following section after considering the vibrational data for RSBH⁺ and retinal pigments.

III. VIBRATIONAL SPECTROSCOPIC CRITERIA FOR CHROMOPHORE-OPSin INTERACTIONS IN THE VICINITY OF THE SCHIFF BASE LINKAGE

As discussed above, the protonated retinal Schiff base in both visual pigments and bacteriorhodopsin may in principle interact with its immediate opsin environment via electrostatic and/or hydrogen bonds with a protein counteranion, with protein dipoles or with residual water. Hydrogen bonds can vary in strength in different pigments and may further be altered by the (double) bond isomerization that takes place following light absorption (see discussion below). In the present section we discuss environmental interactions of the Schiff base in relation to rhodopsin pigments and to model RSBH⁺ in solution. Their relevance to the molecular mechanism of the photocycle will be discussed in Section V.

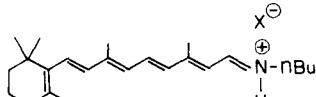
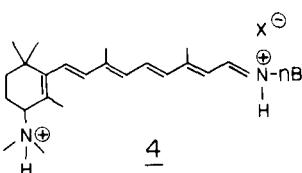
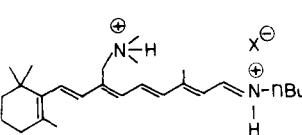
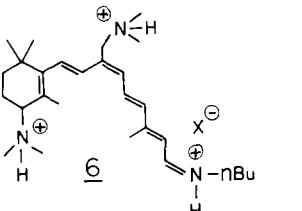
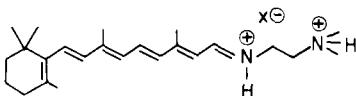
A powerful method for investigating these and other interactions between the retinal polyene and its environment is to measure the C=N and C=C stretching frequencies of the protonated retinal Schiff base by resonance Raman and FTIR spectroscopy.²⁷⁻³⁰ The C=C stretching frequencies of various pigments are inversely correlated with their absorption maxima.³¹ Recently, we showed³² that the inverse correlation also holds for synthetic retinal pyrrolidinium perchlorate salts upon shifting their absorption maxima by interaction with nonconjugated positive charges. The inverse correlation was interpreted in terms of a change

in the π -electron delocalization along the polyene chain that produced changes both in the absorption maxima and in the order of double bonds, which was reflected by different C=C stretching frequencies.³³ A basically different behavior was encountered in the case of the C=N bond. Thus, no systematic correlation was found between the C=N stretching mode and the absorption maxima. For example, bovine rhodopsin and its photochemically induced intermediates all exhibited the same C=N frequency (1660 cm^{-1}) despite the substantial differences in their absorption maxima.^{34,35} In contrast, the C=N stretching frequency in bR (1640 cm^{-1}) did change during the photocycle. Moreover, the hydrogen isotope effects on the C=N frequency in bR and rhodopsin also differed. Following deuteration, the C=N stretching in rhodopsin shifted to lower energies by about 30 cm^{-1} , compared with only about 17 cm^{-1} in bR.³⁵⁻³⁹

In order to interpret these phenomenologically complex phenomena, we have systematically studied⁴⁰ the factors that influence the C=N and C=C modes in RSBH⁺ by using various retinal analogs, as well as different environments in the vicinity of the Schiff base linkage. In Table 3 the effects of nonconjugated positive charges on the C=C and C=N modes of RSBH⁺ are summarized. An inverse correlation between the C=C stretching mode and the absorption maxima, which was similar to the cases of bR and visual pigments³¹ (including their photocycle intermediates), was found in all chromophores. However, nonconjugated positive charges in the vicinity of the β -cyclogeranylidene ring or near C₉, as well as a planar ring-chain conformation (both affecting the absorption spectrum), did not shift the C=N stretching mode. This insensitivity may be rationalized by assuming that the nonconjugated charge or the planar conformation mainly affect the excited state causing only a minor alteration in the π -electron delocalization along the C=N bond in the ground state.

A different situation prevailed when the perturbation was in the vicinity of the C=N bond. In this case a significant change in the C=N stretch was observed; this may be caused by two major effects: (1) the alteration of hydrogen bonding to the N-H moiety, and (2) charge delocalization changes along the C=N bond. The first kind of perturbation is associated with coupling between the C=N stretching and the N-H rock, which has been previously suggested to explain the high frequency of the C=N vibration.⁴¹⁻⁴³ Recently, it has also been proposed that protonation increases the C=N force constant of retinal Schiff bases due to rehybridization of the C=N bond.⁴⁴⁻⁴⁶ Our results with retinal analogs clearly demonstrate that the C=N mode was strongly influenced by hydrogen bond formation with the N-H bond. Thus, a decrease of 11 cm^{-1} in $\nu_{\text{C}=\text{N}}$ was observed following substitution of a Cl⁻ counteranion by excess TFA in CH₂Cl₂. The deuterium isotope effect was decreased as well from 20 cm^{-1} to 15 cm^{-1} . The phenomena are accounted for by the strong hydrogen bonds formed by chloride ions, which are due to the small size and the high density of the negative charge. Excess TFA associates with the anion and diminishes ion-pairing, thereby weakening the hydrogen bond between the N-H moiety and the counteranion and affording a lower energy for the C=N vibration. A possible explanation for the influence of the hydrogen bonds is that strong hydrogen bonding to the N-H bond shifts the C=N-H bend vibration to higher energy⁴⁷ resulting in effective coupling with the C=N mode due to close proximity of the energy levels. (A weak hydrogen bond shifts the C=N-H bend frequency to a lower energy, weakening the interaction with the C=N mode and shifting the C=N frequency closer to its “pure” value.) A similar explanation might also account for the smaller isotope effect observed when hydrogen bonding is weakened. Because deuteration eliminates C=N/N-H coupling due to a significant shift to the N-D frequency, which in turn affects C=N stretching, the isotope effect will be smaller in cases where coupling has already been diminished because of weak hydrogen bonding. Results and conclusions similar to the above were obtained by varying the solvent. Methanol formed strong hydrogen bonds with the N-H bond, resulting in a high C=N frequency (1656 cm^{-1}). In addition, due to the effective

TABLE 3
C=N Stretching Frequencies and Absorption Maxima (in Chloroform) of
Chromophores Carrying Nonconjugated Positive Charges and a Chromophore with
s-Trans Conformation

Chromophore	$\nu_{C=C}$ (cm ⁻¹)	$\nu_{C=NH}$ (cm ⁻¹)	$\nu_{C=ND}$ (cm ⁻¹) ^a	λ_{max} (nm)
	1552	1652	1632 (20)	456
<u>3</u>				
	1566	1653	1632 (21)	432
<u>4</u>				
	1568	1654	1632 (22)	425
<u>5</u>				
	1572	1655	1633 (22)	398
<u>6</u>				
	1538	1646	1625 (21)	480
<u>8</u>				
(<i>n</i> -butyl) iminium salt of 13b	1540	1651	1631 (20)	482

^a Values in parentheses indicate the deuterium isotope effects.

C=N/N-H coupling, a large isotope effect was observed (23 cm⁻¹). Hydrogen bonding to the N-H moiety can be weakened by using fluorinated alcohols as solvents. These solvents form weak hydrogen bonds with the N-H moiety, lowering C=N stretch and reducing the isotope shift. In TFE and HFIP the C=N mode was observed at 1650 and 1644 cm⁻¹, respectively.⁴⁰

The second kind of perturbation of the C=N bond involves charge redistribution along the bond, which was achieved by introducing a nonconjugated positive charge in the vicinity of the Schiff base linkage. Such interaction shifted the C=N stretch to a lower energy but did not change the deuterium isotope effect (Table 3). This insensitivity indicates that C=N/

N–H coupling was not affected by the nonconjugated charge. Our studies revealed that a change in the absorption maximum of RSBH⁺ occurring without C=N stretching alteration should imply a perturbation in the ring region or close to it along the polyene chain (around C₅ to C₁₀). Alteration of both the absorption spectrum and the C=N stretching mode should point to perturbations in the vicinity of the C=N linkage.

We now consider the implications of the above results on bR and on visual pigments. First we conclude that in bR the retinal-protein interactions in the vicinity of the chromophore ring induce a red shift in the absorption maximum but cannot affect C=N stretching. Consequently, the C=N shift of bR relative to RSBH⁺ in methanol (1640 cm⁻¹ compared with 1656 cm⁻¹) may be attributed to weaker hydrogen bonding of the N⁺–H moiety with its counteranion and/or with protein dipoles or residual water.

This conclusion is directly supported by the observations that shifts in the C=N frequency of the order of 12 cm⁻¹ (due to diminishing H-bonds), observed in the previously described model systems, were accompanied by a shift of about 2400 cm⁻¹ in the absorption maximum. Similarly, the 5000 cm⁻¹ opsin shift in the absorption of bR (out of which about 3000 cm⁻¹ is attributed to the weaker H-bond^{22–25}) was accompanied by a ~16 cm⁻¹ shift in the C=N frequency relative to methanol. The claim that the above environmental effects in the region of the Schiff base in bR was due to weaker H-bonding, rather than to other kinds of electrostatic interactions, finds support in the ~17-cm⁻¹ deuterium isotope effect on the C=N frequency of bR. The latter was similar to that found in weakly H-bonded model systems, such as RSBH⁺ in methylene chloride with an excess of TFA. Moreover, evidence for weak hydrogen bonding in bR was also suggested by ¹⁵N-NMR studies.⁴⁸

The C=N stretching frequency observed in bovine-rhodopsin (1660 cm⁻¹) indicates C=N/opsin interactions differing from those in bR. This value suggests that solvation of the positively charged nitrogen is similar, or even stronger, than that which prevails for RSBH⁺ in methanol. The isotope effect of about 30 cm⁻¹ following deuteration supports the conclusion that in the visual pigment H-bonding of the Schiff base was as effective as in methanol. Because such a C=N environment should lead to an absorption maximum of ~440 nm (or even lower), one must also conclude that the red shift found in bovine rhodopsin ($\lambda_{\max} = 498$ nm) originates from interactions that do not affect the C=N stretching. Our model studies in solution revealed that electrostatic interactions in the vicinity of the chromophore ring shifted the absorption maximum without affecting the C=N frequency. However, substantial opsin shifts were observed for a 5,6-dihydroretinal artificial visual pigment¹⁰ and for the pigment derived from a (ring) bicyclic chromophore.⁴⁹ Thus, the presence of a nonconjugated charge in the vicinity of the ring (or that of a planar ring-chain structure) should be discarded as a mechanism for the opsin shift in bovine rhodopsin. This conclusion is supported by recent ¹³C NMR data.⁵⁰ The external point charge model⁵¹ proposed a nonconjugated negative charge in the vicinity of C₁₂ to C₁₄ as a source for the red shift in bovine rhodopsin. It seems to us that such a charge (or dipole) is more likely to be located closer to C₉ and C₁₀ than to C₁₂ to C₁₄, because in the latter case a negative charge will influence the ground state more, introducing a blue shift in the spectrum,¹³ and because it is also expected to affect C=N stretching. On the other hand, a nonconjugated charge in the vicinity of C₉ and C₁₀ will satisfactorily account for both optical and vibrational spectroscopic data.

IV. THE pK_a OF THE PROTONATED SCHIFF BASE OF bR AND ITS ALTERATION DURING THE PHOTOCYCLE

As shown in Figure 1, following light absorption bR undergoes a photochemical cycle including an intermediate (M₄₁₂) in which the Schiff base is not protonated. The deprotonation of the Schiff base at this stage is most probably directly associated with the operation of the bR proton pump. It was suggested^{52,53} that the loss of the proton results from a marked

reduction of the pK_a of the protonated Schiff base as a consequence of light absorption. Thus, understanding the factors influencing the pK_a of model $RSBH^+$ is a prerequisite for clarifying the retinal-opsin interactions that affect the pK_a of the Schiff base in bacteriorhodopsin and, consequently, for understanding the mechanism of the proton pump.

Several suggestions have been made to account for the high apparent pK_a and for its reduction during the photocycle. Warshel et al.⁵⁴ proposed that a relatively polar protein environment surrounding the protonated Schiff base stabilizes the Schiff base-counteranion pair. Hildebrandt and Stockburger⁵⁵ attributed the stabilization to hydrogen bonding with trapped water molecules. Schulten et al.^{56,57} suggested a reduction in the pK_a during the photocycle due to twisting around the C_{14} to C_{15} single bond of the retinal chromophore. Destabilization of the ion-pair due to charge separation was proposed by Honig et al.⁸ whereas Scheiner and Hillenbrand⁵⁸ proposed a change in the relative orientation of the protonated Schiff base and its counterion. The influence of an electric field upon transfer of a proton from a protein donor to an acceptor was proposed by Mertz and Zundel.⁵⁹ Recently, El-Sayed et al.^{60,61} suggested the possibility of reducing the pK_a by a positive charge approaching the Schiff base linkage.

The apparent pK_a of bR in the dark was established⁶² as 13.3 ± 0.3 . Although the formation of a high-pH nonprotonated Schiff base species was clearly established, it was pointed out that in principle (e.g., if the Schiff base is inaccessible to H_3O^+ and OH^-) the reversible spectral changes observed upon titration may not represent the direct titration of the Schiff base but rather that of other groups in bR. Upon titration such groups may induce protein conformational changes exposing the Schiff base and causing its deprotonation. We have approached this uncertainty²¹ by using an artificial pigment based on 13-CF₃-all-*trans*-retinal, a chromophore which was synthesized previously.^{63,64} Upon incubation with bacteriopsin, the chromophore yielded a red-shifted pigment (bR₆₂₅). The red shift is attributed to the trifluoromethyl group located in the vicinity of the Schiff base bridge, which causes destabilization of the ground state due to its electron-withdrawing character. This effect is also reflected in the pK_a value. Titration experiments showed that the apparent pK_a of the 13-CF₃-retinal protonated Schiff base was markedly reduced by ~ 5 units to $pK_a = 1.8 \pm 0.1$ relative to that (7.4 ± 0.1) of all-*trans*- $RSBH^+$. Moreover, titration of the 13-CF₃ artificial bR pigment (27, Table 4) indicated that the CF₃ substituent induced a comparable decrease of ~ 5 units ($pK_a = 8.0 \pm 0.2$) with respect to that of native bR. Thus, the intrinsic change induced in the pK_a of the free retinal Schiff base chromophore was faithfully reflected in that of the corresponding bR pigment. These results provide direct support for the suggestion that the reversible spectral change induced by raising the pH in bR (with an apparent pK_a value of 13.3) is associated with the direct titration of the Schiff base nitrogen.

An additional important aspect of studying the artificial pigment derived from 13-CF₃-retinal is the effect of the intrinsic pK_a change on the photocycle. As described above, the bR photocycle is characterized by a nonprotonated intermediate, M₄₁₂. If proton transfer from the Schiff base to a protein acceptor is rate-determining in the above deprotonation process, then the rate of M₄₁₂ formation is expected to be markedly affected by reduction of the pK_a . However, an analysis of the formation process indicates that the rate observed for the artificial 13-CF₃ pigment was close to that of native bR. It can therefore be concluded that proton transfer from the Schiff base to the protein acceptor is not the rate-determining step in the Schiff base deprotonation process. Consequently, the formation of M must be controlled by a distinct process involving changes in the protein. A plausible alternative for this rate-determining process based on changes of the NH⁺-protein interactions will be discussed.

The high apparent pK_a of the protonated bR Schiff base should be attributed to factors associated with the close Schiff base environment rather than to retinal-opsin interactions along the polyene or in the β -cyclogeranylidene ring region. As discussed above, the latter

TABLE 4
Spectral Data For Artificial Pigments 20 to 27 and Their Protonated Form (RSBH^+) Derivatives

Pigment	Absorption maxima (nm)			Spectral Shifts (cm^{-1})		
	RSB (MeOH)	RSBH^+ (MeOH)	Pigment	OS_{bR}	$\Delta\nu$ (bR/K)	OS_M^*
	20 367	436	565	5240	—	—
	21 364	435	570	5600	1520	—
	22 395	480	518	1340	2800	625
	23 360	448	570	4800	1900	3400
	24 358	436	556	5000	2000	3540
	25 366	438	525	3780	—	—
	26 367	467	625	5400	1900	3440
	27 363	428	480	2500	1600	1900

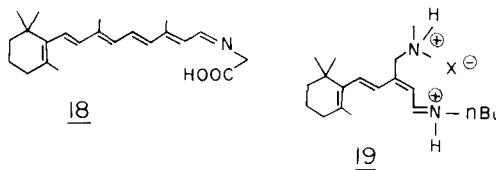
* Whenever $\Delta\nu$ (bR/K) or OS_m are not specified, it implies that K or M intermediates were not observed.

^b With the exception of pigment 25 having a 13-cis isomer, the pigments have the all-trans polyene chain.

have a marked influence on the absorption maximum of bR. This conclusion has been suggested by the pK_a s found for artificial pigments having bulky substituents at the C₄ position of the retinal.¹⁹ The absorption maxima of such pigments were considerably blue-shifted relative to that of bR due to elimination of the (red-shifting) chromophore-opsin interactions in the ring region. However, the apparent pK_a s of the pigments (about 12.5) were close to that of native bR.

The capability of altering the pK_a of RSBH^+ in solution by changing the environment in the vicinity of the Schiff base linkage was demonstrated by using fluorinated alcohols, such as TFE.¹⁴ The protonation of RSB in TFE was interpreted in terms of the pK_a of RSBH^+ in this solvent, which was substantially increased due to strong hydrogen bonding that stabilized the ions. [In view of the pK_a values of TFE and of RSBH^+ in a MeOH/H₂O solution (12.5⁶⁵ and 7.4,⁶⁶ respectively), direct protonation of RSB by TFE is not feasible.] The effect of environment-stabilization was further demonstrated by studying the condensation product **18** between retinal and glycine.¹⁴ In chloroform, the Schiff base linkage was

not protonated by the carboxylic acid, whereas in trifluoroethanol complete protonation by the acid was observed.



An alternative way of altering (reducing) the effective pK_a of RSBH^+ is by introducing positive charges in the vicinity of the Schiff base bridge. This was demonstrated by using synthetic retinal analogs bearing nonconjugated positive charges.¹⁴ The nonconjugated charge was more effective as the distance from the Schiff base bridge was reduced. Thus, the pK_a of chromophore **8** was affected (relative to that of retinal) by about 2 units (in 50% $\text{H}_2\text{O}-\text{MeOH}$), whereas the pK_a of **4** changed by only 0.5 units. Further enhancement of the above positive charge effect was observed in fluorinated alcohols, which do not effectively solvate positive charges.¹⁷ Such a weak solvation caused a strong interaction between the positive charge and the NH^+ moiety in HIPP so that in **19**, a reduction of more than 5 pK_a units was observed.

In conclusion, our work with model RSBH^+ and with the 13- CF_3 artificial pigment, is clearly indicative that both the apparent pK_a of bR (13.3 ± 0.3) and its reduction during the photocycle are controlled by interactions with the protein in the vicinity of the Schiff base. Primarily, it was possible to increase considerably the effective pK_a of RSBH^+ by stabilizing the ions via hydrogen bonding. Accordingly, the pK_a can be reduced by eliminating hydrogen bonds or by introducing a charge in the vicinity of the Schiff base bridge. It is therefore feasible that reduction of the bR pK_a value takes place at an early stage of the photocycle (possibly, following the primary event) due to dislocation of the positively charged Schiff base nitrogen from a stabilizing (hydrogen bonding) protein environment. In this case, the rate-determining step in the deprotonation process (i.e., M_{412} formation) will involve opening of a proton channel to a suitable H^+ acceptor. Alternatively, as discussed by Hanamoto et al.,⁶⁰ Baasov and Sheves,¹⁴ and Sheves et al.,²¹ the rate-determining step might involve the approach of a positive charge that reduces the pK_a value at the stage of the L_{550} intermediate. It should be noted that any quantitative specific mechanism for the deprotonation process should also account for the observation^{53,60,67-69} that the rate of M_{412} formation is catalyzed by a deprotonated form of a protein moiety undergoing titration with a $\text{pK}_a \approx 9.5$.

V. THE PRIMARY PHOTOCHEMICAL EVENT IN bR AND IN BOVINE RHODOPSIN

Bovine rhodopsin and bacteriorhodopsin exhibit photocycles with remarkably similar features¹⁻⁴ primarily, as shown in Figure 1, the occurrence of early red-shifted photointermediates, the number of stages in the photocycle and their respective time scales, the photoreversibility of most intermediates, and the deprotonation of the Schiff base on a $\sim 10^{-4}$ s time scale. The interpretation of such phenomena in terms of the molecular changes occurring in the retinal moiety and its protein environment is an essential prerequisite for understanding the mechanism by which retinoid pigments convert, store, and subsequently utilize solar radiation.

We first consider the primary events in Rh and bR, which are associated with the generation of the red-shifted phototransients bathorhodopsin (BATHO) and K_{610} , respectively (for reviews see References 1 to 4). A feature common to these two intermediates are their

evolution kinetics as monitored by picosecond absorption spectroscopy. Thus, it was recognized that both BATHO⁷⁰ and K₆₁₀⁷¹⁻⁷³ are generated from further red-shifted species denoted⁷⁴ as prebathorhodopsin (PBATHO) and J₆₂₅, respectively. J₆₂₅ grows in on a sub-picosecond time scale from a precursor denoted as I. An analysis of the picosecond and femtosecond excitation data⁷² indicated that J₆₂₅ is red-shifted by approximately 10 nm relative to K₆₁₀, whereas I is markedly blue-shifted ($\lambda_{\text{max}} < 500$ nm). The time constants associated with the I \Rightarrow J₆₂₅ and the J₆₂₅ \Rightarrow K₆₁₀ processes were established as 0.43 + 0.05 and 5 ps, respectively. A growing in of the PBATHO, parallel to the I \Rightarrow J₆₂₅ step in bR, has not yet been observed in visual pigments. It appears, however, that the PBATHO \Rightarrow BATHO process is analogous to the J₆₂₅ \Rightarrow K₆₁₀ transition. Primarily, as in the case of the bR intermediates, PBATHO (or photorhodopsin, as termed by Yoshizawa and co-workers⁷⁵) is red-shifted by approximately 10 nm relative to bathorhodopsin. The rate constants for the PBATHO \Rightarrow BATHO processes are 40 ps for bovine rhodopsin and approximately 200 ps for squid and octopus rhodopsin.⁷⁵ The nature of BATHO and K₆₁₀ in terms of intrinsic changes in the retinyl moiety, as well as the interactions between the chromophore and its protein binding site is discussed later.

A. BOND ISOMERIZATIONS AND ROTATIONS

The above phenomena strongly suggest an essentially identical model for the primary photochemical events, i.e., for the structure of the batho intermediates (BATHO and K₆₁₀) and their precursors, in both rhodopsin and bacteriorhodopsin. Early models (for a review see Reference 7), based primarily on the isotope effects reported for the PBATHO \Rightarrow BATHO⁷⁰ and J₆₂₅ \Rightarrow K₆₁₀⁷⁶ processes, invoked structures for BATHO and K₆₁₀ involving proton translocation to the Schiff base or in the surrounding protein. No changes, or only small distortions, of the polyene chain were invoked. Numerous arguments rejecting this approach have been presented and extensively discussed.^{2,3} In the case of bR, they are now further supported by the lack of an isotope effect in the steps leading to K₆₁₀.⁷¹⁻⁷³ Consequently, models for the structures of BATHO and K₆₁₀ were presented based on primary polyene photoisomerization processes: 11-cis(Rh) \rightarrow all-trans(BATHO) and all-trans(bR) \rightarrow 13-cis(K₆₁₀), respectively.⁷² The early evidence supporting isomerization at the stages of BATHO and K₆₁₀ was indirect being based on the photoequilibria attainable between bathorhodopsin, rhodopsin (11-cis), isorhodopsin (9-cis), and rhodopsin (7-cis).⁷⁷⁻⁷⁹ Further evidence was later obtained from Raman data indirectly supported (distorted) isomerized chromophores, all-trans in the case of BATHO⁸⁰ and 13-cis in the case of K₆₁₀.⁸¹

On the basis of the above information, we have systematically studied the photocycles of a series of artificial bR pigments with blocked bond rotations and isomerizations⁸² to establish the role of the C₁₃=C₁₄, and possibly of other C=C bonds, in the primary event and to investigate the possible involvement of C-Crotations in the generation of K₆₁₀.

Our experimental results have indicated^{22,82} that with the exception of pigment **20** a photocycle associated with a primary red-shifted "K" intermediate is observed for all artificial pigments shown in Tables 2 and 4. This applies to derivatives **21** to **25** with blocked C=C rotations up to C₁₃, as well as **17** (Table 2) characterized by only one C=C double bond (analogous to C₁₃=C₁₄). Such observations are of primary importance because they obviously restrict the possible conformational changes associated with the primary photo-processes to the C₁₃-to-N region of the polyene molecule. In other words, photoisomerization may only involve the C₁₃=C₁₄ and C₁₅=N double bonds. NMR⁸³ and resonance Raman⁸⁴ experiments strongly suggest that the C₁₅=N conformation is "anti" in both bR₅₇₀ and K₆₁₀. This conclusion, along with the failure to observe a photocycle in the case of **20** provides direct evidence confirming the suggestion that the generation of the K₆₁₀ photoproduct of bR₅₇₀ is associated with a *trans-cis* isomerization involving only the C₁₃=C₁₄ bond. This is in keeping with the lack of any proton-pumping activity⁸⁵ and, as recently reported,⁸⁶ with

the lack of light-induced photochemical changes in a bR pigment based on a synthetic chromophore in which the $C_{13}=C_{14}$ rotation was blocked by a five-membered ring. Moreover, it is also evident that rotations about specific single bonds up to C_{13} are not required for initiating the photocycle. An exception may be the $C_{14}-C_{15}$ rotation, which was suggested to take place in the primary event^{56,87,88} as part of a concerted double-bond ($C_{13}=C_{14}$) and single-bond rotation mechanism. This argument is still subject to controversial experimental evidence.^{89,90}

The involvement of the $C_{11}=C_{12}$ isomerization in the primary event of bovine rhodopsin is strongly supported by the observed photochemical stability of a synthetic pigment with a blocked $C_{11}=C_{12}$ bond.⁹¹ However, comprehensive information based on artificial visual pigments (similar to that described above for bR), excluding the (unlikely) participation of other $C=C$ bonds in the primary event in vision, is still unavailable. FTIR and resonance Raman spectroscopy data appear, however, to exclude the $C=N$ photoisomerization in bovine rhodopsin.^{35,92}

Considerable attention has been devoted to theoretical models for the generation of BATHO (similarly for K_{610}) involving rotations about several⁹³ or one specific^{56,88,94,95} single bond(s). The mechanism invoked in the case of visual pigments⁹⁵ is based on a concerted double-bond isomerization — single-bond rotation about the adjacent $C_{10}-C_{11}$ and $C_{11}-C_{12}$ bonds. The main argument favoring the concerted motion approach involves a minimal mass motion, i.e., minimal disturbance of the protein cavity. Experimental evidence based on an artificial C_9-C_{11} -locked rhodopsin has been recently presented by us,⁹⁶ indicating that the $C_{10}-C_{11}$ rotation is not a prerequisite for the occurrence of a normal visual photocycle.

B. RETINAL-OPsin INTERACTIONS

We have approached the question of the changes occurring in the primary steps of the bR photocycle, which are due to interactions between the retinyl moiety and its protein environment by considering the shift in the optical spectrum associated with formation of the K_{610} species. These are quantitatively measured by the parameter — $\Delta\nu$ (bR/K) — which is defined as the energy difference in cm^{-1} between the minimum in the laser-induced difference spectrum, due to the depletion of bR, and the maximum, due to the generation of K_{610} . Working with a series of artificial pigments differing in their basic opsin shift, it was found^{19,21,22} that not only was a K species observed in most cases but that the parameter $\Delta\nu$ (bR/K) showed relatively little sensitivity to the nature of the specific pigment involved (Tables 2 and 4). Thus, the spectral shift associated with the $bR \rightarrow K$ transition, which is $\sim 2500 \text{ cm}^{-1}$ in bR, did not show a trend parallel to that of OS_{bR} . Moreover, a substantial value of $\sim 1500 \text{ cm}^{-1}$ was observed even when a single double bond, such as in **17** was present. [The smaller values of $\Delta\nu$ (bR/K) observed for the shortened aromatic derivatives **16** and **17** and for the substituted analogs **21** and **27**, which are characterized by a different polyene electronic system, may be attributed to a different sensitivity to environmental factors in the vicinity of the Schiff base nitrogen.] These observations led to the conclusion that the main contribution to the spectral shift in K cannot be due to changes in steric or electrostatic interactions between the opsin and the ring, or other polyene sections up to C_{13} . It is thus evident that the primary event is directly associated with changes in the Schiff base environment of the chromophore.

Evidence supporting the above model may also be derived from studies monitoring the $C=N$ stretch frequency at the stage of K_{610} by resonance Raman⁹⁷⁻¹⁰⁰ or FTIR^{28,100,101} spectroscopy. The latter parameter underwent a substantial drop (20 to 30 cm^{-1}) upon the conversion of bR to K_{610} . This observation should be considered in light of the analysis of the factors that affect the $C=N$ stretch frequency of protonated Schiff bases of retinal (PRSB) in solution, described in Section III. Analysis of FTIR data from a variety of retinal analogs in different solvents showed that the above frequency was sensitive to nonconjugated charges

in the vicinity of the NH^+ moiety but not to those located near the ring or along the polyene chain. The C=N frequency was also markedly affected by hydrogen bonding of the nitrogen to its counterion or to solvent molecules. Comparison of the C=N frequencies of model systems with those of the biological pigments indicates that in bacteriorhodopsin the frequency ($\sim 1640 \text{ cm}^{-1}$) was similar to that prevailing in a weakly H-bonded PRSB. Also comparable are the respective drops induced by deuteration (14 to 17 cm^{-1}). This picture is in keeping with the conclusion derived from ^{15}N NMR experiments.⁴⁸ Accordingly, the event associated with the bR $\xrightarrow{\text{h}\nu}$ K₆₁₀ transition may be interpreted in terms of the C₁₃=C₁₄ isomerization that induces a charge separation between the NH⁺ moiety and its counterion and/or weakens the H-bond with neighboring opsin residues or H₂O molecules. Such a model will (qualitatively) account for the bathochromic shift in K₆₁₀ [i.e., for most of the value of $\Delta\nu$ (bR/K)], as well as for the storage of ~ 15 kcal/mol out of the initial proton energy at the stage of K₆₁₀.^{102,103}

Difficulties arise, however, when attempting to apply the above model to visual (bovine) rhodopsin. First, a systematic study of the parameter $\Delta\nu$ (Rh/BATHO) — analogous to $\Delta\nu$ (bR/K) — in a variety of artificial pigments is still unavailable. Second, both resonance Raman^{92,104,105} and FTIR³⁵ data indicate that the C=N stretch frequency in BATHO is identical to that of the parent pigment. Such observations that may imply that the Schiff base environment in visual pigments does not change after photoisomerization question the validity of any general model for the primary event that is common to visual rhodopsins and to bacteriorhodopsin. Specifically, they call for a model for the visual photocycle that will account for both spectral shift and energy storage in BATHO without requiring changes in the Schiff base environment, such as charge separation from its counterion. The picture in this respect is still obscure. One possibility is that charge separation takes place (as in bR), accounting for energy storage and for the (optical) red shift. The fact that the C=N frequency does not change in BATHO may be attributed to strong H-bonds (with protein moieties or with H₂O) counterbalancing the effect caused by separation from the counterion. Alternatively, it is possible that the Schiff base environment does not change (no charge separation). In such a case, nonelectrostatic energy storage, such as conformational distortion of the polyene chromophore, must be invoked.^{92,106-108} Obviously, further work will be required for reaching a clear model for the structure of BATHO on a molecular basis.

We finally note that, although the work with artificial pigments does not bear directly on the nature of J₆₂₅ and PBATHO, a variety of indirect evidence suggests that they are ground state species with polyene conformations identical to the (isomerized) structures of BATHO and K₆₁₀, respectively, but with a nonrelaxed protein structure. This is in keeping with the failure to observe J₆₂₅ and PBATHO in artificial pigments in which the photocycle is blocked by appropriate ring structures.^{72,75} A clear identification of the I species in the bR photocycle (a similar precursor has not yet been observed in visual systems) is still unavailable. One possibility^{7,24} is that it corresponds to the “common excited state” along the reaction (isomerization) coordinate between the *trans* (bR) and 13-*cis* (J₆₂₅) configurations. Obviously, picosecond and subpicosecond studies of artificial bR pigments may shed new light on the nature of the I intermediate.

VI. SUBSEQUENT STAGES IN THE PHOTOCYCLE OF bR

The basic questions related to the nature of the molecular changes in the photocycles of bR and Rh, beyond the respective stages of K₆₁₀ and BATHO, are similar to those associated with the primary event. Namely, how may the various stages be described in terms of the retinal conformation, changes in the protein structure, and changes in the retinal-protein interactions. Substantial data concerning acid-base dissociation processes in the protein during the photocycle has been obtained on bR from FTIR experiments.¹⁰⁹⁻¹¹¹ Res-

onance Raman spectroscopy has provided qualitative information on the conformation of the polyene and its interactions with the opsin at the stages of L, M, and O. As far as information derived from artificial pigments is concerned, our work has indicated that while the freedom to rotate about $C_{13}=C_{14}$ appears to be a prerequisite for the formation of K, it seems (see pigments 15 to 17) that parts other than the C_{13} -to-N region must be associated with the formation of the L_{550} and M_{412} intermediates. Thus, as shown by us,²² at least two C=C bonds (those corresponding to $C_{11}=C_{12}$ and $C_{13}=C_{14}$) are required for the formation of L_{550} . The M_{412} intermediate, which is directly related to the initiation of proton translocation, (for a review see Reference 3), appears to be found only in analogs retaining at least three C=C bonds ($C_{13}=C_{14}$, $C_{11}=C_{12}$, and $C_9=C_{10}$). However, the work with pigments 22 to 24 has demonstrated that specific isomerizations and rotations of bonds other than those of the C_{13} -to-N region of the chromophore were not required for generation of these photocycle intermediates.

The use of artificial pigments is also relevant to the spectrum of the M intermediate, characterized by an unprotonated Schiff base structure.¹¹² Here, too, we may consider an *opsin shift* (OS_M), to be defined as the energy difference between the absorption maximum of the (unprotonated) Schiff base of the chromophore in solution and that of the M intermediate in the photocycle of the corresponding synthetic pigment. The data in Tables 2 and 4 show that part of the value of OS_M may be attributed to a planar ring-chain conformation (e.g., the difference between the OS_M value of bR and those of 13a, 27, and the “blue” pigments of 9 to 11. However, OS_M maintains a considerable value of $\sim 2100\text{ cm}^{-1}$, even in those derivatives in which the opsin shift of the basic pigment (OS_{bR}) is markedly affected by the lack of ring-opsin interactions (see discussion above). This observation suggests that the red-shifted spectrum of M is also due to interactions associated with the Schiff base nitrogen, such as exposure to a protein charge or to the effects of hydrogen bonding.

We have already pointed out (see Section IV) that important insights into the bR photocycle, based on the use of artificial pigments, may be obtained by investigating the photochemical behavior of pigments in which the pK_a values of their Schiff base have been intrinsically modified. As previously discussed, the introduction of a $C_{13}\text{-CF}_3$ substituent decreased the pK_a value of the Schiff base by an amount (~ 5 units) that was additive to the increase (~ 6 units relative to a model Schiff base in solution) caused by its interactions with the protein environment. Based on the assumption that such additivity also holds during the photocycle (when new retinal-protein interactions are present, reducing the pK_a to below ~ 5)⁵³ it was concluded²¹ that proton transfer to a protein acceptor is not the rate-determining step in the Schiff base deprotonation process leading to M_{412} . Consequently, the formation of M must be controlled by a distinct process involving changes in the protein, as discussed in Section IV. A similar approach may prove to be helpful in elucidating the formation of the deprotonated species M_{II} in the visual photocycle.

VII. THERMAL ISOMERIZATIONS OF RETINAL ISOMERS

The M_{412} intermediate of the bR photocycle consists of a 13-*cis*-retinal isomer originating in the all-*trans*- to 13-*cis* isomerization that occurs during the primary event in the photocycle. To complete the photocycle, the M_{412} intermediate undergoes a rapid (millisecond) thermal process, regenerating the original bR_{570} all-*trans* chromophore. Such a process is substantially faster than the corresponding thermal isomerization of model retinals or their Schiff bases in solution and also the thermal isomerization to 13-*cis* during the dark adaption of all-*trans*- bR_{570} .³ Similarly, visual pigments consisting of 11-*cis*-retinal undergo an extremely slow thermal isomerization to all-*trans*,⁸ thus accounting for their high thermal stability (i.e., low dark thermal-noise), compared to the efficient photochemical isomerization of the 11-*cis* bond following light absorption. Thus, the mechanism through which retinal isomers ex-

perience thermal isomerization and the factors controlling this process are of important significance.

Several suggestions have been made to account for the fast “catalyzed” thermal isomerization of the retinal chromophore in the protein binding site during the $M_{412} \rightarrow bR_{570}$ step in the bR photocycle, as well as in the process of dark adaption. Nucleophilic attack by dihydroflavin was suggested by Futterman and Rollins,¹¹³ and a base attack at the C₁₃ position of the retinal skeleton was suggested by Mowery and Stoekenius.¹¹⁴ Rando et al.^{115,116} have further stressed the possibility of a nucleophilic attack mechanism as the driving force for thermal isomerization. Theoretical studies carried out by Schulten et al.^{87,94} and Warshel⁹³ proposed that thermal isomerization is controlled by π -electron delocalization along the retinal polyene skeleton. By applying a series of environmental changes, we have experimentally studied¹¹⁷ factors affecting the rate of the thermal isomerization of 13-*cis*-RSBH⁺ in solution. We have shown that the thermal isomerization is controlled by π -electron delocalization, which enhances bond alteration, reducing the energy barrier, and thereby, inducing fast isomerization.

π -Electron delocalization in RSBH⁺ was modified in solution in three major ways: (1) by diminishing ion-pairing between the positively charged Schiff base nitrogen and its counterion, (2) by modifying stabilization of the positively charged nitrogen via hydrogen bonding with various solvents, and (3) via interaction with a nonconjugated positive charge in the vicinity of the ring moiety. For example, it was shown^{13,15} that excess TFA red-shifted the absorption maximum of protonated retinal Schiff bases in nonprotic solvents. The excess acid hydrogen bonds to the anion associated with the positive ion and diminishes ion-pairing. Weakening of ion-pairing deshields the positive charge, enhancing charge delocalization, so that a rapid thermal isomerization is expected. In fact, the addition of excess TFA to a 13-*cis*-retinal Schiff base, dissolved in chloroform solution, caused thermal isomerization with a rate that correlated with the TFA concentration. The effect of counteranion association can also be induced by other compounds capable of effective hydrogen bonding, such as TFE. Addition of TFE to a chloroform solution of 13-*cis*-RSBH⁺ caused thermal isomerization, similar to (although slower than) the case of TFA, supporting the hydrogen bonding mechanism. An alternative, nucleophilic attack mechanism is unlikely because addition of a large excess of trifluorocarboxylate anion (pyridinium trifluorocarboxylate) instead of TFA did not enhance thermal isomerization. Furthermore, substitution of chloroform by methanol, which effectively solvates the positively charged nitrogen, prevented thermal isomerization even in the presence of a large excess of TFA.

The rate of isomerization of RSBH⁺ may also be enhanced in chloroform solution by increasing the size of the counteranion. A larger counteranion increases charge delocalization due to its weaker interaction with the positively charged nitrogen. Thus, the half-life of the 13-*cis*-to-all-*trans* process was 8 h with Cl⁻ as the counteranion, compared to 50 min with ClO₄⁻. The rate of isomerization was also found to be controlled by the nature of the solvent. Slow isomerization of 13-*cis*-PRSB was observed in methanol (12 h), whereas a half-life of 5 min was observed in HFIP. Methanol effectively solvates the positively charged nitrogen, preventing charge delocalization, in contrast to HFIP, which only weakly interacts with the nitrogen moiety.

Finally, charge delocalization and, consequently, the rate of thermal isomerization of RSBH⁺, may be controlled via the interaction of the polyene with nonconjugated charges. Very slow isomerization was observed in the 13-*cis* isomer of chromophore **4**, carrying a nonconjugated positive charge in the vicinity of the ring moiety, even with excess TFA in chloroform. The nonconjugated charge reduces charge delocalization, thereby reducing the rate of thermal isomerization. A positive charge, located in the vicinity of the Schiff base linkage, will enhance the isomerization rate due to repulsion between the positive charges, which increases bond alteration. This effect was observed in the case of the 13-*cis* isomer of chromophore **8**.

In the ground state, charge delocalization along the polyene is diminished as the distance from the positively charged nitrogen is increased. Thus, one would expect the rate of isomerization to follow the order 13-*cis* > 11-*cis* > 9-*cis*. Such a trend was indeed observed in our model studies, as well as by Rando et al.¹¹⁶

In conclusion, our experimental results provided direct evidence, supporting the theoretical prediction,^{87,94} that the thermal isomerization of 13-*cis*-RSBH⁺ is controlled by charge delocalization, which may be induced by environmental factors such as hydrogen bonds and external charges. It is highly probable that such factors, in the form of appropriate retinal-opsin interactions, control the process in the photocycle of bR in which the back, 13-*cis* → *trans* thermal isomerization allows the reversion of the primary *trans* → 13-*cis* photoreaction on a millisecond time scale. In other words, it is suggested that during the last stages of the photocycle, 13-*cis*-bR rapidly isomerizes to all-*trans* due to appropriate modulation of π-electron delocalization via weak interactions of the Schiff base linkage with its counteranion and/or protein dipoles, as well as with nonconjugated charges along the polyene. We note that this mechanism implies that the 13-*cis* nonprotonated intermediate in the bacteriorhodopsin photocycle experiences protonation prior to isomerization, a fact which has not yet been confirmed experimentally. Moreover, the exact nature of the protein catalytic effects on the thermal isomerization of the bR chromophore are still undetermined. In addition to the above electrostatic effects, other factors such as steric hindrance imposed by the protein and including effects on ring-chain planarity, might also affect the barrier for thermal isomerization. For example, the light → dark adaption of bR, which involves a *trans* → 13-*cis* process, occurs on a time scale of ~10⁴ s. Such a slow isomerization, relative to that associated with the M₄₁₂ decay process, might be attributed to a weaker (catalytic) π-electron delocalization or to steric hindrance imposed by the protein.

We finally note the extremely slow thermal isomerization in pigments, which is responsible for their high thermal stability (i.e., low thermal noise). This behavior, which is in contrast to the relatively faster 13-*cis*↔all-*trans* equilibration processes in bR, can (at least partially) be attributed to strong hydrogen bonding of the Schiff base linkage with its protein environment. As discussed in Section III, the C=N stretching frequency of bovine rhodopsin is indicative of strong hydrogen bonds of the N-H moiety with its immediate environment, similar to those prevailing in RSBH⁺ methanol solutions. The effective solvation of the positively charged nitrogen decreases π-electron delocalization along the polyene, decreasing the isomerization rate, as observed with model 13-*cis*¹¹⁷ and 11-*cis*¹¹⁸ RSBH⁺ in methanol. It is, thus, possible that the differences in the C=N stretching of bovine rhodopsin and bacteriorhodopsin, account for the observed difference in their respective thermal isomerization rates.

VIII. SUMMARY AND CONCLUSIONS

In this chapter, we have highlighted our work on the chemistry and photochemistry of bacteriorhodopsin and (bovine) visual pigments. We have clarified experimentally the influence of nonconjugated charges on the absorption maxima of RSBH⁺ and evaluated the direction and magnitude of the spectral shifts induced by them. Furthermore, we have provided support for the hypothesis that a significant part of the red shift observed in bR is due to interactions that occur between the retinal and the opsin in the region of the Schiff base nitrogen. Future investigations should clarify the exact electrostatic interactions prevailing between the retinal and the opsin in the region of the retinal ring and along the polyene skeleton in bR and, especially, in visual pigments. ¹³C NMR studies might prove a powerful tool in this respect.

Our results with models in solution indicated a correlation between the C=C stretching frequency of various protonated retinal Schiff bases and their absorption maxima. No such

correlation was found for the C=N mode. The latter is changed due to alteration of its close environment either by modification of hydrogen bonding with the N–H bond or by introducing external charges in the vicinity of the Schiff base linkage. External charges in the vicinity of the β -cyclogeranylidene ring or ring-chain planarity affect the absorption maximum without changing the C=N stretching. The results shed light on the different C=N environments prevailing in bR and in bovine rhodopsin. Weaker hydrogen bonds probably exist in the former. Further diminishing in hydrogen bonding occurs in bR following light absorption and generation of K₆₁₀. The situation in this respect is still unresolved in the rhodopsin system. Further studies will have to clarify the insensitivity of the C=N frequency to the changes occurring in the retinal chromophore following light absorption by the 11-*cis* pigment and the generation of the all-*trans* BATHO intermediate. Any such molecular mechanism should also account for the energy storage (~ 30 kcal) and the absorption maximum change taking place following this step.

We have shown that the apparent pK_a of bacteriorhodopsin is 13.3, which is significantly different from that found for protonated retinal Schiff bases in solution. We explain this difference by effective hydrogen bonding mainly with the Schiff base counterion, with protein dipoles, or with residual water. We found that proton transfer from the protonated Schiff base chromophore to a protein acceptor is not the rate-determining step in the Schiff base deprotonation process in the bR photocycle. It is proposed that such deprotonation is controlled by a distinct process in which the pK_a is reduced either by elimination of a stabilizing environment or by introducing a positive charge in the vicinity of the Schiff base linkage. Here again, further investigation is needed to clarify the specific mechanism and the protein groups involved. Amino acid substitutions by site-specific mutagenesis might be very helpful in this respect.

By studying the photocycles of artificial pigments, we have found that the primary event in bR is mainly associated with changes in the Schiff base–opsin interactions. Following light absorption, the retinal chromophore experiences isomerization of the C₁₃=C₁₄ polyene double bond only. Rotation about all the single bonds with the possible exception of the C₁₄–C₁₅ bond (which still awaits final clarification) does not occur. We also observed that, rather than the C₁₃-to-N region, other parts of the retinal chromophore must be associated with the formation of the L₅₅₀ and M₄₁₂ photocycle intermediates. Furthermore, we have found that in bovine rhodopsin a rotation about the C₁₀–C₁₁ single bond is not a prerequisite for generating the photoprocess or for the bleaching sequence of rhodopsin.

Finally, we have studied the thermal isomerization of protonated retinal Schiff base isomers in solution and found that the energy barrier for isomerization is controlled by π -electron delocalization along the polyene. Increased π -electron delocalization reduces the energy barrier and allows fast isomerization. The results may account for the relatively efficient isomerization of 13-*cis* to all-*trans* (or vice-versa) in the bacteriorhodopsin system, as compared with the high stability of the 11-*cis*, 9-*cis*, and 7-*cis* isomers in rhodopsin.

Clarification of the function of bR and visual pigments, apart from bearing directly on the mechanism of the corresponding photoprocesses, is expected to be of value in the general area of protein structure and dynamics. The presence of a light-sensitive chromophore, such as retinal, which can be used to induce fast protein reactions, offers a unique way of applying rapid triggering and detection methods to biophysics. In addition, the high sensitivity of the spectroscopic and photophysical properties of retinal to its protein microenvironment may lead to a better understanding of the steric and electrostatic interactions between small ligand molecules in their protein binding site.

NOTE ADDED IN PROOF

We suggested (see Section VII) that a 13-*cis* protonated intermediate exists in bacter-

iorhodopsin photocycle following M intermediate. We note, in this respect, that such intermediate (N) was characterized recently.^{119,120}

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Chapter 6

**ANALOG PIGMENTS OF RHODOPSIN AND
BACTERIORHODOPSIN; RETINAL BINDING SITE AND ROLE
OF THE CHROMOPHORE IN THE FUNCTION OF THESE
PIGMENTS**

Rosalie K. Crouch

TABLE OF CONTENTS

I.	Introduction	126
II.	Rhodopsin Analogs	126
A.	Nature of Chromophore-Binding Site.....	126
1.	Acyclic Retinals	126
2.	Spin-Labeled Rhodopsin Pigments	129
B.	Photointermediates in Rhodopsin Bleaching — Probes Using Retinal Analogs	130
1.	Bathorhodopsin	130
2.	Metarhodopsin	131
C.	Function of Analog Pigments.....	133
1.	The Intact Skate Retina	133
2.	Vitamin A-Deprived Rats	133
III.	Bacteriorhodopsin Analog Pigments	135
A.	Binding Site Tolerance for Alteration of the Ring	136
B.	Pigments with Varied Length of the Polyene Side Chain.....	136
C.	Pigments with Acyclic Polyene Aldehydes	138
D.	Spin-Labeled Pigment Analogs of Bacteriorhodopsin.....	140
IV.	Summary	142
	Addendum.....	143
	Acknowledgments	144
	References.....	144

I. INTRODUCTION

Since the preparation of the first analog pigment with the substitution of the 9-*cis* isomer of retinal (**1**) for the native 11-*cis* isomer into bovine opsin,¹ several laboratories have actively pursued the use of retinal derivatives as tools for describing the nature of the chromophore binding site, for predicting particular aspects of the chromophore interaction with the protein and for ascertaining the chromophore's role in the physiological activity of both rhodopsin and, more recently, bacteriorhodopsin. By variation of certain steric and electronic features of the chromophore, it has been feasible to determine the limitations of the chromophore binding site and the specific protein-chromophore interactions that control the light absorption of these pigments. The approach of studying analog pigments has likewise yielded data on the various intermediates in the photocycle/bleaching processes that could not have been obtained by other methods. Finally, these analog pigments have been used to ascertain the chromophore's role in the particular physiological activity of the respective pigment. We discuss here the contributions of our laboratory to this knowledge on the structure and function of rhodopsin and bacteriorhodopsin.

II. RHODOPSIN ANALOGS

A. NATURE OF CHROMOPHORE BINDING SITE

Rhodopsin contains 11-*cis*-retinal as its native chromophore.² Hubbard and Wald¹ established that the addition of 11-*cis*-retinal to the pigment that had been exposed to light resulted in regeneration of the rhodopsin pigment. These workers further demonstrated that 9-*cis*-retinal likewise formed a photosensitive pigment with "bleached" rhodopsin (opsin) and thereby created the first artificial rhodopsin pigment. Since that time, several laboratories have shown that all the *cis* isomers of retinal, with the exception of 13-*cis*, will form pigments of varying stability (see, for example, Crouch et al.³ and deGrip et al.⁴). Much of this work will be presented in subsequent chapters. Likewise, numerous structural derivatives of retinal are reported to form pigments (for reviews, see Balogh-Nair and Nakanishi,⁵ Crouch,⁶ and chapters within this volume). Presented below are our approaches to this problem as illustrated by two retinal analog systems, which are among those we have studied.

1. Acyclic Retinals

In order to determine which structural features of the retinal molecule are essential to fill the binding site of rhodopsin and, thus in turn to use this knowledge in the design of a retinal analog that has the minimum structure required to anchor the retinal into the binding site, competition studies were undertaken by following the rate of formation of pigments in the presence of small molecules that mimic portions of the retinal molecule.⁷

Studies of the regeneration reaction have previously been shown to follow pseudo first-order kinetics in the presence of excess 11-*cis*-retinal.^{8,10} The binding site is well protected as shown by our studies demonstrating that the rate of regeneration of both rhodopsin and isorhodopsin (9-*cis*-retinal pigment) is amazingly consistent in a wide variety of solvents.¹¹ Other workers have also demonstrated the lack of accessibility of small hydrophilic reagents.^{13,14} The rates of reaction were therefore followed, assuming psuedo first-order kinetics, by observing the appearance of product as measured by absorption at 500 nm. Compounds resembling the aldehyde portion of the retinal do not inhibit this rate of reaction and therefore the formation of the Schiff base linkage may not be the rate-determining step. Compounds modeled after the ring portion of the retinal but lacking one or two of the methyl groups likewise fail to retard the rate of reaction. However, if two or three of the ring methyl groups are present, the reaction rate is attenuated (Figure 1). A compound with only the ring present, i.e., 1,3 dimethylcyclohexane, reduces the K_{app} ; it is of interest that the *trans*

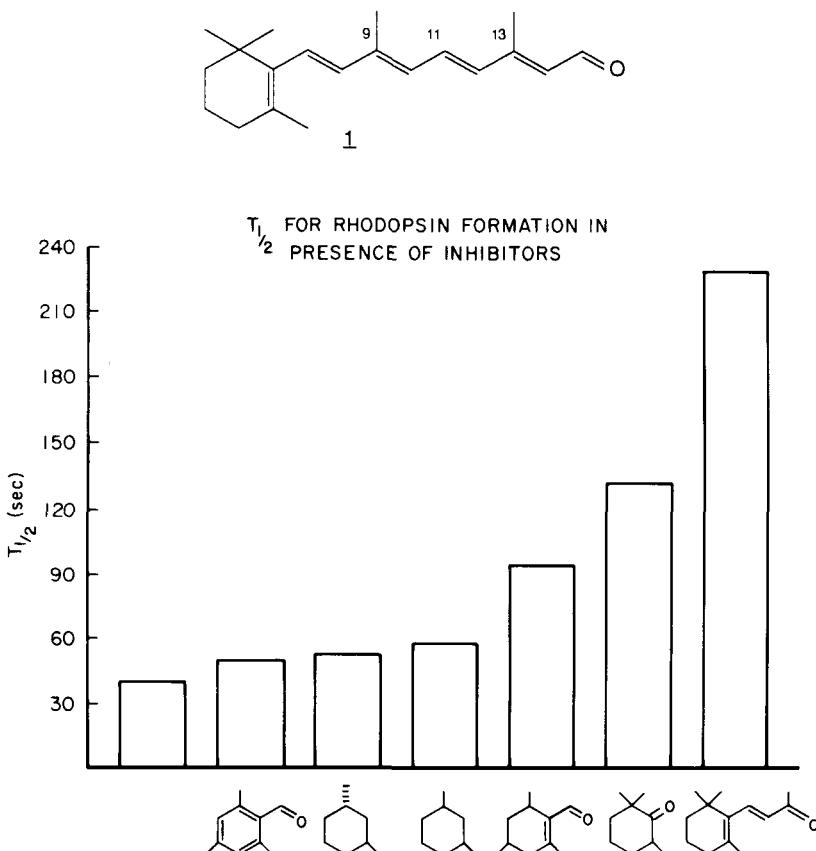


FIGURE 1. Inhibition of rhodopsin formation by cyclohexyl compounds. Rhodopsin formation followed by increase in absorption at 500 nm; opsin (12 μM), 11-cis-retinal (37.4 μM), inhibitor (670 μM) at 20°C in 20 mM phosphate buffer, pH 7.4. Reaction quenched with 5 mM hydroxylamine, pH 7.0.

isomer is more effective than the *cis* (Figure 2). There is no synergistic effect on the rate of reaction in adding both a compound mimicking the ring and a compound resembling the carbonyl portion of the molecule.

Matsumoto and Yoshizawa¹² had observed previously that β -ionone inhibits the regeneration of rhodopsin. Compounds with longer side chains are shown to be more effective inhibitors,¹⁵ and Towner et al.¹⁶ have demonstrated that the most effective inhibitors are those that can be superimposed on retinal up to C₁₁.

Our results led us to speculate that the ring methyls are key for anchoring the chromophore in the binding site and consequently, these methyls may represent the "minimum" requirements for filling the binding site. Therefore, it appeared feasible that an analog containing only groups representing the ring methyls rather than the complete ring might form a pigment that is biologically active.

Based on the results obtained from the competition experiments, it was decided to synthesize compounds (**2** to **4**) in order to test for the importance of the ring methyls.^{17,18} These compounds lack the cyclohexyl ring, but are designed to have groups that can fill none (**2**), one (**3**), or two (**4**) of the ring methyl-binding sites. The 6-*cis* and 2,6-di-*cis* isomers of **3** and **4** were found to form photosensitive pigments with bovine opsin.^{17,18} The absorption spectra of these pigments are blue shifted from those of the 9-*cis*-retinal and 9,13-di-*cis*-retinal pigment. Some shift is expected due to the lack of the 5,6-double bond. However,

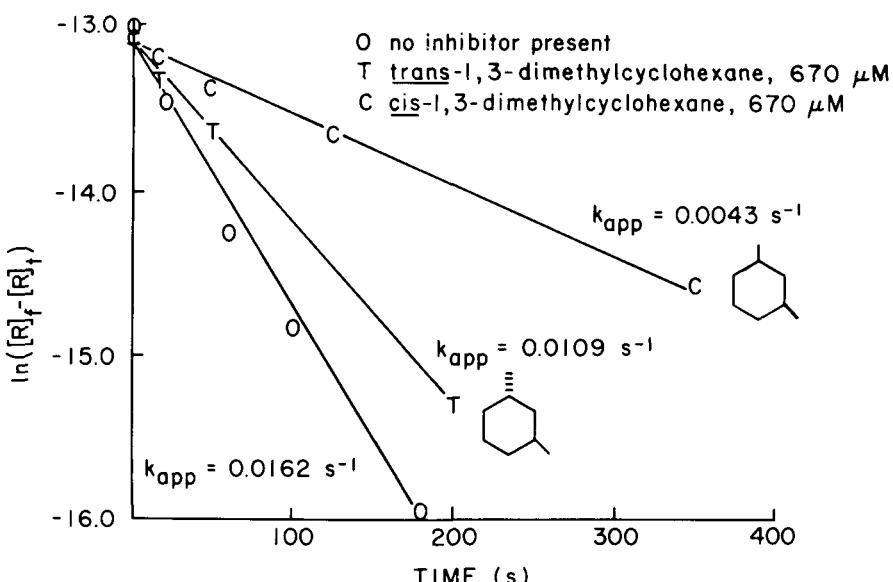
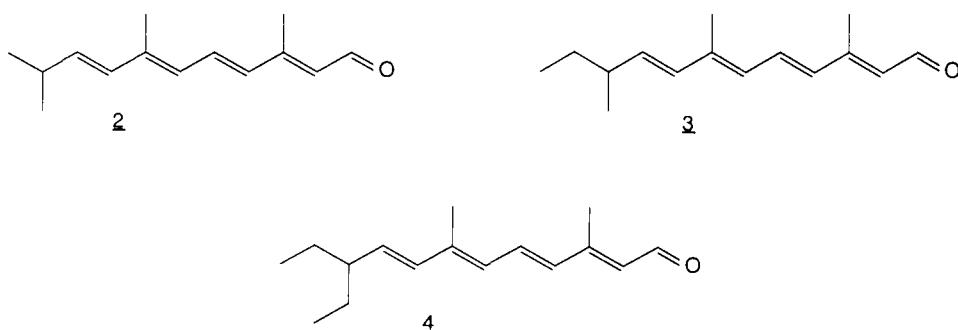
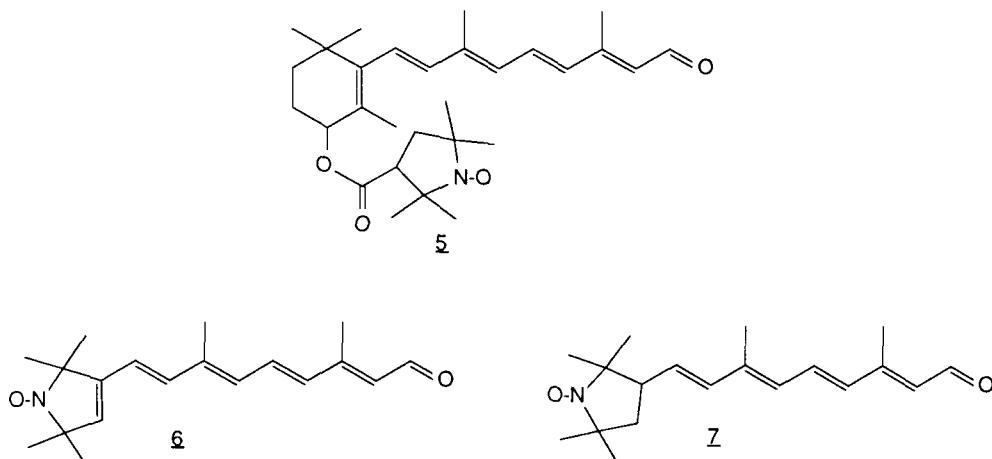


FIGURE 2. Inhibition of rhodopsin formation by dimethylcyclohexanes. Rhodopson formation monitored by increase in absorption at 500 nm. Reaction in 20 mM phosphate buffer, pH 7.4, at 20°C, opsin 12 (μM), 11-cis-retinal (37.4 μM) and dimethylcyclohexanes (670 μM). Reaction quenched with 5mM hydroxylamine, pH 7.0.



these acyclic chromophores may not be interacting in the binding site with the secondary point charge site proposed by Nakanishi, Honig, and co-workers.¹⁹ These pigments formed at a somewhat slower rate than those pigments with the corresponding retinal isomers. The acyclic pigments are stable to the addition of hydroxylamine and 11-cis-retinal over several hours, which demonstrates that the analogs are in the binding site. The pigment of **4** with the “two ring methyl groups” is more stable than the pigment of **3**. Analog **2** containing no ring “methyl groups” did not form pigment, supporting the hypothesis that ring methyls are essential to pigment formation.

From the data on the inhibition studies and from the acyclic pigments, we conclude that there is precise requirement for the ring methyl groups. In surveying analog data from other laboratories, we find that this correlation holds. For example, 11-cis-5,6-dihydroretinal forms a stable pigment with bovine opsin.^{20,21} However, no pigment is observed with the 11-cis-5,6-dihydro analog **13** lacking ring methyl groups.¹¹ In addition, the phenyl derivative with two methyl groups shows formation of a stable pigment in high yield, whereas the analog with no methyl groups yields 10% of an unstable pigment.²²



2. Spin-Labeled Rhodopsin Pigments

In selecting analogs to be synthesized, consideration is given to: (1) selectively altering the structure to test some particular interaction with the protein or (2) incorporating a reporter group into the structure that can measure some aspect of the chromophore-protein interaction. Obviously, in the latter analogs, it is desirable to have as little disruption of the natural protein-chromophore interaction as possible.

Nitroxide groups allow the use of electron spin resonance (ESR) spectroscopy, which is a nondestructive, nonphotolytic method for observing protein environment. This method is ideally suited for these pigments, provided the correct analog can be synthesized.

Three spin-labeled retinals have been synthesized in our laboratory. Compound 5 contained the spin label connected to the retinal molecule by an ester linkage. This derivative proved to be unsuitable in these studies due to the labile allylic ester linkage at C₄ and the large steric bulk in the ring portion which prevents binding.²³ The pyrrolinyl derivative (6) eliminated the problems with steric bulk and the labile ester linkage.²⁴ However, pigment was not formed between the 9-*cis* or 11-*cis* isomer and bovine opsin, possibly due to the lack of ring mobility which should allow it to twist into the native binding site. The conjugation of the double bond in the ring with the double bonds in the side chain may prevent significant rotation of the ring and apparently, even the limited size of this small tetramethylated cyclopentene presents too much bulk for the ring binding cavity.

The spin-labeled analog 4 containing the five-membered saturated ring proved to be ideal for rhodopsin pigment studies. As a consequence of the method of synthesis (shown in Figure 3), the 9-*cis* isomer was the *cis* isomer obtained in good yield and was therefore chosen for these studies.²⁵ Good pigment formation is obtained from the 9-*cis* or 9,13-di-*cis* isomer. The pigment is stable to either the addition of hydroxylamine or 11-*cis* retinal which demonstrates that the chromophore occupies the site filled by 11-*cis*-retinal in the native pigment.

The ESR spectrum of the spin-labeled retinal shows the classic triplet expected for a nitroxide in a mobile environment. The ESR signal of the pigment is highly immobilized with a rotational correlation time of 10⁻⁷s. Addition of small, hydrophobic molecules, capable of reducing the nitroxide molecule of the pigment (i.e., sodium ascorbate or nickel ion), did not affect the ESR spectrum, again confirming that the chromophore binding site is highly sequestered.

Many biophysical and biochemical studies on rhodopsin have been conducted with the pigment solubilized in various detergents. As a large number of these studies address the interaction between chromophore and protein, the detergent's effect on this reaction is ex-

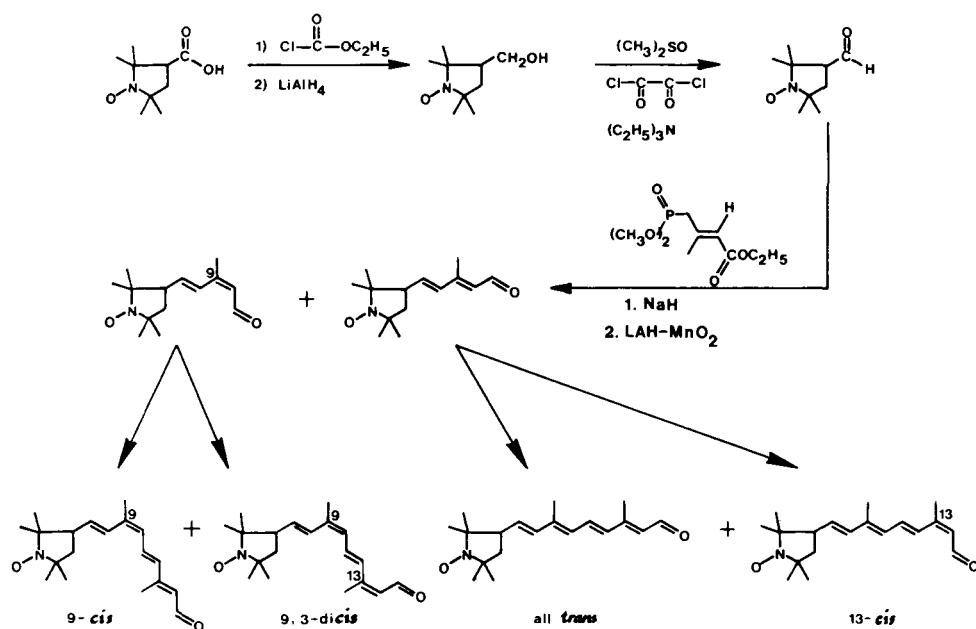


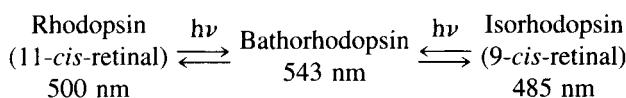
FIGURE 3. Route of synthesis for spin-labeled retinal. Compound 7 synthesized as shown.

tremely important. The ESR technique is an ideal method for addressing this question. In comparing the spectra of pigment in various detergents, it was observed that the stronger detergents (i.e., Triton X-100 and Ammonyx LO) markedly affected the spectra, whereas spectra of pigment solubilized in weaker detergents (i.e. CHAPS and digitonin) closely approximated that of the unsolubilized pigment in suspension. It must also be noted that this pigment has decreased stability in the stronger detergents. These results imply that stronger detergents may not be ideal choices for rhodopsin studies as the protein structure may be significantly affected.²⁵

B. PHOTointermediates in Rhodopsin Bleaching—Probes Using Retinal Analogs

1. Bathorhodopsin

The bleaching of rhodopsin starts with the absorption of light by pigment. The pigment then passes through a series of intermediates characterized by changes in the absorption spectra.²⁶ The original configuration of the chromophore is the 11-cis isomer. At the metarhodopsin state, the chromophore is still attached to the protein via a Schiff base.²⁷ However, the precise point of chromophore isomerization and specific interactions with the protein are still not completely understood and have been the subject of controversy for many years. Bathorhodopsin is the first photoproduct observed in this bleaching process at 77 K. With appropriate wavelengths of light, the mixture can be converted into primarily a pigment containing 9-cis-retinal.



Several workers have suggested that the chromophore is in the all-trans configuration while in the bathorhodopsin state.^{28,29} In order to determine if the chromophore is indeed in the

TABLE 1
Absorbance of Metarhodopsins of Analog Pigments

Chromophore	Pigment λ_{\max} (nm)		
	Rhodopsin state ^a	Meta I state ^b	Meta II state ^c
9-cis-retinal 1	485	478	380
11-cis-retinal 1	500	478	380
11-cis-13-desmethyl retinal 8	490	485	380
9-cis-13-desmethyl retinal 8	477	485	380
9-cis-spin-labeled-retinal 7	448	440	380*
11-cis-4-hydroxyretinal 9a	470	480	390

^a Potassium phosphate buffer (pH 7.4).

^b Tris acetate buffer (pH 8.5).

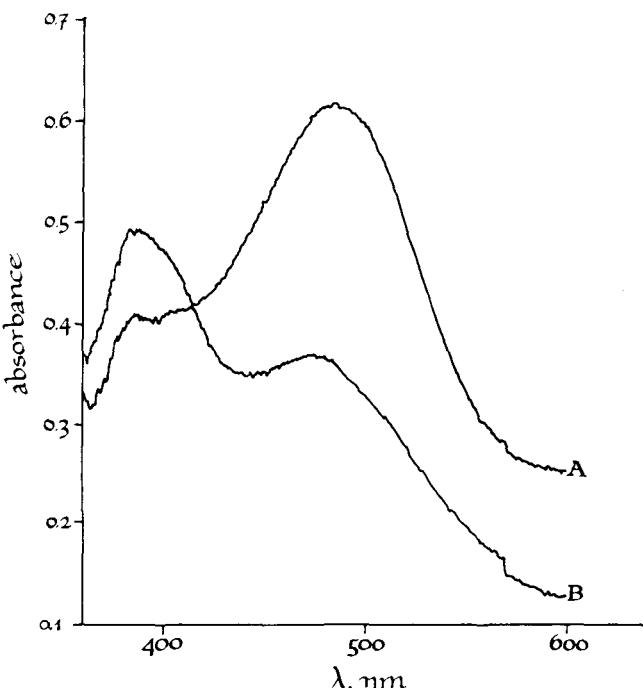
^c NaAc/HAc (pH 5.1 or *4.5).

all-trans state, our laboratory in collaboration with T. G. Ebrey and his group (University of Illinois) examined the bathorhodopsins prepared from pure 9-cis-retinal and from 9,13-di-cis-retinal.³⁰ The batho formed from 9-cis-retinal is found to be identical in λ_{\max} , bandwidth, and extinction coefficient with the batho formed from rhodopsin. However, the batho formed from the 9,13-di-cis pigment is quite different from the batho of either the 9-cis pigment or rhodopsin. The absorption maximum is blue shifted (532 nm), the bandwidth broader, and the extinction coefficient lower. These results are strong evidence for the hypothesis that the chromophore in rhodopsin has isomerized to the *all-trans* configuration by the bathorhodopsin state.

2. Metarhodopsins

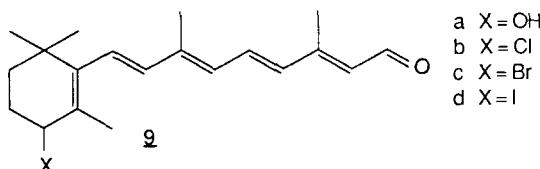
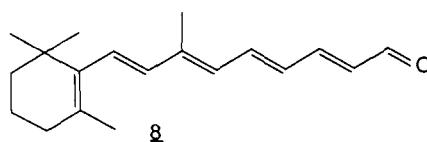
The metarhodopsin intermediates are late photoproducts in the bleaching process. The electrophysical events at the plasmalemma are related in time (millisecond range) to the formation of the metarhodopsin I and II states. Spectroscopic evidence indicates that metarhodopsin II, containing the chromophore in the *all-trans* form linked to the protein by a nonprotonated Schiff base, serves as the activated rhodopsin form that catalyzes the G protein.^{31,32} The transition from the metarhodopsin II state to metarhodopsin III, which is then hydrolyzed to opsin and *all-trans* retinal, occurs over a period of minutes. Little is known about the interaction of the chromophore with the protein in these three states. As the meta I and meta II states have distinct absorption characteristics and can be easily converted to each other, these are ideal states to study with analog pigments (Table 1).

When rhodopsin is irradiated at 0°C, pH 8.5, the bleaching sequence proceeds to the meta I and meta II states in an equilibrium that favors meta I. Irradiation at 0°C, pH 5.1, yields an equilibrium mixture favoring the meta II intermediate. The equilibria between these two states can be shifted by changing the pH.^{33,34} The behavior of isorhodopsin is identical to that of rhodopsin, which is to be expected since the chromophore is in the *all-trans* configuration (Figure 4). The 11-cis- and 9-cis-desmethyl retinals (**8**) also have identical metarhodopsin states, and the meta I and meta II equilibria are obtained under the same conditions as for rhodopsin. The 11-cis-4-hydroxyretinal (**9a**) likewise shows a meta I and meta II state. However, the shifts are not as well-defined as in the other pigments, possibly due to dehydration of the labile allylic hydroxyl group. The spin-labeled retinal is of particular interest as the changes in the nitroxide can be followed through various states. The meta II state is observed at pH 8.5, but for equilibrium to be moved to the meta II state, a more acidic pH of 4.5 is required. The acyclic retinal **4** does not form a meta I or meta II state that is detectable by absorption changes. However, the pigment is photosensitive. This result suggests that specific interactions of the ring portion of the chromophore (lacking in this analog) with the protein may give rise to the absorption changes in these states.³⁵



Meta formation from isorhodopsin

FIGURE 4. Metarhodopsin formation from isorhodopsin. (A) Iisorhodopsin in 10 mM potassium phosphate buffer at pH 7.0. (B) Metarhodopsin II formed at 0°C, pH 5.1, in 10 mM sodium acetate/acetic acid at pH 5.1.



The ESR spectrum of the spin-labeled rhodopsin in the meta I state shows no significant changes from the rhodopsin state. However, in the meta II state, the ESR spectrum shows a small increase in the separation of the hyperfine extrema and some minor line shape changes. Introduction of nickel ion, which undergoes spin-spin interactions with the nitroxide causing spin broadening of the signal,³⁶ to the spin-labeled meta II rhodopsin did not affect the signal. Therefore, the chromophore's ring end remains deeply buried within the protein throughout the meta II state.

These results are supported by our studies of this labeled chromophore with labeled membrane lipids.³⁷ ¹⁵N spin labels in double-labeling experiments have been used to study

lipid protein interactions.³⁸ Feix and co-workers³⁹ have used electron-electron double resonance (ELDOR) technique with ¹⁴N:¹⁵N spin-labeled pairs in liposomes. Using our spin-labeled retinal and ¹⁵N-spin-labeled stearic acids (labeled at the 5- and 16-positions), we have probed for interaction between the lipid and the chromophore ring at the rhodopsin, meta I and meta II states. In none of these states was any interaction between the ¹⁴N-retinal and ¹⁵N-stearic acid observed. The results again demonstrated that the chromophore ring is strongly sequestered within the protein.

C. FUNCTION OF ANALOG PIGMENTS

Although extensive research by several laboratories has shown that pigments can be formed *in vitro* between numerous retinal analogs and opsins of various species, the ability of these analogs to form pigment *in vivo* and to function in the visual process is another issue. We have studied both the incorporation of retinal analogs and functionality of the resulting pigments in two models: the isolated skate and bullfrog retina (in collaboration with David Pepperberg, University of Illinois, Chicago) as measured by the sensitivity of the retina to irradiation; and the vitamin A-deprived rat by measuring the electroretinogram. These experiments were coupled with isolation of the pigments and identification of the extracted chromophore by high-pressure liquid chromatography.

1. The Intact Skate Retina

The isolated skate retina has been shown by Pepperberg to be a useful model system for studying the effects of externally applied retinals on light sensitization.⁴¹ In the intact eyecup preparation, visual sensitivity parallels rhodopsin concentration.⁴⁰ When 11-*cis*-retinal is applied to the partially bleached aspartate-treated retina, a recovery in sensitivity is likewise observed. Pepperberg et al.⁴¹ have also shown that 9-*cis*-retinal can restore sensitivity to the system.

9,13-di-*cis*-Retinal was of interest to study because the bleaching intermediates (e.g., the bathorhodopsin) of this pigment are not identical to the rhodopsin system. When 9,13-di-*cis*-retinal was applied to the isolated skate retina, the photoreceptor threshold was reduced significantly.⁴² The increased sensitization proceeded more slowly than with the addition of 9-*cis*- or 11-*cis*-retinal. *In vitro*, a slower rate of pigment formation has likewise been observed.³ In order to establish unequivocally that the 9,13-di-*cis* is incorporated in the pigment and that this pigment is the one responsible for the increase in sensitivity, the chromophore was extracted from the pigment and shown by high-pressure liquid chromatography to be mainly 9,13-di-*cis*-retinal with essentially no 9-*cis*- or 11-*cis*-retinal present. These experiments demonstrate that if an "unnatural" chromophore, capable of meeting the binding site requirements, can be delivered to the site and if the resulting pigment is photosensitive, one may obtain thresholds that approximate the same values found for native rhodopsin.

2. Vitamin A-Deprived Rats

Vitamin A deprivation is known to deplete the retina of retinal and rhodopsin of its chromophore. The amplitude of the electroretinogram (ERG) b-wave has been shown by Dowling⁴³ to correlate with the rhodopsin content in the eye. Vitamin A deprivation of weanling rats reduces this b-wave amplitude (Figure 5) and the amplitude is restored to normal on the administration of retinol.^{44,45} Intraperitoneal injection of radiolabeled 11-*cis*- or all-*trans*-retinal into a vitamin A-deprived rat leads to incorporation of the label in the visual pigment.⁴⁶ Rat opsin forms pigments *in vitro* with vitamin A analogs.⁴⁷ Therefore, the vitamin A-deprived rat appears to be an excellent model for testing the functionality of these unnatural chromophores. The two isomers that occur physiologically, namely 11-*cis* and all-*trans*, are both incorporated into the retina and restore the threshold of light sensitivity. The pigments were isolated and had the identical absorption spectra to that of rhodopsin.

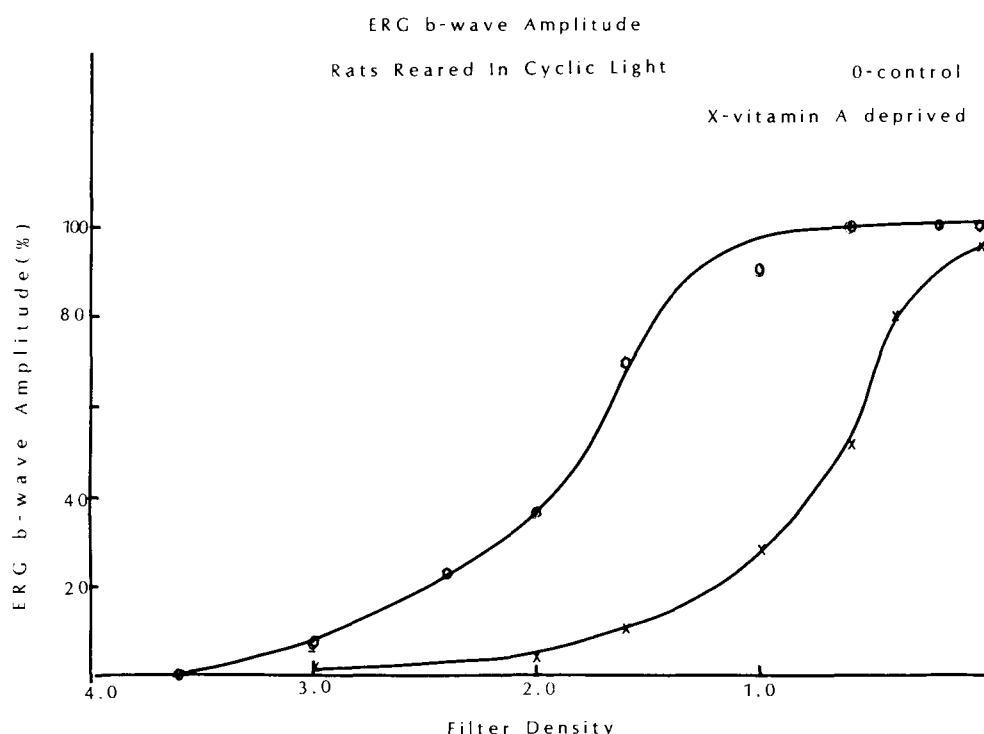


FIGURE 5. ERG b-wave amplitude of control and vitamin A-deprived rats. Black-hooded Long Evans male rats maintained from weaning for 12 weeks on vitamin A-deficient diet or control food. Abscissa describes the logarithmic attenuation by neutral density filters of the 10- μ s stimulating flash. Maximum flash was 2.7×10^4 l. Each point is the mean of data obtained from 3 animals.

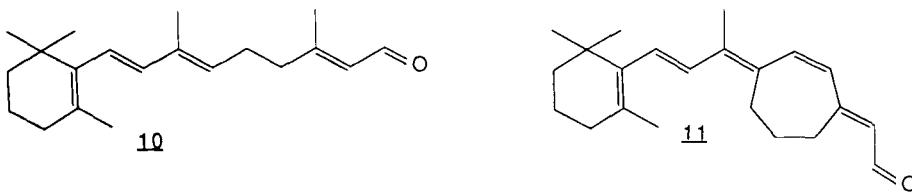
To confirm that the *trans* isomer had indeed isomerized, the chromophore was extracted from the pigment and found to be 11-*cis*-retinal.

The 9-*cis*-retinal likewise restores the ERG threshold after intraperitoneal injection. The pigment was again extracted and found to contain the 9-*cis* isomer. The pigment spectrum had an absorption maximum blue shifted from that of rhodopsin and characteristic of iso-rhodopsin. Therefore, the 9-*cis* isomer is incorporated into the pigment intact and this "unnatural" pigment is physiologically active. Witkovsky et al.⁴⁸ have obtained a similar result with 9-*cis*-retinal in *Xenopus*.

The 13-*cis* isomer of retinal does not affect the ERG threshold or pigment content when injected intraperitoneally. This isomer is neither active with opsins *in vitro* nor is it found naturally in the visual system. On studying the effect of isomers injected intraperitoneally on the visual system, caution must be exercised in interpreting results as negative since this may be due to failure of the isomer or analog to be transported to the eye. For example, Futterman et al.⁴⁹ have shown that 9-*cis*-retinal binds with at least one protein in the retina, whereas 13-*cis* does not bind and hence may not be transported.

When this method was extended to other analogs, those that form pigments *in vitro* will generally form pigments *in vivo*. For example, both 13-desmethylretinal (7) and 4-hydroxy-retinal (9) induced an increased sensitivity threshold. The results on the latter retinal were somewhat complicated by the dehydration reaction leading to 3,4-dehydroretinal, which likewise forms a pigment. This system does present a useful model for testing the physiological properties of these analogs.

In collaboration with Koji Nakanishi and his group at Columbia University, two analogs, which do not undergo the traditional 11-*cis* to all-*trans* isomerization, were tested for phys-



iological activity. The tritium-labeled 11,12-dihydroretinal **10**⁵⁰ was found incorporated in the pigment of the rod outer segments of vitamin A-deprived rats following intraperitoneal injection as determined by the presence of tritium in the rod outer segments and by identification of the extracted retinal using high-pressure liquid chromatography.⁵¹ However, the amplitude of the ERG b-wave was not affected. In order to determine if this chromophore forms pigment but is incapable of inducing the phototransduction process, one must prove that the chromophore is linked to the opsin protein. Rod outer segments from 11,12-dihydroretinal-treated rats generate 0.6 nmol of rhodopsin when incubated with 11-cis-retinal. Untreated vitamin A-deficient rats generated 1.4 nmole rhodopsin, showing that indeed the 11,12-dihydroretinal blocked the incorporation of retinal into pigment. Likewise, treatment of a vitamin A-deficient rat first with 11,12-dihydroretinal followed 24 h later with retinal failed to restore visual sensitivity. Therefore, dihydroretinal occupies the same binding site as the natural 11-cis-retinal, but this occupation is not sufficient to restore visual sensitivity.

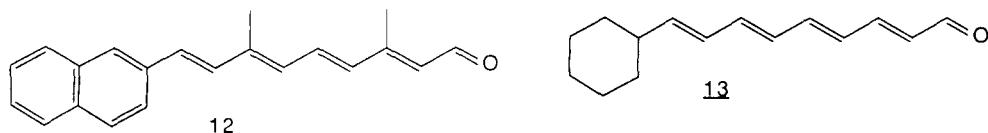
The flaw in the above experiment is that the chromophore does not contain a fully conjugated side chain and therefore does not absorb in the visible wavelength region. A second analog was therefore tested. The cycloheptatrienylidene retinal derivative **11**, which contains the fully conjugated side chain, has the 11-12 double bond locked in a *cis* configuration by a seven-membered ring. Akita et al.⁵² had shown this analog to form a pigment with bovine opsin. The pigment had an absorption near that of rhodopsin, making it suitable for *in vivo* studies, and the pigment neither bleached nor exhibited the bathorhodopsin product when illuminated.⁵³

The tritium-labeled *cis*-locked analog **11** was injected into vitamin A-deprived rats⁵⁴ and then the analog was incorporated into the rod outer segments as shown by the presence of tritium. The chromophore was in the intact form, shown by analysis of the extracted chromophore by high-pressure liquid chromatography, and again occupied the binding site as demonstrated *in vitro* by competition experiments with 11-cis-retinal. However, formation of the analog pigment appeared to have no significant affect on the ERG b-wave amplitude. Furthermore, administration of all-trans-retinol 24 h after administration of **11** did not result in the sensitization of the retina observed when retinol was administered to deprived rats. If presented after seven days, retinol did restore the photosensitivity in the retina. Likewise, the quantity of **11**, determined by tritium levels in the extracted pigment, decreased markedly over seven days. These data are in agreement with the known turnover rate of the rat rod.⁵⁵ These results were confirmed by experiments in the isolated bullfrog retina in which, upon the addition of **11**, the formation of an analog pigment is observed. This pigment with a $\lambda_{\max} = 497$ nm is relatively resistant to photolysis (in collaboration with Dr. Pepperberg).

These data obtained from these two retinal derivatives demonstrate that occupation of the rhodopsin binding site and the ability to absorb light at the rhodopsin λ_{\max} is insufficient for the physiological activity of rhodopsin, namely the transduction process. These results support the hypothesis that a *cis-trans* isomerization is essential for the activity of the pigment (see Addendum).

III. BACTERIORHODOPSIN ANALOG PIGMENTS

Bacteriorhodopsin functions to transport protons across the *Halobacterium halobium* membrane and has as its chromophore all-trans-retinal.⁵⁶ On dark adaptation, the chro-



mophore mixture of all-*trans*- and 13-*cis*-retinal which converts totally to all-*trans* with light.⁵⁷ However, the protonated Schiff base linkage does not hydrolyze upon exposure to light. Bacteriorhodopsin binds only these two isomers of retinal and has rather different constraints on the binding site than does rhodopsin. Retinal analogs have been used extensively to probe this binding site (for a review, see Mitsner et al.⁵⁸).

The physiological functionality of bacteriorhodopsin pigments can be readily tested by measuring the proton pumping of the analog pigment. The work reported here was performed in collaboration with T. G. Ebrey and his group at the University of Illinois. Our studies on bacteriorhodopsin, which have helped define the binding pocket of this pigment and the retinal structure essential for functionality, are summarized below.

A. BINDING SITE TOLERANCE FOR ALTERATION OF THE RING

The binding site of bacteriorhodopsin is far less restrictive than that of rhodopsin as shown by the large number of retinal derivatives, some with extensive modification, that can form pigments with bacterioopsin, the form of bacteriorhodopsin which has had the chromophore removed. Specifically, we elected to study the ring portion of the molecule by synthesizing analogs with significant electronic and steric alterations in that portion of the molecule.⁵⁹

The 4-halo-retinals (**9b-d**) all form pigments that show a large shift in absorption from the native pigment (560 to 460 nm) and are unstable to the addition of all-*trans*-retinal or hydroxylamine. The data suggest that the electronegative halogens are preventing the chromophore from being positioned firmly in the binding site and interfering with the secondary interaction proposed⁶⁰ to explain the absorption properties of the native membrane. This allylic C₄ position is very reactive and upon standing or on exposure to light, the halogens on the retinal are readily displaced by a hydroxyl group *in situ*, thereby forming the 4-hydroxy pigment (**9a**, λ_{max} 530).

Considerable bulk can be tolerated in the bacterioopsin binding site as shown by pigment formation with the naphthyl derivative **12**.⁵⁹ The pigment λ_{max} has again been shifted, but the pigment has physiological properties similar to those of the native pigment. The ring double bond of the chromophore does not control the photochemistry or proton pumping functions of bacteriorhodopsin as shown by the result that 5,6-dihydroretinal forms a stable pigment that pumps protons.⁶¹ The completely demethylated chromophore **13** likewise forms a pigment, which in turn can pump protons, showing that bacteriorhodopsin does not have the same methyl group requirement as rhodopsin for pigment formation and that the ring double bond and methyl group features are not involved in the proton pumping process.⁶⁰ These data offer strong evidence that the ring portion of the chromophore in bacteriorhodopsin does not play a role in the function of pigments except to control the wavelength absorption and that the binding site is quite nonrestrictive in this region (Table 2).

B. PIGMENTS WITH VARIED LENGTH OF THE POLYENE SIDE CHAIN

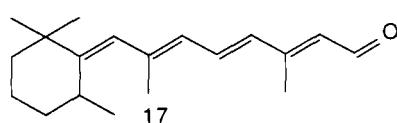
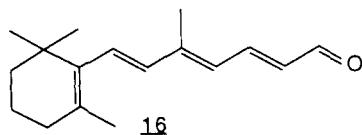
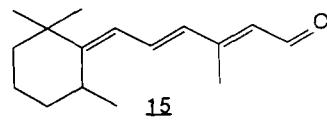
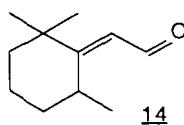
Because the ring binding site appears to be so undefined, we hypothesized that the key feature of the retinal structure for this pigment may be the side chain, and we synthesized a series of acyclic polyenes and cyclohexyl derivatives with side chains of varying length⁶² to study this premise.

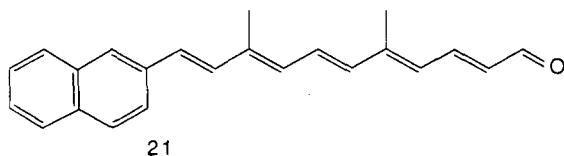
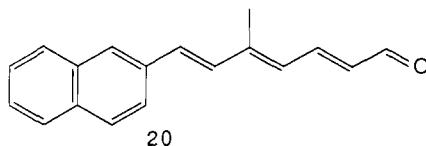
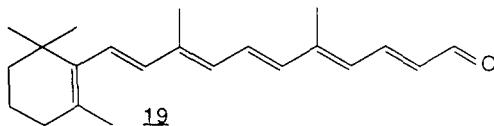
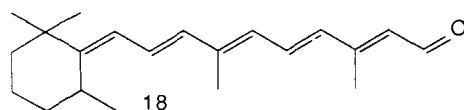
A series of analogs with shortened polyene side chains were prepared (**14** to **17**). The small derivative **14** showed no association with bacterioopsin. The analog with three ethylene

TABLE 2
Absorption Data of Bacterioopsin Analog Pigments

Chromophore	λ_{\max} (nm)	Pigment λ_{\max} (nm)		
		da	la	M
Retinal 1	380	560	570	412
4-Hydroxyretinal 9a	370	530	540	400
4-Chlororetinal 9b	348	462	NA	NA
4-Bromoretinal 9c	352	460	NA	NA
4-Iodoretinal 9d	340	455	NA	NA
Naphthylretinal 12	395	492	497	395
5,6-Didehydro-1,1,5,9,13-desmethylretinal 13	340	460	460	350
6-(2,2,6-Trimethylcyclohexylidene)-3-methyl-2,4,-hexadienal 15	320	416	420	NO
C ₁₇ -Aldehyde 16	340	413	415	NO
8-(2,2,6-Trimethylcyclohexylidene)-3,7-dimethyl-2,4,6-octatrienal 17	334	458	365	357
10-(2,2,6-Trimethylcyclohexylidene)-3,7-dimethyl-2,4,6,8-decatetraenal 18	382	518	524	392
C ₂₂ -Retinal 19	384	500,540	500,540	400
5-Methyl-7-(2'-naphthyl)-2,4,6-heptatrienal 20	362	482	485	370
5,9-Dimethyl-9-(2'-naphthyl)-2,4,6,8-nonatetraenal 21	405	460—480	460—480	405
3-Methyl-2-butenal 22	257	NO	NO	NO
3-Methyl-2,4-hexadienal 23	268	NO	NO	NO
3,7-Dimethyl-2,4,6-octatrienal 24	312	422	425	NO
3,7-Dimethyl-2,4,6,8-decatetraenal 25	347	462	466	365
3,7,11-Trimethyl-2,4,6,8,10-dodecapentaenal 26	400	513	519	NM
7,11-Dimethyl-2,4,6,8,10-dodecapentaenal 27	390	520	522	NM
3,7,10-Trimethyl-2,4,6,8-undecatetraenal 2	364	487	487	365
3,7,10-Trimethyl-2,4,6,8-dodecatetraenal 3	364	487	487	363
3,7-Dimethyl-10-ethyl-2,4,6,8-dodecatetraenal 4	373	487	487	373
3,7-Dimethyl-9-(2,2,5,5-tetramethyl-1-oxy-3-pyrrolin-3-yl)-2,4,6,8-nonatetraenal 6	374	454	459	NO
3,7-Dimethyl-9-(2,2,5,5-tetramethyl-1-oxy-3-pyrrolin-3-yl)-2,4,6,8-nonatetraenal 7	360	440	450	365

Note: Data presented for all-*trans* isomers. All chromophore absorption data are in ethanol and all pigment absorption data are in phosphate buffer, pH 7.0; $\lambda_{\max} \pm 3$ nm; NA = not applicable; NO = not observed; NM = not measured; da, dark-adapted, la, light-adapted pigment; M-intermediate formed on flash of actinic light >450 nm.





bonds in the side chain **15** did form a complex with bacteriorhodopsin and the λ_{\max} shifted upon light exposure, but no photocycle or light-induced proton release was observed. The C₁₇ aldehyde **16** formed a pigment that was somewhat stable to hydroxylamine but no photocycle was detected.⁶³ The analog one carbon shorter than retinal **17** formed a stable pigment with both the all-*trans* and 2-*cis* isomer. The pigment did undergo a slow photocycle and light-induced proton release although in low yield.

Analog **18**, with one carbon more than retinal in the polyene chain, likewise formed a stable pigment with both the 2-*cis* and all-*trans* isomers. This pigment underwent photo-cycling and released protons in good yield. The compound extended by two carbons (**19**) formed a pigment with a λ_{\max} at 500 nm and a shoulder at 540 nm.⁶² Light adaptation and pH studies suggest that there were two species present.⁵⁹ A distinct intermediate and a small proton pumping function were observed. The compound extended by four carbons did not form a pigment (Oesterhelt and Christoffel⁶⁴ and unpublished results). The naphthyl compounds **20** and **21**, which contain increased bulk in the ring, but a polyene sidechain two carbons less (**20**) and two carbons more (**21**) than retinal, form pigments which pump protons weakly in contrast to **12** which has the naphthyl ring but contains a polyene chain identical to that of retinal.⁷⁰ We again interpreted these results to support the premise that bacteriorhodopsin has great flexibility in the ring portion of the chromophore binding site.

We therefore have prepared a series of analog pigments in which the side chain of the chromophore was altered. The chromophore side chains were varied from 6 to 11 carbons in length (compared to the native retinal side chain of nine) and all these derivatives retained the cyclohexyl ring. Although all formed "pigments" with bacteriorhodopsin, only retinal itself and the compounds that vary by one carbon showed full proton-pumping function. Therefore, the length of the polyene chain appears to be key in maintaining this function.

C. PIGMENTS WITH ACYCLIC POLYENE ALDEYDES

A series of acyclic polyene aldehydes (**22** to **27**) was synthesized to test the selectivity

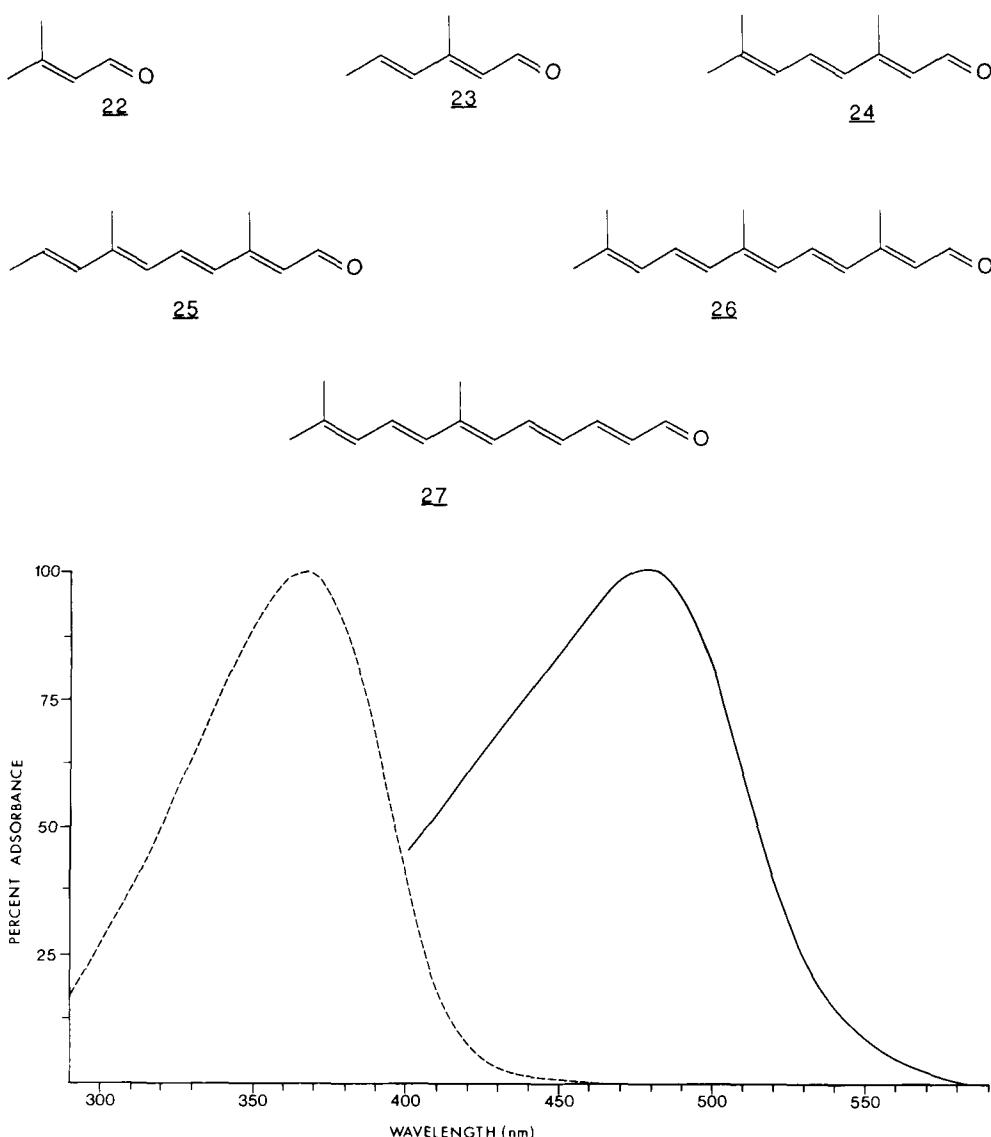


FIGURE 6. Absorption spectra of acyclic retinal and the acyclic bacteriohodopsin pigment. Retinal derivative **4** in ethanol (---); pigment in water (—).

of the binding site for simple polyenes and to determine the minimum structure required for pigment formation and function.^{65,66}

The polyenes **22** and **23** have short chains of four to six carbons and do not form pigments with bacterioopsin. However, the polyene **24** with eight carbons did form a pigment in low yield, which demonstrates no light-induced absorption changes or proton-release. Compound **25**, which contains what might be considered the "intact polyene chain", did form a stable pigment (λ_{max} 460 nm) and exhibited photocycling and proton release.

A series of acyclics, which can be thought of as containing the complete polyene side chain and a portion of the ring structure, has been studied. Compounds **2**, **3**, **4**, **26**, and **27** all formed stable pigments with bacterioopsin (Figure 6) and all show light-induced absorption and pH changes (Figure 7). Compound **27**,⁶⁶ identical to **26**⁶⁵ except for the C₁₃ methyl group, formed pigment but the proton pumping efficiency was decreased. Consequently,

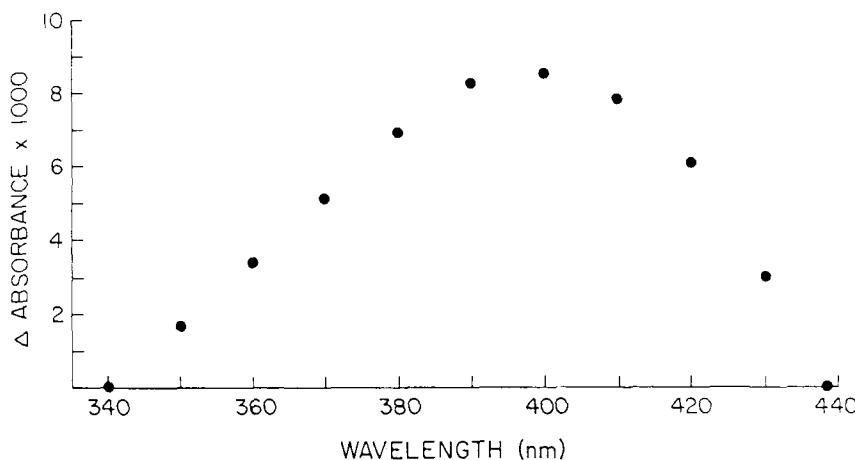
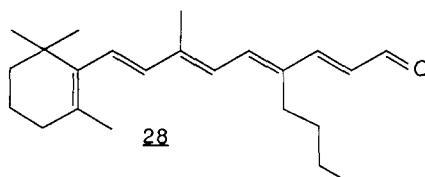


FIGURE 7. Absorption spectrum of the M intermediate for an acyclic bacteriohodopsin derivative. Flash induced difference spectrum of pigment with **4** in 150 mM KCl, pH = 6.7, 20°C, actinic flash ≥ 470 nm (xenon flash plus Corning Cs 3-71 filter).



the 13-methyl group was apparently not key for pigment formation but the proton pumping efficiency was affected in the absence of this group. It is of interest that the 12-*n*-butyl-13-desmethylretinal **28** failed to form a bacteriorhodopsin pigment.⁷⁰

Therefore, the cyclohexyl ring is not required for pigment formation. The results of the competition experiment reported by Towner et al.¹⁶ show that bacterioopsin did not have a specific cyclohexyl binding site. Our results demonstrate that the cyclohexyl ring is also not required for the photocycle or light-induced proton pumping.

An interesting feature of the acyclic compounds is that in cases where the 6-*cis* isomer is available, pigment formation is observed with polyenes longer than seven carbons. These pigment "complexes" absorb at a shorter wavelength than the corresponding all-*trans* derivatives. These pigments are generally less stable than the corresponding "trans" pigments (see Figure 8). However, upon exposure to light, the chromophores are readily isomerized to the all-*trans* isomer and on dark adaptation (see Figure 9), the all-*trans*/13-*cis* mixture generally observed for dark-adapted bacteriorhodopsin pigments is obtained.

D. SPIN-LABELED PIGMENT ANALOGS OF BACTERIORHODOPSIN

Three spin-labeled retinals have been incorporated into the binding site of bacterioopsin. The bulky derivative **5** is incorporated but hydrolyzed *in situ* to the 4-hydroxyretinal **9a** pigment.²³ Compound **6** containing the nitroxide incorporated in the ring formed a stable pigment, as did the corresponding unsaturated ring retinal (**7**). Both pigments showed a small shift in λ_{max} on exposure to light (Figure 10).^{25,67} Interestingly, for the unsaturated derivative only a small photocycle and limited proton pumping could be detected. However, the saturated derivative both photocycled and pumped protons efficiently. We have no firm explanation for this dissimilarity. It is possible that the fixed conformation of the ring with

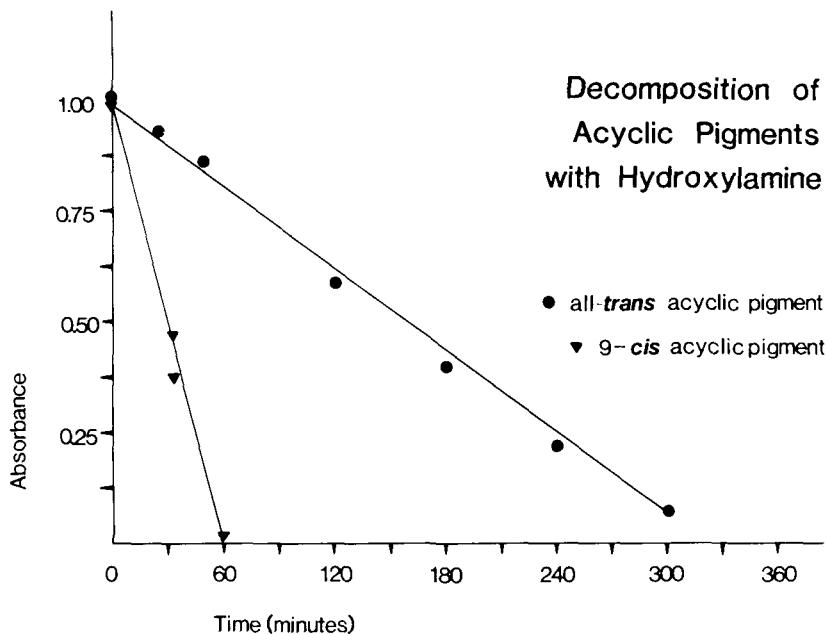


FIGURE 8. Decomposition of acyclic pigments with hydroxylamine. Rate of decomposition of all-*trans* and 9-*cis* acyclic (3) pigments by the addition of hydroxylamine (10 mM, pH 7.0, 20°C); measured by change in absorption at pigment λ_{\max} .

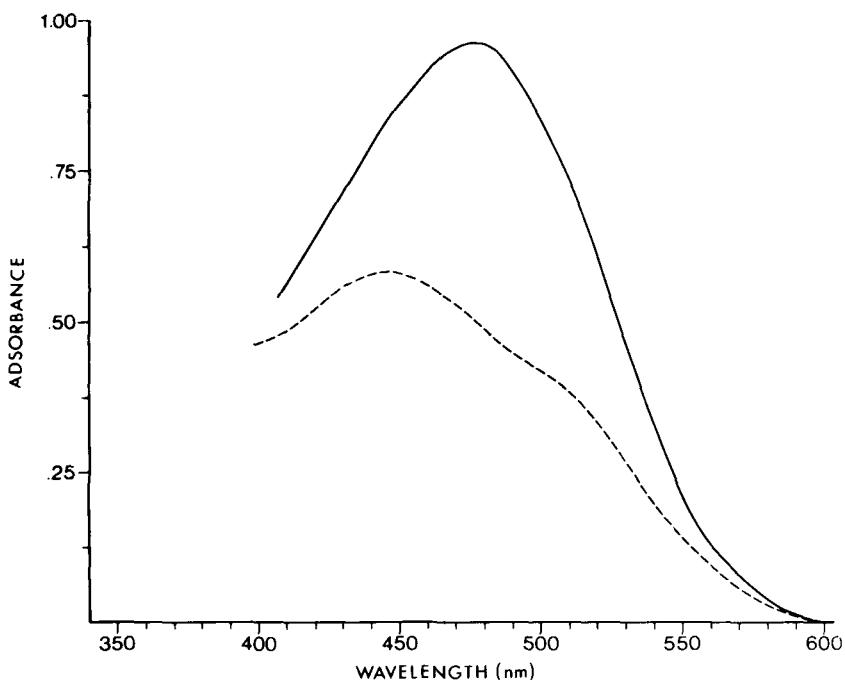


FIGURE 9. Absorption spectra of pigments formed with 9-*cis* acyclic retinal derivative. Pigment formed with derivative 3 in dark (---) and after exposure to light (—), pH 7.4, 20 mM potassium phosphate buffer.

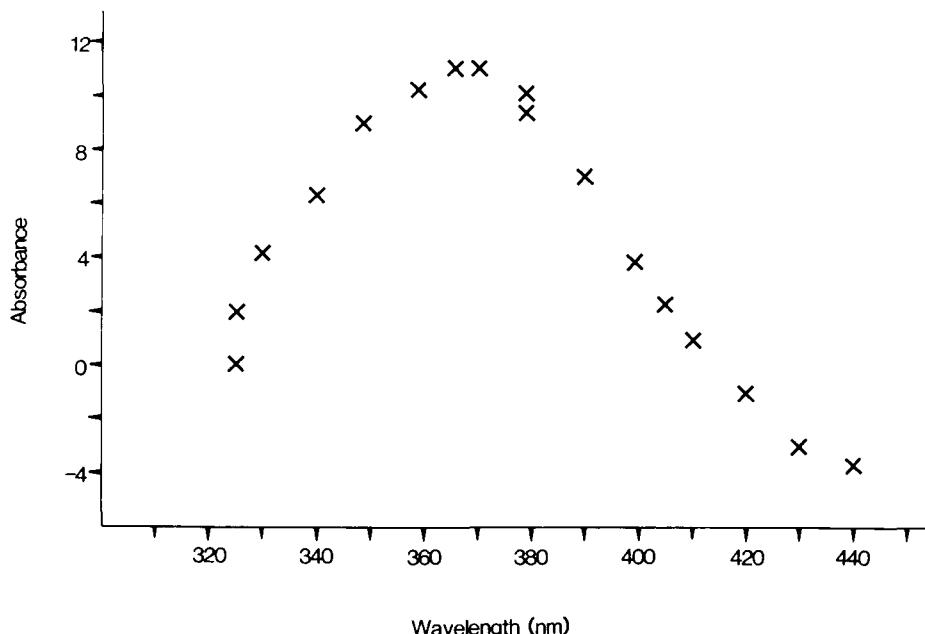


FIGURE 10. Absorption spectrum of M intermediate of spin-labeled bacteriorhodopsin. Flash-induced difference spectrum of M intermediate of 7 in 150 mM KCl, pH = 6.7, 20°C actinic flash ≥ 470 nm (Xenon flash plus Corning Cs 3-71 filter).

respect to the polyene side chain may prevent the chromophore from sitting in the binding site in the required manner to render the pigment functional.

The ESR spectra of both pigments showed a highly immobilized signal with a slight decrease in mobility on light adaptation. The label is highly oriented and inaccessible to water-soluble reducing agents (Figure 11). After the removal of ions, a blue shift of 30 nm from the pigment formed with 7 resembles that of the "blue membrane" of bacteriorhodopsin.⁶⁸ The ESR spectrum suggests that the deionized pigment may exist in two forms: one immobilized and a second with a mobile label. These data suggest that the blue membrane may contain a more open binding site than purple membrane and thus the specific protein structure in the ring protein of the binding site may be strongly affected by the presence of certain ions.

IV. SUMMARY

The research described here has been aimed at illustrating the structure-function relationships of the chromophores of rhodopsin and bacteriorhodopsin. As a means for studying the interaction of the chromophore with the protein, we have used synthetic retinals in specific ways to test some key function. Our results are summarized in Table 3. In general, the key limitations for rhodopsin binding appear to be the distance between the ring methyl groups and the aldehyde, an idea proposed using the retinal ring as the limiting factor by Matsumoto and Yoshizawa.⁶⁹ The ring binding site appears to have specific requirements for the ring methyls, but not the ring itself. *Cis-trans* isomerization appears to be essential for the functioning of rhodopsin. For bacteriorhodopsin, the polyene chain appears to be the key feature. The ring methyls are not essential for pigment formation or function and there is great tolerance for ring substitution. Our results show that the length of the polyene chain is critical for the function of this pigment. These two photoactive proteins containing



FIGURE 11. ESR spectrum of light- and dark-adapted, spin-labeled bacteriorhodopsin. Pigment analog 7 in water, 20°C, light-adapted (—), dark-adapted (---). Sample in flat cell irradiated for 5 min in ESR spectrometer cavity with light through ≥ 450 nm sharp cut-off filter. Time constant 1.0 s, gain 2000; scan time, 8 min; modulation amplitude, 2.0 G; microwave power 7 mW.

TABLE 3
Comparison of Binding Restraints of Rhodopsin and Bacteriorhodopsin

	Rhodopsin	Bacteriorhodopsin
Isomeric specifications	All- <i>cis</i> isomers except 13- <i>cis</i>	13- <i>cis</i> , all- <i>trans</i> only
Ring portion: methyl groups	At least <i>one</i> required	None required
Cyclic ring	Not required	Not required
Bulky substituents	Limits binding	No limitations
Side chain: length	Length between aldehyde and ring methyl groups critical to pigment formation	Side chain lengths of eight to eleven carbons adequate
C ₉ and C ₁₃ methyl group	Not required; absorption sensitive to C ₉ substitution	Not required; C ₁₃ methyl may effect proton pumping
Bulky substituents	Moderate tolerance	Low tolerance

retinal therefore have different ring binding requirements and limitations in addition to distinct protein-chromophore interactions that induce their respective functions.

ADDENDUM

The *cis*-locked analog 11 has also been used to study the mechanism of bleaching adaptation in isolated rods of the tiger salamander.⁷¹ A number of researchers have shown that the sensitivity loss resulting from bleaching by bright light is much greater than the loss of sensitivity due to pigment depletion alone. With D. W. Corson (Medical University of South Carolina) and M. C. Cornwall and E. F. MacNichol (Boston University), we have investigated this loss of sensitivity and the formation of analog pigments by physiological and microspectrophotometric methods using 11 (synthesized by Koji Nakanishi et al.). Microspectrophotometric measurements demonstrated that this analog forms a pigment in

isolated bleached rods and that this pigment is not bleached by visible light. Surprisingly, sensitivity is partially restored by **11**. However, spectral sensitivity measurements showed that in the resensitized cells the pigment producing excitation is the residual native pigment. We suggest that **11** relieves some inhibitory constraint on the residual native pigment and that **11** and other analogs provide a powerful tool for the investigation of excitation and adaptation in receptors.

ACKNOWLEDGMENTS

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Chapter 7

VISUAL PIGMENT AND BACTERIORHODOPSIN ANALOGS**Valeria Balogh-Nair and Koji Nakanishi****TABLE OF CONTENTS**

I.	Introduction	148
II.	Analogs of Bovine Rhodopsin	150
A.	Studies with 11- <i>cis</i> -Locked Chromophores	150
1.	Seven-Membered Ring Containing Rhodopsin Rh7	151
2.	Eight and Nine-Membered Ring-Containing Rhodopsins, Rh8 and Rh9.....	157
B.	Studies with Side-Chain Modified Retinals	157
1.	The Effect of Side-Chain Alkylation.....	157
2.	The External Point-Charge Models	160
C.	Studies with Retinals Modified at the Ring-Binding Site	161
1.	Allenic, Acyclic, and Aromatic Analogs.....	161
2.	Labeling of the Ring-Binding Site	161
III.	Bacteriorhodopsin Analogs.....	163
A.	Studies with Retinals Containing Modified Side Chains	166
1.	13- <i>trans</i> - and 13- <i>cis</i> -Locked Chromophores	166
2.	Other Side-Chain Modifications.....	166
B.	Studies with Retinals Modified at the Ring-Binding Site	169
	Acknowledgments	171
	References.....	171

I. INTRODUCTION

Rhodopsin (Rh), the visual pigment of vertebrates, and the purple pigment of *Halobacterium halobium* have retinal chromophores bound to apoproteins (opsins) through Schiff base linkages. Their similarity at the molecular level is further apparent in that both Schiff bases are protonated species, and that in the primary event of light absorption, the retinal moieties in both pigments isomerize to a distorted form. However, the quantum yield of the isomerization is much lower for the bacterial rhodopsin.^{1,2} Although both opsins consist of a single polypeptide chain containing seven transmembrane helices and a similar number of buried, charged residues, they do not show a statistically significant sequence homology.³ The differences in the primary^{4,5} and tertiary^{3,6} structures of the opsins, yet to be clarified experimentally, are considered to be responsible for the requirement of an 11-*cis* geometry for the retinal of rhodopsins and the all-*trans*-retinal form for light-adapted bacteriorhodopsin (bR^{LA}). The differences in the structures of the visual opsins also enable them to bind to the same 11-*cis*-retinal and yet yield pigments of widely varying absorption maxima. Rhodopsin (Rh) and bacteriorhodopsin (bR) have fundamentally different biological roles to fulfill. Rhodopsins are exquisitely sensitive photon detectors while bacteriorhodopsin provides the hypoxic cell with an alternate source of energy, namely adenosine triphosphate (ATP) generated by a proton gradient across the bacterial cell membrane. In order to provide a structural basis for these differences in function, the protonated Schiff bases must interact with the apoproteins (opsins) in a highly specific manner. Thus, the retinals not only must be able to form specific Schiff bases with the appropriate lysine residues of the apoproteins, Lys-296 in rhodopsin⁷ and Lys-216 in bacteriorhodopsin,^{8,9} but also these Schiff bases must fit the binding sites in opsins in such a manner as to permit highly specific interactions to be initiated.¹⁰ These interactions upon absorption of light evolve to allow the formation of bathochromically shifted, isomerized photoproducts, bathorhodopsins and K intermediates, respectively. The retinal moiety is a distorted all-*trans* isomer in bovine bathorhodopsin, and a distorted 13-*cis* isomer in the K intermediate of bR. The energy of the photon stored in these batho intermediates drives the subsequent events,^{11,12} i.e., the bleaching process in rhodopsins and the photocycle in the bR¹³ (Figure 1). The bleaching sequence of bovine Rh is characterized by the thermal decay of the batho product through spectrally distinct intermediates until the opsin separates from the isomerized chromophore. In the photocycle of bR, the K intermediate decays back to bR, through spectrally distinct intermediates of the photocycle. The structures of the intermediates so far have defied precise definition due to the difficulty in establishing the tertiary structures of the proteins and monitoring the changes in them initiated by the isomerization of the retinal chromophores. It is, however, clear that the Meta II intermediate in Rh and the M₄₁₂ intermediate in the bR photocycle are the crucial, activated forms of the proteins, which directly trigger the events leading to their biological responses.¹⁴

In order to better understand the molecular details of the visual process and the proton pump, it will be necessary to establish the precise chemical structures of rhodopsins and the bacteriorhodopsin. Moreover, the structures of the intermediates produced during the bleaching process and the photocycle must also be determined. Synthetic analogs of rhodopsin and bacteriorhodopsin can be prepared by reacting the opsins with synthetic retinals that mimic the natural chromophores. These pigment analogs have been used extensively and successfully to gain information on the structure and function of the natural systems.¹⁵⁻¹⁹ In spite of the wealth of information already available from studies on pigment analogs, several important questions still need to be addressed. For example, why does dark-adapted bR (bR^{DA}) consist of a mixture of all-*trans*,15-anti (*E*) and 13-*cis*,15-syn (*Z*) isomers, and what is the role of the 6-*s-trans* conformation of the retinal in its interaction with the opsin point-charges at the ring-binding site? Yet another fundamental question is to what extent

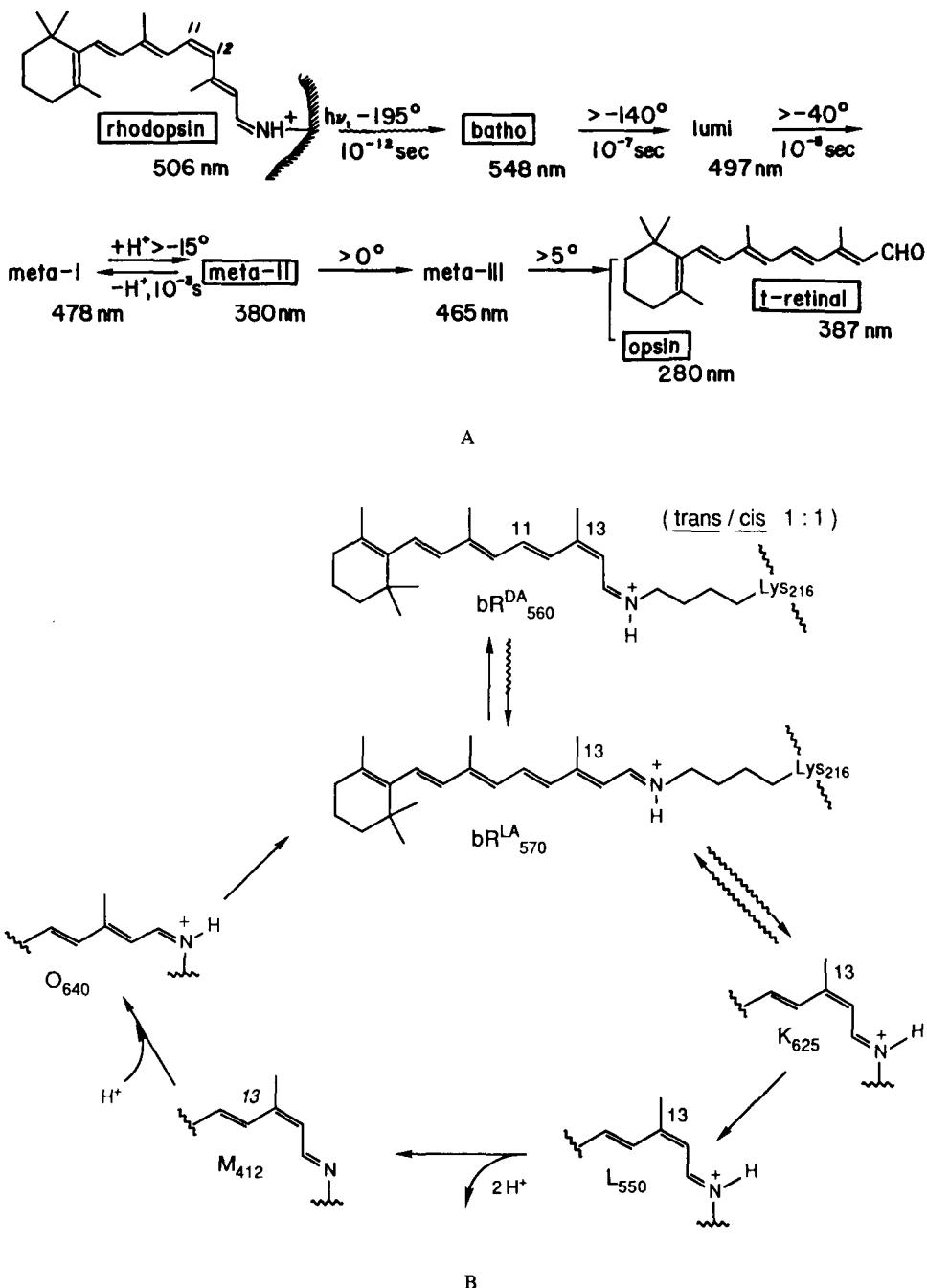


FIGURE 1. (A) The bleaching of bovine rhodopsin (Rh). (B) The photocycle of bacteriorhodopsin (bR).

are the Schiff bases protonated and to what extent hydrogen bonding in them contributes to wavelength regulation in the pigments? In this context, it will be also essential to ascertain the number, the exact location (with respect to the retinal moiety), and the identity of the point-charges on the opsins. These point-charges are considered responsible for the regulation of the absorption maxima of the pigments and their photoproducts, and they are also central to energy storage via charge separation in the primary events. This review will discuss how

recent studies with visual pigments and bacteriorhodopsin analogs have contributed to a better understanding of some of the problems addressed earlier.

II. ANALOGS OF BOVINE RHODOPSIN

There are several ways to obtain visual pigment analogs: (1) by *in vitro* regeneration of the rhodopsin from a synthetic retinal and opsin, the purified opsin being prepared from the bleached rod outer segment (ROS) membranes, (2) by *in vitro* displacement of a bound synthetic retinal from the binding site by another retinal to which the opsin has a higher affinity, (3) by *in vitro* incubation of bleached photoreceptors with a medium containing a synthetic retinal, (4) by *in vitro* incubation of bleached, perfused retinae with synthetic retinals, and (5) by *in vivo* delivery (i.p.) of synthetic retinals to animals maintained on a vitamin A-deficient diet. The majority of pigment analogs discussed here were obtained *in vitro*, by incubation of synthetic retinals with bleached ROS, either in suspension or with ROS solubilized in a suitable detergent. The importance of the solubilizing agent cannot be underestimated. It can affect not only the success of pigment formation but can also influence the outcome of the studies carried out with the artificial pigments. Solubilizing in detergents can cause varying degrees of delipidation and ensuing changes in the structure of the apoprotein used for regeneration of the pigments. Further, solubilizing agents can affect the structural features and stability of the reconstituted systems. Another important aspect is that the isomeric purity of the synthetic retinals used for the formation of pigment analogs must be checked by high-performance liquid chromatography (HPLC) directly prior to their incubation with opsin, and their stability in the incubation medium must also be ascertained. The bound retinals can be retrieved from the pigment analogs, via denaturation of the protein in such a condition that does not lead to the isomerization of the synthetic retinals, and their integrity can be checked by HPLC.²⁰ The latter technique employing a diode array detector in the UV/VIS region of the spectrum, combined with microprocessor control, allows quick and efficient data comparison especially suitable for this purpose. The synthesis of retinal analogs and their binding studies will not be detailed here but references to earlier studies will be provided instead.

Among the methods used to study the rhodopsin analogs, spectroscopic investigations occupy a prominent place. In addition to the classical approaches, such as UV/VIS, CD, resonance Raman, and FTIR spectroscopy,^{2b} recent advances include the use of difference FTIR,²¹ two-photon absorption,²² and ¹³C solid state NMR (MASS)²³ spectroscopic techniques as well as innovations in the use of picosecond pulse techniques in monitoring the absorption and fluorescence of the pigments and bleaching intermediates.

A. STUDIES WITH 11-CIS-LOCKED CHROMOPHORES

The purpose of synthesizing retinal analogs in which the 11,12-double bond is part of a ring was to create rhodopsins that would allow evaluation of the 11-*cis* to 11-*trans* isomerization in the bleaching of natural rhodopsins. Specifically, a rhodopsin analog in which this isomerization is completely prohibited by virtue of ring size, as in **1** (7-membered ring), was expected to clarify the role of *cis* to *trans* isomerization in the primary event of vision. Analogs in which the 11,12-bond is part of a larger ring that permits a partial twist, as in **2** (8-membered ring), or a more complete rotation, as in **3** (9-membered ring), of the double bond then would be mimics suitable to study the structural changes of the retinal when rhodopsin undergoes bleaching (Figure 2).

Synthetic retinals **1**, **2**, and **3** were bound to bovine opsin to yield pigment analogs. The seven-membered pigment could be made by incubation of the retinal with a suspension of bleached ROS, but efficient formation of pigments from the larger ring-containing retinals **2** and **3** required the use of the detergents, CHAPS²⁴⁻²⁶ and CHAPSO, respectively. These

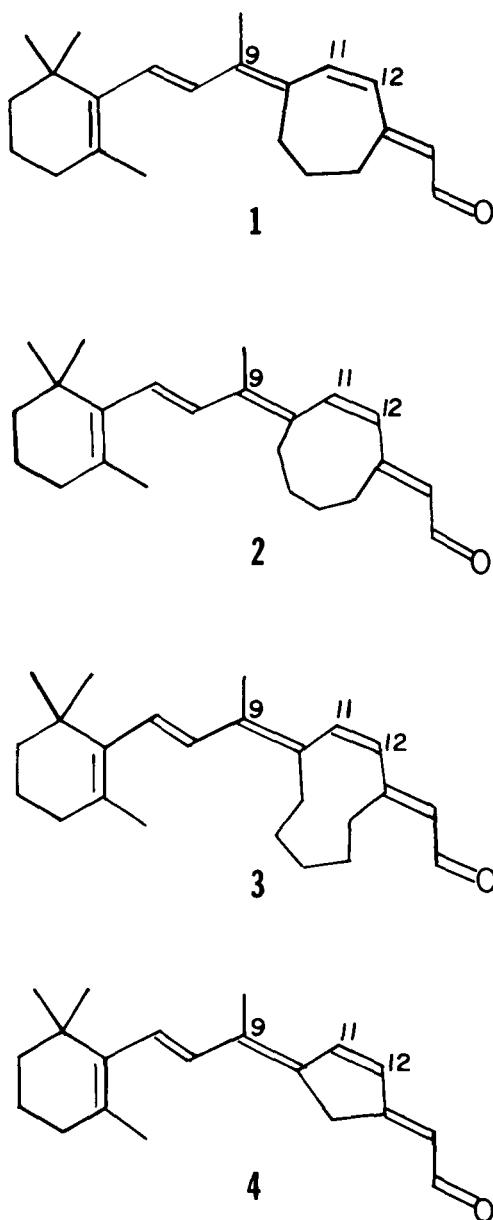


FIGURE 2. 11-*cis*-Locked-retinals.

cholate-based zwitterionic detergents are commercially available in chemically pure form and have good optical transparency. We have developed an efficient method using CHAPS to overcome the necessity of using excess retinal, employed in other regeneration methods of pigments (Figure 3). CHAPSO has the added advantage that it also allows the facile purification of the regenerated pigment, as shown in the case of bovine rhodopsin (Figure 4).

1. Seven-Membered Ring-Containing Rhodopsin, Rh7

Rh7, obtained by incubation of retinal 1 with bleached bovine ROS, in 2% digitonin solution had an absorption spectrum with a rhodopsin-like broad band and maximum at 490

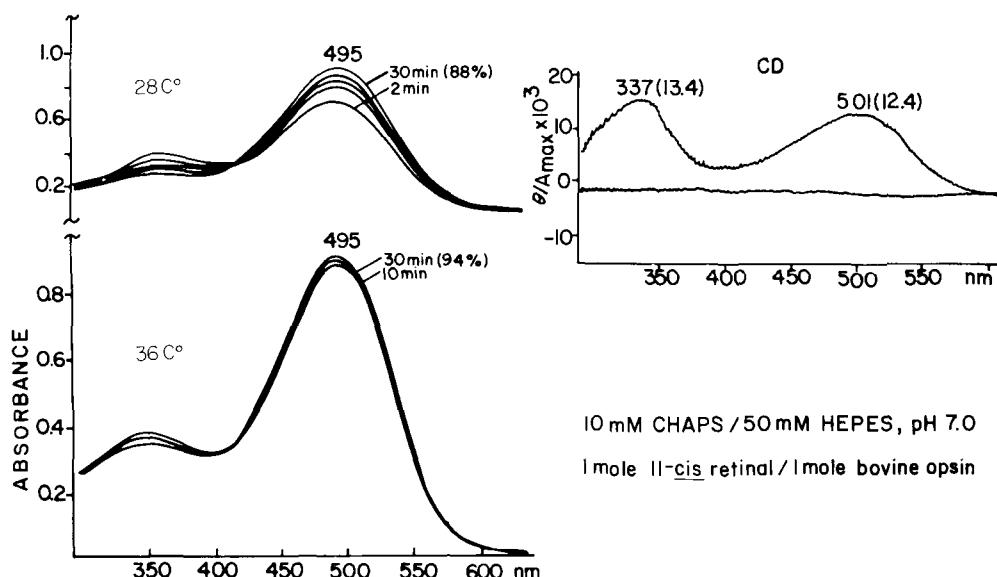


FIGURE 3. Efficient regeneration of rhodopsin in CHAPS.

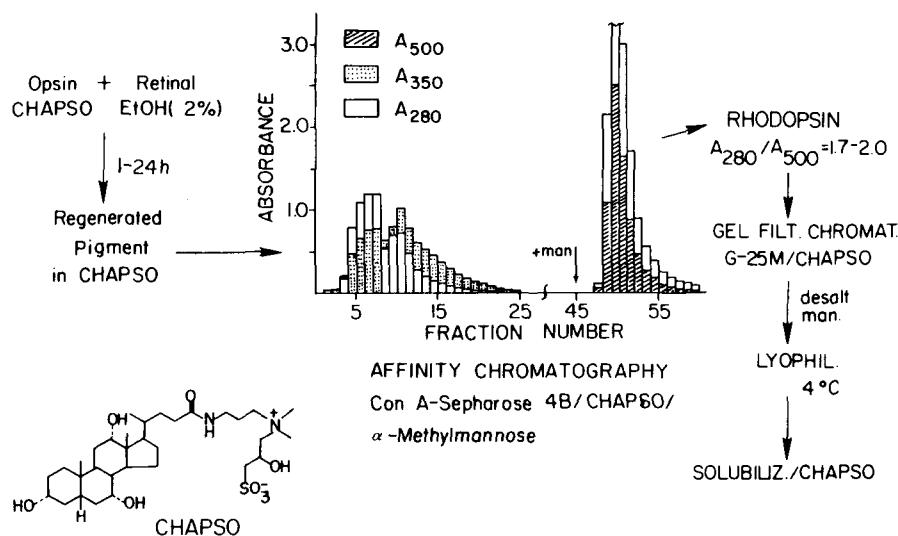


FIGURE 4. Purification of rhodopsin and rhodopsin analogs in CHAPSO.

nm.²⁷ The CD spectrum had maxima at 330 and 488 nm (Figure 5), remarkably similar to those of bovine rhodopsin (340 and 490 nm). The Cotton effects seen in the spectrum of natural rhodopsins were interpreted as due to a chirally twisted chromophore, the twist being imposed on the retinal by the polypeptide chain, which folds into its active site configuration. Alternately, it has also been proposed that a coupled oscillator mechanism may operate, resulting from the interaction of aromatic amino acid dipole(s) with the transition dipole(s) of the bound retinal. In the absence of adequate measurements, which would also include the CD bands in the far UV region, it is difficult to ascertain the extent to which each mechanism is involved.¹⁵ However, since in the retinal analog **1** the cycloheptatrienylidene ring can force the chromophore to adopt a noncoplanar shape, the characteristic CD spectra

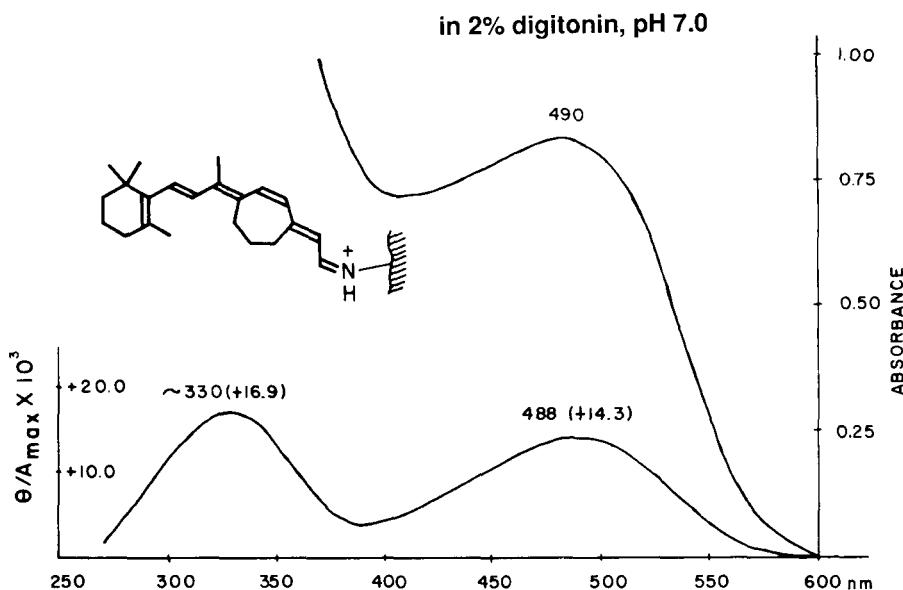


FIGURE 5. Absorption and circular dichroic spectra of Rh7 rhodopsin.

of Rh7 can be considered as evidence that the mechanism involving a chirally twisted chromophore plays an important role in Rh7 and perhaps also in bovine rhodopsin.

A rhodopsin analog prepared from retinal **4**,²⁸ which has a locked 11-*cis* and fixed 12-*s-trans* geometry, and hence a completely planar configuration of the C₉-C₁₂ side-chain moiety, showed a CD spectrum with rhodopsin-like β -band at 336 nm. However, no α -band was observed corresponding to the pigment's absorption maximum at 498 nm. This further supports the necessity of a twist of the chromophore side chain to account for the optical activity of its long wavelength absorption band.²⁹

The Rh7 pigment could be regenerated from retinal **1** not only in bovine ROS suspension but also in bullfrog ROS and isolated retinas, as well as *in vivo*, in the photoreceptors of vitamin A-deprived rats.³⁰ In each system, the locked 11-*cis* chromophore fulfilled the role for which it was designed, i.e., to yield a nonbleachable pigment analog. Detailed studies were carried out to determine at which stage the bleaching sequence of bovine rhodopsin was inhibited by the seven-membered ring-containing chromophore in bovine Rh7 rhodopsin. Flash photolysis experiments showed that Rh7 cannot form a Meta I intermediate at 20.8°C and also that at 77 K, where the batho product of natural rhodopsin can be observed, irradiation of Rh7 led to no changes in its absorption spectrum (Figure 6).³¹ This confirmed the original premise that 11-*cis* to *trans* isomerization is a prerequisite to rhodopsin¹ activation in the primary event of vision.

Picosecond kinetic absorption and fluorescence measurements further demonstrated that although Rh7 can undergo photochemical changes at the picosecond timescale,³² none of the species formed from Rh7 was the equivalent of the batho intermediate found in the bleaching sequence of natural rhodopsin but was instead a species which could have been obtained also from natural rhodopsin if the primary event in vision did not involve the crucial 11,12-double bond isomerization (Figure 7). The absorbance data shown in Figure 7 was obtained using an actinic 530-nm pulse with a density of 2×10^{16} photons/cm². In the fluorescence measurements power densities from 2×10^{15} to 2×10^{16} photons/cm² were used. All these experiments gave similar results, but these were different from the rhodopsin data obtained employing pulses with comparable characteristics. However, Yoshizawa et al.³³ observed that the saturation effect on rhodopsin can be induced by laser pulses of much

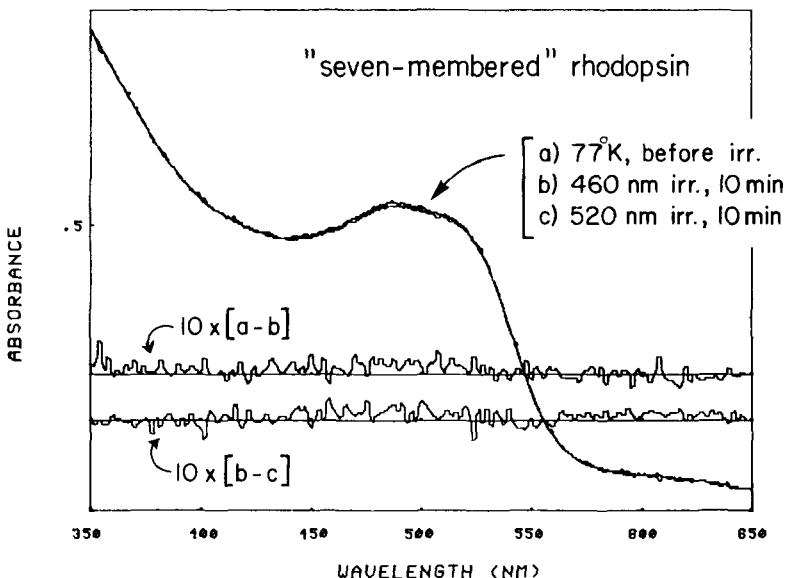


FIGURE 6. Photolysis of Rh7 at 77 K.

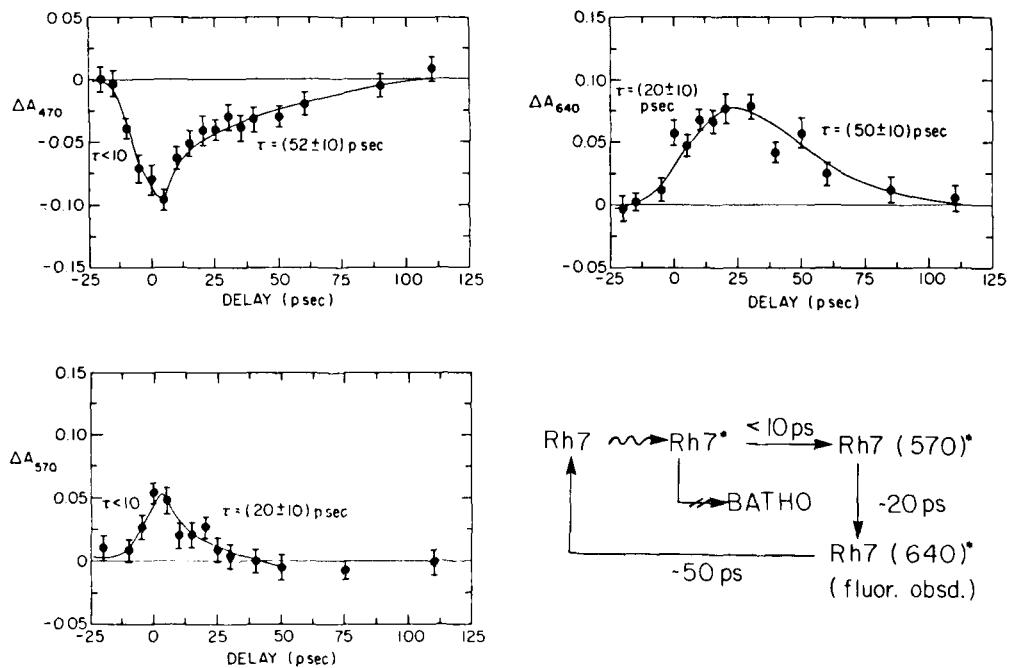


FIGURE 7. Picosecond absorption and fluorescence of Rh7 at room temperature.

less intensity, e.g., 20 μ J. Accordingly, they found that when less than 20 μ J pulses were used for excitation of rhodopsin, formation of bathorhodopsin was preceded by another species which they named photorhodopsin. Based on this report the possibility that the 570 nm species in Figure 7 resulted from a one photon absorption and the 640 nm species from absorption of more than one photon cannot be precluded. However, because both the rhodopsin and Rh7 measurements were carried out employing identical pulse intensity ranges,

the important conclusion drawn from these studies that 11,12-double bond isomerization is a prerequisite primary event in the formation of the batho intermediate remains established.

Picosecond measurements also revealed that photochemical formation of bathorhodopsin (<6 ps, unresolved at room temperature) is a more complicated process than was thought originally.³⁴ It consists of at least two distinct processes, the fast and efficient 11,12-bond isomerization at a subpicosecond timescale,³⁵ and the slower, and unspecified proton motion process, first seen by Rentzepis et al.³⁶ The proton translocation step was too slow to be considered responsible for competing with rhodopsin's fluorescence. Hence, the proton-independent, efficient, subpicosecond isomerization step was held responsible for rhodopsin's low fluorescence quantum yield.³⁷ The fluorescence characteristics of Rh7 differed considerably from those of rhodopsin. These include the slower rise time (25 ps) and decay time (55 ps) of its emission (<12 ps for both, in Rh),³⁸ as well as its much larger fluorescence quantum yield (almost two orders of magnitude larger than that of rhodopsin). These observations were found consistent with a slowing of the rotational motion in Rh7 attributable to the three-carbon bridge that hinders rotation around the 11,12-bond.³² In the case of rhodopsin, theoretical calculations predict that emission should occur within the first 45° of the rotation, and a larger barrier to rotation in the first 45° should increase the fluorescence quantum yield.³⁹ The observed fluorescence quantum yield with Rh7 is in good agreement with these predictions.

In order to explain the efficient 11-*cis* to *trans* isomerization observed in rhodopsin, but not seen in the corresponding protonated Schiff base models, a precise knowledge of the excited state level ordering of rhodopsin needs to be elucidated. The location of a ${}^1\text{Bu}^{*+}$ -like state has never been a problem as it could be easily determined from the electronic absorption spectrum of rhodopsin. However, the location of a nearby "forbidden" ${}^1\text{Ag}^{*-}$ state remained controversial until Birge et al.⁴⁰ demonstrated that two-photon spectroscopy is the most reliable technique to locate ${}^1\text{Ag}^{*-}$ -like states, especially, in molecules such as rhodopsin that display severe inhomogenous broadening. The two-photon thermal lensing technique has a serious drawback in that it requires the use of high intensity light pulses over extended periods of time, which can lead to sample degradation. This problem can, however, be circumvented by working at low temperatures or by employing light-stable rhodopsin analogs. The light-stable Rh7 pigment, therefore, was particularly suitable for two-photon measurements (Figure 8).⁴¹

Figure 8 shows that the separation between the Franck-Condon maxima of the one-photon absorption and two-photon thermal lens spectra is $2250 \pm 850 \text{ cm}^{-1}$, thus, the more covalent ${}^1\text{Ag}^{*-}$ state lies at about 2000 cm^{-1} above the ionic ${}^1\text{Bu}^{*+}$ state. Although, technical difficulties precluded recording the entire two-photon spectrum, a comparison of the one and two-photon maxima clearly indicate the reversal of excited state level ordering in rhodopsin. This follows the trend observed on protonation of the model retinal Schiff bases, where the protonation was observed to stabilize preferentially the ionic ${}^1\text{Bu}^{*+}$ state relative to the covalent ${}^1\text{Ag}^{*-}$ state. Excited state level ordering is known to be a sensitive probe of the molecule's environment and therefore can be used to probe the binding site environment of retinal.⁴² If the retinal was bound to Lys-296 via an unprotonated Schiff base linkage, even drastic dispersive and electrostatic effects on this unprotonated Schiff base by the opsin environment would not bring about the excited state level ordering reversal seen in rhodopsin. We can therefore conclude from the excited state reversal seen with the Rh7 pigment that the Schiff base linkage in rhodopsin must be protonated. Moreover, the excited state level ordering observed indicated that the binding site in rhodopsin must be a neutral environment containing only one minus charge and the positive charge of the protonated Schiff base. This finding is not in conflict with the external point charge model of rhodopsin,⁴³ which proposed two point charges in the retinal binding site, because these point charges were considered to be members of a salt-bridge or a neutral polar group. On the basis of the one-

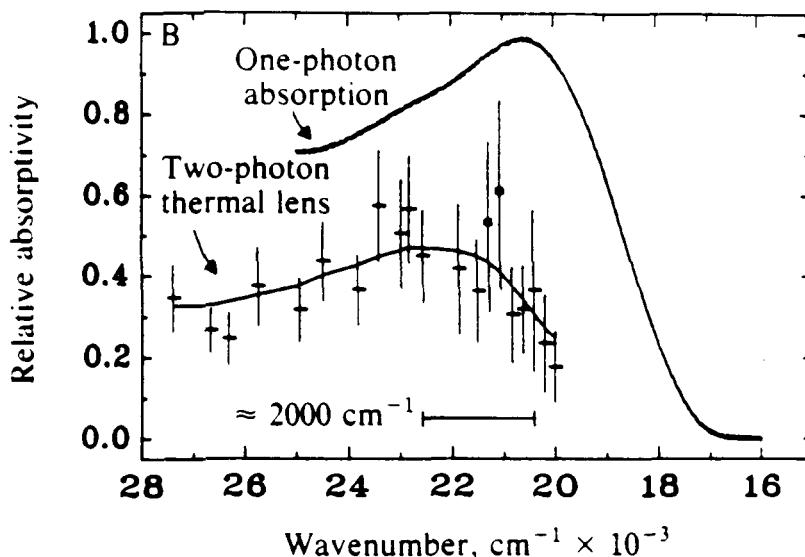


FIGURE 8. Comparison of one-photon absorption and two-photon thermal lens spectra of Rh7 at room temperature.

and two-photon data, and in agreement with the external point charge model, two possible binding site geometries were proposed, as shown in Figure 9. In the single counterion model, the full minus charge of the neutral binding site is provided by a glutamate (or aspartate) residue beneath the polyene chain, providing both the counterion to the protonated Schiff base and the wavelength regulating point charge close to the end of the polyene chain. In the two-counterion model, two glutamate (or aspartate) residues are placed as shown, to be shared by the protonated Schiff base and the polyene side chain.

An interesting feature of the two-counterion model is that it can provide a mechanism for the ground state proton translocation that is detected by the observed deuterium isotope effect, a step which follows retinal isomerization in the primary event. The charge separation resulting from isomerization of the 11,12-bond will change the retinal's environment in such a way as to stabilize the 11-transoid configuration by the transfer of a proton from one glutamic residue to the other. In this process the residue that transferred its proton is proposed to become the primary counterion in bathorhodopsin.

However, the excited state level ordering reversal in rhodopsin has by no means gained universal acceptance as the explanation for the photochemistry of Rh. For instance, it has been proposed that level mixing rather than level ordering reversal is responsible for the photochemical properties of rhodopsin's excited state and that protonation of the Schiff base does not induce level reversal.⁴⁴ More recent results,⁴⁵ however, support the conclusion from the studies with Rh7 rhodopsin, i.e., that level ordering reversal is the key photophysical event responsible for the high quantum yield (0.67) and the fast (<3 ps) isomerization of rhodopsin. These studies using two-photon, and ¹³C and 2-D ¹H NMR spectroscopic data of retinyl Schiff bases, protonated Schiff bases, and Schiff base salts provide evidence for a protonation-induced $\pi\pi^*$ excited state level ordering reversal and demonstrate that relative state level ordering is highly sensitive to counterion location. These findings were in agreement with the previous theoretical studies⁴⁶⁻⁴⁸ that suggested that protonation of the Schiff base induces level ordering reversal and that the interaction of a fixed counterion and lowest lying ${}^1\text{Bu}^{*+}$ -like state provides the barrierless excited state potential surface required for efficient 11-*cis* to *trans* isomerization.

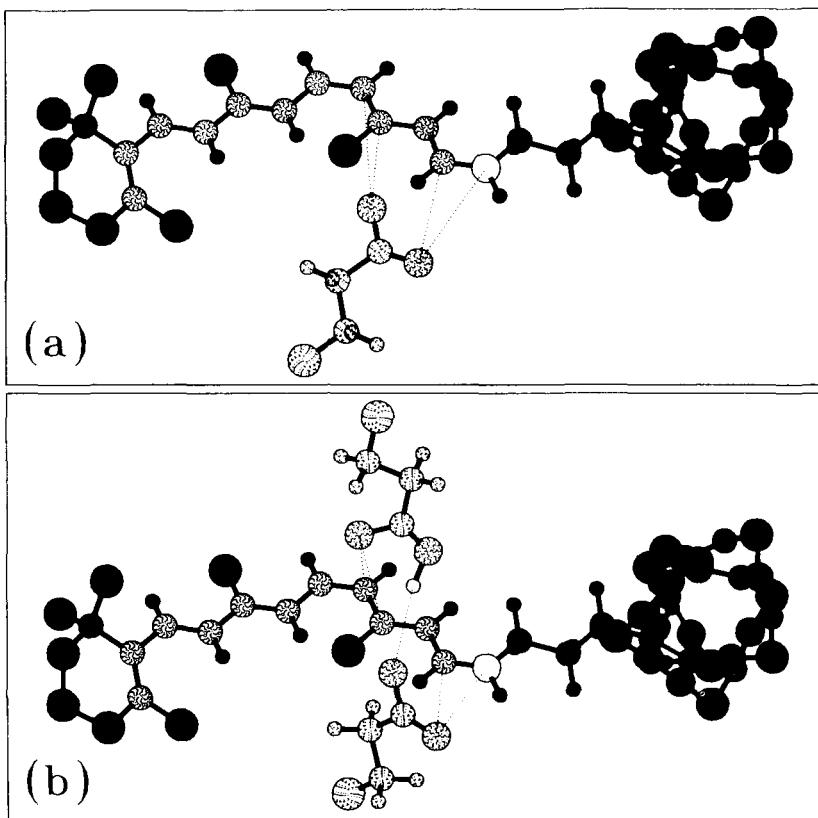


FIGURE 9. (a) Single-counterion model for rhodopsin showing a glutamic residue underneath the plane of the polyene. (b) Two-counterion model for rhodopsin showing two glutamic residues below the polyene.

2. Eight- and Nine-Membered Ring-Containing Rhodopsins, Rh8 and Rh9

The larger ring-containing rhodopsin analogs, Rh8 and Rh9, were prepared by incubation of retinals 2 and 3 with bleached ROS solubilized in the detergent CHAPSO.¹⁷⁻¹⁸ Incubation of retinal 2 at room temperature yielded a photolabile species with a broad absorption and a maximum at 430 nm as determined from its second derivative spectrum (Figure 10). At 37°C, however, a rhodopsin-like pigment Rh8, absorbing at 515 nm, was also obtained together with the 430-nm species. Irradiation at wavelengths longer than 410 nm “bleached” the 430-nm species but the absorption of Rh8 at 515 nm remained unchanged. Thus these preliminary data indicate that Rh8, as was the case with Rh7, is a nonbleachable rhodopsin analog suitable for further spectroscopic studies. The retinal 3 with the 9-membered ring produced a 407-nm absorbing and light-sensitive species when incubated with bovine ROS solubilized in CHAPSO (Figure 11). The retinal 3 has a distorted chromophore in which the full conjugation of the retinal side chain is disrupted as indicated by its absorption maximum at 280 nm. It is therefore possible that the 407-nm, light-sensitive species produced from it by incubation with opsin is the expected light-sensitive Rh9 analog. Ongoing spectroscopic investigations using Rh8 and Rh9 should lead to further insights into the structure of the intermediates involved in the bleaching of natural rhodopsin.

B. STUDIES WITH SIDE CHAIN-MODIFIED RETINALS

1. The Effect of Side-Chain Alkylation

Among the very first synthetically modified retinals employed to prepare rhodopsin

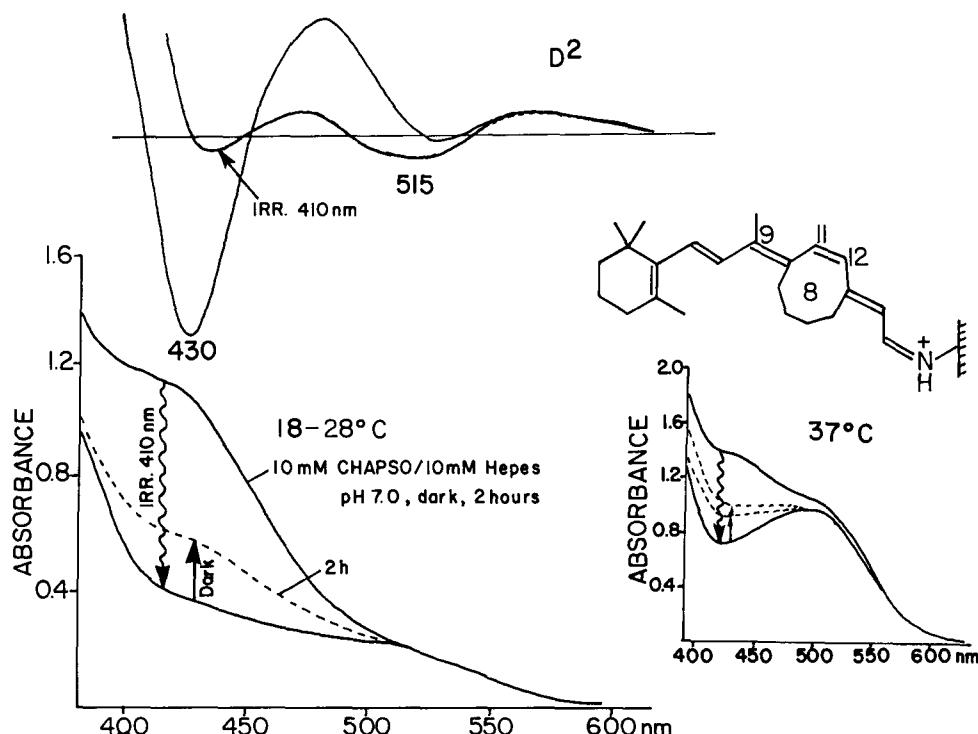


FIGURE 10. Formation of photosensitive 430-nm species, and nonbleachable Rh8 from retinal 2 and bleached bovine ROS in CHAPSO solution.

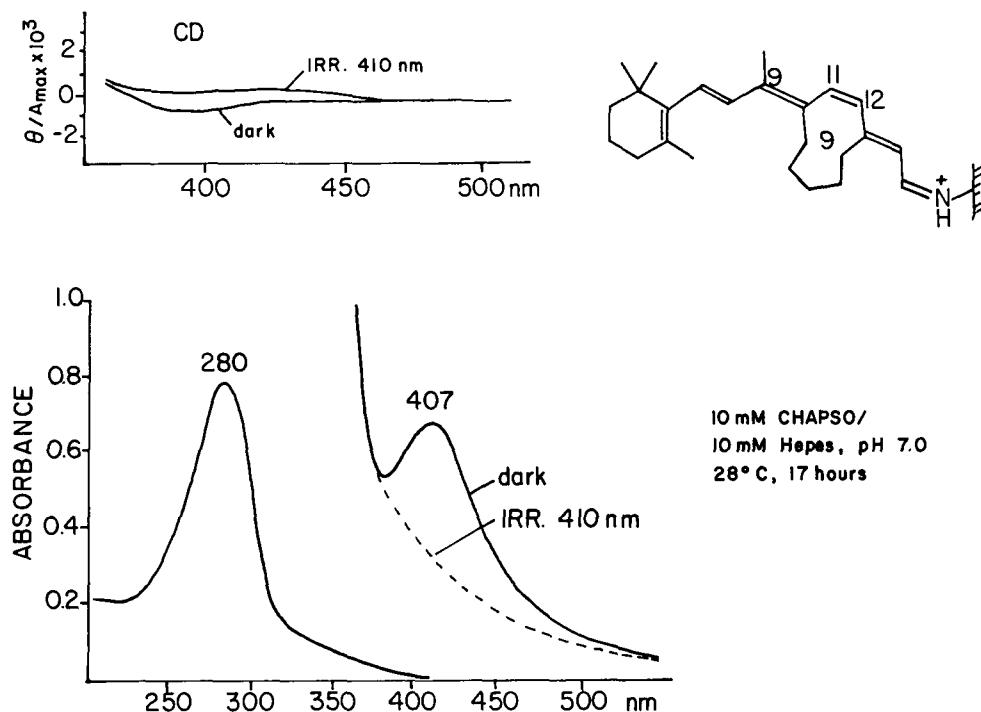
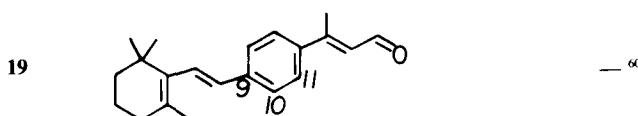
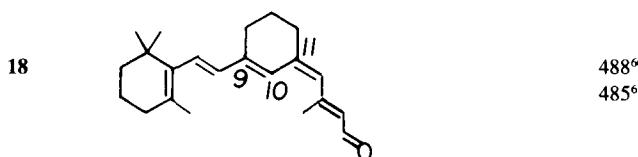


FIGURE 11. Formation of light-sensitive, 407-nm species by incubation of retinal 3 with bleached bovine ROS in CHAPSO.

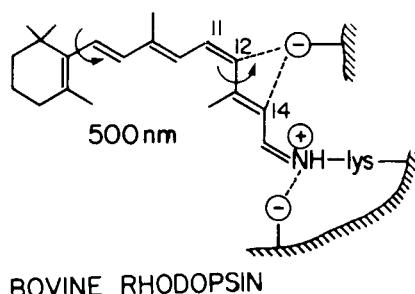
TABLE 1
**Alkylated and Dealkylated Retinals that Bind to Bovine Opsin,
and the Absorption Maxima of the Pigment Analogs Formed**

Entry	Retinal	Absorption Maxima
5	11-cis-9-desmethylretinal	465 ⁴⁹⁻⁵¹
6	11-cis-13-desmethylretinal	495 ^{49,50,52-54}
7	11-cis-9,13-desmethylretinal	483 ⁴⁹⁻⁵¹
8	9-cis-13-desmethyl-14-methylretinal	492 ⁵⁵
9	9-cis-14-methylretinal	502 ⁵⁵
10	9-cis-10-methylretinal	485 ⁵⁶
11	9-cis-10,14-dimethylretinal	500 ⁵⁶
12	11-cis-9-ethylretinal	497 ⁵⁷
13	11-cis-11-methylretinal	490 ¹⁷
14	11-cis-12-methylretinal	500 ^{17,59}
15	11-cis-11,12-dimethylretinal	480 ¹⁷
16	9-cis-13-ethylretinal	495 ¹⁷
17	9-cis-13-propylretinal	490 ¹⁷



analogs were the side-chain dealkylated and alkylated chromophores such as 9-desmethylretinal **5**, and 13-desmethylretinal **6**,⁴⁹⁻⁵¹ and others, which are listed in Table 1 together with the absorption maxima of bovine rhodopsin analogs formed from them.

The conclusion from studies with the set of retinals listed in Table 1 was that side-chain alkylation or dealkylation of the retinal has no significant effect on the absorption maxima of pigment analogs formed from them, but the rate of formation of the analogs, as well as their stability towards heat, hydroxylamine and detergents was reduced. In addition, among these early analogs the 9- and 13-desmethyl derivatives, **5** and **6**, by forming batho intermediates, demonstrated that these methyl groups are not important in the formation of bathorhodopsin. Furthermore, the 14-methyl analogs **9** and **11**, which cannot assume an 11-cis, 12-s-cis conformation helped to clarify the conformation of the 12,13-single bond in natural rhodopsin as 12-s-trans.⁵⁵⁻⁵⁶ More recent alkyl modifications of the side chain include the 9- and 11-cis isomers of 12-methylretinal **14**,^{17,58} where the lower yield of pigment analog from the 11-cis isomer compared with that of the 9-cis was compatible with the existence of a forbidden zone in the binding site.⁵⁹ Studies in progress of the bleaching process of rhodopsin analogs, prepared from retinals that contain multiple alkylation, e.g., **15** (11,12-dimethyl),¹⁷ or larger alkyl groups, e.g., **16** (13-ethyl) or **17** (13-propyl),¹⁷ on the side chain should not only help to clarify the more subtle steric requirements of the binding site but should also be expected to affect the rate of formation and the structures of the bleaching intermediates. This, in turn, will contribute to our knowledge of the structure of these intermediates in the natural pigment. The rhodopsin analog prepared from the side chain-modified retinal **18**⁶⁰⁻⁶¹ with the C₉=C₁₀,C₁₀=C₁₁-locked trans configuration, formed batho-, lumi-, and meta-I-like intermediates with properties similar to those of the natural



<u>opsin shifts, in cm⁻¹</u>	
retinal	2700 (11-cis)
	2110 (9-cis)
5,6 - dihydro	1800 (9-cis)
7,8 - dihydro	1700 (9-cis)
9,10- dihydro	2100
11,12 - dihydro	5300

FIGURE 12. The external point-charge model of bovine rhodopsin, and opsin shifts of hydorhodopsins.

bleaching intermediates.⁶¹ These experimental data did not support the proposed significant rotation around the C₁₀-C₁₁ bond, presumed to be a prerequisite for bleaching of rhodopsin during the primary event, as envisaged by the hula twist model (H.T.-n, hula-twist at center n; also known as C.T.-n, concerted twist at center n) put forward recently by Liu et al.⁶²⁻⁶³ However, these authors propound that the data could be accommodated by a new pathway involving a H.T.-12 process,⁶⁰ instead of the H.T.-11 mechanism originally proposed. Furthermore, the all-trans,10-s-cis configuration, proposed for bathorhodopsin, was found to be in agreement with the observation that retinal 19 modified by the incorporation of an aromatic ring in the side chain that fixes this particular configuration, could not be accommodated by bovine opsin, i.e., it did not form a Rh analog.

2. The External Point-Charge Models

Another important family of side chain-modified retinals we investigated was a set of hydrogenated analogs. 5,6-2H, 7,8-2H, 9,10-2H, 11,12-2H, and 9,10,11,12-4H retinals (2H, dihydro; 4H, tetrahydro). Among these, the dihydroretinal in which the saturation of the 11,12-double bond eliminates the possibility of 11-cis to 11-trans isomerization formed the first known nonbleachable rhodopsin analog,⁶⁴⁻⁶⁶ thus supporting the important role attributed to the isomerization of this bond in the bleaching of rhodopsin. The opsin shifts of bovine rhodopsins formed from hydroretinals constituted the experimental basis for the external-point charge model.⁴³ This model was proposed to explain the color of bovine rhodopsin as well as to account for a general mechanism by which opsins regulate the absorption maxima of visual pigments in nature (Figure 12).

According to the external point charge model, charged or polar groups on opsin via through-space interaction with the bound retinal chromophore regulate the absorption maxima of the visual pigments by varying the number and orientations of these charges with respect to the retinal moiety within the opsin binding site. Continuing studies of rhodopsin analogs formed by incorporation of dihydroretinals into opsins derived from various other species, such as vitamin A-deprived rats,⁶⁷ chicken (cones),⁶⁸ and the blind mutant of *Chlamydomonas*

*reinhardtii*⁶⁹⁻⁷⁰ are expected to help to elucidate of the molecular mechanism by which nature fine-tunes the sensory perception of light.

The opsins shifts of rhodopsin analogs derived from 9- and 13-bromoretinals afforded further support to the point-charge model of bovine rhodopsin by confirming the higher sensitivity of the C₁₁-C₁₅ moiety of the retinal side-chain to stereoelectronic effects as compared with that of the C₇-C₁₁ region.⁷¹ Other, recently prepared side-chain halogenated derivatives (Liu et al.) as well as novel synthetic routes to side-chain-alkylated and trimethylsilyl-substituted retinals (Okamura and Hopf and their co-workers) will be discussed elsewhere in this volume.

C. STUDIES WITH RETINALS MODIFIED AT THE RING-BINDING SITE

1. Allenic, Acyclic, and Aromatic Analogs

The very first modifications of the ring-binding site in bovine rhodopsin were the formation of rhodopsin analogs from 3,4-dehydroretinal and 5-desmethylretinal. This was followed by a plethora of synthetic analogs prepared by various groups.¹⁵ Among these were adamantyl allenic derivatives,⁷² which by virtue of forming a rhodopsin analog pointed to a lenient ring-binding site. Studies with acyclic analogs⁷³⁻⁷⁴ demonstrated that the whole cyclohexyl ring of the retinal is not required for binding to bovine opsin and that the ring-methyl groups enhance the recognition of the analog by the ring-binding site. The cyclohexyl ring could also be replaced with aromatic and heteroaromatic rings in several modified retinals without affecting the regeneration yield of rhodopsin analogs, provided that the aromatic ring had methyl substituents in positions *ortho* to the polyene side chain (Figure 13). Methyl substitution of the aromatic rings assured good regeneration yields of rhodopsin analogs and also contributed to the stability of the latter.

Table 2 lists absorption maxima of aromatic 9-*cis*-rhodopsin analogs⁷⁵ together with their opsin shifts (OS).⁷⁶ These values are comparable to the opsin shift seen in 9-*cis*-rhodopsin. Therefore, these analogs also support the external point charge model of bovine rhodopsin, which places the wavelength-determining interaction with the opsin at a site remote from the ring-binding site. An interesting observation with this aromatic series was that the unmethylated analogs had absorption maxima red shifted from those seen for the methylated ones. This was interpreted as a result of ring-side chain planarization, possible only in the unmethylated analogs, a phenomenon which is also observed in the corresponding model retinal Schiff bases, which displayed fine structures in their long wavelength absorption band.

2. Labeling of the Ring-Binding Site

Enzymatic⁷⁷ as well as fluorescence labeling⁷⁸⁻⁷⁹ techniques were invaluable tools in determining the topography of rhodopsin in the ROS membranes. However, to determine the topography of the rhodopsin, itself, for instance the location of its retinal chromophore within the seven helical segments of the opsin, it is important to determine the exact site of the amino acid residues that surround the retinal when it is bound to Lys-296 of the opsin. X-ray⁸⁰ and other diffraction techniques, e.g., neutron diffraction,⁸¹ could not provide such information because rhodopsin is an integral membrane protein and hence is not prone to yield crystals suitable for higher resolution studies.

The photoaffinity labeling technique pioneered by Westheimer⁸² and Knowles⁸³ to investigate enzyme structure and function employs substrate mimics that can be activated by light. In the case of rhodopsin it is a particularly suitable technique for the determination of the amino acid make-up of the retinal-binding site. Concerns about "unwanted" pseudoaffinity labeling, which arise from competing diffusion of the substrate from the active site during the labeling process, can be disregarded since in rhodopsin the substrate (retinal) is permanently fixed by covalent bonding as long as rhodopsin is maintained in the dark (or

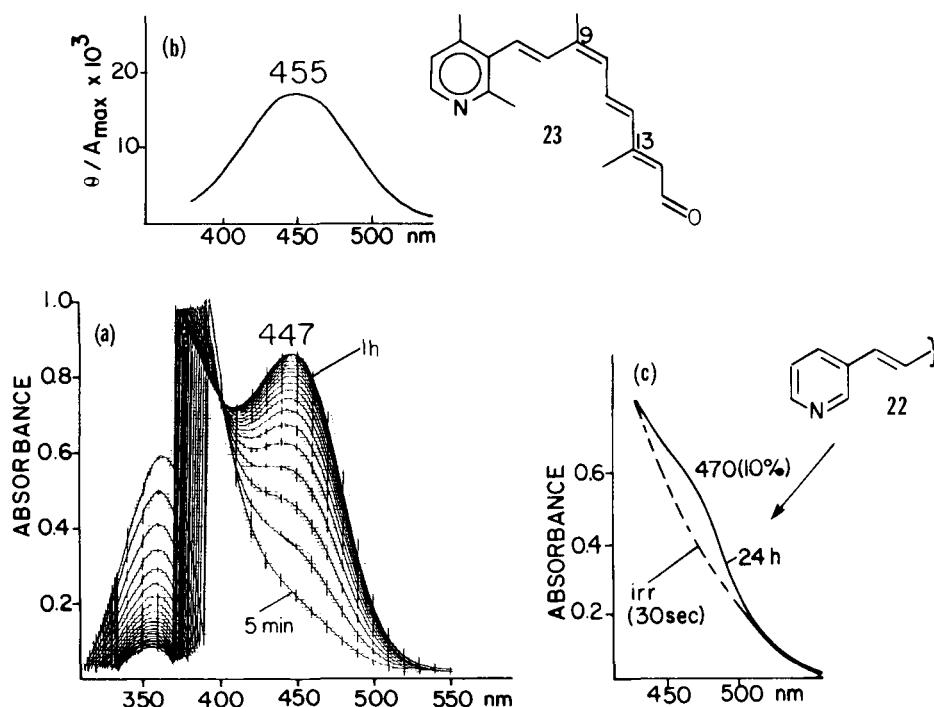


FIGURE 13. The effect of *ortho* methyl groups on the regeneration yield of aromatic 9-*cis*-rhodopsins. (a) absorption curves showing the formation of 9-*cis*-rhodopsin from retinal **23** and bleached ROS in CHAPS; (b) circular dichroism curve of 9-*cis*-rhodopsin from retinal **23**, in CHAPS; (c) absorption curve of 9-*cis*-rhodopsin from retinal **22** and bleached ROS in CHAPS, before (—) and after (---) bleaching.

irradiated only with light that does not trigger its bleaching). We found that incubation of the 9-*cis* isomers of 3-diazoacetoxy- and 9-methylenediazaoacetoxyretinals with bleached ROS in CHAPS detergent allowed incorporation of these label-bearing retinals in the opsin binding site (Figure 14).²⁶

The 3-diazoacetoxyretinal gave a higher yield of the rhodopsin analog than the 9-methylenediazaoacetoxy derivative, and accordingly, further studies were carried out using this retinal. Moreover, the CH_2Cl_2 denaturation/extraction procedure followed by HPLC separation⁸⁴ and binding studies indicated for the first time that, although the ring-binding site in opsin is lenient, it is also capable of chiral discrimination. Thus, one of the enantiomers of a mixture of the 3-diazoacetoxy-9-*cis*-retinals was preferentially bound when the racemic mixture was incubated with bovine opsin. Activation of the photoaffinity label must be carried out in an efficient manner so as to obtain the highest possible yield of crosslinks but also in such a manner as to preserve the integrity of the system to be labeled. We have solved the problem of the activation of the label, which was shown in the case of other systems to destroy the protein integrity, by using UV light (254 nm) of low intensity for efficient and selective activation of the diazoacetate label.⁸⁵ During this procedure, we encountered no significant, concomitant destruction of the aromatic amino acid residues of the protein, nor destruction of the active site structure. Employing this methodology, labels inserted into the binding site could be selectively activated to yield rhodopsin analogs crosslinked via the C₃ and C₁₅ sites of the retinal chromophore. Experiments with retinals bearing radiolabel(s) in addition to the photoaffinity label, followed by analysis of the cross-linking patterns obtained, should not only reveal the identity of the amino acid residues that

TABLE 2
The Absorption Maxima and Opsin Shifts of 9-cis-Rhodopsins Formed from Aromatic Ring Containing Retinals 20 to 23

Entry	9-cis-rhodopsin	Absorption maxima	Opsin shifts
20		485	1850
21		457	1650
22		465	2530
23		447	2380

constitute the active site of rhodopsin, but may also yield information on the spatial arrangement of seven alpha helical segments of rhodopsin.

III. BACTERIORHODOPSIN ANALOGS

There are three ways to prepare bacteriorhodopsin analogs: (1) by incubation of the synthetic retinal with a suspension of bleached purple membranes (or with bleached purple membranes solubilized in a detergent). (2) by incubation of the synthetic retinal with white membranes obtained from retinal-minus mutant cells, and (3) by growing retinal-minus mutant cells in the presence of the synthetic retinal.

The examples discussed in this review were prepared according to the first method, followed by an additional step to eliminate retinal oxime from the bleached membranes. Thus, the purple membranes isolated from *H. halobium* strains and bleached according to

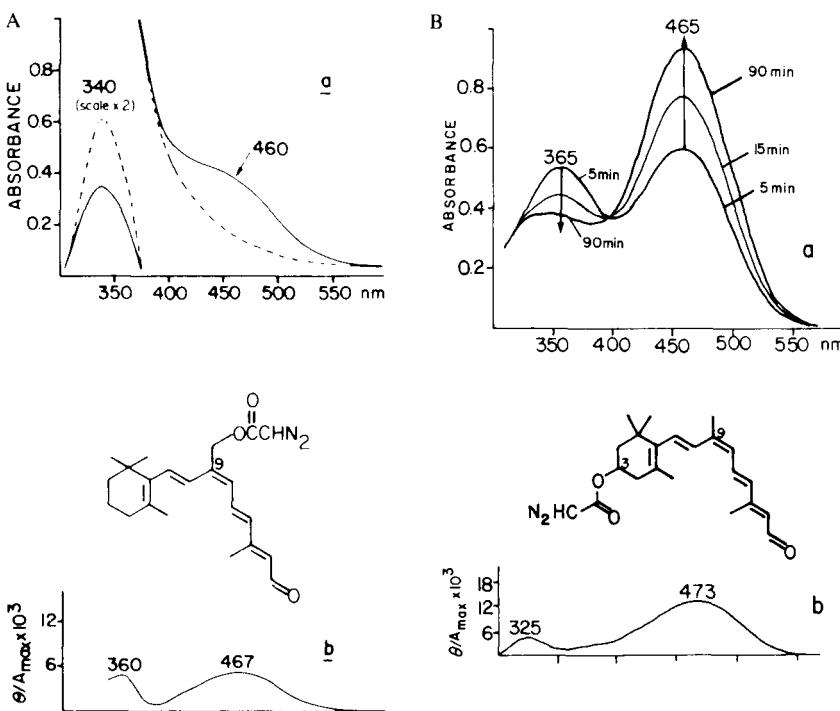


FIGURE 14. (A) (a) Absorption curve of 9-methylenediazoacetoxy-9-cis-rhodopsin before (—) and after (---) bleaching; (b) circular dichroism curve of 9-methylenediazoacetoxy-9-cis-rhodopsin. (B) (a) Formation of 3-diazoacetoxy-9-cis-rhodopsin in 10 mM CHAPS/10 mM HEPES buffer, pH 7.0, dark, rt; (b) circular dichroism curve of 3-diazoacetoxy-9-cis-rhodopsin in 10 mM CHAPS/10 mM HEPES buffer, pH 7.0, dark, rt.

standard procedures,⁸⁶ were treated with a 2% solution of fatty acid-free bovine serum albumin (BSA),⁸⁷ followed by several washes with distilled water. The membrane-bound retinal oxime did not seem to interfere with the regeneration of a bacteriorhodopsin analog, but the oxime released retinal in the presence of light or even upon standing of the bleached membranes in the dark. Therefore, the experiments that involve testing the proton pumping ability of the bR analogs or other studies related to bR function can be successfully carried out with analogs regenerated by method 1 followed by BSA washes of the membrane suspension, or even better, by following methods 2 or 3. As was the case with the preparation of Rh analogs, the isomeric purity of the synthetic retinals must be ascertained prior to reconstitution experiments. Only the all-*trans* or 13-*cis* isomers of the synthetic retinal form bR analogs. The bR analogs formed from an all-*trans* isomer may undergo light-dark adaptation, which can be easily observed by monitoring the reversible shift of the long wavelength absorption maxima (560 and 570 nm in natural bR^{LA} and bR^{DA}, respectively).

Bacteriorhodopsin analogs can be incorporated into phospholipid vesicles suitable for

testing their proton-pumping ability. Vesicles can be prepared by the method of Racker⁸⁸ or other procedures that do not involve the use of a detergent. Alternately, detergents can be employed, for instance as in the procedure by Khorana et al.,⁸⁹ which employs Triton X-100 to delipidate the membranes prior to renaturation in a deoxycholate environment. The bR incorporated in the vesicles has an inside-out orientation; therefore, alkalinization of the medium — instead of acidification as with natural purple membrane (PM) — is observed when the vesicles are irradiated to pump protons. The size of the vesicles is of importance, and can be measured by electron microscopy; larger vesicles have larger internal volumes and hence have superior proton-pumping capacity. The photochemical properties of bR incorporated into the vesicles are similar to those of the purple membrane except that the “O₆₄₀” intermediate of the photocycle cannot be observed. Apparently, this intermediate disappears when the photocycle is slower than in native purple membrane (PM).⁹⁰ Another consequence of detergent solubilization of PM is that some detergents dissociate the two-dimensional hexagonal lattice of bR trimers present in the native membrane. For instance, solubilization in Triton X-100 yields bR monomers lacking the exciton coupling effects in the visible CD spectrum, characteristic of the native PM, as well as that of bR analogs regenerated from bleached membrane suspensions. Bacteriorhodopsin monomers do pump protons, but when other detergents, such as Ammonyx LO, CTAB, DTAB, cholate, deoxycholate, Emulphogene, and Tween 80 are used, they either denature the bacteriorhodopsin or do not yield monomers.^{2b} The synthetic analogs designed to test this activity therefore must be prepared according to procedures which preserve the native structure. Moreover, the absorption spectrum of PM is drastically modified by changes in the pH and the ionic composition of the medium. Thus, upon acidification to pH 1 to 3 or upon deionization of the membrane, the color changes from purple to blue⁹¹ with an absorption maximum at 603 nm. Irradiation of the blue species gives a pink membrane with absorption maximum at 491 nm.⁹² These modified membranes also have an isomeric composition different from that of the native membrane. The blue membrane contains a 32:68 mixture of 13-*cis* and all-*trans*-retinal and the chromophore of the pink membrane is entirely 9-*cis*. In the native membrane, the dark-adapted and inactive form contains a 6:4 mixture of 13-*cis* and 13-*trans* isomers, and the active, light-adapted state has only the all-*trans* isomer. Moreover, native PM has a net negative surface charge, and recent conductivity studies have revealed dramatic changes in the surface potential when PM is solubilized, e.g., in Triton X-100. It has been suggested that these changes are due to differences in counterion binding to the membrane's surface, and that this may contribute significantly to the proton pumping ability of PM.⁹³ Accordingly, appropriate precautions must be taken when preparing samples of bR analogs so that they match the properties of the native membrane.

As was the case with rhodopsin analogs, spectroscopic investigations of bR analogs provided new insight into our knowledge of the structure of bacteriorhodopsin. Among the recent spectroscopic studies, prominent place is taken by FTIR, resonance Raman, and solid state NMR spectroscopic studies. For instance, FTIR studies provided evidence for protonation of internal carboxylic groups of the opsin during the photocycle of bR.⁹⁴⁻⁹⁵ FTIR difference spectroscopic studies using bR analogs containing ¹³C-amino acids and ²H-labeled retinal demonstrated that the bR photocycle involves at least four Asp residues but that the Glu groups do not play a role in the proton-pumping mechanism.⁹⁶ These findings are particularly interesting because they are consistent with neutron diffraction data obtained using ²H-labeled amino acids, with models proposed fitting these data and with the density map of Henderson and Unwin.⁹⁷ Tyrosine residues also play a role⁹⁸ since the following protonation/deprotonation steps were observed during the photocycle:^{99,100} bR^{DA}(Tyr-OH)→bR^{LA}(Tyr-O⁻)→(Tyr-OH)→L(Tyr-OH)→M(Tyr-O⁻). The change bR^{LA}(Tyr-O⁻)→K(Tyr-OH) has been independently observed.¹⁰¹

Resonance Raman data has been useful for monitoring the light-induced structural changes

of the retinal chromophore in bR.¹⁰² For example, resonance Raman measurements with labeled bR analogs supported the presumed deprotonation of the retinal Schiff base in the M₄₁₂ intermediate of the photocycle. It also helped to recognize the intermediate O₆₄₀ as a protonated 13-*trans* structure. Resonance Raman data has shown recently¹⁰³ that both the intermediates K₆₂₅ and L₅₅₀ have a protonated 13-*cis*,14-*s-trans*-retinal Schiff base; hence, the proton pump involves isomerization of the 13,14-double bond but not that of the 14,15-single bond.

Valuable information on the structure of the opsin-bound retinal in bR was obtained from solid state ¹³C magic angle spinning (MASS) experiments on bR analogs. Investigation of a series of bR analogs containing ¹³C labels revealed that the opsin-bound retinal is in a perturbed 6-*s-trans* conformation.¹⁰⁴ ¹⁵N solid state NMR experiments employing (ϵ -¹⁵N)-Lys bR confirmed that in bR the retinal Schiff base is protonated and weakly hydrogen bonded.¹⁰⁵ Other ¹³C MASS experiments on bR analogs containing ¹³C-enriched retinals showed that the configuration of the Schiff base in dark-adapted bR is a mixture of 13-*cis*,15-syn and all-*trans*,15-anti isomers.¹⁰⁶

A. STUDIES WITH RETINALS CONTAINING MODIFIED SIDE CHAINS

1. 13-*Trans*- and 13-*cis*-Locked Chromophores

A fundamental aspect of bR chemistry that needed clarification was the elucidation as to whether the isomerization of the 13,14-double bond was a prerequisite for the formation of the photocycle intermediate(s) responsible for the translocation of protons. Modified retinals **24** and **25**, in which isomerization at C₁₃=C₁₄ was prevented by virtue of a structural feature of the retinal chromophore, were therefore designed, synthesized, and bound to bacteriorhodopsin to form bR analogs.¹⁰⁷ The bR analogs obtained from the 13-*trans*-locked retinal **24** and 13-*cis*-locked chromophore **25** showed UV/VIS absorption and CD spectra comparable to those of bR (Figure 15 and 16).

The opsin shift for **24** (4140 cm⁻¹) was similar to the opsin shift in bR (4800 cm⁻¹) and that for **25** was the same (4480 cm⁻¹) as for the pigment regenerated from 13-*cis* retinal. Furthermore, binding studies indicated that **24** and **25** occupy the natural sites. However, a significant difference was that the regeneration intermediates with the characteristic fine structures are observed only when the bR is regenerated at 0°C, but with these bR analogs they could be observed even at room temperature. Thus, the regeneration intermediate from the incubation of **25** was stable at room temperature for more than 2 h and the intermediate from **24** took 15 d to yield the desired bR analog. The fundamental difference, however, with these bR analogs was that when vesicles (prepared from them and soybean phospholipids)⁸⁸ were irradiated, no proton translocation was observed. This clearly indicated that isomerization of C₁₃-C₁₄-double bond is essential for the proton translocation activity by bR.

2. Other Side-Chain Modifications

Following the same approach that was described for the hydrogenated rhodopsin analogs, a series of selectively hydrogenated retinals such as 5,6-2H, 7,8-2H, 9,10-2H, and 11,12-2H were synthesized and then bound to bacteriorhodopsin to yield bR analogs.¹⁶ The opsin shifts were determined in each case, and on the basis of the trend seen in the opsin shifts, the first external point-charge model that explained the purple color of bR was proposed.¹⁰⁸ In this model, in addition to the counterion of the Schiff base, the negative end of a protein dipole served as the second point-charge close to C₅ of the ionone ring-binding site. In the course of our studies to elucidate the role of the polyene side chain length and that of the point-charge at the ring-binding site in the proton pumping of bR, we found that the opsin shift of the 7,8-2H analog was larger than reported originally.¹⁰⁹ Studies of sR (sensory rhodopsin) analogs and analogs containing 5,6,7,8-4H, *cis*-5,6-, and *trans*-5,6-2H retinals led to a modified point-charge model (Figure 17).¹¹⁰⁻¹¹¹ In this model both the negative and the

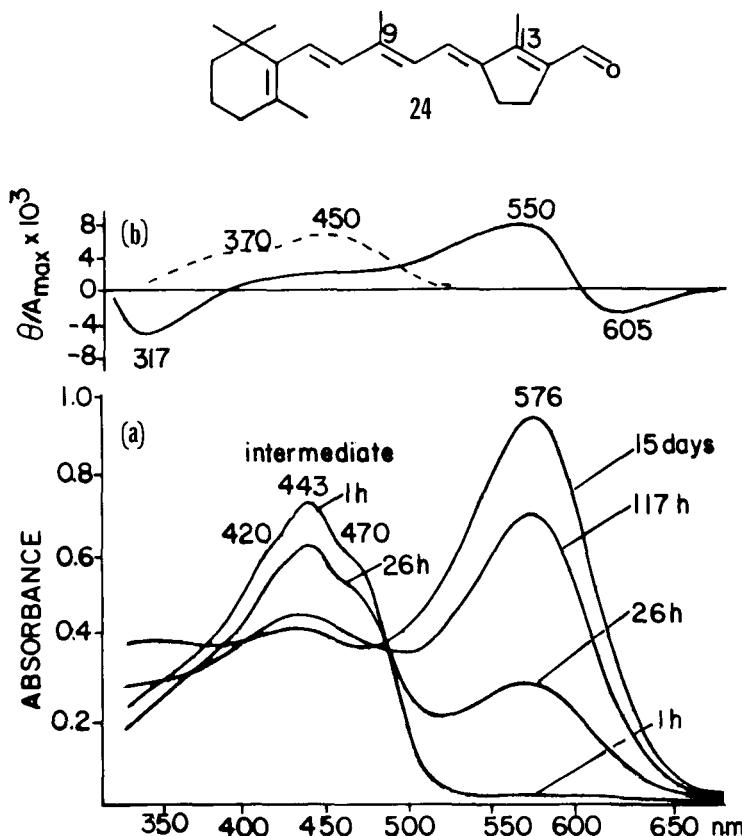


FIGURE 15. (a) Formation of bR analog from a 1:1 OD ratio of apomembrane and retinal **24**, in 10mM HEPES buffer pH 7.0 dark, 22°C; (b) circular dichroism spectrum of bR analog after 15 d of regeneration; dotted line shows the spectrum of the regeneration intermediate.

positive ends of the ion-pair of the opsin interact with the ionone ring. Moreover, the modified model incorporates a planar *s-trans* configuration of the retinal. This is in agreement with recent NMR studies, which established that bacterioopsin preferentially binds the 6-*s-trans* isomer of the retinal.¹⁰³ A similar modification of the point-charge model, differing slightly in that the positive end of the dipole is placed close to C₇ of the side chain, has been proposed to account for the larger opsin shift of the 7,8-2H analog ($3,500 \text{ cm}^{-1}$) compared to that of the 5,6-2H analog ($2,300 \text{ cm}^{-1}$).¹¹² It is yet to be established which amino acid residue on the opsin provides the ion-pair close to the retinal at the ionone ring-binding site, and also the extent of the contribution of this ion-pair to the opsin shift.

Among sterically fixed retinals that formed bR analogs was the 9,12-phenylretinal having a chromophore which could be isomerized in solution from the 13-*trans* to the 13-*cis* form. However, picosecond studies indicated that when the trans isomer was bound to opsin, isomerization did not occur. Hence this hindered bR analog did not yield a K intermediate nor did it translocate protons.¹¹³ Studies with other bR analogs in which structural features prevented rotation around specific double or single bonds demonstrated that rotations around the C₁₀-C₁₁ and C₁₂-C₁₃ single bonds and the C₁₁=C₁₂ and C₉=C₁₀ double bonds are not relevant to the primary event in bR nor are these rotations required for the formation of the M₄₁₂ intermediate. These observations lend further support to the critical role attributed to the isomerization of the C₁₃=C₁₄ double bond in the photocycle of bR.¹¹⁴

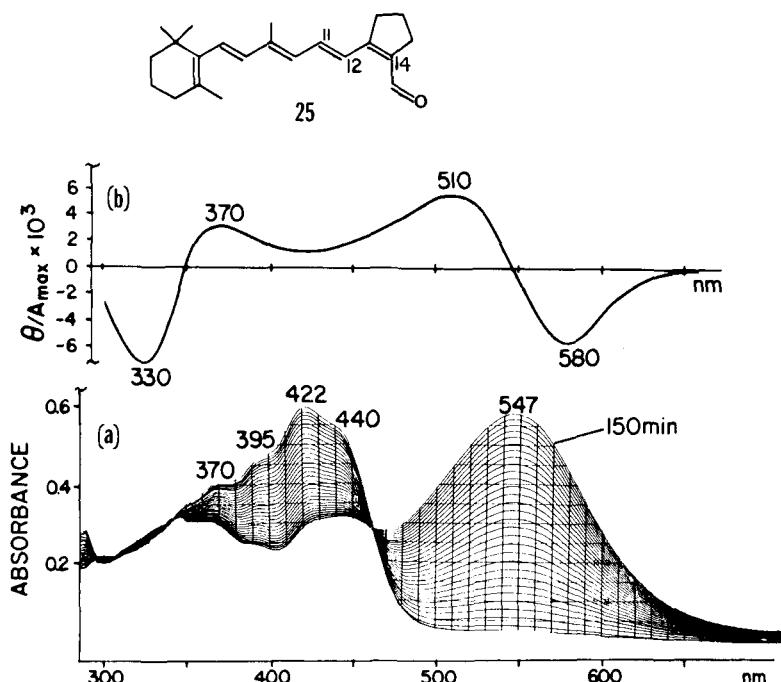


FIGURE 16. (a) Formation of bR analog from a 1:1 ratio of apomembrane and retinal 25, in 10 mM HEPES buffer pH 7.0, dark, 22°C; the maximum pigment yield is achieved after 66 h; (b) circular dichroism spectrum of bR analog after 66-h incubation.

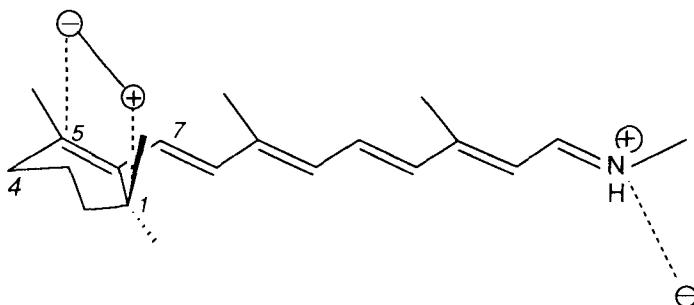


FIGURE 17. The modified point-charge model of bacteriorhodopsin.

Other side chain-modifications were the 9-bromo- and 13-bromo analogs which had opsin shifts in agreement with the point-charge model. The 14-bromoretinal did not form a pigment.¹⁶ Side chain modifications not yielding bR analogs are tabulated in Reference 16. The syntheses of both the 13-*trans* and *cis* isomers of 9-trifluoromethyl- and 13-trifluoromethylretinals were reported by several groups, and the effect of the strongly electron-withdrawing trifluoromethyl group on the pK_a of the Schiff base and the photocycle of bR has been investigated.¹¹⁵ The 10-methyl, 13-desmethyl, 14-methyl, and 10,14-dimethyl analogs and 5,6-2H-bR were employed to investigate the influence of the -C=N-H bend on the -C=NH- stretch vibration of the opsin-bound retinal by resonance Raman spectroscopy. The purpose of this investigation was to rationalize the experimentally observed -C=NH-stretch, which is lower in bR than in Rh, whereas a higher force constant was predicted for bR.¹¹⁶ Another explanation postulated for this apparent discrepancy was that the electronic

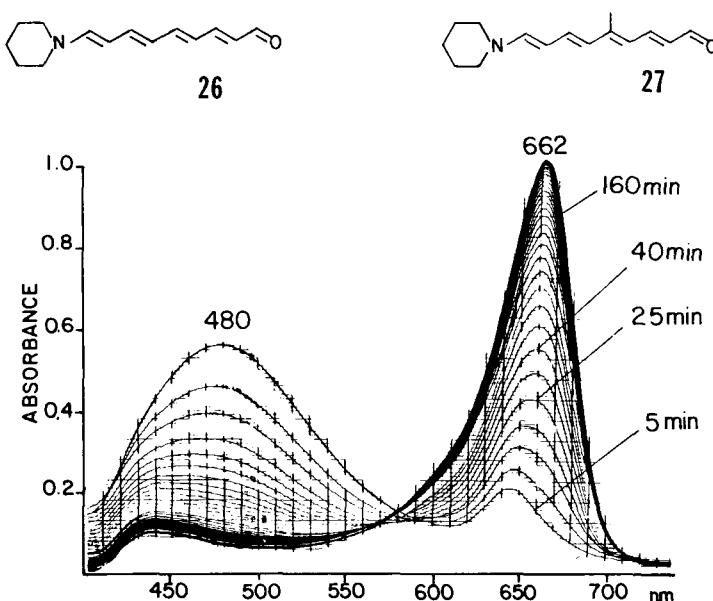


FIGURE 18. The structures of merocyanines **26** and **27**, and absorption spectra obtained during incubation of 11-methylmerocyanine, **27**, with apomembranes, in 10 mM HEPES buffer, pH 7.0, dark, 22°C.

rearrangement occurring on protonation strengthens the C=N stretching force constant. In agreement with this postulate was that the observed C=N stretching frequency in Schiff base/Lewis acid complexes increases relative to that of the free Schiff base by an amount similar to that observed on protonation.¹¹⁷

The syntheses and studies with conformationally-locked retinals, such as the 12-s-*cis*-locked analogs, are discussed in Chapter 9 by Okamura et al.

B. STUDIES WITH RETINALS MODIFIED AT THE RING-BINDING SITE

Bacteriorhodopsin analogs in which the ionone ring was modified were designed to obtain information on retinal-opsin interactions responsible for the color of the purple membrane and also to determine the amino acid residues that constitute the ring-binding site of the opsin. These should help to clarify the role of the ring-binding site in the molecular mechanism of proton pumping by the purple membrane.

Merocyanines, when bound to the opsin via protonated Schiff base linkages are expected to yield cyanine structures occupying the retinal-binding site in the opsin. Synthetic merocyanines **26** and **27** on incubation with bacteriorhodopsin afforded bR analogs with absorption maxima at 662 nm.¹¹⁸ Further, as expected for cyanines, these absorption bands were narrow, with half-band widths ($W_{1/2}$) of ca. 1000 cm^{-1} (Figure 18). The observation that cyanine structures are formed from merocyanines when the latter are bound to opsin via the Schiff base linkage presents strong evidence for the protonated nature of the Schiff base linkage in bR. Further, the observed red shifted absorption maxima of the cyanine analogs were in good agreement with the location of point-charge of opsin being quite close to the ring end of the polyene chain of the retinal. Theoretical calculations fully support the observed presence of cyanine structures whose absorption maxima were regulated by the point-charge at the ring-binding site.¹¹⁹

Studies with aromatic bR analogs also support the presence of wavelength-regulating point-charge at the ring-binding site. Thus, the phenyl analog was investigated by several groups.^{16,71,75,120-123} The reported absorption maxima of phenyl-bR varied depending on the

conditions employed for its preparation, e.g., pH, salt concentration, temperature. However, in all of the studies a significant blue shift of the absorption maximum was seen (as compared to that of bR), which fully supported the presence of opsin point-charge at the ring-binding site.¹⁰⁸ In addition, these studies ruled out proton abstraction from the ionone ring of the retinal as a step in the catalytic cycle of bR.¹²¹ Other aromatic bR analogs prepared include the *p*-dimethylaminophenyl,⁷¹ bromobenzyl,¹²² *o*-tolyl, mesityl, 2-chloro-6-fluorophenyl, and piperonyl,¹²³ and *p*-dimethylaminonaphthyl¹²⁴-containing congeners. The 13-*cis* isomers yielded pigments with a single absorption maximum, while the spectra of the bR analogs from the *trans* retinal derivatives were composed of two or more absorption bands. It was also noticed that bulky groups on the phenyl ring resulted in slower formation of the bR analog and that some of the bulky *trans* retinal derivatives isomerized to the shorter 13-*cis* forms during the incubations, apparently to more optimally occupy the retinal-binding site. In accordance with the point-charge model, all the aromatic analogs reported exhibited opsin shifts smaller than that of natural bR.

Bacteriorhodopsin analogs from retinals containing locked 6-*s-cis* and 6-*s-trans* conformations have also been prepared.¹²⁵ When bound, their pigments had absorption maxima close to that of natural bR, at 564 and 596 nm, respectively. However, only the 6-*s-trans* analog showed light-dark adaptation and translocated protons efficiently, thus supporting the requirement for a 6-*s-trans* conformation in natural bacteriorhodopsin. Numerous bR analogs are also known in which the entire cyclohexenyl ring or parts of it is lacking. It has been observed that an analog possessing the full side chain but no ring, nevertheless, pumps protons efficiently.¹²⁶ Further requirements for efficient pigment formation and photocycling were a chain length of at least nine carbons, and for efficient proton release ten carbons. Furthermore, the methyl groups on the ring did not contribute significantly to the opsin shift in bR, and the C₁ and C₅ methyl groups, even when present on an incomplete ring, generated absorption spectra and opsin shifts similar to that in natural bR.¹²⁷ However, electronic perturbations of the point-charges near the ionone ring caused by substituents on the ring, such as the 5-trifluoromethyl group, have a dramatic influence on both the absorption spectrum and the proton-pumping capacity of the bR analog.¹²⁸ The small opsin shift (2,400 cm⁻¹) suggests that the 5-trifluoromethyl group does not permit proper locking of the modified retinal into the binding site, and this lack of fit precludes proton translocation.

The ionone ring of the retinal has also been employed for attachment of reporter groups to investigate the environment of the ring-binding site in bR. Thus, a nitroxide spin label attached to the ring indicated a rigid environment of the retinal's ring at the binding site.¹²⁹ Photoaffinity labels were also attached to the ring.¹³⁰ For example, 3-diazoacetoxyretinal formed a bR analog with an absorption maximum at 532 nm. The light-adapted form of the analog translocated protons with about 50% efficiency. The incorporation of the diazoacetoxy label in the active site was ascertained by binding studies and also with the help of difference FTIR measurements. As was the case with the photoaffinity label-bearing Rh analog, the label in the 3-diazoacetoxy-bR could also be selectively activated to yield about 25% cross-links; hence, this analog and similar ones will be suitable for elucidation of the nature of amino acid residues in the bR binding site. Selective and multiple substitutions of the cyclohexylidene ring protons of retinal with deuterium followed by incorporation of the modified chromophores into growth cultures of JW5 mutant bacteria allowed neutron diffraction measurements at 8.7-Å resolution. Based on these experiments, the ionone ring was located between helices 3, 4, 5, and 6 and the lysine-216 attachment site of the retinal was placed on helix 2 or 6.¹³¹ Considering that high resolution electron diffraction data has now been recorded not only for bR but also for its M₄₁₂ intermediate at 3.5 Å resolution,¹³² the labeling experiments will be particularly valuable in establishing the identity of the few amino acid residues that move to new positions following the *trans* to *cis* isomerization of the retinal and the subsequent repositioning of the lysine side-chain.

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Chapter 8

ACETYLENIC INTERMEDIATES IN RETINOID SYNTHESIS**Henning Hopf and Norbert Krause**

I.	Introduction	178
II.	Acetylenic Vitamin A Analogs — Structures and Known Syntheses	178
III.	Preparation of 19-nor-9,10,Didehydroretinal (2b), 19-nor-7,8,9,10-Tetra-dehydroretinal (5b), and 19-nor-9,10,11,12-Tetradehydroretinal (6b)	182
IV.	Addition Reactions to Acetylenic Retinoids	186
V.	Novel Acetylenic Building Blocks for Retinoid Synthesis	190
	References.....	197

I. INTRODUCTION

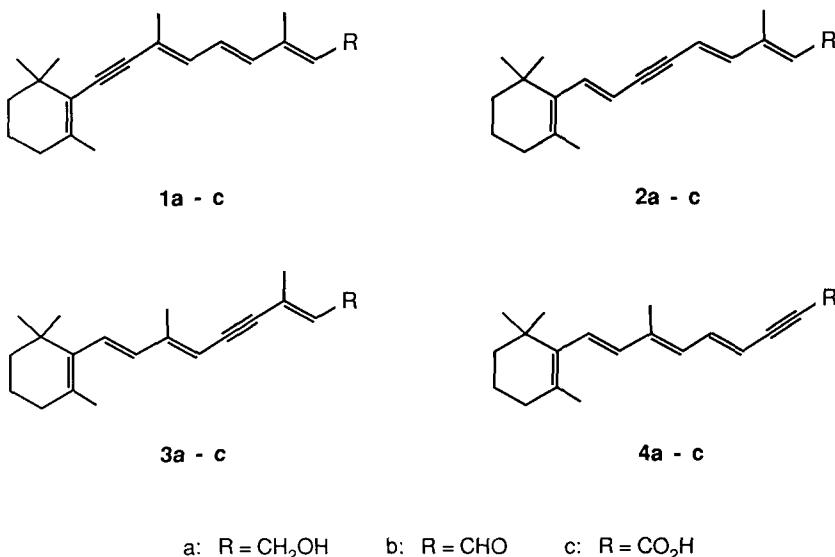
Ever since the first syntheses of vitamin A and its derivatives were reported by Arens, Heilbron, Inhoffen, Isler, Karrer, Kuhn, Milas and many others^{1,2} alkynes have played a prominent role in retinoid synthesis. The reason for this widespread use is twofold. For the construction of the carbon framework of the future vitamin A derivative the high acidity of sp-bonded hydrogen atoms may be exploited. The carbanions formed on treatment of terminal alkynes with numerous bases are excellent nucleophiles that react with all kinds and types of electrophilic reagents — carbonyl compounds, epoxides, and halides, to name but a few. In its simplest form, acetylide anion — as the Grignard reagent, or sodium or lithium salt — has been employed numerous times as a C₂-unit for chain elongation. From the earliest days on, more complex alkynes, some of them carrying functional groups, were used as building blocks. Isler's famed first industrial retinol synthesis³ during which a C₆-chain extension is carried out with *cis*-3-methyl-2-penten-4-yn-1-ol may be cited as a specific example. Having completed the carbon skeleton, the triple bond can subsequently be used as a precursor for the eventually required double bonds. In most cases this means either catalytic hydrogenation over a Lindlar-type catalyst or reduction by lithium aluminum hydride, the latter reducing agent being of great value for the preparation of *trans*-allylic alcohols from their propargylic precursors.^{2b}

Whereas the acidity of an ethynyl function is still employed in more or less the same manner as during the days of the classical syntheses (*vide infra*), addition reactions to the triple bonds have become much more varied. As a matter of fact, there nowadays seems to be hardly an organic functional group or substituent that cannot be added to an alkyne.⁴ Furthermore, since, these reactions may often be carried out with a high degree of stereochemical control, providing both the required regioisomers and/or diastereomers, their importance has grown rapidly in recent years. As will be shown in this chapter, alkynes are particularly useful intermediates for the deliberate introduction of various types of substituents into the polyenic side chain. Thus, many of the retinoids required for biochemical studies have or may be prepared by this addition route.

Having worked with simple acetylenes for some time,⁵ we became interested in applying our know-how to more complex cases, vitamin A chemistry being a particularly attractive area. On the following pages we will present our synthetic work completed prior to the end of 1986. After an introductory section on the structural types to be dealt with and a brief summary of the literature emphasizing the more important synthetic methods rather than attempting to be comprehensive,^{1,2a,2b} we will discuss in the third section the preparation of novel vitamin A derivatives containing triple bonds. The fourth part will describe novel addition reactions to acetylenic intermediates and/or retinoids. And in the last section we finally present some new acetylenic building blocks that we hope will be of importance in future preparative work in this field.

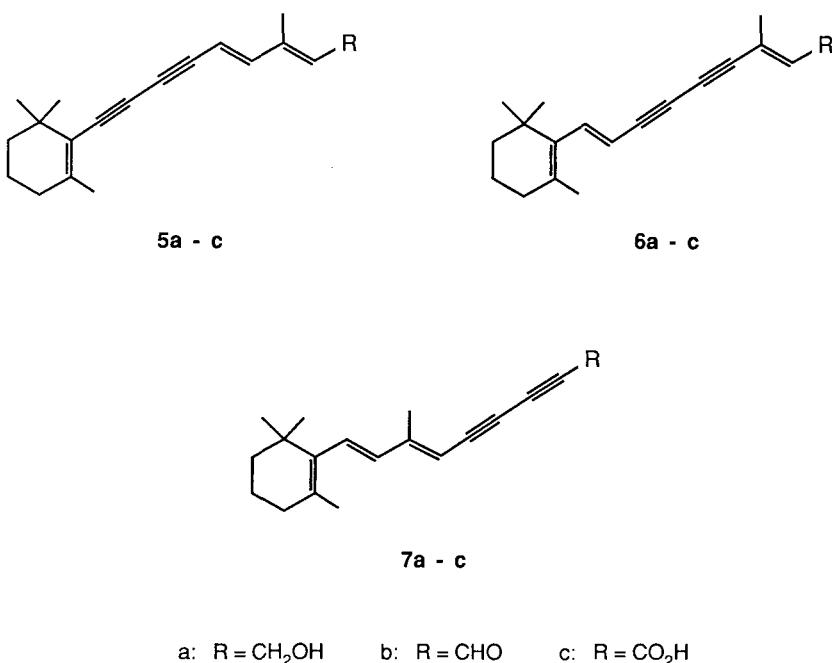
II. ACETYLENIC VITAMIN A ANALOGS — STRUCTURES AND KNOWN SYNTHESES

In a completed C₂₀-skeleton there are four ways of incorporating a triple bond into the retinoid, viz. **1** to **4**:



These molecules are of interest from structural as well as synthetic viewpoints. Structurally, the introduction of two sp-hybridized carbon atoms leads to a “linearization” of the polyene chain, while maintaining its conjugated nature. Considerable deviations from the geometry of the native derivatives are thus to be expected. Furthermore, no *cis/trans*-isomerization can take place at a triple bond. Since geometric isomerizations are of utmost importance in biological processes involving vitamin A and its derivatives,⁶ it is obvious from the very beginning that **1** to **4** must differ in their biochemical behavior from their all-olefinic counterparts.

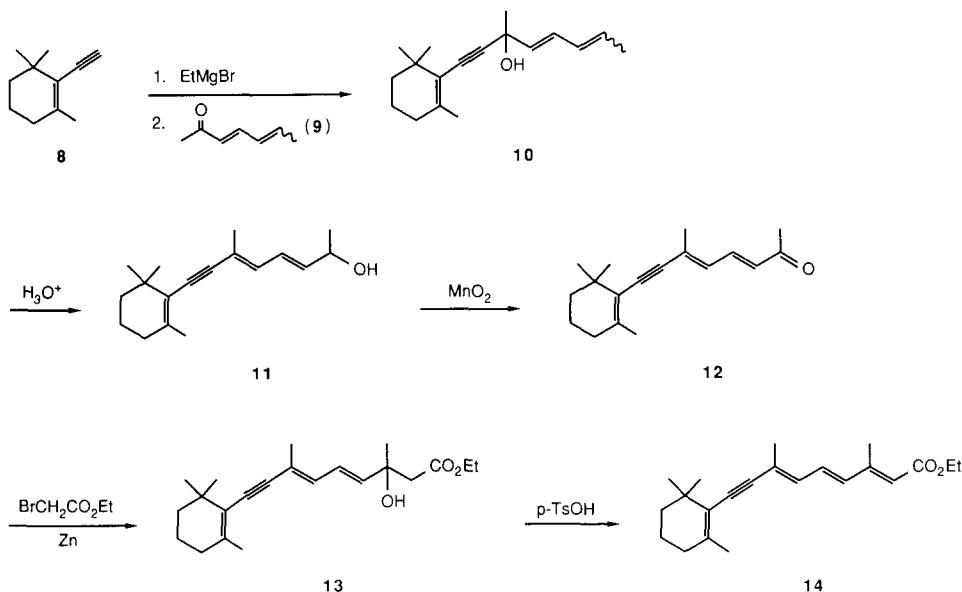
Chemically, the question arises whether **1** to **4** can be made to undergo chemo- and stereoselective reactions at the triple bond. Or will, for example, addition reactions to the double bonds also present in these molecules compete favorably with the desired attack at the acetylenic function? If **1** to **4** carry polarizing substituents like the formyl (**b**) or the carboxy group (**c**), one would predict that their influence will decrease with increasing distance between triple bond and the R group. A further linearization of the side chain may be caused by the incorporation of additional acetylenic units. We were particularly interested in preparing conjugated dialkynes like **5**, **6**, or **7** since the coupling of two terminal alkynes — either directly or *via* appropriate intermediates — is a very well established method in acetylene chemistry:⁷



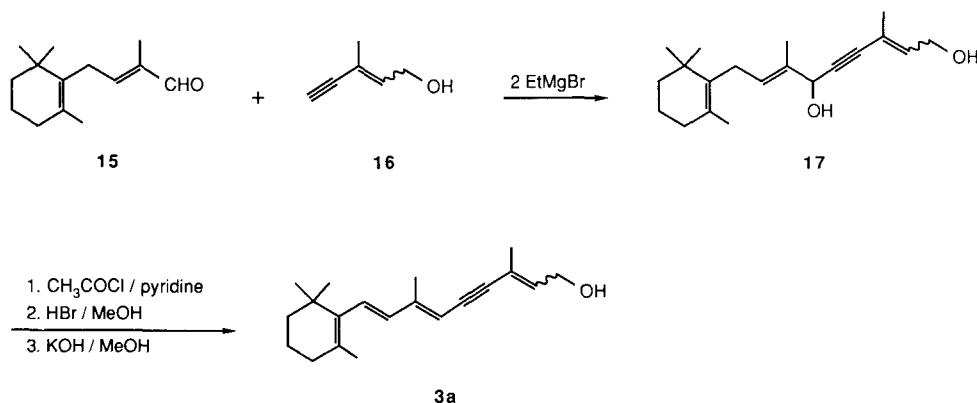
Reactions like the Eglinton, Glaser, Cadot-Chodkiewicz coupling^{7,8} have to our knowledge not been applied previously to vitamin A chemistry. For the case of 7 with its diyne unit as close as possible to the end of the molecule one might expect decreased stability since it is known that conjugated diynals lose carbon monoxide at room temperature.

Of the seven structures shown in the above formulae three — 1, 3, and 4 — were known at the beginning of our investigations in this area. Since their syntheses demonstrate several important principles of acetylenic vitamin A chemistry, they are briefly discussed here.⁹

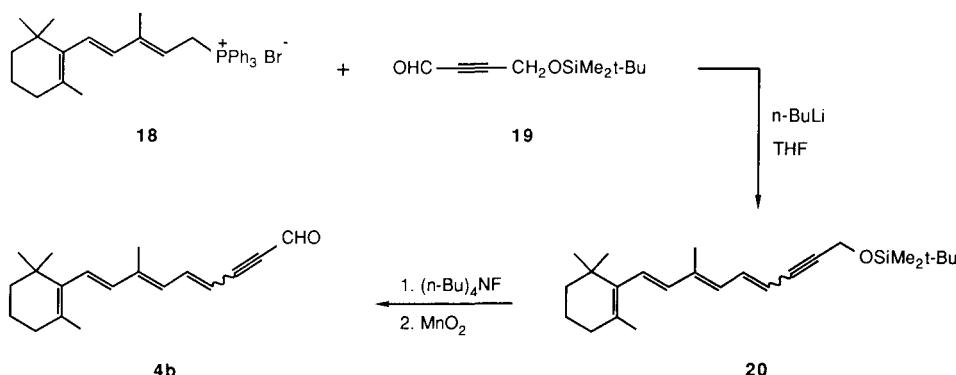
In the Attenburrow synthesis of **1a**¹⁰ as well as the ethyl ester of **1c** (**14**) the synthetic sequence begins with ethynylcyclohexene **8**. These enynes were originally introduced by Heilbron and co-workers¹¹ and are — as will be shown in Section V — versatile educts in vitamin A chemistry until the present day. The Grignard derivative of **8** is allowed to react with crotonylideneacetone (**9**) to provide the tertiary alcohol **10**. Upon acid treatment, **10** isomerizes in a doubly allylic rearrangement — another classical process in vitamin A chemistry^{1,2} — to the conjugated secondary alcohol **11**. Oxidation with manganese dioxide and chain elongation of the resulting ketone **12** by a Reformatsky reaction provides **13**, which is finally dehydrated to **14** with *p*-toluenesulfonic acid; lithium aluminum hydride reduction of **14** leads to **1a**:



The preparation of the acetate of **1a** has also been reported,¹² as has the synthesis of all-*trans*-7,8-didehydroretinoic acid (**1c**),^{13,14} To synthesize the 13-*cis*- and the all-*trans*-isomers of **3a** Oroshnik¹⁵ and later Isler¹⁶ used a C₁₄ + C₆-approach. The C₁₄-aldehyde **15** was first condensed with the bisanion of the acetylenic alcohol **16**; the resulting isomeric diols **17** were monoacetylated and dehydrated to provide the isomeric dehydroretinols **3a**. Geometric isomers of **3b** have also been prepared;¹⁷ a novel route to these aldehydes will be presented in Section III.



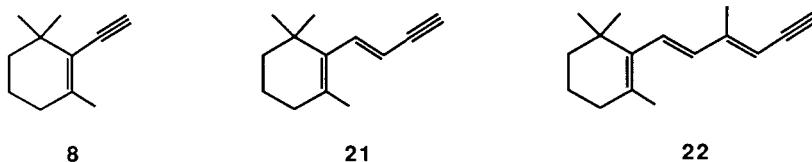
The third known monoacetylenic retinoid mentioned in the introduction, the aldehyde **4b**, has been synthesized by Nakanishi and his co-workers.¹⁸ The carbon skeleton of this isomer is obtained by a Wittig reaction between the C₁₅-salt **18** and the silylated aldehyde **19**, demonstrating another of the fundamental carbon-carbon bond forming processes of vitamin A chemistry.



To obtain **4b** the ether **20** was first deprotected by fluoride treatment and the resulting propargylic alcohol **4a** finally oxidized with manganese dioxide. Derivative **4b** served as the starting material for 20-nor-13-bromoretinal as will be discussed later (see Section IV).

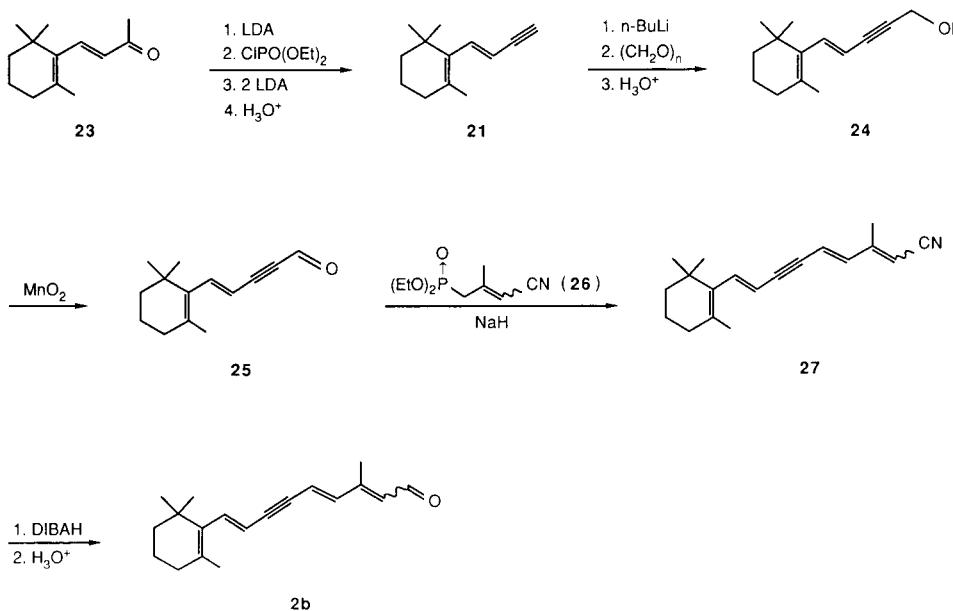
III. PREPARATION OF 19-NOR-9,10-DIDEHYDRORETINAL (2b), 19-NOR-7,8,9,10-TETRADEHYDRORETINAL (5b), AND 19-NOR-9,10,11,12-TETRADEHYDRORETINAL (6b)

It has already been pointed out that 2-ethynyl-1,3,3-trimethylcyclohexene (**8**) is a useful starting material for the synthesis of retinoids.^{10,11} The same should be true for its vinylogs **21** and **22**.



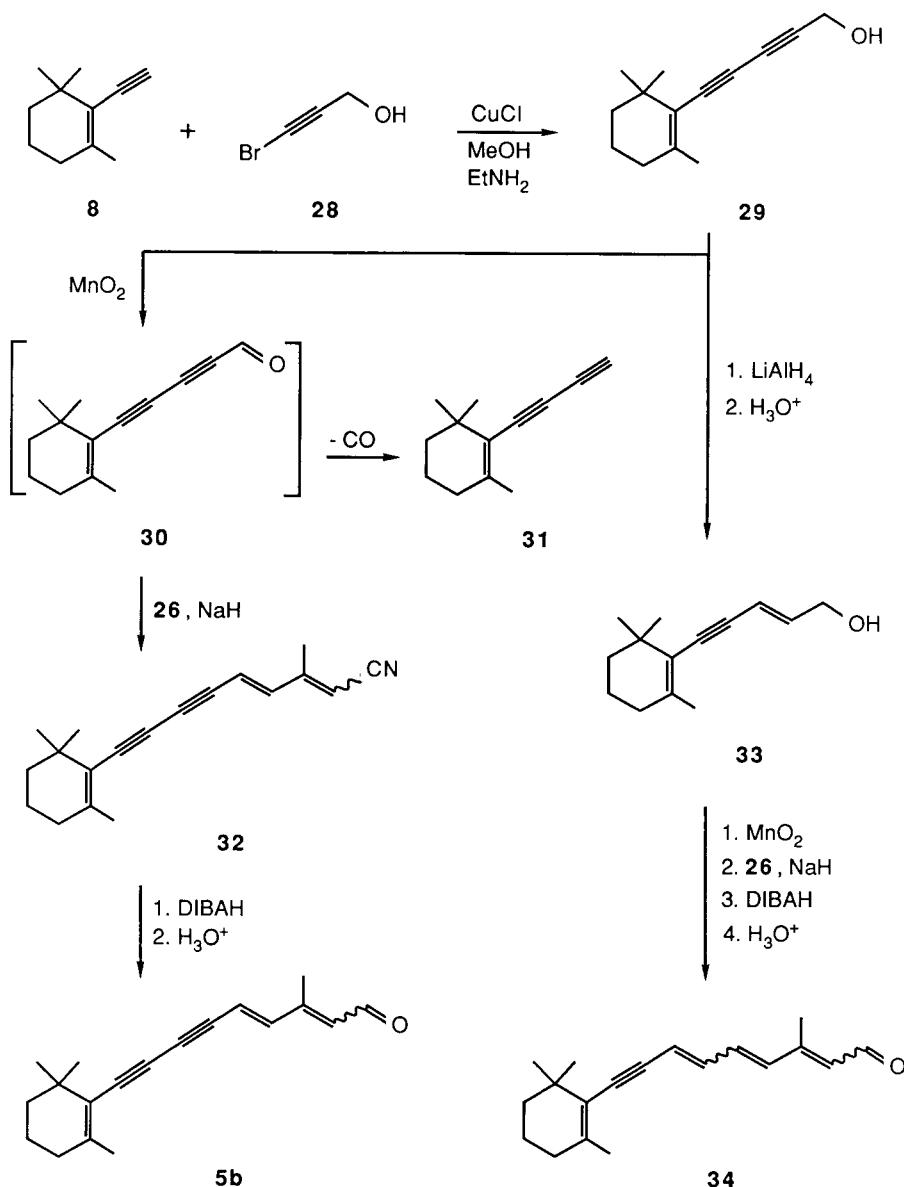
In fact, these three terminal alkynes were used to prepare the “missing” retinoids **2b**, **5b**, and **6b**.

The monoacetylenic aldehyde **2b** was obtained from **21**, which, in turn, can be prepared readily in gram quantities by Negishi’s dehydration method from β -ionone (**23**).¹⁹ This two-step process involves the interception of a terminal enolate by diethyl chlorophosphosphate and base-induced elimination of the enol phosphate intermediate thus obtained, the latter normally not being isolated.



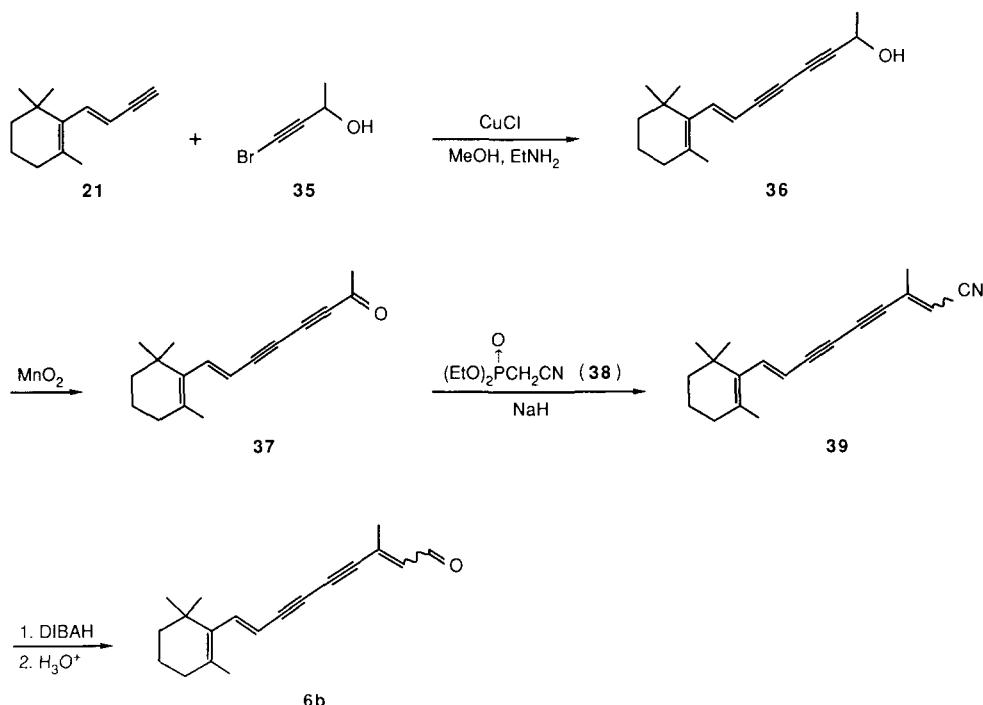
The steps connecting **21** with the target molecule **2b** are standard procedures in vitamin A chemistry and require no further explanation.²⁰ The overall yield of the synthesis, beginning with **21**, was 12.7%, and the product mixture consisted — as was shown by HPLC and high-field NMR spectroscopy — of 65% 13-cis- and 35% all-trans-**2b**.

For the preparation of the diacetylenic retinal **5b** the alkyne **8** was coupled with 3-bromo-propyn-1-ol (**28**) by a Cadiot-Chodkiewicz coupling reaction.²¹ The diynol **29**, obtained in 38% yield, was not only converted to the desired retinoid **5b** but served also as the educt for the preparation of the 9-desmethyl derivative of **1b** (**34**).



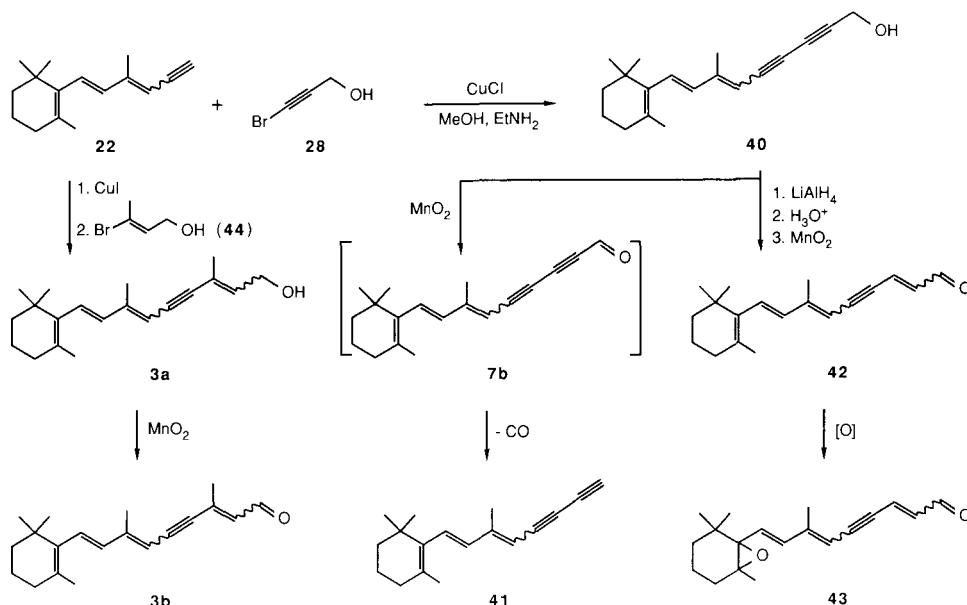
As shown in the scheme, oxidation with manganese dioxide converted **29** to the aldehyde **30**. On standing, this highly unstable compound decarbonylated to the diyne **31**. However, freshly prepared samples were chain-elongated with **26** by the Wittig-Horner reaction; the resulting nitrile **32** was readily reduced to **5b** (77% all-*trans* and 22% 13-*cis* configuration). When **29** was reduced with lithium aluminum hydride the *trans*-allylic alcohol **33** was isolated in excellent yield (81%). Its further transformation to **34** (25% 13-*cis*-, 11% 9,13-di-*cis*-, 22% 9-*cis*-, and 42% all-*trans*-isomer) posed no difficulties.

Essentially the same methodology was employed for the preparation of the diynal **6b**.²⁰



The 51% yield of **36** in the coupling step was again satisfactory. After oxidation, the resulting methylketone **37** was chain-extended with **38**, and the nitrile **39** was reduced to **6b** with diisobutylaluminum hydride. The total yield from **21** to **6b** amounted to 8.3%, with the target molecule being isolated as a mixture of the all-*trans* (84%) and the 13-*cis* isomer (16%).

After these successful syntheses, it came as a surprise that the last member of the diyne series, **7**, could not be prepared by this approach. Although coupling of the known^{17,22} trienyl **22** with **28** took place readily, the resulting alcohol **40** (45% yield) could not be oxidized to **7b**. Rather, hydrocarbon **41** was isolated, an obvious decarbonylation product of the desired **7b**. All attempts to prevent this process failed. On the other hand, selective reduction of one of the triple bonds of **40** could be accomplished with lithium aluminum hydride. The alcohol, formed in 59% yield, may be reoxidized to the 13-desmethyl derivative of **3b** (aldehyde **42**) (83%; 2% 13-*cis*-, 2% 9,13-di-*cis*-, 46% 9-*cis*-, and 34% all-*trans*-**42** as shown by HPLC analysis). On standing **42** was slowly converted to the epoxide **43** by air oxidation.

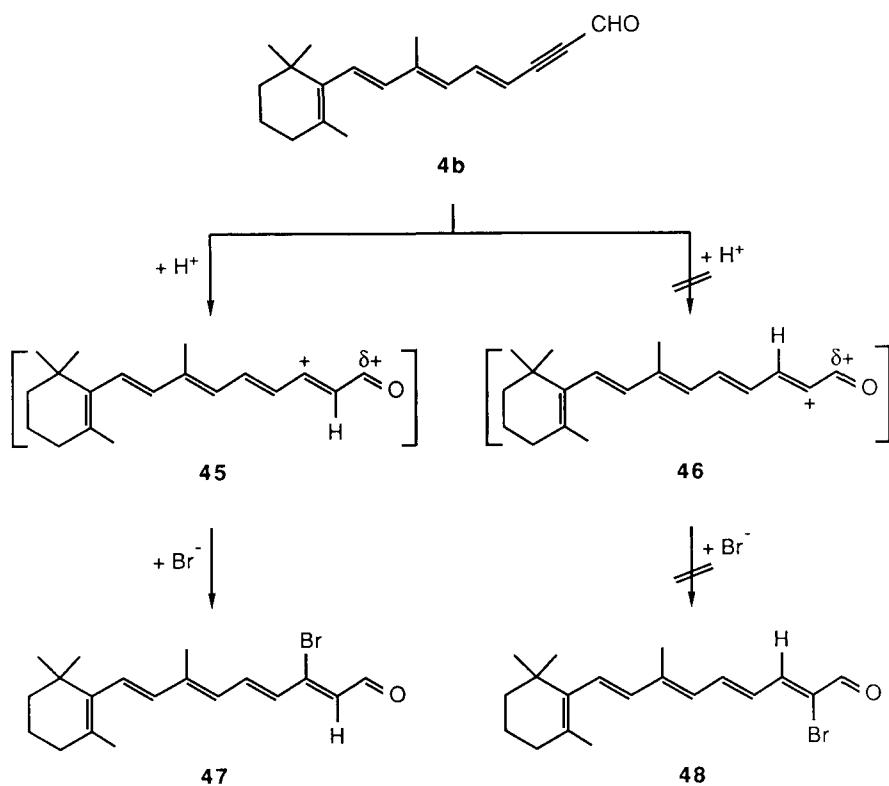


In our opinion the formation of carbon-carbon bonds by coupling reactions like the Cadiot-Chodkiewicz process deserves further attention in retinoid synthesis, especially since this reaction is not restricted to 1-bromoalkynes but may be extended to vinyl halides as well. A case in point is furnished by the coupling of **22** with *E*-3-bromo-2-buten-1-ol (**44**). The acetylenic alcohol **3a**¹⁷ was obtained effortlessly (49%) as was the aldehyde **3b** (50%).²¹

IV. ADDITION REACTIONS TO ACETYLENIC RETINOIDES

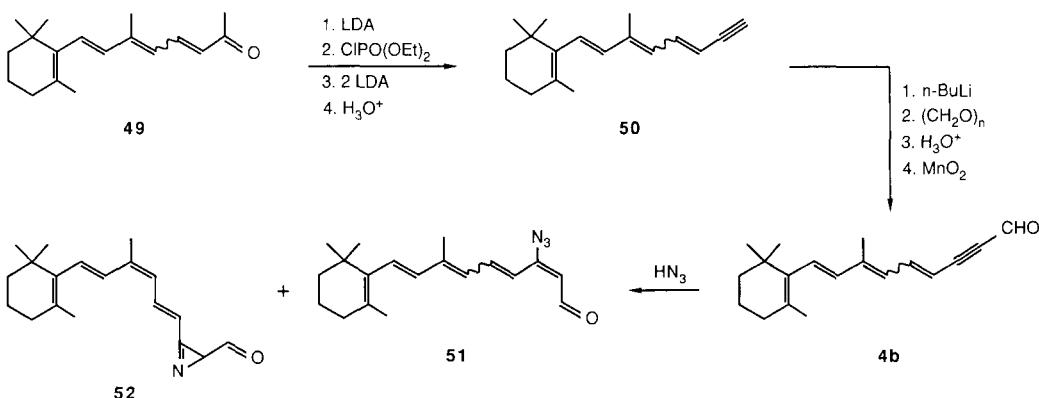
As pointed out above, in the traditional approach comparatively small alkynes were used as building blocks for the construction of the polyene chain of vitamin A and its derivatives. Having served its function, the triple bond is removed by hydrogenation. However, other atoms or groups than hydrogen may be added to an alkyne,⁴ and, therefore, the question arises whether the introduction of a specific functional group may not be “saved” until the very last step, that is, until the whole carbon framework containing at least one triple bond has been completed. This approach appears to be particularly attractive for the incorporation of highly reactive substituents since it is unlikely that these would survive the preparative manipulations during the stepwise construction of the polyene chain.

One of the first applications of this strategy was provided by Nakanishi’s synthesis of 13-bromoretinal (**47**).¹⁸ Retinoids carrying groups other than methyl in the 13-position are of particular importance in mechanistic studies aimed at unraveling the molecular details of energy-converting processes like the vision process in vertebrates²³ or the mechanism of the light-driven proton pump in the purple membrane of halobacteria.^{18,24} When Nakanishi treated the monoacetylenic aldehyde **4b** with hydrobromic acid in benzene at room temperature the extremely unstable 13-bromo retinoid **47** was obtained in 40% yield. Extensive chromatography was necessary to furnish the shown all-*trans* isomer.



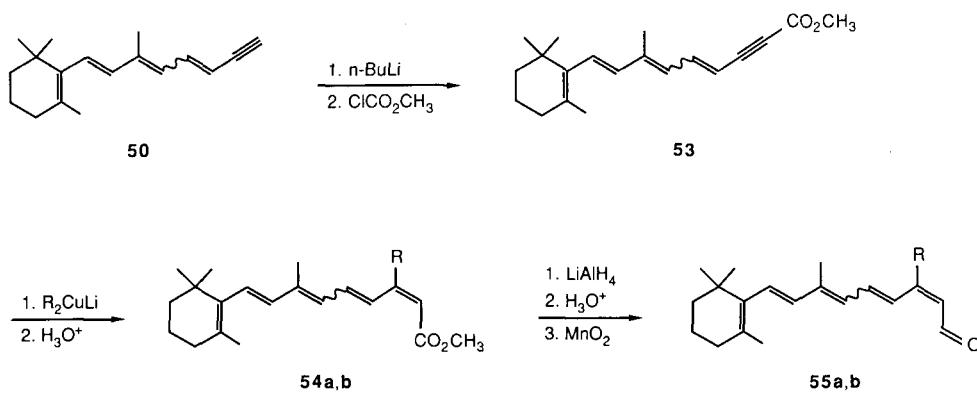
The observed regioselectivity may be rationalized by assuming an ionic addition mechanism. Initial attack of a proton at the triple bond could lead to either of two vinyl cations, **45** and **46**. The former should be the more stable one since the positive charge of the cation and the partial positive charge at the carbonyl group are further apart than in the alternative cation **46**. Cation **45** would hence form more rapidly and its combination with bromide ion would lead to the observed regiosomer **47**.

Another polar reagent that has been added to a “complete” acetylenic retinoid is hydrazoic acid. Instead of preparing **4b** by the Nakanishi route and in view of the successful application of terminal alkynes (**8**, **21**, **22**) in retinoid synthesis (*vide supra*), we decided to use the tetraenye **50** as the starting material. This hydrocarbon is readily obtained from the known ketone **49**²⁵ by the Negishi method^{19,26} (42% yield).



Aldehyde **4b** was prepared from **50** by routine steps, and the addition of hydrazoic acid in chloroform led to a mixture of the expected azide **51** and the azirine **52** (overall yield from **50** was 8%, consisting of 8% **52**, 48% 13-cis-, 44% 9,13-di-cis-**51**). The various adducts could be separated by HPLC and characterized spectroscopically.²⁷ When the chloroform solution of hydrazoic acid was replaced by an aqueous sodium azide solution no **52** was formed, whereas **51** was produced in 7% yield (again from **50**).²⁷ Azides like **51** are of interest as substrates for photoaffinity-labeling experiments.^{28,29} The formation of **52**, an addition/isomerization product of the nitrene generated from the azide by nitrogen loss, indicates that the intramolecular reaction with the polyene chain may well compete with the intended (intermolecular) nitrene insertion into neighboring functional groups.

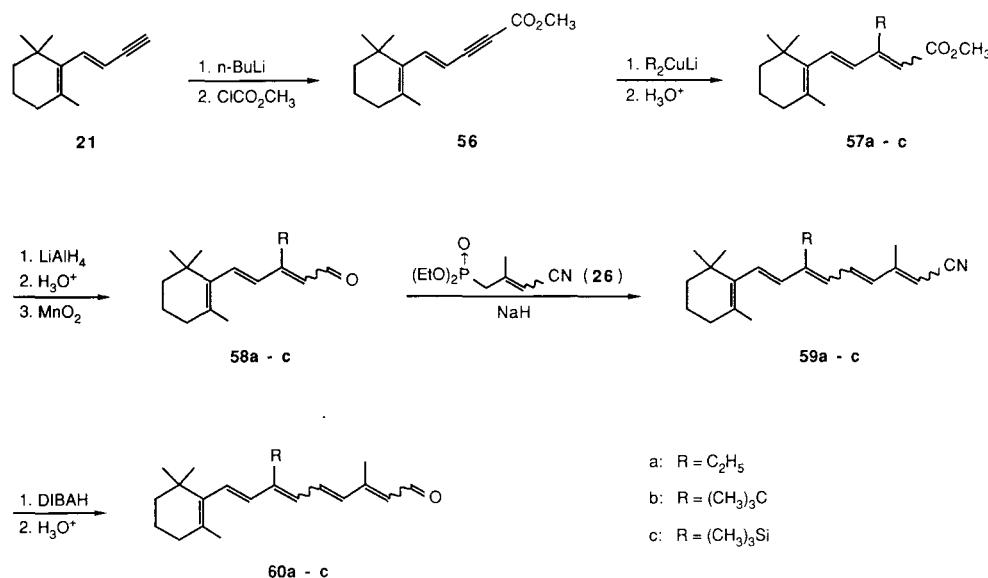
Although 20-nor-13-methoxyretinal was prepared by a building-block strategy (see Section V), we believe that the above approach may be applied to the synthesis of many other retinoids carrying electron-donating or -attracting substituents at the 13-position. To exert steric control at this end of the molecule methods are required for the introduction of large, chemically more or less inert substituents, i.e., bulky alkyl groups. As it turns out, the addition of cuprates to the acetylenic ester **53**, prepared from **50** as shown, is the method of choice.²⁶



a: R = C₂H₅ b: R = (CH₃)₃C

The reaction of **53** with excess lithium diethylcuprate at -78°C provided the ethyl group-carrying retinoate **54a** in 89% yield. Reduction/reoxidation subsequently led to **55a**, isolated in 49% yield as a mixture of the 13-*cis*- (55%) and 9,13-di-*cis*-isomers (45%). The addition of the cuprate to **53** thus proceeded stereoselectively *cis* under these conditions. A mixture of the various isomers of **55a** has recently also been obtained by Bestmann and co-workers, who used a step-wise approach and the Wittig reaction for connecting the building blocks.³⁰ Although successful for the preparation of 20-nor-13-propylretinal as well,³⁰ this route failed when applied to the synthesis of 20-nor-13-*tert*-butylretinal (**55b**).³¹ On the other hand, no difficulties were encountered preparing this sterically overcrowded system by the cuprate addition process.²⁶ When excess lithium di-(*tert*-butyl)cuprate was added to **53** at -78°C the ester **54b** was obtained in 77% yield after 2 h. The conversion to **55b** was carried out in the usual fashion (59%) leading to a mixture of 13-*cis*- and 9,13-di-*cis*-**55b** (product ratio 1:2). Surprisingly, lithium bis(trimethylsilyl)cuprate did not react with **53**; even after several hours at 0°C , the substrate remained unchanged. This observation is astonishing in view of the observation that the introduction of this particular substituent into the 9-position could be accomplished readily.

As shown in the following scheme 19-nor-9-ethylretinal (**60a**), 19-nor-9-*tert*-butylretinal (**60b**), and 19-nor-9-(trimethylsilyl)retinal (**60c**) could all be obtained from one common precursor, the ester **56**, by adding the appropriate homocuprates and extending the polyene chain by the usual steps:²⁶



The yields of the various steps are in all cases satisfactory, falling into the 50 to 80% range. Thus, the different isomers of **60a-c** were isolated in amounts sufficient for further studies.³²

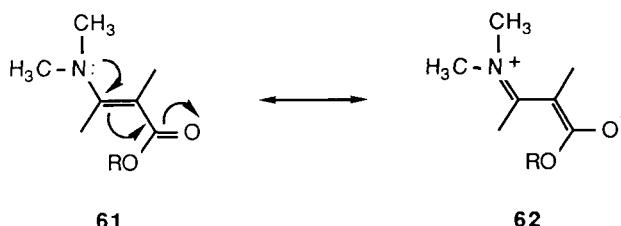
It should be pointed out that the stereochemistry of the crucial addition process can be influenced by both solvent and temperature effects. For example, when lithium diethylcuprate was added to **56** in tetrahydrofuran at -78°C and the reaction mixture was quenched after 1 h by the addition of methanol more than 99% of the *cis*-addition product was formed. On the other hand, reaction in diethyl ether at 0°C provided an equimolar mixture of the *cis*- and *trans*-adduct after 5-min reaction time, the isolated yield being in excess of 80% in both cases.²⁶

To summarize this section, we think that addition reactions to acetylenic retinoids constitute a considerable extension of the methods available for the modification of the polyene chain. Both inert and reactive substituents may be introduced by this approach under mild reaction conditions. The “delay” of the addition step to a late stage of the synthesis offers the advantage of employing common intermediates for a vast variety of derivatives. In a step-wise approach every single substituent would require its own building block. The “naked” triple bond function thus serves as a generally usable functional group.

V. NOVEL ACETYLENIC BUILDING BLOCKS FOR RETINOID SYNTHESIS

Although the importance of the complete carbon-skeleton approach has been stressed in the previous sections, the building-block strategy has its obvious merits, and there is no reason to abandon it. It is the intention of the following examples — all taken from our recent work — to show that even after so many decades of preparative vitamin A chemistry new acetylenic building blocks can still be imagined and successfully applied to synthesis. As with the examples discussed above the aim of these investigations is to provide tailor-made retinoids carrying substituents in clearly defined positions that influence the electronic and steric nature of retinal in a predictable way.

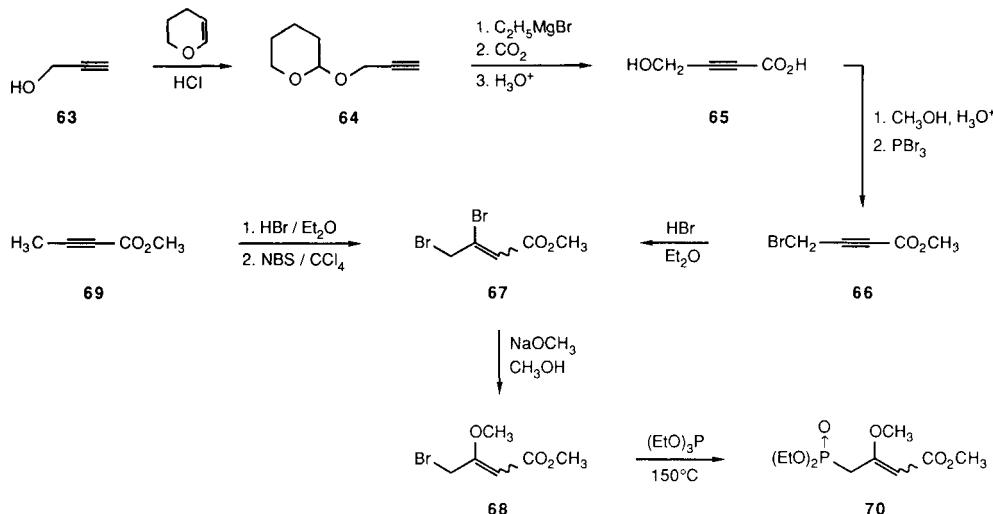
Having prepared 13-trifluoromethylretinal by a step-wise approach — not employing acetylenic intermediates³³ we were interested in obtaining a retinoid carrying an electron-donating substituent in this position (C_{13}) and selected the methoxy group. It is well known from numerous examples that the barrier for *cis/trans* isomerization of alkenes may be dramatically influenced by substituents. Thus the isomerization barrier in ethylene-1,2-d₂ is 65.5 kcal/mol at 450°C,³⁴ and that in 2-butene is 57.9 kcal/mol at the same temperature.³⁵ On the other hand, en amino acid derivatives like **61** — to quote but one example from the vast literature —³⁶ show rotational barriers far below 20 kcal/mol.³⁶



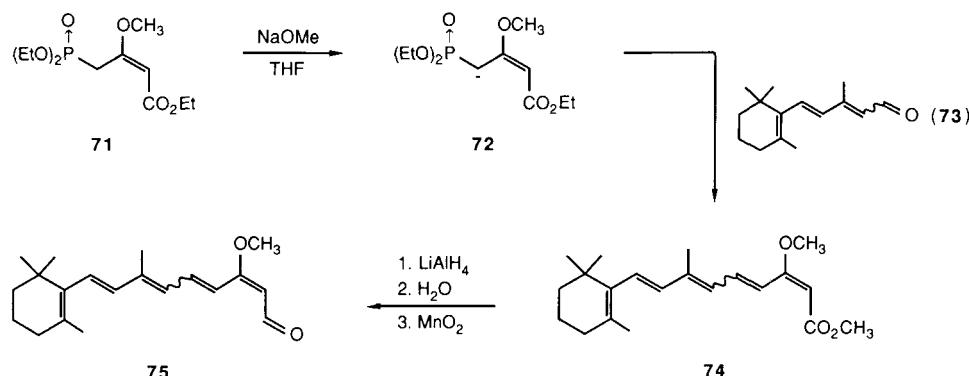
The reason for this very low barrier becomes obvious on inspection of the resonance structure **62**. Because of the polarity of the two substituents the double bond character of the central bond is greatly diminished, approaching that of a single bond in the extreme case. Systems like **61/62** are commonly called “push-pull olefins”, and the question arises whether a retinoid may be constructed possessing push-pull character. Obviously, this will strongly influence any type of isomerization process of the polyene chain, and the properties of a retinoid depending on this isomerization. The synthesis of 13-methoxyretinal (**75**) was designed as a first step towards “push-pull-retinoids.”³⁷

As the starting point for **75**, we first selected 4-hydroxytetrolic acid (**65**), easily available from propargyl alcohol (**63**) via the tetrahydropyranyl ether **64**. When the bromide **66**, prepared from the methyl ester of **65** by phosphorous tribromide treatment, was allowed to react with hydrobromic acid in ether at room temperature the dibromoester **67** (*E/Z*-mixture)

was formed in quantitative yield. The regioselectivity of the addition process may be explained by similar arguments as discussed for the hydrobromination of **4b**. When these isomers were treated with sodium methoxide in methanol, the vinylic bromine substituent was replaced rather than the allylic one, and the methoxy ester **68** was produced (82%).

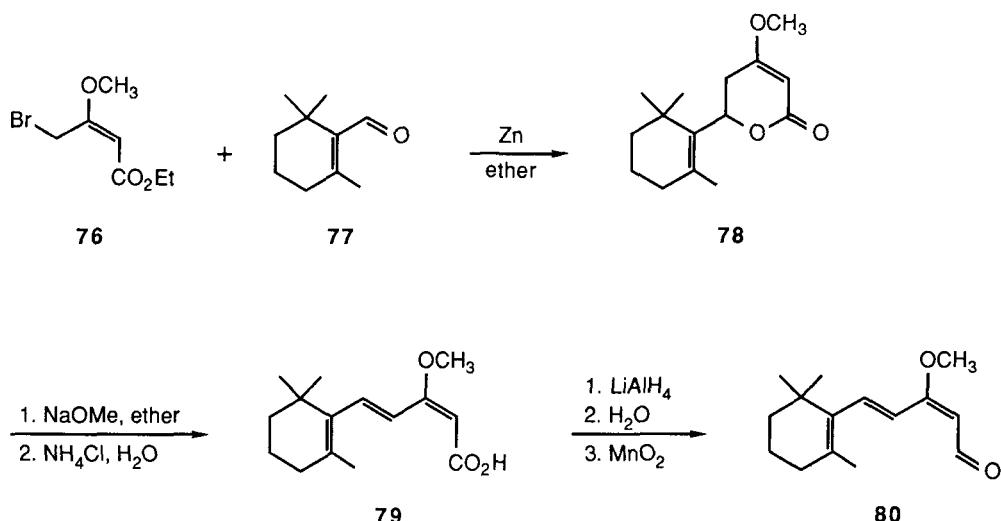


This at first-sight surprising result may be rationalized by postulating a Michael-type attack of methoxide on **67** followed by reformation of the α,β -unsaturated ester system with concomitant bromide expulsion. Later, it was found that **67** could be prepared in a shorter synthesis by first treating the (commercial) methyl tetrolate (**69**) with hydrobromic acid in ether, and then subjecting the formed vinylic bromide to an allylic bromination by *N*-bromosuccinimide (70%).³⁸ When, finally, **68** was submitted to the Arbuzov reaction with triethylphosphite, the phosphonate **70** was produced in 87% yield.³⁹ This very versatile C₄-unit has also been prepared from ethyl acetoacetate^{39,40} as well as methyl 2,3-butadienoate.^{38,39} Wittig-Horner reaction of **72**, the anion of the ethylester **71**, with the long-known C₁₅-aldehyde **73**⁴¹ provided 13-*cis*- and 9-*cis*,13-*cis*-**74**, in 70% yield and a 2:1 isomer ratio, respectively.^{38,39}

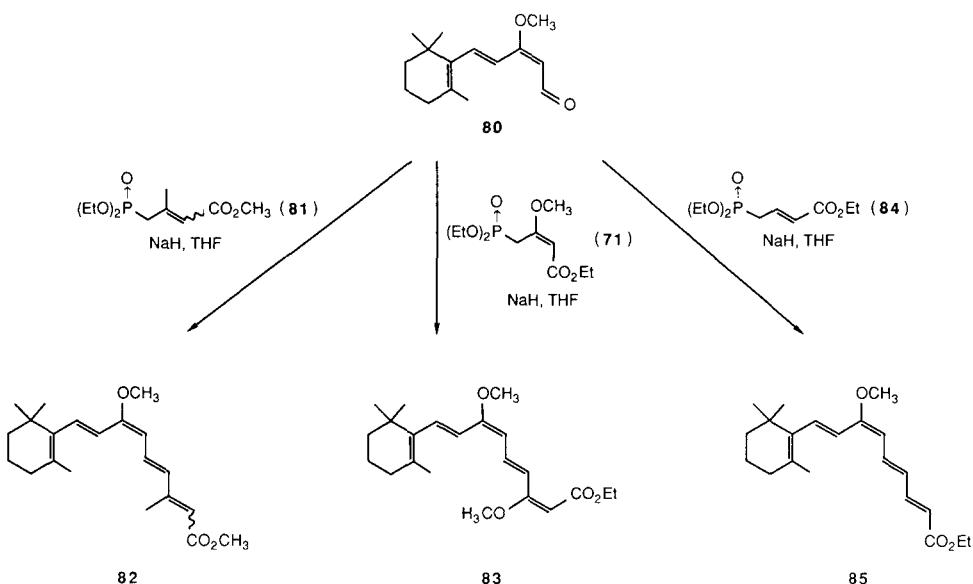


(Note that during the bond forming step transesterification took place.) When **74** was reduced with lithium aluminum hydride in ether, the very sensitive methoxy-retinols resulted; manganese dioxide oxidation converted these into a mixture of the aldehydes **75** (33% yield for the reduction/oxidation steps) from which the 13-*cis* isomer could be isolated in analytically pure form.

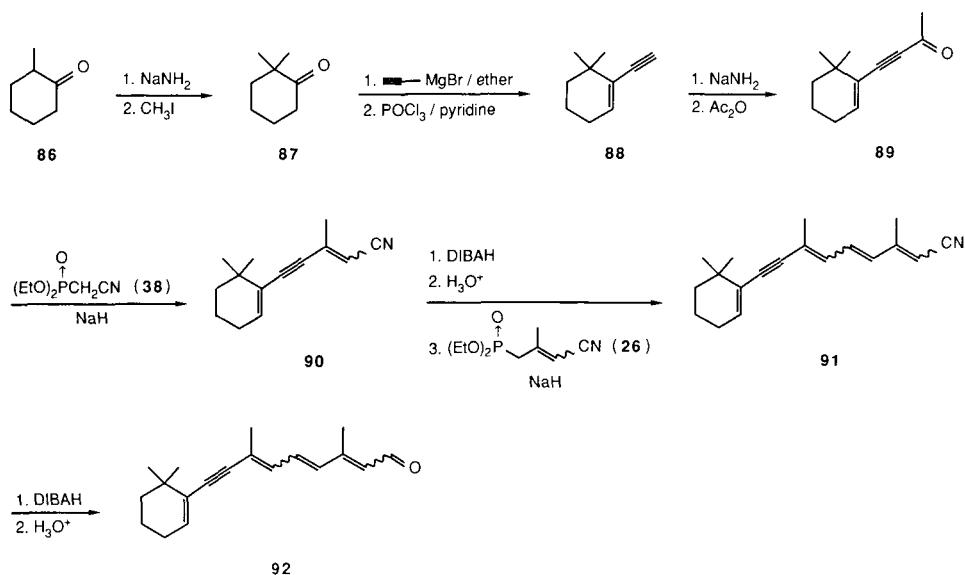
Although somewhat beyond the scope of this review, it should be mentioned that esters like **68** are useful intermediates for the preparation of many other methoxy-substituted retinoids.^{38,42} When, for example, a Reformatsky reaction between the ethyl ester **76** and β-cyclocitral (**77**) was carried out, the lactone **78** was obtained in excellent yield (85%). By base-induced ring opening it was converted to the isomerically pure methoxy acid **79** (84%)



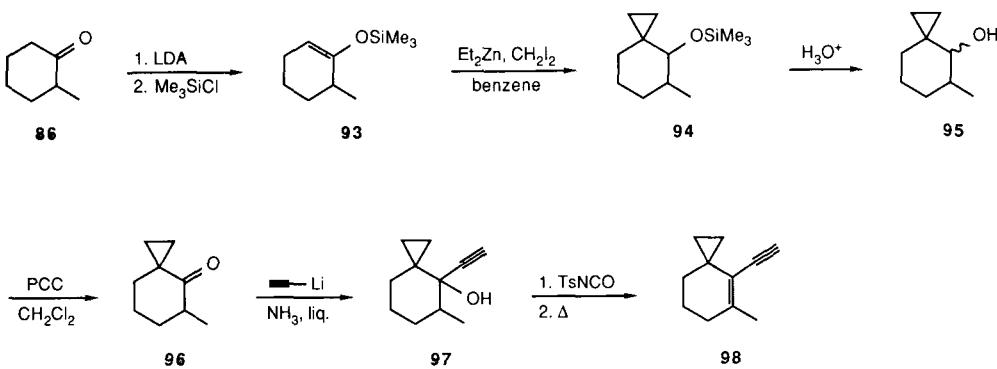
Having converted **79** to the aldehyde **80** by the usual reduction/oxidation sequence, an intermediate was at hand that can be coupled with many other building-blocks as demonstrated by the following selection (yields: **82**: 57%, **83**: 85%, **85**: 50%).⁴²



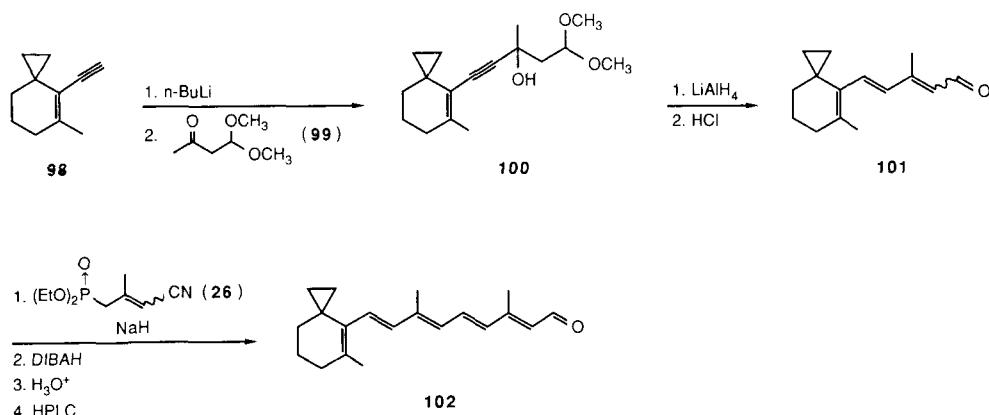
All modifications of the retinal framework discussed so far in this chapter concerned the polyene chain, where certain of its double bonds were replaced by one or two triple bonds, or where the substituents and/or the substitution pattern were changed. The last part of this section will present alterations of the cyclohexene nucleus. And again, we will only discuss examples from our work in which alkynes intermediates were employed during the synthesis. The usefulness of terminal alkynes **8**, **21**, **22**, and **50** for the preparation of retinoids has already been demonstrated several times. Since several acetylenic retinoids of the general type **1b** but with a reduced number of methyl groups at the six-membered ring were required for reconstitution experiments with bovine opsin and bacteriopsin, respectively, these retinoids were prepared according to a modified Attenburrow synthesis (*vide supra*).⁴³ To prepare 18-nor-7,8-dehydroretinal (**92**) the enyne **88** was first synthesized from 2-methylcyclohexanone (**86**) via **87** (overall yield 28.3%).



Three standard chain elongation steps — to the ketone **89** (40%), the nitrile **90** (76%), and finally the C₁₉-nitrile **91** (80%) — furnished the desired carbon skeleton, and diisobutyl aluminum hydride reduction the aldehyde **92** (56%). By an analogous approach 16,17,18-trinor-7,8-dehydroretinal, i.e., a retinoid lacking all three ring methyl groups, was obtained.⁴³ Whereas the preparation of ethynylcyclohexenes like **88** was straightforward, considerably more effort was required to obtain the spiro compound **98**. This acetylenic building-block was selected as the starting point for the spiroretinal **102**, a compound that sterically should not differ significantly from native retinal since the three-membered ring may be regarded as a “short-circuited” *gem*-dimethyl group. Electronically, however, a cyclopropane ring is equivalent to a double bond, and, thus, **102** may formally be considered as a crossconjugated molecule. To put it in other words, while possessing comparable steric requirements, **102** should differ electronically from retinal. To obtain the spiro-enzyme **98**, 2-methylcyclohexanone (**86**) was converted under kinetically controlled conditions⁴⁴ to the trimethylsilyl enol ether **93** (95%). Cyclopropanation according to a procedure developed by Murai⁴⁵ provided the spiro compound **94** (92%) from which the alcohol **95** was liberated by citric acid treatment (94%). Oxidation with pyridinium chlorochromate afforded the ketone **96** (70%), which was ethynylated to the propargyl alcohol **97** (82%, mixture of diastereomers). After many unsuccessful attempts⁴⁶ the dehydration of **97** was accomplished by tosylisocyanate treatment followed by thermal decomposition of the carbamate formed as the initial product (46%).⁴⁷

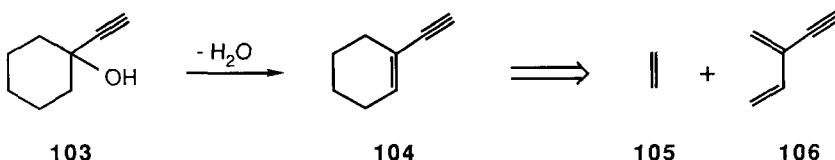


For the chain extension of **98**⁴⁸ various approaches were applied,⁴⁶ the shortest one is shown in the following scheme.

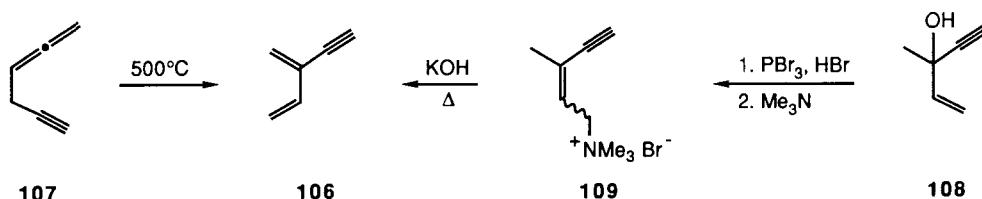


The acetylide derived from **98** by *n*-butyllithium treatment was allowed to react with the C₄-unit **99**⁴⁹ to provide the propargyl alcohol **100** (58%). Reduction with lithium aluminum hydride, dehydration, and deacetalization subsequently lead to the spiro-C₁₅-aldehyde **101** (45%), which was transformed to the desired **102** by routine steps. From the isomer mixture, isolated in 41% yield, the all-*trans*-isomer **102** was separated by HPLC; besides this diastereomer (product contribution 37%) the 13-*cis* (31%), 9-*cis* (20%), and 9,13-di-*cis* isomers (12%) were separated and characterized spectroscopically.^{46,48}

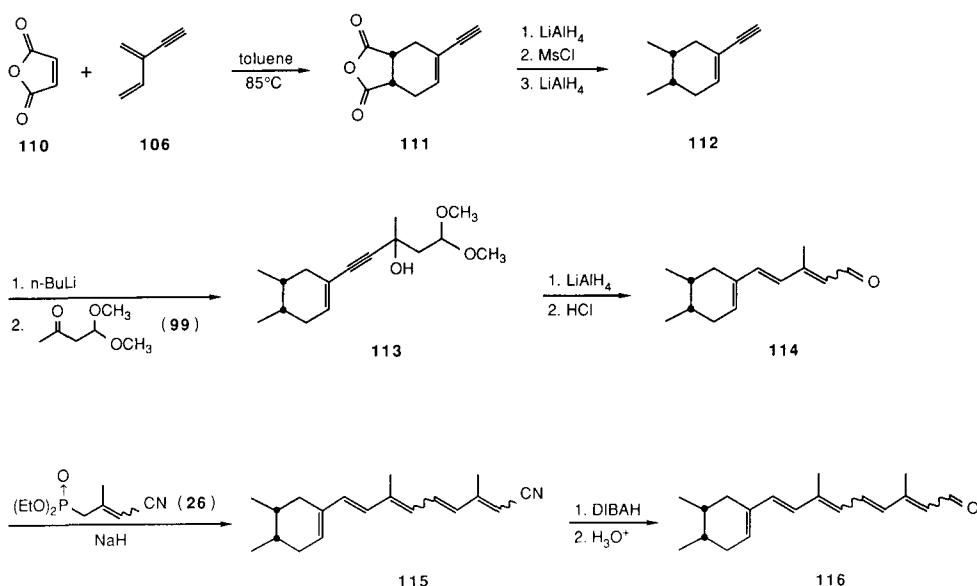
The 1-ethynylcyclohexenes (**104**) involved in all syntheses, were generated by dehydration of the corresponding cyclohexanols **103** or derivatives thereof, that is, from substrates already possessing the complete carbon skeleton of the future building block.



Since the most general and preparatively most useful method for the synthesis of six-membered ring compounds is the Diels-Alder addition reaction,⁵⁰ it occurred to us that this process might also be employed for retinoid synthesis. As shown by the retroreaction, the intended [2 + 4] cycloaddition would require a double bond dienophile (**105**) and a diene carrying a 2-ethynyl substituent, 2-ethynyl-1,3-butadiene (**106**) being the simplest possible addition partner. Having prepared this C₆H₆-hydrocarbon by the high temperature pyrolysis of 1,2-hexadien-5-yne (**107**) more or less accidentally several years ago,⁵¹ a more rational and efficient method of preparation was required if this novel diene should ever be of synthetic use.



In the so far best preparative method providing gram quantities of spectroscopically pure **106**, 3-methyl-1-penten-4-yn-3-ol (**108**), which is one of the classical substrates of vitamin A chemistry, was used as the starting material.⁵² Bromination of **108** was accompanied by allylic rearrangement,⁵³ and trimethylamine treatment converted the conjugated bromide to the Hofmann base **109** (92%).⁵⁴ When the latter was heated in 55% aqueous potassium hydroxide solution at 120°C, hydrocarbon **106** was formed in up to 50% yield.⁵⁴ The new diene system reacted with numerous double and triple bond dienophiles, yielding the expected [2 + 4] cycloadducts in good yields in most cases.⁵⁴ The C₆-unit **106** found its first preparative uses in the synthesis of *cis*-2,3-dimethyl-16,17,18-trinorretinal (**116**), which is a retinoid with a completely new substitution pattern at the cyclohexene ring.



The decisive intermediate, the enyne **112**, was prepared via the Diels-Alder adduct **111** (obtained from **106** and maleic anhydride (**110**) in 78% yield) by reduction of the anhydride ring to two *cis*-oriented methyl groups (30% overall yield for the three steps). From here on, the rest of the synthesis was routine, and followed the chain elongation sequence described above for the preparation of **102**. Both **113** and **114** were isolated in good yields (88 and 74%, respectively). After the last coupling step to the nitrile **115** (42%), reduction furnished the desired aldehyde **116** (68%). According to HPLC analysis **116** was a mixture of six isomers in which the 13-*cis*-, 9-*cis*-, and all-*trans*-diastereomers predominated strongly (relative ratio 1.9:1:5.5; the other isomers were produced in trace amounts).⁵⁴

One amusing aspect of this synthesis was the use of a C₆-unit that traditionally is used for the construction of the polyene chain — the acetylene **108** — as a building block for the cyclohexene nucleus. A more important aspect concerns the stereochemical properties of the new retinoid. It is a molecule with a six-membered ring that is relatively unhindered at one face, and sterically shielded at the other. This is obviously not the case in vitamin A itself. Furthermore, **116** is a chiral molecule. The 1- to 4-positions in vitamin A are prochiral: by appropriate substitution they become centers of chirality. The Diels-Alder addition is cherished for its high degree of stereoselectivity.⁵⁰ Provided that a general method for the preparation of (substituted and derivatized) 2-ethynyl-1,3-butadienes can be found, we see many new applications of this route for the preparation of chiral and possibly optically active vitamin A derivatives. The corresponding investigations are presently under way.⁵⁵

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Chapter 9

SYNTHESIS AND STUDIES OF 12-S-CIS CONFORMATIONALLY LOCKED RETINOIDS

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TABLE OF CONTENTS

I.	Conformational Analysis of the Retinoids.....	202
A.	The Cyclohexene (β -Ionylidene) Ring.....	202
B.	The Polyene Chain	202
1.	Conformationl Studies	202
2.	Absorption Spectra of 11- <i>cis</i> -Retinoids	203
II.	Synthetic Retinoids with Conformational and/or Configurational Restrictions: The 12-s- <i>cis</i> -Locked Retinoids	204
A.	The C ₁₄ -Methyl Retinoids	204
B.	The "Locked" Retinoids.....	204
1.	The 12-s- <i>trans</i> -Locked Retinoids	205
2.	The 12-s- <i>cis</i> -Locked Retinoids	206
C.	The 12-s- <i>cis</i> Conformation in Acyclic Systems.....	206
D.	The Significance of the 12-s- <i>cis</i> Conformation in Biological Actions of Retinoids.....	207
III.	Strategy Directed Towards the Synthesis of Retinoids and 12-s- <i>cis</i> -Locked Retinoids: The Thermal Rearrangement of a Vinylallene	207
A.	The [1,j]-Sigmatropic Hydrogen Shift	207
1.	Concept, Scope, and Limitations	207
2.	Selected Synthetic Applications.....	209
3.	Synthetic Variations in the Retinoid Skeleton.....	210
B.	The Elaboration of the Vinylallene Functionality.....	210
1.	The Vinylcuprate Approach.....	210
2.	The Alkylcuprate Approach.....	213
3.	The Wittig Condensation of an Allene Aldehyde	214
C.	Thermal Rearrangement of Vinylallen.....	214
1.	Primary Thermal Pathways	214
2.	Secondary Thermal Pathways	216
IV.	The 12-s- <i>cis</i> Conformationally Locked Retinals.....	217
A.	Primary Retinol-Derived Aldehydes	217
B.	Thermal and Photochemical Interconversions.....	217
C.	Spectroscopic Properties of 12-s- <i>cis</i> -Locked Retinals.....	219
1.	Nuclear Magnetic Resonance	219
2.	UV Absorption	220
Addendum.....		223
Acknowledgments		223
References.....		224

I. CONFORMATIONAL ANALYSIS OF THE RETINOIDS

Soon after the pioneering investigations by Wald, Hubbard, and colleagues concerning the direct role played by 11-*cis*-retinal (**1**) in the functioning of the visual pigments,¹ research focussed on the special features of the retinal structure that might be relevant to its function as the chromophore of the vertebrate visual pigments.

Two aspects of the conformation of the retinal isomers deserve special attention: the orientation of the cyclohexene ring relative to the polyene chain and the conformation adopted by the chain itself in order to alleviate the steric hindrance unique to the 11-*cis* isomer. The steric hindrance is usually relieved by rotation(s) around one or more single bonds along the polyene chain, while maintaining the maximum level of conjugation, which gives rise to conformational isomers, denoted as *s-cis* (dihedral angle ranging from 0° to 90°) and *s-trans* (from 90° to 180°). For example, Figure 1 depicts the 12-*s-trans* and 12-*s-cis* conformers of 11-*cis*-retinal (**1**) as well as those of the 14-methyl analog (**2**) (*vide infra*).

A. THE CYCLOHEXENE (β -IONYLIDENE) RING

With regard to the β -ionylidene ring, it has been predicted by semi-empirical calculations²⁻⁵ that **1** adopts a nonplanar 6-*s-cis* conformation (Figure 1) relative to the polyene chain (50° for all-*trans*- and 44° for 11-*cis*-retinal). The angle of twisting was also estimated from a parallel ¹H NMR study⁵ to be approximately 30° in 11-*cis*-retinal (**1**). Both values were consistent with its theoretical potential function^{2,5} and with the X-ray data of a number of closely related compounds available at the time.⁶

The crystal structure of the visual chromophores⁷⁻⁸ established definitively a skewed structure with a torsional angle of 59° in all-*trans*-retinal and 40° in 11-*cis*-retinal (**1**) from a planar 6-*s-cis* arrangement. However, the existence of conformational polymorphism in the solid state exhibited by retinal derivatives⁸ allowed the study of both 6-*s-cis* and 6-*s-trans* conformers (e.g., all-*trans*-retinoic acid) by solid state ¹³C NMR (MAS, magic angle sample spinning).^{9,10} A comparison of the values of the carbon resonances in retinal derivatives in solution with those exhibited in the solid state by both 6-*s-cis* and 6-*s-trans* conformers (specially the downfield shift of C₅ and the upfield shift of C₈ in the 6-*s-trans* conformer with respect to the 6-*s-cis* form) suggested an equilibrium population of 6-*s-trans* and 6-*s-cis* conformations in solution.⁹ The equilibrium value deduced from this data (28 ± 5% of 6-*s-trans* in solution at 25°C)⁹ contrasted with the conclusion stated above⁵ based on long-range coupling constants and nuclear Overhauser enhancement (NOE) studies, which ruled out a significant population of 6-*s-trans* in solution. Moreover, the same technique, when applied to bacteriorhodopsin, the purple pigment of *Halobacterium halobium*, established a perturbed 6-*s-trans* chromophore (all-*trans*-retinal) in the protein,¹⁰ in contrast to the 6-*s-cis* conformation energetically favored for retinals in solution. This finding has been corroborated by parallel studies of retinal analogues with locked 6-7 conformations.¹¹

B. THE POLYENE CHAIN

1. Conformational Studies

The existence of considerable steric hindrance in the side chain as a consequence of steric interaction between the C₁₃-methyl and H₁₀ is unique to the 11-*cis* isomers. The molecule must, therefore, twist about either the C₁₀–C₁₁ or C₁₂–C₁₃ bond. Earlier theoretical studies^{2,4} found low energy conformations with large torsions (40 to 50°) about the C₁₀–C₁₁ single bond, with the C₁₂–C₁₃ bond close to planar. However, subsequent molecular orbital calculations^{12,13} and ¹H NMR spectroscopy studies^{13,14} (including measurements of long-range nuclear spin-coupling constants, chemical shifts, spin-lattice relaxation times, and nuclear Overhauser enhancements¹³ established a slight twisting from planarity around the C₁₀–C₁₁ bond and a primary model of twisting of the polyene chain around the C₁₂–C₁₃

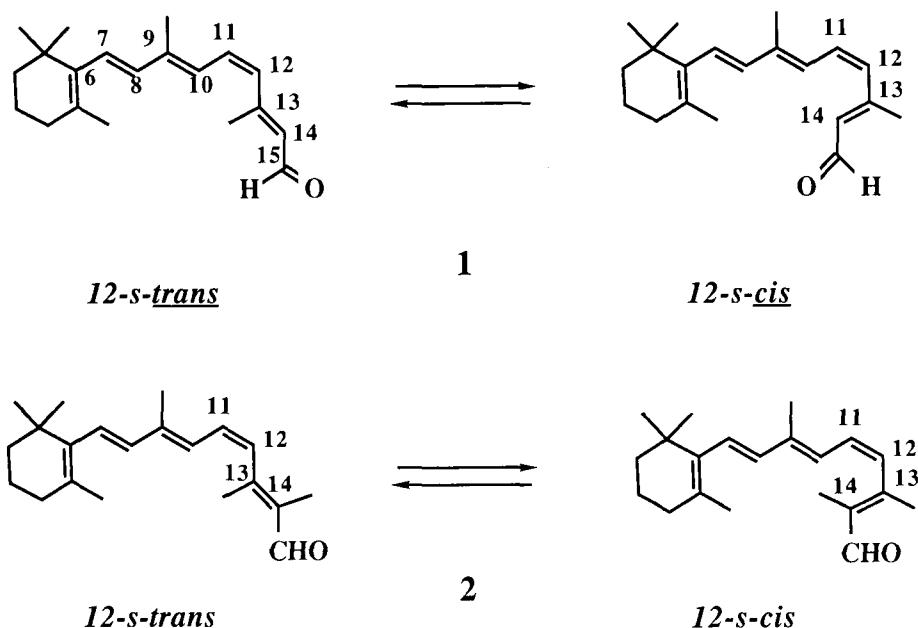


FIGURE 1. The 12-*s-trans* and 12-*s-cis* conformers of 11-*cis*-retinal (**1**) and 11-*cis*-14-methylretinal (**2**). A more complete conformational description of, for example, 12-*s-trans*-**1** should be 6-*s-cis*,8-*s-trans*,10-*s-trans*,12-*s-trans*,14-*s-trans*.

bond. This bond was found to be highly flexible in such a way that 11-*cis*-retinal (**1**) exists in solution as an equilibrium between two low energy conformers very similar in energy (within 1 kcal/mol), distorted *s-trans* **1** and distorted *s-cis* **1**, about the C₁₂–C₁₃ single bond. Earlier resonance Raman studies also led to the suggestion that both conformations exist in solution.^{15,16}

By contrast, the X-ray study⁷ of 11-*cis*-retinal (**1**) established a single conformation in the crystalline state, 12-*s-cis*, with a 39° twist from a planar *s-cis* conformation. However, it should be emphasized that preferential crystallization of a substance in a single conformation, even a less stable isomer, cannot be considered surprising. Moreover, caution must be exercised in deducing the topography of a protein-bound biological substrate from either solution or solid state structural information concerning the free substrate.

2. Absorption Spectra of 11-*cis*-Retinoids

One of the most surprising properties of 11-*cis*-retinoids found by Wald, Hubbard, and co-workers was the anomaly in their absorption spectrum.⁶ The absorption maxima of 11-*cis* isomers of retinal, retinol, and retinaldehyde oxime are shifted only slightly towards shorter wavelengths (e.g., 381 nm for all-*trans*-retinal and 376 nm in ethanol for 11-*cis*-retinal) from those of the all-*trans* isomer and also do not differ appreciably from the sterically unhindered 9-*cis* and 13-*cis* isomers. By contrast, the molar absorptivity of the various absorptions for 11-*cis*-retinal (**1**) are lower than those for the unhindered isomers, but increase to the level of the unhindered mono-*cis* forms upon cooling to –190°C.¹⁷ This effect was initially attributed to the relief of the intramolecular crowding responsible for the steric hindrance.⁶

A convincing explanation of this anomalous absorption spectrum was put forward by Honig and Karplus¹² who suggested that the expected shift to shorter wavelengths due to twisting of the π-electron system (which raises the energy of the π* orbital relative to that of the π orbital) would cancel the shift to longer wavelengths due to the 12-*s-cis* conformation

(the "red shift" effect, well known in polyene chemistry¹⁸), resulting in an absorption maximum in the vicinity of the unhindered isomers.

To test this proposal, Nakanishi, Ebrey, and co-workers^{19,20} synthesized the sterically hindered 11-*cis* isomer of 14-methylretinal (**2**) (Figure 1) in which the 12-s-*cis* conformation accessible to the parent 11-*cis*-retinal (**1**) is precluded by severe steric repulsion between the 14-methyl and the C₁₀-hydrogen. The absorption maximum in ethanol of this compound (350 nm) is blue-shifted by 31 nm from that of the corresponding all-*trans* isomer, and about 26 nm from that of the parent 11-*cis*-retinal, which is in agreement with the elimination of the 12-s-*cis* conformation-induced red shift. As a consequence, it was proposed that the conformation for 11-*cis*-retinal that predominates at room temperature is 12-s-*cis* **1**. The same authors proposed that the increase in extinction coefficients experienced by 11-*cis*-retinal upon lowering the temperature cannot be attributed to a predominant 12-s-*trans* conformation^{12,13} because 11-*cis*-14-methylretinal (**2**) shifts from 350 to 353 nm upon cooling to -105°C with an increase in ϵ of only 1.13 (compared to 11-*cis*-retinal: shift from 376 to 387 nm and an increment of 1.31 in ϵ). Instead, a redistribution of intensities within the main band of the 12-s-*cis* conformer²¹ was proposed to be a more likely possibility for the parent 11-*cis*-retinal.

II. SYNTHETIC RETINOIDS WITH CONFORMATIONAL AND/OR CONFIGURATIONAL RESTRICTIONS: THE 12-S-CIS-LOCKED RETINOIDS

A. THE C₁₄-METHYL RETINOIDS

The properties of the 14-methyl retinoids discussed above illustrate the utility of retinal analogues in the determination of the conformation of the retinoids (11-*cis*-retinal in visual pigments and all-*trans*-retinal in the purple membrane of *Halobacterium halobium*) when bound to the corresponding protein (opsin and bacteriopsin, respectively) via a protonated Schiff base linkage. The retinal-protein interaction results in a change in the properties of the protein and, at the same time, the conformation and electronic properties of the retinal chromophore are modulated by the protein.

The exact topography of the retinal chromophore when bound to the opsin is not known, but its conformation is likely to differ from that of the free retinal in solution. As emphasized earlier, the conformational flexibility of retinal itself and the steric restrictions in the binding site of the protein can force the π -extended Schiff base to adopt a conformation different from that in solution.²²

In order to obtain more information on the conformational and/or steric requirements of the protein on its chromophore, it can be extremely useful to synthesize and study retinal analogs incorporating new features or lacking some of the characteristics of the retinal system. The visual pigment analog, if formed, can provide valuable information ultimately concerning the nature of the molecular events that take place during and following the primary photochemical event of both the processes of vision and the light-driven proton pumping activity of *Halobacterium halobium*.²²

One of the main findings of the earlier studies was that the binding site of opsin (contrary to earlier predictions) is not very restrictive either with respect to the retinal configuration²³ or to the orientation of the β -ionone ring.²²

In this connection, the pigment formed from 11-*cis*-14-methylretinal (**2**)^{19,20} exhibited a similar absorption and CD spectrum as well as the photosensitivity as native rhodopsin did suggesting that opsin can accommodate a 12-s-*trans* conformation in the chromophore.

B. THE "LOCKED" RETINOIDS

A somewhat different approach to retinoid analogs involves the imposition of certain

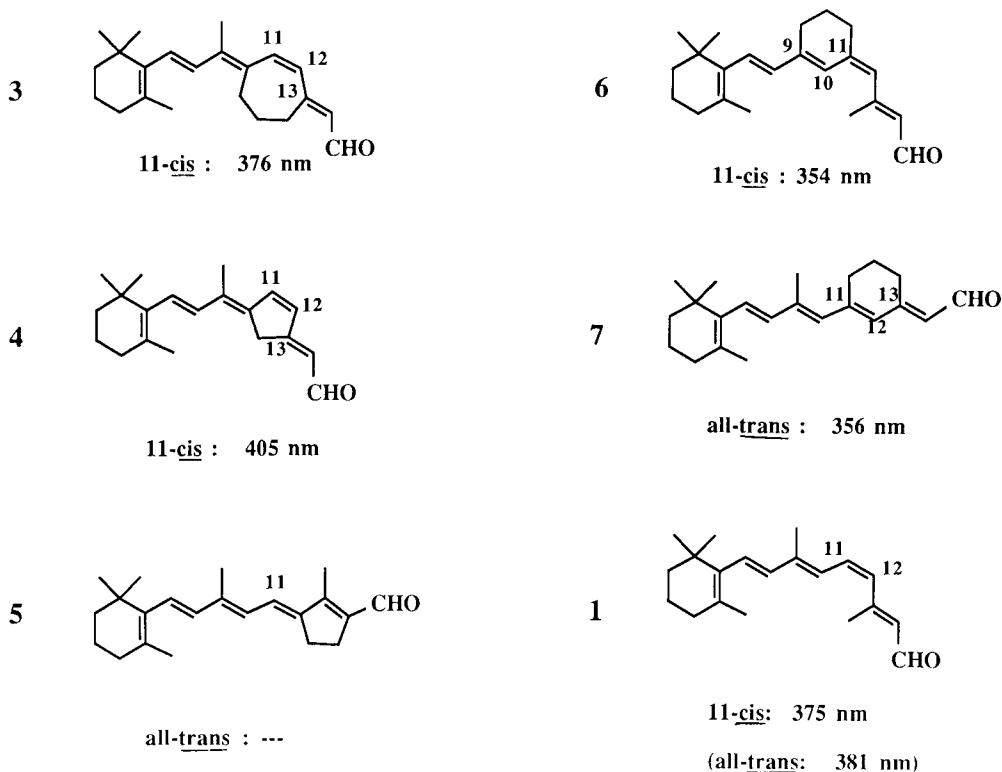


FIGURE 2. Examples of 12-s-*trans*-locked retinals. Electronic absorption data for selected isomers of 3 to 7 are compared with that for 11-*cis*-retinal (1).

configurational and/or conformational restrictions to the retinoids by “locking” the polyene chain between two of its positions by means of a cyclic, relatively rigid, structure.

1. The 12-s-*trans*-Locked Retinoids

Selected examples, including the absorption maxima of some of the important isomers, are shown in Figure 2. Among them, the 10,13-trimethylene bridged retinal 3,²⁴ which is 10-s-*trans*,11-*cis*,12-s-*trans*-locked, is forced into a noncoplanar 9,11,13-triene conformation by the seven-membered ring. The blocking of the 11-*cis*-to-*trans* isomerization caused the corresponding pigments to be stable when exposed to light at their absorption maxima. Interestingly, the absorption maximum of the synthetic retinals is very similar to that of the parent retinal. The more conformationally restricted 5-membered ring analog 4^{25,26} retaining a fully coplanar chromophore also formed an artificial pigment that was resistent to bleaching, supporting the *cis*-*trans* isomerization hypothesis as the primary photochemical event. A different isomer 5, with a 12-s-*trans*,13-*trans*-locked chromophore was designed²⁷ to evaluate the primary photochemical event in bacteriorhodopsin. The failure of its derived pigment to pump protons showed the necessity of 13-ene isomerization to initiate the subsequent dark reactions leading ultimately to the synthesis of ATP. The 6-membered ring analogs 6 and 7,²⁸⁻³⁰ one with the 11-*cis* configuration but 9-*trans*,10-s-*trans*-locked (6)^{28,29} and the other with the 11-*trans* configuration but 12-s-*trans*-locked (7), showed a normal photocycle, with photolysis intermediates similar to those of the native rhodopsin^{28,29} or bacteriorhodopsin,³⁰ respectively.

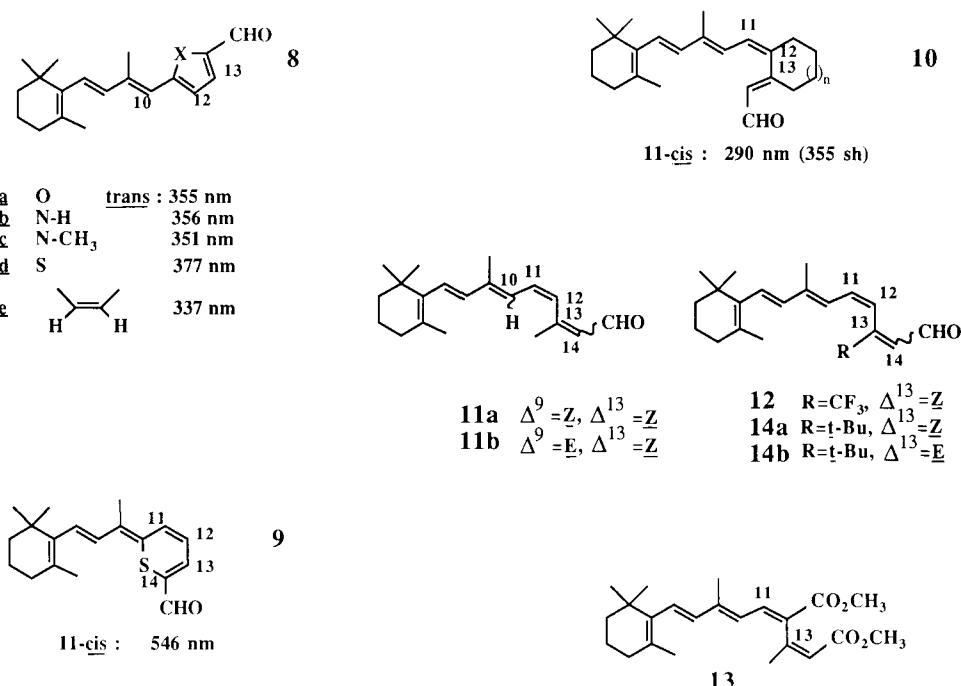


FIGURE 3. Examples of 12-s-cis-locked retinoids. Electronic absorption data are given for some of the isomers.

2. The 12-s-cis Locked Retinoids

By contrast to the relatively large number of synthetic retinoids with 12-s-trans-fixed conformation described above, there are only a few examples of 12-s-cis-locked retinoids (Figure 3). Besides our own work^{31,32} with this system (Sections III and IV, below), only the heterocyclic analogs **8** and **9** synthesized by Lugtenburg et al.³³⁻³⁶ have been reported to our knowledge. Retinoid **8a** formed a bacterial pigment analog with proton-pumping activity,³⁴ which questions the results described earlier,²⁷ and indicates that a 13-ene isomerization must not be strictly required for the proton pump action. The failure of the other analogs **8b-e** to form a pigment with bacterioopsin³⁵ has been ascribed to either an electronic perturbation of the bridging group or to steric hindrance.

C. THE 12-S-CIS CONFORMATION IN ACYCLIC SYSTEMS

As can be deduced from the data accompanying the retinoid structures in Figure 2, there is a close similarity in the absorption maxima of the 12-s-trans-locked retinoids with those of the parent system, the exception being the 5-membered ring analog **4** which, due to its coplanar chromophore absorbs to the red of all-trans-retinal.

The situation is different for the 12-s-cis-locked retinal **10** synthesized by our group.^{31,32} Surprisingly, the absorption maxima of the several isomers maintaining the 11-cis geometry reveal (see the discussion in Section IV of the chapter) a dramatic reversal of the intensity of the retinal absorption bands. In fact, the 12-s-cis-locked aldehydes exhibit their main maximum (288 to 300 nm) to slightly lower wavelengths than the corresponding alcohols (298 to 309 nm). The same anomaly was also detected when studying the absorption properties of the highly strained 9,11,13-tri-cis-**11a**^{37,38} and 11,13-di-cis-**11b** retinals, to which we assigned a twisted 12-s-cis conformation (Figure 3). A similar result has been reported³⁹ for 13-CF₃-13-cis-retinal **12**. The ¹H NMR NOE was used to demonstrate the existence⁴⁰ of a 12-s-cis conformation in an open chain retinoid, 12-(carbomethoxy)retinoic acid (**13**), and using the same NMR technique we recently observed that the 13-t-Bu-11,13-di-cis-

(**14a**)⁴¹ and 13-*t*-Bu-13-*cis*- (**14b**)^{41,42}-retinals exist in solution in a predominant 12-*s-cis* conformation.

D. THE SIGNIFICANCE OF THE 12-S-CIS CONFORMATION IN BIOLOGICAL ACTIONS OF RETINOIDS

Given these initial observations on the unusual blue-shifted absorption maxima characteristic of 12-*s-cis* retinal analogs, we embarked on a more detailed study of the influence of this conformation on the absorption properties of retinal chromophores. Our purpose is to draw a clear-cut picture of the variation in the absorption maximum of the retinals as the dihedral angle about the 12,13-single bond varies gradually with the size of the anchoring cycle, as does the number of conformations and mobility available to this seemingly topographically crucial segment of the polyene side chain.

Two other reasons behind this study have biological implications. It has recently been reported⁴³ that certain 12-*s-cis*-locked analogs of retinoic acid, the arotinoids (analogs incorporating aromatic residues as part of the polyene chain), function effectively in the regression of papillomas. The second reason deals with the structure of the primary photoproduct in the process of vision, the so-called bathorhodopsin. Recently, Liu and co-workers⁴⁴ proposed a new mechanism ("hula-twist") for the isomerization of polyenes in an restrictive protein environment, assigning structures for the intermediates in both the visual cycle and the photocycle of bacteriorhodopsin. Accounting for the photochemical behavior of the retinal molecule, the effect of the medium (protein) and the longitudinal restrictions to the reorientation of the chromophore, they assigned bathorhodopsin the 10-*s-cis,all-trans* structure. As indicated before, the synthetic retinal **6** (Figure 2) showed normal behavior, thus ruling out the 10-*s-cis* conformation for its bathorhodopsin analogue. As Liu pointed out,²⁸ new pathways could be operating in this case, which would produce a different primary photoproduct, with 12-*s-cis* structure, as first suggested by Honig and Karplus¹² based on the higher double bond character of the C₁₂–C₁₃ bond in the π* excited state.

III. STRATEGY DIRECTED TOWARDS THE SYNTHESIS OF RETINOIDS AND 12-S-CIS-LOCKED RETINOIDS: THE THERMAL REARRANGEMENT OF A VINYLALLENE

A. The [i,j]-SIGMATROPIC HYDROGEN SHIFT

1. Concept, Scope, and Limitations

By contrast to the general applications of carbon-carbon bond forming pericyclic reactions (Diels-Alder, Claisen Rearrangements, etc.) in organic synthesis, the [i,j]-sigmatropic hydrogen shifts have been scarcely used as part of a synthetic sequence.⁴⁵ The fact that this process is not commonly used as a C–C bond forming process and the observation that often times high temperatures are required to effect the [i,j] process precluded its wide applicability.

The [i,j]-sigmatropic rearrangement was first defined by Woodward and Hoffmann as "the migration of a σ bond, flanked by one or more π-electron systems, to a new position whose termini are i-l and j-l atoms removed from the original bonded loci, in an uncatalyzed intramolecular process".⁴⁶ As prototypes for the processes described in this section, Figure 4 depicts a sigmatropic rearrangement of order [1,5] in a pentadiene **15** and a sigmatropic rearrangement of order [1,7] in a 1,3,5-heptatriene **16** to give **17** and **18**, respectively. Of course, the formation of **17** and **18** are detectable only by the presence of functional group or isotope labels, and even then, with respect to **15** and **16**, respectively, they are nearly degenerate from a thermodynamic standpoint.

At an elementary theoretical level, the [i,j]-hydrogen migration may be considered in terms of interacting a hydrogen atom and the HOMO (highest occupied molecular orbital) of a polyene radical. The stereochemical course (Figure 4) of the migration (shown for the

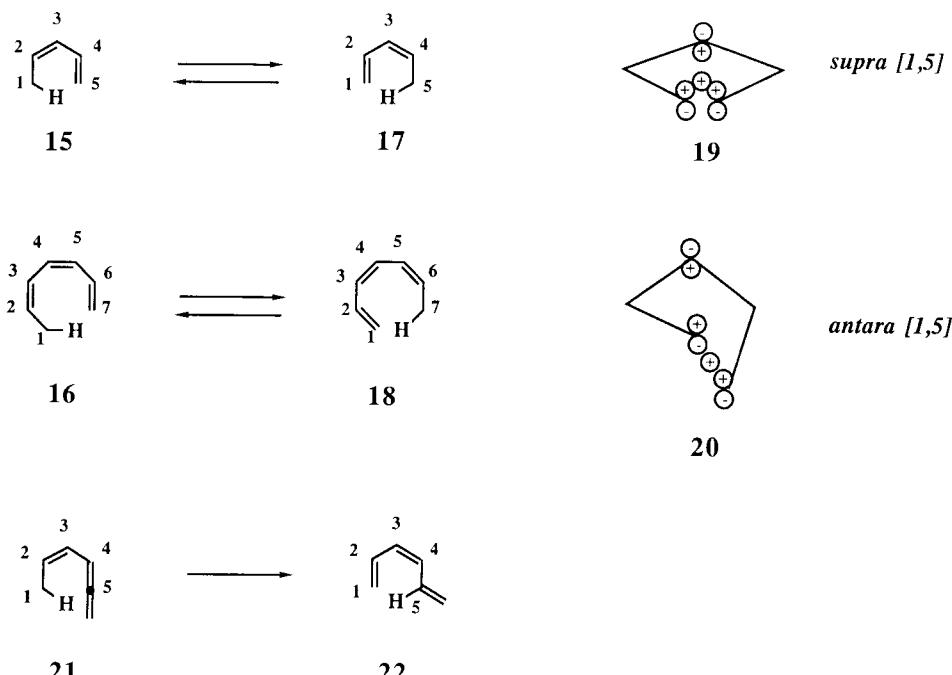


FIGURE 4. [1,5]- and [1,7]-Sigmatropic hydrogen shifts.

[1,5]-process) can be defined as suprafacial, **19** with the transferred H atom associated at all times with the same face of the π system in a transition state possessing a plane of symmetry, or antarafacial, **20** in which the transferred atom passes from one face at one carbon terminus, to the opposite face of the other terminus in a transition state characterized by a twofold axis of symmetry, C_2 .⁴⁶

The selection rules for the thermally-allowed processes predict the [1,5]-sigmatropic hydrogen shift to be suprafacially allowed and antarafacially forbidden.⁴⁶ By contrast, the [1,7]-sigmatropic hydrogen shift is predicted to be antarafacially allowed, but suprafacially forbidden.⁴⁶ Recently, an *ab initio* self-consistent field (SCF) method has been used to study the simplest [1,5]- and [1,7]-sigmatropic hydrogen shift (**15**→**17** and **16**→**18**, respectively).⁴⁷ The ground state energy and the two transition state energies (suprafacial and antarafacial) were compared for each process and the calculations were in good agreement with the stereospecificities (antarafacial or suprafacial) predicted by the elementary Woodward-Hoffmann theory and selection rules. Experimental evidence supports the suprafacial character of the [1,5]-sigmatropic hydrogen shift⁴⁸ and the antarafacial nature of the [1,7]-sigmatropic hydrogen shift counterpart.⁴⁹

The kinetic data reported to date for the [1,5]-hydrogen shift in acyclic systems indicate that these processes require relatively high temperatures, with an activation energy of 30 to 36 kcal/mol (i.e., **15**→**17** in Figure 4).⁵⁰ By contrast, the thermally induced rearrangement of a 1,2,4-triene (vinylallene) **21** to (Z)-3-hexa-1,3,5-triene **22** in Figure 4 exhibits a typical activation energy of only about 24 kcal/mol.⁵¹ Translated into experimental conditions, typical reaction temperatures for rearranging 1,3-pentadienes range from 250 to 300°C, whereas 110°C is required for isomerizing vinylallenes related to **21**. It was proposed⁵² that this energy difference resulted from increased conjugative stabilization of the transition state for the conversion of **21** to **22**, coupled with a 3 to 5 kcal/mol gain in bond energy associated with hydrogen migration from an sp^3 to an sp^2 carbon center. Furthermore, the s-*cis* conformation necessary for this rearrangement would be made sterically more accessible by the linearity of the allene linkage leading to an increase in the observed rate of reaction.

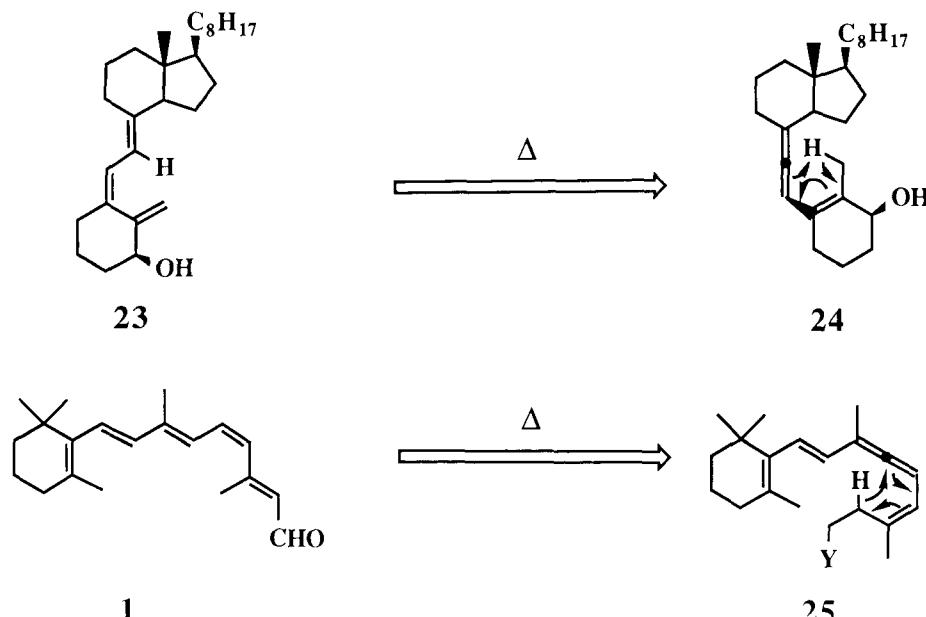


FIGURE 5. [1,5]-Sigmatropic hydrogen shifts of vinylallenes utilized by this laboratory in the synthesis of biologically active molecules (vitamin D **23** and the retinal **1**).

By virtue of the intramolecular nature of the sigmatropic rearrangement involving a cyclic transition state, the hexatriene resulting as product of the thermolysis of a vinylallene has a *cis* or (*Z*)-configuration about the central double bond (Δ^3). However, the stereochemistry about the termini of the triene (Δ^1 and Δ^5 double bonds) is uncertain, and only exhaustive experimentation with carefully designed molecules can reveal the factors imparting stereoselectivity at the terminal double bond (*vide infra*).

2. Selected Synthetic Applications

The Δ^3 -*Z*-hexatriene unit resulting from the thermal rearrangement of a vinylallene (Figure 4) is a unique structural feature of the calcium-regulating steroid hormone vitamin D (**23**)⁵³ (see Figure 5). Also, this structural unit can be envisaged to be present in the pentaene skeleton of 11-*cis*-retinal (**1**). This fact provides a rationale for approaching the syntheses of both biologically important molecules **23** and **1** by using as the key step the thermal rearrangement of vinylallenes **24** and **25**, respectively.⁵⁴

The two possible trajectories (a and b) of the migrating hydrogen in the vinylallene **26** (Figure 6) can give rise to the four possible isomeric Δ^3 -(*Z*)-hexatrienes, namely **27a-d**. In the course of our studies on the sigmatropic rearrangement of **24** and other model compounds, we discovered an antidirecting effect of the hydroxyl group, favoring the trajectory occurring opposite the face of the cyclohexene bearing the hydroxyl group.⁵⁵ Furthermore, we recently reported a sulfoxide-induced acceleration with enhancement of geometric selectivity in the [1,5]-sigmatropic hydrogen shift of a vinylallene sulfoxide, favoring migration of the hydrogen anti to the sulfoxide group.⁵⁶ It is planned to pursue the investigation along these lines in order to gain further information on the control of the geometry at the termini of the hexatriene, which is an important consideration of the well-defined stereochemistry of some retinal isomers in eliciting their biological function. However, the possibility of obtaining the important 11-*cis*-retinoids (or some other retinoids with well-defined stereochemistry) by using the vinylallene approach makes this method highly attractive. These studies would also test the scope and limitations of the vinylallene approach because of the

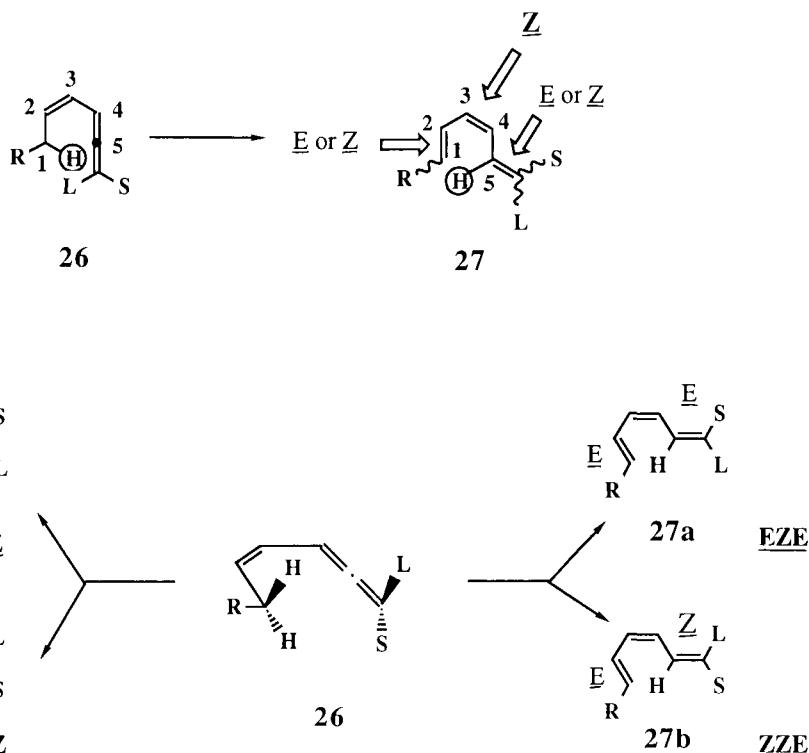


FIGURE 6. The suprafacial [1,5]-sigmatropic hydrogen shift of general vinylallene structure **26** can afford four possible geometric isomers **27a-d**, all of which possess a *cis*-geometry at the central double bond.

known thermal instability of polyenes of this kind. Finally, the allenic retinoids can be considered side-chain analogs of normal retinoids or retro-ene type retinoid⁵⁷ valence isomers, formally interrelated by [1,j]-sigmatropic hydrogen shifts. This study of the polyene-disrupted system can provide some useful information on the absorption properties of the retinoids.

3. Synthetic Variations in the Retinoid Skeleton

Allenic retinoids **28a-d** and **29a-d** have been prepared by Nakanishi^{58,59} and several (**28b,d** and **29b,d**) were observed to form pigments with bovine opsin. This constitutes further evidence that the binding site of opsin is not overly restrictive as to the size and/or the orientation of the β -ionylidene ring.

The polyenic nature of the retinoid offers different alternatives as to the location of the allenic unit in the side chain. Three of them (**25**, **30**, and **31**; **28** and **29** represent yet a fourth possibility) are depicted in Figure 7. At first sight, only the [1,5]-sigmatropic hydrogen shift of a 9,10-allene **25** would afford the corresponding 11-*cis*-retinoids, and our main efforts have been directed towards studies of this allenic isomer.^{37,38} However, other sigmatropic rearrangements from the isomeric allenes (specifically [1,7]-sigmatropic hydrogen shifts, which are known to occur at lower temperatures than the [1,5]-shift in analogous systems)⁴⁹ can occur in tandem (i.e., as secondary steps) with the primary [1,5]-shift process to give the same kind of isomers as would result directly from **25**. This fact makes the other isomeric allenes such as **30** and **31** interesting alternatives to the 9,10-allene **25** as synthetic targets.

B. THE ELABORATION OF THE VINYLALENE FUNCTIONALITY

1. The Vinylcuprate Approach

Among the several methods devised for the generation of a vinylallene functionality,

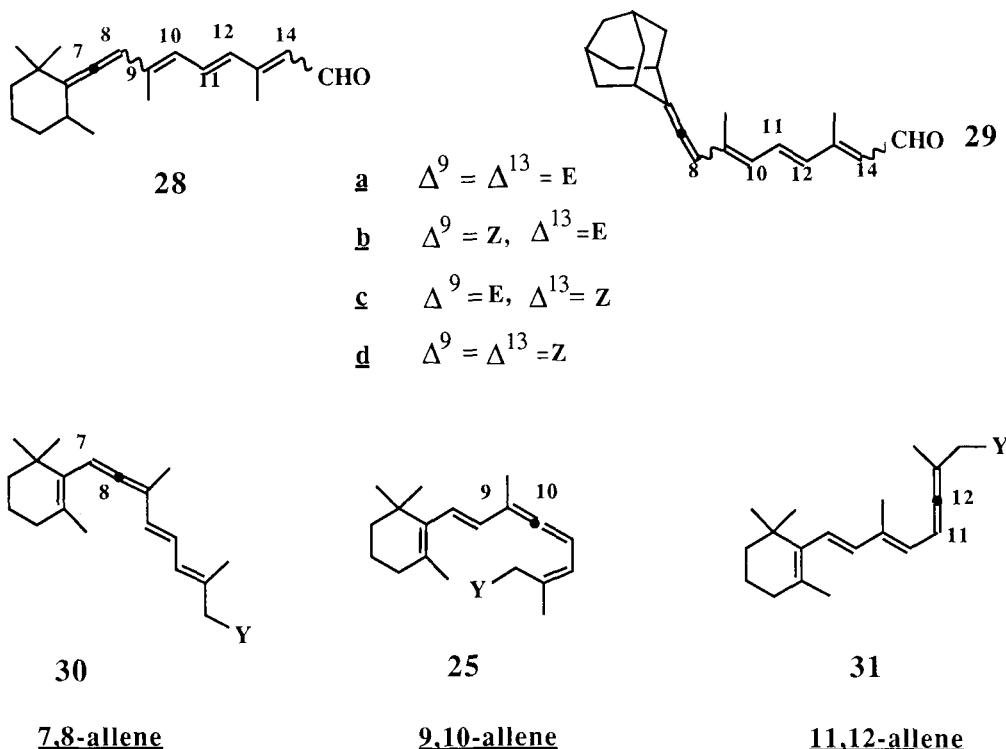


FIGURE 7. Allenic retinoids.

the propargylic rearrangement (S_N2' -type displacement of a suitable functionalized propargylic derivative by an organocuprate) stands as one of the mildest methods compatible with the known lability of these polyenic compounds.⁶⁰ Paralleling our first synthesis of vinylallenes related to the vitamin D system,⁶¹ we first devised a protocol for the generation of the 9,10-allenes **36**³⁷ and **37**³¹ that featured the displacement of a propargylic benzoate **32** (derived from **33**) by vinylcuprates **34** and **35**, respectively.

The choice of the cuprate reagent **34** or **35** was the subject of exhaustive studies during the development of the vinylallene approach for the parent system **36**.³⁸ The best non-transferable ligand was found to be MMB [$\sim C\equiv C-C(CH_3)_2-OCH_3$] and the generation of the active cuprate species required the use of *t*-BuLi. Severe competition reaction problems were found with this key reaction, as deduced from the isolation of the allene **38** and the alcohols **39** and **40** as side products from the reaction of the benzoate **32** with the cyclic cuprate **35**.³² Moreover, the reaction of vinylcuprates with propargylic esters proved to be capricious. The synthetic scheme, however, provided the vinylallenes **36** and **37**, which became the targets for our first thermal experiments.

The preparation of the vinylcuprates for both systems is also summarized in Figure 8 above. The C-5 fragment **34** was prepared starting from isopentenyl alcohol, which was brominated (Br_2/CCl_4) and then dehydrobrominated (DBU/benzene) to give the known bromides **41** and **42** (1:2 *Z:E* mixture). On the other hand, reaction of the cycloakanone with DMF/PBr₃ gave the bromoaldehyde **45**, which was subjected to Wittig condensation to give the bromodiene **46**. Hydroboration, followed by oxidation gave the bromo alcohol **47**. The alcohols were protected as their TBDSM ethers **43** and **48**. Metal-halogen interchange with *t*-BuLi followed by reaction with MMB-Cu afforded the mixed cuprates **34** and **35**, respectively, from which the desired vinylallenes were obtained.

The vinylcuprate approach proved to be less than satisfactory, not only because of the

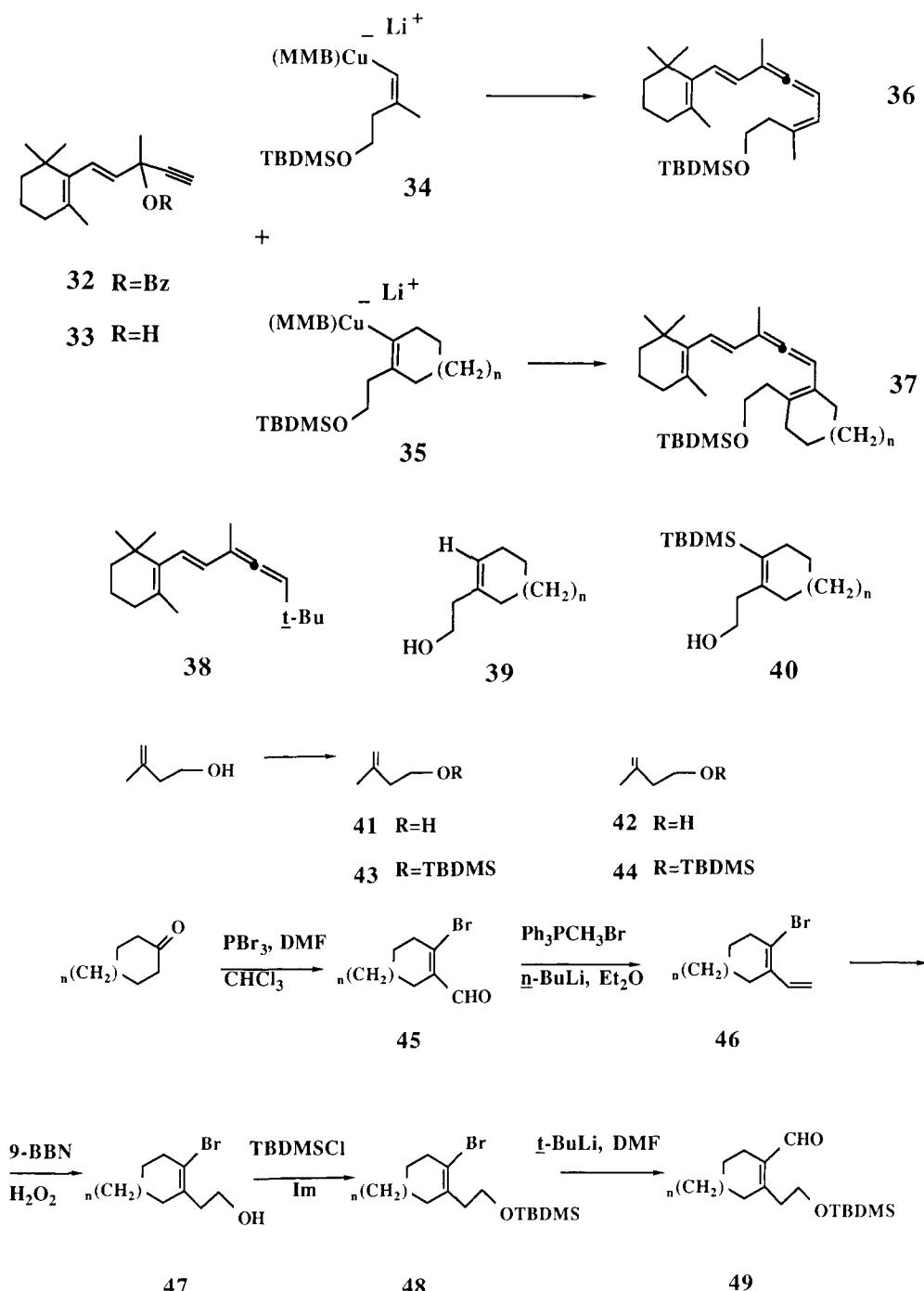


FIGURE 8. Synthesis of 9,10-allenic retinoids and precursors.

side products obtained in the coupling step, but also because the method was found to be restrictive. Attempts to prepare the cuprate derived from the *E*-bromide **44** proved to be unsuccessful under the same conditions found highly effective for its isomer **43**.

It should be mentioned that Schlosser has made use of a vinylcuprate derived from 3-chloro-4,5-dihydro-2-furyllithium (**51**) in a synthesis of the 11,12-allene **53**, which was

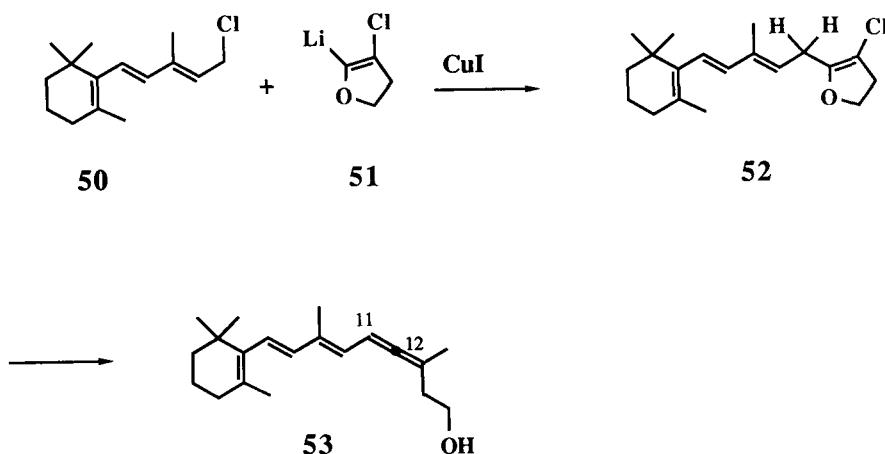


FIGURE 9. Synthesis of 11,12-allenic retinoids. See Figure 11 for an alternative synthesis.

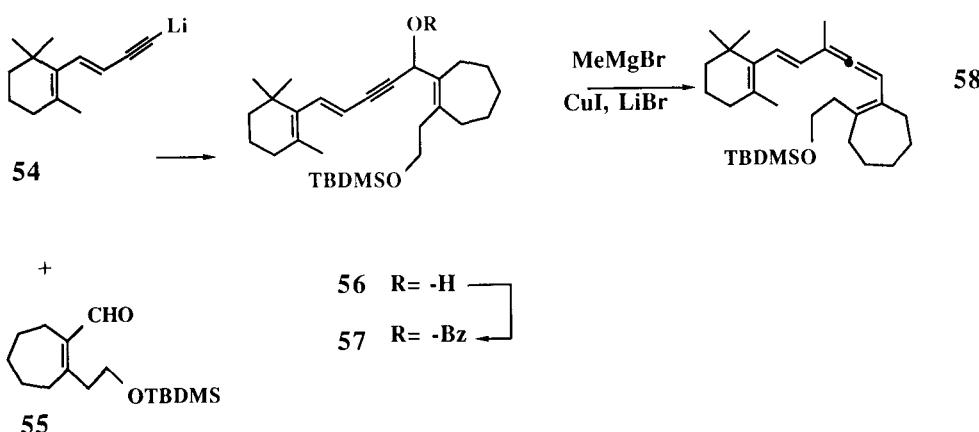


FIGURE 10. The alkylcuprate approach to 12-s-cis-locked 9,10-allenic retinoid 58. This approach is superior to the complementary vinylcuprate approach outlined in Figure 8.

prepared by reacting the chloride **50** with **51** via the dihydrofuran **52**.⁶² Addition of two equivalents of methylolithium to **52** caused ring opening and substitution of the chlorine to give the 11,12-allenol **53**. Our earlier synthesis of **53** following a more classical Wittig-type approach⁶³ will be described later in this section.

2. The Alkylcuprate Approach

The capriciousness of the vinylcuprate route described above along with the production of unwanted side products derived from competitive processes, prompted us to develop a new route to the key vinylallenes **36** and **37** (Figure 8). This new approach has recently been reported⁶⁴ for the 12-s-cis-locked-9,10-allene **58** (via **54**→**57**) although it has been further developed for the parent system 9,10-allene **36** as well as different allenes with bulky (*t*-Bu) groups at the C₉ and C₁₃ positions of the side chain.⁶⁵

The new route entails the regioselective S_N2'-type displacement of a retinoid-like propargylic benzoate **57** by a suitable copper species derived from a commercially available lithium reagent. Although the reaction between **57** and cuprate could have resulted in a competition between allylic (at C₁₃ or other allylic sites) and propargylic (at C₉) attack, it

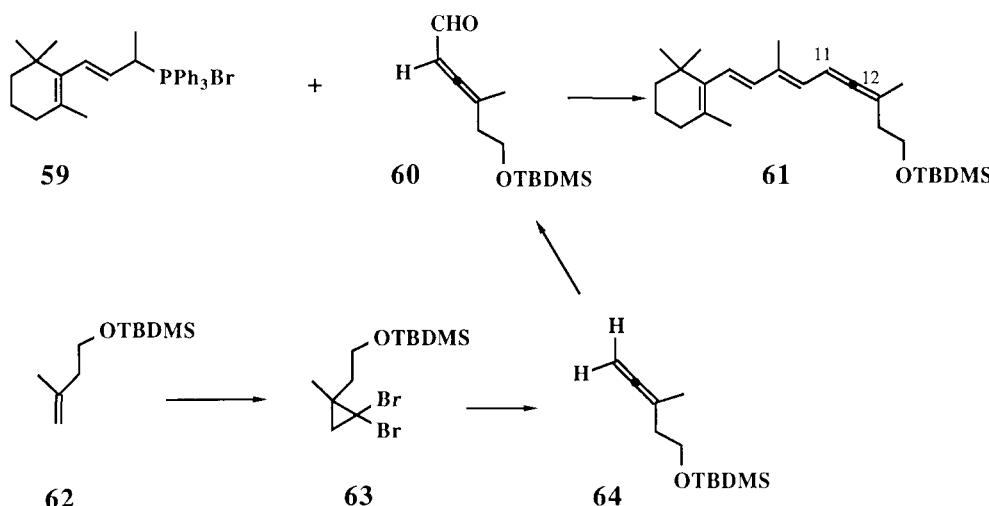


FIGURE 11. The Wittig approach to 11,12-allenic retinoid **61**. See the alternative synthesis outlined in Figure 9.

was found to be highly regioselective at C₉ to give the desired 12-s-*cis*-locked-9,10-allenic retinoid **58**. The required propargylic benzoate was prepared by benzoylation of the propargylic alcohol **56**, which, in turn, was obtained by coupling of the lithium derivative of the dienyne **54** with the aldehyde **55** (obtained by lithiation of **48** and subsequent treatment with DMF).

An additional advantage of this approach is the possibility of modifications of the composition of the cuprate species to get the best yield and/or regioselectivity. In this regard, the introduction of a methyl group was found to be highly efficient by using MeMgBr in the presence of LiBr/CuI as a promoting agent. By contrast, the introduction of a *t*-Bu group required the preparation of the Lipshutz-type mixed heterocuprate derived from *t*-BuLi and CuCN.⁶⁵

3. The Wittig Condensation of an Allene Aldehyde

Although the alkylcuprate approach has been recently successfully used for the preparation of C₁₃-substituted 11,12-allenes of the type **31** (See Figure 7), our first approach to this system made use of the more classical Wittig chemistry in the key step of the synthesis. This involved the coupling of the ylid derived from the phosphonium salt **59** with the allene aldehyde **60**. This was obtained by a sequence involving dibromocyclopropanation of the protected isopentenyl alcohol **62** to give **63** followed by elimination to the allene **64**. The latter by treatment with *t*-butyllithium and then DMF afforded **60**.

The lack of stereoselectivity in the Wittig reaction compared to the highly regioselective S_N2'-displacement method described in the preceding section makes the latter operationally more convenient for preparing these sensitive vinylallenes. Accordingly, no further effort was made to pursue the Wittig-type approach.

C. THERMAL REARRANGEMENT OF VINYLALLENES

1. Primary Thermal Pathways

The key step in the vinylallene approach to retinoids, including 12-s-*cis*-locked retinoids, features as a primary process a thermal [1,5]-sigmatropic hydrogen rearrangement of the vinylallene functionality. Figure 12 depicts the products resulting from thermolysis followed by deprotection of the vinylallenes **36**, **37**, and **67**. The required reaction conditions were largely dependent on the structure of the vinylallene and the results are summarized in Table

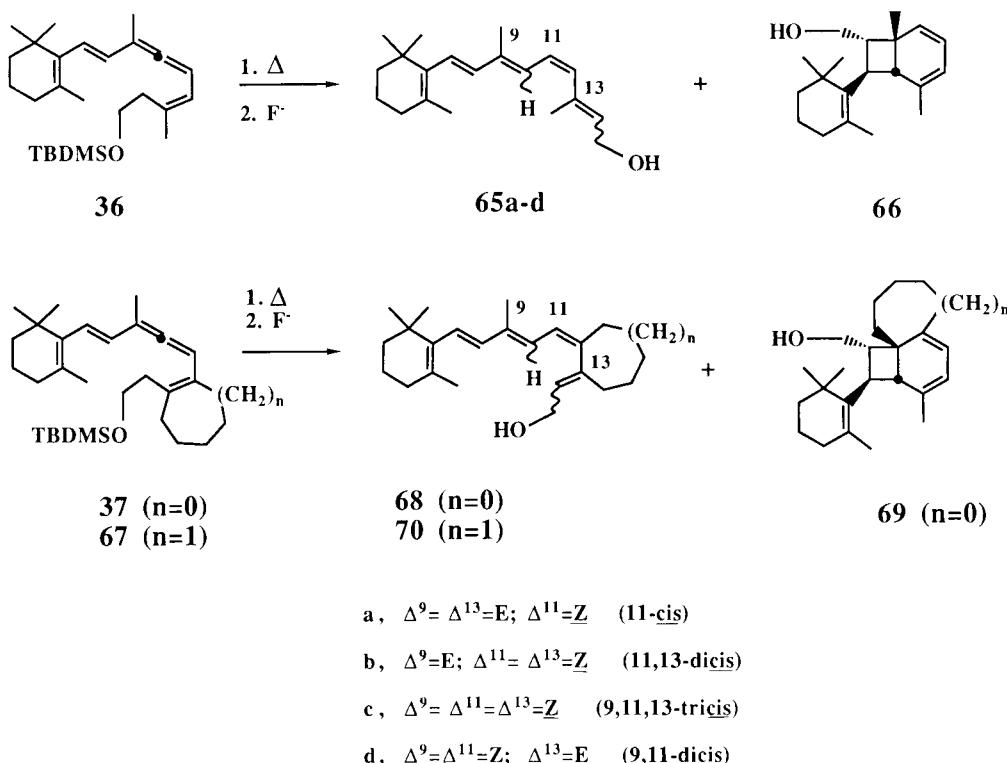


FIGURE 12. Thermolysis of 9,10-allenic retinoids.

TABLE 1
The Thermal Rearrangement of the Vinylallene — Reaction Conditions and Stereochemical Course.

Vinylallene	Reaction conditions	11,13-di- <i>cis</i>	11- <i>cis</i>	9,11,13- <i>tri-cis</i>	9,11- <i>di-cis</i>	9Z/9E	13Z/13E
36	69°C, 2 h	12	10	14	14*	1.25/1	1.1/1
37	69°C, 4 h	33	14	23	12*	0.75/1	2/1
67	rt, 3 h	12	5	27	6	2/1	3.5/1

* Isolated as electrocyclized product **66** or **69**.

1. The thermolysis of the 9,10-allene **36** required heating for 2 h at 69°C.³⁷ In the case of the 12-*s-cis*-locked-9,10-allene we observed a profound influence of the ring size on the reaction conditions as indicated in Table 1.

As expected, there is little or no stereodifferentiation at the 9,10-position. In general, as was discussed earlier (Figure 6), 9,10-allenic retinoids may be anticipated to produce four geometric isomers (11-*cis*; 11,13-*di-cis*; 9,11-*di-cis*; and 9,11,13-*tri-cis*) of the retinol (or secondary rearrangement products) specific for the Δ^{11} -Z configuration but not so for the Δ^9 or Δ^{13} double-bond configurations. The origin of the lack of influence by substituents at the allene terminus (methyl and vinyl groups at C₉ in this case) is not known, but it is not due to the difference in size between the substituents (methyl and vinyl groups) at C₉.⁵⁶ However, we observed the preferential formation of the sterically more congested 13-*cis* isomers, which we attributed to the preferred conformation adopted by the vinylallenols in

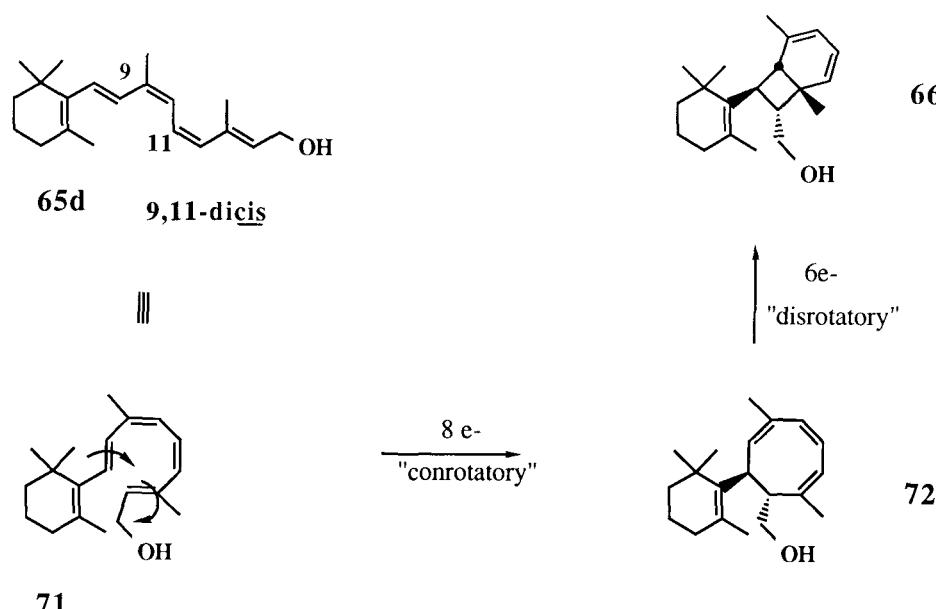


FIGURE 13. Thermal electrocyclizations characteristic of 9,11-di-*cis*-retinols. Cyclooctatrienes and bi-cyclo[4.2.0]octa-2,4-dienes.

order to alleviate the steric interactions between C₁₅ and C₂₀ methylenes during the [1,5]-sigmatropic shift.

2. Secondary Thermal Pathways

Whenever the thermal reactions require relatively higher temperatures (about 70°C) we observed the absence of the 9,11-di-*cis* retinol **65d** or **68d** in the thermolysis mixture.^{31,38} This result became clearer when we observed that the [1,5]-sigmatropic hydrogen shift of the vinylallene **67**, locked across the 12-13 bond with a 7-membered ring, occurred at room temperature.³² The milder conditions required for this seven-membered ring system allowed us to isolate for the first time all four possible "expected" geometric isomers **70a-d** (11-*cis*; 11,13-di-*cis*; 9,11,13-tri-*cis*; 9,11-di-*cis*) and study their individual behavior. From these experiments we further confirmed the structure of the fourth component in the reaction mixture of **36** and **37** to be the bicyclic compound **66** and **69**, respectively. Their formation from 9,11-di-*cis* **65d** and **68d** parallels the behavior exhibited by octatetraenes possessing *trans-cis-cis-trans* geometries, which are well-known⁶⁶ to undergo very facile eight-electron conrotatory electrocyclizations to give cyclooctatrienes followed by six-electron disrotatory electrocyclizations to afford the bicyclo[4.2.0]octa-2,4-dienes. Thus, the 9,11-di-*cis* isomer **65d** can be considered to electrocycliclyze via its 8-s-*cis*, 10-s-*cis*, 12-s-*cis* conformer **71** to afford the putative cyclooctatriene **72**, which affords by further electrocyclization the bi-cyclooctadiene **66**. A similar rationale has been forwarded for the isomerization of 9,11-di-*cis* **68d** to bicyclooctadiene **69**. More recent studies⁶⁵ suggest that this behavior may be general for all retinols possessing 9,11-di-*cis* geometry including the 9,11,13-tri-*cis* isomer although elevated temperatures are required for the latter.

By contrast, the thermolysis of the 11,12-allene **61** resulted in a [1,5]-sigmatropic hydrogen shift from the C₁₉-CH₃ to C₁₂ affording the putative 19,14-retro-retinols **73** and **74** which, under the reaction conditions, are considered to undergo spontaneous [1,7]-sigmatropic hydrogen shifts affording, after deprotection, the 20,14-retro-retinol **76**, the 11-*cis*-retinol **65a**, and the 11,13-di-*cis*-retinol **65b** as shown in Figure 14. This represents a different route to 11-*cis*-retinoids with 9-*trans* geometry.⁶³

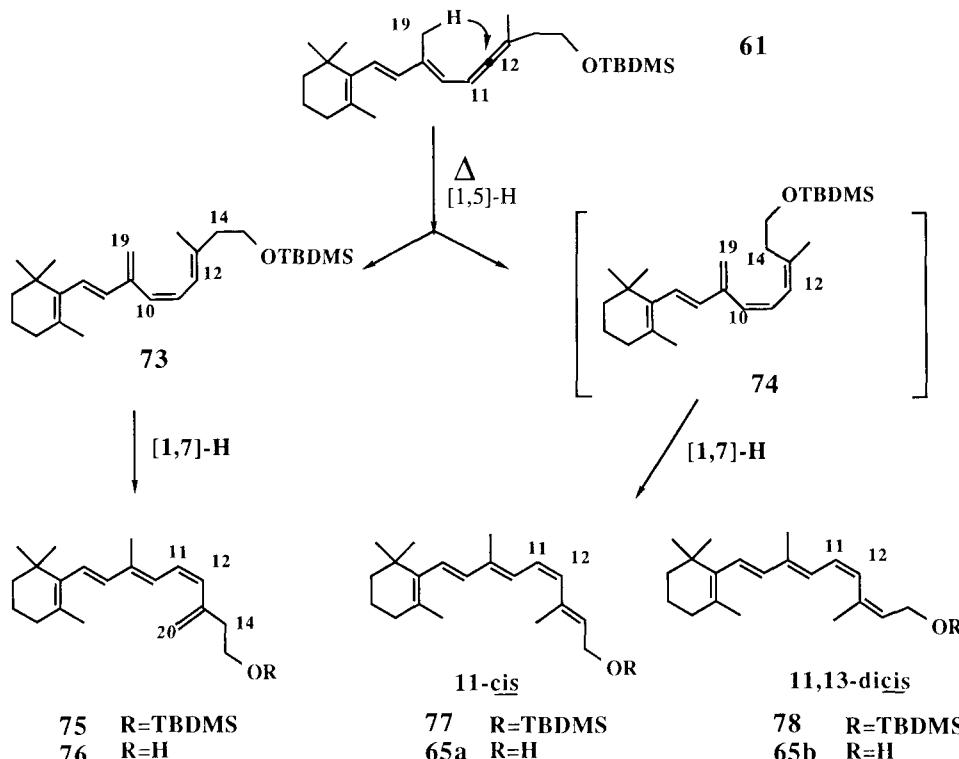


FIGURE 14. Thermolysis of 11,12-allenic retinoids.

IV. THE 12-S-CIS CONFORMATIONALLY LOCKED RETINALS

A. PRIMARY RETINOL-DERIVED ALDEHYDES

The milder conditions needed for effecting the [1,5]-sigmatropic hydrogen shift of the 12,20-tetramethylene-9,10-allene **67** allowed the isolation of all four of the possible retinols (Figure 12 and Table 1), free of secondary bicyclooctadiene products from secondary thermal isomerizations. This fact enabled us to study more completely the chemical and spectroscopic properties of the retinoids possessing this particular seven-membered ring-fused skeleton.³²

The transformation of each of the retinols **70a-d** to the corresponding aldehydes **79a-d** took place by using the classical procedure (MnO_2 , hexanes, 0°C , 1 h). The method of synthesis and spectral data established a *7-trans,11-cis* geometry for all four 12-s-cis conformationally locked retinals. Their spectroscopic properties will be the subject of the remaining section.

B. THERMAL AND PHOTOCHEMICAL INTERCONVERSIONS

It has been established that in dienones⁶⁷ and retinal^{32,38,68,69} possessing α,γ -di-cis configuration (relative to the carbonyl function), geometrical isomerization of the γ -bond from *cis* to *trans* by a series of successive six electron electrocyclicizations occurs readily. Initial electrocyclicization to an α -pyran followed by its retro-electrocyclization in the same disrotatory sense accounts for the loss of the original γ -*cis* configuration. We sought to utilize these thermal electrocyclic processes to prepare some of the isomers possessing 11-*trans* (γ -*trans*) stereochemistries. In accordance with this mechanistic scheme, the thermolysis of the 11,13-di-*cis* **79b** and the 9,11,13-tri-*cis* isomers **79c** (refluxing hexanes, argon, dark, 1 h) resulted in an equilibrium mixture containing 47% **79b** and 53% **79e**, and 38% **79c**, and 62% **79f**,

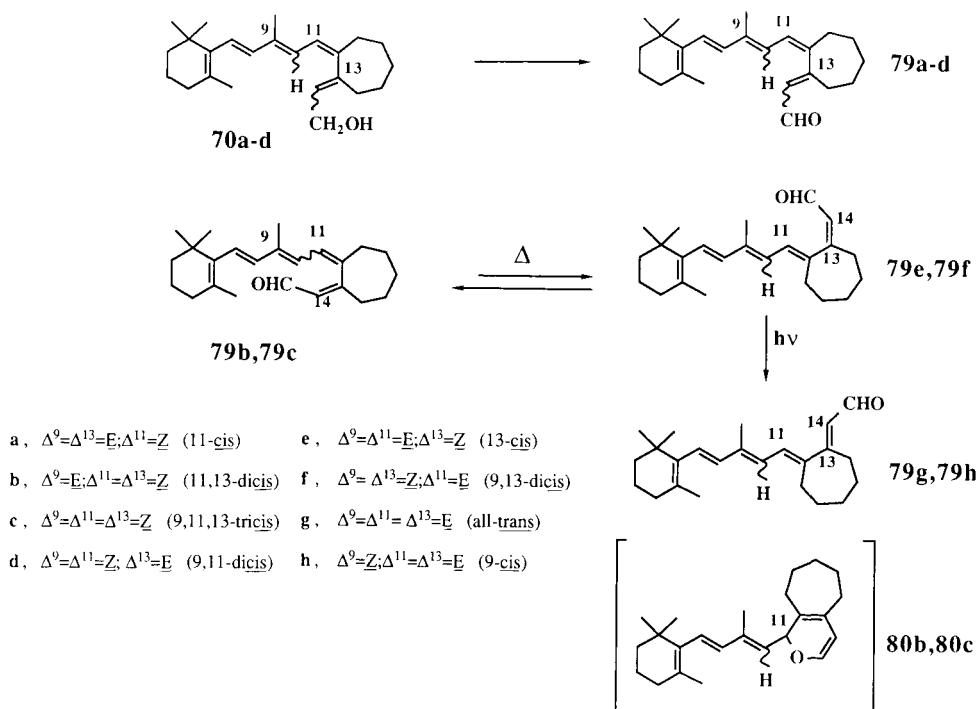


FIGURE 15. The 12-s-*cis*-locked retinals possessing seven-membered ring fusion synthesized and characterized in this laboratory. The 11-*cis* isomers **79a-d** were prepared by oxidation of the corresponding alcohols **70a-d**, respectively (Figure 12). The remaining isomers were prepared either thermally (**79e** and **79f** from **79b** and **79c**, respectively) or photochemically (**79g** and **79h** from **79e** and **79f**, respectively).

respectively (through the intermediacy of the putative dihydropyran tautomers **80b** and **80c**, respectively).⁶⁹ The same mixture was obtained by heating **79e** or **79f** under identical conditions, thus establishing that these compositions represent true equilibrium mixtures. This can be expressed as K_{eq} (69°C) = 1.1 for **79b** to **79e** and K_{eq} (69°C) = 1.7 for **79c** to **79f**. The behavior of **79b** (**79e**) and **79c** (**79f**) contrasts with the results obtained for similar transformations of parent 11,13-di-*cis* and 9,11,13-*tri-cis*-retinals (**11b** and **11a**, respectively; see Figure 3),³⁸ in which the less hindered retinal (13-*cis* and 9,13-*di-cis* isomers) was irreversibly obtained starting from the more hindered precursor isomer.

This selective thermal interconversion was complemented by a selective photochemical isomerization on exposure of the isomers possessing 13-*cis* geometries to light (100 W tungsten light source – 10°C). Photolysis of **79e** and **79f** resulted in the isolation of all-*trans* **79g** and 9-*cis* **79h**, respectively.⁶⁹ The remarkable affinity for photoisomerization about the 13-double bond exhibited by **79e** and **79f** can be rationalized by applying the Dillinger-Kasha model to the regioselective isomerization of retinoids. Liu⁷⁰ has previously applied this model to related photochemical processes. This model states that the relative amount of primary photoproduct is largely dependent on the ease of rotation about equivalently substituted double bonds in the excited singlet state, which, in turn, is dependent on the volume of displacement accompanying such a rotation. Geometric isomerization of the 13,14-double bond in the vitamin A series should be preferred because displacement of fewer solvent molecules is involved, compared to the rotation of either end of the remaining double bonds. This bias towards photoisomerization of the 13-double bond exhibited by the parent retinal is further reinforced in the 12-s-*cis*-locked retinals by the introduction of the 12,20-tetramethylene bridge which increases the spatial demand for twisting about the 9- and 11-bonds.

TABLE 2
¹H NMR Data for 79a-h

	H₇	H₈	H₁₀	H₁₁	H₁₄	H₁₅	2(CH₃)_{16,17}	(CH₃)₁₈	(CH₃)₁₉
79a	6.19 (16.4)	6.05 (16.4)	6.18 (11.7)	6.46 (11.7)	5.96 (8.0)	10.11 (8.0)	1.02	1.70	1.95
79b	6.17 (16.1)	6.00 (16.1)	5.95 (11.7)	6.57 (11.7)	5.99 (8.2)	9.51 (8.2)	1.01	1.68	1.94
79c	6.19 (15.8)	6.62 (15.8)	5.86 (12.0)	6.65 (12.0)	6.00 (8.2)	9.51 (8.2)	1.04	1.75	1.89
79d	6.21 (16.0)	6.64 (16.0)	6.08 (11.9)	6.55 (11.9)	5.97 (8.1)	10.12 (8.1)	1.05	1.76	1.93
79e	6.31 (16.4)	6.17 (16.4)	6.29 (11.7)	6.47 (11.7)	5.98 (8.2)	9.64 (8.2)	1.04	1.74	1.95
79f	6.28 (15.8)	6.58 (15.8)	6.20 (12.3)	6.54 (12.3)	5.95 (7.6)	9.60 (7.6)	1.02	1.71	2.04
79g	6.30 (15.8)	6.17 (15.8)	6.29 (12.0)	6.80 (12.0)	6.18 (8.2)	10.04 (8.2)	1.04	1.74	2.00
79h	6.23 (16.2)	6.66 (16.2)	6.20 (11.8)	6.87 (11.8)	6.15 (8.2)	10.04 (8.2)	1.04	1.74	2.04

^a Chemical shift data in δ (ppm) in CDCl₃ relative to (CH₃)₄Si.

^b Methyl groups appear as singlets and all other protons listed appear as doublets; coupling constants in Hz are in parentheses.

C. SPECTROSCOPIC PROPERTIES OF 12-S-CIS LOCKED RETINALS

1. Nuclear Magnetic Resonance

The ¹H NMR data for the eight isomeric Δ^7 -trans-12,20-tetramethylene retinals **79a-h** are given in Table 2. Extensive NOE measurements have been made to confirm the stereochemical assignments already suggested on the basis of the method of synthesis, namely the selective thermal ([1,5]-sigmatropic hydrogen shift leading to 11-cis geometry and thermal interconversions of 11,13-di-cis to 13-cis stereoisomers) and photochemical interconversions discussed thus far. The 13-cis geometry assigned to compounds **79b, c, e, and f** was further confirmed by the observation of a NOE between H₁₅ and H₁₀. This NOE between these protons was not detected in the other set of compounds with 13-trans geometry (they exhibit enhancement of H₁₅ when the cisoid C₂₀-CH₂ was saturated).

Several salient features of the ¹H NMR chemical shifts for the 12-s-cis conformationally locked retinals were observed. These are analyzed as effects at the end of the polyene (H₁₅) and for the C₉-C₁₄ hexatriene region (the behavior of H₇ and H₈ upon changing the configuration of the side chain is similar to that exhibited in the parent system).⁷¹

There is an upfield shift (about 0.5 ppm) of the aldehydic resonances in compounds with 13-cis geometry. This effect can be attributed to the absence of the deshielding interaction between the aldehyde proton and the C₂₀-methylene group present in the 13-trans geometry. In the 12-s-trans-locked analogs (Figure 2) synthesized by Nakanishi,²⁴ the steric interaction between H₁₂ and H₁₅ causes deshielding of the H₁₅ resonance. Moreover, in the 13-cis series of the parent system, only 11,13-di-cis **11b** and 9,11,13-tri-cis **11a** isomers present an upfield shift of the same magnitude due to the predominant 12-s-cis conformation³⁸ (which is the one “locked” by the bridge across C₁₂ and C₂₀) exhibited by these hindered isomers in solution. Note that when the 11-bond is trans in the parent system, the 9,13-di-cis isomer shows a downfield shift for H₁₅ compared to its analogue with 13-trans geometry.⁷¹ The trend of upfield shift for the aldehydic signals in compounds with 13-cis geometry and 12-s-cis conformations and downfield shifts in analogs with 13-cis geometry and 12-s-trans conformations seems to be quite general.

The protons in the C₉-C₁₄ region experience, in general, upfield shifts upon changing

the *trans* (*E*) to the *cis* (*Z*) configuration as a result of moving to a more sterically congested structure. The value of the upfield shift depends mainly on the deviation from planarity, but the conformational mobility of the C₈—C₉ and C₁₀—C₁₁ single bonds makes the comparative analysis of the chemical shift values very difficult. In the parent system,⁷¹ it is worth mentioning the downfield shift exhibited by H₁₀ when changing the configuration at the C₉ and C₁₁ double bonds ($\Delta\delta = +0.34$ ppm in 11-*cis* and $\Delta\delta = +0.2$ ppm in 9,11-di-*cis*). The existence of the 12,20-tetramethylene bridge in the analogs **79a** and **79d** causes twisting of the polyene system such that the H₁₄ and H₁₀ signals are shifted upfield apparently due to π -shielding.

The above observations can be more easily visualized with the aid of the ¹H NMR simulated spectra depicted in Figure 16, which contains the signals for the protons on the side chain of compounds **79a-h**.

2. UV Absorption

The absorption spectra of *unhindered* parent retinals in the visible and near UV are dominated by three bands: a main one (α) around 380 nm and two weak transitions near 280 and 250 nm, denoted as β and γ bands, respectively, whose assignments have been the subject of theoretical debate.^{72,73}

Retinyl polyenes belong to the C₁ point group, but it is very useful to describe the excited states by reference to the idealized C_{2h} point group of, for example, s-*trans*-butadiene.⁷⁴ In this respect, transitions are expected from the ¹Ag*⁻ ground state to ¹Bu*⁺, ¹Bu*⁻, ¹Ag*⁺, and ¹Ag*⁻ excited states.

α -Band — The main band in all retinal isomers has been calculated to derive mainly from the allowed transition to the lowest “¹Bu*⁺” state, although recently a contribution by the “¹Ag*⁻” state has been suggested.^{75,76} This additional contribution would provide a satisfactory explanation to the fact that 11-*cis*-retinal, without changing its absorption maximum, exhibits a lower extinction coefficient than the other isomers¹² at room temperature (see also Section I.B.2). The 12-s-*trans* conformation suggested to predominate at 77 K has been assumed to have a different level ordering than the 12-s-*cis* conformation (the lowest excited state would be “¹Bu*⁺” in the latter and “¹Ag*⁻” in the former). Upon the increase of the relative proportion of the 12-s-*trans* conformer at low temperature, there is a compensation between the blue shift undergone by the transitions to the “¹Bu*⁺” state (s-*cis* contribution) and the red shift of that to the “¹Ag*⁻” state (s-*trans* contribution), resulting in a maximum in the vicinity of the other isomers with no steric strain. An alternative rationale has been proposed by Nakanishi as discussed earlier (see Section I.B.2).

β - and γ -Bands — The β -band has been assumed to derive from the “¹Ag*⁺” state,⁷⁷ a transition well known for polyene hydrocarbons with *cis*-geometry. Although for the lower symmetry retinals this selection rule is no longer valid, the corresponding band is still referred to as a “*cis*” band. It has been observed for retinals possessing more centrally located *cis* double bonds (i.e., 9-*cis* or 11-*cis* isomers) that the β -bands are more intense.⁷⁸

With regard to the γ -band, the current assumption is that it can be associated with either the second “¹Bu*⁺” state or the second “¹Ag*⁺” (*cis*) state, by accordance with its characterization in *trans* linear polyenes.⁷⁹⁻⁸¹

The UV spectra of the 12-s-*cis* conformationally locked retinals **79a-h** is represented in Figure 17. From a comparative study of the isomeric retinals, in this 12-s-*cis*-locked series some of the following points can be noted in regard to assignment of the bands to particular conformations.

The most striking feature of the absorption spectra of retinals **79a-d** with 11-*cis* configuration is the presence of very prominent β -bands and weak α -bands, which supports the assignment of the β -bands to the “¹Ag*⁺” state. As the population of the 6-s-*trans* conformers would not be expected to change in these retinals, the proposal that the β -bands

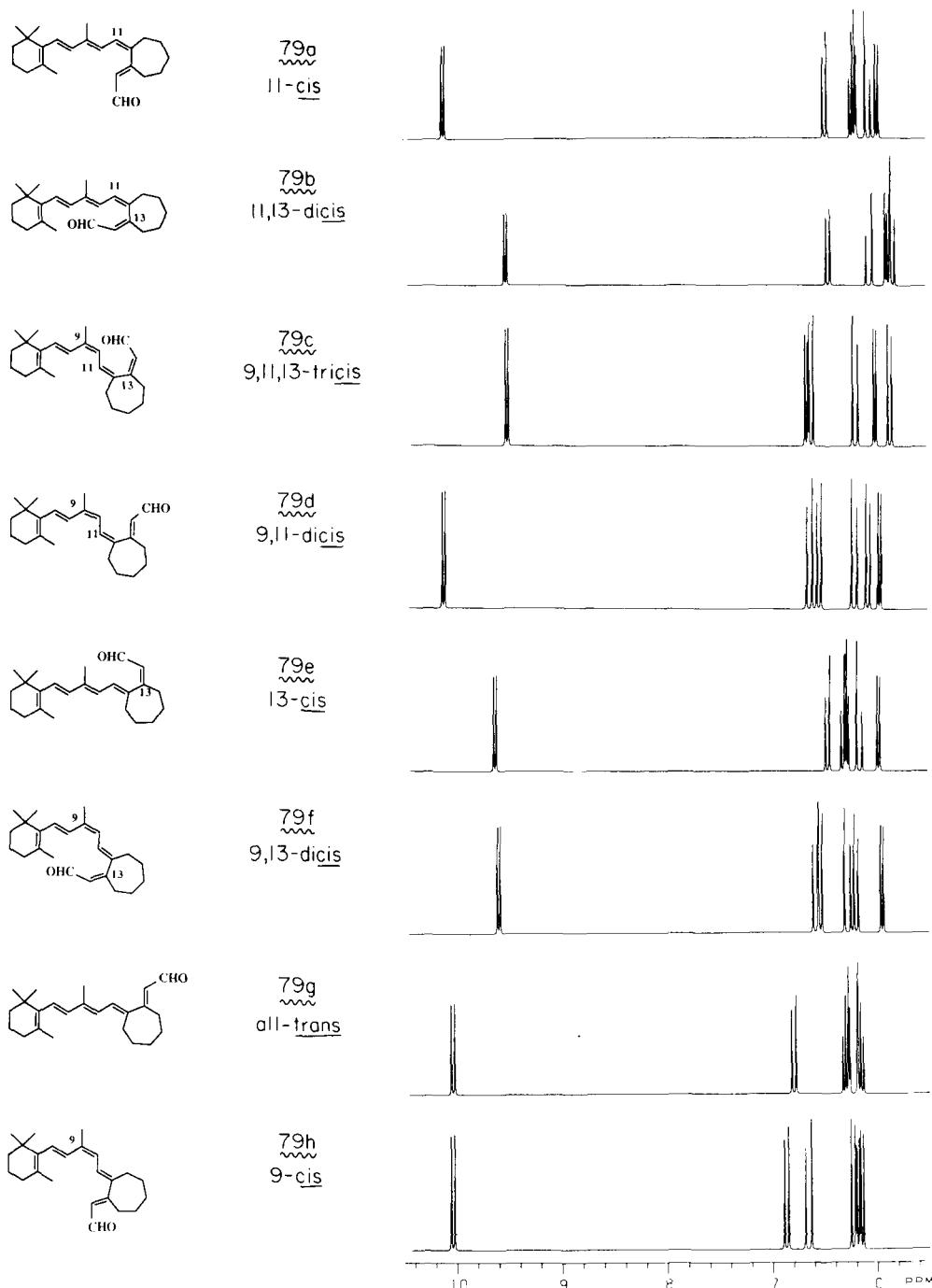


FIGURE 16. Simulated ¹H NMR data for 12-s-cis-locked 12,20-tetramethyleneretinals **79a-h**. The data from Table 2 were evaluated without iteration.

are conformeric in origin⁷⁹ (due to the “¹Bu*⁺” state of 6-s-*trans* conformers, present in solution to the extent of ~10%, in equilibrium with the predominant 6-s-*cis* conformers, which would be responsible for the main α -band) is invalidated.

The absorption maxima for the β -band in the same compounds is red-shifted (~295 to 300 nm) with respect to its normal value in retinal isomers. If the low energy position of

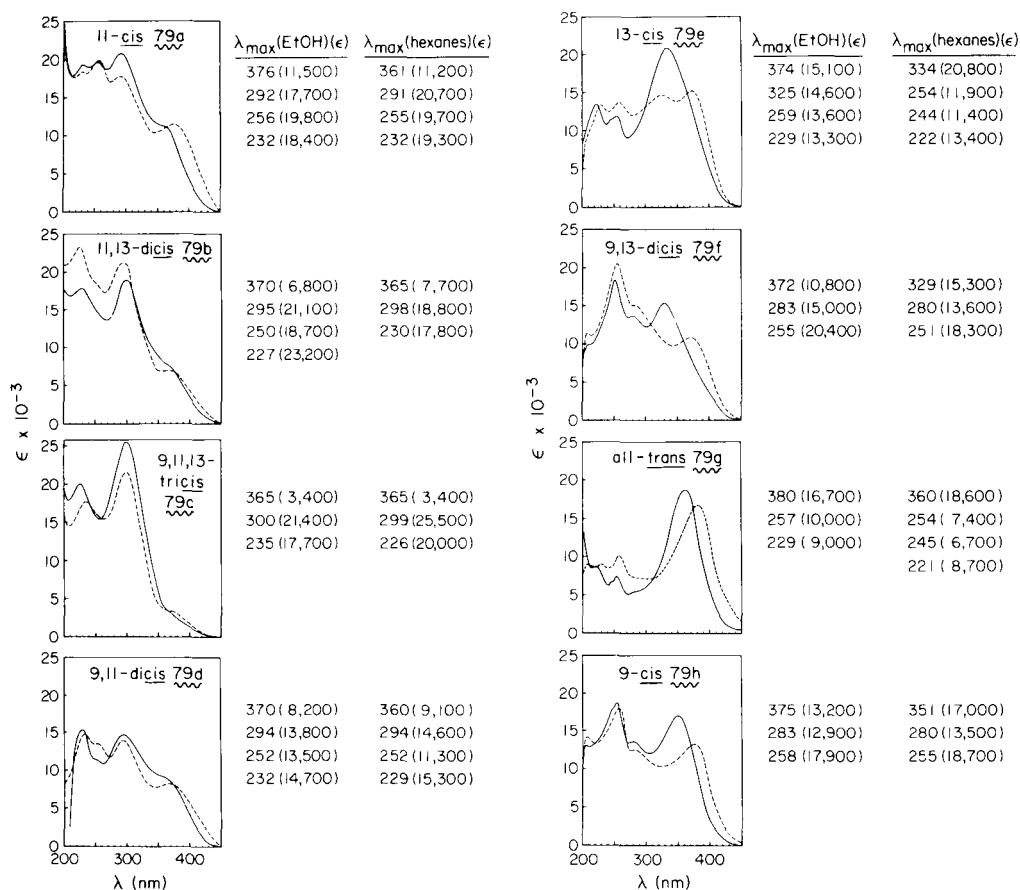


FIGURE 17. Absorption spectra and quantitative data of the 12-s-cis-locked retinals 79a-h in hexanes (—) and 95% ethanol (---) at room temperature.

the β -band was explained as derived from 6-s-cis conformers,⁷⁷ the data for 79a-d points to a contribution of both 6-s-cis and 12-s-cis conformations, thereby maximizing a double s-cis-induced red shift in the position of the β -band.

Removal of the 11-cis geometry causes increased α -band intensity, resulting in a progressive red shift of the low energy maximum, with concomitant decrease in the intensity of the β -band when lowering the “cis” character by changing the geometry at the internal C₉ and C₁₁-double bonds (in agreement with its assignment as the *cis*-band).

The 12-s-cis-locked retinals exhibit prominent γ -bands (which, in fact, are the most intense maxima in the UV spectra of 9--cis 79h and 9,13-di-cis 79f isomers) with progressive decreases in their intensity with successive loss of *cis* geometry. This behavior, parallel to that described for the β -bands, can lead to the assumption that the γ -band is associated with a second “¹Ag*+” *cis* band. However, theoretical calculations^{75-77,82} do not predict the existence of a second “¹Ag*+” state with a wavelength above 200 nm. One possibility for the assignment of the γ -band is to produce a C₅-C₁₂ “fragment chromophore” by distorting the chromophore around the 12,13-single bond to near orthogonality. The need of accounting for the lower lying transitions from the manifold of calculated states will require that only a small proportion of distorted chromophores generate the γ -band, while the remaining population with more planar 12-s-cis conformations contribute to the α - and β -bands.

The 12-s-cis-locked retinals exhibit a red shift of the α -band in polar solvents (\sim 20 nm) of greater magnitude than that observed for the parent retinols. This effect could be explained

if the change in dipole moment upon excitation is greater in 12-s-*cis* than in 12-s-*trans* conformers. Also, the enhanced bathochromic solvent shift could be due to enhancement of more planar 12-s-*cis* conformers in polar solvents due to stabilization of conformers with higher dipole moments, and both arguments can be used with many of the isomers examined.

The UV spectra of the 13-*cis* isomer **79e** deserves further comments. In hexanes, the spectrum has a maximum at 334 nm, with an undefined β -band. In ethanol the maxima are observed at 376 and 325 nm. The “ ${}^1\text{Bu}^{*+}$ ” state is a polar state in the valence bond sense, and is better depicted by resonance structures with large charge separation. The resolution of the 374-nm band is explicable on the basis of a red shift of the “ionic ${}^1\text{Bu}^{*+}$ ” state. That explanation is not valid for the red shift of the “ ${}^1\text{Ag}^{*+}$ ” state, assigned previously to the 325-nm band. An interesting possibility, which needs to be tested by two-photon spectroscopy is that the 325-nm band is formed by superposition of “ ${}^1\text{Bu}^{*+}$, ${}^1\text{Ag}^{*-}$, and ${}^1\text{Ag}^{*+}$ ” states.

One of the goals of the present study was the finding of an explanation for the unusual electronic absorption spectra of 11-*cis*-retinal (**1**), which displayed temperature-dependent behavior, showing at low temperatures increased intensity of the α -band and decreased intensity of the β - and γ -bands, especially in polar solvents. Theoretical calculations utilizing singly and doubly excited configurations in the PPP formalism predict that the oscillator strength for the “ ${}^1\text{Bu}^{*+}$ ” absorption (α -band) is increased for the 12-s-*trans* conformation of 11-*cis*-retinal while the oscillator strength for the “ ${}^1\text{Ag}^{*+}$ ” absorption (β -band) is increased in the planar 12-s-*cis* conformation.⁸² Our results⁸³ on the 12-s-*cis*-locked analog **79a** (strong β - and γ -bands and moderate α -band) together with those obtained by Nakanishi²⁴ in the 12-s-*trans*-locked series **3** (strong α -band and moderate β - and γ -bands) are in accord with the calculations and confirm the original assumption that 11-*cis*-retinal exists in solution as an equilibrium mixture of 12-s-*cis* and 12-s-*trans* conformers, the latter being predominant at lower temperature. However, we are aware of alternative views on this matter.^{19,20,22}

ADDENDUM

Since submitting the original manuscript, several additional studies have been conducted in this laboratory concerning 12-s-*cis* locked retinoids. Investigations of model Schiff base derivatives of 13-*cis*-retinal analogs in these and related systems have been initiated. It has been discovered that *n*-butylamine derived Schiff bases of 12-s-*cis*-locked 13-*cis*-retinals as well as the parent 13-*cis*-retinal and its substituted derivatives are prone to undergo side chain electrocyclizations to form stable 1,2-dihydropyridine derivatives. In view of the extensive use of Schiff base model systems in investigations of bacteriorhodopsin and related pigments, it is evident that caution must be exercised in interpreting electronic and vibrational data of 13-*cis*-retinal derived Schiff base systems because of dihydropyridine contamination. It is intriguing to ask whether formation of 1,2-dihydropyridines or the occurrence of other related pericyclic processes are pertinent to the photocycle of bacteriorhodopsin and other Schiff base derived pigments of biological significance.^{84,85}

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Chapter 10

THE BIOGENESIS OF RETINOIC ACID: A PHYSIOLOGICALLY SIGNIFICANT PROMOTER OF DIFFERENTIATION**Joseph L. Napoli****TABLE OF CONTENTS**

I.	Introduction	230
II.	Physiological Occurrence of Retinoic Acid	230
III.	Biosynthesis of Retinoic Acid	232
IV.	Assay of F9 Embryonal Carcinoma Cell Differentiation	235
V.	Role of Retinoic Acid as an Inducer of EC Cell Differentiation	237
A.	Retinol vs. Retinoic Acid	237
B.	Retinoic Acid vs. Retinoic Acid Metabolites	238
VI.	Structure/Activity Relationships of Aromatic Synthetic Retinoids	240
VII.	Retinoids as Photoaffinity Probes	245
VIII.	Summary	246
	Addendum.....	246
	References.....	247

I. INTRODUCTION

Retinoic acid (RA) can support the systemic functions of vitamin A *in vivo*, but unlike retinol, orally or peritoneally dosed RA cannot support the visual or the reproductive functions of vitamin A in mammals.^{1,2} As expected from its activity *in vivo*, RA induces the differentiation of a broad variety of cells and tissues in culture; generally, RA is a more potent promoter than retinol of differentiation *in vitro*.³ Although the pharmacological utility of RA is appreciated, its physiological significance has been less certain. The apparent partial vitamin A activity of RA *in vivo*, early conclusions that RA levels *in vivo* were quantitatively insignificant, and a lack of information about the biogenesis of RA, has limited acceptance of RA as a naturally occurring retinoid with a physiologically significant role in vitamin A-dependent differentiation.

This chapter discusses the evidence that RA is an endogenous retinoid in the pathway of retinol metabolism under physiological conditions. Data consistent with the notion that RA is physiologically essential to specific vitamin A-dependent functions include the widespread tissue distribution of RA derived from endogenous retinol, rather than pharmacologically dosed retinol, the synthesis of RA by a variety of tissues and cell lines, and concentrations of RA *in vivo* sufficient to support vitamin A-dependent differentiation. A direct role for RA, rather than a metabolite, is supported by its nuclear localization, the enhancement of its activity by agents that arrest its metabolism, and by structure-activity relationship studies with a class of potent synthetic aromatic retinoids that undergo metabolism less readily than RA. The latter class of compounds provides the opportunity of obtaining retinoids with enhanced activity and/or decreased toxicity, as well as generating agents useful for investigating which retinoids are physiologically essential. Finally, synthetic retinoids with appreciable biological activity can serve as parent molecules for the synthesis of azidoretinoids to be used as photoaffinity probes.

II. PHYSIOLOGICAL OCCURRENCE OF RETINOIC ACID

The conclusions of several reports that RA was produced *in vivo* from dosed retinol or exists in significant concentrations as an endogenous metabolite were made based on analytical techniques that could not distinguish RA from the plethora of polar retinoids that are generated, some artifactually, from retinol and/or RA. Sephadex and thin-layer chromatography are not capable of the resolution required to separate RA from the pool of polar retinoids. Even the usually superior technique of HPLC has its potential problems. F9 cells convert retinol into a metabolite that is methylated by diazomethane and comigrates with RA through an HPLC system developed with toluene/hexane. Analysis of the recovered sample with an HPLC system developed with methyl *t*-butyl ether/hexane revealed that only 33% of the material was RA; the remainder was an unidentified metabolite.⁴ Attempts by two different groups to measure RA in human blood by potentially more specific selective ion monitoring by GC/MS used relatively insensitive conditions and produced contradictory and equivocal results. In one study, a very small amount of "retinoic acid" was detected in an indeterminate number of human plasma samples, but no adequate blank was found, perhaps indicating that the assay was producing false positives.⁵ In the second study, endogenous RA was not detected in ten human plasma samples, perhaps reflecting its instability during handling.⁶

Endogenous RA has been purified from rat tissue and rigorously identified.⁷ In two separate experiments, pools of retinol in rat tissues were radiolabeled by oral administration of [³H]retinyl acetate (Expt. 1) or [³H]retinol (Expt. 2) for 5 d. Both the route of administration and the amount dosed (4 µg/day/rat) were physiological. RA and retinol were extracted from kidney and were purified by elution through multiple HPLC systems. The specific

TABLE 1
Specific Radioactivities of Retinoids
Isolated from the Kidneys of Rats⁷

Retinoid	Specific Radioactivity	
	Expt. 1	Expt. 2
	(dpm/ μ mol)	
Original dose	3.3×10^8	4.3×10^9
Retinol	5.7×10^6	9.3×10^7
RA	4.6×10^6	4.5×10^7

Note: Rats were dosed orally for 5 consecutive days with either [³H]retinyl acetate (experiment 1) or [³H]retinol (experiment 2) (4 μ g/d). Twenty-four h after the last dose, retinol and RA were isolated from kidney.

activities of retinol and RA recovered in each experiment were similar (Table 1) and were much less than the retinol dosed, demonstrating that the RA was derived from the total pool of endogenous retinol. Finally, the structure of RA was established unequivocally by GC/MS analysis with comparison of the entire spectrum to that of authentic standard.

RA and RA derivatives have now been measured in human plasma or serum by three different groups. Napoli et al.⁸ developed a GC/MS assay sensitive to 0.25 pmol and determined the concentration of total retinoic acids in male human plasma to be 4.9 ± 1.2 ng/ml (mean \pm SD, n = 12), of which about 75% was all-*trans*-RA. De Leenheer et al.⁹ obtained a mean of 3.5 ± 0.4 ng/ml (n = 37) for all-*trans*-RA. Barua and Olson¹⁰ reported a total of 4.2 ± 1.5 (n = 6) for the sum of RA and retinoyl beta-glucuronide in human serum, of which 43% was underivatized RA. RA has also been detected in rat and fetal calf serum at a concentration for each of 2 ng/ml.¹¹ These results clearly demonstrate that RA is an endogenous retinoid of blood, and that multiple derivatives circulate, including all-*trans*-RA, retinoyl beta-glucuronide, and perhaps 13-*cis*-RA.

The GC/MS assay used for human blood was applied to quantify endogenous RA in the tissues of male rats fed a stock diet.¹² The tissues examined all contained appreciable concentrations of all-*trans*-RA [nmol/g tissue, mean \pm SE, (the number of animals assayed are indicated in parentheses)]: bladder, 1.5 ± 0.4 (8); kidney, 0.13 ± 0.15 (9); liver 0.08 ± 0.03 (9); lung, 0.04 ± 0.004 (6); pancreas, 0.58 ± 0.04 (6); prostate, 2.0 ± 0.05 (15). In addition, RA has been measured in the placenta and fetus during gestation of the rat.¹³ The RA concentration in the placenta was 0.06 nmol/g. In contrast, its concentration in the fetus was relatively high immediately before organogenesis (2 nmol/g) but decreased to 0.01 nmol/g after induction of organogenesis — suggesting a function for RA in initiating organogenesis.

These data not only demonstrate that RA is a naturally occurring retinoid but also show that RA is distributed in many tissues *in vivo* and that the concentrations of RA in tissues are sufficient to induce differentiation. The latter inference is supported by comparing the concentrations of RA in tissues to the concentrations of RA that produce half-maximum differentiation in several assays, i.e., 0.001 to 0.01 nmol/ml medium.^{3,14-16} In fact, these values probably underestimate inherent RA activity because RA has an elimination half-life as short as 3 h in culture.^{4,17} Moreover, RA is notorious for sticking to plastic. Both factors would rapidly lower the effective RA concentration in media and result in an underestimation of RA biological potency.

TABLE 2
Rates of RA Synthesis in Rat Tissues Relative to the
Concentrations of CRBP and CRABP

Tissue	RA synthesis (pmol/h/mg protein) ^a	CRBP		CRABP (pmol/mg protein)
		(pmol/mg protein) ^b	(pmol/mg protein) ^b	
Adrenal	43 ± 8 (n = 3)	—, ND ^c , —	—	ND
Kidney	200 ± 100 (n = 9)	67, 32, 8	—	0.27
Liver	250 ± 100 (n = 15)	20, 30, 7	—	ND
Lung	210 ± 100 (n = 9)	11, 9, 5	—	0.06
Mucosa	100 ± 40 (n = 11)	26, + ^d , 0.5 ^e	—	ND
Spleen	ND (n = 9)	10, 4, 1.5	—	0.1
Testes	330 ± 140 (n = 9)	32, 15, 6	—	5.7

^a Data were obtained from Reference 18 and indicate the rates of RA synthesis from 10 μM retinol in homogenates. Mean ± SD (n = number of animals) representing animal variation.

^b Data for CRBP were calculated from References 21 — 23, respectively. Data for CRABP were calculated from Reference 22. To obtain the first set of values for CRBP, an assumption was made that the amounts of protein/g tissue for Reference 22 were the same as Reference 21.

^c Not detected.

^d Although CRBP was detected in the small intestinal mucosa in this report, no concentrations were given.

^e Average for ileum and jejunum measured separately.

III. BIOSYNTHESIS OF RETINOIC ACID

Two complimentary approaches have been taken in this laboratory to study the biogenesis of RA from retinol, the parent endogenous retinoid. Homogenates have been used to evaluate co-factor requirements, to study the characteristics of enzymes and to determine the tissue distribution of RA synthesis activity.¹⁷ Cells in culture have been used to examine RA synthesis relative to the other routes of endogenous retinoid metabolism and to determine what regulates RA concentrations.¹⁸

A spectrum of vitamin A-dependent tissues converts retinol and retinal into RA.¹⁷ RA synthesis from retinol was detected in homogenates of rat adrenal, testes, liver, lung, kidney, and small intestinal mucosa, but not spleen (Table 2). Several established mammalian cell lines also convert retinol into RA, including cells derived from human carcinomas, dog and pig kidney, mouse teratocarcinoma, and rat ileum, hepatoma, and osteosarcoma (Table 3).¹¹ Note the substantial rate of RA synthesis from 10 μM retinol, a near physiological concentration for several tissues. This rate of synthesis would appear to be sufficient to saturate cellular-retinoic acid binding protein (CRABP) and to provide the concentrations of endogenous RA measured in tissues (Table 4). These data suggest that retinoid target tissues meet their RA needs predominantly by synthesis *in situ*, rather than by sequestering blood-borne RA, which has been synthesized at limited distal sites. It is not clear from these data what types of cells in a given tissue synthesize RA. Do daughter cells produce RA and secrete it to initiate stem cell differentiation? Is induction of RA biogenesis an obligatory and/or hormone-stimulated step in stem cell differentiation?

Mammals fed retinol-deficient, RA-supplemented diets are incapable of spermatogenesis and suffer testicular atrophy.¹⁹ These data have been interpreted as demonstrating that RA does not support the functions of vitamin A in mammalian testes. The conversion of retinol into RA by rat testes, however, suggests that mammalian testes do require RA to support vitamin A-dependent functions. The Sertoli cells in mammals form a blood-testes barrier

TABLE 3
RA Synthesis from Retinol by Established Mammalian Cell Lines¹¹

Cell Line	Species	Type	Relative rate ^a
MDCK	Dog	Kidney epithelia	0.8
T24	Human	Bladder carcinoma	1.0 ¹²
JEG-3		Choriocarcinoma	ND ^b
BeWo		Choriocarcinoma	ND
A431		Epidermal carcinoma	0.5
U-937		Monoblast-like	ND
SCC-9		Squamous cell carcinoma	0.3
SCC-15		Squamous cell carcinoma	0.3
F9	Mouse	Embryonal carcinoma	ND ^c
LLC-PK ₁	Pig	Kidney epithelia	1.0
IEC-18	Rat	Ileum epithelia	0.1
IEC-6		Small intestine epithelia	ND
H4-II-E-C3		Hepatoma	0.04 ^d
RLC		Hepatoma	0.4
Rice		Leydig tumor	ND
ROS 17/2		Osteosarcoma	0.2

^a A relative rate of 1 is roughly equivalent to 150 pmol/h/100-mm plate of confluent cells, obtained at a retinol concentration of 10 μ M.

^b ND, not detected by HPLC assay; less than 10 pmol/h/plate.

^c Not detected with HPLC assay; but detected by using 60 Ci/mmol of [³H]retinol.

^d Detected by combining two plates of cells.

TABLE 4
Subcellular Distribution of RA Synthesis Activity in Rat Tissues^{11,17}

Expt.	Tissue	Units/g ^a in P + S	% Activity ^b in S
1	Liver	3.1	88
2	Liver	1.9	97
3	Kidney	1.1	100
4	Mucosa ^c	0.045	97

^a Virtually all of the activity was recovered in the P + S (particulate + supernatant) fraction. A unit is nmol of RA formed/min from retinol.

^b Fraction of the total activity recovered in the cytosol.

^c Small intestinal mucosa.

that may prevent uptake of RA from the interstitium. On the other hand, RA synthesized in the Sertoli cells may be available to the CRABP that is localized in the spermatocytes and spermatids.²⁰ In support of this hypothesis, primary cultures of Sertoli cells, isolated from rat testes, are capable of converting retinol into RA at a rate similar to the rate observed in homogenates of whole testes.¹¹

In rat liver, small intestinal mucosa and kidney, the activity converting retinol into RA was recovered in the post-mitochondrial supernatant (P + S fraction). Separation of this fraction into microsomes (P) and cytosol (S) showed that the activity in kidney is predominantly cytosolic, and the major, but not exclusive subcellular locus in liver and intestine is cytosol. This is interesting because cytosol is the locus of the two cellular retinoid-binding proteins, cellular retinol-binding protein (CRBP) and CRABP.

There is no obvious relationship, however, between the presence or concentrations of

CRBP and CRABP in tissues and their ability to synthesize RA (Table 2).²¹⁻²³ The observation in tissue homogenates is supported by results in established cell lines. The mouse embryonal carcinoma cell line F9 has detectable CRBP (0.28 to 0.75 pmol/mg protein) and CRABP (0.44 to 0.55 pmol/mg protein),²⁴⁻²⁶ but a relatively low rate of RA synthesis from retinol (about 0.025 pmol/h/10⁷ cells, at a retinol concentration of 150 nM).^{4,27} In contrast, the pig kidney cell line LLC-PK₁ has no detectable CRBP (less than 10 fmol/mg protein) and relatively low levels of CRABP (0.07 pmol/mg protein), but a relatively high rate of RA synthesis (about 100 pmol/h/mg protein, 10 μM retinol).¹⁸ Although CRBP and CRABP do not appear to be obligatory to RA synthesis, it is quite possible that they are involved in the process. It is reasonable to anticipate that RA synthesis *in vivo* is initiated by direct transfer of retinol from CRBP to retinol dehydrogenase, and that CRABP is charged by direct transfer of RA from retinal dehydrogenase.

It is sometimes assumed that retinol dehydrogenase is cospecific with alcohol dehydrogenase — an enzyme for which ethanol has been assumed to be the preferred substrate. It is now clear that alcohol dehydrogenase in human consists of at least 20 isozymes;²⁸⁻³⁰ in rat at least four isozymes exist.³¹ All oxidize ethanol poorly relative to numerous other alcohols of widely varying structures, although some oxidize ethanol far more efficiently than others. Given this diversity, it would seem simplistic to assume that retinol dehydrogenase and “alcohol dehydrogenase” are cospecific. Moreover, Koen and Shaw³² have shown that staining with retinol or ethanol produced different bands on starch gel electrophoresis of rat liver cytosol. Consistent with this observation, ethanol was neither an effective inhibitor of RA biosynthesis by LLC-PK₁ cells,¹⁷ nor of rat intestinal retinal reductase, which is presumably the retinol dehydrogenase working in the reverse direction.³³ In rat liver and kidney homogenates, an ethanol concentration of 100 mM, 10⁴-fold greater than the concentration of retinol, was required to inhibit the synthesis of RA 50 and 30%, respectively.¹⁷ Such a high ethanol concentration could be working by nonspecific means—solvent effects, competition for NAD, or by generating NADH, a potent inhibitor of RA synthesis.¹⁷ It is likely that retinol dehydrogenation is catalyzed by multiple isozymes, including some that are sensitive to and/or may also recognize ethanol. The physiological importance and potential toxicity of RA, however, argue for specificity and control in the biogenesis of RA from retinol.

The recent observation that beta-carotene serves as a substrate for RA synthesis in cytosol prepared from rat liver, intestine, lung, kidney and testes adds another dimension to RA biosynthesis.^{11,34} Carotenoids are well-established dietary sources of vitamin A. The conversion of beta-carotene into retinol in intestine and the integration of the retinol into the various pathways of vitamin A metabolism is well-accepted, but not understood is the role of beta-carotene as a source of RA, or the importance of extra-intestinal carotenoid metabolism to vitamin A homeostasis. Because humans accumulate carotenoids in many tissues, often in large quantities, the recent results showing that beta-carotene is a RA precursor reveal a fascinating possibility that certain tissues may support RA-dependent processes without requiring retinol as the RA precursor. Another aspect of this interesting prospect is the mechanism of the potential anticarcinogenic activity of carotenoids. Whether or not a carotenoid requires provitamin A activity to decrease the incidence of tumors in lab animals, seems to depend on the nature of the carcinogen used in the experiments. Carotenoids that do reduce the incidence and numbers of tumors by virtue of provitamin A activity may be effective through an ability to raise steady-state levels of RA.

Quite oddly, in incubations in which beta-carotene is converted into roughly similar proportions of retinol and RA, retinal is converted predominantly, if not exclusively, into RA.¹¹ Clearly, retinal in solution is metabolized differently than is beta-carotene over broad concentration ranges of either substance. In other words, the putative retinal derived from beta-carotene does not appear to equilibrate with preformed retinal added to the incubation.

TABLE 5
Laminin Secreted into the Medium of F9 Cells During the
Final 24 h of a 96 h Incubation¹⁴

Dose ^a	μg/ml medium	μg/10 ⁶ cells
None	0.5 ± 0.2	0.2 ± 0.1
Dibutyryl cAMP	0.7 ± 0.1	—
Retinoic acid	1.5 ± 0.1	2.1 ± 0.2
Retinoic acid + dibutyryl cAMP	9.0 ± 1.0	7.4 ± 0.5

^a Cells were untreated or were treated with 1 mM dibutyryl cAMP, 100 nM RA, or both for 96 h.

This observation suggests that carotenoid cleavage may not be a simple case of producing and releasing retinal into solution and indicates that further studies are in order.

IV. ASSAY OF F9 EMBRYONAL CARCINOMA CELL DIFFERENTIATION

Embryonal carcinoma (EC) cells, or teratocarcinoma stem cells, are used extensively as models for differentiation and embryonic development. Several of these established cell lines are advantageous for studying retinoid structure/function relationships and the mechanism of retinoid action because of their homogeneous cell populations, their production of quantifiable indicators of differentiation and their sensitivity to retinoids. Retinoids, for example, are the only naturally occurring substances that promote differentiation of the F9 EC cell line and, on a molar basis, are the most potent inducers of F9 cell differentiation known.³⁵⁻⁴⁰ Retinoids, in the presence of dibutyryl cAMP, induce monolayers of F9 cells to differentiate into parietal endoderm.³⁷ The differentiated cells are no longer tumorigenic, have altered morphology, and synthesize many proteins different from the stem cells,⁴¹ including laminin and plasminogen activator, which are secreted into the medium.^{37,42,43}

A precise and technically convenient method of evaluating retinoid potency in F9 cells has been developed with an ELISA for quantifying laminin.¹⁴ The typical assay involves treating F9 cells growing on nongelatinized plates with 1 mM dibutyryl cAMP and graded concentrations of retinoids for a total of 96 h with medium changes every 24 h although a single 24 h treatment is sufficient to achieve a response 80% of maximum. The amount of laminin secreted into the medium in the final 24 h is measured by a nonequilibrium ELISA,⁴⁴ modified for use with medium from cultured cells. Data generated by the ELISA are statistically analyzed and fitted to dose-response curves by the Allfit program,^{45,46} rewritten for use with an IBM personal computer. The Allfit program fits data to dose-response curves by non-linear regression analysis and compares multiple dose-response curves by analysis of variance. The Allfit program tests whether each curve is sigmoidal, determines the concentration producing a half-maximum response, compares the slopes of several curves, and determines whether maximum responses are equivalent. Thus, the F9 cell/laminin ELISA/Allfit combination offers a well-characterized model system, convenient technology, and statistical treatment of experimental data. This approach should provide the opportunity to examine more subtle aspects of retinoid mechanism and/or structure and should help minimize the discordant results often reported in retinoid assays, particularly those with EC cells.

As expected from previous work,³⁶ dibutyryl cAMP alone had little effect on laminin secretion in F9 cells (Table 5). Exposure to RA alone increased laminin secretion/10⁶ cells tenfold. Simultaneous exposure to both dibutyryl cAMP and RA caused a 37-fold increase in laminin secreted/10⁶ cells. This quantity of laminin secreted into the medium in the final 24 h of a 96 h exposure to RA and dibutyryl cAMP represents over 1% of the total protein in the cells (about 13 μg/mg cellular protein).

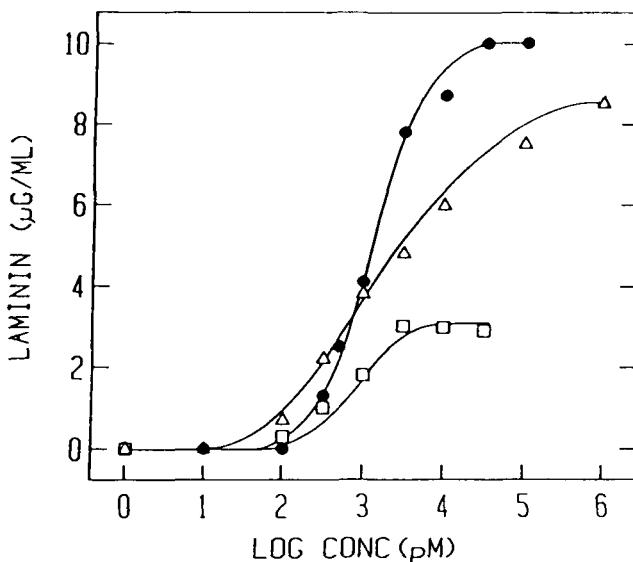


FIGURE 1. RA dose-response curves determined by the F9 cell/laminin/ELISA. F9 cells were incubated with graded concentrations of RA for 96 h with dibutyryl cAMP (closed circles) or without dibutyryl cAMP (open squares), or with RA for the first 24 h only and with dibutyryl cAMP for the entire 96 h (open triangles). The data are expressed as μg of laminin/ml in the medium of the last 24 h of the incubation.

Dose-response curves for RA alone and RA in the presence of dibutyryl cAMP were obtained (Figure 1). In the presence or absence of dibutyryl cAMP, the concentration of RA that produced a half-maximum response was 1.3 nM, i.e., dibutyryl cAMP amplified the magnitude of the RA effect on laminin production but did not change the inherent activity of RA.

The laminin/ELISA assay appears to be a more sensitive assay for F9 cell differentiation than the more commonly used plasminogen activator assay, which measures the degree of fibrinolysis. The RA concentration that induced a half-maximum response in F9 cells, determined with the laminin/ELISA assay,^{14,27} was two orders of magnitude lower than that estimated from the plasminogen activator assay.^{36,47} Moreover, 24 h of retinoid treatment induced differentiation, as indicated by changes in morphology, and induced laminin secretion that was 80% of maximum; maximum laminin secretion was observed by 48 h (Figure 2). In contrast, the response observed with the plasminogen activator assay after 24 h of retinoid treatment was only slightly above background—48 h seemed to be required for a marked response. The response at 48 h was about 25% of the response at 96 h and the response at 96 h has not been shown to be the maximum response.³⁶ Since no maximum has been determined, it is probably inappropriate to apply the concept of “half-maximum response” to data generated by measuring fibrinolysis. These differences between the two assays could arise from the nature of the measurements. The plasminogen activator assay relied on the accumulation of plasminogen activator in the medium of embryonal carcinoma cells, but did not quantify the amount of plasminogen activator synthesized and secreted. Instead, the activity of plasminogen activator is measured by assessing the conversion of plasminogen to plasmin. This assessment is indirect; plasmin-mediated fibrinolysis of [¹²⁵I]fibrin is used to determine the degree of plasminogen activation. The dependence of the assay on enzyme kinetics is likely confounding the estimation of the actual amount and rate of plasminogen activator synthesis. The assay may require accumulation of a critical (and larger) amount of plasminogen activator before fibrinolysis can be observed by the

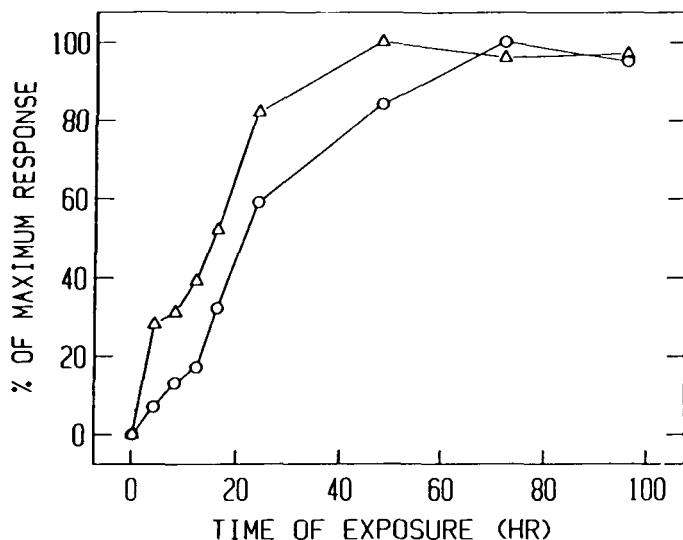


FIGURE 2. Degree of F9 cell differentiation versus time of retinoid exposure. F9 cells were incubated with dibutyryl cAMP for 96 h and with either 100 nM RA (triangles) or 1 μ M retinol (circles) for the times indicated.

techniques used. Another factor to consider is the use of gelatinized plates in the estimate of RA potency with the plasminogen activator assay. Gelatin affects have not been examined on the concentration of RA available to the cells. Alternatively, it is possible that RA has fundamentally different affects on the rates and amounts of production of laminin and plasminogen activator. The expressions of the genes for these two proteins are likely to be results, rather than causes, of differentiation.

V. ROLE OF RETINOIC ACID AS AN INDUCER OF EC CELL DIFFERENTIATION

A. Retinol vs. Retinoic Acid

F9 cells provided an opportunity to compare the rates at which retinol and RA induce differentiation. The onset for RA was faster than that of retinol (Figure 2). As little as 6 h of exposure to RA produced a significant response and a response that was fivefold greater than that achieved after 6 h of exposure to retinol. Maximum response required 72 h of exposure to retinol, in comparison to 48 h for RA. Not only was retinol slower acting, it was also 0.6% as potent as RA (Figure 3), inducing a half-maximum response at 226 nM.¹⁴ Retinol was the least active naturally occurring retinoid tested—it was an order of magnitude less active than the catabolites 18-hydroxy- and 13-cis-4-oxo-retinoic acids (Table 8). On the other hand, the elimination half-life of retinol was fivefold longer than that of RA, and cell-associated retinol was 150- to 3700-fold higher than RA, at concentrations of each that produce equivalent responses (Table 6). Despite its advantages in metabolic stability and intracellular concentrations, retinol was 175-fold less potent than RA. These results extend results obtained in numerous test systems that demonstrate that RA is more potent than retinol in promoting differentiation, with differences *in vitro* between the two as large as three orders of magnitude.

Contrary to initial conclusions, F9 cells did convert retinol into RA, albeit at the low rate of 54 fmol/2 h/10⁷ cells. Even though this rate is low, it is potentially significant. When F9 cells are exposed to RA for 12 h, a response of 40% of maximum was detected (Figure 2). Exposure to retinoic acid for 24 h doubled the response. During the second 12 h period,

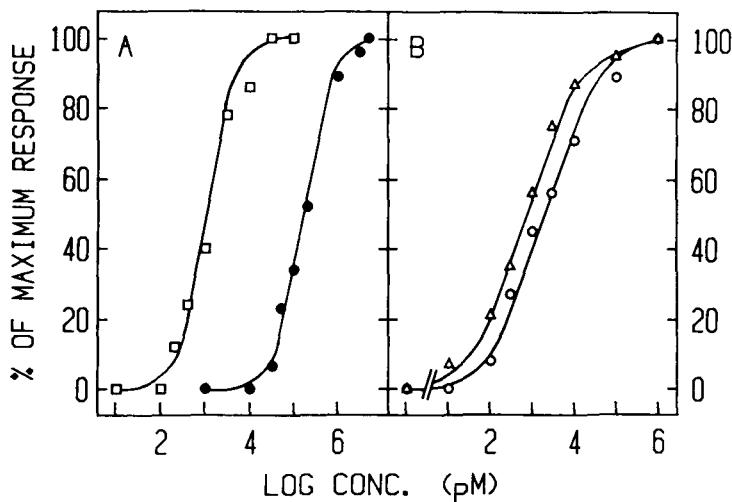


FIGURE 3. Relative potencies of RA and retinol in F9 cell differentiation: the effect of ketoconazole on RA potency. (A) Cells were incubated with RA (squares) or retinol (closed circles) and dibutyryl cAMP for 96 h. (B) Cells were incubated with dibutyryl cAMP and RA for 24 h in the presence of ketoconazole (triangles) or in its absence (open circles).

TABLE 6
Comparison of RA and Retinol Activity, Rates of Catabolism and Uptake by F9 EC Cells^{4,27}

Retinoid	ED ₅₀ (nM)	t ^{1/2} ^a (h)	Concentration ^b (pmol/10 ⁶ cells)
Retinol	226	16	147 — 148
RA	1.3	3.5	0.013 — 1

^a Elimination half-life was determined at concentrations that produced maximum responses, 50 nM and 1 μ M for RA and retinol, respectively.

^b These data represent the range of concentrations in cells from the time of maximum accumulation through 24 h after addition of 50 nM RA or 1 μ M retinol.

the average concentration of RA in cells treated with 50 nM RA at time zero was less than 10 fmol/10⁷ cells. Clearly, concentrations of RA as low as fmol/10⁷ cells had decisive effects. Another perspective is provided by the report of 2500 copies of CRABP/F9 cell.⁴⁸ As much as 50% of this concentration of CRABP could be saturated every 2 h at the rate of RA synthesis observed. In support of these results, Sherman⁴⁹ obtained mutants of EC cells that did not respond to retinol but did respond to RA. In contrast, mutants that did not respond to RA also did not respond to retinol. These data suggest that either RA, or a metabolite of RA, is an activated form of retinol in F9 cells.

B. Retinoic Acid vs. Retinoic Acid Metabolites

EC cells rapidly convert RA into a plethora of polar metabolites.^{4,50} To examine whether metabolites are responsible for RA effects, RA metabolism was studied during the differentiation of F9 cells.^{4,27} RA itself was the major retinoid in the cells at all times analyzed (0 to 24 h); the metabolites were excreted into the medium (Table 7). Notably, the sole

TABLE 7
Nature of Retinoids in F9 Cells and Their Medium
after Incubation with RA^{4,27}

% Retinoids as RA

	Time (h)	
Locus	6	24
Medium	44	0.5
Cells	76	60
Nuclei	94	

Note: Cells were incubated with 50 mM all-*trans*-RA for the times incubated. The proportion remaining as RA was determined by HPLC. At all times 99% of the total retinoids was found in the medium.

retinoid in the nucleus was RA. This is consistent with a model of cellular uptake of RA, specific transfer of RA to the nucleus, catabolism of "excess" RA (perhaps that not protected from degradation by CRABP), and excretion of the catabolites into the medium.

The results discussed above indicate that RA *per se* acts to stimulate differentiation and suggest that the potency of RA is stinted by its rapid degradation. An opportunity to test this hypothesis was afforded by the observation that imidazole antimycotics inhibit RA metabolism.⁵¹ Ketoconazole, clotrimazole and miconazole, at concentrations of 10 μM, inhibited the metabolism of RA by F9 cells 84, 62, and 12%, respectively, during 4 h of incubation. All were more potent than the well-known cytochrome P-450 inhibitor metyrapone, which inhibited about 2% during the same experiment.

Ketoconazole (10 μM) increased the elimination half-life of RA in F9 cells 3-fold to 11.6 h (Figure 4). Ketoconazole alone did not stimulate differentiation, but decreased the concentration of RA required to produce a half-maximum response from 1 to 0.3 nM (Figure 3). This data indicate that RA acts without modification to induce differentiation.

The failure to date to identify any naturally occurring retinoid with activity greater than that of RA, using a variety of *in vivo* or *in vitro* assay systems, including EC cells, is consistent with the demonstration that arresting RA metabolism enhances its potency. Although there are claims of metabolites and/or derivatives, notably 13-cis-RA and the glucuronide of RA, having activity equivalent to RA, the conclusions depend upon test systems capable of converting both compounds into all-*trans*-RA. It would seem to be a formidable task to establish that these compounds are not acting by generating RA, given the minute amounts of RA needed to initiate differentiation.

Structure/activity studies with the F9/laminin/ELISA confirm and extend the conclusion that RA metabolism is catabolic in nature. Oxidation, isomerization and covalent modification of the carboxyl group, all diminish the potency of RA (Table 8). Multiple metabolic alterations diminish activity synergistically. For example, 4-oxo-RA was 0.5-fold as active as RA and 13-cis-4-oxo-RA was 0.04-fold as active as RA. Moreover, the effect of 13-cis-isomerization was consistent: 13-cis-RA was 10-fold less potent than RA; 13-cis-4-oxo-RA was 10-fold less potent than 4-oxo-RA. These data, considered with the demonstration that the major metabolites of 13-cis-RA are derived from all-*trans*-RA,⁵² suggest that 13-cis-RA provides a reservoir *in vivo* for the release of all-*trans*-RA.

The results with 13-cis-*N*-ethylretinamide and *N*-4-(hydroxyphenyl)retinamide in the F9/laminin/ELISA assay exemplify an advantage of this test system in determining structure/activity relationships and examining the mechanism of retinoid action. These compounds

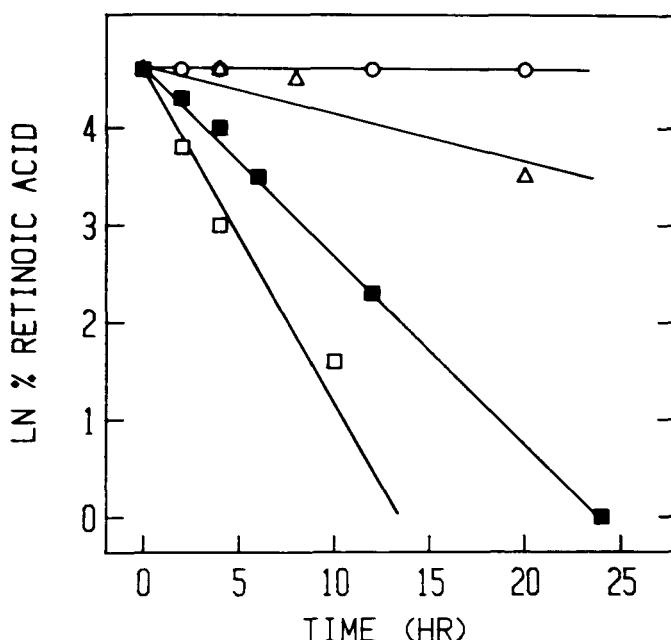


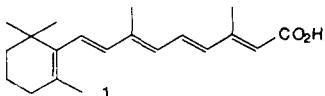
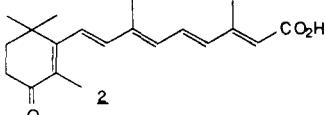
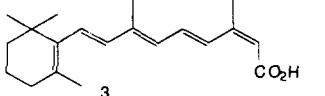
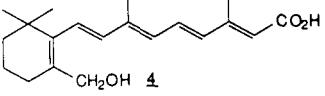
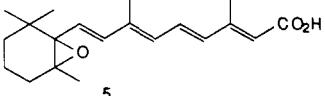
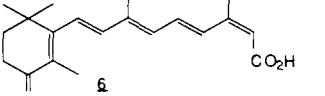
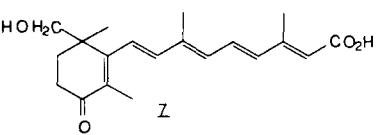
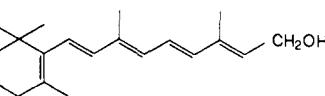
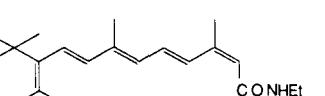
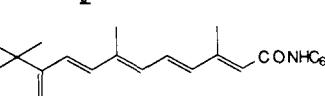
FIGURE 4. Rates of RA metabolism in F9 cells. The amount of 50 nM RA remaining with incubation time was determined in medium alone (circles), in the presence of cells (closed squares), in the presence of cells and ketoconazole (triangles), and in the presence of cells that had been preincubated with RA (open squares).

are both effective, but tissue specific, chemopreventive agents *in vivo*.⁵³ Their inactivity in the F9 system suggests either that they act as prodrugs and have little inherent activity before metabolism, or the mechanism by which they act as chemopreventive agents is different from the mechanism by which RA induces differentiation. The last contention is not unreasonable because retinoids exert plural effects and there is evidence that retinoids have extranuclear as well as nuclear sites of action.⁵⁴ Testing compounds such as these carboxyl derivatives in culture systems that convert them into RA tends to obscure potential insight into differences in mechanisms of action and does not always allow unequivocal conclusions concerning structure/activity relationships.

VI. STRUCTURE/ACTIVITY RELATIONSHIPS OF AROMATIC RETINOIDS

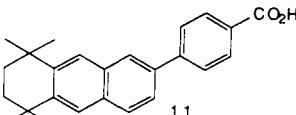
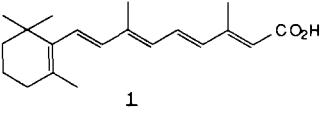
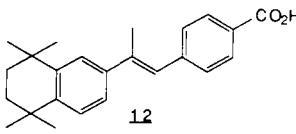
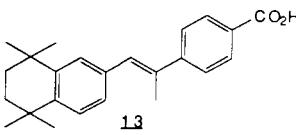
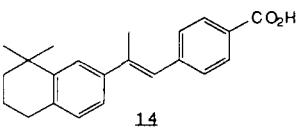
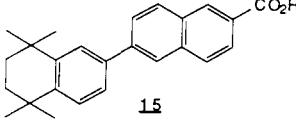
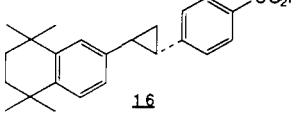
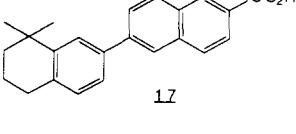
The F9/laminin/ELISA assay was used to evaluate a series of aromatic retinoids, synthesized by Dawson and colleagues in ongoing studies of the relationship between retinoid structure and activity (see Chapter 16, this volume). Retinoid potency may depend on a combination of two properties—metabolic stability and affinity of binding to receptors that mediate their effects. *Evidence that an inverse relationship exists between the elimination half-life of RA and its potency is essential support for the hypothesis that retinoids with increased metabolic stability may be more potent than RA.* The demonstration that arresting RA metabolism *does* amplify RA potency,¹⁴ discussed earlier, provides the foundation for the contention that metabolism affects potency. In addition to the potency derived from increased metabolic stability, a synthetic retinoid with a configuration that mimics closely the conformation assumed by RA upon receptor binding might have increased potency because of enhanced receptor affinity. Although RA is normally depicted as shown in Table

TABLE 8
Activity of Nonaromatic Retinoids in F9 Cell Differentiation¹⁴

Retinoid	Structure	ED ₅₀ (nM)	Potency (%)
RA		1.3	100
4-Oxo-RA		2.3	50
13-cis-RA		12	10
18-Hydroxy-RA		16	8
5,6-Epoxy-RA		17	8
13-cis-4-Oxo-RA		34	4
4-Oxo-16-hydroxy-RA		77	1
Retinol		226	0.6
13-cis-N-Ethylretinamide		Inactive	
N-4-(Hydroxyphenyl)retinamide		Inactive	

9, this conformation is not necessarily the one assumed upon binding to receptors. The analogs in Table 9 were synthesized to continue testing this hypothesis, i.e., to determine the effects of resistance to metabolism and restricted rotation about the single bonds of retinoids. The new analogs⁵⁵ and a previously tested aromatic retinoid,⁵⁶ compound 12, were compared to RA. The aromatic retinoids fell into three categories by activity: high, ED₅₀ < 100 nM (Table 9); modest, ED₅₀ > 100 nM (Table 10); negligible, no activity observed

TABLE 9
Activities of Aromatic Retinoids in the
Induction of F9 Cell Differentiation

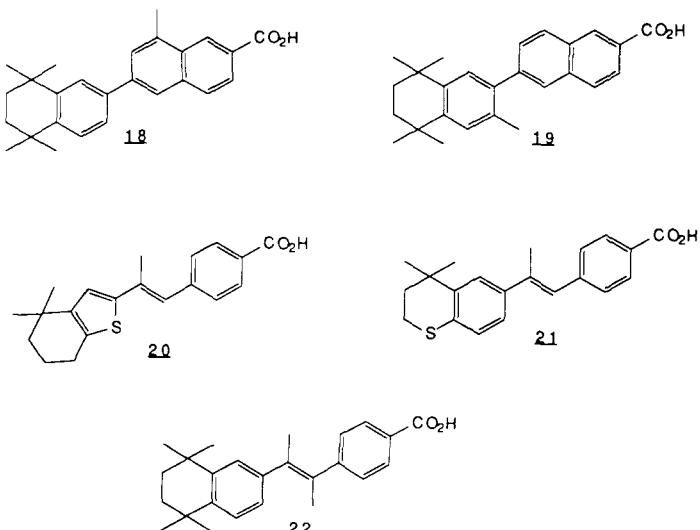
Retinoid	$ED_{50}(nM)$
	0.2
	1.5
	1.5
	1.5
	8
	31
	90
	184

up to $5\mu M$ (Table 10). Three of the synthetic retinoids, compounds **11**, **12**, and **13** had activity greater than or equal to that of RA. Each of these three compounds, however, behaved somewhat differently.

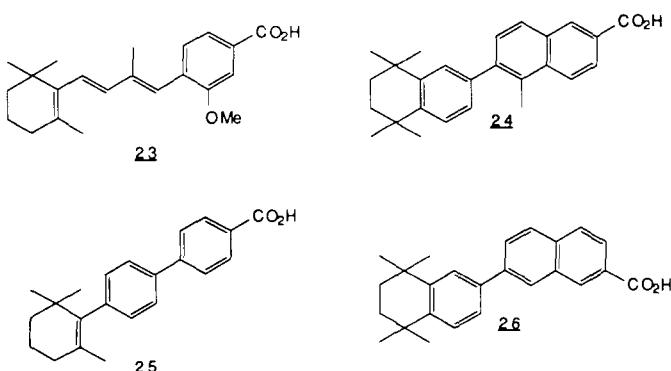
In compound **11** the double-bond system of RA has been replaced by aromatic rings. This severely restricts the conformation assumed upon receptor binding; the only remaining point of flexibility is the single bond joining the terminal benzene ring to the tetrahydroanthracene ring. Compound **11** was about 7.5-fold more potent than RA and was also more potent than **12**, the most potent synthetic retinoid reported previously. Perhaps even more interesting, compound **11** produced a maximum response 30% greater than that of RA (p

TABLE 10

Aromatic Retinoids with Modest Activity ([Half-Max. Response] Greater than 100 nM)



Aromatic Retinoids with Negligible Activity (No Activity Observed with Concentrations as High as 5 μM)



<0.05) (Figure 5). Compound **12**, a much less restricted analog of RA than **11**, was equipotent with RA, but also was 30% more efficacious than RA; i.e., had a greater maximum response. Of the retinoids tested, only compounds **11** and **12** had efficacies greater than RA and the other active retinoids tested. Compound **13**, an analog of **12**, which was as potent as RA (i.e., had the same ED₅₀), was no more efficacious than RA.

Compound **11** was metabolized slowly if at all—virtually all of it was recovered unchanged after 16 h of incubation with F9 cells.⁵⁵ Compound **12** also was not appreciably metabolized by F9 cells.⁵⁷ This could explain the potency of these two analogs. Presently one can only speculate on the mechanism of enhanced efficacy. Inhibiting RA metabolism did not increase its efficacy (Figure 3). It is possible that the phenomenon is caused by recruitment of CRABP and/or enhanced stability of a retinoid receptor complex, perhaps because the configuration of the analog does mimic the conformation that RA assumes upon binding to a receptor that directly mediates retinoid action. Although binding affinity is

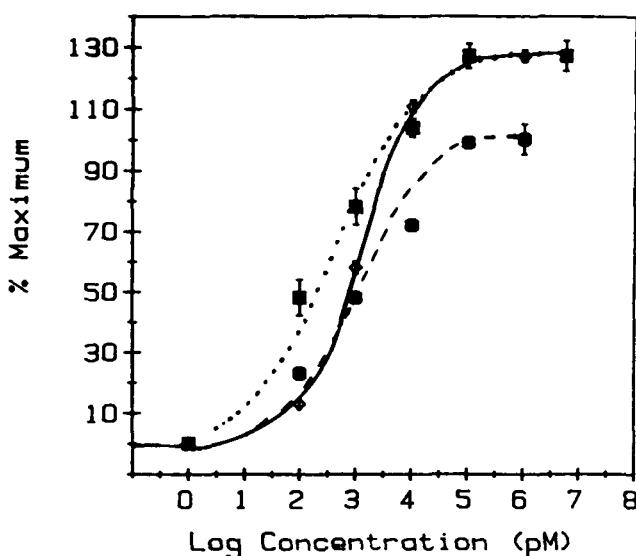


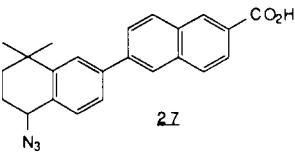
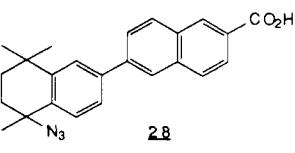
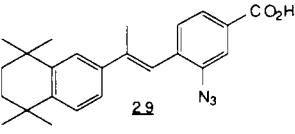
FIGURE 5. Dose-response curves of retinoids in the induction of F9 EC cell differentiation: aromatic retinoid 1 (dotted line, squares); 2 (solid line, triangles); RA (dashed line, circles). Each point is the mean \pm SE of three plates assessed in triplicate. Structures are shown in Table 9.

usually related to affinity to CRABP,⁵⁸ the measurements are approximations because radiolabeled analogs were not generally available for these experiments. Moreover, CRABP apparently transports RA into the nucleus but does not participate directly in genotypic changes.^{59,60} A nuclear receptor exists that presumably affects gene expression after activation by binding to RA.^{61,62} A nuclear acceptor as an obligatory mediator of retinoid action is consistent with the fact that not all cells that differentiate in response to retinoids have detectable CRABP.

The biological data suggest that compound 11 mimics well the conformation that RA assumes upon binding to receptors and may even have higher affinity for the receptors than RA. Compound 13 is an analog of 11 with part of the tetrahydroanthracene ring of 11 removed. This allows more flexibility and diminishes both potency and efficacy, but still provides a compound equipotent with RA. Compound 12 could be considered either as an analog of 11 lacking a greater portion of the anthracene ring of 11 but having a methyl group or as an isomer of 13 about the olefinic bond. Either way, the placement of the methyl group has the interesting effect of increasing efficacy but not potency. Restricting the configuration of the substituents about the olefinic bond by replacing it with a cyclopropane ring, compound 16, diminished potency 90-fold, but did not decrease efficacy relative to RA.

Compound 15, a 2-naphthalenecarboxylic acid (naphthanoic acid) joined by a single bond at C₆ to the C₂ of a tetrahydronaphthalene ring, was about 30-fold less potent than 11 and was only as efficacious as RA. In other words, fixing the configuration about the olefinic bond of 12 and 13 by extending the system into a naphthalene ring system was not as effective as extending the ring system to produce an anthracene-based derivative as in 11. Alterations to the naphthalenecarboxylic acid further diminished potency and possibly efficacy. Analog 18 with a methyl group on C₁ of the naphthalenecarboxylic acid had modest activity (Table 10). Placement of a methyl group on C₅ of the naphthalenecarboxylic acid ring virtually eliminated activity (compound 24), as did substituting a 3-carboxylic acid group for the 2-carboxylic acid group (compound 26).

TABLE 11
Activities of Azido Retinoids in the Induction of F9
EC Cell Differentiation

Retinoid	ED_{50} (nM) (rel. to RA = 1)
 27	31
 28	31
 29	20

Placement of substituents on the aromatic section of the tetrahydronaphthalene ring system was crucial to activity. Compound **19**, an analog of **15** with a methyl group at C₃ of the tetrahydronaphthalene ring, had only modest activity. The aliphatic portion of this second naphthalene ring was also important to optimal activity. Replacement of the 5,5-dimethyl groups by hydrogen atoms reduced activity two- to fivefold, but provided compounds with efficacy equivalent to RA and relatively high potency—for example, the activity of compound **14** compared to that of **12** and that of analog **17** to that of **15**. Replacement of carbon atoms in the partially saturated naphthalene ring with sulfur atoms, compounds **10** and **11**, greatly diminished, but did not eradicate activity.

Radical departure from the tetrahydroanthracene-benzoic acid or tetrahydronaphthalene-naphthalenecarboxylic acid structures, such as the *para*-phenylbenzoic acid **25**, resulted in nearly complete loss of activity, as did adding substituents *meta* to the carboxyl group on the aromatic ring system, compound **23**.

RA and compounds **12**, **14**, **15**, **19**, **21**, and **25** have been tested by Dawson et al.²⁵ in the tracheal organ culture assay. Despite the vast differences in these two assay systems, the results were quite similar. The order of decreasing potency in the tracheal organ culture assay was (the ED₅₀ in pM is in parenthesis): **12** (1), RA (10), **15** (3), **14** (30), **19** (20), **21** (50), and **25** (3000). The order in the F9/laminin/ELISA test system is (the ED₅₀ is given in nM): **12** (1.5), RA (1.5), **14** (8), **15** (31), **19** and **21** (both greater than 100), and **25** (negligible).

VII. RETINOID AS PHOTOAFFINITY PROBES

Retinoids containing photolabile azide groups should be useful photoaffinity probes for studying the mechanism of retinoid action, particularly in the identification of nuclear retinoid receptors. Three azido-substituted aromatic retinoids retained appreciable activity in the F9/laminin/ELISA assay (Table 11). Azides **27** and **28** competed with [³H]RA for binding to calf testes CRABP;⁶³ azide **29** has not yet been tested. Both azides **27** and **28** bound specifically to CRABP upon photolysis, but the secondary azide, **27**, was twice as efficient,

most likely because of lesser steric hindrance. Thirty-seven percent of the available CRABP binding sites were bound by azide **27**. These results demonstrate the feasibility of synthesizing photoaffinity probes from aromatic retinoids that retain biological activity and efficiently occupy the site of at least one retinoid binding protein. Current work is aimed at obtaining radiolabeled azides with improved biological potency and higher binding affinity.

VIII. SUMMARY

Although RA has been used extensively in the past eight years as an agent to induce differentiation *in vitro*, its physiological occurrence and consequently a physiological role for RA had been unresolved. The cumulative evidence of recent years, however, should dispel doubt about the physiological importance of RA. RA has been unequivocally identified as a metabolite of retinol *in vivo* and *in vitro* and as a metabolite of beta-carotene *in vitro*. RA circulates in the blood of several species and has been measured in the tissues of rats. Its activity is rapidly and severely diminished by its rapid rate of catabolism, and yet it is one to three orders of magnitude more potent than its more slowly metabolized precursor, retinol. Moreover, cells retain RA specifically against a concentration gradient of polar retinoids that accumulate in the medium as RA is degraded. The exclusive retinoid localized in the nucleus of F9 cells is RA. These data strongly support the view that RA is an activated metabolite of retinol that has a physiological role in supporting vitamin A-dependent differentiation. The observations that RA potency is increased in the presence of antimycotic imidazole agents that inhibit its metabolism and the activity of an array of aromatic retinoids that attain potency and efficacy greater than RA are further testaments to a direct role for RA in the induction of differentiation. The identification of a RA receptor, similar in structure to the family of receptors for steroids and other hormones, also provides direct evidence of a specific physiological role for RA.

ADDENDUM

RA biogenesis from retinol has been demonstrated in cytosol prepared from the liver, kidney, testes, lung and small intestinal mucosa of the alcohol dehydrogenase negative (ADH⁻) deer mouse.⁶⁴ Because the cytosolic alcohol dehydrogenase associated with ethanol metabolism in the alcohol dehydrogenase positive (ADH⁺) deer mouse is encoded by a single genetic locus, these results indicate that cytosolic retinol dehydrogenase(s) occur which are distinct from the dehydrogenases that catalyze ethanol metabolism. On the other hand, the rate of retinol dehydrogenation was much higher in the ADH⁺ deer mouse strain than in the ADH⁻ strain, indicating that retinol metabolism *in vitro* is catalyzed by more than one class of dehydrogenase. The enzyme(s) significant to retinoic acid biogenesis most likely belongs to the class(es) that is common to both ADH⁺ and ADH⁻ deer mouse strains.

Beta-Carotene is a metabolic precursor of RA, not only in rat tissue fractions, but also in the established human intestinal cell line HT29.^{65,66} The maximum rate of RA production in the presence of a bile-acid type detergent was about 160 to 210 pmol/h/100-mm plate of HT29 cells. The beta-carotene concentration that afforded a half-maximal rate was 20 μM . This indicates that RA is a quantitatively minor metabolite of beta-carotene, which is perhaps why RA has been detected as a product of beta-carotene metabolism only recently. The rate of production, however, is consistent with a significant contribution to physiological RA requirements being met by beta-carotene metabolism.

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Chapter 11

**INHIBITION OF MELANOMA CELL GROWTH BY RETINOIDS:
STRUCTURE-ACTIVITY RELATIONSHIPS****Reuben Lotan and Dafna Lotan****TABLE OF CONTENTS**

I.	Introduction	252
II.	Murine Melanoma Growth Inhibition by RA	252
III.	Effects of Synthetic Retinoids and Related Compounds on Melanoma Cell Growth	252
IV.	Relationship between Effects of Retinoids on Cell Growth and Binding to Cellular Retinoic Acid-Binding Protein (CRABP).....	260
V.	Effects of Retinoids on the Growth of Retinoic Acid-Resistant Melanoma Cells.....	262
VI.	Enhancement of Melanogenesis by Retinoids	262
VII.	Summary and Conclusions.....	264
	Acknowledgments	272
	References.....	272

I. INTRODUCTION

Retinoids constitute an interesting family of biologically active compounds. Of particular importance is their ability to modulate the proliferation and differentiation of various normal and malignant cells *in vivo* and in culture.¹⁻⁴ It is thought that these activities are responsible for the efficacy of retinoids in the treatment of dermatological disorders, in prevention or suppression of carcinogenesis, and in therapy of some neoplastic diseases. The exposure of various tumor cells to certain retinoids in culture has been shown to result in growth inhibition and induction or enhancement of differentiation. Although attempts have been made at elucidating the mechanism(s) of action of retinoids, their mode of action is still unknown.

We have been interested in the antiproliferative action of retinoids ever since we found that retinyl acetate and β -all-*trans*-retinoic acid (RA) can inhibit the growth of many rodent and human tumor cells of different histopathologic origin and modes of transformation.⁵ The cloned murine melanoma S91-C2 was one of the most sensitive cell lines; therefore, we have used these cells to characterize the anti-proliferative activity of RA.⁶⁻¹¹ This chapter describes the effect of RA and numerous synthetic analogs of vitamin A and related compounds on the growth of the S91-C2 melanoma cells in culture.

II. MURINE MELANOMA GROWTH INHIBITION BY RA

The exposure of S91-C2 mouse melanoma cells to RA results in dose- and time-dependent changes in cell morphology (Figure 1) and cell proliferation rate (Figure 2). The cells become elongated, extend long dendrite-like processes, and their growth is inhibited.⁶⁻⁹ The reduced growth rate is a reflection of a prolongation of the G₁ phase of the cell cycle,⁹ which is caused presumably by a suppression of protein synthesis activity.⁸ The effects of RA on the proportion of cells in G₁ and on protein synthesis can be detected 24 h after exposing the cells to this retinoid,^{8,9} whereas the changes in cell number are detectable by 48 h. No cytotoxicity could be detected with RA concentrations lower than 50 μM .

The ability of the S91-C2 cells to synthesize DNA while suspended in a liquid growth medium above nonadhesive poly(hydroxyethylmethacrylate)-coated plastic dishes, a property characteristic of transformed cells, was suppressed by RA.¹¹ Further, RA inhibited the formation of tumor cell colonies in semisolid medium containing 0.5% agarose (Figure 1, C, F). This inhibition was also dose-dependent, with 1 μM RA causing a complete suppression of colony formation (Figure 3). Inhibition of colony formation by RA required continuous exposure of the cells to the retinoid, and there was no inhibition of cells exposed to RA for 1 h only.⁹

III. EFFECTS OF SYNTHETIC RETINOIDS AND RELATED COMPOUNDS ON MELANOMA CELL GROWTH

We have used the S91-C2 melanoma cells to compare the growth inhibitory effects of more than 150 retinoids and related compounds. These synthetic compounds were dissolved in ethyl alcohol or in dimethylsulfoxide (DMSO) and added to the growth medium of the melanoma cells at concentrations in the range of 0.01 nM to 10 μM . The cells were grown for 5 or 6 d, detached and counted using an electronic particle counter. Cell viability was determined by exclusion of 0.1% trypan blue. Although some retinoids were toxic to cells at 10 μM , no compound was toxic at 1 μM .

Dose-response curves were plotted and most showed a linear increase in inhibition with increasing doses. Examples are presented in Figure 4. From such curves we determined by interpolation the retinoid concentrations required for 50% inhibition. The results obtained with 151 compounds are presented in Tables 1 through 16. Previously we published on the

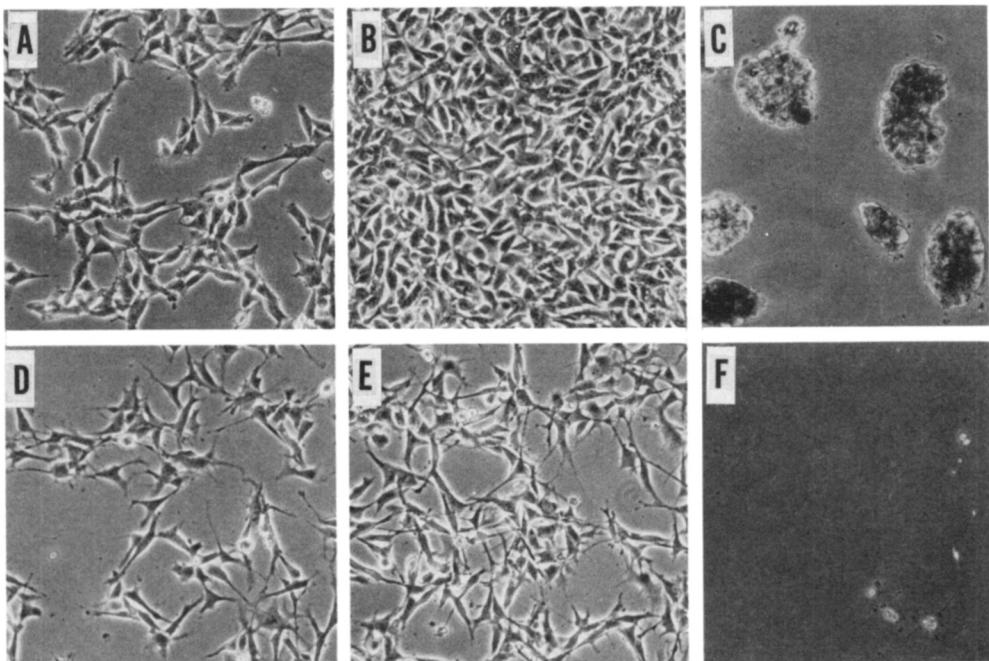


FIGURE 1. Photomicrographs of S91-C2 melanoma cells grown for 3 d (A, D), 5 d (B, E), or 10 d (C, F) on plastic dishes (A, B, D, E) or in 0.5% agarose (C, F) in the absence (A-C), or presence of 10 μ M all-trans-retinoic acid (D, F). Phase contrast, $\times 120$.⁷

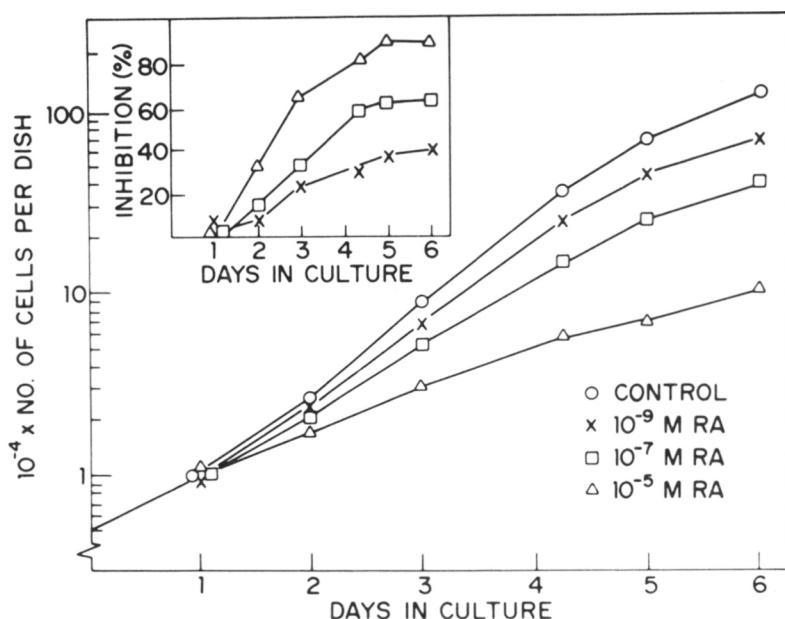


FIGURE 2. Effect of various concentrations of all-trans-retinoic acid on the growth rate of S91-C2 melanoma cells. Cells were seeded at 5×10^3 cells/dish in 3.5-cm diameter dishes in control medium or in medium containing retinoic acid and re-fed on alternate days. After 1 d, and at 24-h intervals thereafter, the cells were detached and counted. Inset: kinetics of growth inhibition. The percentages of growth inhibition were calculated by the following equation: % inhibition = $100 - (R/C) \times 100$, where R and C are the numbers of cells in retinoic acid-treated and control cultures, respectively.⁸

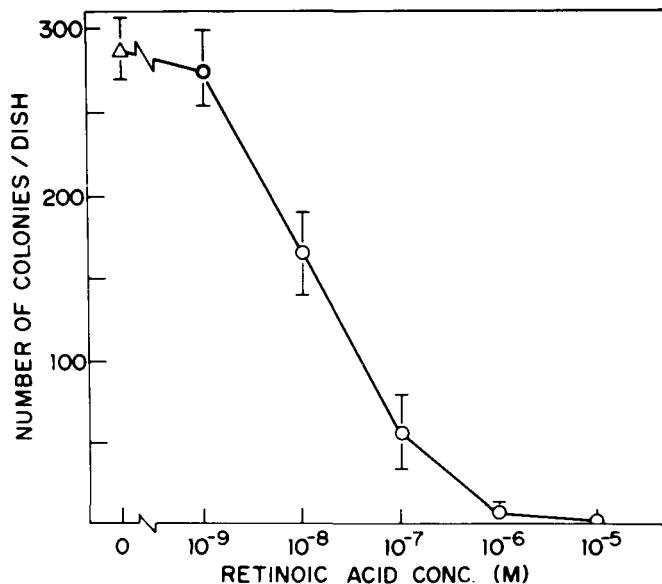


FIGURE 3. Suppression of S91-C2 melanoma colony formation in semisolid medium by all-trans-retinoic acid. Cells were suspended at 1×10^5 cells/ml in medium without or with the indicated retinoic acid concentrations. After a 1-h incubation at 37°C the cells were suspended in medium containing 0.5% agarose with or without retinoic acid, and 1-ml aliquots containing 1×10^3 cells were placed in 3.5-cm dishes on top of a precast 0.5% agarose layer. The cultures were refed every 72 h by placing 1 ml of fresh medium on top of the cell-containing agarose layer to replenish growth factors or retinoic acid. After 12 d the number of colonies was determined using a microscope at $\times 40$ magnification.⁹

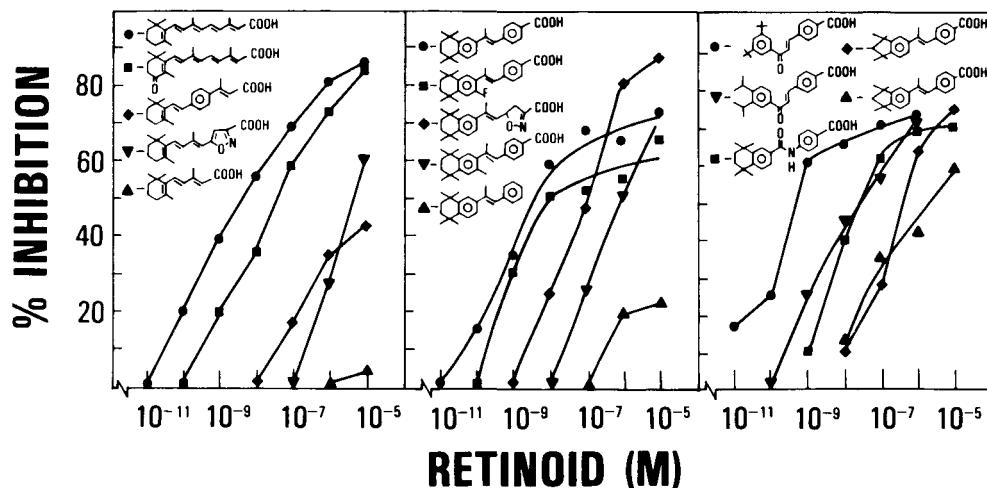


FIGURE 4. Dose-response curves for retinoid-induced inhibition of S91-C2 melanoma cell proliferation. Cells were seeded at 5×10^3 cells/dish in 3.5-cm diameter dishes and incubated in regular growth medium for 24 h. The medium was then replaced either with control medium, containing 0.1% DMSO, or medium containing different retinoids at the indicated concentrations. The medium was replaced with fresh medium after 72 h of incubation and the cells were detached and counted after an additional 48 h of growth. Percentages of inhibition were calculated as in Figure 2. The results are the mean of 3 independent experiments, each performed in duplicate. S.D. was <10%.

activities of 27 retinoids,¹² 11 retinoidal benzoic acid derivatives,¹³ 2 terephthalic anilides,¹⁴ and 4 alkyl-substituted chalcone-4'-carboxylic acids.¹⁴ The results presented here include the above compounds as well as unpublished data on 107 other retinoids. Details on the synthesis of these retinoids, which is out of the scope of this chapter, have been reviewed previously,¹⁵ and some are described in other chapters of this book. In discussing these results we refer to the retinoids by table number and compound number. For example, retinol is compound **1.2**. We defined as inactive all the compounds that produced less than 20% inhibition of growth at 10 μM . Compounds that inhibited growth by between 20 and 45% at 10 μM were assigned an IC_{50} value of >10 μM .

RA is a natural metabolite of retinol^{1,16} and is very potent in many *in vitro* assay systems.¹² We compared the activities of all the retinoids to this compound, which exhibits an IC_{50} of 0.004 μM (Table 2, compound **2.1**), by including a reference compound in all the assays of new retinoids.

Tables 1 to 9 present results obtained with analogs of the so-called classical retinoids, which are synthetic variations on the basic structure comprised of a ring, a dimethyl nonatetraene side chain and a polar group, or derivatives thereof, at carbon 15 (see top of Table 1).

Retinol (**1.2**) and retinal (**1.3**) were both moderately active; however, axerophthene, or deoxiretinol (**1.1**), an analog lacking a polar group, exhibits low activity. Retinyl acetate (**1.7**) was more active than retinol, presumably because the latter compound is less stable. It is known that retinol and retinal can be metabolized to RA *in vivo*,^{1,16} however, we do not know whether the melanoma cells possess the required enzymes for this metabolism. Ethers (**1.5** and **1.6**) and thioether (**1.4**) were almost inactive, whereas retinylamine derivatives exhibited low activity (**1.10**, **1.12**, **1.14**).

Analogs of RA with modified ring structures (Table 2) were all less potent than the parent compound with its trimethylcyclohexenyl ring (**2.1**). Some ring structures reduced the growth inhibitory activity by 10- to 20-fold compared to RA (e.g. compounds **2.4**, **2.5**, **2.6**, and **2.7**). 4-Oxoretinoic acid (**2.7**) is the major metabolite of pharmacological doses of RA *in vivo*.¹⁶ This compound is at least one order of magnitude less active than RA, in agreement with the suggestion that it is not an "active metabolite" of RA but rather a product of inactivation reactions.¹⁶ Other ring structures such as those in the pyridyl (**2.3**) and the phenyl (**2.2**) analogs of RA almost abolished activity. The TMMP (trimethylmethoxyphenyl) analog of RA (**2.10**) and its ethylester (**2.11**) showed modest activity. The trimethylhydroxyphenyl analog of RA (**2.9**), a metabolite of the TMMP analog,¹⁶ was almost inactive.

Modifications of the carboxylic group at carbon 15 were usually detrimental to activity on melanoma cells (Tables 3 to 5). Retinoylamine (**3.1**) and a few retinamides terminating with a hydroxyl (**3.2**, **3.3**, **3.4**) or a carboxyl group (**3.5**, **3.6**) exhibited a modest-to-low activity as did other retinamides containing various groups. It is possible that the retinamides have to be hydrolyzed to RA before they can affect cell growth. In this case, the low activity might reflect a low rate of metabolism to RA. In this context it is interesting to note that compound **4.7**, which can be hydrolyzed to yield RA either by an amidase or by an esterase exhibits only 1% of the activity of RA.

As shown in Table 6, isomerization of RA to 13-*cis*-RA (**6.A.1**) is a naturally occurring event *in vivo* and in organ culture.¹⁶ The potency of 13-*cis*-RA in inhibiting melanoma cell growth is equivalent to that of RA, however the efficacy of ring-modified 13-*cis*-RA analogs such as the 5,6-epoxy (**6.B.1**) and TMMP (**6.B.2**) was lower than the efficacy of the same analogs of RA (**2.6** and **2.10**). Various amides of 13-*cis* RA (Table 6A) exhibited low activity.

Modification of the nonatetraene side chain (Table 7), such as its shortening by the introduction of a C≡C bond between carbons 7 and 8 (**7.1**), decreased efficacy to 20% of

TABLE 1

Compound No. **Structure R** **IC₅₀ (μM)**

Compound No.	Structure R	IC ₅₀ (μM)
1	H	8
2	CH ₂ OH	0.8
3	CHO	0.7
4	CH ₂ SCH ₃	>10
5	CH ₂ OCH ₃	10
6	CH ₂ O(CH ₂) ₃ CH ₃	>10
7	CH ₂ OOCCH ₃	0.4
8		8
9		>10
10		8
11		10
12		5
13		0.2
14		2
15		2

that of RA. The activity of the aryltriene analog of 7,8-didehydro-9-cis-RA (**7.3**) was much lower than that of **7.1**, indicating the importance of the spatial relationship between the ring and the carboxylic group.

Retinoids with a longer side chain than that of RA (e.g. **1.11**) or a shortened chain terminating with a keto (β -ionone, **8.1**) or a carboxyl (**8.2**) moiety are inactive or poorly active, as are most other derivatives shown in Table 8 including some that have a terminal carboxylic group on structures that are apparently distinct in spatial arrangement from RA.

RA has been shown to antagonize the tumor-promoting effects of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) as well as various other effects of TPA on cultured cells.¹ Interestingly, when added to the growth medium of S91-C2 melanoma cells TPA inhibited growth in a dose-dependent fashion with an IC₅₀ of 0.002 μ M.¹⁷ The combination of 0.02 μ M TPA with 1 μ M RA resulted in a greater growth inhibition than by each agent alone.¹⁷ Phorbol,

TABLE 2

Compound No.	Structure R ₁	Structure R ₂	IC ₅₀ (μM)
1		H	0.004
2		H	10
3		H	>10
4		H	0.05
5		H	0.04
6		H	0.07
7		H	0.05
8		CH ₃	0.1
9		H	>10
10		H	0.3
11		C ₂ H ₅	0.7

which is not active in tumor promotion, failed to inhibit cell proliferation even at 50 μM.¹⁷ It is therefore surprising that an ester of RA with phorbol — a 12-O-retinoylphorbol-13-acetate (**9.2**), was the most potent compound among the ones that we tested so far with an IC₅₀ of 0.00002 μM. This is 100 times more active than TPA alone and 200 times more than RA alone. At present we have no explanation for this high activity. Because the retinoylphorbol-acetate exhibited tumor-promoting activity, it is highly unlikely that it will ever be considered for clinical applications: therefore, its activity is just an *in vitro* curiosity.

Aromatic retinoids (Tables 10 to 16) have been synthesized in which double bonds corresponding to selected bonds of the *E*-tetraene chain of RA are held in a planar cisoid conformation by inclusion in one or more aromatic rings without or with additional alterations in the trimethylcyclohexenyl ring.^{18,19} These compounds, and especially those that have the basic structure shown on top of Table 10 (**10.1**, 4-[*E*-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)propenyl]benzoic acid, TTNPB), are called arotoninoids¹⁸ or retinoidal benzoic acid derivatives.²⁰ TTNPB (**10.1**) was almost as active as RA (**2.1**). Modifications of ring B (Table 10, top) such as the introduction of a methyl (**10.3**), an ethyl (**10.4**), or an isopropyl (**10.5**) group at carbon 3 decreased activity dramatically, whereas the introduction of a fluorine atom (**10.2**) in the same position had only a small effect on activity.

TABLE 3

Compound No.	Structure R	IC50 (μM)
1	H	0.3
2	$\text{CH}_2\text{CH}_2\text{OH}$	0.3
3	$(\text{CH}_2)_3\text{CH}_2\text{OH}$	0.4
4	$\text{C}_6\text{H}_4\text{OH}$	0.2
5	$2\text{-C}_6\text{H}_4\text{COOH}$	0.2
6	$4\text{-C}_6\text{H}_4\text{COOH}$	2
7		4
8		0.2
9		8
10		2
11		2
12		10
13		>10
14		2

compared with TTNPB (10.1). A free carboxylic group on ring C (Table 10) is important for activity. Derivatives of TTNPB such as esters (10.6, 10.7) and some amides (14.4, 14.5, 14.6) exhibited lower activity. Further, a decarboxylated TTNPB (11.1), a nitrile analog (11.2), a methylamine analog (11.5), and a tetrazolyl analog (11.10) were almost inactive. The activity of TTNPB requires the carboxyl group in ring C to be in the *para* position (10.1); when the carboxyl is moved to the *meta* position (13.1), the activity was almost completely lost. The replacement of the carboxylic group by sulfur-containing groups such as sulfonate (13.5), sulfinate (13.6), or sulfone (13.7) resulted in a large decrease in activity.

The structure of ring A (Table 10) also contributes to the activity of aritinoids. The removal of two methyl groups from position 5 to yield a tetrahydromethylnaphthalene ring (12.2) led to an almost complete loss of activity. In contrast, replacement of the benzylic

TABLE 4

Compound No.	Structure R	IC ₅₀ (μ M)
1		3
2		0.5
3		2
4		>10
5		2
6		3
7		0.4

group in position 5 of ring A by oxygen (**12.1**) decreased activity by no more than 10-fold, and this residual activity is still significant. Replacement of the tetrahydrotetramethyl-naphthalenyl group by a pentamethylindanyl group (**15.1**) also resulted in a marked decrease in activity (67-fold).

Ring C of TTNPB (Table 10), a benzoic acid, is an important determinant of activity not only by virtue of the carboxylic group alone because its replacement by an isoxazole 3-carboxylic acid (**16.2**) reduced activity 17-fold.

Recently two new types of compounds related to retinoids have been synthesized: one group includes terephthalic anilides²¹ and the other consists of alkyl-substituted chalcone-4'-carboxylic acids.²² The terephthalic anilides (**12.6**, **12.7**), which differ from TTNPB in the group that links ring B to ring C (an amide instead of a propenyl) are about 3.5 times less active than TTNPB in inhibition of melanoma cell proliferation. The analysis of chalcone carboxylic acid analogs in the melanoma system resulted in surprising results. Whereas the tetramethyl-3,4-tetramethylenechalcone carboxylic acid (**12.3**) and 3,4-di-isopropylchalcone carboxylic acid (**12.4**) were less active than TTNPB, the 3,5-di-*tert*-butyl chalcone carboxylic acid (**12.5**) was about one order of magnitude more potent than TTNPB and RA.

It is beyond the scope of this chapter to compare the potency of each of the numerous retinoids described here with structure-activity relationships obtained in other systems *in vitro* and *in vivo*. Such comparisons have been reviewed^{12,23} and they show that results obtained using the S91-C2 melanoma cells are in good agreement with results obtained in other assay systems. This similarity is also true for the activities of terephthalic anilides and chalcone carboxylic acid derivatives.¹⁴

TABLE 5

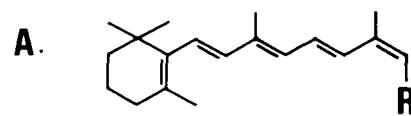
Compound No.	Structure R	IC ₅₀ (μM)
1	CH ₂ CH ₃	3
2	CH ₂ CH ₂ OH	0.6
3	CH ₂ CH ₂ SCH ₂ CH ₂ OH	5
4	C ₆ H ₅	2
5		0.3
6		0.4
7		0.7
8		3
9		0.8
10		2
11		10

Our ability to interpret accurately the structure-activity results is limited because we do not know enough about factors such as the half-life time of the retinoids in growth medium, the rate of their uptake by the cells, and the metabolic activation or inactivation reactions. Further, because ultimately the *in vitro* analyses are expected to indicate which retinoids might be useful *in vivo*, they must be accompanied by *in vivo* studies to determine important aspects such as the pharmacology of the compounds, their tissue distribution, metabolism and toxicity. The main advantages of the melanoma system are the ease of evaluation of numerous compounds and the broad range of compounds that exhibit activity compared with the other *in vitro* assays such as the murine embryonal carcinoma or the human promyelocytic leukemia HL-60.^{12,23}

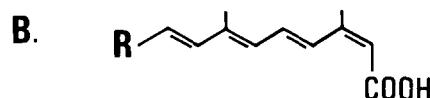
IV. RELATIONSHIP BETWEEN EFFECTS OF RETINOID ON CELL GROWTH AND BINDING TO CELLULAR RETINOIC ACID-BINDING PROTEIN (CRABP)

The finding of very specific structural requirements for activity of retinoids on the S91-C2 melanoma cells implies that the cells possess a mechanism for distinguishing active from

TABLE 6



Compound No.	Structure R	IC ₅₀ (μM)
1	COOH	0.005
2		0.09
3		>10
4		2
5		2
6		10
7		>10



Compound No.	Structure R	IC ₅₀ (μM)
1		0.3
2		0.9

inactive compounds. One of the most plausible candidates for this role is the CRABP, which has been proposed to act as a mediator of the effects of retinoids on cells *in vitro* and *in vivo*.²⁴ The S91-C2 melanoma cells contain both a cellular retinol-binding protein (CRBP) and CRABP.²⁵ CRABP is detected by incubating cell extracts with [11,12-³H]RA in the absence or presence of excess of unlabeled RA followed by centrifugation in sucrose gradients. The CRABP is detected as a 2S peak and the specific binding is determined by comparing the peak size in the absence and presence of unlabeled RA (Figure 5A). The CRABP is sensitive to pronase and to the thiol reagent *p*-chloromercuribenzenesulfonic acid (PCMBS) (Figure 5A). The ability of different retinoids to compete with [³H]RA for binding to CRABP was evaluated and the results are presented in Figure 5 B, C and in Table 17. Among the retinoids that possess a free carboxyl at carbon 15, those that inhibit the growth

TABLE 7
7,8-Didehydroretinoids

Compound No.	Structure	IC ₅₀ (μM)
1		0.02
2		0.2
3		3

of the melanoma cells invariably compete well for binding to CRABP. Only the phenyl and the pyridyl analogs, which are almost inactive in growth inhibition, fail to bind to CRABP (Table 17). Retinamides (3.5 and 3.7) that inhibit growth fail to bind to CRABP, presumably because the binding requires a free carboxyl group. These analogs might be hydrolyzed by the cells to RA, which could then bind to CRABP. The requirement for a free carboxylic group at carbon 15 is evidenced by the inability of retinol, retinal or axerophthene to bind to CRABP (Table 17). These results support a role for CRABP in mediation of the growth inhibitory effects of certain retinoids on the S91 melanoma cells.^{9,12}

V. EFFECTS OF RETINOIDS ON THE GROWTH OF RETINOIC ACID-RESISTANT CELLS

The study of drug-resistant mutants of mammalian cells provided important clues for understanding the mechanism of drug action. We have isolated from S91-C2 melanoma cells several mutant cell clones that are resistant to the growth inhibitory effects of RA.¹⁰ The mutants were selected to grow in 0.5% agarose in the presence of 1 μM RA. The uptake of [³H]RA by these mutants is not significantly different from the uptake by the parental S91-C2 clone and most of the mutants contained CRABP. Thus, their mechanism of resistance is not a simple one. The presence of CRABP in the mutants at levels comparable to that in S91-C2 cells raises the question whether CRABP is playing any role in the growth inhibitory action of retinoids in the S91-C2 cells in spite of the correlation described earlier (Section IV). To determine whether the mutant clones that were selected for resistance to RA were also resistant to other retinoids, we analyzed the effects of all-*trans*-retinol (1.2), 13-*cis*-RA (6.1) and TTNPB (10.1) on their growth. Figure 6 shows that the mutants are cross-resistant to the other retinoids. The finding that the acquisition of resistance to RA was accompanied by acquisition of resistance to retinoids with distinct structural features strongly suggests that all these compounds share a similar mechanism of antiproliferative action in the melanoma cells. In contrast, TPA, which inhibits the growth of the S91-C2 melanoma cells also inhibits the growth of RA-resistant cells,⁷ suggesting that TPA and RA act via distinct mechanisms.

VI. ENHANCEMENT OF MELANOGENESIS BY RETINOIDS

In addition to suppressing the proliferation of S91-C2 melanoma cells RA also enhanced

TABLE 8

Compound No.	Structure R	IC ₅₀ (μ M)
1		inactive
2		inactive
3		4
4		>10
5		inactive
6		inactive
7		5
8		>10
9		>10
10		inactive
11		10

the expression of their melanotic phenotype.^{17,26} The cells produced only small amounts of melanin at low cell density and the level of the enzyme tyrosinase was also low. When they reached higher densities ($>2 \times 10^5$ cells per square centimeter) the cells produced more melanin and tyrosinase activity increased.¹⁷ These two markers of melanocytic differentiation increased several fold in S91-C2 cells that were grown in the presence of RA under conditions that enabled the treated cells to reach a final density similar to untreated cells (by seeding cells destined for RA treatment at higher initial densities than control cells). The increased melanogenesis did not require an increase in the level of cyclic AMP in contrast with melanogenesis induced by α -melanocyte stimulating hormone.^{17,26}

Analyses of the effects of several retinoids on melanogenesis (Table 18) indicate that, in addition to RA, some other retinoids were able to stimulate this process. 13-*cis*-RA was equivalent to RA in potency, whereas retinyl esters exhibited low activity. There is no tight

TABLE 9

Compound No.	Structure R	IC ₅₀ (μ M)
1		0.1
2		0.00002

TABLE 10

Compound No.	Structure R ₁	Structure R ₂	IC ₅₀ (μ M)
1	H	OH	0.006
2	F	OH	0.01
3	CH ₃	OH	1
4	CH ₂ CH ₃	OH	6
5	CH(CH ₃) ₂	OH	3
6	H	OCH ₂ CH ₃	0.1
7	F	OCH ₂ CH ₃	1
8	CH ₃	OCH ₂ CH ₃	1
9	H	CH ₂ SOCH ₃	0.5
10	CH ₃	O-N ₂	9

correlation between the effects of the retinoids on growth and on melanogenesis. For example, the phenyl analog of RA (2.2) was a considerably less potent growth inhibitor than the TMMP analog (2.10) or retinyl acetate (1.7) but it stimulated melanogenesis better than they did.

VII. SUMMARY AND CONCLUSIONS

Retinoids are capable of inhibiting the proliferation of murine S91-C2 melanoma cells and stimulating morphological and biochemical differentiation *in vitro*. The efficacy with

TABLE 11

Compound No.	Structure	IC50 (μM)	
	R ₁	R ₂	
1	H	H	>10
2	H	CN	>10
3	CH ₃	CH ₂ OH	>10
4	CH ₃	CH ₂ OCOCH ₃	inactive
5	H	CH ₂ NH ₂ xHCl	>10
6	CH ₃	CH ₂ NH ₂ xHCl	>10
7	H	CH ₂ NHCOC ₆ H ₅	10
8	CH ₃	CH ₂ NHCOC ₆ H ₅	inactive
9	H	CH ₂ NH C(=O) N	inactive
10	H	+ N=N-N= +	8
11	F	+ N=N-N= +	>10
12	CH ₃	+ N=N-N= +	>10
13	OCH ₃	+ N=N-N= +	>10
14	H	[N+] Cl ⁻	0.9

which different retinoids inhibited cell growth depended on the structure of the compound. Similarly, the binding of retinoids to CRABP and their ability to stimulate melanogenesis required certain specific structural characteristics. As reported for many other systems, the effectiveness of retinoids in the melanoma system required a free carboxyl group at carbon 15 of the classical retinoids, or on the benzene ring (ring C) of the arotinoids. The structure of the ring was also important for activity as was the structure of the polyene chain. Some new synthetic compounds with structures vastly different from retinoids or arotinoids exhibited high potency. The S91-C2 melanoma cell system is useful for screening new retinoids.

TABLE 12

Compound No.	Structure R	IC_{50} (μM)
1		0.08
2		10
3		0.01
4		0.03
5		0.0005
6		0.02
7		0.02

for biological activity because results obtained with these cells compare favorably with other systems. However, these *in vitro* results have to be considered only preliminary, because they do not provide any information on how a given retinoid will behave *in vivo* in terms of pharmacodynamics, metabolism, tissue distribution, effects on the immune response, and toxicity, which are all important factors in the efficacy of retinoids in prevention or therapy of neoplastic disease.

TABLE 13

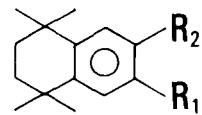
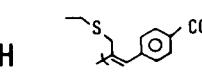
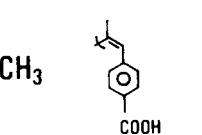
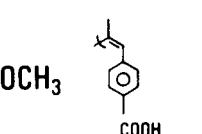
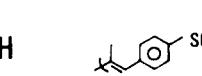
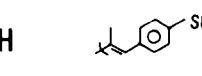
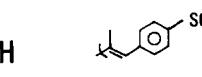
Compound No.	Structure		IC ₅₀ (μM)
	R ₁	R ₂	
1	H		>10
2	H		inactive
3	CH ₃		10
4	OCH ₃		inactive
5	H		7
6	H		6
7	H		inactive

TABLE 14

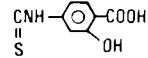
Compound No.	Structure R ₁	Structure R ₂	IC ₅₀ (μM)
1	H	CH ₂ CH ₃	inactive
2	H	CH ₂ CH ₃ OH	>10
3	CH ₃	CH ₂ CH ₂ N(CH ₃) ₂	inactive
4	H	C ₆ H ₄ OH	0.5
5	H	C ₆ H ₄ COOH	0.3
6	H		0.3
7	H		8

TABLE 15

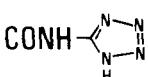
Compound No.	Structure R	IC ₅₀ (μM)
1	COOH	0.4
2	CN	>10
3	COOC ₂ H ₅	0.4
4	CONHC ₆ H ₄ COOH	1
5	CONHC ₆ H ₄ OH	0.3
6	CONHC ₆ H ₄ COOCH ₃	>10
7	CONH 	0.1

TABLE 16

A.

Compound No. Structure IC₅₀ (μM)

Compound No.	R ₁	R ₂	IC ₅₀ (μM)
1	H	CH ₃	9
2	H	COOH	0.1
3	H	COOC ₂ H ₅	1
4	H	CH ₂ OH	10
5	H	CH ₂ OCH ₃	10
6	F	COOH	0.2
7	F	COOC ₂ H ₅	0.03
8	H	CONHCH ₂ CH ₂ OH	>10

B.

Compound No. Structure IC₅₀ (μM)

Compound No.	R ₁	R ₂	IC ₅₀ (μM)
1	CH ₃	COOH	0.2
2	H	COOH	3
3	CH ₃	COOC ₂ H ₅	0.8
4	H	COOC ₂ H ₅	2
5	CH ₃	COCH ₂ SOCH ₃	>10
6	CH ₃	CONH_N-_N-H	6
7	CH ₃	CH ₂ OOCCH ₃	>10
8	CH ₃	CONHCNHS-C(=O)-_C(OH)-COOH	2

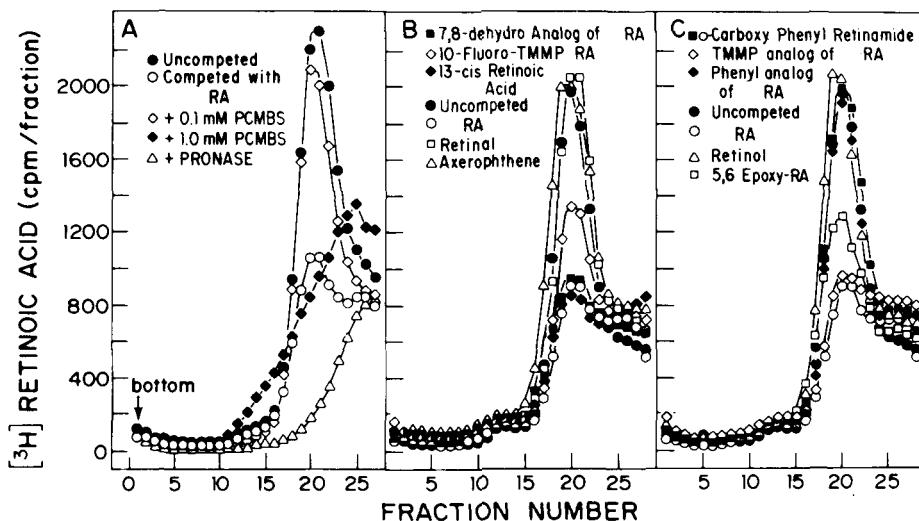


FIGURE 5. Cellular retinoic acid-binding protein (CRABP) of S91-C2 melanoma cells. Chemical characteristics and retinoid binding specificity. Cells were suspended at 10^8 cells/ml in 50 mM Tris-HCl buffer, pH 7.2, and homogenized in a Dounce homogenizer placed on ice. Cell extracts, containing 0.8 mg protein in 0.2 ml of the Tris buffer, were incubated for 4 h on ice with 0.1 μM [$^{11,12}\text{-}^3\text{H}$]retinoic acid (10 Ci/mmol) added in 5 μl of isopropyl alcohol. The presence of a 2S binding protein was detected in fractions collected after high-speed centrifugation of charcoal-treated incubation mixtures on 5 to 20% sucrose gradients. (A) Effect of a protease and a thiol-blocking reagent on binding of all-trans-retinoic acid (RA): Aliquots of cell extracts (0.2 ml) were incubated with 1 mg pronase P/ml for 2 h at 25°C or with *p*-chloromercuribenzenesulfonate (PCMBS) at 0.1 or 1 mM for 1 h at 4°C, or in Tris buffer without additives (uncompeted), or with a 100-fold excess of unlabeled RA. The samples were then incubated with [^3H]RA and analyzed as described above. (B) and (C), Competition of various retinoids for binding to CRABP was performed by adding a 100-fold excess of retinoids to the [^3H]RA binding mixture prior to the 4-h incubation.⁹

TABLE 17
Comparison Between Inhibition of Cell Proliferation and Binding to CRABP
in S91-C2 Cells¹²

Retinoid (compound designation) ^a	IC_{50} (μM)	Competition for binding to CRABP ^b
RA (2.1)	0.004	+
13-cis-RA (6.1)	0.005	+
7,8-Didehydro-RA (7.1)	0.02	+
7-Oxo analog of RA (2.7)	0.05	+
DACP analog of RA (2.5)	0.04	+
TMT analog of RA (2.4)	0.05	+
5,6-Epoxy-RA (2.6)	0.07	+
TMMP analog of RA (2.10)	0.03	+
Phenyl analog of RA (2.2)	10	-
Pyridyl analog of RA (2.3)	>10	-
<i>N</i> -(2-Carboxyphenyl)retinamide (3.5)	0.2	-
<i>N</i> -(2,2-Dimethyl-4-dioxolanyl)methylretinamide (3.7)	4	-
Retinol (1.2)	0.8	-
Retinal (1.3)	0.7	-
Axerophthene (1.1)	8	-

^a The structures of these retinoids can be seen in the appropriate tables.

^b + and - represent the ability and inability, respectively, of a retinoid to inhibit the binding of [^3H]RA to CRABP in cell extracts.

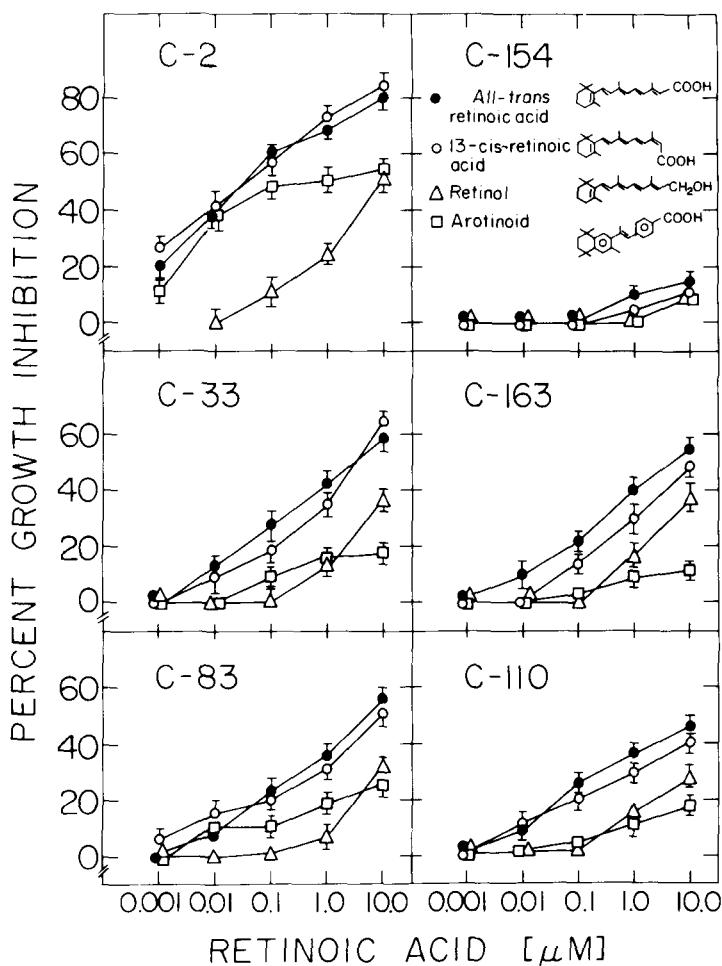


FIGURE 6. Effects of various retinoids on the proliferation of S91-C2 cells and on RA-resistant mutant clones derived from them. The cells were seeded at $1 \times 10^4/35\text{-mm}$ dish and incubated for 24 h before feeding fresh medium without or with different retinoids, described in the upper right panel, at the indicated concentrations. The cultures were fed fresh medium every 48 h and detached and counted on day 5, except for clones-33 the control culture of which became nearly confluent only on day 6. The percentages of growth inhibition were calculated as in Figure 2.¹⁰

TABLE 18
Effects of Various Retinoids on Growth and Melanogenesis in Mouse
S91-C2 Cells¹⁷

Retinoid	% Growth inhibition at $10 \mu\text{M}^a$	Melanogenesis stimulation (T/C) ^b
RA (2.1)	90 ± 2	3.2 ± 0.2
13-cis-RA (6.1)	90 ± 2	3.3 ± 0.3
TMMP analog of RA (4.20)	78 ± 4	1.8 ± 0.2
Phenyl analog of RA (2.2)	40 ± 3	2.2 ± 0.2
Pyridyl analog of RA (2.3)	<10	1.0 ± 0.1
Retinyl acetate (1.7)	70 ± 5	1.3 ± 0.1
Retinyl palmitate	30 ± 2	1.0 ± 0.1

^a Cells were cultured in 10-cm dishes such that they reached confluence after 6 d in the absence or presence of the respective retinoids.

^b Ratios of relative melanin content in treated (T) and control (C) cultures.

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Chapter 12

**STRUCTURE-ACTIVITY RELATIONSHIPS OF A NEW SERIES
OF SYNTHETIC RETINOIDS (RETINOBENZOIC ACIDS)****Koichi Shudo and Hiroyuki Kagechika**

I.	Introduction	276
II.	Design of New Retinoids and Their Bioassay.....	276
III.	Benzanilides	277
	A. Terephthalic Anilides.....	277
	B. Benzoylaminobenzoic Acids.....	278
	C. Structural Links to Retinoic Acid.....	280
IV.	Azobenzenes and Stilbenes	280
	A. Azobenzenecarboxylic Acids	280
	B. Stilbenecarboxylic Acids	280
V.	Chalcones and Flavones	281
	A. Chalconecarboxylic Acids	281
	B. Flavonecarboxylic Acids	282
VI.	Conformations of the Linking Chains	282
VII.	Biological Activities	284
VIII.	Conclusion.....	285
	References.....	285

I. INTRODUCTION

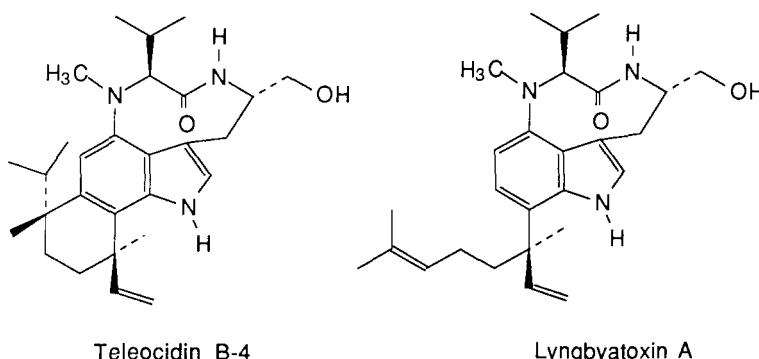
The fundamental role of retinoids in controlling the state of cellular differentiation and preventing excessive proliferation raises the possibility that these compounds may be useful for inhibiting carcinogenesis and for the treatment of cancer, dermatological diseases such as psoriasis, and possibly rheumatoid arthritis. Extensive chemical modification of retinoic acid led to the discovery of etretinate, which has been used clinically to treat psoriasis. The idea of incorporating a part of the olefinic side chain into one or two aromatic ring systems led to a new class of retinoids, the stilbene-4-carboxylic acids (i.e., arotoninoids). This approach has been well documented.¹ Though retinoids might be valuable as noncytotoxic chemotherapeutic agents, their high toxicity prevents their use conventionally or for a prolonged period. In addition, the retinoids thus far known are lipophilic and are very slowly eliminated from the body, causing serious prolonged toxicity, and in particular, teratogenic effects.

We will describe here how we have modified the structure of retinoic acid and what the consequences are. The structure of retinoic acid was greatly altered to obtain a new series of active benzoic acid derivatives. The structure-activity relationships are also discussed.

II. DESIGN OF NEW RETINOIDs AND THEIR BIOASSAY

In an effort to obtain still more potent biological activity we focused our attention on modification of the carbon skeleton of retinoic acid. As an initial guideline for structural alterations, we referred to three structure-activity relationship studies from our previous work:

1. 4-(*N,N*-Dimethylamino)azobenzene and 4-(*N,N*-dimethylamino)stilbene are both potent chemical carcinogens.² Several heteroaromatic amines such as 2-aminodipyridimidazole are also potent carcinogens and may be regarded as aza-analogs of 4-*N*-acetylaminofluorene.^{3,4} Thus, we wished to introduce a nitrogen atom(s) instead of a carbon atom(s) in the retinoid skeleton; C=C can also be replaced by N=N and other functional groups.
2. A retinal analog that does not contain an intact cyclohexene ring combines with opsin in an analogous manner to retinal.⁵ Similarly, the dipeptide alkaloids teleocidin B-4 and



Teleocidin B-4

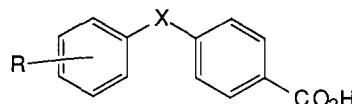
Lyngbyatoxin A

lyngbyatoxin A are strong tumor promoters, like the phorbol esters although the important hydrophobic group of teleocidin is cyclic and that of lyngbyatoxin A is acyclic.⁶ Thus, we decided to modify the cyclohexene ring of retinoic acid to an acyclic moiety for the first time. In addition, because the biological activity of teleocidin is

closely related to the activity of retinoids, we extracted the amide structure of teleocidin, which defines the conformation of the ring system, as a partial structure of the designed retinoids.⁷

3. Our past experience in the development of plant growth hormones suggested that we should be ambitious and not overly rational in the modification of chemical structures.^{8,9}

Thus, we designed a general structure for new compounds represented by the indicated formula, where the link X that bridges



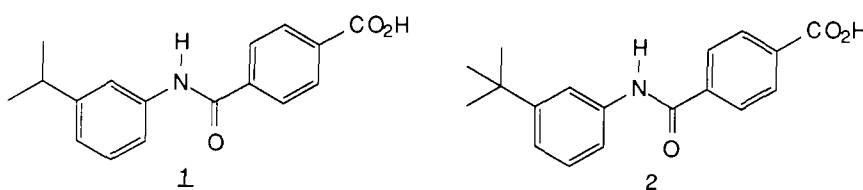
the two aromatic rings is variable. The use of a wide range of functional moieties for the linking group in the structure yields a series of compounds of interest. We propose the generic name retinobenzoic acid for the active compounds represented by the formula and for related benzoic acid derivatives.

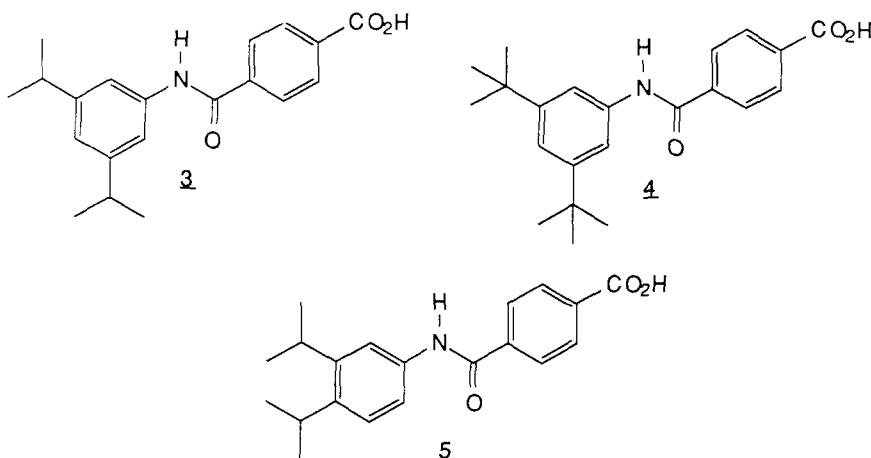
The development of new retinoids requires effective methods for assaying biological activity. Retinoids are active in a wide variety of test systems. It has been shown that retinoids can induce terminal differentiation of several types of neoplastic cells and that the measurement of such a response can be used for rapid and quantitative assays of activity. The degree of differentiation of the human promyelocytic leukemia HL-60 cell line can be quantified both on the basis of morphological criteria and biochemically by using the ability of mature cells to reduce nitroblue tetrazolium chloride to blue formazan.¹⁰ This system is very sensitive to retinoidal acids, and the relative activity obtained correlates well with the results in many *in vivo* and *in vitro* test systems. We therefore adopted the HL-60 system for the initial bioassay. We always examined retinoic acid and other reference compounds at the same time during the assay of a test sample, and all results were reproducible. Thus, we could estimate the relative potencies of the test compounds very reliably, which provided a sound basis for the design of new modified analogs.

III. BENZANILIDES

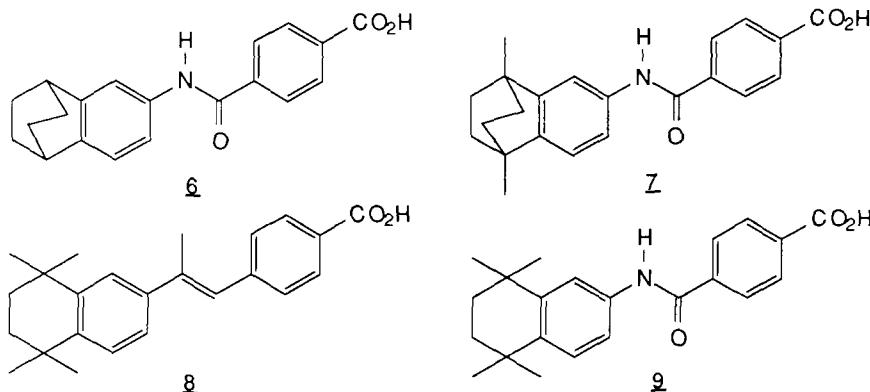
A. TEREPHTHALIC ANILIDES

The simplest terephthalic anilide was completely inactive in the HL-60 bioassay. Although compounds having an alkyl substituent at the *ortho* or *para* position of the benzene ring of the aniline moiety were inactive, the effect of a *meta*-alkyl group was pronounced. Thus, compounds having a *meta*-isopropyl (**1**) or a *meta*-*t*-butyl (**2**) group exhibited significant activity, though higher concentrations were required for activity ($\times 10^2$) compared with that of retinoic acid. Among compounds having two alkyl groups, the 3,5-diisopropyl (**3**) and 3,5-di-*t*-butyl (**4**) compounds were as potent as retinoic acid. Though the *para*-alkyl group had little effect by itself, it enhanced the potency of the *meta*-alkyl compounds; thus, the 3,4-di-isopropyl compound (**5**, Am 68) was highly potent ($ED_{50} = 2.1 \times 10^{-9} M$; ED_{50} for retinoic acid = $3.0 \times 10^{-9} M$). The introduction of an *ortho*-alkyl group in any dialkyl compound diminished activity.¹¹

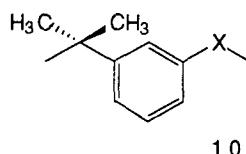




When the two isopropyl groups of **5** were combined as a ring in such a way as to form a bicyclic system (compound **6**), the strong activity was abolished. Introduction of two methyl groups at the bridgehead positions (as in **7**) regenerated the activity.¹² The combination of the two isopropyl groups to form a ring in such a way as in the stilbenecarboxylic acid **8** (arotinoid Ro 13-6298) increased the activity further; for example, the ED₅₀ of 4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid (Am 80, **9**) was $3.0 \times 10^{-10} M$, whereas that of retinoic acid was $7.1 \times 10^{-10} M$. The dose-response relationship of Am 80 in the HL-60 bioassay together with that of retinoic acid is illustrated in Figure 1.



The structure-activity relationship study revealed that the presence of a medium-sized alkyl group at the *meta* position was crucial for activity and that it was also preferable that the hydrophobic moiety is oriented towards the *ortho* side as shown in **10**. This orientation also accounts for the enhancing effect of the *para*-alkyl group.



B. BENZOYLAMINOBENZOIC ACIDS

The synthesis of these acids was undertaken in an attempt to evaluate the electronic

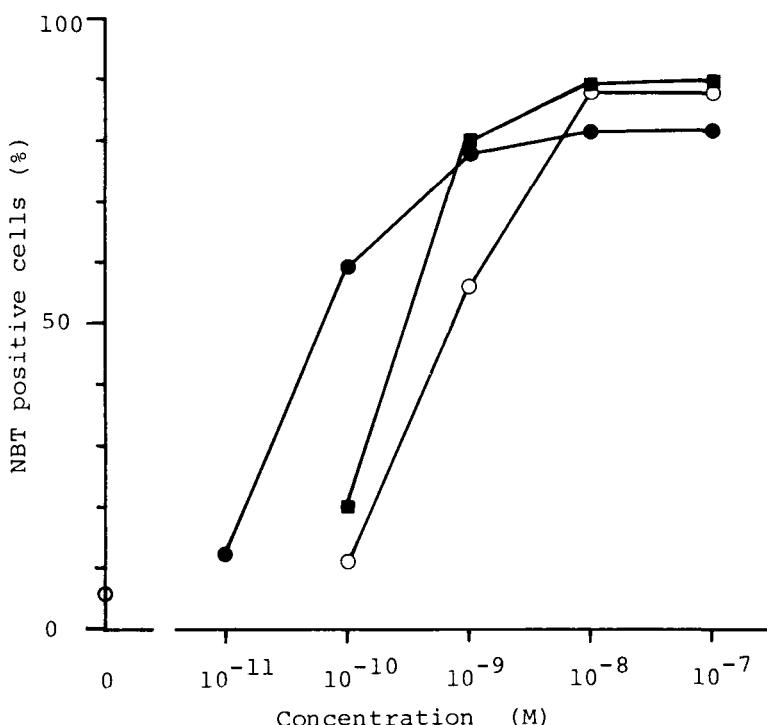
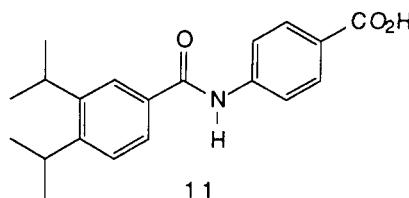
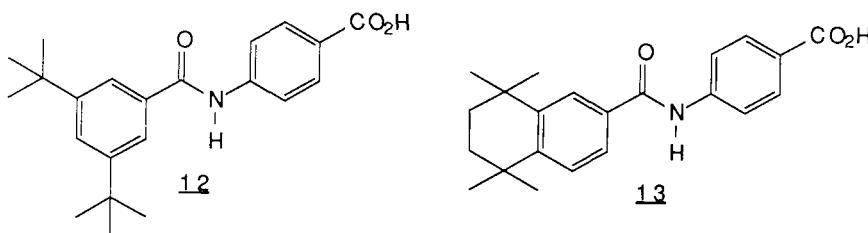


FIGURE 1. Dose-response curves for retinobenzoic acid Am 80 (7) and Am 580 (13). Differentiation induced in HL-60 cells by various concentrations was measured by the ability to reduce nitroblue tetrazolium to formazan in the presence of TPA (200 nM), and represented by percentage of NBT positive cells. The results correlated very well to the degree of morphological change. -○-: retinoic acid; -■-: Am 80; -●-: Am 580.

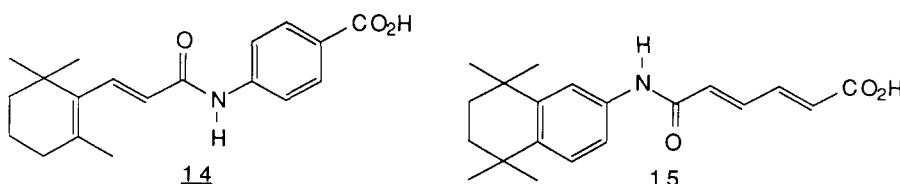
effect of the amide functional group. For this purpose, we prepared benzoates of 4-aminobenzoic acid where the amide bond was inversely positioned compared to the case of terephthalic anilides. In this case, too, the simple 4-benzoylaminobenzoic acid was completely inactive. Introduction of a medium-sized alkyl group generated biological activity. Introduction of two alkyl groups led to an increase of the activity; the 3,4-di-*t*-isopropyl (11) and 3,5-di-*t*-butyl (12) derivatives had activity similar to that of retinoic acid. Cyclization of the isopropyl groups of 11 gave 13 [4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalenyl-2-carboxamido)benzoic acid, Am 580], which had the strongest activity among amide compounds tested (ED_{50} , $5.9 \times 10^{-11} M$; ED_{50} for retinoic acid, $7.1 \times 10^{-10} M$). The dose-response relationship curve for 13 is shown in Figure 1. A very similar structure-activity relationship concerning the effect of alkyl substitution was observed though the inversion of the amide bond must alter the electronic character of the two aromatic rings as compared with the terephthalic anilides.¹³ This result indicated that the intervening group could be replaced by a variety of functional groups, and this idea led to the discovery of a new series of active compounds.





C. STRUCTURAL LINKS TO RETINOIC ACID

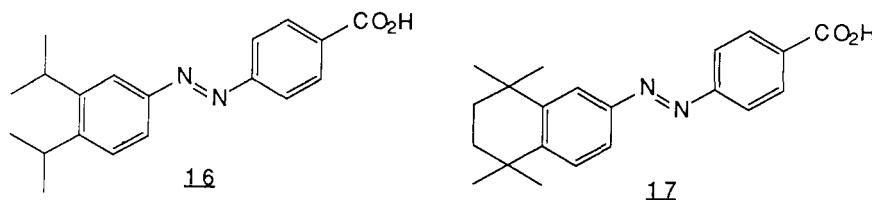
Since the structure of the above amide compounds seemed superficially to differ from that of retinoic acid, hybrid compounds of retinoic acid and these amido compounds attracted our attention. Combination of the left half of retinoic acid and the right half of Am 580 (13) led to 14. On the other hand, the combination of the right half of retinoic acid and the left half of Am 80 (9) gave a muconic acid derivative (15). The differentiation-inducing activities — converting HL-60 cells to granulocytes — of these hybrid compounds were found to be about one-tenth of that of retinoic acid. The significant activity of this class of amides strongly supports the idea that these amides and retinoic acid are structurally related agonists.¹³



IV. AZOBENZENES AND STILBENES

A. AZOBENZENECARBOXYLIC ACIDS

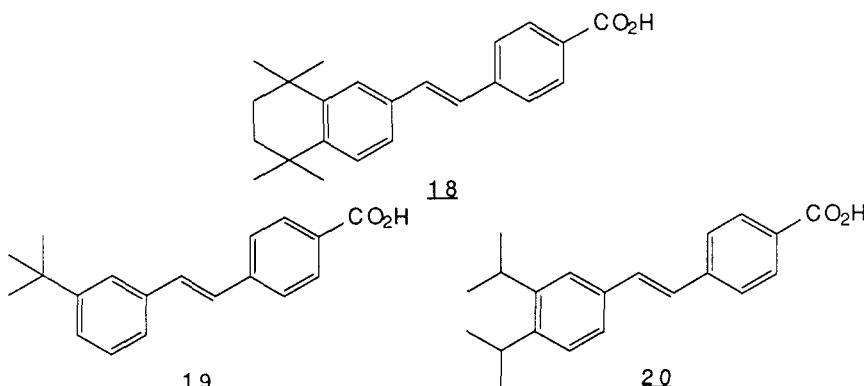
Azobenzencarboxylic acids are another series of active substances. In this case again, a similar structure-activity relationship was found concerning the effect of the alkyl group(s) on the benzene ring. The unsubstituted azobenzene-4-carboxylic acid was inactive at 10^{-6} M. The presence of a *meta*-isopropyl or *t*-butyl group generated the activity. A *para*-alkyl group did not have any effect, but it did enhance the effect of the *meta* substituent. The activity of 3',4'-diisopropylazobenzene-4-carboxylic acid (16) was as high as that of retinoic acid, and the tetramethyltetrahydronaphthalene derivative 17 was more active than retinoic acid.¹⁴



B. STILBENECARBOXYLIC ACIDS

A stilbene derivative, tetrahydrotetramethylnaphthalenyl-propenylbenzoic acid (8, Ro 13-6298), has been reported to inhibit chemical carcinogenesis and to induce the differentiation of HL-60 cells.¹⁵ Azobenzene and stilbene are isosteric. From the results on azobenzencarboxylic acids and amido benzoic acids, it appears that the cyclohexenyl ring system of 8 may not be essential for activity. Therefore, the activity of several alkyl-

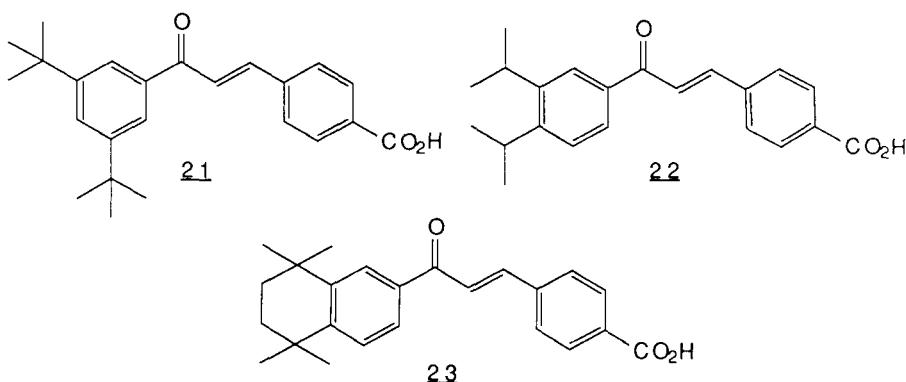
substituted stilbene derivatives without the cyclohexenyl ring were examined.¹⁴ The methyl group on the ethylene moiety of **8** can be removed without loss of activity, compound **18** being as active as **8** (ED_{50} , $7 \times 10^{-10} M$; ED_{50} of retinoic acid, $2.1 \times 10^{-9} M$). In this case again, the parent unsubstituted stilbene-4-carboxylic acid was completely inactive, and compounds having an alkyl group at the *meta* position were active (e.g., **19** was active at ED_{50} , $1.2 \times 10^{-8} M$; ED_{50} of retinoic acid, $1.9 \times 10^{-9} M$). 3',4'-Diisopropylstilbene-4-carboxylic acid (**20**) had activity similar to that of retinoic acid. These results show that the cyclohexenyl ring of the aritinoids is not essential for inducing activity. This moiety seems to act only as a bulky hydrophobic group. Among the four methyl groups, the 8,8-dimethyl groups (methyl groups at the *meta* position to the linking olefinic bond) were more important in determining the magnitude of activity.



V. CHALCONES AND FLAVONES

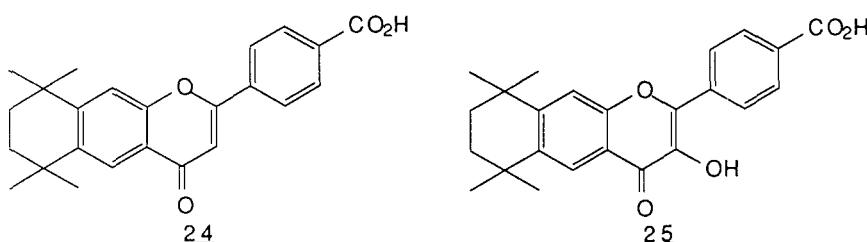
A. CHALCONECARBOXYLIC ACIDS

As part of a program to synthesize retinoids with new structures and to study structure-activity relationships, we prepared 3',5'-di-*t*-butylchalcone-4-carboxylic acid (**21** (*E*)-4-[3-(3,5-di-*t*-butylphenyl)-3-oxo-1-propenyl]benzoic acid, Ch 55), which was one of the most active compounds we had so far obtained (ED_{50} , $6.0 \times 10^{-11} M$; ED_{50} of retinoic acid, $1.3 \times 10^{-9} M$). The activity was definitely higher than that of retinoic acid, Am 80, and the other compounds we had synthesized. The structural effect of the alkyl group on the aromatic ring was similar to that in the cases of amides, azo compounds, and stilbenes. The unsubstituted compound was inactive, while a *meta*-*t*-butyl group had an enhancing effect. The 3',4'-diisopropyl compound (**22**) and tetramethylnaphthalenyl compound (**23**) were 10 times more active than retinoic acid. However, in this case a *para*-*t*-butyl group generated activity even when it was present alone, reflecting the greater flexibility of the C₃ unit of the linking chain of the chalcones.¹⁶



B. FLAVONECARBOXYLIC ACIDS

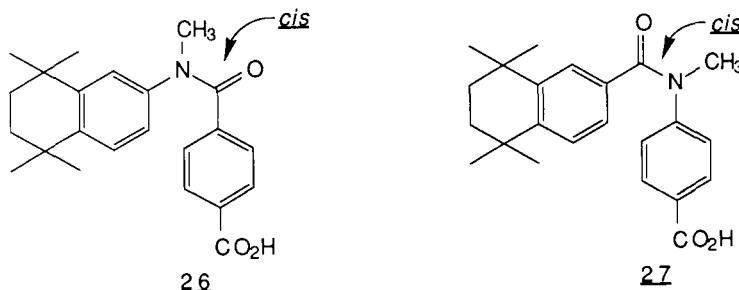
Though the stable conformation of chalcones is believed to be *s-cis*, which is very different from that of the rigid flavones, flavones and chalcones appeared to be structurally related compounds. In fact, some flavone carboxylic acids were highly active. The most potent compound in this series was the tetramethyltetrahydronaphthyl derivative **24** (Fv 80), the ED₅₀ of which was $6.3 \times 10^{-11} M$ (ED₅₀ of retinoic acid, $4.1 \times 10^{-9} M$). The flavonol **25** was less active, but was as active as retinoic acid. The correspondence of the alkyl groups between flavones and chalcones, however, might not be straightforward. The most important alkyl substituent appeared to be different from that of the chalcones. Because flavonecarboxylic acids are rather structurally rigid compounds with highly potent activity, the molecular shape and other physical properties of these molecules may give us important information on the biological receptor of retinoids.¹⁷

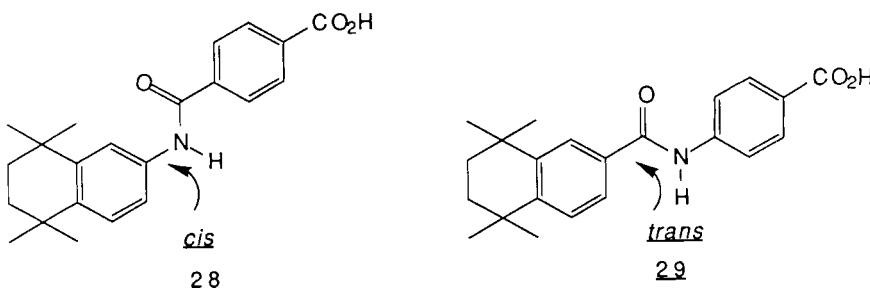


VI. CONFORMATIONS OF THE LINKING CHAINS

The potent activity of the flavone-carboxylic acids seems to suggest that the stereochemistry of the linking chain between the two aromatic rings is plausibly *trans* or *transoid*. This idea is supported by the fact that *trans*-stilbene-carboxylic acid **8** is highly active, while the *cis* isomer is a hundred times less active in the HL-60¹⁸ and other bioassays.¹⁹ The azobenzene must also exist in the *trans* conformation. The amide derivatives, i.e., Am 80 and Am 580, were deduced to have a *trans* NH-CO configuration from the results of NMR and UV spectroscopy and X-ray crystallographic analyses.²⁰ Interestingly, the *N*-methylation of these amides, Am 80 and Am 580, to give Am 90 (**26**) and Am 590 (**27**), respectively, dramatically reduced the activity to about 0.001.¹⁸ This almost complete loss of the activity is considered to be due to changes of the amide conformations because NMR, UV, and X-ray analyses indicated that the amide stereochemistry in both cases was *cis*.²⁰

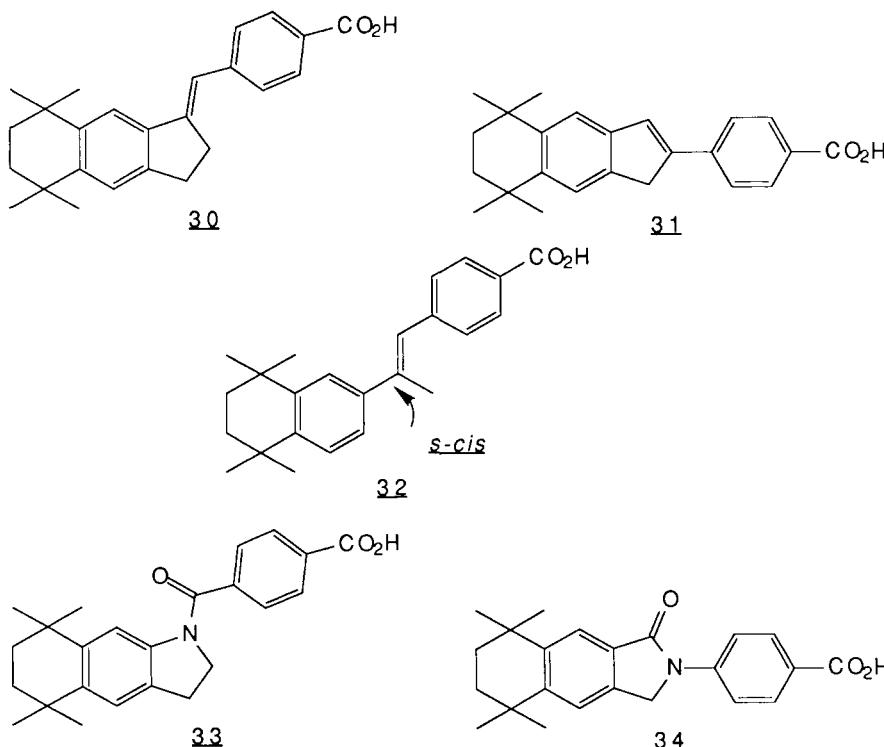
During this study, it was found that the bond between the tetramethyltetrahydronaphthalene ring and the NHCO group could exist in two possible forms, *s-trans* and *s-cis*.²⁰ In the crystalline state, this bond exists in the *s-cis* form (**28**) in the case of Am 80, and in the other conformation (**29**) in the case of Am 580. The NMR data suggested that both conformations are rapidly interchangeable in solution. Since a *meta*-alkyl group rather than a *para*-alkyl group is important for the activity, it would be interesting to know which conformation is more favorable for inducing the activity.





In order to differentiate these stereochemistries, we prepared two series of stilbenes the single bond of which was fixed in conformations corresponding to the *s-trans* and *s-cis* structures. For this purpose, two indane derivatives were prepared, one (**30**) having an *exo*-olefinic bond and the other (**31**) an *endo*-olefinic bond. The *exo*-olefinic compound (**30**) was as potent as compound **8**, while the *endo*-olefinic compound (**31**) was far less potent than **8** though slight activity was observed.¹² Therefore, we suggest that the *cis* stereochemistry of the Ar-C bond of the stilbene **8**, represented by **32**, is closer to the active conformation of the stilbene derivatives.

The activity of *N*-benzoyldihydroindole (**33**), which is regarded as a derivative of Am 80 conformationally fixed in its *s-cis* state, decreased to 0.02 of that of Am 80. The active *N*-phenyldihydroisoindol derivative **34** is regarded as a derivative of Am 580 conformationally fixed in the *s-cis* state. The activity of **34** was less than that of **33**, and 0.1 to 0.2% of that of Am 580. In both cases, the activity decreased significantly. This effect of cyclization of the amides may reflect the larger torsion angle of the nitrogen-Ar bond (in the case of **34**), or the ready *cis-trans* isomerization of the amide bond (in solution, **33** exists in equilibrium with the *cis* isomer). The decrease of activity in going to **33** from Am 80 is smaller than that in going to **34** from Am 580.¹²



This is consistent with the above conclusion that the *s-cis*-conformation is preferred for activity, although the situation is not straightforward since the strong activity of flavone-carboxylic acids suggests that the *s-trans* structure of the stilbenes and the corresponding conformers of amidobenzoic acids are active. It will be necessary to extract common structural units required for activity or to superimpose active conformations of a variety of compounds to identify the necessary molecular shape at the receptor site.

VII. BIOLOGICAL ACTIVITIES

The biological activities of the new retinobenzoic acids have been evaluated in terms of the induction of differentiation of HL-60 cells. Other representative biological activities of retinoids have been also assayed for some retinobenzoic acids (in particular, for Am 80, Am 580, Ch 55, and Fv 80).

These compounds induce differentiation of mouse embryonal teratocarcinoma F9 cell line; a characteristic change in morphology and induction of plasminogen activator were observed as with conventional retinoids.^{21,22} Retinoids are potent inhibitors of melanoma S91 cell proliferation. The Am- and Ch-series of retinobenzoic acids effectively inhibited the growth of the melanoma cells in the nanomolar concentration range.²¹ These compounds, like retinoic acid, did not induce differentiation of K562 leukemia cells or mouse Ml cells.²³ Growth of U937 and MLI leukemia cells was suppressed at nanomolar concentrations.²⁴

Retinobenzoic acids inhibited the induction of ornithine decarboxylase by TPA in 3T6 fibroblast cells²¹ and by the teleocidins on mouse skin;²⁵ their order of activity correlated reasonably well to the order of the activity observed in HL-60 cells.

Retinobenzoic acids also inhibited the proliferation and keratinization of rat bladder cancer cell line BES2OB²⁶ at nanomolar concentrations.²⁷ This activity was very high for Ch 55 and Fv 80, in particular, and was observed at $10^{-10} M$.

Some biochemical studies related to the mechanisms of action of new retinobenzoic acids have been undertaken. In the course of the terminal differentiation of the HL-60 cells to granulocytes, the enhanced expression of *c-myc* of the cells was strongly suppressed by these retinobenzoic acids before the appearance of morphological and biochemical indications of differentiation, as found with retinoic acid.²⁸ This effect can be considered as one of crucial early events required for differentiation. One of the new compounds, Ch 55, suppressed the proto-oncogene *c-mos*, accompanied with early marker changes associated with cell differentiation.²⁹

An important effect of some retinobenzoic acids was observed in a nonneoplastic rat kidney fibroblast cell line (NRK 49F).³⁰ These compounds are also potent enhancers of the binding of EGF to the cellular receptor. This enhancement of the growth factor is possibly caused by the increase of EGF receptor. Ch 55 is particularly active, being the most active compound so far tested.

It is expected that retinoids, including the new retinobenzoic acids, would share a common mechanism of action. A possible mode of action is similar to that of steroids, where the binding proteins play a role in the translocation of active compounds to specific sites on the chromatin, resulting in the specific modulation of gene transcription. In relation to this hypothesis, specific binding of the new retinobenzoic acids to cellular retinoic acid-binding protein (CRABP) was studied.^{21,26,31} Some of the Am series of analogs exhibited reasonable binding to CRABP. However, the most active compound (Ch 55) did not bind to CRABP from rat testis,²¹ from rabbit tracheal epithelial cells²¹ and from bovine adrenal glands.²⁶ There was no direct correlation between biological activity and binding ability to CRABP. The results bring into question the role of these binding proteins in the mechanism of action of retinoids.²¹

VIII. CONCLUSION

New benzoic acid derivatives named generically retinobenzoic acids are potent inducers of the HL-60 promyelocytic leukemia cells to granulocytes. Structure-activity relationship studies showed the importance of a *meta*-alkyl substituent and the geometry of the linking group of the two benzene rings. These compounds also showed several important biological activities of retinoids including growth-promoting action in vitamin A-deficient rats (illustrated for Ch 55).³² Because the most potent compound, Ch 55, did not bind to CRABP, this compound should be an important tool for the elucidation of the mechanism of action of retinoids.³³⁻³⁷

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Chapter 13

**REGULATION OF SQUAMOUS DIFFERENTIATION IN
TRACHEOBRONCHIAL EPITHELIAL CELLS BY SYNTHETIC
RETINOIDS****Anton M. Jetten****TABLE OF CONTENTS**

I.	Introduction	288
II.	Multistep Process of Squamous Cell Differentiation.	288
III.	Effect of Retinoic Acid on Squamous Cell Differentiation	291
IV.	Structure-Activity Relationships.....	291
V.	Mechanisms of Action	302
VI.	Summary	303
	References.....	304

I. INTRODUCTION

The pseudostratified tracheobronchial epithelium consists of a variety of cell types whose frequency varies from species to species.¹ In tracheas of most species the ciliated cell is the most abundant cell type, often representing 40 to 60% of the total cell population. The ciliated cell is considered to be a terminally differentiated cell — a cell that has irreversibly lost its ability to divide. The secretory cells (Clara, mucous, and serous cells) and basal cells all have proliferative capacity. The Clara and mucous cells can further differentiate into ciliated cells.^{3,4} The function of the basal cell is not yet established. Some investigators have suggested that it functions as the stem cell for several differentiated cells.

Retinoids are a class of naturally occurring compounds that are known to influence the differentiation of the tracheobronchial epithelium. As early as 1920, Wolbach and Howe⁵ observed in rat tracheas that during vitamin A-deficiency the mucociliary epithelium disappears and is replaced by a squamous metaplastic epithelium. Inclusion of vitamin A in the diet reverses this condition and a normal mucociliary epithelium is re-established. Later studies *in vivo* and in tracheal organ cultures have confirmed and extended these observations.⁶⁻¹⁶ Vitamin-A deprivation is not the only condition that results in squamous metaplasia. It can also be induced by mechanical or toxic injury of the tracheal epithelium^{4,9,11,17-20} and can be observed in squamous cell carcinomas. Retinoids have also been shown to inhibit and reverse squamous metaplasia induced by toxic injury. For example, Lasnitzki and Bollag²¹ reported inhibition and reversal by etretinate of 3,4-benzpyrene- and cigarette smoke condensate-induced hyperplasia and metaplasia. Moreover, increased retinoid intake has been reported to reverse and reduce the number of squamous metaplastic foci in the lungs of smokers.²²

The tracheal organ culture system has been used extensively to test the efficacy of a wide variety of chemically synthesized retinoids in reversing squamous metaplasia.²³⁻²⁶ The reasons for the extensive use of this system were twofold, namely, (a) the tracheal organ culture system is very sensitive to retinoids, and (b) the trachea is a target tissue for retinoid action and therefore physiologically relevant. The tracheal organ culture assay has been a most valuable procedure for initial evaluation of the biological activity of new retinoids.

This chapter describes the multistep process of squamous differentiation in rabbit tracheal epithelial cells cultured *in vitro* and the effect that retinoic acid and several series of different synthetic analogs have on this process.

II. MULTISTEP PROCESS OF SQUAMOUS CELL DIFFERENTIATION

Despite the fact that the effects of retinoids on the differentiation of tracheobronchial epithelial cells have been studied intensively, there still exists a considerable uncertainty as to what the target cells are in the tracheobronchial epithelium and what the mechanism of action is of these agents. Since tracheal organ culture systems do not lend themselves very well to a molecular approach of the study of differentiation, *in vitro* cell culture systems have been developed.²⁷⁻³⁰ In our studies, cultured epithelial cells from the rabbit trachea were used as an *in vitro* model system.^{27,31-33} Depending on the substratum and the presence of retinoids in the medium, these cells express either a squamous cell phenotype or a mucosecretory phenotype.³¹⁻³³

Squamous cell differentiation of rabbit tracheal epithelial cells is a multistep process consisting of at least the following three steps: (1) commitment to terminal cell division, (2) expression of the squamous differentiated phenotype, and (3) cornification.³⁰⁻³³ A schematic view of the multi-step process of squamous cell differentiation is presented in Figure 1. The commitment to terminal cell division is characterized by the irreversible loss of the

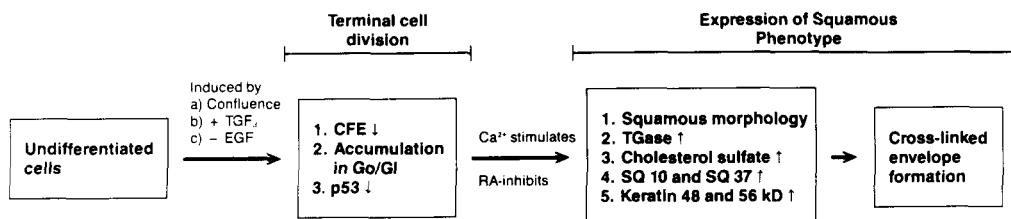


FIGURE 1. Schematic view of the multistep process of squamous cell differentiation in rabbit tracheal epithelial cells *in vitro*. In the first, retinoid-insensitive stage, cells undergo terminal cell division. This is accompanied by an accumulation of cells in G₀/G₁ phase of the cell cycle and a drop in colony-forming efficiency (CFE). Terminal cell division is followed by the expression of the squamous cell phenotype and finally cornification. These stages are stimulated by high Ca²⁺ concentrations and inhibited by retinoids.

ability to undergo cell division and is accompanied by an accumulation of the cells in the G₀/G₁ phase of the cell cycle.³⁴ After terminal cell division, cells begin to express a squamous cell phenotype and several squamous cell-specific biochemical markers are expressed. Finally, cells form cross-linked envelopes. This process of squamous cell differentiation has certain similarities to the differentiation of epidermal keratinocytes.³⁵ In both systems, cross-linked envelopes are formed by synthesis of a layer of cross-linked proteins just beneath the plasma membrane. At least three factors are involved in this process: specific proteins, which are the precursors of the cross-linked envelope; the enzyme transglutaminase, which cross-links these proteins; and Ca²⁺ ions, which are necessary to activate transglutaminase. Squamous cell differentiation of rabbit tracheal epithelial cells in culture is accompanied by a 20- to 30-fold increase in Type I (epidermal) transglutaminase.³⁴ Immunohistochemical staining of vitamin A-deficient tracheas with a monoclonal antibody against Type I transglutaminase has shown that this transglutaminase is associated solely with the squamous, cornifying cell layers in the trachea, indicating that the expression of transglutaminase Type I is also a marker for squamous differentiation of these cells³⁶ *in vivo*.

An accumulation of cholesterol sulfate has also been correlated with the expression of the squamous cell phenotype.^{37,38} In rabbit tracheal epithelial cells levels of cholesterol sulfate are increased 100- to 200-fold during squamous differentiation. This increase in cholesterol sulfate appears almost solely related to an increase in the activity of the biosynthetic enzyme cholesterol sulfotransferase. While the function of cholesterol sulfate is not known, the fact that its formation is closely associated with the formation of cross-linked envelopes suggests that it may be involved in this process.

Other changes that occur during squamous differentiation of rabbit tracheal epithelial cells are alterations in keratin expression (in particular the increased expression of keratin 13), and a reduction in the release of hyaluronic acid in the medium.²⁷

To study the regulation of gene products differentially expressed during squamous differentiation of tracheal cells, a cDNA library was constructed from poly(A)⁺ RNA present in squamous differentiated rabbit tracheal epithelial cells.³⁹ Screening of the cDNA library was aimed at the identification of RNAs that are abundant in squamous cells but that are expressed only at low levels in undifferentiated cells. Two different recombinants, designated SQ10 and SQ37, were obtained containing inserts (0.86 and 0.77 kb in size, respectively) that hybridize to mRNAs 1.0 and 1.25 kb in length, respectively. These RNAs are present at approximately 50-fold higher levels in squamous cells than in undifferentiated cells (Figure 2). The increase in the levels of the 1.0 and 1.25 kb RNAs correlates closely with the onset of squamous differentiation and is not related to the induction of terminal cell division. Therefore, these two RNAs appear to be squamous cell-specific RNAs.

It is not clear what the molecular mechanism is that initiates the program of squamous cell differentiation at confluence. It is possible that there is a link between the cessation of

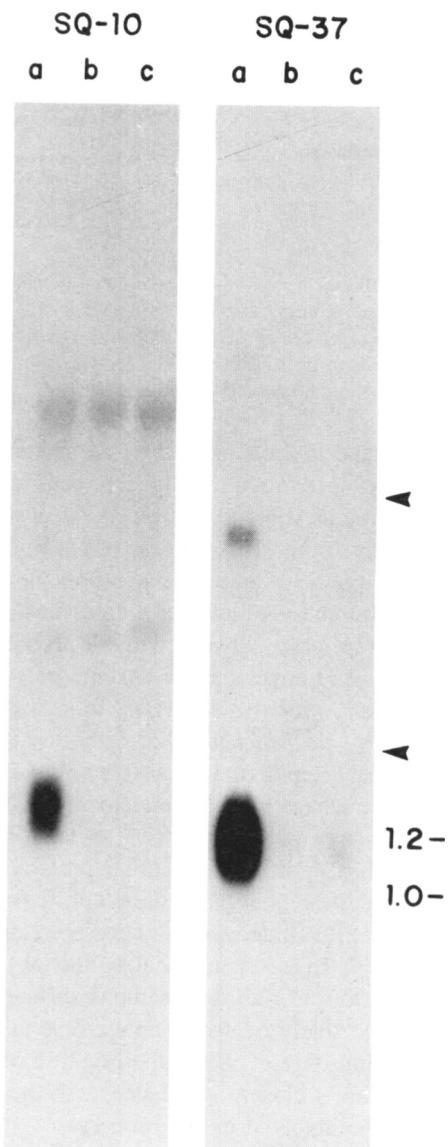


FIGURE 2. Expression of squamous cell-specific RNAs during differentiation of rabbit tracheal epithelial cells. Total cellular RNA isolated from squamous differentiated cells (lane a), undifferentiated cells (lane b), and cells treated with $3 \times 10^{-7} M$ retinoic acid (lane c) were electrophoresed through a 1.2% agarose formaldehyde gel, transferred to nitrocellulose, and hybridized to the nick-translated probes SQ10 and SQ37. Arrowheads indicate 2.0 and 4.2 kb RNA standards. (From Smits et al., *Mol. Cell Biol.*, 1987. With permission.)

proliferation and the induction of differentiation. Cellular proliferation and differentiation may be coordinately regulated. The latter is supported by our findings on the effects of epidermal growth factor (EGF) and transforming growth factor β (TGF β) on rabbit tracheal epithelial cells. Decreasing EGF concentrations result in a reduction of cell proliferation and an increase in the levels of transglutaminase Type I activity suggesting an inverse relationship between proliferation and differentiation. The addition of TGF β commits the cells to terminal

cell division and induces the expression of the squamous cell phenotype.⁴⁰ Calcium ions affect the program of squamous cell differentiation in a different manner than EGF and TGF_β. A high calcium ion concentration in the medium (1.8 mM instead of 0.3 mM) has little effect on cell growth and does not effect the commitment to terminal cell division.^{32,34} However, a high calcium concentration stimulates the expression of the squamous differentiated phenotype as indicated by a stimulation of transglutaminase activity and an increase in the rate of crosslinked envelope formation.

III. EFFECT OF RETINOIC ACID ON SQUAMOUS CELL DIFFERENTIATION

Retinoids, which inhibit squamous cell differentiation both *in vivo* and in organ cultures, also inhibit this pathway of differentiation in rabbit tracheal epithelial cells *in vitro*.^{30-34,36-40} The addition of retinoic acid at concentrations up to 10⁻⁸ M has little effect on the growth of rabbit tracheal epithelial cells. Higher concentrations inhibit cell proliferation. When cultures reach confluence, the presence of retinoic acid does not prevent the cells from undergoing terminal cell division. These findings suggest that this first step in the process of squamous cell differentiation is insensitive to retinoids. However, retinoids inhibit the subsequent steps in this pathway of differentiation. This is indicated by a decrease in several biochemical markers that are associated with the expression of the squamous cell phenotype such as transglutaminase activity, cholesterol sulfate levels, cholesterol sulfotransferase activity, changes in keratin synthesis, the expression of the two squamous cell-specific RNAs hybridizing to the SQ10 and SQ37 recombinant DNAs (Figure 2), and the formation of cross-linked envelopes.³⁶⁻⁴⁰ The differential effect of retinoic acid on the process of terminal cell division and the expression of the squamous cell phenotype suggests that cell proliferation and differentiation are regulated separately. The fact that the commitment to terminal cell division appears to proceed the expression of the differentiated phenotype may indicate a link between these two controls. The findings on the effects of EGF and TGF_β, described above, seem to support this hypothesis.

IV. STRUCTURE-ACTIVITY RELATIONSHIPS

To examine the structure-activity relationships of retinoids, the effect of several synthetic analogs on squamous differentiation in rabbit tracheal epithelial cells was determined. The chemical structures of the derivatives used in this study are shown in Tables 1 and 2. The retinoids were synthesized and provided by Hoffmann-La Roche, Inc., Nutley, NJ, (compounds **1** through **7**), Dr. M. Dawson, SRI International, Menlo Park, CA, (compounds **8** through **22**), and Dr. K. Shudo, University of Tokyo, Japan, (compounds **23** through **28**). The effect of these analogs on rabbit tracheal epithelial cells was determined by measuring the inhibition of the induction of transglutaminase Type I activity, the increase in cholesterol sulfate levels, and crosslinked envelope formation, all of which accompany squamous cell differentiation.^{34,37} The efficiency with which the retinoids inhibit transglutaminase activity correlates well with their ability to inhibit cholesterol sulfate levels and crosslinked envelope formation (Tables 1 and 2). In particular, the measurement of cholesterol sulfate is an easy and very sensitive assay and appears an excellent tool to screen the activity of retinoids (Figure 3). Previous studies^{24-26,41-45} in different cell systems have shown that substitution of the carboxylic acid group of a retinoic acid derivative by an alcohol, aldehyde, ether, or amide generally reduces the biological activity, indicating the importance of the carboxylic acid group in the activity of retinoids. The findings presented in Tables 1 and 2 are in agreement with this conclusion. Substitution of the COOH-group of compound **7c** by hydrogen (compound **7a**) leads to an inactive analog, whereas replacement by a SO₃Na group

TABLE 1
Comparison of the Inhibition of Transglutaminase Activity, Cholesterol Sulfate Accumulation, and Cross-linked Envelope Formation by Various Retinoids with their Binding to CRABP

Retinoids	R group	TGASE (% control)		Cholesterol sulfate (% control)		Cross-linked envelopes (% control)	Inhibition of [³ H]RA binding (%)
		$10^{-8} M$	$10^{-10} M$	$10^{-8} M$	$10^{-10} M$		
1a		COOH	15	5	110	<1	91
b		$\text{CH}_2\text{OOCCH}_3$	87	11	155	100	0
2			63	20	118	49	50
3			2	44	175	<1	89
4			58	36	66	61	57
5			100	138	128	100	0
6			100	82	118	100	0

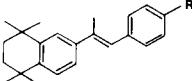
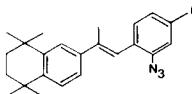
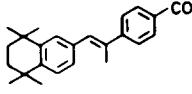
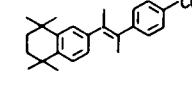
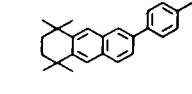
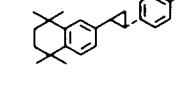
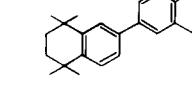
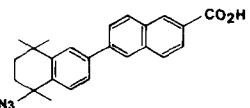
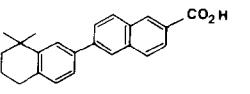
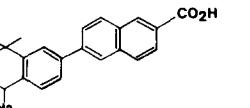
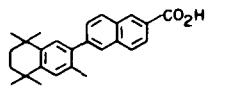
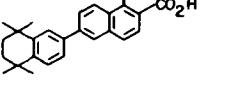
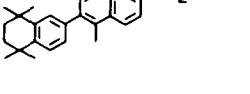
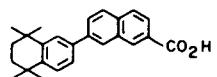
7a		H	100	89	127	100	0
b		SO ₃ Na	<1	2	31	<1	100
c		COOH	<1	1	6	<1	99
8			49			64	27
9			5	10	1	2	0
10			68	131	33	161	100
11			23	130	2	73	<1
12			14	12	2	3	<1
13			9		2	15	<1
							91

TABLE 1 (continued)

Comparison of the Inhibition of Transglutaminase Activity, Cholesterol Sulfate Accumulation, and Cross-linked Envelope Formation by Various Retinoids with their Binding to CRABP

Retinoids	R group	TGASE (% control)		Cholesterol sulfate (% control)		Cross-linked envelopes (% control) $10^{-8} M$	Inhibition of [3 H]RA binding (%)
		$10^{-8} M$	$10^{-10} M$	$10^{-8} M$	$10^{-10} M$		
14		8				<1	84
15		7				<1	63
16		27				<1	
17		11	37	2	12	<1	
18		7	91	1	38	<1	
19		47	114	14	187	5	

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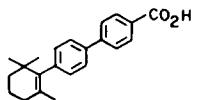
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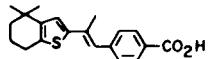
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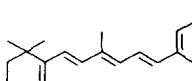
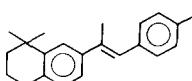
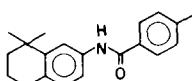
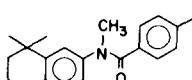
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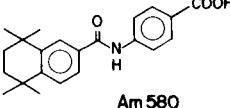
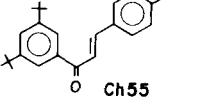
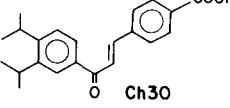
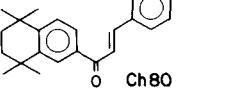
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Note: Rabbit tracheal epithelial cells were grown to confluence in the presence of the indicated molarity of retinoid and the incorporation of radioactive sulfate into cholesterol sulfate,³⁷ the activity of transglutaminase³⁴ and the formation of cross-linked envelopes³⁴ were measured. Values were expressed as percent of the comparable value of control cells that received no retinoid. Competition for binding [³H]retinoic acid ([³H]RA) to CRABP isolated from rabbit tracheal cells was carried out at $3 \times 10^{-6} M$ unlabeled retinoids. Value is expressed as percent inhibition. Derivatives 1 through 7 provided by Dr. P. Sorter, Hoffman-La Roche; Derivatives 8 through 22 provided by Dr. M. Dawson, SRI International.

TABLE 2
**Comparison of the Biological Activity and Binding to CRABP of Retinoic Acid,
Ro 13-7410 and Benzoic Acid Analogs from the Am- and Ch-Series**

Cell system parameter	Analog	RbTE Cells		F9 Plasminogen activator	HL-60 ^a			3T6 ODC	Binding to CRABP		
		TGase type I	EC ₅₀		EC ₅₀	EC ₅₀	NBT				
1a RA	 RA		0.4	EC ₅₀	EC ₅₀	EC ₅₀	250	1.0	EC ₅₀	EC ₅₀	K _I
7 Ro 13-7410	 Ro 13-7410	0.1	0.01		0.2		NT	0.3	6.0	NT	0.18
23 Am80	 Am 80	1.1	NT		6.3		850	0.4	16	15	3.6
24 Am90	 Am 90	>100	>100		>100		>100	>100	NT	NT	>100

25	Am580		Am 580	1.0	0.2	3.8	950	NT	22	26	4.0
26	Ch55		Ch 55	0.02	0.03	0.26	300	0.04	0.5	1.0	540
27	Ch30		Ch 30	4.0	0.5	0.8	NT	0.8	30	NT	>100
28	Ch80		Ch 80	0.4	0.1	0.6	700	0.7	10	NT	>100

Note: The concentrations at which half-maximum inhibition or stimulation (EC_{50}) were obtained are expressed in nM. The inhibitory constant K_i is expressed in μM . NT = not tested. TGase = transglutaminase activity; Chol-SO₄ = cholesterol sulfate levels; NBT = nitroblue tetrazolium reduction; RA = retinoic acid; ODC = ornithine decarboxylase activity.^{21,22} The analogs in the Am- and Ch-series were provided by Dr. K. Shudo, University of Tokyo. (From Jetten et al., *Cancer Res.*, 47, 3523, 1987. With permission).

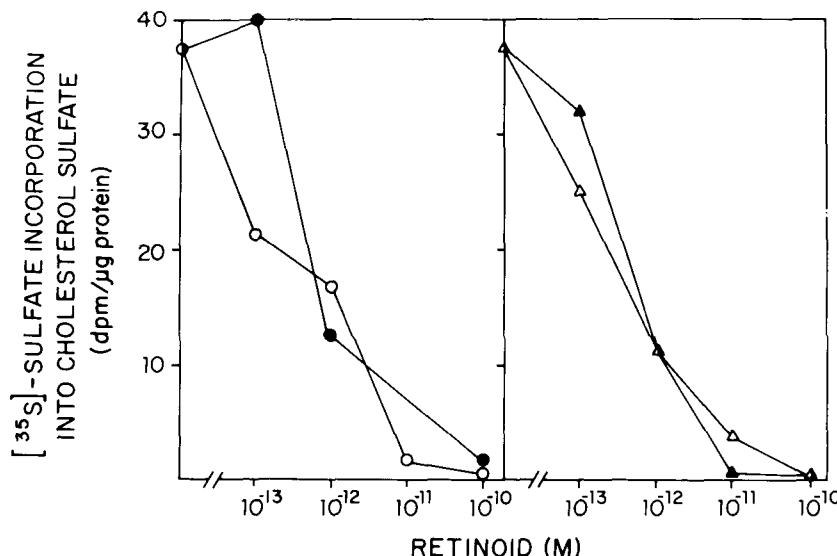


FIGURE 3. Concentration dependence of retinoid inhibition of cholesterol sulfate accumulation by rabbit tracheal epithelial cells. ○, compound 9; ●, compound 17; △, compound 21; ▲, compound 21.

(compound 7b) yields a still very active compound. Replacement of the 11,13*E*-dienic-bond system by a naphthalene ring (compound 13) yields very active compounds. Certain substitutions by methyl groups have little effect whereas others decrease the activity significantly (compare compounds 7c, 9, and 10). Substitution by an azide group (compound 8) markedly reduces the activity. Comparison of the activity of Am 80 and Am 90 shows that methylation of the amide nitrogen group results in the complete elimination of the biological activity. Thus, minimal modification of the chemical structure can have dramatic effects on the biological activity of the retinoid and indicate that for high activity a certain structural specificity is required. These structural requirements are dictated by the binding site of retinoid receptor(s), the putative mediators of retinoid action. A good illustration of the relationship between geometrical shape and biological activity was provided by Frickel.⁴⁶ The geometrical conformation was determined by X-ray crystallography. Comparison of the geometrical structure of all-trans-retinoic acid with that of compound 7c, which has the carboxyl group in the *para* position, shows that the structures closely overlap each other (Figure 4a). However that *meta*-compound cannot bring its carboxyl group into a spatially adjacent position to the carboxyl group of all-trans-retinoic acid (Figure 4B). The similarity and dissimilarity of the geometrical conformation of the *para*- and *meta*-compounds with that of retinoic acid may explain why the *para*-compound exhibits a level of biological activity compared with that of retinoic acid and why the *meta*-compound does not.

Recently, we made an extensive study of the biological activity of several newly synthesized benzoic acid acid derivatives (Table 2) of the Am- and Ch-series, which are structurally different from retinoic acid and arotoninoids.⁴⁷ The Am-series consists of terephthalic anilides, whereas the Ch-series consists of chalcone-4'-carboxylic acid derivatives.⁴⁸⁻⁵⁰ The chemical structures of several analogs are presented in Table 2. Like retinoic acid, these derivatives inhibit the induction of Type I transglutaminase during squamous differentiation of rabbit tracheal cells in a concentration-dependent manner (Figure 5A). The EC₅₀ values for Am80, Am580, Ch55, and retinoic acid were 1.1, 1.0, 0.02, and 0.4 nM, respectively (Table 2). The increase in cholesterol sulfate also was inhibited by these agents in the nanomolar to picomolar range (Table 2).

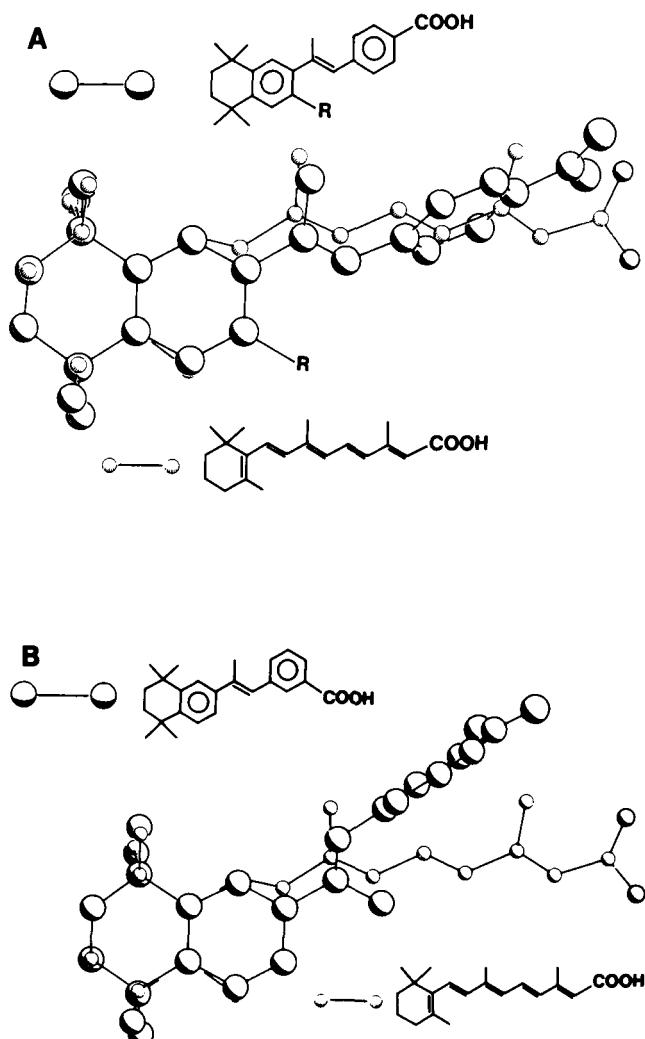


FIGURE 4. Jointly projected structures of all-trans-retinoic acid and a *p*-carboxy analog (A), and a *m*-carboxy analog of retinoidal benzoic acid. (From Frickel, F., in *Retinoids. Differentiation and Disease*, CIBA Foundation Symposium 113, Nugent, J., and Brown, S., Eds., Pitman, London, 1985, 21. With permission.)

These derivatives were also tested in several other experimental cell systems known to be responsive to retinoic acid.⁴⁷ Like retinoic acid, derivatives from the Am- and Ch-series can induce differentiation of embryonal carcinoma F9 cells into parietal endoderm-like cells as indicated by the irreversible change in cellular morphology and the induction of plasminogen activator production (Figure 5B). These compounds also induce differentiation of the human promyelocytic leukemia HL-60 cells.^{48,49} Davies et al.⁵¹ have shown that retinoic acid induces transglutaminase Type II activity in HL-60 cells. This action is highly specific for retinoids because other compounds that induce differentiation do not increase this enzymatic activity. Treatment of HL-60 cells with Ch55, Am80, or Am580 also induces Type II transglutaminase activity (Table 2). The concentrations of Am80, Am580, and Ch55 required to induce transglutaminase activity are significantly higher than those needed to induce differentiation of HL-60 cells into granulocytes (Table 2) as has been reported

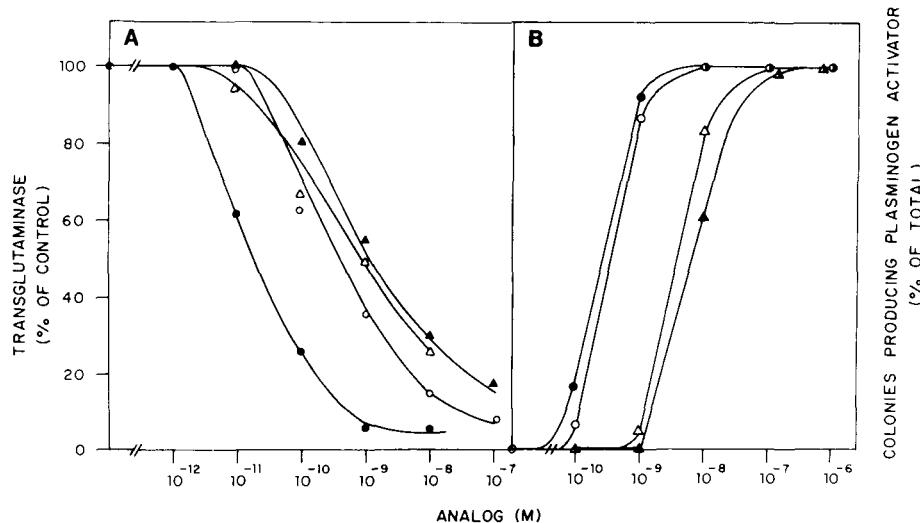


FIGURE 5. Effect of Am80, Am580, Ch55, and retinoic acid on the differentiation of rabbit tracheal epithelial cells and embryonal carcinoma F9 cells. (A) Inhibition of transglutaminase Type I activity in rabbit tracheal epithelial cells. (B) Stimulation of plasminogen activator production in F9 cells. ○, retinoic acid; ●, Ch55; ▲, Am80; △, Am580. (From Jetten, A. M. et al., *Cancer Res.*, 47, 3523, 1987. With permission.)

previously for retinoic acid.^{51,52} These results may indicate that in these cells the induction of differentiation and the induction of transglutaminase activity are regulated by two different mechanisms.

In addition to their effects on differentiation, retinoids also affect various parameters associated with cellular proliferation.⁴⁷ The Am- and Ch-derivatives inhibit the growth of melanoma S91 cells and inhibit that induction of ornithine decarboxylase activity induced by 12-O-tetradecanoylphorbol-13-acetate in 3T6 fibroblasts (Table 2). And recently, it has been shown⁵⁰ that these derivatives can increase the binding of epidermal growth factor in NRK fibroblasts, as has been reported previously for retinoic acid.⁵³

Comparison of the action of the Am- and Ch-analogs with that of retinoids shows that these groups of compounds elicit very similar biological and biochemical responses in various different cell types (Table 2). Many of the Am- and Ch-analogs are active at very low concentrations and some, such as Ch55, appear more active than retinoic acid. Although the Am- and Ch-compounds are structurally different from retinoids and arotinoids, they have certain structural similarities with retinoic acid and the arotinoid Ro 13-7410 (Table 2). The fact that these compounds have such a wide variety of biological, biochemical and structural similarities to retinoids makes it likely that the Am- and Ch-analogs exert their biological effects through mechanism(s) similar to that of retinoic acid.

Retinoids have been defined as compounds related to vitamin A (retinol) and as "diterpenoids derived from a monocyclic parent compound containing five carbon-carbon double bonds and a functional group at the terminus of the acyclic portion" (IUPAC-IUB). This definition, however, excludes the large number of tricyclic and tetracyclic derivatives, such as the arotinoids and Am- and Ch-derivatives (compounds 23 through 28), which exert biological and biochemical activities indistinguishable from the "classical" retinoids.^{23,23,47,54} Sporn et al.⁵⁰ have proposed an alternative definition: "A retinoid is a substance that can elicit biological responses by binding to and activating a specific receptor or set of receptors". As mentioned above, the structural specificity is dictated by the binding site of retinoid receptors and it is likely that the receptor is involved in determining the response as is the case for other ligand-receptor systems. Although this definition is not

very specific, it puts the emphasis on the receptor and response rather than the structure of the retinoid. Until better knowledge is obtained about the characteristics of the receptor, this definition appears satisfactory for now.

V. MECHANISMS OF ACTION

Although the biological and biochemical responses to retinoids have been studied intensively in a variety of different cell systems,⁵⁵⁻⁵⁷ the mechanism of action is not fully established. Possibly, retinoids elicit their responses via more than one independent mechanism. Considerable evidence has been accumulated to support the idea that retinoic acid and retinol influence genomic expression by activating or repressing specific genes. The synthesis of many gene products in a variety of cell systems has been reported to be affected by retinoids. In tracheobronchial and epidermal epithelial cells retinoids inhibit squamous cell differentiation as indicated by the reduction in transglutaminase type I, sulfotransferase and keratin expression.^{33,34,38,58,59} In tracheobronchial epithelial cells retinoids enhance the production of mucin-glycoproteins.^{29,36} Induction of differentiation in embryonal carcinoma F9 cells by retinoids is accompanied by changes in laminin, entactin, and collagen IV synthesis.⁵⁶ In a variety of systems retinoic acid increases the synthesis of epidermal growth factor receptor.⁵⁷ Retinoids could affect the expression of these genes at a transcriptional or post-transcriptional level. The high structural specificity exhibited by retinoids and the low concentrations at which retinoids act are indicative that these compounds operate via high-affinity receptor proteins. Chytil and Ong^{60,61} hypothesized that retinoids, like steroid hormones, act via specific receptors that bind to specific sites in the chromatin thereby altering gene expression. They proposed that the cellular retinoic acid- and retinol-binding proteins (CRABP and CRBP, respectively) may be involved in such a mechanism of action. Several findings appeared in agreement with this idea. Translocation of the holoprotein to the nucleus and specific interactions with chromatin have been demonstrated.⁵²⁻⁵⁴ Moreover, mutants have been isolated from embryonal carcinoma cells that do not respond to retinoids and lack CRABP.^{65,66} However, other studies appeared inconsistent with a role of CRABP in the mechanism of action of retinoic acid. Several cell systems that are very responsive to retinoid lack any detectable levels of CRABP or CRBP.^{67,68} In addition, certain benzoic acid derivatives of retinoic acid (Ch-series), which are more than or as equally active as retinoic acid, do not bind to CRABP (Figure 6).^{47,48} In the light of our current knowledge about retinoid-metabolism, it appears unlikely that these analogs are metabolized to compounds able to bind to CRABP. These studies seem to question a role for CRABP/CRBP in the mechanism of action of retinoids and seem to suggest that other receptors are involved.^{47,50} Recently, several laboratories⁶⁹⁻⁷² have identified nuclear retinoic acid receptors (RARs), which belongs to a family of receptors that include the receptors for steroid hormones, thyroid hormones and vitamin D3. These receptors share a highly conserved DNA-binding region. These retinoic acid receptors have a high affinity for retinoic acid and a 100-fold lower affinity for retinol and are present at low abundance (several thousand molecules/cell). These receptors appear to represent retinoic acid-dependent, transacting enhancer factors that interact with specific sequences in the DNA thereby inducing changes in the rate of transcription of specific genes (Figure 7). Some of these genes might represent transcriptional factors themselves that then regulate the transcription of other genes. Studies are now focussed on the identification of genes that are under a direct control by this receptor and the analysis of the enhancer sequence.

One of the questions remaining is what is the function of CRABP/CRBP? It could be possible that CRABP translocates retinoic acid to the nucleus and transfers retinoic acid to the nuclear retinoic acid receptor. However, as discussed above, it appears that CRABP is not required in the mechanism of action of retinoids. CRABP could play a role in the

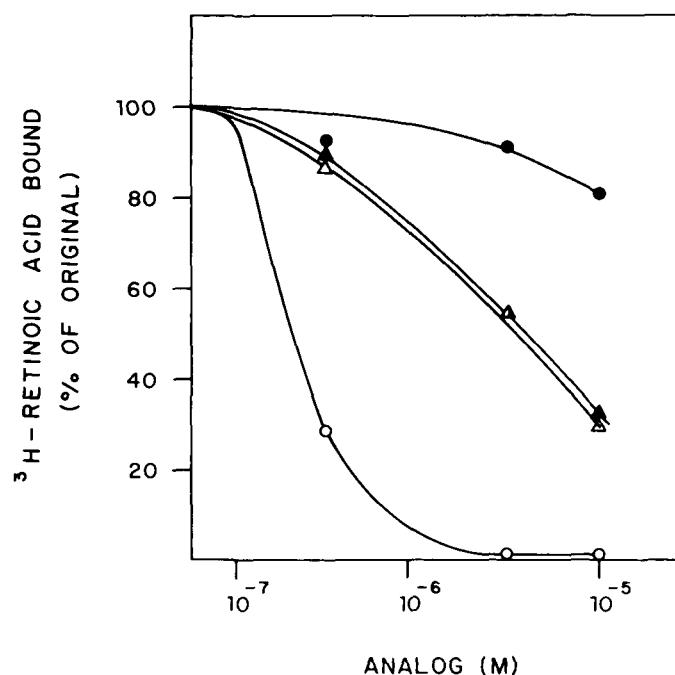


FIGURE 6. Competition Am80, Am580, Ch55 and retinoic acid with the binding of labeled retinoic acid to CRABP. ○, retinoic acid; ●, Ch55; ▲, Am80; △, Am580. (From Jetten, A. M. et al., *Cancer Res.*, 47, 3523, 1987. With permission.)

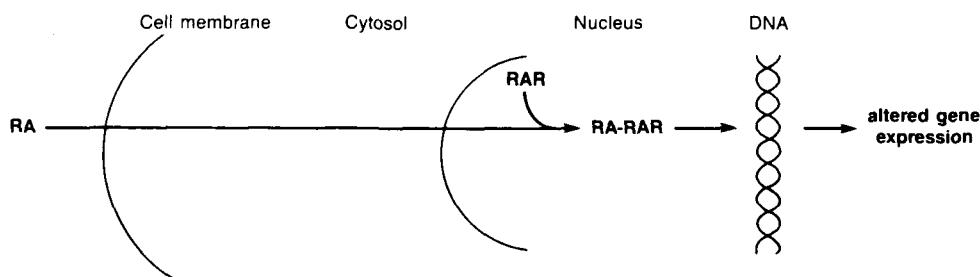


FIGURE 7. Tentative mechanism of action of retinoic acid. After binding of retinoic acid (RA) to the nuclear retinoic acid receptor (RAR), the complex interacts with specific enhancer sequences in the DNA and induces alterations in the rate of transcription of specific genes.

metabolic pathway of retinoids by transporting retinoids to the endoplasmic reticulum, the site of retinoid metabolism.

VI. SUMMARY

The tracheobronchial epithelium is a target for retinoid action. Retinoids affect the differentiation program of these cells at different stages. Retinoic acid promotes the program of mucosecretory differentiation and inhibits the program of squamous cell differentiation as indicated by the inhibition of transglutaminase type I, sulfotransferase, and keratin synthesis. We propose that the effects are mediated via a nuclear RAR. Binding of the RAR-retinoic acid complex to specific sequences in the chromatin can suppress genes associated

with the expression of the squamous cell phenotype or enhance the transcription of genes such as mucins, associated with the expression of the mucosecretory phenotype. Whether these genes are controlled directly by RAR-RA complex or indirectly via other transcriptional factors has to be established.

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Chapter 14

**STRUCTURE-BIOLOGICAL ACTIVITY RELATIONSHIPS OF
NEW SYNTHETIC RETINOIDS ON EPITHELIAL
DIFFERENTIATION OF CULTURED HAMSTER TRACHEA****Leonard J. Schiff, William H. Okamura, Marcia I. Dawson, and Peter D. Hobbs****TABLE OF CONTENTS**

I.	Introduction	308
II.	Retinoid Control of Cell Differentiation	308
III.	<i>In Vitro</i> Assay of Retinoids Using Hamster Tracheal Organ Culture.....	309
IV.	Design and Synthesis of Retinoids	310
	A. Retinoids in the Benzoic Acid Series.....	311
	B. Retinoids in the Naphthalenecarboxylic Acid Series.....	318
	C. Polar Terminus Modification	323
V.	Retinoid Structure-Biological Activity Relationships	324
	A. Background	324
	B. SRI Retinoids	325
	C. UC Riverside Retinoids	356
VI.	Conclusions	357
	Acknowledgments	358
	References	359

I. INTRODUCTION

Although advances have been made in the traditional approaches to cancer prevention and therapy, these diseases still remain one of the major problems in medicine. New therapies involving immunological methods and biological response modifiers are in the early stages of development. Because retinoids (vitamin A and its natural and synthetic derivatives and analogs) have a vital role in growth and differentiation of many types of epithelial cells, and therefore have potential value for chemoprevention of epithelial cancers, they have pharmaceutical importance. The activity of an individual retinoid, however, is dependent on pharmacokinetic properties, including metabolism.

Retinoic acid can be modified at the hydrophobic ring, the polyene side chain, or the polar terminus to reduce toxicity and alter tissue distribution, yet still maintain the ability to control normal epithelial cell differentiation.

In this chapter we examine the ability of a variety of structurally modified retinoids to reverse keratinizing squamous metaplasia in vitamin A-deficient hamster tracheas in culture.

II. RETINOID CONTROL OF CELL DIFFERENTIATION

The first study showing epithelial tissue changes resulting from vitamin A deficiency was reported by Mori in the early 1920s.¹ A few years later, Wolbach and Howe,^{2,3} using vitamin A-deficient rodents, demonstrated the importance of vitamin A in controlling proliferation and normal differentiation in a variety of epithelial tissues. In their studies, differentiation of stem cells into mature epithelial cells did not occur. Abnormal cellular differentiation was characterized by keratin accumulation and excessive cellular proliferation. Several *in vivo* and *in vitro* studies have since confirmed these observations.⁴⁻⁹ Consequently, it was reasonable to suggest that retinoids may have an application in the prevention of cancer.

The importance of retinoids in chemoprevention of cancer received attention with the findings that retinoids can prevent cancer of the bladder, lung, and skin in experimental animals¹⁰⁻¹⁶ and suppress *in vitro* expression of malignant transformation.¹⁷⁻²⁰ Certain retinoids have shown the capacity to suppress the proliferation⁹ or reverse the neoplastic transformation of cells with a neoplastic phenotype to terminal differentiation.²¹⁻²⁴

The development of organ culture methods has significantly enhanced our understanding of the mechanisms of action of retinoids. The first report of retinoid activity in organ culture involved chick epidermis, which changed from keratinization to mucus production when treated with retinol or retinyl acetate.²⁵ Studies by Lasnitzki²⁶ showed that hyperplasia and squamous metaplasia produced by 3-methylcholanthrene in mouse prostate in organ culture was reversed or inhibited by natural and synthetic retinoids. Furthermore, in prostatic epithelium treated with carcinogenic polycyclic hydrocarbons and vitamin A-deficient prostatic epithelium, normal cellular differentiation was restored by retinoid treatment, further substantiating the relationship between the mechanisms of action in differentiation and carcinogenesis.²⁷

When laboratory animals were deprived of vitamin A in their diet, their tracheobronchial epithelium lost its normal pattern of differentiation (pseudostratified mucociliary epithelium), and squamous metaplasia and keratinization developed.²⁸⁻³⁰ Tracheobronchial epithelium from vitamin A-deficient hamsters that was maintained in organ culture without retinoids underwent epidermoid transformation similar to that seen *in vivo*.^{31,32} Mucociliary epithelium was restored when vitamin A was given either to the animal^{2,28,29,33} or to trachea in organ culture.³⁴⁻³⁷

The replication potential of different cell types and the characterization of morphological changes in hamster tracheal epithelium that occur during vitamin A deprivation and reversal

of vitamin A deficiency have been investigated in detail by McDowell and co-workers.^{38,39} The morphological changes in vitamin A-deprived tracheal epithelium varied from animal to animal and from area to area within a tracheal ring.³⁸ This was also the case in vitamin A-deficient hamster trachea in organ culture.^{31,32} Vitamin A deprivation decreased the replication of those basal cells and mucous cells in hamster tracheal epithelium that showed minimal morphological changes.³⁸ Mitotic rates and labeling indices dropped approximately 4-fold in basal cells and 14-fold in mucous cells, compared with control epithelium from hamsters fed a normal diet. The mucosa of tracheas from vitamin A-deficient hamsters showed a range of epidermoid metaplasia. In focal areas of stratification and epidermoid metaplasia, cell replication increased over that of controls, with over 70% of the mitotic activity being associated with "nonbasal cells". It was concluded from these studies that both a *direct effect* (repression of cell replication coinciding with the minimal changes in the epithelium) and an *indirect effect* (increase in cell replication coincident with metaplasia) depended on and were initiated by cell death and loss resulting from vitamin A deficiency.³⁸ McDowell et al.³⁹ then investigated the effects of vitamin A on the replication of basal cells and mucous cells during the reversal of the vitamin A-deprivation condition. When dietary vitamin A was fed to vitamin A-deprived hamsters, the basal cell mitotic rate remained below that of the control level throughout the seven-day experimental period. The mucous cell rate increased starting on day 2 of the vitamin A restoration and was within the control range on day 3. By day 7 of reversal, mucociliary epithelium was restored. Thus, mitotic division of mucous cells appears to be a prerequisite for ciliogenesis and new mucous cells and for restoration of mucociliary epithelium. Chopra⁴⁰ reported similar results following retinoid treatment of hamster tracheal organ cultures.

We have reviewed both *in vivo* and *in vitro* reports linking retinoids to the control of epithelial cell replication and differentiation. Because cancer is in many respects a disease of abnormal cell differentiation,⁴¹ the development of safe and effective synthetic retinoids offers the possibility of their practical therapeutic application for prevention of human cancer.

III. IN VITRO ASSAY OF RETINOIDS USING HAMSTER TRACHEAL ORGAN CULTURE

The standard hamster tracheal organ culture (TOC) assay permits measurement of the ability of biologically active retinoids to reverse keratinization (Figure 1).^{34,35,42} Tracheas were taken from hamsters that were in the very early stages of vitamin A deficiency (29- to 31-day-old hamsters maintained on a vitamin A-free diet) and placed in organ culture. At the time of culture, the hamsters were still gaining weight and the tracheal epithelium was generally low columnar or cuboidal, with only patches of squamous metaplasia.³⁵ Tracheas were removed by sterile techniques. Each trachea was then opened along the membranous dorsal wall and cultured in a 60 × 15-mm culture dish containing approximately 2 ml of a serum-free medium consisting of CMRL Medium 1066 with crystalline bovine insulin, 1.0 µg/ml; hydrocortisone hemisuccinate, 0.1 µg/ml; glutamine, 2 mM; penicillin, 100 U/ml; and streptomycin, 100 µg/ml. The cultures were placed in a controlled atmosphere chamber and gassed for 5 min at 15 l/min with a water-saturated atmosphere of 50% oxygen, 45% nitrogen, and 5% CO₂ before incubation at 36°C. The chamber was then rocked ten times per minute. For each set of retinoid bioassays with controls, tracheas were maintained in culture medium containing no retinoid for the first 3 d. At the end of 3 d, seven tracheas were removed from the culture dishes and fixed in 4% formaldehyde-1% glutaraldehyde. The remaining tracheas were divided into groups and treated with either (1) culture medium without retinoid; (2) retinoid test compounds dissolved in dimethyl-sulfoxide (DMSO) at concentrations ranging from 10⁻⁸ to 10⁻¹² M (final concentration of DMSO in culture medium was 0.1%); (3) all-trans-retinoic acid (RA, reference substance) dissolved in DMSO

***In Vitro Assay of Retinoids
Using Hamster Tracheal Organ Culture***

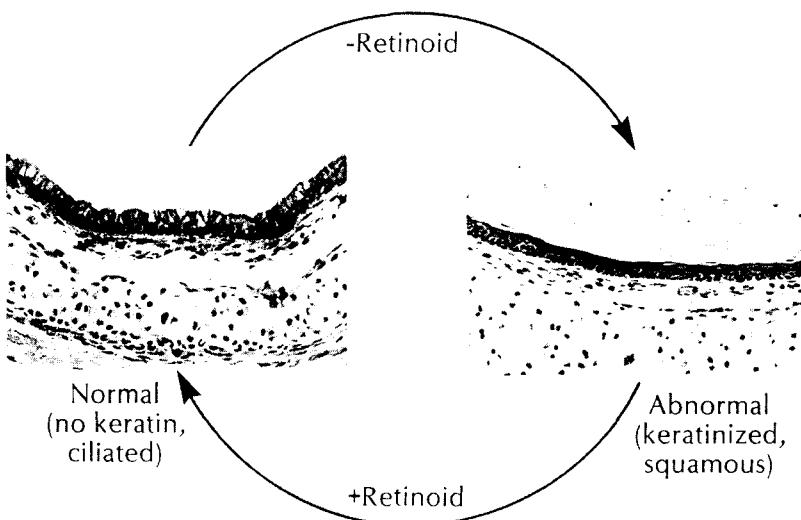


FIGURE 1. Appearance of hamster tracheal tissue in presence and absence of retinoid.

at concentrations of 10^{-10} , 10^{-11} , and $10^{-12} M$; or (4) an equivalent amount of DMSO alone. The culture medium and gas atmosphere were changed three times a week. At the end of 10 d in culture (7 d in medium containing no retinoid, retinoid test compound, RA, or DMSO alone), all remaining tracheas were fixed, dehydrated, cleared, and embedded in paraffin.

For histological evaluation, each trachea was divided into two equal portions. Cuts were made straight across the trachea so that a true transverse section could be mounted. The two portions were processed together and the anterior and posterior portions were identified. The two portions of trachea were embedded as close together as possible in the middle of the block, making sure that they were level and standing so that transverse sections could be microtomed. Eight cross-sections of $5 \mu\text{m}$ were made from the middle of each trachea and stained with hematoxylin and eosin-phloxine.

The histological status of the tracheal epithelium was assessed with respect to the extent of squamous metaplasia and the presence of keratin and keratohyaline granules, both of which have been present in approximately 90% of control cultures, i.e., tracheas evaluated at the end of 10 d in culture medium with no vitamin A.³⁰ Cultures were scored as active if neither keratin nor keratohyaline granules were seen, or as inactive if both keratin and keratohyaline granules were present. The ED₅₀, defined as the molarity of retinoid required to reverse keratinization in 50% of the cultures, was determined for each new retinoid.

IV. DESIGN AND SYNTHESIS OF RETINOID

The routes to several new aromatic retinoids that we have recently synthesized are outlined in Charts 1 through 15. The syntheses of these compounds are presented because they give a general overview of the methodology that can be used to prepare analogs of this type and because they present some interesting synthetic chemistry. Also described is the rationale for the design of these compounds. This topic is further elaborated in Section V of this chapter, in which the results of screening these retinoids in the TOC assay and structure-activity relationships are described.

A. RETINOIDS IN THE BENZOIC ACID SERIES

The methylene group at the 4-position of the retinoic acid skeleton undergoes oxidative metabolism, leading to deactivation.⁴³ Aromatic retinoids that are unsubstituted at the corresponding position of their skeleton may be similarly deactivated after administration. A methyl group at this position could prevent oxidation by replacing the hydrogen atom that would be abstracted or by blocking the abstraction of a hydrogen on the same carbon for steric reasons. Proton abstraction could be an enantioselective process, in which case one optical isomer may possess greater biological activity than its antipode. Racemic 5,6,7,8-tetrahydro-5,8,8-trimethylnaphthalenyl retinoid **32** was synthesized first to establish that the removal of a methyl substituent at the 5-position of the tetrahydronaphthalene ring of the highly active retinoid **30** would afford a retinoid (**32**) having biological activity before the syntheses of the chiral analogs **33** and **34** were undertaken. The chiral analogs were designed to determine if an enantioselective metabolic deactivation could occur by oxidation at the position of the skeleton corresponding to the 4-position of RA.

Tetralone **108** was used as the key intermediate for the preparation of **32** (Chart 1) because it had been used as a precursor in the syntheses of other retinoids that we had prepared. Friedel-Crafts acylation⁴⁴ of 1,2,3,4-tetrahydro-1,1-dimethylnaphthalene, which was prepared by treatment of tetralone **106** with dimethylzinc-titanium tetrachloride, gave a mixture of **107** and the 6-acetyl isomer, from which a low yield (17%) of **107** was isolated by crystallization and converted in seven steps to the unstable benzylic chloride **109**. Reaction of **109** with the cuprate reagent derived from methylolithium and cuprous cyanide⁴⁵ afforded a mixture of the methylated intermediate **110** and by-products derived from elimination and reduction of the halide. Intermediate **110** was produced in far more satisfactory yield by Wittig olefination of tetralone **108** followed by hydrogenation of the *exo*-olefin **111**. The synthesis of **32** was completed in the standard manner by conversion of **110** to the phosphonium salt **112**, Wittig reaction of the phosphonium salt, separation of the *E* isomer of the ester product, and saponification of the ester group. The configuration of the double bond of **32** and the other target retinoids shown in Charts 2 to 6 was determined by comparison of the 400 MHz ¹H NMR spectra of the *E* and *Z* isomers. Large differences in the chemical shifts of signals for the protons of each isomer pair are characteristic⁴⁶ and are similar to the differences reported for *E*- and *Z*-stilbene.⁴⁷

Because the 5-methyl group of the racemic retinoid **32** was best introduced by hydrogenation of the *exo*-methylene group of **111**, the corresponding synthesis was investigated for the preparation of the chiral retinoids **33** and **34**. Hydroboration of the *exo*-methylene derivative of ketone **114** using chiral boranes prepared from (+)- α -pinene⁴⁸ or (+)-longifolene⁴⁹ gave products of low optical purity (30% enantiomeric excess). Chirality at the 5-position of the tetrahydronaphthalene ring was introduced by reduction of ketone **114** with chiral hydroboranes produced by reaction of the chiral amines **115** and **116** with borane as reported by Itsuno⁵⁰ (Chart 2). The optical purities of the products, **117** and **118**, respectively, were determined to be 95% in each case from the ¹⁹F NMR spectra of the diastereomeric mixtures obtained by reaction of the alcohols with Mosher's reagents, (+)- and (-)- α -methoxy- α -(trifluoromethyl)phenacetyl chloride.⁵¹ A variety of other chiral reducing agents, such as *B*-isopinocampheyl-9-borabicyclo[3.3.1]nonane,⁵² gave far lower optical purities. The chiral alcohols **117** and **118** were converted to their mesylates, which were reacted *in situ* with lithium dimethylcuprate. Reaction of a chiral alkyl mesylate with a cuprate had been reported⁵³ to proceed with inversion of configuration. In addition to the desired chiral 5-methyl products **119** and **121**, an elimination product (**120**) was also formed. It was readily removed from the product mixtures after conversion to an alcohol by hydroboration and oxidation. Standard techniques were used to elaborate the aryl halides **119** and **121** to the chiral retinoids **33** and **34**. The optical rotations of **33** $[\alpha]_D^{23} + 4.0^\circ$ (1.00, CH₂Cl₂) and **34** $[\alpha]_D^{23} - 3.8^\circ$ (1.00, CH₂Cl₂) indicated almost equal enantiomeric purities

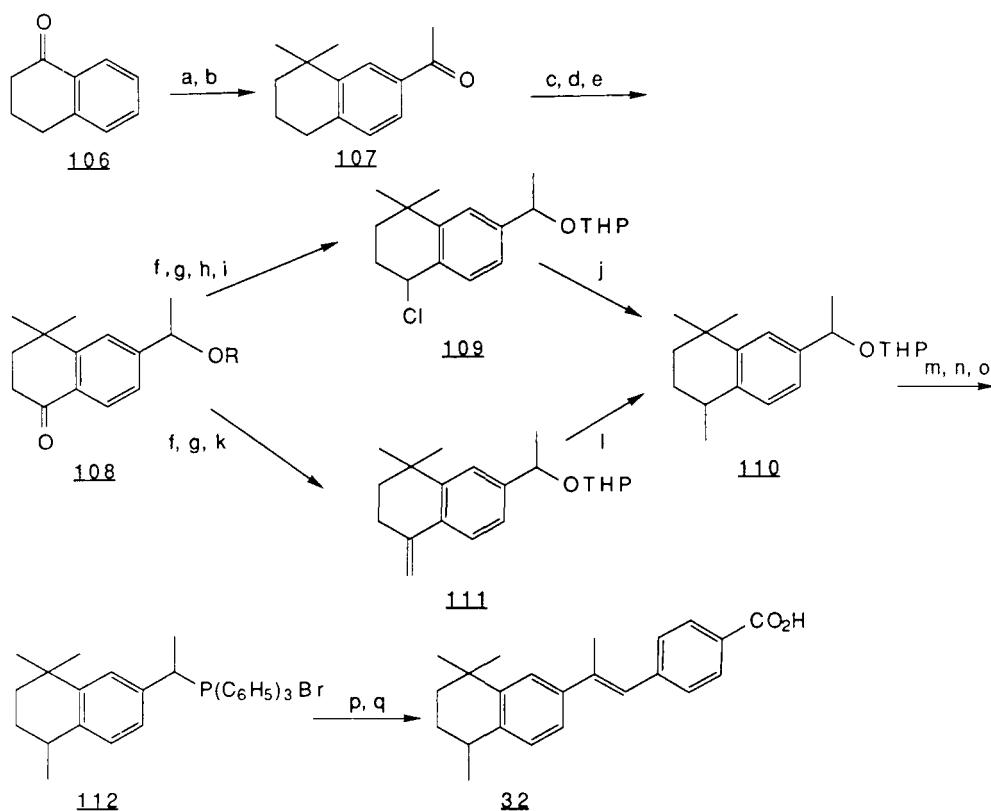


Chart 1. (a) TiCl_4 , Me_2Zn , CH_2Cl_2 , -40°C , 15 min; -10°C , 4 h, then 20°C , 16 h (95%); (b) AcCl , AlCl_3 , 20°C , 2 h (17%); (c) NaBH_4 , MeOH , 0°C , 1.25 h (89%); (d) Ac_2O , $\text{C}_5\text{H}_5\text{N}$, 20°C , 72 h (99%); (e) CrO_3 , Ac_2O , HOAc , 0°C , 48 h (73%); (f) K_2CO_3 , MeOH , 65°C , 1 h (98%); (g) DHP, TsOH , CH_2Cl_2 , 0°C , 1 h, then 20°C , 16 h (90%); (h) LiAlH_4 , THF , 0°C , 2 h (95%); (i, j) SOCl_2 , $\text{C}_5\text{H}_5\text{N}$, Et_2O , 0°C , 1.5 h; CuCN , MeLi , THF , -50°C ; 109, 0°C , 24 h (13%); (k) $(\text{C}_6\text{H}_5)_3\text{P}=\text{CH}_2$, THF , 20°C , 16 h (64%); (l) H_2 , Pd/C , EtOAc , 20°C , 1.5 h (65%); (m) TsOH , MeOH , 20°C , 16 h (77%); (n) PBr_3 , $\text{Et}_2\text{O}/\text{hexane}/\text{C}_5\text{H}_5\text{N}$, 0°C , 27 h (76%); (o) $(\text{C}_6\text{H}_5)_3\text{P}$, xylene , 100°C , 96 h (92%); (p) $\Delta\text{-BuLi}$, hexane/THF , -35°C to 0°C , 0.5 h, then 0°C , 1.5 h; $4\text{-EtO}_2\text{CC}_6\text{H}_4\text{CHO}$, THF , 20°C , 16 h (50%); (q) KOH , $\text{EtOH/H}_2\text{O}$, 55°C , 4 h; aq. HCl (74%).

for these products. Their absolute configurations were verified by comparing the rotation of the tetrahydronaphthalene **123** prepared by hydrolysis of the Grignard reagent derived from intermediate **121** with that of **123** prepared from (+)-(S)-2-phenylpropionic acid (**122**). Samples of tetrahydronaphthalene **123** prepared by both methods were levorotatory although the rotation of the sample prepared from **122** was lower {**122**-derived **123**: $[\alpha]_D^{23} -4.6^\circ$ (1.14, CHCl_3); **121**-derived **123**: $[\alpha]_D^{23} -9.1^\circ$ (1.0, CHCl_3)} because some racemization occurred during the Horner-Emmons olefination (Step 1).

The dihydrobenzothiopyran **37** is an isomer of the 6-substituted 3,4-dihydro-2*H*-1-benzothiopyran **40**, a retinoid that is much less toxic than **30** or other carbocyclic analogs.⁵⁴ In addition to being a positional isomer, the heteroatom of **37** is not effectively conjugated with the stilbenecarboxylic acid system. Because the heteroatom directs electrophilic substitution *para*, the methodology used in the synthesis of **40**⁴⁶ could not be employed. Instead, a bromo group that could be elaborated to an acetyl group was selected as the *meta* substituent

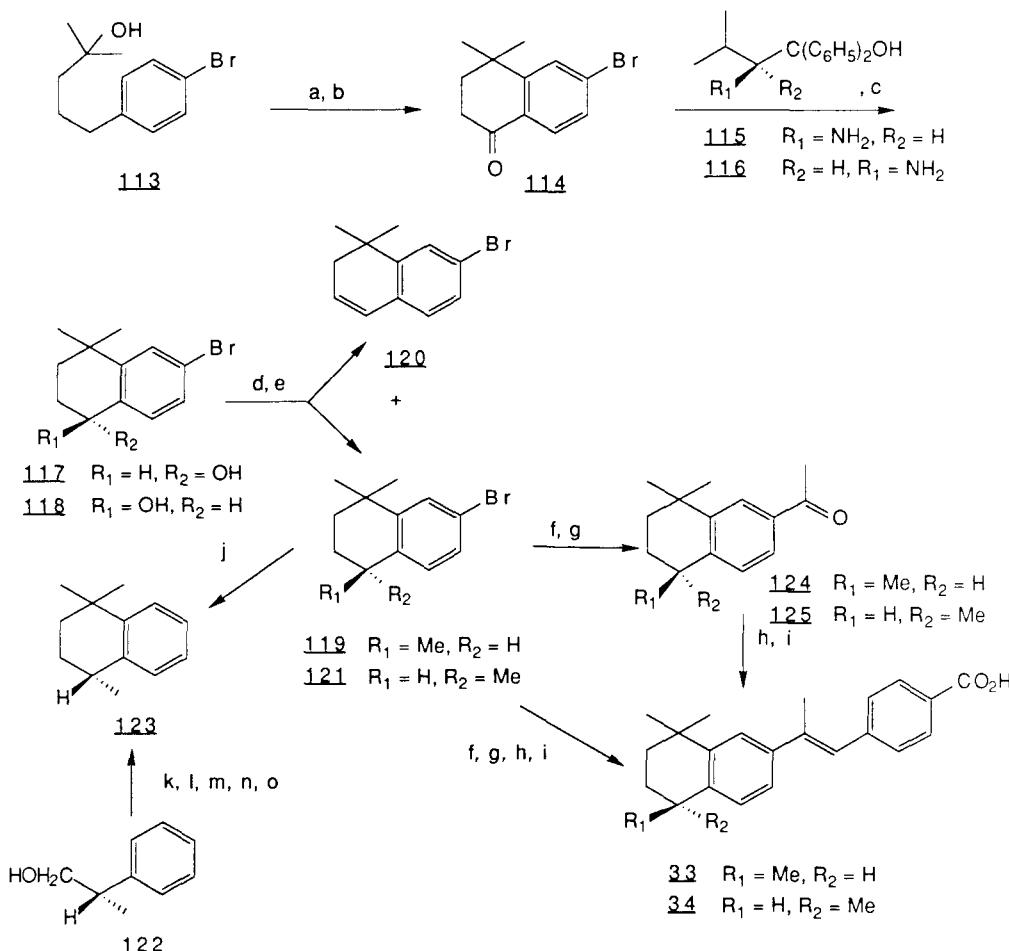


Chart 2. (a) H_2SO_4 , 0°C , 30 min (97%); (b) CrO_3 , $\text{Ac}_2\text{O}/\text{HOAc}$, 5°C , 18 h (85%); (c) **115**, $\text{BH}_3\text{-THF}$, THF, -78°C to 20°C , 1.5 h, then 30°C , 8 h; **114**, THF, 0°C , 6 h, (95% **117**, 92% **118**); (d, e) MsCl , Et_3N , Et_2O , -20°C to -3°C , 1 h; Me_2CuLi , Et_2O , -70°C , 15 min, then -16°C , 5 h; $\text{BH}_3\text{-THF}$, THF, 20°C , 1.5 h; aq. NaOH , H_2O_2 , 20°C , 1.5 h (29% **119**, 37% **121**); (f) Mg , THF, 20°C , 30 min; CH_3CHO , -20°C to 15°C , 1 h (70% from **119**, 88% from **121**); (g) CrO_3 , H_2SO_4 , aq. Me_2CO , 20°C (96% **124**, 99% **125**); (h) $\text{CH}_3\text{SOCH}_2\text{Na}$, $4\text{-Et}_2\text{O}_2\text{CC}_6\text{H}_4\text{CH}_2\text{P}(\text{O})(\text{OEt})_2$, Me_2SO , 20°C , 0.5 h; **124**, Me_2SO , 20°C , 12 h; NaOEt , $\text{EtOH/Me}_2\text{SO}$, 20°C , 2 h; aq. NaH_2PO_4 (54%); **125** (67%); (i) KOH , $\text{EtOH/H}_2\text{O}$, 70°C , 1 h; aq. NaH_2PO_4 (86% **33**, 77% **34**); (j) Mg , THF, 65°C , 2 h; aq. NH_4Cl (90%); (k) $(\text{COCl})_2$, CH_2Cl_2 , Me_2SO , -78°C , 10 min; **122**, CH_2Cl_2 , -78°C , 20 min; Et_3N , -78°C , 1 h, then -40°C , 1 h; aq. NH_4Cl (71%); (l) $(\text{EtO})_2\text{P}(\text{O})\text{CH}(\text{Na})\text{CO}_2\text{Et}$, THF, -78°C , 1.25 h (61%); (m) H_2 , Pd/C , EtOAc , 20°C , 6 h (80%); (n) MeMgBr , Et_2O , 0°C , then 35°C , 1 h (97%); (o) H_2SO_4 , -78°C , then 0°C , 30 min (70%).

in the starting material **126** (Chart 3). The bromodihydrobenzothiopyran **128** was prepared by a two-phase, polyphosphoric acid-catalyzed cyclization⁵⁵ of either olefin **127** or tertiary alcohol **129** derived from **126**. In each case the product was an 80:20 mixture of **128** and the isomer 5-bromo-3,4-dihydro-4,4-dimethyl-2*H*-1-benzothiopyran. These isomers were best separated after conversion to the corresponding methyl aryl ketones, which were prepared by reaction of the Grignard reagents derived from the aryl bromides with 2-pyridylthioacetate.⁵⁶ Horner-Emmons olefination⁵⁷ of methyl ketone **130** followed by base equilibration of the isomeric *E* and *Z* esters, chromatographic separation, and saponification afforded **37**.

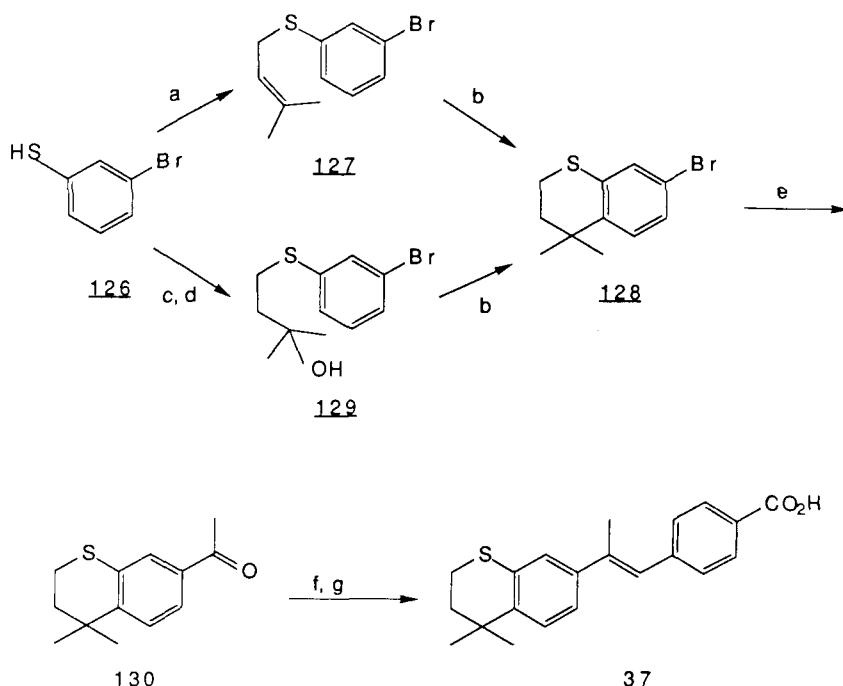


Chart 3. (a) NaH , THF , 0°C , 40 min; $\text{Me}_2\text{C}=\text{CHCH}_2\text{Br}$, THF , 20°C , 16 h (100%); (b) **127**, P_2O_5 , H_3PO_4 , C_6H_6 , 80°C , 128 h, then 120°C , 4 h (71%); or **129**, P_2O_5 , H_3PO_4 , C_6H_6 , 80°C , 20 h (53%); (c) $\text{H}_2\text{C}=\text{CHCO}_2\text{Et}$, Et_3N , Et_2O , 0°C , 35 min (97%); (d) MeMgBr , Et_2O , 35°C , 1.5 h (100%); (e) Mg , THF , 50°C , 35 min; 2-pyridyl thioacetate, THF , 0°C , 35 min (71%); (f) $\text{CH}_3\text{SOCH}_2\text{Na}$, Me_2SO , $4\text{-EtO}_2\text{CC}_6\text{H}_4\text{CH}_2\text{P}(\text{O})(\text{OEt})_2$, 20°C , 30 min; **130**, Me_2SO , 20°C , 18 h; EtONa , $\text{EtOH}/\text{Me}_2\text{SO}$, 20°C , 5 h (68%); (g) NaOH , $\text{EtOCH}_2\text{CH}_2\text{OH}/\text{H}_2\text{O}$, 20°C , 3 h; aq. H_2SO_4 (90%).

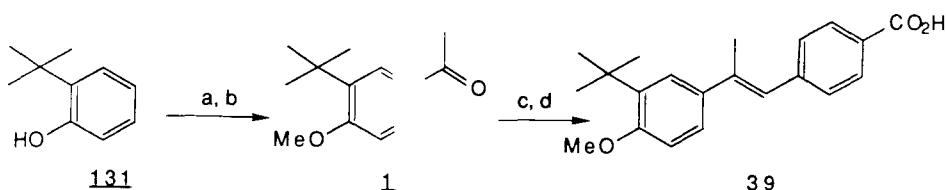


Chart 4. (a) Me_2SO_4 , CH_2Cl_2 , $\varepsilon\text{-NaOH}$, $(\text{d-Bu})_4\text{NI}$, 20°C , 3.5 h; NH_4OH , 20°C , 17 h (99%); (b) AcCl , SnCl_4 , CS_2 , 20°C , 2 h; aq. NaHCO_3 , CH_2Cl_2 (85%); (c) $\text{CH}_3\text{SOCH}_2\text{Na}$, Me_2SO , $4\text{-EtO}_2\text{CC}_6\text{H}_4\text{CH}_2\text{P}(\text{O})(\text{Et})_2$, 20°C , 35 min; **132**, Me_2SO , 20°C , 18 h; NaOEt , $\text{EtOH}/\text{Me}_2\text{SO}$, 20°C , 3.5 h (61%); (d) NaOH , $\text{MeOCH}_2\text{CH}_2\text{OH}/\text{H}_2\text{O}$, 20°C , 18 h; aq. HCl (94%).

The methyl phenyl ether **39** is an acyclic analog of the 6-substituted 3,4-dihydro-4,4-dimethyl-2*H*-1-benzopyranyl retinoid **38**, which had an ED_{50} in the tracheal organ culture assay of $2 \times 10^{-10} \text{ M}$ and low toxicity.⁵⁴ The acyclic retinoids have the advantage that they are readily synthesized from commercially available precursors. A phase-transfer procedure⁵⁸ was used to methylate 2-*t*-butylphenol (**131**), and the resultant methyl ether was converted to the methyl ketone **132** (Chart 4). Horner-Emmons olefination of **132** again gave an *E/Z* isomeric mixture of olefins, which were base-equilibrated to maximize the yield of the more

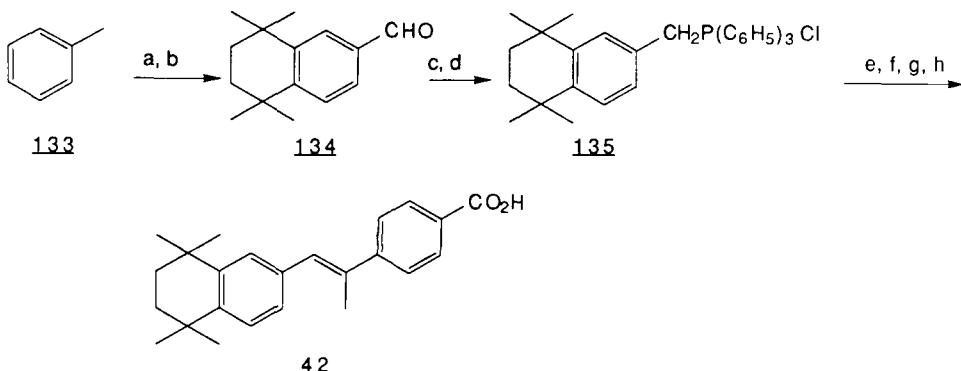


Chart 5. (a) $\text{CICMe}_2\text{CCH}_2\text{CH}_2\text{CMe}_2\text{Cl}$, AlCl_3 , $\text{C}_6\text{H}_5\text{CH}_3$, 5°C , 25 min (98%); (b) $(\text{NH}_4)_2\text{Ce}(\text{NO}_3)_6$, $\text{HOAc}/\text{H}_2\text{O}$, 100°C , 2.25 h (78%); (c) NaBH_4 , $\text{EtOH}/\text{Et}_2\text{O}$, 10°C , 1.5 h (100%); (d) HCl , CHCl_3 , -30°C , 15 min, then 20°C , 20 h; $(\text{C}_6\text{H}_5)_3\text{P}$, CH_2Cl_2 , 25°C , 16 h, then 40°C , 24 h (67%); (e, f, g) NaH , Me_2SO , 20°C , 20 min; 4- $\text{NCC}_6\text{H}_4\text{COCH}_3$, Me_2SO , 20°C , 16 h; KOH , $\text{EtOH}/\text{H}_2\text{O}$, 80°C , 20 h; aq. HCl ; EtI, NaHCO_3 , DMA, 20°C , 112 h; hv , hexane, 0°C , 15 min (56%); (h) NaOH , $\text{MeOCH}_2\text{CH}_2\text{OH}/\text{Et}_2\text{O}/\text{H}_2\text{O}$, 20°C , 2 h; aq. HCl (99%).

thermodynamically stable *E* isomer, which was isolated by crystallization (EtOH). Saponification afforded **39**.

Retinoid **42** is the methyl isomer of the extremely active, but very toxic, retinoid **30**⁵⁹ it is the first reported member of a potentially large class of analogs. We prepared members of this class to study the effects that perturbations in the propenyl group region of **30** would have on activity. Interestingly, while we were preparing **42**, a structure-activity study by Niculescu-Duvăz appeared that predicted that **42** would have high activity in controlling cell differentiation.⁶⁰ The synthesis of **42** is shown in Chart 5. The aromatic aldehyde intermediate **134** was prepared by Ce(IV) oxidation⁶¹ of 1,2,3,4-tetrahydro-1,1,4,4,6-pentamethyl-naphthalene,⁶² which was synthesized from toluene (**133**). The alcohol formed on reduction of **134** with NaBH_4 was converted to the phosphonium chloride **135**. Alternatively, the tetrahydropentamethylnaphthalene intermediate was brominated under radical conditions (*N*-bromosuccinimide, benzoyl peroxide) and the product converted to the phosphonium bromide. Reaction of the ylid, derived from either phosphonium salt, with 4-acetylbenzonitrile gave an unfavorable ratio of *E/Z* isomeric nitriles (20:80). This mixture was converted to the more readily separable ethyl esters by hydrolysis and esterification. In this case, base-catalyzed equilibration⁵⁶ could not be employed to increase the *E/Z* isomer ratio because **42** lacked the methyl group at the appropriate position that would permit abstraction of a proton to give a stabilized, delocalized anion required for equilibrations of this type. However, photochemical equilibration of the isomer mixture, using a Pyrex-jacketed, medium-pressure mercury immersion lamp and an ice-cooled hexane solution of the ester mixture, afforded an equilibrium *E/Z* isomer ratio of 70:30, from which the *E* isomer was isolated in 56% yield by crystallization (EtOH). The *E* configuration was assigned to **42** after comparison of its ^1H and UV spectra with those of its *Z* isomer. The spectral differences of these isomers were similar to those observed for retinoid **30**, several related α -methylstilbene retinoids,⁴⁶ and *E*- and *Z*-stilbene.⁴⁷ Shielding by the aryl groups in the *Z* isomer of **42** caused upfield shifts in the ^1H NMR spectrum relative to that of **42** for the signals of the vinylic proton (Δ 0.44 ppm), the aromatic protons *ortho* to the vinylic group (Δ 0.30 to 0.53 ppm), and the methyl groups at the 5-position of the tetrahydronaphthalene ring (Δ 0.30 ppm). The more highly conjugated *E* isomer (**42**) had a UV (EtOH) λ_{max} at 296 nm (ϵ 2.4×10^4), whereas that for the *Z* isomer appeared at 270 nm (ϵ 1.2×10^4).

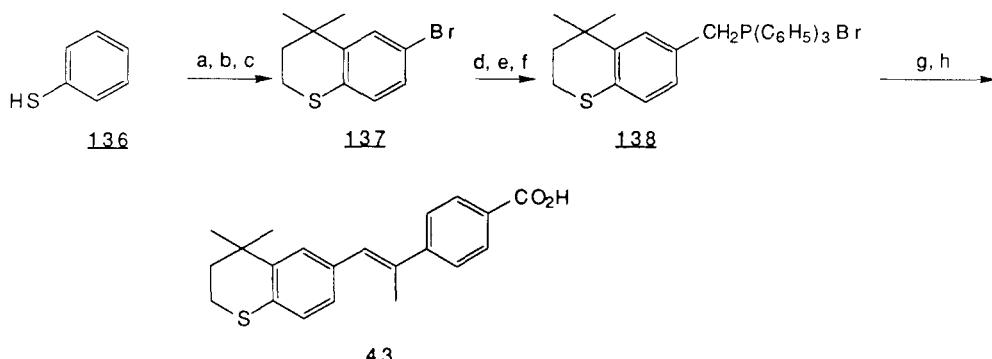


Chart 6. (a) NaH, THF, 0°C, 40 min; $\text{Me}_2\text{C}=\text{CHCH}_2\text{Br}$, THF, 0°C, 45 min, then 20°C, 1.25 h (88%); (b) SnCl_4 , CH_2Cl_2 , 0°C, 20 min, then 20°C, 16 h (67%); (c) Br_2 , Fe, CS_2 , 20°C, 20 h (83%); (d) Mg, THF, 50°C, 10 min, then 20°C, 15 min; N-formylpiperidine, THF, 0°C, 10 min, then 20°C, 30 min (81%); (e) NaBH_4 , EtOH, 0–20°C, 15 min (99%); (f) PBr_3 , Et_2O , 0°C, 15 min, then 20°C, 2 h; $(\text{C}_6\text{H}_5)_3\text{P}$, xylenes, 80°C, 20 h (95%); (g) $n\text{-BuLi}$, THF, -35°C to -10°C, 30 min, then 0°C, 30 min; 4-Et₂OCC₆H₄COCH₃, THF, -35°C to 20°C, 15 min, then 20°C, 6 h (32%); (h) KOH, EtOH/H₂O, 80°C, 3 h; aq. H_2SO_4 (94%).

Retinoid **43** is an analog of the potent retinoid **41** and the 3,4-dihydro-4,4-dimethyl-2*H*-1-benzothiopyranyl retinoid **40**, which we found to have a low therapeutic ratio (dose of retinoid in the antipapilloma assay/dose in toxicity assay).⁴⁶ It was prepared by a straightforward Wittig olefin synthesis, employing condensation of the ylid derived from phosphonium salt **138** and ethyl 4-acetylbenzoate rather than the nitrile used to prepare **42** (Chart 6). The *E/Z* isomer ratio was again unfavorable (36:64). In this case, photochemical equilibration of the mixture gave predominantly the *Z* isomer (*E/Z* ratio 16:84). Therefore, the *E* isomer was directly isolated in 32% yield from the reaction mixture by fractional crystallization (ethyl acetate/hexane).

Because both **30**, which has a vinylic methyl group at a position corresponding to the 19-methyl group of RA, and **42**, which has its methyl group shifted on the vinylic bond to the carbon corresponding to the C₁₀ of RA, were potent inhibitors of epidermal ornithine decarboxylase activity induced by 12-*O*-tetradecanoylphorbol-13-acetate, we undertook the synthesis of the *trans*-dimethyl-substituted retinoid **44** having methyl groups on both vinylic carbons joining the aromatic ring systems. In this analog, steric interactions between both methyl groups and the *ortho* protons on the neighboring aromatic rings could prevent effective conjugation of the aromatic systems and also prevent the rings from assuming the orientation required for activity. This sterically congested retinoid was prepared by the route shown in Chart 7 because standard Wittig and Horner-Emmons olefination procedures would proceed in only low yield. The hindered, tetrasubstituted olefin was generated by an acid-catalyzed isomerization of the less hindered olefin **141**. Retrosynthetic analysis suggested that alkylation of a dithiane would be an effective route to ketone **140**, the precursor for **141**. Aldehyde **139** was converted to the dithiane by standard methods;⁶³ the dithiane was metallated (*n*-BuLi)⁶⁴ and alkylated with 4-bromo-1-(1-chloroethyl)benzene. The alkylated dithiane was not readily purified. Therefore, the dithioketal group was removed from the crude dithiane using oxidative cleavage methods [CuCl₂, CuO, aqueous acetone,⁶⁵ or Ce(IV) oxidation⁶⁶], which were the only successful cleavage methods. Ketone **140** was elaborated to the desired olefin (**141**) by Wittig methylation, followed by introduction of the carbethoxy function by reaction of the aryl Grignard reagent, derived from the bromo-olefin Wittig product, with ethyl chloroformate at -60°C. The entrainment technique (EtBr, THF, 40 to 50°C)⁶⁷ was required to generate the Grignard reagent. The *exo*-olefin **141** was isomerized to an *E/Z*

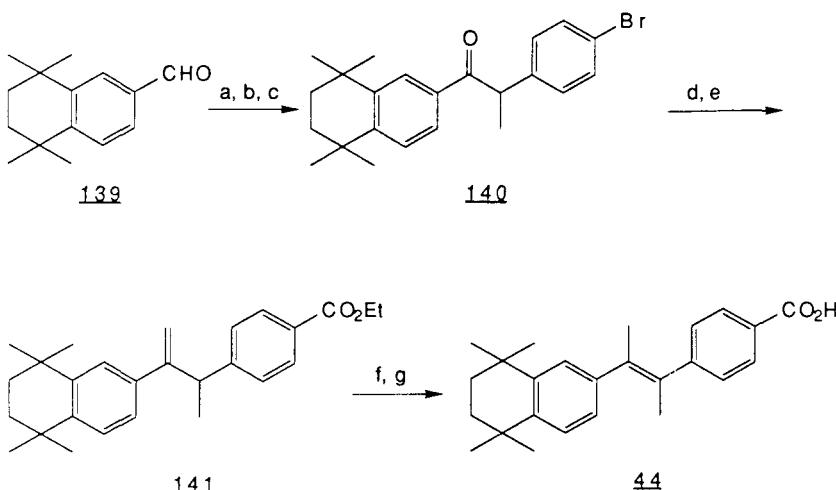


Chart 7. (a) $\text{HS}(\text{CH}_2)_3\text{SH}$, HCl , CHCl_3 , -20°C , 15 min; 20°C , 16 h (97%); (b, c) $\alpha\text{-BuLi}$, THF , -78°C , 1.5 h; $4\text{-BrC}_6\text{H}_4\text{CH}(\text{Cl})\text{CH}_3$, THF , -78°C , 3 h, then -20°C , 16 h; CuO , CuCl_2 , $\text{Me}_2\text{CO}/\text{H}_2\text{O}$, 56°C , 50 min (34%); (d) $(\text{C}_6\text{H}_5)_3\text{P}=\text{CH}_2$, THF , -40°C to -20°C , 35 min (39%); (e) Mg , EtBr , THF , 65°C , 3 h; ClCO_2Et , THF , -60°C , 30 min, then 0°C , 15 min (53%); (f, g) TsOH , C_6H_6 , 80°C , 4 h; hv , hexane, 0°C , 30 min; NaOH , $\text{MeOCH}_2\text{CH}_2\text{OH/Et}_2\text{O/H}_2\text{O}$, 20°C , 2 h; aq. HCl (48%).

mixture (42:58) of tetrasubstituted olefins on heating with *p*-toluenesulfonic acid in benzene.⁶⁸ Photochemical equilibration of this isomer mixture gave an *E/Z* equilibrium mixture (82:18), from which the *E* acid (**44**) was isolated by crystallization (hexane) after saponification. Photochemical equilibration of the crystallization mother liquor yielded additional **44**.

The UV spectrum of **44** [λ_{max} 252 nm (ϵ 1.7×10^4)] revealed that the aryl groups were not effectively conjugated. Suzuki⁶⁹ made a similar observation for α,α' -dimethylstilbene. Therefore, the assignment for the configuration of the double bond of **44** was made from the ^1H NMR spectra alone and was based on the observation of upfield shifts of certain signals in the spectrum of the *Z* isomer relative to that of the *E*, as observed for retinoids **30** and **42**. The *gem*-dimethyl group signals of the *Z* isomer of **44** were at 0.87 and 1.20 ppm, compared with 1.31 ppm for the *E* isomer. Upfield shifts of up to 0.50 ppm for the aromatic protons of the *Z* isomer relative to those of **44** were also observed.

In retinoid **45** a *trans*-substituted cyclopropane ring replaces the propenyl group of **30**. This compound was designed to assess the effect of the lack of planarity of the C_8-C_{11} bond system on activity. Retinoid **45** was prepared by a route similar to that used for a cyclopropylbenzonorbornenyl retinoid that we prepared earlier.⁴⁶ Aldehyde **139** was converted to olefin **142**, which was allowed to react with the carbene generated *in situ* by thermal decomposition of ethyl 4-diazomethylbenzoate (Chart 8).⁴⁶ This unstable diazoester was prepared immediately prior to use by oxidation (HgO)⁷⁰ of 4-carbethoxybenzaldehyde hydrazone. The product was obtained as a *cis/trans* mixture (24:76) of esters, which were separated by silica gel chromatography. Saponification afforded **45**. The stereochemistry about the cyclopropane ring was established from the ^1H NMR and UV spectra. Comparison of the ^1H NMR and UV spectra of the ethyl esters of the *cis*- and *trans*-substituted cyclopropanes showed differences that were similar to those reported for *cis*- and *trans*-1,2-diphenylcyclopropanes⁷¹ and the corresponding isomers of a benzonorbornenyl retinoid analog of **45**.⁴⁵ In each case, the chemical shifts of the benzylic methine cyclopropane protons were downfield in the *cis* isomer (multiplets at 2.4 and 2.49 ppm) relative to those in the *trans* isomer (multiplet at 2.2 ppm). Because of shielding by the noncoplanar aromatic groups, the signals for the

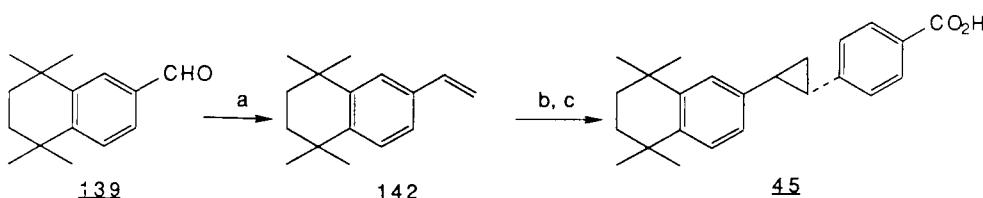


Chart 8. (a) $(C_6H_5)_3P=CH_2$, THF, $-40^\circ C$, 15 min, then $0^\circ C$, 1 h (90%); (b) $4-EtO_2CC_6H_4CH=NNH_2$, HgO , Na_2SO_4 , KOH , $EtOH/Et_2O$, $0^\circ C$, 15 min, then $25^\circ C$, 2 h; **142**, hexane, $80-100^\circ C$, 20 min (20%); (c) KOH , $EtOH/H_2O$, $80^\circ C$, 15 min; aq. $HOAc$ (97%).

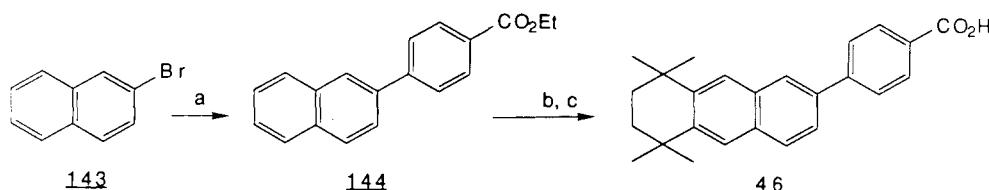


Chart 9. (a) Mg , THF , $35-40^\circ C$, 2 h; $ZnCl_2$, THF , $-10^\circ C$, 3 h; $4-BrC_6H_4CO_2Et$, $Ni(0)$ catalyst solution prepared by $(i-Bu)_2AlH$ reduction of $[(C_6H_5)_3P]_2NiCl_2$ in the presence of $(C_6H_5)_3P$, THF , $0^\circ C$, 0.5 h, then $20^\circ C$, 16 h (84%); (b) $ClMe_2CCH_2CH_2CMe_2Cl$, $AlCl_3$, CS_2 , $25^\circ C$, 0.5 h (75%); (c) KOH , $MeOH/H_2O$, $70^\circ C$, 45 min; aq. HCl (86%).

gem-dimethyl groups in the *cis* isomer (0.96, 1.00, 1.15, and 1.16 ppm) were upfield relative to those of the *trans* (1.30 ppm). The UV spectra also demonstrated greater conjugation⁷¹ of the aryl groups in the *trans* isomer [λ_{max} 263 nm (ϵ 1.8×10^4)] than in the *cis* isomer [λ_{max} 256 nm (ϵ 1.4×10^4)].

The tetrahydroanthracene retinoid **46** is sterically related to the aromatic retinoid **42**, which has a methyl group at the position corresponding to C_{10} of RA. However, conformational mobility in **46** is more restricted than that in **42** because rotation in the chain region of the molecule is restricted to the bond corresponding to the $C_{10}-C_{11}$ bond of RA. The route to **46** shown in Chart 9 gave higher yields than an alternative method in which the cycloalkylation step⁶² was performed first on 2-bromonaphthalene and then the second ring system was introduced using the Negishi coupling procedure.⁷² The Friedel-Crafts reaction conditions used for the cyclo-alkylation also produced side products derived from migration of the bromo group.

B. RETINOID IN THE NAPHTHALENECARBOXYLIC ACID SERIES

Methylnaphthalenecarboxylic acid analogs of retinoid **50**⁷³ were designed to assess the effect that methyl groups at positions corresponding to the 19- and 20-methyl groups of RA would have on activity. Conformational freedom in the chain region of these retinoids is restricted to rotation about the bond corresponding to C_8-C_9 in RA. Methyl groups in this region would further restrict rotation about this bond. A common precursor (**147**) to **57** and **59** was employed to reduce the number of synthetic steps. Indene **147** was prepared from 5-bromoindanone **146**.⁷⁴ Although the 2- and 6-positions of 1-methylnaphthalene could be selectively functionalized by using 2-bromo-6-chloro- and 2-chloro-6-bromo-1-methylnaphthalenes as intermediates, the preparation of the former starting from a mild Friedel-Crafts acylation of chlorobenzene with 3-chloropropionyl chloride failed. 2-Bromonaphthalenes have been prepared from indenes by dibromocarbene ring-expansion methods using

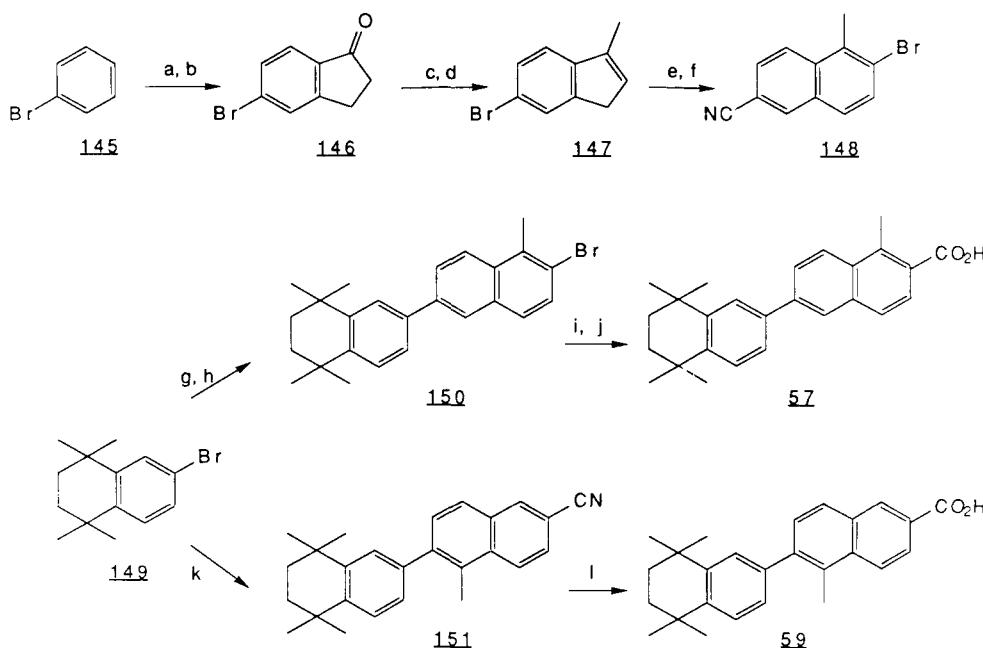


Chart 10. (a, b) $\text{ClCH}_2\text{CH}_2\text{COCl}$, AlCl_3 , CH_2Cl_2 , 20°C , 24 h; $\text{AlCl}_3/\text{NaCl}$, 185°C , 1.25 h (63%); (c, d) MeMgBr , Et_2O , 35°C , 1 h; aq. H_2SO_4 , 120°C , 40 min (83%); (e) CuCN , DMF, 160°C , 23 h; FeCl_3 , aq. HCl, 70°C , 25 min (65%); (f) $\text{C}_6\text{H}_5\text{HgCBr}_3$, C_6H_6 , 80°C , 16 h (80%); (g) Mg, THF, 45°C , 15 min; ZnCl_2 , THF, 20°C , 30 min; **147**, Ni(0) catalyst solution, THF, 20°C , 18 h (58%); (h) $\text{C}_6\text{H}_5\text{HgCBr}_3$, C_6H_6 , 80°C , 18 h (60%); (i) CuCN , DMF, 160°C , 17 h; FeCl_3 , aq. HCl, 75°C , 25 min (76%); (j) KOH, $(\text{HOCH}_2\text{CH}_2)_2\text{O}/\text{H}_2\text{O}$, 145°C , 30 h; aq. HCl (81%); (k) Mg, THF, 50°C , 20 min; ZnCl_2 , THF, 20°C , 30 min; **148**, Ni(0) catalyst solution, THF, 20°C , 20 h (56%); (l) NaOH, EtOH/ H_2O , 80°C , 24 h; aq. HCl (83%).

either $\text{CHBr}_3/\text{KOr-Bu}^{75}$ or $\text{C}_6\text{H}_5\text{HgCBr}_3$ in benzene at reflux.⁷⁶ Because 3-methyl-1*H*-indene is thermodynamically stable relative to its 1-methyl isomer,⁷⁷ reaction of the related 6-substituted indene with dibromocarbene was expected to give only the 1,2,6-trisubstituted naphthalene. 6-Bromo-3-methyl-1*H*-indene (**147**) was first converted to the nitrile by reaction with CuCN in DMF at reflux (Chart 10).⁷⁸ An oxidative [Fe(III)] workup destroyed the Cu(I)-nitrile complex formed during the reaction. Reaction of the nitrile with the dibromocarbene reagent $\text{C}_6\text{H}_5\text{HgCBr}_3$ gave 2-bromo-1-methylnaphthalene-6-carbonitrile (**148**). Completion of the synthesis of the 1-methyl-2-naphthalenecarboxylic acid **57** was achieved by coupling the bromoindene **147** and the arylzinc halide prepared from **149** using the Negishi biaryl coupling procedure,⁷² followed by ring expansion using dibromocarbene, displacement of the bromide by cyanide, and hydrolysis. Hydrolysis of this hindered nitrile required vigorous conditions (KOH, aqueous diethyleneglycol at reflux).⁷⁹ Mild hydrolysis conditions (NaOH, aqueous EtOH at reflux) afforded the primary amide. The 5-methyl-2-naphthalenecarboxylic acid **59** was prepared by a similar sequence, namely, coupling of the arylzinc reagent with aryl bromide **148** followed by base hydrolysis.

The 3- and 7-methyl-2-naphthalenecarboxylic acids **58** and **60**, respectively, were prepared in a similar fashion (Chart 11). However, because 6-bromo-5-methyl-1*H*-indene (**153**), the common intermediate for these retinoids, readily equilibrates⁷⁷ with its 5-bromo-6-methyl isomer under mildly basic or thermal conditions, difficult to separate mixtures of isomeric bromonaphthalenes would be expected after the dibromocarbene ring expansion.^{75,76} 5-Bromo-6-methylindanone was prepared from 2-bromotoluene (**152**) by the Friedel-Crafts sequence that had been used to prepare indanone **146**. Both the acylation and alkylation

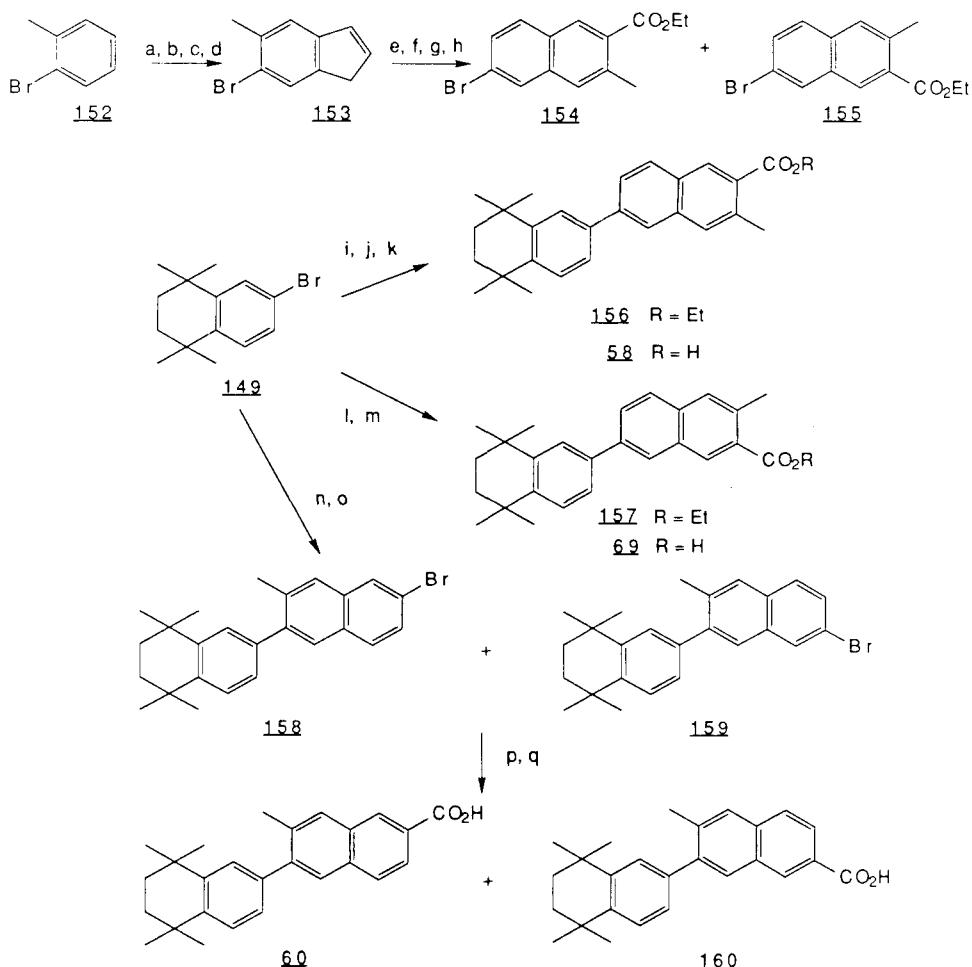


Chart 11. (a, b) $\text{ClCH}_2\text{CH}_2\text{COCl}$, AlCl_3 , CH_2Cl_2 , 20°C , 24 h; $\text{AlCl}_3/\text{NaCl}$, 180°C , 50 min (20%); (c, d) NaBH_4 , EtOH/THF , 0°C , 1.25 h; MgSO_4 , 110°C , 3.5 h (51%); (e) **153**, CuCN , DMF , 160°C , 22 h; aq. NaCN , C_6H_6 , 40°C , 30 min (67%); (f, g, h) $\text{C}_6\text{H}_5\text{HgCBr}_3$, C_6H_6 , 80°C , 16 h; H_2SO_4 , $\text{HOAc}/\text{H}_2\text{O}$, reflux, 20 h; **EtI**, K_2CO_3 , DMF , 20°C , 72 h (53% **154** and **155**); (i) Mg , **149**, THF , 50°C , 30 min; ZnCl_2 , THF , 20°C , 20 min; **154** and **155**, Ni(0) catalyst solution, THF , 20°C , 2.5 h (44% **156/157**, 65:35); (j, k) KOH , $\text{EtOH}/\text{H}_2\text{O}$, 80°C , 2 h; aq. HCl ; (42% **58**); (l, m) Mg , **149**, THF , 50°C , 30 min; ZnCl_2 , THF , 20°C , 10 min; **155**, Ni(0) catalyst solution, 20°C , 2.5 h; KOH , $\text{EtOH}/\text{H}_2\text{O}$, 80°C , 1 h; aq. HCl (24% **69**); (n, o) Mg , **149**, THF , 50°C , 15 min; ZnCl_2 , THF , 20°C , 15 min; **153**, Ni(0) catalyst solution, THF , 20°C , 18 h (52%); $\text{C}_6\text{H}_5\text{HgCBr}_3$, C_6H_6 , 80°C , 15 h (49% **158** and **159**); (p, q) CuCN , DMF , 160°C , 30 h; FeCl_3 , aq. HCl , 70°C , 20 min (69%); KOH , $\text{EtOH}/\text{H}_2\text{O}$, 80°C , 47 h; aq. HCl (32% **60**).

steps gave complex isomer mixtures, which were separated by fractional crystallization and chromatography. Reduction of the indanone gave the related indanol, which was converted to indene **153** using MgSO_4 as the mild catalyst⁷⁴ for the thermal elimination of water. Very little (5%) double-bond isomerization occurred in this process. In contrast, pyrolysis (180°C) of the corresponding acetate gave a mixture containing 20% of the double-bond isomer of **153**. Reaction of **153** with CuCN in DMF at 160°C ⁷⁸ gave, as expected, a mixture of two nitriles because of double-bond isomerization. This mixture was used to prepare a 60:40 mixture of esters **154** and **155** by dibromocarbene ring expansion,⁷⁶ followed by hydrolysis

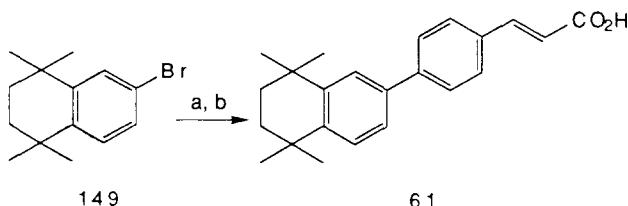


Chart 12. (a) Mg, THF, 50°C, 15 min; ZnCl₂, THF, 20°C, 15 min; (E)-4-BrC₆H₄CH=CHCO₂Et, Ni(0) catalyst solution, THF, 20°C, 18 h; aq. HCl (25%); (b) NaOH, MeOCH₂CH₂OH/H₂O, 20°C, 6 h; aq. HCl (98%).

of the nitriles and esterification. Crystallization (EtOH) of this mixture gave a small sample of the less soluble 7-bromo-3-methyl-2-naphthalenecarboxylate **155**, which was used to prepare the 7-aryl-3-methyl-2-naphthalenecarboxylic acid **69** by biaryl coupling⁷² and ester hydrolysis. This reaction sequence on the mixture of **154** and **155** gave a mixture of **156** and **157**, which after hydrolysis was separated by crystallization (EtOH) and silica gel chromatography (MeOH/CHCl₃). Nickel(0)-catalyzed coupling⁷² of the arylzinc chloride prepared from **149** with indene **153** gave a mixture of indene isomers, which after dibromocarbene ring expansion afforded an 80:20 mixture of bromonaphthalenes **158** and **159**, which was not readily separated by high-performance liquid chromatography (HPLC) or crystallization. Therefore, this mixture was used to prepare acids **60** and **160** by displacement of the bromide by cyanide using CuCN in DMF and base hydrolysis. Crystallization (EtOH) gave **60**. An alternative route using a Pd(0)-catalyzed carbonylation of bromonaphthalenes **158** and **159** at 110°C in *n*-butanol gave only a very low yield of the *n*-butyl ester of **60**.

The aromatic proton signals in the ¹H NMR spectra of **57**, **58**, **59**, and **60** were unequivocally assigned by successive decoupling of all coupled signals in the aromatic systems and by 2D NOE NMR spectroscopy. Comparison of these spectra with that of the parent retinoid **50**⁷³ revealed that a methyl group *ortho* to the biaryl bond (compounds **59** and **60**) reduced the conjugation of the biaryl system.⁸⁰ In addition, the signals for the other *ortho* protons were shifted upfield by 0.41 to 0.54 ppm relative to those for **50**. The UV spectra (EtOH) also indicated that in **59** (λ_{\max} 294 nm) and **60** (λ_{\max} 290 nm) conjugation was reduced compared with **50** (λ_{\max} 307 nm), **57** (λ_{\max} 301 nm), and **58** (λ_{\max} 296.5 nm) because of the lower absorption maxima.^{81,82} The 2-methyl-3-naphthalenecarboxylic acid **69** was readily identified from its UV absorption maximum (λ_{\max} 261 nm, EtOH) because the naphthalenecarboxylic acid group is not effectively conjugated with the tetrahydronaphthalene ring system.

Modifications were made in the naphthalene ring region of **50** to assess the effect that increasing the conformational freedom of the region adjacent to the polar terminus would have on activity. The first compound in this series was the *trans*-propenoic acid **61**, which lacks the methyl group corresponding to the 20-methyl of RA. Retinoid **61** was prepared by a Negishi Ni(0)-catalyzed biaryl coupling reaction⁷² between ethyl 4-bromocinnamate and the arylzinc reagent derived from bromide **149** (Chart 12). This reaction was slow and incomplete after 18 h at 20°C, probably because of reversible conjugate addition of the arylzinc species to the propenoate function.

Compound **62** was prepared to assess the effect that conformational restrictions would have on activity in a rigid analog of 13-*cis*-retinoic acid. A reported method for the preparation of *Z*-cinnamic acids by the acid-catalyzed (polyphosphoric acid, 100°C) isomerization of *E*-cinnamic acids⁸³ was not effective because of the low solubility of **61** in the acid. In addition, when (*E*)-4-bromocinnamic acid was subjected to these isomerization conditions, the coumarin resulting from cyclization of the acid was obtained rather than the intermediate *Z*

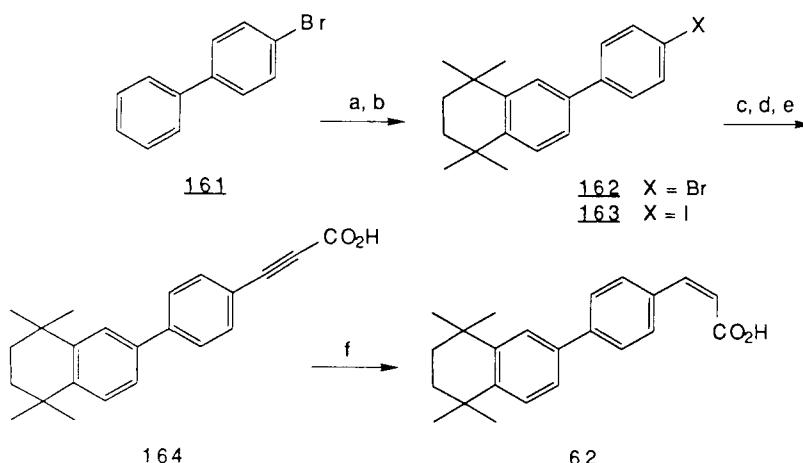


Chart 13. (a) $\text{ClMe}_2\text{CCH}_2\text{CH}_2\text{CMe}_2\text{Cl}$, AlCl_3 , CH_2Cl_2 , 0°C , 1 h (35%); (b) Mg , THF , 20°C , 0.5 h, then 50°C , 15 min; I_2 , THF , $0\text{--}20^\circ\text{C}$, 20 min (75%); (c) Et_2NH , $[(\text{C}_6\text{H}_5)_3\text{P}]_4\text{Pd}$, $\text{HC}\equiv\text{CCH}_2\text{OH}$, Et_2NH , CuI , 20°C , 67 h (93%); (d) MnO_2 , NaCN , MeOH , HOAc , 20°C , 90 h (90%); (e) NaOH , $\text{MeOCH}_2\text{CH}_2\text{OH}/\text{H}_2\text{O}$, 20°C , 4 h; aq. H_2SO_4 (98%); (f) H_2 , $\text{Pd/BaSO}_4/\text{quinoline}$, MeOH , 22°C , 23 min (64%).

isomer. The *cis*-propenoic acid was readily obtained by partial hydrogenation⁸⁴ of arylpropenoic acid **164** (Chart 13). Acid **164** was prepared starting from cycloalkylation⁶² of 4-bromobiphenyl (**161**) to give in moderate (35%) yield arylbromide **162**, together with products resulting from migration of bromine. The Pd(0)-catalyzed coupling^{85–87} of **162** to propargyl alcohol at 100°C in the presence of diethylamine as the base and CuI^{85,87} gave low yields of the arylpropynol because propargyl alcohol readily polymerized⁸⁸ in the presence of the catalyst. Therefore, bromide **162** was converted to the more reactive iodide **163** by reaction of the Grignard reagent with iodine in THF.⁸⁹ Iodide **163** readily reacted with propargyl alcohol at 20°C in the presence of Pd(0), CuI, and diethylamine to give the propynol (93% yield). Direct oxidation of this alcohol to the propenoic acid **164** was not successful. Therefore, Corey's oxidation procedure⁹⁰ was used to convert the alcohol to the methyl ester of **62**. Alkaline hydrolysis and hydrogenation (Pd/BaSO₄/quinoline⁸⁴) gave, in up to 65% yield, the *cis*-propenoic acid, which was readily separated from the fully hydrogenated by-product by extraction into CCl₄.

The double-bond geometries of **61** and **62** were assigned from ¹H NMR and UV spectra. Compound **61** displayed a typical coupling constant ($J = 16$ Hz) indicative of an *E*-double bond, whereas **62** had a *Z*-double-bond coupling constant ($J = 13$ Hz). The UV spectra (EtOH) supported these assignments. The *E* isomer (**61**) was more highly conjugated, having a λ_{max} at 310 nm ($\epsilon 3.6 \times 10^4$), whereas the λ_{max} for the *Z* isomer was at 293 nm ($\epsilon 2.7 \times 10^4$).

Compounds **63** and **168** are butenoate analogs of **61** and **62**, respectively, having a methyl group at the side-chain position corresponding to the 20-methyl group of retinoic acid. Retinoid **63** was obtained as a byproduct in one attempted stereospecific route to **168** (Chart 14). Compound **168** was prepared by a method related to that used for the synthesis of **62**. (*Z*)-3-Iodo-2-butanol (**166**)⁹¹ was prepared by a reported method.⁹² A Pd(0)-catalyzed coupling^{93–95} of organometallic reagents to iodoalkenes was adapted to the synthesis of *Z*-allylic alcohol **167** from aryl bromide **162** and iodoalkenol **166**. The Pd(0) catalyst was prepared by $(i\text{-Bu})_2\text{AlH}$ reduction of a mixture of bis(triphenylphosphine)palladium(II) dichloride and triphenylphosphine in benzene. Direct oxidatton of the coupling product to the

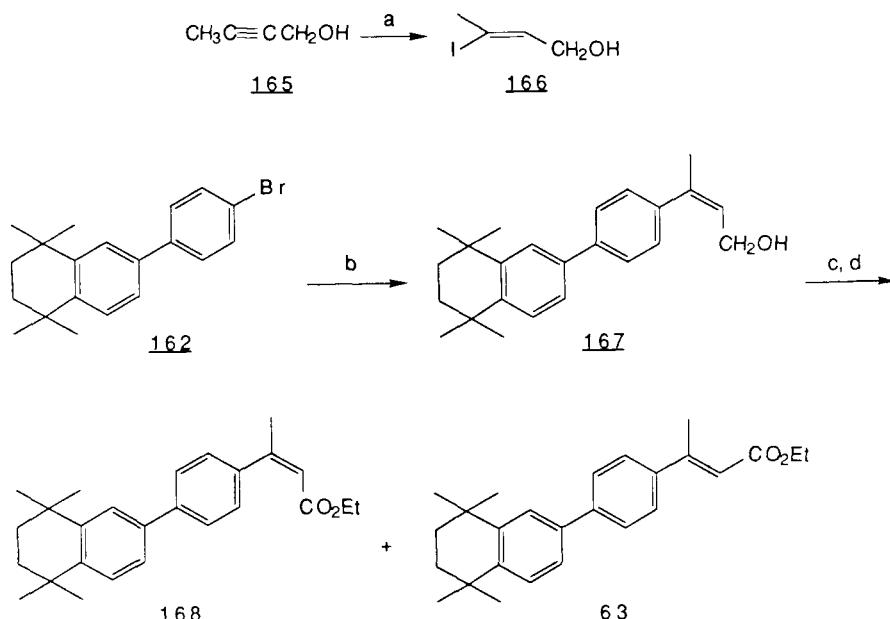


Chart 14. (a) LiAlH₄, NaOMe, THF, 65°C, 2 h; I₂, THF, -78°C, 20 min, then to 20°C, 16 h (54%); (b) Mg, THF, 50°C, 45 min; ZnCl₂, THF, 20°C, 30 min; 166, C₆H₆, Pd(0) catalyst solution, reflux, 30 min, then 20°C, 15 h (87%); (c, d) (C₅H₅NH)₂Cr₂O₇, CH₂Cl₂, 20°C, 7 h; MnO₂, NaCN, EtOH, HOAc, 20°C, 43 h (12% 63 and 43% 168).

Z-butenoic acid was not successful. A two-step oxidation (pyridinium dichromate in CH₂Cl₂, followed by NaCN, MnO₂, HOAc in EtOH⁹⁰) gave a mixture of *Z* and *E* esters (**168** and **63**, respectively) together with a methyl ketone resulting from double-bond cleavage. Direct Corey oxidation⁹⁰ of **167** to **168** in EtOH was very slow. *Z* Ester **168** was obtained by a stereospecific conjugate addition^{96,97} of Me₂CuLi to the ethyl ester of propynoic acid **164** at -78°C. Hydrolysis of the *Z* ester under a variety of conditions afforded a mixture of the *E/Z* acids, which were difficult to separate. In addition, on standing, the *Z* acid readily isomerized to the *E* and therefore was not isolated. Because of the lability of the *Z* acid, the esters were submitted for screening. The ¹H NMR spectrum of *Z* isomer **168** displayed upfield shifts, relative to the corresponding signals of *E* isomer **63**, of 0.39 ppm for the vinylic methyl group, 0.26 ppm for the vinylic proton, and 0.30 ppm for the aromatic protons *ortho* to the 3-(2-butenoate) group. These shifts are consistent with the reports that in (*Z*)-cinnamic acids and their derivatives, the alkenoic acid system is not coplanar with the aryl group.^{98,99} The UV spectra (EtOH) support the isomer assignments,^{100,101} with the *E* isomer having a higher λ_{max} [300 nm (ϵ 2.6 × 10⁴)] than the *Z* isomer [280 nm (ϵ 1.8 × 10⁴)].

C. POLAR TERMINUS MODIFICATION

The enolic β -diketone **70** is a polar terminus-modified analog of RA. In the keto-enol tautomer (**70**) illustrated in Chart 15, the acidic enolic hydrogen is equivalent in position to the carboxylic acid hydrogen of RA. Compound **70** was readily prepared by reaction of the lithium enolate of acetone¹⁰² with all-*trans*-retinoyl chloride¹⁰³ at -78°C. The olefinic protons of **70** were assigned unequivocally by two-dimensional ¹H NMR, and the *E* configuration of the double bonds was determined by comparison with reported spectra for (*E*)-retinal and (*E*)-retinoic acid.¹⁰⁴ The keto-enol structure was evident from the signal at 5.49 ppm for the C₃ proton.

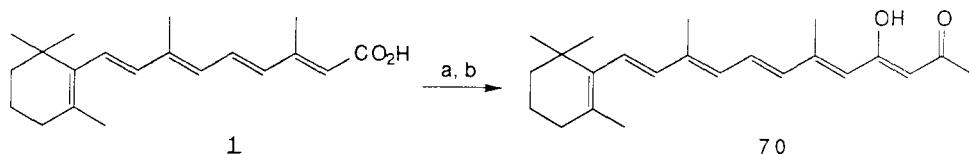


Chart 15. (a, b) SOCl_2 , $\text{C}_5\text{H}_5\text{N}$, THF/ Et_2O , -25°C to 0°C , 1 h; Me_2CO , LDA, -78°C , 30 min; aq. NaH_2PO_4 (43%).

V. RETINOID STRUCTURE-BIOLOGICAL ACTIVITY RELATIONSHIPS

A. BACKGROUND

In the past few years, many new synthetic retinoids have been prepared for testing as potential cancer chemopreventive agents and screened in a wide variety of *in vivo*^{105,106} and *in vitro*^{107,108} assays. The hamster TOC bioassay has been one of the most valuable for providing information on retinoid structure-activity relationships.^{42,46,73,109} In this assay system, an alternate differentiation pathway is followed in the tracheobronchial epithelium of vitamin A-deficient hamsters, i.e., the basal cells differentiate into squamous cells that become cornified and produce keratohyaline granules.¹¹⁰ When these tracheas are placed in organ culture containing a medium supplemented with biologically active retinoids, the reversal of keratinization occurs. In the hamster TOC bioassay, the progression of squamous metaplasia in tracheal cultures maintained *in vitro* in medium without retinoid has been demonstrated. At day 3, 85% of the cultures showed some degree of squamous metaplasia and 30% had keratinized lesions containing keratohyaline granules. Of the tracheas receiving no retinoid for the entire 10-d culture period, 99% showed squamous metaplasia and 88% had keratin and keratohyaline granules.³² These findings have led to the development of this organ culture system for reproducibly assaying large numbers of new compounds in a relatively short time.

Newton et al.³¹ have summarized the structure-activity relationships of 87 retinoids using the TOC assay to measure biological activity. Modifications were made in the ring, side chain, and polar terminus regions of the natural retinoid skeleton to give such compounds as the esters and amides of all-*trans*-retinoic acid (RA), analogs of all-*trans*-retinylamine having modifications of the side chain or both the ring and side chain, and 13-*cis*-retinoic acid or its derivatives. The data from this study indicated that a wide range of modifications of the retinoid skeleton could be made that allowed the expression of biological activity. For example, the retinyl nitrones, a new class of retinoids having a polar nitrone terminus, were shown to be effective in reversing keratinization in hamster tracheal organ cultures.¹¹¹ The *N*-methyl derivatives were the most active compounds in this series, having activity comparable with that of RA.

Structure-function relationship studies have also been performed on retinoidal benzoic acid derivatives in the TOC assay.^{31,112} In addition to the parent structure, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-benzoic acid (**30**), ring-modified analogs, and analogs with polar terminal group modifications were tested. Compared with the reference substance RA, 11 of the 13 benzoic acid derivatives tested were equal to or more potent in reversing keratinization. Many of the analogs showed activity at 10^{-12} M, making these compounds some of the most active retinoids in this assay.

The 3-alkyl and 3-alkoxy derivatives of **30** were also evaluated *in vitro* using the F9 teratocarcinoma and HL-60 leukemia assays,¹⁰⁷ which measure the ability of retinoids to induce terminal differentiation. In the F9 assay, **30** was more active than RA, but the 3-

methyl derivative of **30** was less active. In the HL-60 assay, the 3-methyl derivative was more active than RA, whereas **30** was less active. Other derivatives were less active than RA in both the F9 and HL-60 assays. Therefore, relative activity is dependent on the assay system used.

Because the TOC bioassay employs an organ culture system that has the capacity for metabolizing compounds and possesses a full complement of other enzymes, any assessment of biological activity — and therefore structure-activity correlations — must take these factors into account. For example, retinoid amides are inactive in the epidermal ornithine decarboxylase assay for retinoid activity but show high activity in the TOC assay, indicating that the latter system possesses the necessary enzymes to convert the amides to the parent acids. Esters also possess significant activity. For example, the ethyl ester (**2**) of RA (**1**, Table 1) had activity comparable with that of RA. The 13-*cis* isomer (**3**) of RA had comparable activity. Although it has not yet been established whether 13-*cis*-retinoic acid is inherently active or is a prodrug that must be isomerized to the active all-*trans* form, studies indicate that the 13-*cis* and all-*trans* isomers of retinoic acid were interconverted *in vivo* and in the presence of mercaptans.¹¹³ Therefore, bond isomerization to the all-*trans* isomer could be occurring in organ culture. This would serve to explain the activity of **3** and the lack of activity of the conformationally restricted 13-cisoid compound **9**.

It has been hypothesized, although not proved, that the highest oxidation state of vitamin A — namely, retinoic acid — is the active form of the vitamin that is responsible for controlling cell differentiation,¹¹⁴ and that to show this type of biological activity, the lower oxidation states (retinol and retinaldehyde) must first be oxidized to the acid and therefore are less active than the acid. For example, all-*trans*-retinol (**5**), which is oxidized to RA *in vivo*, was less active than RA in the TOC assay by over two orders of magnitude. Retinyl nitrones could be hydrolyzed in culture to the parent retinaldehyde, which could then be oxidized to RA.

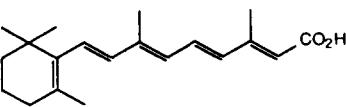
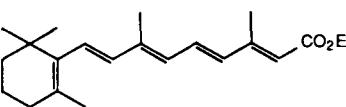
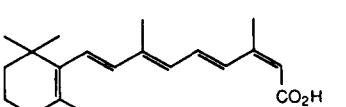
In addition to metabolic activation, metabolic deactivation must be considered. RA is oxidatively metabolized on the trimethylcyclohexenyl ring to introduce hydroxyl and then oxo groups at the 4-position of the retinoid skeleton.^{43,115} It has not been established if a similar pathway occurs in tracheal organ culture. However, the dihydrobenzothiopyrans **37**, **40**, **43**, and **55** (Table 1), which have a thia group at the corresponding position, are oxidized in air to the corresponding sulfoxides and sulfones, compounds which have low activity in the TOC assay.¹¹⁶ Therefore, deactivation pathways should also be considered, especially in an assay system taking ten days. Other oxidations could also occur. For example, on exposure to air in organic solvent, the trienyl benzoate **12** underwent epoxidation of the cyclohexenyl ring to give the epoxy-dienyl benzoate **13**, which retained biological activity. Similar oxidations could occur in organ culture with the polyolefinic retinoids. The 5,6-epoxide of RA has been isolated from intestinal tissue by McCormick et al.¹¹⁷

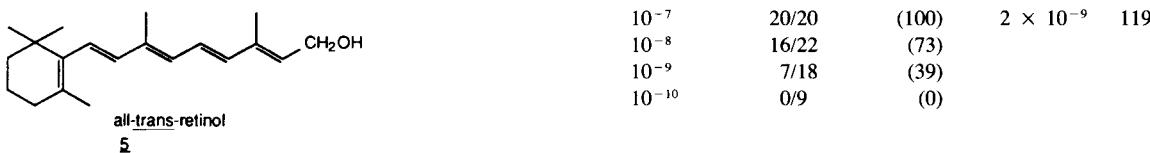
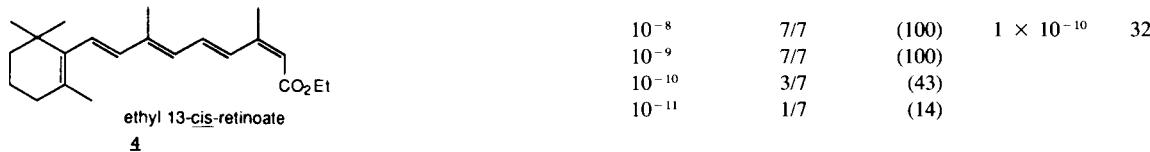
The physical properties of the retinoids affect their biological activity. These compounds, especially the polycyclic retinoids, have low solubility in the culture medium to which they are added dissolved in dimethylsulfoxide. Their insolubility and their affinity for proteins, such as serum albumin,¹¹⁸ may influence results. Transport into the tracheal epithelial cells will also be affected by retinoid structure and lipophilicity. All these factors may perturb the biological results in the TOC assay, and may also serve to explain the low correlation found on comparing retinoid activities in some assays.¹¹⁹ Nevertheless, the activities found in the TOC assay do show various trends that provide valuable insights about the structure of the putative retinoid receptor.

B. SRI RETINOIDS

The structure and TOC assay activities of the series of retinoids that we have synthesized are presented in Tables 1 (control compounds and SRI retinoids) and 2 (UC Riverside

TABLE 1
Retinoid Structure, Code Number, and Activity in the TOC Assay

Retinoid		TOC assay			
Structure	Code number	Conc., M	Active/total cultures (%)	ED ₅₀ , M*	Ref.
I. Control compounds					
					
<i>all-trans</i> -retinoic acid 1 *					
		10 ⁻¹⁰	267/312	(86)	1 × 10 ⁻¹¹
		10 ⁻¹¹	131/263	(50)	
		10 ⁻¹²	63/336	(19)	
					
ethyl <i>all-trans</i> -retinate 2					
		10 ⁻⁸	7/7	(100)	7 × 10 ⁻¹²
		10 ⁻⁹	7/7	(100)	
		10 ⁻¹⁰	10/13	(77)	
		10 ⁻¹¹	4/7	(57)	
		10 ⁻¹²	1/7	(14)	
					
13- <i>cis</i> -retinoic acid 3 *					
		10 ⁻⁸	7/7	(100)	2 × 10 ⁻¹¹
		10 ⁻⁹	7/7	(100)	
		10 ⁻¹⁰	6/7	(86)	
		10 ⁻¹¹	2/7	(29)	



II. Ring modifications

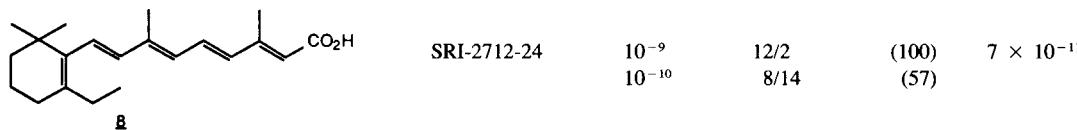
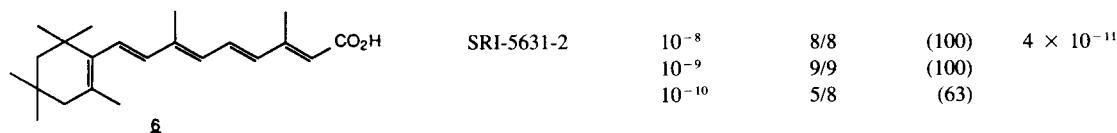
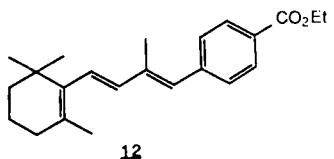


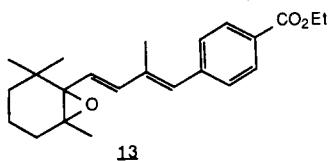
TABLE 1 (continued)
Retinoid Structure, Code Number, and Activity in the TOC Assay

Retinoid		TOC assay			
Structure	Code number	Conc., M	Active/total cultures (%)	ED ₅₀ , M ^a	Ref.
III. Chain Conformational Restrictions					
A. 13Z-Cisoid					
	SRI 3204-91	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰ 10 ⁻¹¹	2/7 1/7 1/7 0/7	(29) (14) (14) (0)	>1 × 10 ⁻⁸ 32
B. 7E,9E-Cisoid					
	SRI 4657-47	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰	2/7 1/7 1/7	(29) (14) (14)	>1 × 10 ⁻⁸ 32 46
C. 11E,13E-Cisoid					
1. Compounds 11 and 12					
	SRI 2965-38			2 × 10 ⁻¹⁰	31 32

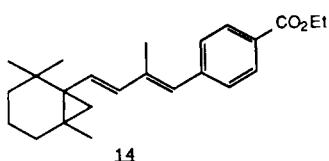


SRI 3498-95	10^{-8} 10^{-9} 10^{-10} 10^{-11} '	6/6 13/13 4/13 0/7	(100) (100) (31) (0)	2×10^{-10}	32
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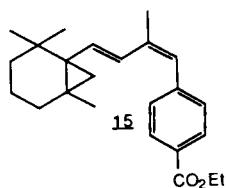
2. Trimethylcyclohexenyl Ring Analogs of 11



SRI 3204-84	10^{-8} 10^{-9} 10^{-10} 10^{-11}	5/5 8/12 5/14 2/7	(100) (67) (36) (29)	4×10^{-10}	32
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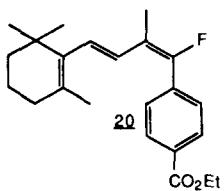
SRI 3618-93B	10^{-8} 10^{-9} 10^{-10} 10^{-11}	7/7 10/13 4/14 2/7	(100) (77) (29) (29)	5×10^{-10}	32
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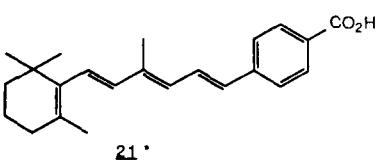
SRI 3618-93C	10^{-8} 10^{-9} 10^{-10}	5/13 2/12 1/12	(38) (17) (8)	$>1 \times 10^{-8}$	32
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TABLE 1 (continued)
Retinoid Structure, Code Number, and Activity in the TOC Assay

Structure	Retinoid	TOC assay				Ref.
		Code number	Conc., M	Active/total cultures (%)	ED ₅₀ , M ^a	
3. Side Chain Analogs of 11 and 12						
		SRI 4657-55B	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰	7/7 7/7 0/6	(100) (100) (0)	3 × 10 ⁻¹⁰ 32 46
		SRI 5193-27	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰ 10 ⁻¹¹	7/7 14/15 4/15 1/8	(100) (93) (27) (12)	3 × 10 ⁻¹⁰ 32 46
		SRI 3809-83	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰	13/13 12/13 5/13	(100) (92) (38)	2 × 10 ⁻¹⁰ 32
		SRI 3809-79B	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰	11/12 10/12 6/13	(92) (83) (46)	1 × 10 ⁻¹⁰ 32

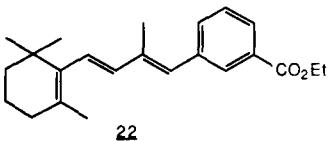


SRI 3809-79A	10^{-8} 10^{-9} 10^{-10}	2/12 1/13 0/12	(17) (8) (0)	$>1 \times 10^{-8}$	32
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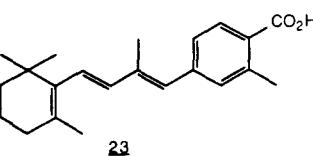


SRI 4092-68	10^{-8} 10^{-9} 10^{-10}	6/7 4/7 3/7	(86) (57) (43)	4×10^{-10}	32
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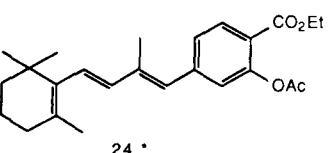
4. Substitutional Variations of the Aromatic Ring



SRI 4092-52	10^{-8} 10^{-9} 10^{-10}	1/7 1/6 2/7	(14) (17) (29)	$>1 \times 10^{-8}$	32
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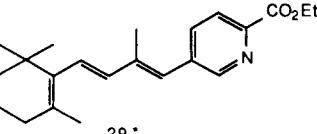
SRI 3204-87	10^{-8} 10^{-9} 10^{-10}	7/7 8/19 2/6	(100) (42) (33)	1×10^{-9}	32
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SRI 5193-7	10^{-8} 10^{-9} 10^{-10}	7/7 4/7 0/7	(100) (57) (0)	8×10^{-10}	32
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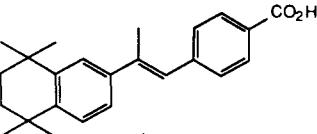
TABLE 1 (continued)
Retinoid Structure, Code Number, and Activity in the TOC Assay

Retinoid	TOC assay					Ref.
	Code number	Conc., M	Active/total cultures (%)	ED ₅₀ , M ^a	Ref.	
 25	SRI 4657-10	10 ⁻⁸	6/6	(100)	2 × 10 ⁻¹⁰	32
		10 ⁻⁹	12/12	(100)		109
		10 ⁻¹⁰	3/11	(27)		
		10 ⁻¹¹	0/6	(0)		
 26	SRI 4592-48	10 ⁻⁸	5/6	(83)	1 × 10 ⁻⁹	32
		10 ⁻⁹	3/6	(50)		109
		10 ⁻¹⁰	1/6	(17)		
5. Aromatic Ring Variations						
 27	SRI 4445-40	10 ⁻⁸	5/11	(46)	>1 × 10 ⁻⁸	32
		10 ⁻⁹	5/13	(39)		109
		10 ⁻¹⁰	4/13	(31)		
 28	SRI 3920-59	10 ⁻⁸	15/15	(100)	1 × 10 ⁻¹⁰	32
		10 ⁻⁹	14/15	(93)		109
		10 ⁻¹⁰	7/15	(47)		

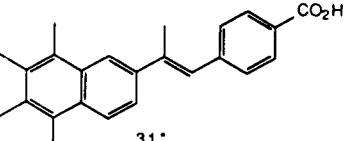
	SRI 4445-55	10^{-8} 10^{-9} 10^{-10} 10^{-11}	7/7 8/14 4/14 1/7	(100) (57) (29) (14)	6×10^{-10}	32 109
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D. 5,7*E*- and 11*E*,13*E*-Cisoid

1. Compound 30

	Ro 13-7410	10^{-10} 10^{-11} 10^{-12}	15/15 15/15 6/15	(100) (100) (40)	2×10^{-12}	46
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2. Modifications of the Tetrahydronaphthalene Ring

	SRI 5193-55	10^{-8} 10^{-9} 10^{-10}	7/7 4/7 1/7	(100) (57) (14)	8×10^{-10}	
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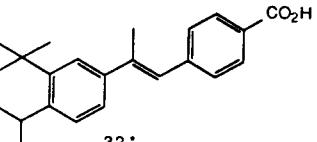
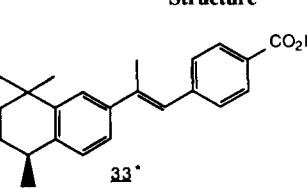
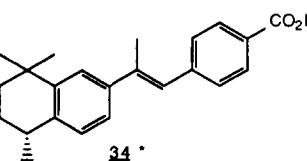
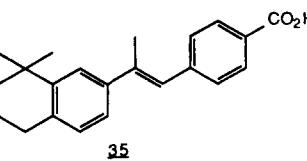
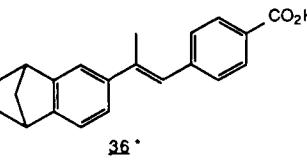
	SRI 6124-47	10^{-9} 10^{-10} 10^{-11}	2/7 1/7 0/7	(29) (14) (0)	2×10^{-9}	
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TABLE 1 (continued)
Retinoid Structure, Code Number, and Activity in the TOC Assay

Retinoid	TOC assay					Ref.
	Code number	Conc., M	Active/total cultures (%)	ED ₅₀ , M*	Ref.	
	SRI 6910-29	10 ⁻¹⁰ 10 ⁻¹¹ 10 ⁻¹²	6/7 2/7 0/7	(86) (29) (0)	2.5 × 10 ⁻¹¹	
	SRI 6910-50	10 ⁻¹⁰ 10 ⁻¹¹ 10 ⁻¹²	3/6 1/7 1/7	(50) (14) (14)	1 × 10 ⁻¹⁰	
	SRI 5639-27	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰ 10 ⁻¹¹ 10 ⁻¹²	7/7 7/7 9/11 2/7 1/7	(100) (100) (82) (29) (14)	3 × 10 ⁻¹¹	32
	SRI 4445-86B	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰	8/8 8/14 3/14	(100) (57) (21)	1 × 10 ⁻⁹	32 46

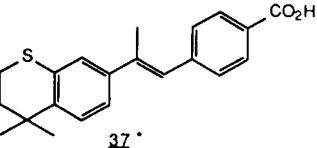
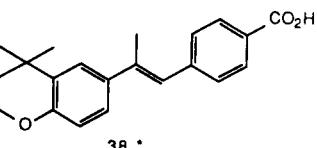
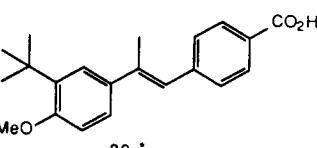
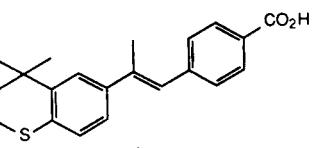
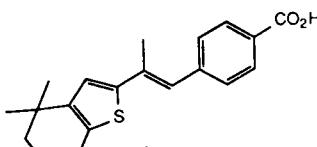
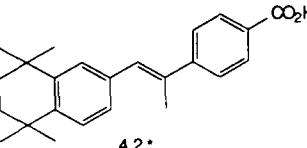
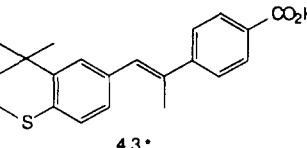
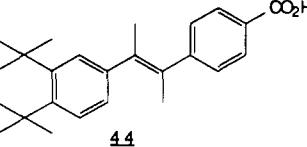
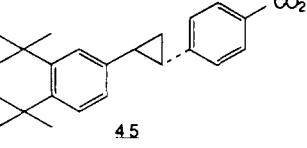
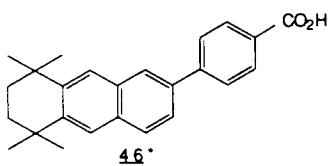
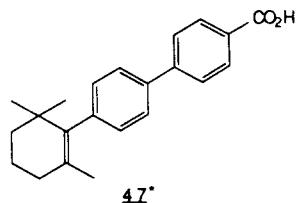
	37	SRI 6575-26	10^{-9} 10^{-10} 10^{-11}	6/6 4/6 2/7	(100) (67) (29)	5×10^{-11}	.
	38	SRI 5387-12	10^{-8} 10^{-9} 10^{-10} 10^{-11}	7/7 7/7 4/13 1/7	(100) (100) (31) (14)	2×10^{-10}	32 46
	39	SRI 6575-38	10^{-9} 10^{-10} 10^{-11}	4/6 3/7 1/7	(67) (43) (14)	6×10^{-10}	
	40	SRI 5896-39	10^{-8} 10^{-9} 10^{-10} 10^{-11}	7/7 7/7 12/14 1/7	(100) (100) (86) (14)	5×10^{-11}	32 46
	41	SRI 5442-28	10^{-9} 10^{-10} 10^{-11}	8/8 9/12 1/7	(100) (75) (14)	5×10^{-11}	

TABLE 1 (continued)
Retinoid Structure, Code Number, and Activity in the TOC Assay

Retinoid	TOC assay					Ref.
	Structure	Code number	Conc., M	Active/total cultures (%)	ED ₅₀ , M ^a	
3. Modifications of the Side Chain of 30						
	SRI 6409-40	10^{-10} 10^{-11} 10^{-12}	7/7 4/6 1/6	(100) (67) (17)	7×10^{-12}	
42*						
	SRI 7323-78	10^{-9} 10^{-10} 10^{-11}	5/7 1/7 0/7	(71) (14) (0)	4×10^{-10}	
43*						
	SRI 6751-62	10^{-8} 10^{-9} 10^{-10}	3/7 1/7 0/7	(43) (14) (0)	$>1 \times 10^{-8}$	
44						
	SRI 6751-94	10^{-10} 10^{-11} 10^{-12}	3/7 1/7 0/7	(43) (14) (0)	$>1 \times 10^{-10}$	
45						



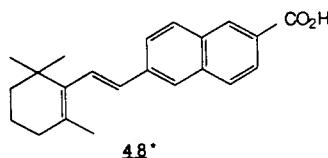
SRI 6751-84	10^{-10}	7/7	(100)	1×10^{-11}
	10^{-11}	3/6	(50)	
	10^{-12}	1/6	(17)	



SRI 4529-19	10^{-8}	7/7	(100)	5×10^{-9}	32
	10^{-9}	2/12	(17)		46
	10^{-10}	2/12	(17)		

F. 7*E*,9*E*- and 11*E*,13*E*-Cisoid

1. Compound 48

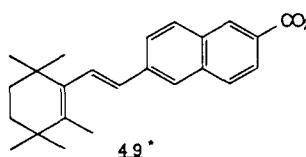


SRI 4445-75	10^{-8}	7/7	(100)	1×10^{-10}	32
	10^{-9}	11/12	(92)		46
	10^{-10}	6/13	(46)		
	10^{-11}	1/5	(20)		

TABLE 1 (continued)
Retinoid Structure, Code Number, and Activity in the TOC Assay

Retinoid		TOC assay			
Structure	Code number	Conc., M	Active/total cultures (%)	ED ₅₀ , M ^a	Ref.

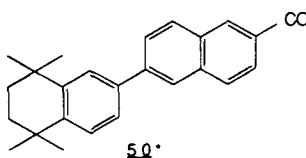
2. Ring Modification



SRI 5397-30	10 ⁻⁸	8/8	(100)	3 × 10 ⁻¹⁰
	10 ⁻⁹	6/8	(75)	
	10 ⁻¹⁰	0/8	(0)	

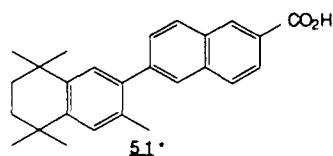
G. 5,7E- and 9E,11E,13E-Cisoid

1. Compound 50

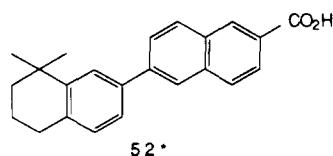


SRI 5898-52	10 ⁻⁹	7/7	(100)	3 × 10 ⁻¹²	32
	10 ⁻¹⁰	21/22	(95)		46
	10 ⁻¹¹	17/22	(77)		
	10 ⁻¹²	4/15	(27)		

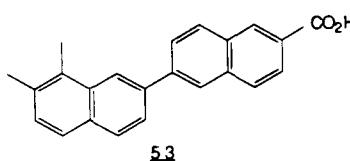
2. Modifications of the Tetrahydronaphthalene Ring



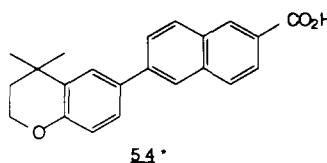
SRI 5193-43	10^{-9}	7/7	(100)	2×10^{-11}
	10^{-10}	12/14	(86)	
	10^{-11}	3/7	(43)	
	10^{-12}	1/7	(13)	



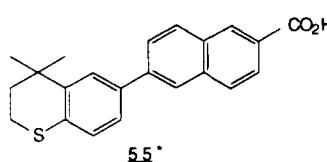
SRI 6153-40	10^{-8}	8/8	(100)	2×10^{-9}
	10^{-9}	3/8	(38)	
	10^{-10}	0/7	(0)	



SRI 5387-29	10^{-8}	4/8	(50)	$\sim 1 \times 10^{-8}$	32
	10^{-9}	1/8	(17)		
	10^{-10}	2/8	(25)		

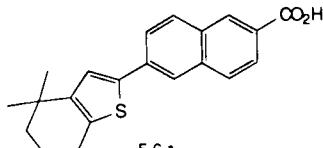
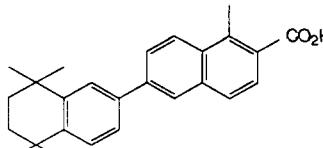
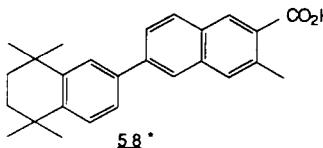


SRI 5387-30	10^{-8}	7/7	(100)	2×10^{-10}	32
	10^{-9}	7/7	(100)		
	10^{-10}	4/13	(31)		
	10^{-11}	1/7	(14)		



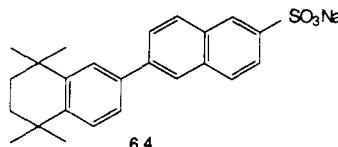
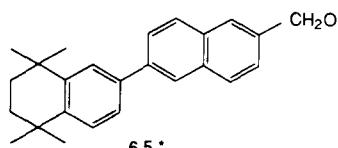
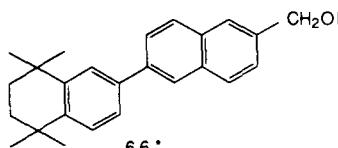
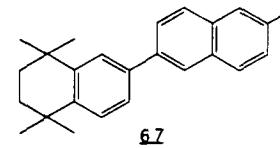
SRI 5387-31	10^{-8}	5/8	(63)	5×10^{-10}	32
	10^{-9}	3/8	(33)		
	10^{-10}	2/7	(29)		

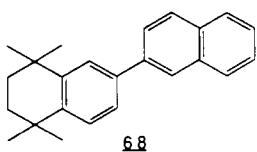
TABLE 1 (continued)
Retinoid Structure, Code Number, and Activity in the TOC Assay

Retinoid	TOC assay				Ref.
	Code number	Conc., M	Active/total cultures (%)	ED ₅₀ , M*	
Structure 	SRI 5442-30	10 ⁻⁹ 10 ⁻¹⁰ 10 ⁻¹¹	4/7 3/8 1/7	(57) (38) (14)	4 × 10 ⁻¹⁰
5.6*					
3. Modifications of the Naphthalene Ring					
Structure 	SRI 6787-88	10 ⁻⁹ 10 ⁻¹⁰ 10 ⁻¹¹	4/6 1/7 1/7	(67) (14) (14)	4 × 10 ⁻¹⁰
5.7*					
Structure 	SRI 7567-65	10 ⁻⁹ 10 ⁻¹⁰ 10 ⁻¹¹	6/7 3/7 0/7	(86) (43) (0)	2 × 10 ⁻¹⁰
5.8*					

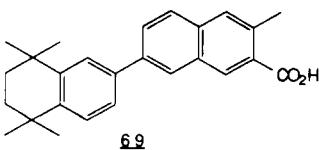
	SRI 6787-81	10^{-9} 10^{-10} 10^{-11}	7/7 4/7 0/7	(100) (57) (0)	8×10^{-11}
	SRI 7567-53	10^{-9} 10^{-10} 10^{-11}	7/7 5/7 2/7	(100) (71) (29)	3×10^{-11}
	SRI 7101-27	10^{-9} 10^{-10} 10^{-11}	5/7 2/7 0/7	(71) (29) (0)	3.2×10^{-10}
	SRI 7101-53	10^{-9} 10^{-10} 10^{-11}	3/7 0/7 0/7	(43) (0) (0)	1.3×10^{-9}
	SRI 7101-94A	10^{-9} 10^{-10} 10^{-11}	2/7 1/7 0/7	(29) (14) (0)	2×10^{-9}

TABLE 1 (continued)
Retinoid Structure, Code Number, and Activity in the TOC Assay

Retinoid	TOC assay					Ref.
	Structure	Code number	Conc., M	Active/total cultures (%)	ED ₅₀ , M ^a	
4. Polar Terminus Modifications						
	6.4	SRI 5942-92	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰	8/8 9/17 3/17	(100) (53) (17)	8 × 10 ⁻¹⁰
	6.5*	SRI 5442-11	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰ 10 ⁻¹¹	7/7 13/14 6/14 2/7	(100) (93) (43) (29)	2 × 10 ⁻¹⁰
	6.6*	SRI 5442-12	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰ 10 ⁻¹¹	7/7 11/14 6/15 1/6	(100) (79) (40) (17)	2 × 10 ⁻¹⁰
	6.7	SRI 5193-67	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰ 10 ⁻¹¹	6/7 6/14 1/14 1/7	(86) (43) (7) (14)	2 × 10 ⁻⁹

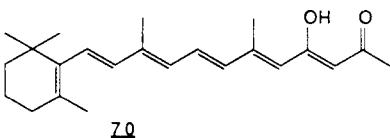


SRI 5193-71	10^{-8}	2/7	(29)	$>1 \times 10^{-8}$
	10^{-9}	1/15	(7)	
	10^{-10}	1/15	(7)	
	10^{-11}	0/7	(0)	



SRI 7567-67	10^{-9}	0/7	(0)	$>1 \times 10^{-9}$
	10^{-10}	0/7	(0)	
	10^{-11}	0/7	(0)	

IV. Polar Terminus Modification

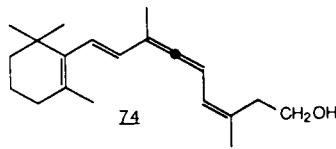


SRI 6567-60	10^{-10}	3/7	(43)	$>1 \times 10^{-10}$
	10^{-11}	1/7	(14)	
	10^{-12}	0/6	(0)	

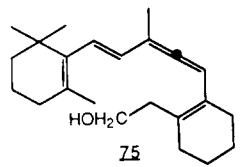
^a ED₅₀: calculated estimated dose inhibiting keratinization by 50%, determined by cubic regression analysis.

TABLE 2
**UC Riverside Chain-Modified Retinoids: Structure, Code Number, and Activity in
the TOC Assay**

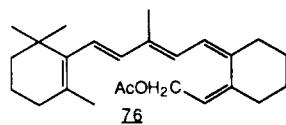
Retinoid		TOC assay		
Structure	Code number	Conc., M	Active/total cultures (%)	ED ₅₀ , M
I. Chain Modifications				
A. Retinol Analogs				
	Okamura-1	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰ 10 ⁻¹¹	6/6 10/11 4/12 0/5	(100) (91) (33) (0)
	Okamura-5	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰ 10 ⁻¹¹	7/7 9/12 7/12 2/6	(100) (75) (58) (33)
	Okamura-6	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰ 10 ⁻¹¹	7/7 9/12 5/11 1/5	(100) (75) (45) (20)



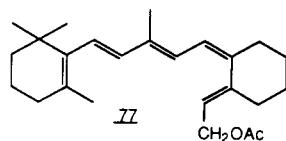
Okamura-7	10^{-8} 10^{-9} 10^{-10}	5/7 2/6 1/7	(71) (33) (14)	4×10^{-9}
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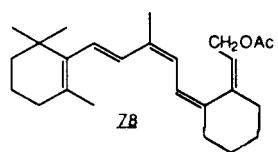
Okamura-2	10^{-7} 10^{-8} 10^{-9} 10^{-10}	2/6 2/11 2/12 0/5	(33) (18) (17) (0)	$>1 \times 10^{-7}$
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Okamura-8	10^{-8} 10^{-9} 10^{-10}	3/7 2/7 2/7	(43) (29) (29)	$>1 \times 10^{-8}$
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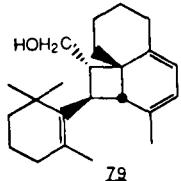
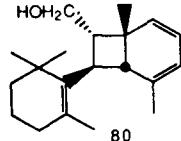
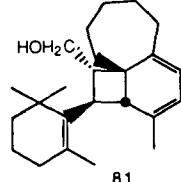
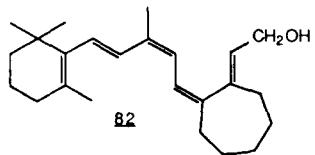


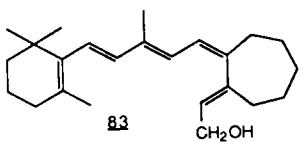
Okamura-9	10^{-8} 10^{-9} 10^{-10}	2/7 2/7 2/7	(29) (29) (29)	$>1 \times 10^{-8}$
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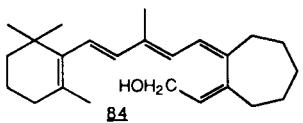
Okamura-10	10^{-8} 10^{-9} 10^{-10}	4/7 2/7 1/6	(57) (29) (17)	7×10^{-9}
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TABLE 2 (continued)
UC Riverside Chain-Modified Retinoids: Structure, Code Number, and Activity in the TOC Assay

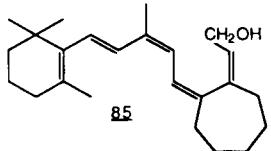
Retinoid	TOC assay			
	Code number	Conc, M	Active/total cultures (%)	ED ₅₀ , M
 79	Okamura-20	10 ⁻⁸	2/7	(29)
		10 ⁻⁹	0/7	(0)
		10 ⁻¹⁰	1/6	(17)
 80	Okamura-21	10 ⁻⁸	3/7	(43)
		10 ⁻⁹	0/7	(0)
		10 ⁻¹⁰	1/6	(17)
 81	Okamura-22	10 ⁻⁸	3/7	(43)
		10 ⁻⁹	3/7	(43)
		10 ⁻¹⁰	1/7	(14)
 82	Okamura-25	10 ⁻⁸	2/7	(29)
		10 ⁻⁹	1/7	(14)
		10 ⁻¹⁰	1/7	(14)



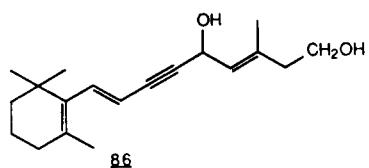
Okamura-26	10^{-8} 10^{-9} 10^{-10}	4/7 4/7 2/6	(57) (57) (33)	3×10^{-10}
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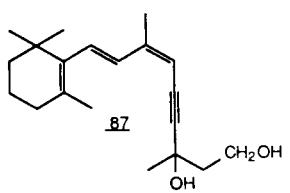
Okamura-27	10^{-8} 10^{-9} 10^{-10}	4/6 3/7 2/6	(67) (43) (33)	4×10^{-9}
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Okamura-30	10^{-8} 10^{-9} 10^{-10}	1/7 1/7 1/7	(14) (14) (14)	$>1 \times 10^{-8}$
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Okamura-31	10^{-8} 10^{-9} 10^{-10}	3/8 4/7 1/7	(37) (57) (14)	5×10^{-10}
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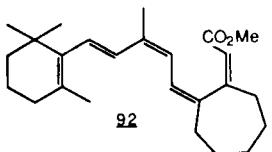


Okamura-32	10^{-8} 10^{-9} 10^{-10}	4/7 3/7 1/6	(57) (43) (17)	4×10^{-9}
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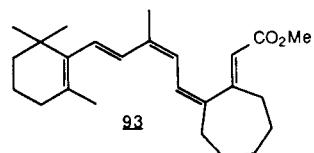
TABLE 2 (continued)
UC Riverside Chain-Modified Retinoids: Structure, Code Number, and Activity in the TOC Assay

Structure	Retinoid	TOC assay		
		Code number	Conc, M	Active/total cultures (%)
			ED ₅₀ , M	
	Okamura-33	10 ⁻⁸	3/7	(43)
		10 ⁻⁹	2/7	(29)
		10 ⁻¹⁰	1/7	(14)
	Okamura-34	10 ⁻⁸	5/7	(71)
		10 ⁻⁹	3/7	(43)
		10 ⁻¹⁰	2/7	(29)
	Okamura-35	10 ⁻⁸	3/7	(43)
		10 ⁻⁹	1/7	(14)
		10 ⁻¹⁰	1/7	(14)
	Okamura-36	10 ⁻⁸	4/7	(57)
		10 ⁻⁹	0/7	(0)
		10 ⁻¹⁰	1/7	(14)

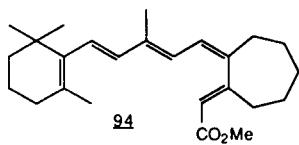
B. Retinoic Acid Ester Analogs



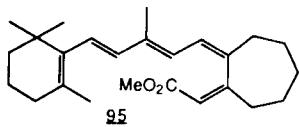
Okamura-23	10^{-8} 10^{-9} 10^{-10}	2/7 2/7 1/7	(29) (29) (14)	$>1 \times 10^{-8}$
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Okamura-24	10^{-8} 10^{-9} 10^{-10}	2/7 3/7 2/7	(29) (43) (29)	$>1 \times 10^{-8}$
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Okamura-28	10^{-8} 10^{-9} 10^{-10}	2/7 5/7 1/7	(29) (71) (14)	$>1 \times 10^{-8}$
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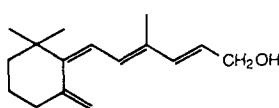
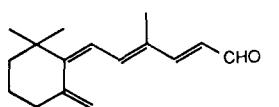
Okamura-29	10^{-8} 10^{-9} 10^{-10}	2/7 1/7 1/7	(29) (14) (14)	$>1 \times 10^{-8}$
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TABLE 2 (continued)
UC Riverside Chain-Modified Retinoids: Structure, Code Number, and Activity in the TOC Assay

Structure	Retinoid Code number	TOC assay		
		Conc, M	Active/total cultures (%)	ED ₅₀ , M
II. Ring and Chain Modifications				
 96	Okamura-3	10 ⁻⁸	3/13 (23)	>1 × 10 ⁻⁸
		10 ⁻⁹	1/11 (9)	
		10 ⁻¹⁰	0/10 (0)	
 97	Okamura-4	10 ⁻⁸	2/13 (15)	>1 × 10 ⁻⁸
		10 ⁻⁹	0/10 (0)	
		10 ⁻¹⁰	0/11 (0)	
 98	Okamura-11	10 ⁻⁸	1/7 (14)	>1 × 10 ⁻⁸
		10 ⁻⁹	1/7 (14)	
		10 ⁻¹⁰	1/7 (14)	
 99	Okamura-12	10 ⁻⁸	3/7 (43)	>1 × 10 ⁻⁸
		10 ⁻⁹	1/7 (14)	
		10 ⁻¹⁰	1/7 (14)	

	Okamura-13	10^{-8} 10^{-9} 10^{-10}	1/7 1/7 1/7	(14) (14) (14)	$>1 \times 10^{-8}$
	Okamura-14	10^{-8} 10^{-9} 10^{-10}	4/6 3/7 1/7	(67) (43) (14)	2×10^{-9}
	Okamura-15	10^{-8} 10^{-9} 10^{-10}	1/7 0/7 0/6	(14) (0) (0)	$>1 \times 10^{-8}$
	Okamura-16	10^{-8} 10^{-9} 10^{-10}	0/7 1/7 0/6	(0) (14) (0)	$>1 \times 10^{-8}$
	Okamura-17	10^{-8} 10^{-9} 10^{-10}	2/7 2/6 1/6	(29) (33) (17)	$>1 \times 10^{-8}$

TABLE 2 (continued)
**UC Riverside Chain-Modified Retinoids: Structure, Code Number, and Activity in
 the TOC Assay**

Structure	Retinoid	TOC assay			ED_{50}, M
		Code number	Conc, M	Active/total cultures (%)	
 <u>105</u>	Okamura-18	10^{-8}	4/7	(57)	6×10^{-9}
		10^{-9}	2/7	(29)	
		10^{-10}	2/6	(33)	
 <u>106</u>	Okamura-19	10^{-8}	2/7	(29)	$>1 \times 10^{-8}$
		10^{-9}	1/6	(17)	
		10^{-10}	1/6	(17)	

retinoids). Retinoid analogs that were closely related structurally were synthesized and screened by Dawson and coworkers to provide a data base for future SAR studies (Table 1). As discussed earlier, the activities of all-*trans*-retinoic acid, its 13-*cis* isomer, their ethyl esters (**2** and **4**, respectively), and retinol are also listed in this table for comparison purposes. Activities are given as ED₅₀ — the estimated dose of retinoid required to reverse keratinization in 50% of the organ cultures. An overview of the testing results in Table 1 indicates that structural modifications in one region of the retinoid skeleton will influence the effect of modifications in other regions. For example, the introduction of two methyl groups at the 4-position of the retinoic acid skeleton (compound **7**), which would block oxidative metabolism at this position, decreased activity by 50% compared with that of RA; and introduction of two methyl groups at the 3-position of the cyclohexenyl ring of naphthalenecarboxylic acid **48** afforded **49**, which had one third the activity of **48**; whereas removal of these methyl groups from the corresponding positions of (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)propenyl]benzoic acid (**30**) and 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-2-naphthalenecarboxylic acid (**50**) gave retinoids **35** and **52**, the activities of which were decreased by over an order of magnitude and approximately three orders of magnitude, respectively. Therefore, replacement of the 18-methyl group and the 5,7*E*-double bond system of RA by an aromatic ring influenced the effect that substitution of methyl groups at the 4-position of the retinoid skeleton had on activity. Placement of a *gem*-dimethyl group at the 3-position of RA also decreased activity in the TOC assay compared with RA. However, a similar substitution on the aromatic retinoids may have a quite different effect.

As part of our research program to develop more effective retinoids for cancer chemoprevention and to investigate the nature of the putative retinoid receptor, we prepared a series of conformationally restricted retinoids in which selected double bonds of RA were replaced by aromatic ring systems. Benzoic acid **9**, an aromatic analog of 13-*cis*-retinoic acid, was found to be essentially devoid of activity. This was also the case for **10**, in which the 19-methyl group and 7*E*,9*E*-double-bond system of RA were replaced by a 1,4-disubstituted 2-methylphenyl group, and for **47**, in which the 7*E*,9*E*-and 11*E*,13*E*-double-bond systems were replaced by a 4,4'-disubstituted biphenyl group. In contrast, benzoic acid **11**, the first aromatic retinoid that we synthesized in which the 11*E*,13*E*-double bond system was replaced by a 1,4-disubstituted phenyl ring, and the naphthalenecarboxylic acid **48** had 5 and 10% of the activity of RA, respectively. However, replacement of the cyclohexenyl ring system and 7*E*-double bond of **11** and **48** by the 2-substituted tetrahydrotetramethyl-naphthalene ring system afforded **30**, which was first synthesized by Loeliger and co-workers,⁵⁹ and **50**, which were five and three times more active than RA, respectively. Analogs of **11**, **30**, and **50** were synthesized and screened.

Testing of analogs of the ethyl ester of **11** (**12**) indicated that the 5- and 7*E*-double bonds could be replaced by cyclopropane rings (**14** and **16**) with no appreciable decrease in activity. In addition, saturation of the 7*E*-double bond or replacement of the C₁₀ vinylic hydrogen by fluorine had little effect on activity in this series. The vinylic homolog **21** of **11** had one half the activity. Shifting the carboxyl group from the *para*-substituted position on the phenyl ring to the *meta* (**22**) abolished activity. Incorporation of a methyl group (**23**) or an acetoxy group (**24**) on the phenyl ring *ortho* to the carboxyl group of **11**, which corresponds to the 20-methyl of RA, decreased activity by over an order of magnitude. In contrast, a *meta*-fluoro group (**25**) had no effect on activity, but a *meta*-methoxy group (**26**) decreased activity by over an order of magnitude. The 1,4-disubstituted phenyl ring could be replaced by a 1,5-disubstituted thiophene ring (**28**) with retention of activity, whereas replacement with a furan ring (**27**) resulted in loss of activity. Replacement by a 2,5-disubstituted pyridine (**29**) decreased activity by over an order of magnitude.

Retinoid **30** proved to have the highest activity of any retinoid screened in the TOC assay. The effects on activity of modifications of the tetrahydronaphthalene ring, the propenyl

side chain, and the 4-substituted benzoic acid group were assessed. Replacement of the tetrahydrotetramethylnaphthalene ring with a 5,6,7,8-tetramethylnaphthalene ring (**31**) decreased activity by over two orders of magnitude. Evidently, for high activity the lipophilic bulk of the methyl substituents must lie out of the plane of the naphthalene ring. Removal of one methyl group from the 5-position of the tetrahydronaphthalene ring of **30** (**32**) led to a severe reduction in activity. To determine which methyl group at C₅ was essential for activity, the enantiomeric retinoids **33** and **34** were prepared. The S-retinoid (**33**) proved to be more active than the R-retinoid (**34**); both were more active than **32**. At present, we have no explanation for the low activity of **32**. All three compounds were less active than **30**, indicating the importance of the two methyl groups at C₅ for high biological activity. The importance of lipophilic bulk at this position is also supported by the lower activity of **35**, lacking both methyl groups at C₅, and by the decrease in activity of almost three orders of magnitude for the benzonorbornenyl analog **36**.

Replacement of the C₅ methylene group of **35** with an oxa group (**38**) led to over a one-log decrease in activity, whereas replacement by the less-polar thia group (**40**) produced only a 60% decrease in activity. Replacement of the C₈ of **30** with a thia group gave dihydromethylbenzothiopyran **37**, which had the same ED₅₀ as **40**. Methyl phenyl ether **39**, the acyclic analog of the dihydromethylbenzopyran **38**, had one third the activity of **38**. Replacement of the tetrahydromethylnaphthalene ring of **35** with a 4,5,6,7-tetrahydro-4,4-dimethylbenzothiophene ring system afforded retinoid **41**, which had 60% of the activity of **35** in the TOC assay.

Shifting the methyl group on the vinylic side chain of **30** from the position corresponding to C₉ of retinoic acid to C₁₀ (**42**) had only a slight negative effect on activity (ED₅₀, 7 × 10⁻¹² M for **42**). A similar lack of effect was seen on comparing the activities of the dihydrobenzothiopyrans **40** and **43**. In contrast, two *trans*-methyl groups on the vinylic bond (**44**) abolished activity. Replacement of the propenyl moiety of **30** with a *trans*-1,2-disubstituted cyclopropane ring (**45**) also had a deleterious effect on activity. Evidently, the 8,9,10-bond system must assume a planar configuration to afford high activity. Replacement of the 2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)propenyl group by a 5,6,7,8-tetrahydro-5,5,8,8-tetramethylanthracene ring system (**46**) also decreased activity; however, **46** still had activity comparable with that of RA.

The effects on activity of modifying the tetrahydronaphthalene ring system in the naphthalenecarboxylic acid series of retinoids were similar to those in the benzoic acid series. However, analogs in this series were generally less active than those in the benzoic acid series of retinoids. A methyl group at the 3-position of the tetrahydronaphthalene ring (**51**), which reduced rotation about the bond joining the two aromatic ring systems, had less than one tenth the activity of **50**. Screening of the benzoic acid series of retinoids in the F9 teratocarcinoma assay indicated that the 3-methyl analog of **30** was less active than **30**. In contrast, in the HL-60 leukemia cell differentiation assay this 3-methyl analog was more active.¹⁰⁷ Removal of the 5,5-*gem*-dimethyl group of **50** (**52**) decreased activity, whereas conversion of **52** to the planar 7,8-dimethylnaphthalene analog **53** abolished activity. Insertion of a heteroatom in the 5-position of the tetrahydronaphthalene ring decreased activity relative to **50**. The dihydrobenzothiopyran (**55**) was again more active than its oxygen-containing analog (**54**). Both hetero analogs were more active than **52**, the low activity of which was surprising and perhaps could be explained by its very low solubility. The tetrahydromethylbenzothiophene **56** was more active than **52** but less active than **50**.

The 1-, 3-, 5-, and 7-methyl-2-naphthalenecarboxylic acid analogs (**57**, **58**, **59** and **60**) were prepared to assess the effect that methyl substituents on the naphthalene ring in positions corresponding to the 19- and 20-methyl groups of retinoic acid would have on activity. All these analogs were less active than **50**. The 1- and 3-methyl substituents reduced activity more than did those at the 5-, and 7-positions. In contrast, removal of the 20-methyl group

of RA reduced activity to one fiftieth of that of RA.¹²⁰ These results again underscore the fact that correlations between substituent effects in different retinoid analog series may be difficult. The 7-methyl substituent had the least effect on activity; in fact, analog **60** had activity comparable with that of the 3,5,5,8,8-pentamethyltetrahydronaphthalene **51**. The acyclic analog **61** of **50** was less active than **50** by over an order of magnitude. Its *cis* isomer, **62**, which was designed as an analog of 13-*cis*-retinoic acid, was even less active. Analog **63**, having a methyl substituent on the propenoate chain corresponding to the 20-methyl group of RA, also had low activity. These results indicate that in addition to conferring conformational rigidity on the retinoid skeleton, the aromatic rings may also enhance binding by enhancing hydrophobic interactions with the retinoid receptor.

The sodium salt of the sulfonic acid analog of **30** was reported to have activity in reversing papilloma formation, having about 0.5% of the activity of **30**.¹²¹ However, the sodium salt of the sulfonic acid analog (**64**) of **50** was found to be less active than **50** in the TOC assay by almost three orders of magnitude. A series of other polar terminus modifications of **50** was prepared for screening as potential prodrug analogs. As expected, the methanol **65**, its methyl ether (**66**), and the 2-methylnaphthalene **67** were less active than **50**. (*E*)-4-[2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzene has been patented as a cancer chemopreventive agent.¹²² This compound may be metabolically oxidized to a polar species such as a phenol that could affect cell differentiation as retinoids do. Our phenolic analog of RA, (*E*)-3-[4-methyl-6-(2,6,6-trimethylcyclohexenyl)-1,3,5-hexatrienyl]phenol, had an ED₅₀ in the TOC assay of $1 \times 10^{-9} M$.¹²⁰ Unfortunately, the naphthalene **68** was inactive in the TOC assay. The 2-methyl-3-naphthalenecarboxylic acid **69**, the positional isomer of **58**, was inactive in this assay at the highest concentration screened ($1 \times 10^{-9} M$), again supporting the requirement for substitution of the polar group and the hydrophobic portion of the retinoid on a plane through the ring system, as was found in the benzoic acid series.

The keto enol analog **70** of RA had less than one-tenth the activity of RA, indicating that compounds having higher pK_a values, such as the aforementioned phenol analog, are less effective at controlling differentiation than analogs having a carboxylic acid terminus.

Linear regression analysis of the ED₅₀ values in the hamster TOC assay calculated for 43 of the retinoids (marked with an asterisk in Table 1) and the calculated ED₅₀ values for these retinoids in the mouse epidermal ornithine decarboxylase assay (ODC) assay (see Table 9 of Chapter 16 in this monograph) indicated no correlation in activity in the two assays (*r* < 0.0). Even when the activities of a closely related series of retinoids (**30** to **34** and **36** to **41**) in the two assays were compared, the correlation was poor (*r* = 0.18). Although both assays test the ability of epithelial tissues to respond to an external dose of retinoids and retinoids having activity in the TOC assay also show activity in the ODC assay, the lack of correlation may be caused by differences in these assays, e.g., (1) *in vitro* vs. *in vivo*, (2) species (hamster vs. mouse), (3) retinoid-deficient compared with normal tissue, (4) application solvent (dimethylsulfoxide vs. acetone), and (5) the solubility of the retinoids in the tissue culture medium compared to the more lipophilic environment of the skin. A similar lack of correlation was found on comparison of the ED₅₀ values of retinoids **1**, **3**, **5**, **30**, **35**, **41**, **42**, **44** to **47**, **50** to **52**, **57**, **59** and **69** in the TOC assay with those determined by Napoli (Table 9 of Chapter 10) for laminin production induced by these retinoids in the embryonal carcinoma F9 cell differentiation assay (*r* = 0.78). Comparison of the TOC ED₅₀ values for retinoids **41**, **42**, **44** to **47**, **51**, **57**, and **59** with the effects that these retinoids at a concentration of $1 \times 10^{-10} M$ had on the expression of transglutaminase in rabbit tracheal epithelial cells in cell culture determined by Jetten (Table 1 in Chapter 13) also showed a low correlation (*r* = 0.36), as did comparison of the ED₅₀ values for these retinoids and retinoid **50** with the effects that the retinoids at $1 \times 10^{-10} M$ had on cholesterol sulfate accumulation in rabbit tracheal epithelial cells (Table 1 of Chapter 13) (*r* = 0.38). Both

the expression of transglutaminase and the accumulation of cholesterol sulfate are markers for squamous differentiation of epithelial cells. Therefore, care should be taken, as Roberts and Sporn¹¹⁹ have indicated, in assessing the biological activity of any retinoid based on its activity in one biological system. Instead, to more adequately profile biological activity, screens should be performed in a variety of cell and tissue systems, and finally in animal models.

C. UC RIVERSIDE RETINOIDS

In Table 2 are listed the structures and activities of a series of retinoids (**71** to **106**) synthesized by Okamura and co-workers and assayed in the hamster TOC assay. The syntheses of most of these compounds have previously been reported.¹²³⁻¹³⁴ A review on this topic appeared in 1983,¹²³ and more recent results are reviewed in Chapter 9 by de Lera, Chandraratna, and Okamura in this monograph.

The 36 analogs of retinol and retinoic acid and their derivatives shown in Table 2 may be subdivided into six groups: (1) normal side-chain retinoids (**71**^{124,125}); (2) allenic retinoids [**73**,¹²⁶ **74**,^{124,125} **75**,¹²⁷⁻¹³¹ and **99** (G. Shen, unpublished synthesis)]; (3) acetylenic retinoids [**86** to **91**,^{132,133} **96**, **97**, and **104**¹³⁴ (A. Haces et al., unpublished syntheses)]; (4) retroretinoids [**72**,¹²⁶ **73**,¹²⁶ **74**,^{124,125} **75**¹²⁷⁻¹³¹ **96**,¹³⁴ **97**,¹³⁴ **99** (G. Shen, unpublished synthesis), and **100** to **106**¹³⁴]; (5) ring-fused retinoids [**75** to **85**,¹²⁷⁻¹³¹ **92** to **95**,¹²⁷⁻¹³¹ and **98** (W. Reischl, unpublished synthesis)]; and (6) side-chain shortened retinoids (**104** to **106**).¹³⁴ Note that some of these analogs are classified in more than one group. For example, by necessity, all allenic retinoids (**73**, **74**, **75**, **99**,) are retroretinoids.

In contrast to the samples prepared by the Dawson group, which were routinely purified by chromatography and/or crystallization, those submitted for testing by the Okamura laboratory were freshly purified by HPLC. Samples from both laboratories were checked for purity by HPLC, UV, and/or ¹H NMR spectroscopy before submission. Labile samples were sealed in glass ampules under argon or nitrogen at reduced pressure. The Okamura retinoids were submitted as dimethylsulfoxide solutions. When necessary, samples were packed in dry ice and shipped by air overnight to the Schiff laboratory, where upon arrival they were diluted to the appropriate volume with dimethylsulfoxide and then stored at liquid nitrogen temperature (77 K) until the bioassay was performed. Precautions of this type were particularly necessary for the less-stable-than-normal retinoid samples prepared by the Okamura laboratory.

Examination of the data in Table 2 indicates that the 36 samples can be subdivided in terms of their relative activities (ID_{50} values) into three groups: (1) those having activity greater than 1% that of RA (**71** to **73**, **83**, and **86**); (2) those having slight activity (<1% of that of RA) (**74**, **78**, **84**, **87**,⁸⁹, **91**, **101**, and **105**); and (3) the remaining samples having activity less than 0.1% that of RA. Thus, in summary, 13 of the 36 compounds exhibited some activity in the TOC assay. An interesting finding was that seven (**71**, **74**, **78**, **83**, **84**, **89**, and **101**) of the 13 active compounds possess a *cis* configuration or conformation about the 12,13-bond of the side chain. Moreover, compound **72**, which proved to be the most biologically active compound assayed in this series, is free to rotate into the 12-s-*cis* conformation. It is of interest then that Dawson and coworkers have drawn attention to the possible biological significance of 12,13-cisoid topography through studies of aromatic retinoids (*vide supra*). Very interestingly, Lewin et al. at the Research Triangle Institute¹³⁵ and Loeliger and co-workers at Hoffmann-La Roche (Basel)⁵⁹ have also focused on side-chain analogs bearing 12-s-*cis* linkages. Thus, the topographical importance of this region of the retinoid side chain to biological activity is apparent.

It should be further emphasized however that most of the analogs listed in Table 2 may better be classified as prodrugs. If in fact these analogs require rate-limiting metabolism to retinoic acid analogs before exerting cell differentiating action in the TOC assay system,

then the data in Table 2 may not be so easy to analyze. Oxidation and/or hydrolysis as well as pharmacokinetics, bond isomerization, other modes of metabolism or catabolism, and compound stability may play important roles. Comparison of the ED_{50} values of the 13 active retinoid analogs in Table 2 with the ED_{50} value of retinol ($2 \times 10^{-9} M$) indicates that five analogs (71 to 73, 83, and 86) are more active than retinol, one (101) has comparable activity, and seven (74, 78, 87, 89, 91, and 105) are two- to fourfold less active. The esters of the retinoic acid analogs screened were inactive. In fact, although retinol analogs 83 and 84 were active, the corresponding esters 94 and 95 were inactive, as was the aldehyde 106 of alcohol 105. If it is assumed that the alcohols must be oxidized to the acids to have higher activity in the TOC assay, other processes such as compound stability, metabolism, and transport as mentioned above must be involved to give this activity profile. Any structure-activity analysis is therefore difficult with the data available. But, the finding that a 12,13-cisoid topography (assuming that this structural feature is important) characterizes a seemingly high proportion (8 of the 13) of the active analogs may reflect a relatively rapid oxidation to the retinoic acid analog before a rate-limiting cell differentiating action is elicited. Clearly, it would be most desirable to prepare and biologically evaluate the carboxylic acid forms of some of the analogs in Table 2. It would be especially desirable if the putative retinoid receptor in the TOC assay system were more clearly identified.

VI. CONCLUSIONS

New retinoids were synthesized by the SRI and UC Riverside groups and biologically evaluated in the hamster tracheal organ culture assay by Schiff and coworkers at IIT.

The 64 SRI retinoid analogs shown in Table 1 had modifications made in the ring, side chain, and polar terminus of the retinoic acid skeleton in order to explore the interrelationships between structural modifications and biological activity and to probe the spatial requirements of the putative retinoid receptor. To build a data base for future QSAR studies, many of these ring and side-chain modifications were closely related in structure, unlike those reported by Shudo and co-workers in Chapter 12, whereas many of the polar terminus modifications were designed as prodrugs that would be less toxic than the related carboxylic acid and that could be metabolically oxidized to the carboxylic acid after administration.

Certain trends in biological activity became evident on inspection of the ED_{50} values in Table 1. A definite three-dimensional spatial relationship appears to exist between the polar terminus and the lipophilic ring region of the retinoid skeleton, e.g., compare 12 and 22. Structural modifications in one region of the retinoid skeleton influenced the effect that modifications in another region had on biological activity, e.g., increasing lipophilic bulk in the ring of retinoic acid by incorporation of methyl groups decreased activity, but enhanced activity in the 6,7-cisoid tetrahydronaphthalene series. The methyl groups on the ring had to be out of the plane of the ring to enhance activity, e.g., the tetramethylnaphthalene 31 was far less active than the tetrahydrotetramethylnaphthalene 30.

The side chain appears to have a definite conformation at the active site. For example, a 8,9-cisoid conformation decreased activity in the TOC assay, whereas a 6,7-cisoid conformation enhanced it. A 12,13-cisoid conformation decreased activity unless the 6,7-bond system was also restricted to a cisoid conformation. The geometry in the C_8-C_{11} region is very important for activity. For example, the 5,6- and 7,8-double bonds could be replaced by *trans*-substituted cyclopropane rings without appreciable loss of activity in the TOC assay, but a similar replacement for the 9,10-double bond abolished activity. The methyl group at the 9-position of the side chain could be shifted to the 10-position without appreciable loss of activity; however, a retinoid having methyl groups at both the 9- and 10-positions lacked activity. The increased steric bulk of these two substituents affected the conformations of the other substituents at C_9 and C_{10} , thereby reducing biological activity. These results

are supported by the reduced biological activities of other tetrahydronaphthalenylnaphthalene carboxylic acid retinoids having methyl substituents in the region corresponding to the 8,9-bond system of retinoic acid. In addition, the 8,9-bond system appears to assume a transoid conformation at the active site, as evidenced by the high biological activity of the tetrahydroanthracene **46**.

The oxidation state of the polar terminus affects activity, with compounds at the lowest oxidation state being less active than those at higher oxidation states. Therefore, the naphthalene carboxylic acid **50** was more active than the naphthalenemethanol **65**, which in turn was more active than the 2-methylnaphthalene **67**. The sulfonic acid analog (**64**) of **50** was far less active than **50**.

Of the 36 analogs listed in Table 2, 13 exhibited some degree of activity in the TOC assay. Of these active analogs, eight possessed or were capable of assuming a 12,13-cisoid side chain topography. These results, together with results from other laboratories, further suggest that the presence of this stereochemical feature or topographical equivalent in the side chain is desirable in the design of new cancer chemopreventive agents in the retinoid series. However, as implied above, if a carboxylic acid end group is required for cell differentiating action in the TOC assay system, then each of the 13 active analogs listed in Table 2 must undergo initial metabolic transformation. It is obvious then that the preparation and biological evaluation of the carboxylic acid forms of these analogs would be most desirable. On the one hand, if the putative metabolic activation process is rate limiting in exerting cell differentiating action, then any structure-activity analysis of the 13 active analogs given in Table 2 is difficult with the data available. On the other hand, if this activation process is rapid, then the data may provide meaningful structure-activity information. However, the prediction regarding the significance of the 12,13-cisoid topography suffers a serious shortcoming in that it implies the presence of specific retinoid receptors not yet identified in the context of the TOC assay system used in this study. This shortcoming obviously applies to any analog evaluated in this assay system. It remains for future studies to more clearly define the detailed molecular events associated with retinoid action.

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Chapter 15

CELLULAR RETINOIC ACID-BINDING PROTEIN AND THE ACTION OF RETINOIC ACID**Brahma P. Sani****TABLE OF CONTENTS**

I.	Introduction	366
II.	Retinoid-Binding Proteins.....	366
III.	Cellular Retinoic Acid-Binding Protein (CRABP)	367
IV.	Retinoids and CRABP	369
V.	Mechanism of Action of Retinoic Acid	371
	References.....	379

I. INTRODUCTION

The molecular function of vitamin A in vision, through the interaction of opsins and retinal, is well established by the brilliant work of Wald.¹ Other important functions of vitamin A are in the control of normal differentiation of epithelial tissues and bone remodeling in maintenance of growth,²⁻⁵ and in reproduction.^{2,3,6} Retinoic acid (RA), which cannot be biologically reduced to either retinol or retinal, can maintain the general well-being of retinol-deprived animals, with the exception of vision and reproduction.⁶⁻⁸ RA in animals is apparently rapidly degraded, and cannot "buffer" the animal against deprivation. Ordinarily, the animal must rely for this transient function of RA or its derivatives on the continuous oxidation of its stores of retinol esters in the tissue.⁹ By the advent of modern extraction, isolation, and detection procedures,¹⁰ it is well established that RA is present in low concentrations as an endogenous retinoid in a number of tissues.¹¹⁻¹⁴ The prophylactic and tumor inhibiting actions, the cutaneous effects, and the ability to promote differentiation are generally more pronounced with RA than with retinol.¹⁵⁻²¹

The concept of vitamin A activity, except in vision, is a constantly changing one, partly because its molecular mode of action is uncertain, but largely because the concept of vitamin A activity, itself, is not static. It is presently clear that retinoids play an important role in the control of differentiation and in the inhibition of carcinogenesis, but their molecular actions at physiological and pharmacological levels are far from being understood. It is also not known whether there is a common mechanism for all the biological effects produced by retinoids. In the early days of retinoid research, the toxic effects of retinoids, particularly their ability to cause the release of proteoglycans from cartilage in organ culture,^{22,23} have been confused with their biochemical potential to control differentiation. Sporn et al.²⁴⁻²⁶ have, however, shown that a large difference exists between the relative activity and toxicity of many of the retinoids. Based on the pronounced inhibitory effects of retinoids on the phorbol ester-induced promotional phase of tumor formation, Verma and Boutwell^{27,28} suggested that, in the classical two-stage system of carcinogenesis, initiation and promotion, retinoids inhibit the second step. Russel and Haddox²⁹ concluded from their studies with Chinese hamster ovary cells that retinol suppresses the rise of ornithine decarboxylase activity during the G₁ phase by direct or indirect action at the level of transcription of the enzyme gene. There are several reports, however, that retinoids are able to reverse the cellular events associated with the pathological processes induced by chemical carcinogens both *in vivo* and *in vitro*,^{15,30-35} which may be indicative that retinoids are functioning at the initiation level. Dickens and Sorof²⁶ reported that in order to express their anticarcinogenic potential retinoids might interfere with the metabolism of chemical agents that require activation to electrophiles. Ludwig et al.³⁷ proposed cyclic AMP-dependent protein kinase as a target for the growth inhibitory effects of RA. However, none of these mechanisms explains entirely the diverse effects of retinoids in the control of differentiation, reproduction, embryonic development and growth, in the reversal of preneoplastic lesions and tumor promotion *in vivo*, and in the suppression of the induction of cellular transformation *in vitro* by carcinogens, viruses, radiation, and growth factors.^{20,21} Currently, there are two major models to explain the mode of action of vitamin A in the above functions. The first model gives emphasis on retinol as a coenzyme in the membrane-mediated synthesis of glycoprotein.^{4,5} The second hypothesis, which is based on the interaction of retinol and retinoic acid with their specific binding proteins and in their ultimate expression of genomic effects, forms the subject matter of this chapter.

II. RETINOID-BINDING PROTEINS

Vitamin A is stored in the liver as retinyl esters. Mobilization and transport of retinol after hydrolysis from the liver to various tissues are mediated by a specific transport protein,

serum retinol-binding protein (RBP).^{39,40} Extensive work has been carried out on the structure, metabolism, and biological roles of this protein.^{41,42} RBP is a single polypeptide chain with a molecular weight of 21,000 and migrates with α -mobility on electrophoresis.^{43,44} RBP has a single binding site for one molecule of retinol and normally circulates mainly as the holoprotein.^{43,44} RBP circulates as 1:1 molar protein complex with another protein, plasma transthyretin,⁴⁵ which is commonly referred to as plasma prealbumin.^{43,44} Transthyretin is a symmetrical tetramer with a molecular weight of 55,000. The RBP-transthyretin complex circulating in plasma has an apparent molecular weight of 60,000 to 70,000. RBP and transthyretin are both synthesized and secreted independently by the liver; they combine in serum to form the RBP-transthyretin complex.

RA, the major oxidative metabolite of retinol, which is transiently formed and rapidly excreted,^{46,47} is a physiological metabolite of vitamin A¹¹⁻¹⁴ and a normal human plasma constituent of the vitamin A reserve in the body.⁴⁸ It was illustrated by means of radiotracer techniques⁴⁹ and immunoprecipitation reactions^{50,51} that RA, like many fatty acids, is transported in the serum bound to albumin. Although RA can bind to apo-RBP⁵²⁻⁵⁶ as efficiently as does retinol this protein does not seem to facilitate the plasma transport of retinoic acid.⁴⁹⁻⁵¹ It was shown that various synthetic analogs of RA compete for the retinoic acid-binding sites on albumin implying that these analogs are also transported bound to serum albumin.⁵¹ The relative binding affinities of these analogs to serum albumin do not correlate with their biological potency in the modulation of epithelial differentiation.⁵¹⁻⁵³

Various attempts designed to determine the molecular mechanism of retinoid actions in the control of epithelial differentiation, growth, and tumorigenesis have centered on intracellular retinoid-binding proteins. It was suggested from our laboratory,^{51,57} as well as from others,⁵⁸ that this action of retinol and retinoic acid may be mediated by their well-known cellular binding proteins, CRBP and CRABP, respectively. These proteins have been detected in a variety of cells and tissues. Several recent reviews on these proteins are available.⁵⁹⁻⁶³

III. CELLULAR RETINOIC ACID-BINDING PROTEIN (CRABP)

The existence of CRABP in chick embryo skin and the evidence that CRABP might be involved in the expression of the biological activity of RA and its synthetic analogs in the control of epithelial differentiation and tumorigenesis were reported from our laboratory.^{51,57} CRABP, which is distinct from CRBP, had an S_{20} value of 2.0 and a molecular weight of about 16,000 based on chromatography on Sephadex G-100 columns.

The specificity of CRABP for binding [3 H]retinoic acid was determined by challenging with 200-fold molar excess of unlabeled RA, retinol, retinal, and a cyclopentenyl analog of retinoic acid (Ro 8-7699) (Figure 1). Both retinol and retinal showed no competition, whereas unlabeled retinoic acid and the acetylcyclopentenyl analog of retinoic acid abolished the 2S [3 H]retinoic acid-binding peak. The same excess of γ -linolenic acid did not compete with [3 H]retinoic acid for the binding sites on CRABP. The cyclopentenyl analog, like retinoic acid, was very active in reversing keratinized metaplasia to mucous producing epithelium in chick embryo skin in organ culture.¹⁸ The high affinity binding of these two retinoids to CRABP could be related to this activity.^{50,51,57} The binding to CRABP occurred at sites specific for vitamin A compounds and analogs with a free carboxyl group because retinol, retinal, and γ -linolenic acid did not compete for these sites. A high-affinity binding to CRABP was inferred because the bound ligand remained attached after dialysis, sucrose gradient sedimentation, and Sephadex gel column chromatography. Based on the above results, it was concluded that because of the similarity between the function of RA and that of steroid hormones, one would expect the binding of RA to CRABP after its entry into target cells. Such a complex may enter the cell nucleus and interact with nuclear components with a resulting alteration in gene transcription.

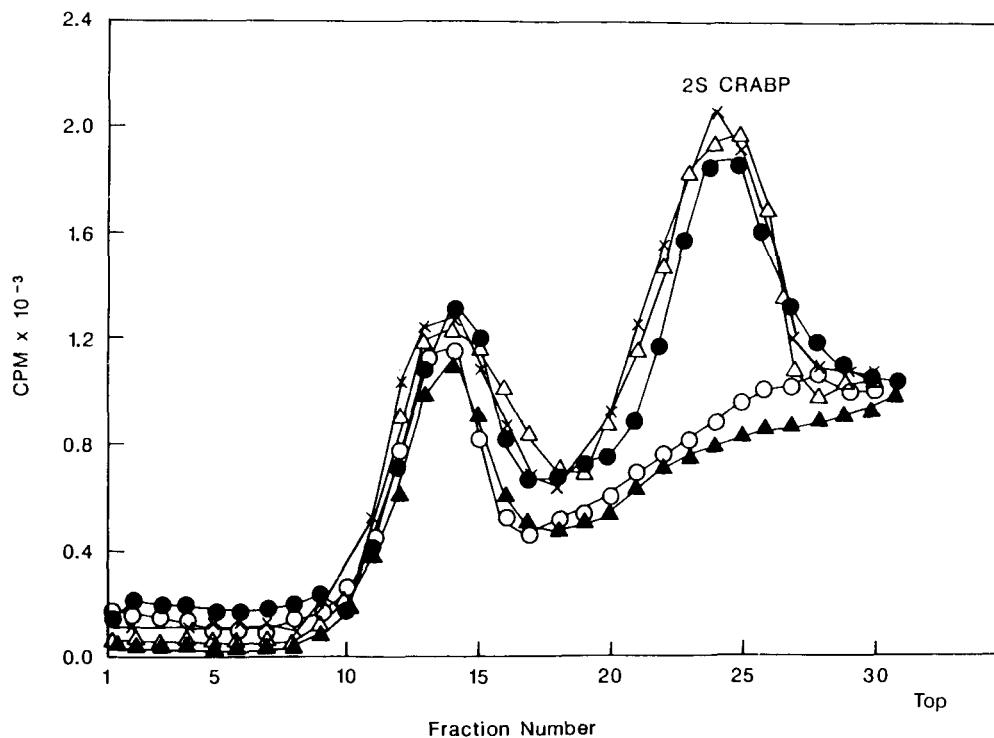


FIGURE 1. Sucrose gradient centrifugation of chick embryo skin cytosol (3 mg of protein) plus [³H]retinoic acid (200 pmol) with or without a 200-fold molar excess of unlabeled retinoids. X, cytosol + [³H]retinoic acid (control); Δ , control + retinol; ●, control + retinal; ○, control + retinoic acid; ▲, control + acetylcylopentenyl analog of RA.

The purification and characterization of CRABP from several sources have been carried out in different laboratories.⁷²⁻⁷⁶ The protein is a single polypeptide chain with a molecular weight of 14,600, determined by sodium dodecyl sulfate-polyacrylamide electrophoresis.⁷³ The protein binds RA with high affinity. The apparent dissociation constant was determined by fluorometric titration to be $4.2 \times 10^{-9} M$. The binding proteins from chick embryo skin⁷² and rat testis⁷⁵ had isoelectric pHs of 4.5 and 4.7, respectively. Both CRBP and CRABP of mouse colon tumor 26 expressed the same isoelectric pH of 4.5.⁷⁴ The two proteins could be resolved from one another by anion-exchange column chromatography⁷³ and by a sedimentation equilibrium technique.⁷⁴ The two proteins were completely resolvable by chromatography on SP-Sephadex⁷⁷ or Mono Q⁷⁸ columns. Chemical modification of thiol groups of CRABP indicated that thiol functions were involved in the binding of RA to the protein.⁷² At low concentrations (0.01 to 0.05 mM) of *p*-chloromercuriphenylsulfonic acid (CMPS) a slight stimulation of [³H]retinoic acid-binding occurred; however, at 0.1, 0.5, and 1 mM mercurial concentration, inhibitions of 10, 50, and 100%, respectively, of the RA binding to CRABP were observed. Such mercurial-inhibited preparations were further exposed to 5 mM dithiothreitol followed by incubation with [³H]retinoic acid. The sucrose-density-gradient sedimentation patterns of these preparations revealed that about 85% of the inhibition was reversed by the thiol compound. In further studies, chick embryo skin CRABP preparations were treated with 2 mM CMPS to completely inhibit RA binding. Part of this preparation was passed through a Sephadex G-25 column (1.5 cm \times 10 cm) to separate free and loosely bound mercurial compounds from the more stable CRABP-mercurial complex. Such a preparation, after incubation with [³H]retinoic acid and sucrose gradient analysis, exhibited only 50% inhibition of the RA-binding sites. This revealed that half of the mer-

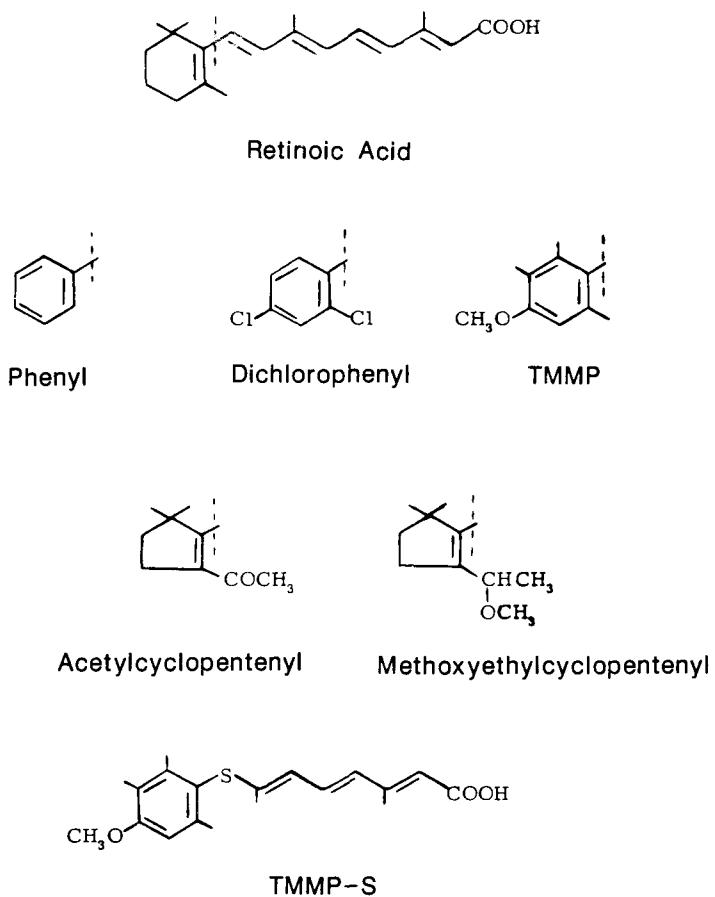
curial-sensitive sites of retinoic acid binding were weak and had lost the mercurial on Sephadex sieving.⁷² Studies on such easily dissociable retinoic acid complexes may reveal the mode of disposition of retinoids for physiological functions. A sensitive and quantitative method for the determination of CRABP in tissue extracts has been developed,⁷⁹ and is based on blocking of the specific retinoic acid-binding sites on CRABP using 1 to 2 mM CMPS. The difference in radioactivity between the control and the mercurial-treated preparation was equivalent to the amount of CRABP present in the preparation.

IV. RETINOIDS AND CRABP

The role of CRABP in mediating the biological effects of RA in the control of epithelial differentiation was further confirmed by the existence of a correlation between the binding affinities of various synthetic analogs of RA to CRABP and their biological potency.^{51,57} Thus, biologically active analogs of RA such as a trimethylmethoxyphenyl analog, a cyclopentenyl analog, and 13-*cis*-retinoic acid also showed good affinity for CRABP, whereas the phenyl and pyridyl analogs, which were biologically less active, were poor binders. Subsequently, more detailed and quantitative studies on the binding affinities of RA analogs to CRABP and their biological activity appeared.^{52,53,64-67}

Structures of some of the retinoids that were tested for their binding affinity for CRABP are shown in Figure 2.⁵² To measure the competitive binding affinity of the retinoids for CRABP, the inhibition of [³H]retinoic acid binding caused by a 100-fold molar excess of unlabeled retinoic acid was regarded as 100% inhibition.^{52,53} The inhibition caused by similar excesses of various analogs was expressed as relative inhibition to the above standard and is shown in Table 1. Sucrose density gradient (5 to 20%) sedimentation of the preparations at 180,000 × g for 18 h, fractionation of the gradients, and estimation of the 2S radioactive peaks were accomplished by standard procedures.^{52,53} Although the trimethylmethoxyphenyl analog showed 95 to 100% inhibition, which is almost as high as RA itself, the analog in which the 7,8-double bond in the chain is replaced by a thiomethylene group showed only 38% inhibition. The dichlorophenyl analog exhibited 55% inhibition, while the phenyl analog had the least competitive effect (30 to 40%). In addition to RA, 100% inhibition was expressed by 13-*cis*-retinoic acid, and the acetylcyclopentenyl and the methoxyethylcyclopentenyl analogs of RA. These retinoids also had pronounced ability to reverse keratinized metaplasia produced by chick embryo or hamster trachea in organ culture.^{18,19} Sani et al.⁵² also reported that various steroids, prostaglandins, cyclic nucleotides, and vitamins did not compete with [³H]retinoic acid for chick embryo skin CRABP. Those RA derivatives in which the free carboxyl group was derivatized, being converted to such groups as esters, amides, oxime, and lactone, showed no affinity for the retinoic acid-binding site. Hence, it is suggestive that the esters and amides of retinoic acid that show biological activity^{27,32,68} may be hydrolyzed to free acids in order to express their receptor-mediated modulating effects in differentiation and anticarcinogenesis. The CRABP of retinoblastoma cytosol showed similar binding affinities for retinoids except that the trimethylmethoxyphenyl analog of RA did not compete for the binding site.⁶³ It has also been observed in different laboratories that retinoids that are highly active in mouse skin papilloma, rat chondrosarcoma, 3T6 cells, and embryonal carcinoma cells also bind tightly to CRABP from rat testes.^{65,66} Lotan²⁰ has observed that the ability of the various analogs of RA to compete with [³H] retinoic acid for CRABP of mouse S91 melanoma correlated well with their effect on cell proliferation, suggesting that the binding protein may be involved in mediating growth inhibition. A similar pattern of inhibition has also been observed with human melanoma cell lines Hs939 and A375 and human breast carcinoma 734B.

A novel synthetic derivative of retinoic acid 4-[*(E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)propenyl]benzoic acid (TTNPB, Ro 13-7410), exhibited biological



Structures of Retinoic Acid and Analogs

FIGURE 2. Structures of retinoic acid and analogs.

TABLE 1
Percent Inhibition of Binding of [³H]Retinoic Acid to CRABP by 100-Fold Molar Excess of Unlabeled Retinoids^a

Retinoid	Percent inhibition
Retinoic acid	100
Phenyl analog	30
Dichlorophenyl analog	55
TMMP analog	95
TMMP-S analog	38
Acetylcylopentenyl analog	100
Methoxyethylcylopentenyl analog	100
13-cis-Retinoic acid	100
Retinal	5
Retinol	0
Methyl retinate	0
<i>N</i> -Diethylretinamide	0

^a See Figure 2 for structures.

activity in the reversal of keratinization in hamster tracheal organ cultures equal to or greater than that of all-*trans*- or 13-*cis*-retinoic acid.⁶⁸ A series of these unusual retinoids was synthesized in a search for new structurally modified retinoids that would possess prophylactic and therapeutic activity.⁶⁹⁻⁷¹ Many of these retinoids were studied for their binding efficiency for CRABP from chick embryo skin and a transplantable mouse colon tumor 26.^{53,67} The binding affinities were compared with the biological potency of the retinoids for reversing keratinization in organ cultures of tracheal epithelium from retinoid-deficient hamsters^{53,67} as described by Sporn et al.²⁴ The structure, code number, and binding efficiency of some of these retinoids are listed in Table 2. The biological activities of these retinoids, as a percentage of the active cultures to the total cultures tested, and their 50% effective dose values in the reversal of keratinization in hamster tracheal organ cultures are also included in the same table. Of the five retinoids that are conformationally restricted to cisoid conformations by aromatic ring systems, the benzoic acid analogue SRI 4657-57, the thiophene carboxylic acid SRI 3920-59, and the naphthalenecarboxylic acid SRI 4445-75 showed high binding affinity to CRABP and high activity in the reversal of keratinization assay. In contrast, the more polar and more labile furoic acid SRI 4445-40 exhibited no binding and low activity in the keratinization assay. The biphenyl analogue SRI 4529-19 showed only low binding to CRABP and low activity in the organ culture assay. Evidently, a cisoid conformational restriction on the 7,9-double bond system reduces biological activity. The same result was found for the *cis*-conformationally locked retinoids SRI 3204-91 and SRI 3618-35. However, the benzoic acid SRI 3204-91 did exhibit slightly more activity than did the corresponding phenol SRI 3618-35. Replacement of the 19-methyl group by a phenyl group also reduced activity. Thus, the binding affinities of these retinoids directly correlate with their biological activity. Retinoids SRI 4445-75, SRI 3920-59, and SRI 4657-57 having a relative percentage of inhibition in the binding assays between 90 and 100% showed appreciable biological activity in the range of 10^{-10} to $10^{-9} M$. About 50% of the tracheal cultures exhibited reversal at about $10^{-10} M$. Retinoids SRI 4445-40 and SRI 3618-35 did not inhibit RA binding to CRABP and did not express any significant biological activity even at a concentration of $10^{-8} M$. Retinoids SRI 3204-91, SRI 4529-19, and SRI 4875-20, which showed intermediate binding affinity for CRABP, also displayed maximal biological potency in the middle range, 10^{-9} to $10^{-8} M$, in the tracheal organ culture assays.

V. MECHANISM OF ACTION OF RETINOIC ACID

It was clear from the original paper by Wolbach and Howe³ that an adequate level of retinoid was necessary for control of normal cellular differentiation and proliferation. However, a satisfactory explanation of the molecular mechanism underlying these effects is still eluding us. An understanding of this mechanism would eventually find practical application in the cancer field, particularly for prevention of cancer. Because chemical carcinogenesis is believed to involve dedifferentiation, it is possible that the anticarcinogenic properties of RA are caused by inhibition of cellular proliferation and enhancement of terminal differentiation of preneoplastic cells,^{80,81} and this process may be mediated by CRABP. Because CRABP levels decrease in the postnatal period and increase in tumors,⁸²⁻⁸⁹ this protein may be considered as a marker for dedifferentiation. Thus, the action of RA through the mediation of CRABP may cause a hormone-like effect involving induction and/or depression of gene activity. As an initial event in its action, RA may complex with CRABP in the cytosol of the target tissues. Subsequently, one would expect this complex to migrate to the nucleus of the target cell to associate with nuclear components or nuclear acceptor sites. This nuclear localization of the receptor-ligand site is thought to stimulate transcription and thus initiate the sequence of biochemical events leading to the overall physiological changes produced by the hormone. In a study of this sequence of events, Sani⁹⁰ demonstrated the localization

TABLE 2

Structures, Binding Efficacy to CRABP, and Biological Potency to Reverse Keratinization

Retinoid Code No.	Structure	Inhibition of [³ H] Retinoic Acid to CRABP			Reversal of Keratinization			
		Chick Embryo Skin CRABP	Colon Tumor CRABP	M	Active/Total Cultures	%	ED ₅₀	Total Cultures
SRI 4657-57		90	92				2 x 10 ⁻¹⁰	24
SRI 3920-59		95	90	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰	15/15 14/15 7/15	100 93 47	1 x 10 ⁻¹⁰	45
SRI 4445-40		0	0	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰	5/11 5/13 4/13	45 38 31	4 x 10 ⁻⁸	37
SRI 4529-19		36	38	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰	7/7 2/12 2/12	100 17 17	4 x 10 ⁻⁹	31
SRI 4445-75		100	100	10 ⁻⁸ 10 ⁻⁹ 10 ⁻¹⁰ 10 ⁻¹¹	7/7 11/12 4/12 2/7	100 92 33 29	2 x 10 ⁻¹⁰	36

SRI 4875-20		40	40	10^{-8} 10^{-9} 10^{-10} 10^{-11}	5/7 5/7 5/14 0/14	71 71 36 0	3×10^{-10}	35
SRI 3204-91		25	22	10^{-9}	2/7	17	$\gg 10^{-9}$	7
SRI 3618-35		0	0	10^{-8} 10^{-9} 10^{-10} 10^{-11}	3/7 4/12 3/12 2/7	43 33 25 29	5×10^{-8}	38
All-trans-retinoic acid		100	100	10^{-10} 10^{-11} 10^{-12}	224/249 141/278 54/241	90 51 36	1×10^{-11}	768

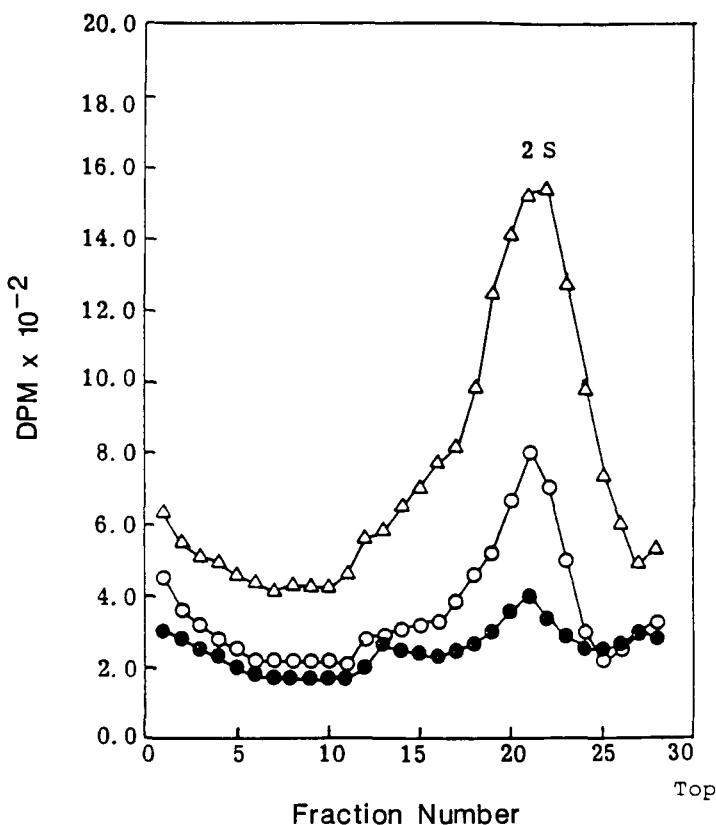


FIGURE 3. Sucrose density gradient centrifugation patterns of cytosol (3 mg of protein) prepared from chick embryo skin after incubation for 2 h in Eagle's modified medium containing 1 μM [3H]retinoic acid at different temperatures, ●, 0°C; ○, 20°C; △, 37°C.

of a binding component of RA in the nuclei of chick embryo skin and transplantable murine tumors. This localization was the first step in delineating the molecular mechanism of RA action at the genetic level.

An early cellular event in RA action will be the entry of the circulating retinoid into the cytoplasm and formation of a complex with the binding protein. To test the effect of temperature on the formation of such a complex, chick embryo skins were incubated in Eagle's modified medium containing 1 μM [3H]retinoic acid for 20 h at 0, 20, or 37°C. The incubations were terminated by quick chilling and immediate centrifugation followed by washing because preliminary experiments indicated that incorporation of the ligand was minimal at 0°C. In Figure 3, the effect of the temperature of incubation on the formation of the binding protein-[3H]retinoic acid complex in the cytosol of chick embryo skin is illustrated.

Based on the size of the 2S CRABP peak, the formation of the binding protein-ligand complex at 37 and 20°C is 10-fold and 3-fold, respectively, higher than at 0°C.⁹² A similar experiment with Lewis lung tumor also exhibited temperature dependency, but to a lesser extent. The time dependence of the incorporation of [3H]retinoic acid by the cytosol binding protein of chick embryo skin was also studied. As indicated by the size of the radioactive 2S peak, retinoic acid incorporation increased almost linearly up to 2 h. When incubation was extended further, there was a considerable decrease in the size of the 2S peak.

After complexing with cytosol CRABP, one would expect this complex to migrate to

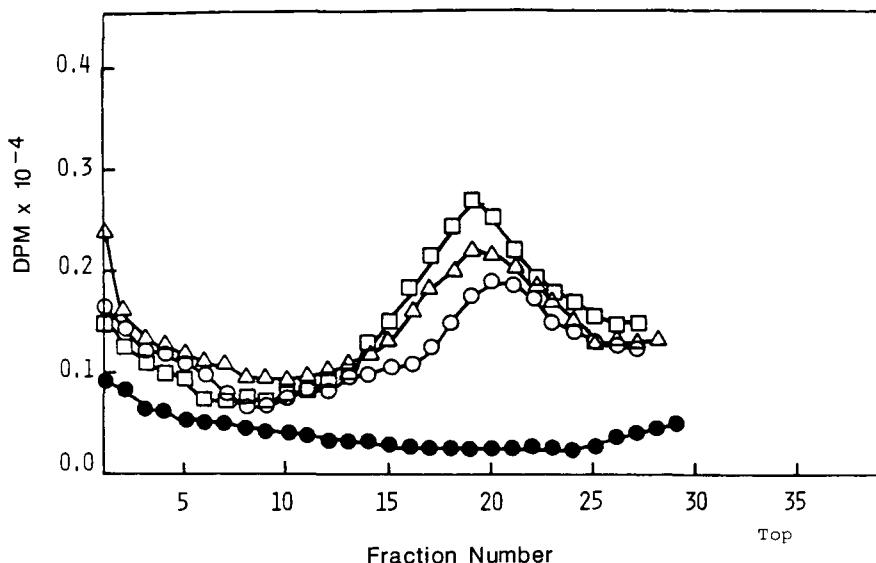


FIGURE 4. Sucrose density gradient profiles showing the effect of DNase, RNase, and pronase on nuclear CRABP-[³H]retinoic acid complex. Nuclear extract (2 mg of protein) from Lewis lung tumor was incubated with [³H]retinoic acid (300 pmol) for 2 h, followed by incubation with DNase, RNase, or pronase for 4 h. The preparations were dialyzed for 22 h and analyzed on 5 to 20% sucrose gradients. □, Lewis lung plus [³H]retinoic acid (control); △, control plus RNase; ○, control plus DNase; ●, control plus pronase.

the nucleus of the target cells to associate with nuclear acceptor sites. In Figure 4 are illustrated the sucrose-density gradient patterns of a nuclear extract from Lewis lung tumor after incubation with [³H] retinoic acid.⁹³ The figure indicates the presence of a 2S binding component in the tumor nuclei. Similar 2S peaks have been detected in the nuclei of other tumor cells as well as chick embryo skin. Whereas pronase completely digested the nuclear component, DNase showed about 40%, and RNase showed negligible digestive action on the complex. It is known that binding of [³H]retinoic acid to cytosol CRABP is inhibited by mercurials and that the inhibition is reversed by dithiothreitol.⁷² Incubation of the nuclear extract with 1 mM CMPS completely inhibited the [³H]retinoic acid-binding sites on the nuclear CRABP. The mercurial-inhibited preparation was further exposed to 5 mM dithiothreitol, followed by incubation with [³H]retinoic acid. The sucrose-gradient sedimentation profile of this mixture showed about 80% recovery from the mercurial inhibition indicating the reversible nature of the binding sites by thiol compounds.

Although the presence of CRABP in the nuclei of cells, which were not preincubated with RA, has been demonstrated, the function of CRABP in the transmigration of RA into the nuclei is not well understood. Incubation of Lewis lung tumor or chick embryo skin with [³H]retinoic acid and subsequent isolation of the tissue nuclei and analysis for nuclear CRABP-[³H]retinoic acid complex in the nuclear extract showed distinct 2S peaks (Figure 5). The presence of unlabeled RA in the initial incubation mixture showed competition for the [³H]retinoic acid-binding sites on nuclear CRABP (Figure 5a). Also illustrated in Figure 5 is evidence that the ligand-protein complex is weak inasmuch as dialysis for 22 h considerably reduced the 2S radioactive peak. The presence of nuclear CRABP has been reported by Wiggert et al.⁹³ and by Jetten and Jetten⁶⁶ in cultured human retinoblastoma and murine embryonal carcinoma cells. Russel et al.⁹⁴ demonstrated by autoradiographic analysis that [³H]retinoic acid taken up by cultured retinoblastoma cells could be detected in the nucleus. Mehta et al.⁹⁵ and Takase et al.⁹⁶ showed that, in mammary tumor cell nuclei and in rat

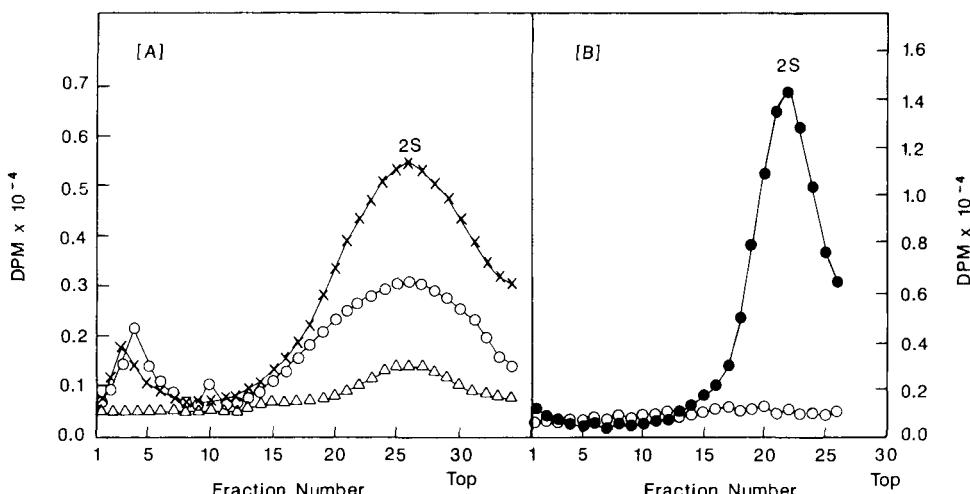


FIGURE 5. Sucrose density gradient centrifugation patterns of Lewis lung tumor (A) or chick embryo skin (B) nuclear extract (2 mg of protein) after incubation of the tissues at 37°C for 2 h as follows; (A) X, 10 μ M [3 H]retinoic acid; O, 10 μ M [3 H]retinoic plus 100 μ M unlabeled RA; Δ , same as X after dialysis for 22 h against PBS; (B) ●, 10 μ M [3 H]retinoic acid; ○, same as ● after dialysis for 22 h against PBS.

testes nuclei, CRABP enabled RA to bind in a specific manner to nuclei. Although Mehta et al.⁹⁵ reported a concomitant appearance of [3 H]retinoic acid-CRABP complex in the nuclei, Takase et al.⁹⁶ did not observe CRABP, itself, being retained in the nuclei after the delivery of the ligand to the nuclear acceptor sites.

Relatively little is known about the plasma transport of RA except that it is bound to serum albumin.⁴⁹⁻⁵¹ The mechanism of transport of RA across the plasma membrane and its cellular uptake by epithelial cells is also not known. In the course of a study of the special surface membrane properties of epithelial cells,^{98,99} Sani located and partially characterized a specific retinoic acid-binding component in the plasma membranes of chick embryo skin and some experimental murine carcinomas. The radioactivity profiles of the plasma membrane extract from chick embryo skin showed a distinct peak with an S_{20} value of 2.0 (Figure 6), which was the same as that for cytosol CRABP. Unextracted plasma membrane, suspended in PBS did not reveal any detectable 2S binding peak; the RA bound to the membrane sedimented to the bottom of the gradient. This result indicated that the binding protein is an integral part of the membrane. Plasma membrane extracts from colon tumor 26 also exhibited pronounced 2S radioactive peaks. Lyophilization of the membrane prior to extraction was essential for maximal release of the binding component. The protein nature of the binding component in the extracts was assessed by treatment with pronase, which completely digested the 2S component. Cope and Boutwell¹⁰⁰⁻¹⁰² also presented evidence for a plasma membrane CRABP from mouse brain with a molecular weight of 15,800, whose binding characteristics mimicked CRABP.

A diagrammatic scheme of a proposed mechanism for the translocation and action of RA in the control of epithelial differentiation is illustrated in Figure 7. The mechanism by which RA is delivered across the cell surface and nuclear membranes, as well as the basic concept of the genomic control mechanism, is not well understood. The detection of RA in human plasma as a normal constituent of the vitamin A reserve in the body,⁴⁸ the facilitation of its transport by serum albumin,⁴⁹⁻⁵¹ and the detection of CRABP as a plasma membrane component⁹⁷⁻¹⁰² may explain the cellular recognition and selective transport of RA for epithelial differentiation and control of tumorigenesis. Subsequently, it appears that the process involves migration of the CRABP-RA complex from the cell membrane through the cyto-

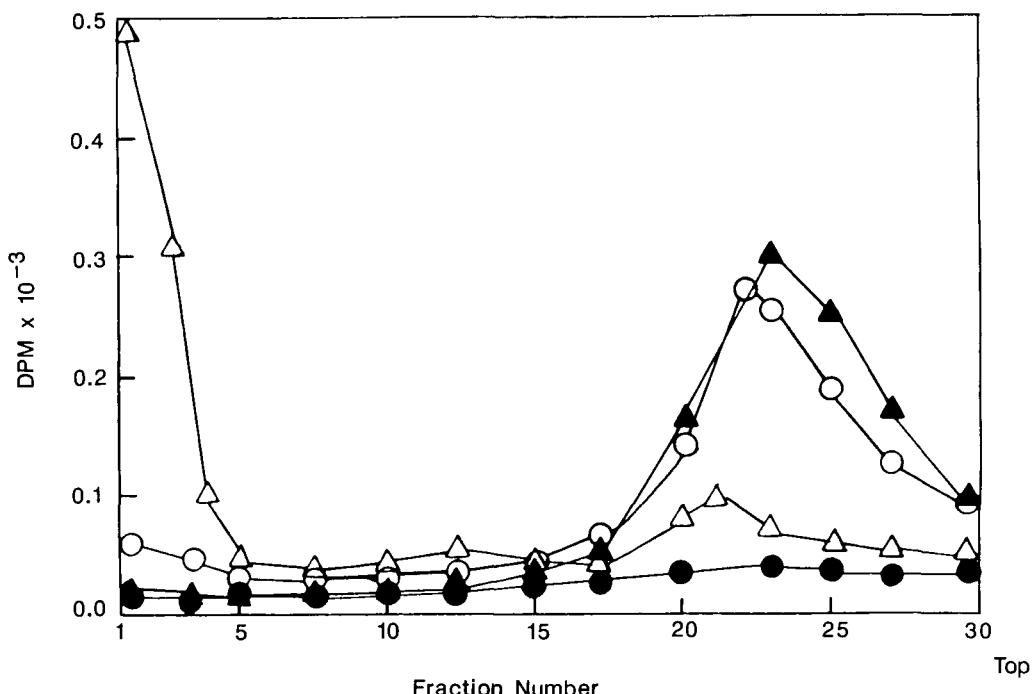


FIGURE 6. Sucrose density gradient sedimentation patterns of plasma membrane preparations plus [³H]retinoic acid (300 pmol). △, plasma membrane suspension from chick embryo skin (1 mg of protein) before lyophilization and extraction; ▲, plasma membrane extract (200 µg of protein) from chick embryo skin; ○, plasma membrane extract (200 µg of protein) from colon tumor 26; ●, plasma membrane extract after treatment with 50 µg of pronase.

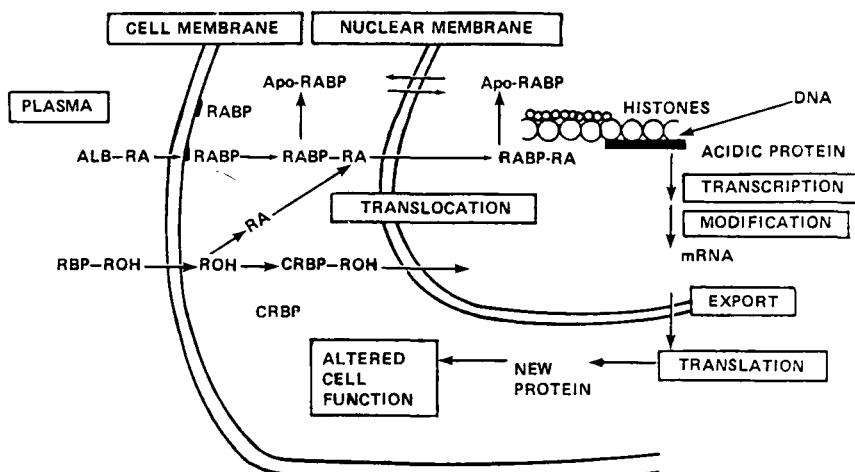


FIGURE 7. Schematic diagram of a proposed mechanism for translocation and action of retinoic acid: ALB, serum albumin; RA, retinoic acid; ROH, retinol; SRBP, serum retinol-binding protein; RABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; Apo-RABP, RABP lacking the ligand.

plasm across the nuclear membrane to the nuclear acceptor sites where it exerts a direct effect upon gene expression and alters transcriptional and translational events. Detection of apo-CRABP in the nuclei of untreated cells supports the hypotheses that the CRABP-retinoic acid complex may transfer RA to the nuclear acceptor sites and that the binding protein may cycle back to apo-CRABP, which in turn equilibrates with cytosol CRABP.

Recently Eichele and Thaller^{103,104} presented evidence that RA acts as an endogenous morphogen. They showed that chick limb buds contain RA and that it forms a concentration gradient across the limb anlage with a high-point in the posterior domains of the limb bud, the part that also contains the zone of polarizing activity. They also showed that the levels of RA at different regions of the limb buds corresponded to the known induction thresholds in response to applied RA. Their data supported that RA is not freely diffusible in the limb rudiment, but interacts with CRABP.¹⁰³ The nuclear acceptor site of RA was, however, unknown until Petkovich et al.¹⁰⁵ and Giguere et al.¹⁰⁶ almost simultaneously reported a nuclear receptor for RA that belonged to the family of nuclear receptors. They cloned cDNA encoding a protein that binds RA with high affinity. The protein, which has a molecular weight of about 54,000, is homologous to the steroid and thyroid hormone receptors. It was suggested that the action of RA on embryonic development, differentiation, and tumor cell growth is mediated by this receptor in a manner similar to the other members of the nuclear family. Recently Brand et al.¹⁰⁷ presented evidence for another nuclear receptor (RAR- β) for RA. This protein, which has a molecular weight of about 51,000, showed 97% amino acid identity with the formerly known receptor (RAR- α).^{105,106} at the DNA binding domains. This suggests that the two receptors may recognize a common RA-responsive element. Brand et al.¹⁰⁷ reported that RAR- β may mediate activation of transcription by RA at concentrations tenfold lower than those necessary for activation by RAR- α , although both receptors respond to RA concentrations within the range observed for RA action *in vivo*. The exact role of CRABP in the transfer of the ligand to the nuclear receptors is presently not known.

We undertook the isolation of RAR from both cytosol and nuclei from chick embryo skin and rat testes. Cytosol and nuclei from these tissues were prepared by our standard procedure.⁹⁰⁻⁹² The nuclear pellets were extracted with 0.3 M KCl, 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, and centrifuged at 100,000 \times g for 1 h. The extract was dialyzed against PBS before use. Both cytosol (50 mg of protein) and nuclear extract (15 mg of protein) were incubated with 3 nmol and 1 nmol, respectively, of [11,12-³H]retinoic acid (1.28 Ci/mmol). The preparations after incubation at room temperature for 1 h and at 4°C for 2 h were filtered through a column of Sephadex G-100 gel (2.3 cm \times 100 cm) in PBS. Both the protein and the radioactivity distributions of the elution profiles were determined. Samples (5 mg of protein each) of bovine serum albumin (mol. wt. 68,000), ovalbumin (mol. wt. 44,000), and myoglobin (mol. wt. 17,800) were run through the same column as standards, and 5-ml fractions were collected.

Figure 8 presents the Sephadex G-100 column chromatographic profile of the radioactivity distribution after chick embryo skin cytosol and nuclear extracts were incubated with [³H]retinoic acid. The first radioactive peak from the cytosol corresponded to a molecular weight of about 68,000 and is probably due to [³H]retinoic acid bound to serum albumin. This peak was absent in the profile of the nuclear extract. The second radioactive peak was evident between fractions 50 to 65, which falls in the molecular weight range of 50,000 to 55,000. Thus, a radioactive peak attributable to RAR-[³H]retinoic acid complex (mol. wt. 54,000) was detectable both in the cytosol and in the nuclei under these conditions. Fractions 50 to 65 from the Sephadex G-100 column chromatographic eluates were pooled and concentrated. About 7 mg of protein was incubated with 1 nmol of [³H]retinoic acid followed by resolution through a Sephadex G-100 column. The radioactivity profile of such cytosolic fractions revealed a sharp radioactive peak corresponding to the molecular size of RAR (Figure 8). The reason that this peak was not detected during the first column chromatography

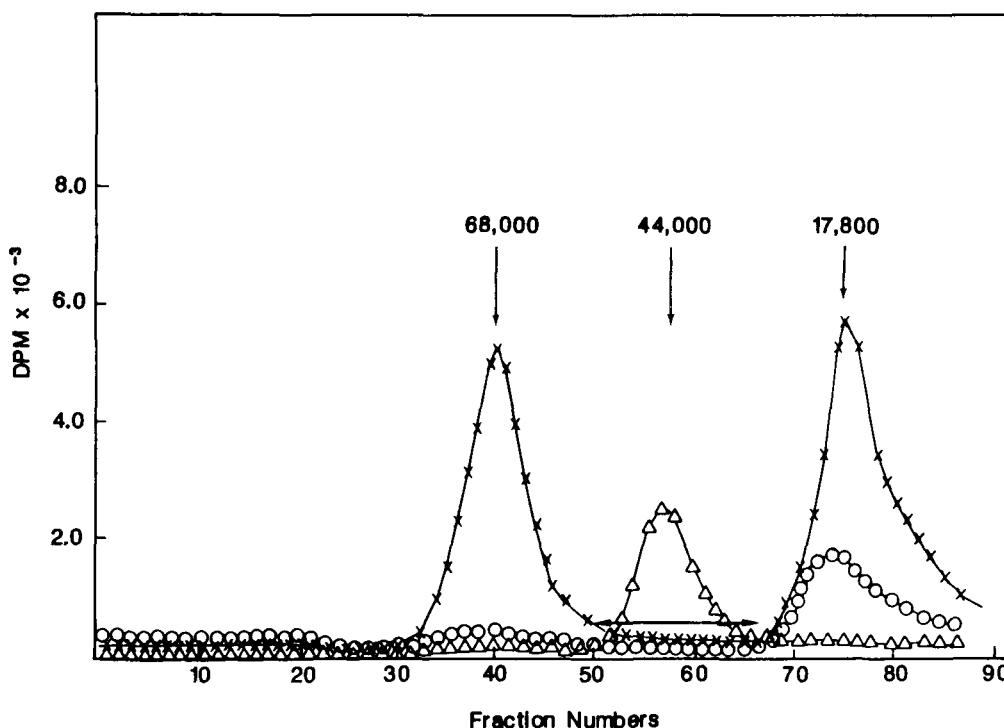


FIGURE 8. Radioactivity distribution of the Sephadex G-100 column ($2.3\text{ cm} \times 100\text{ cm}$) chromatographic elution profiles of chick embryo skin cytosol (50 mg of protein) or nuclear extract (15 mg of protein) after incubation with 3 nmol or 1 nmol of [^3H]retinoic acid, respectively. X = chick embryo skin cytosol plus [^3H]retinoic acid; O, chick embryo skin nuclear extract plus [^3H]retinoic acid. Δ = pooled fractions 50 to 65 (7 mg of protein) from chick embryo skin cytosol-[^3H]retinoic acid chromatographic resolution (X) after incubation with 1 nmol of [^3H]retinoic acid and rechromatography on Sephadex G-100 column. The elution positions of external standards, bovine serum albumin (mol. wt. 68,000), ovalbumin (mol. wt. 44,000), and myoglobin (mol. wt. 17,800) are marked with arrows.

is not known. It may be that RA has a relatively lower affinity for RARs than for CRABP or that RARs were present in too small an amount in the original extracts to be detected. A similar radioactive peak for RAR was also evident after rechromatography of fractions 50 to 65 collected from the eluates after passing the chick embryo skin nuclear extracts through a Sephadex column under the same conditions. We were also able to locate the RAR peak from rat testes cytosol and nuclear extract after rechromatography using Sephadex G-100 columns under similar conditions. The significance for the existence of multiple nuclear receptors for RA, the mode of RA-transfer to the receptors via CRABP, and the mechanism of the morphogenetic concentration gradient formation by RA in presence of the different binding proteins are important scientific questions that need to be addressed.

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Chapter 16

**THE INHIBITORY EFFECTS OF RETINOIDS ON THE
INDUCTION OF ORNITHINE DECARBOXYLASE AND THE
PROMOTION OF TUMORS IN MOUSE EPIDERMIS**

Marcia I. Dawson, Wan-Ru Chao, Peter D. Hobbs, and Thierry Delair

TABLE OF CONTENTS

I.	Introduction	386
II.	Background	386
	A. Ornithine Decarboxylase Induction and Tumor Promotion.....	386
	B. Effect of Retinoids on Ornithine Decarboxylase Induction	388
	C. Effect of Retinoids on Tumor Promotion	389
III.	Effects of Structural Modification on Retinoid Activity	390
	A. Background	390
	1. Inhibitory Effects of Retinoids on Ornithine Decarboxylase Induction and Tumor Promotion	390
	2. Tumor Regression Effects of Retinoids	395
	B. Synthetic Methodology for Retinoid Structural Modification	414
	1. Polyenoic Acid Analogs of Retinoic Acid: Principal Synthetic Routes	414
	2. Less Common Approaches to the Synthesis of Polyenoic Acid Analogs of Retinoic Acid	417
	3. Synthesis of Specific Polyenoic Acid Analogs of Retinoic Acid	420
	4. Retinoids in Which Aromatic Groups Replace Part of the Polyene Chain	422
	5. Retinoids Containing Biaryl Groups	425
	6. Retinoids in Which the Hydrophobic and Polar Termini Are Linked through Other Nonolefinic Groups	427
	7. Synthetic Methodology Overview	427
	C. Assay Protocols	428
	1. Chemicals.....	428
	2. Animals.....	428
	3. Ornithine Decarboxylase Assay	428
	4. Tumor Promotion (Antipapilloma) Assay	428
	D. The Effects of Structural Modifications of the Retinoic Acid Skeleton on Activity in the Ornithine Decarboxylase Assay	429
	E. Correlation of Retinoid Inhibitory Activities in the Ornithine Decarboxylase and Tumor Promotion Assays	457
IV.	Summary and Conclusions	457
	Acknowledgments	458
	References.....	458

I. INTRODUCTION*

With the discovery that retinoids have a prophylactic and therapeutic effect on the transformation of normal cells to the malignant state, interest in the chemistry and biology of retinoids has been considerably enhanced because these compounds are considered to have potential value as cancer chemopreventive agents.¹ Various *in vitro* and *in vivo* models have been established to screen retinoids for their efficacy in preventing and reversing neoplastic transformation. One of the most rapid *in vivo* screening models is the ornithine decarboxylase (ODC) assay, which has been extensively studied by Verma and Boutwell and their co-workers.²⁻⁴ Retinoids inhibit the induction of ODC in mouse epidermis that has been treated with tumor promoters,⁵ and the extent of this inhibition correlates with the ability of retinoids to prevent or inhibit the formation of epidermal tumors caused by tumor promoters.⁴ Therefore, the *in vivo* ODC and tumor promotion assays are excellent models for assessing retinoid structure-activity relationships as a means of discovering the structural modifications that can be made in the retinoid skeleton that enhance chemopreventive activity.

II. BACKGROUND

A. ORNITHINE DECARBOXYLASE INDUCTION AND TUMOR PROMOTION

Increased polyamine biosynthesis is considered to be an essential requirement for cell growth,⁵⁻¹² malignant transformation,^{6,13,14} and the promotion phase of skin carcinogenesis.^{2,15-19} Ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) catalyzes the decarboxylation of ornithine (**1**) to putrescine (**2**), shown in Figure 1. This is the rate-limiting first step in the biosynthesis of polyamines,^{15,20-24} which are required to maintain membrane integrity. Putrescine is the precursor for the biosynthesis of the polyamines spermine and spermidine, which are essential components for ribosome structure and function. The levels of both ODC and polyamines were higher in rodent and human intestinal tumors than in the corresponding normal mucosa.²⁵⁻²⁷ The activity of ODC increased in cells and tissues that were induced to proliferate. For example, the basal level of ODC was much higher in skin tumors that were induced by a tumor initiation-promotion protocol than in normal skin,^{18,28} suggesting that the induction of this activity is important in the process of carcinogenesis.²⁹ ODC activity was induced in rodent colon that had been treated with the carcinogens dimethylhydrazine³⁰ and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine³¹ and in rodent bladders treated with a tumor promoter³² or carcinogens.³³ 2-Difluoromethylornithine, an irreversible inhibitor of ODC, inhibited the promotion of epidermal tumors in a dose-dependent manner³⁴ and the formation of murine mammary tumors induced by dimethylhydrazine.³⁵ Therefore, like enhanced polyamine biosynthesis, enhanced ODC activity is considered to be a marker for tumor promotion. The mechanism by which polyamines and ODC enhance the process of tumorigenesis has not yet been established.

ODC activity was induced over 200-fold above basal levels by topical treatment of mouse epidermis with the potent tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA, **3**, Figure 2), a component of croton oil isolated from the Euphorbiaceae *Croton tiglium* L, which has many different pleiotropic effects on a variety of different cell lines and tissues.^{15,36-45} Verma et al.⁴ established that a correlation exists between the extent of induction of ODC by TPA and the extent of tumor promotion in mouse epidermis. The two-stage

* Abbreviations: AIBN, azobisisobutyronitrile; 13-*cis*-RA, 13-*cis*-retinoic acid; 15-Crown-5, 1,4,7,10,13-pentaoxacyclopentadecane; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; DMBA, 7,12-dimethylbenz[a]anthracene; DME, dimethoxyethane; HMPA, hexamethylphosphoramide; i.p., intraperitoneal; MCPBA, *meta*-chloroperbenzoic acid; NBS, *N*-bromosuccinimide; ODC, ornithine decarboxylase; pet. ether, petroleum ether; PKC, protein kinase C; RA, all-*trans*-retinoic acid; THF, tetrahydrofuran; TMMP, 2,3,6-trimethyl-4-methoxyphenyl; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.



FIGURE 1. Conversion of ornithine to putrescine by ODC.

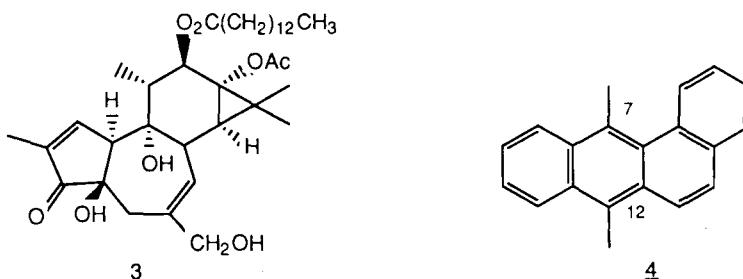


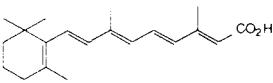
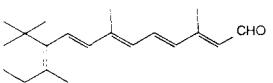
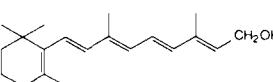
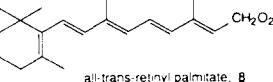
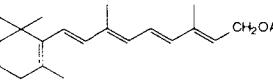
FIGURE 2. Structures of TPA (3) and DMBA (4).

model of skin carcinogenesis is based on these and similar observations.^{39,46-49} In this model, tumors are induced in mouse epidermis by a single topical treatment of a low dose of a carcinogen such as 7,12-dimethylbenz[a]anthracene (DMBA, 4), followed by frequent and repeated applications of a tumor promoter such as TPA. The carcinogen treatment is termed the initiation step. No tumors appear in the epidermis after the initiation treatment until the second step — promotion — has begun.^{39,50,51} Treatment with the promoter alone produces either no tumors or only a few. The initiation-promotion regimen produces many benign tumors (papillomas), only a few of which become malignant. Once promotion with TPA is discontinued, the papillomas regress and eventually disappear. Because of the high proliferation rate of the basal layer of cells and their increased keratinization,⁵² this model using papillomas has proved to be very useful for studying the biochemical events of tumor initiation and promotion^{15,38,39} and for screening the antineoplastic, antikeratinizing, and antipsoriatic properties of biological response modifiers such as retinoids.^{4,52} Neither the mechanism of the neoplastic transformation process nor the role that TPA has in this process has been established although much current work is providing valuable insights into the biochemical pathways involved.

The ability of TPA to induce mouse epidermal ODC correlated with its ability to bind to the TPA receptor in the epidermis⁵³ and its capacity to function as an epidermal tumor promoter in a dose-dependent manner.⁴ TPA has a cellular receptor and that receptor has been shown to copurify with protein kinase C (PKC).³⁶ The activation of PKC is considered to be a major pathway for transmembrane signaling. Activation is mediated by diacylglycerol and calcium(II), suggesting that these components also have a role in the process of promotion. Within 10 to 30 min after treatment with TPA, a redistribution of PKC activity from the cytosol (soluble) fraction to the particulate (membrane) fraction of the cells was observed.⁵⁴ After TPA treatment, PKC in HL-60 cells was translocated from the cytosol to the plasma membrane, whereas this enzyme in fibroblastic 3T3L1, CHO, and E7SKS cells was located in the nucleus.³⁶ The difference in the site of translocation may reflect the difference that TPA has on these cell types. TPA induces differentiation in the former but is mitogenic in the latter. The relationship between PKC activation and the induction of ODC has not yet been established, although it is known that TPA plays a role in both processes. Blumberg⁵⁵ has written an excellent review on the role of PKC as the phorbol ester receptor.

ODC is a labile enzyme that has a rapid turnover of approximately 17 min in the

TABLE 1
Comparison of Abilities of Natural Retinoids and Their Derivatives to Inhibit ODC Induction and Tumor Promotion at 14 Weeks in Female Charles River CD-1 Mice

Retinoid	Dose (nmol)	Inhibition of Tumor Promotion ^b		
		% Inhibition of ODC ^a	% Inhibition of tumors/mouse	% Decrease in number of mice bearing tumors
	1.7 17.0	98 ± 1 56 61	55 61	10 21
	1.7 17.0	58 ± 4	56	14
	1.7 17.0	34 ± 7 43 ± 5	30	4
	1.7 17.0	58 ± 11 82 ± 15	28	7
	1.7 17.0	0 ± 28	34	3

^a Retinoids applied topically to dorsal epidermis of mice 1 h before TPA (17 nmol). Mice sacrificed 4.5 h after TPA. TPA-treated control ODC activity range was 1.36 to 2.76 ± 0.38 nmol CO₂/mg of protein per 30 min.²

^b Mice (30/group) initiated with DMBA (0.2 µmol). After 10 d, mice treated twice weekly with TPA (17 nmol). Retinoids applied 1 h before TPA. All mice in control group had papillomas with the average number being 14.2.²

epidermis. It exists in multiple heterogeneous forms in normal mouse epidermis and its genome is polymorphic.^{56a} O'Brien et al.^{55b-d} reported that the multiple forms of the enzyme found in TPA-induced papillomas were different from those found in normal CD-1 mouse epidermal tissue with respect to four parameters: heat stability, with the papilloma enzyme being far more heat stable; isoelectric point; activation by GTP for papilloma ODC; and a higher Km for ornithine for the papilloma enzyme. TPA induced two different mRNAs for ODC in mouse epidermis, suggesting that TPA regulates transcription of the ODC gene(s).⁵⁷

B. EFFECT OF RETINOIDS ON ORNITHINE DECARBOXYLASE INDUCTION

Retinoids inhibit the induction of ODC and tumor promotion by TPA in mouse epidermis.^{2,3} Application of 17 nmol of all-trans-retinoic acid (RA, 5, Table 1) 1 h before application of 10 nmol of TPA to CD-1 mouse dorsal epidermis inhibited the maximal induction of ODC, which occurred 4.5 h after the application of TPA.² The inhibition of the induction paralleled the inhibition of [³H]2-difluoromethylornithine binding, indicating that RA specifically reduced the increase in active ODC induced by TPA treatment.⁵⁸ O'Brien et al.⁵⁹ quantitatively correlated the increase in epidermal ODC activity with TPA with the increase in the amount of ODC protein measured immunologically. This correlation indicated

that the increase in enzyme activity is the result of new protein synthesis. Northern blot analysis of the murine whole skin supported these results by demonstrating that TPA caused an increase in epidermal ODC mRNA.⁵⁸ A transcriptional site for the action of TPA was supported by the finding that this promoter did not induce ODC activity in enucleated cells.⁶⁰ Employing a method of obtaining RNA from epidermis rather than whole skin, Kumar et al.⁵⁷ reported the presence of two different TPA-inducible ODC mRNAs in SKH/hr-1 female hairless mouse epidermis. On treatment with 17 nmol of TPA, the peak mRNA increased 13-fold — an increase less than that found for ODC (230-fold), suggesting that other mechanisms may be involved in the process of ODC induction. Both the major and minor forms of the ODC mRNA were inhibited in a dose-dependent manner by RA, indicating that mediation of enzyme activity occurred at the transcriptional level. Verma^{61a} reported that RA inhibited TPA-induced mouse epidermal ODC mRNA and the induction of ODC and Stage II skin tumor promotion by diacyl glycerol. Subsequently, he reported that RA inhibits both Stage I and II tumor promotion and that this inhibition is dependent on the duration of RA treatment.^{61b} Because both TPA and RA are reported to affect gene transcription in other systems,^{62,63} similar processes could be operative with ODC induction and tumor promotion.

C. EFFECT OF RETINOIDS ON TUMOR PROMOTION

Retinoic acid and the other natural forms of the vitamin A compounds — retinal (**6**) and retinol (**7**) (Table 1) — are required for both the maintenance and the function of normal differentiation of epithelial cells.^{1,3} This requirement first became evident with the report of Fujimaki et al.⁶⁴ that carcinomas developed in the stomachs of rats fed a vitamin A-deficient diet. Morphological similarities between precancerous lesions caused by treatment with carcinogens and squamous metaplastic changes induced by vitamin A deficiency were noted.⁶⁵ Vitamin A deficiency led to hyperkeratosis of the skin and metaplastic changes in the gastrointestinal, respiratory, and urogenital epithelia.⁶⁶⁻⁷⁰ These metaplasias, the appearance of which was considered to be an initial step in the transformation of normal to neoplastic tissue, led investigators to suggest that retinoids could exert a prophylactic and therapeutic effect on the neoplastic transformation of epithelial tissue.⁷¹⁻⁷³ Lasnitzki⁷² found that RA restored abnormally differentiated prostatic cancer cells in culture to a normal histological appearance. Saffiotti et al.⁷³ found that retinyl palmitate (**8**) inhibited the formation of tracheobronchial squamous metaplasia and carcinomas in hamsters treated with the carcinogen benzo[*a*]pyrene and iron oxide. Retinol retarded the growth and inhibited the induction of both benign and malignant tumors in the Syrian hamster⁷⁴ and the Rhino mouse⁷⁵ treated with carcinogens. Shamberger⁷⁶ found that application of either 0.009% retinyl acetate (**9**) or 0.05% retinol inhibited papilloma formation in ICR Swiss mice initiated with 0.125 mg of DMBA and treated five times weekly over a period of 16 to 18 weeks with a mixture of 0.006% croton resin as the promoter and the test retinoids in acetone. These experiments led Shamberger to suggest that after tumor initiation, external factors acting on the pre-malignant cells in the skin could alter the number of tumors that were induced.

Skin papillomas, which were induced in female Swiss albino mice on days 1 and 15 by 150 µg of DMBA and promoted after an interval of three weeks with twice-weekly applications of 0.5 mg of croton oil for three to four months, regressed after treatment with RA by intraperitoneal (i.p.) injection weekly for 2 weeks or after treatment with retinyl palmitate or RA by stomach intubation daily for 2 to 6 weeks. For example, oral doses of 0.08 to 1.53 mmol/kg of retinyl palmitate over a 2-week period reduced the mean papilloma volume per animal by 46 to 89% and doses of 0.07 to 1.33 mmol/kg of RA decreased the papilloma volume by 60 to 78%.⁷⁷

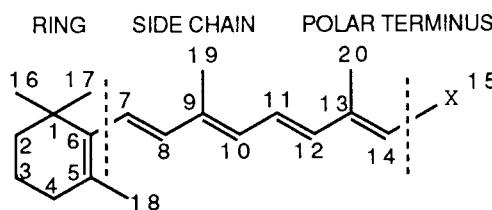


FIGURE 3. Retinoid skeleton showing the three structural regions for synthetic manipulation and standard retinoid numbering.

III. EFFECTS OF STRUCTURAL MODIFICATION ON RETINOID ACTIVITY

A. BACKGROUND

1. Inhibitory Effects of Retinoids on Ornithine Decarboxylase Induction and Tumor Promotion

Verma et al.^{3,4} established that the extent of induction of ODC activity was dependent on the dose of TPA applied to the epidermis of the mouse and correlated with the ability of the dose to promote the formation of papillomas in the epidermis. They² first determined that the ability of a group of natural retinoids to inhibit the induction of ODC by the topical application of 17 nmol of TPA to the dorsal epidermis of female CD-1 mice was consistent with the ability of the retinoids to inhibit skin tumor promotion in these mice initiated with 0.2 μ mol of DMBA and promoted twice weekly ten days later with 17 nmol of TPA for a period of 14 weeks (Table 1). Retinoids were administered topically 1 h before the application of the promoter. For the ODC assay, animals were killed at the time of maximal ODC induction (4.5 h). The inhibitory potency of the retinoids was RA (**5**) > retinal (**6**) ~ retinyl acetate (**9**) > retinol (**7**) > retinyl palmitate (**8**). In the antipapilloma assay protocol, multiple applications of TPA led to a 600-fold increase in the induction of ODC that plateaued between the sixth and eleventh TPA application. Treatment of the epidermis with 1.7 nmol of RA 1 h before TPA treatment led to a 60 to 80% decrease in ODC activity. The retinoids decreased the number of papillomas per mouse and the percentage of mice in a group ($N = 30$) bearing tumors. As Sporn et al.^{78,79} established in other studies, they found that RA was the most active natural retinoid in reversing neoplastic transformation as measured by its effect on ODC activity.

Because the natural retinoids cannot be administered in sufficient doses to have chemopreventive effects without exhibiting toxic systemic side effects,^{80,81} the efforts of medicinal chemists have been directed to the synthesis of structurally modified retinoids. Modifications were made in three regions of the retinoid skeleton — the polar terminus (X), the 2,6-dimethyl-1*E*,3*E*,5*E*,7*E*-octatetraenyl group attached to the polar terminus (the tetraene side chain), and the 2,6,6-trimethyl-1-cyclohexen-1-yl ring attached to the 8-position of the side chain (the β -cyclogeranylidene ring) (Figure 3).

Verma et al.⁴ compared the activities of four synthetic retinoids — 13-*cis*-retinoic acid (13-*cis*-RA, **10**), ethyl 3,7-dimethyl-9-(2,3,6-trimethyl-4-methoxyphenyl)-2*E*,4*E*,6*E*,8*E*-nonatetraenoate (the ethyl ester of TMMP, **11**), ethyl 3,7-dimethyl-9-(4-hydroxy-2,3,6-trimethylphenyl)-2*E*,4*E*,6*E*,8*E*-nonatetraenoate (**12**), and ethyl 3-trifluoromethyl-7-methyl-9-(2,3,6-trimethyl-4-methoxyphenyl)-2*Z*,4*E*,6*E*,8*E*-nonatetraenoate (**13**) — with those of RA in the ODC and antipapilloma assays (Table 2). Although different doses of retinoids were used, the inhibition by retinoids of ODC activity induced by 8 nmol of TPA correlated very well ($r = 0.9778$, $p < 0.005$, $n = 5$) with the inhibitory activity of retinoids on tumor promotion by 8 nmol of TPA over a 20-week period. In this series of experiments, RA proved to be the most effective inhibitor. Perhaps because of the small sample size, this

TABLE 2
Comparison of Abilities of Retinoic Acid and Synthetic Retinoids to Inhibit ODC Induction and Tumor Promotion at 20 Weeks in Female Charles River CD-1 Mice

Retinoid	Dose (nmol)	% Inhibition of ODC ^a	Inhibition of Tumor Promotion ^b	
			% Inhibition of tumors/mouse	% Decrease in number of mice bearing tumors
	34	99	81	45
	34	86 ± 3	62	51
	140	86 ± 3	85	60
	140	6 ± 20	0	3
	140	0 ± 20	3	0

^a ODC level measured 4.5 h after TPA. ODC level in treated control group was 3.5 ± 0.4 nmol of CO₂/mg of protein per 30 min.⁴

^b Mice initiated with DMBA (0.2 μmol); 2 weeks later, mice treated twice weekly with retinoid 1 h before treatment with TPA (8 nmol). Control mice received TPA alone. At 20 weeks, 90% of control mice had tumors with 10.0 as the average number per mouse (estimated from Chart 5 of Ref. 4).

excellent statistical correlation was not found in the study using the five natural retinoids ($r = 0.674$, $p > 0.1$, $n = 5$).² Nevertheless, these results suggested that testing the inhibitory effects of retinoids on TPA-induced ODC activity would prove to be a rapid, preliminary screen for assessing the potential chemopreventive activity of retinoids.

Verma et al.³ extended their studies on the inhibition of TPA-induced ODC activity in CD-1 mouse epidermis by determining the topical doses of a series of 22 synthetic retinoids that would inhibit enzyme activity by 50% (Table 3). These retinoids, which had been prepared by scientists at Hoffmann-La Roche, had modifications in the cyclohexene ring, tetraene side chain, and polar terminus and had demonstrated biological activity in *in vitro* assays.^{78,79,82-87} Mice were treated topically with the retinoids one hour before application of 17 nmol of TPA to the dorsal epidermis. Triplicate determinations of enzyme activity were made from the combined epidermal extracts of four mice using five to seven different retinoid concentrations. Seven of the synthetic retinoids (**10** and **14** to **19**) had ID₅₀ values of the same order of magnitude as that of RA (0.12 nmol). In this assay, shifting the double bond of the trimethylcyclohexenyl ring from the 1-position to the 2-position (**16**) or saturating this bond (**18**) decreased activity only slightly. Replacement of the ring with a 2-acetyl-5,5-dimethyl-1-cyclopentenyl ring system (**19**) had only a slight negative effect on activity and its 2-(1-methoxyethyl) analog (**14**) was more active than RA. The 13-cis isomer of retinal

TABLE 3
ID₅₀ Values for Inhibition of ODC Induction and for Papilloma Regression for a Series of Synthetic Retinoids

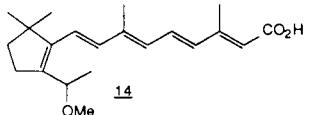
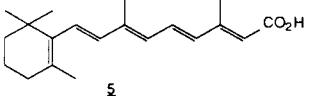
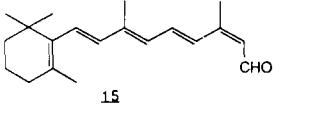
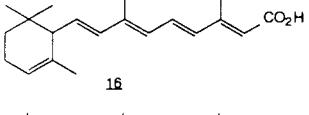
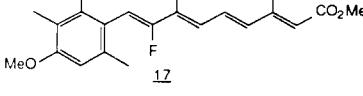
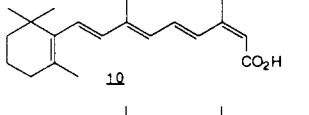
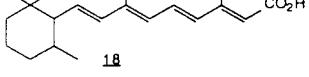
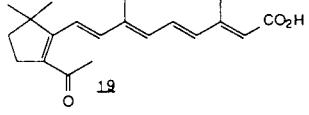
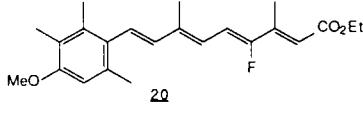
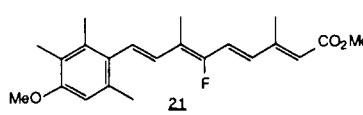
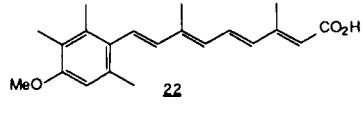
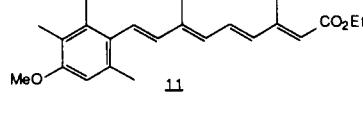
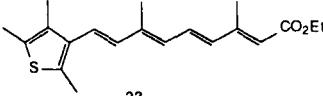
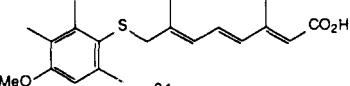
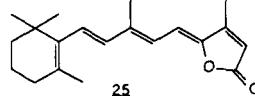
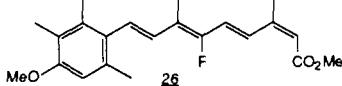
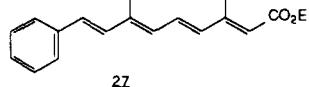
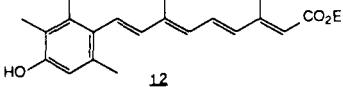
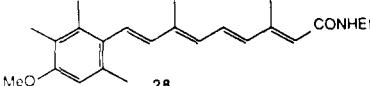
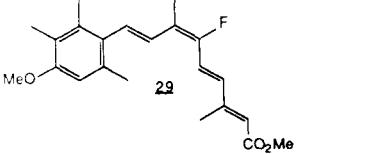
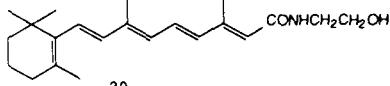
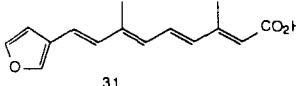
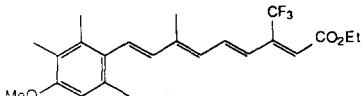
Retinoid	ODC Inhibition ^a ID ₅₀ (nmol)	Papilloma Regression ID ₅₀ (mmol/kg/day)
	0.09	
	0.12	1.33 ^b
	0.14	
	0.20	0.67 ^c
	0.21	0.22 ^d
	0.24	2.67 ^b
	0.43	
	0.54	
	5.00	0.01 ^d
	8.90	0.04 ^d
	12.8	0.04 ^{d,e}
	14.0	0.23 ^{d,e}

TABLE 3 (continued)
ID₅₀ Values for Inhibition of ODC Induction and for Papilloma Regression for a Series of Synthetic Retinoids

Retinoid	ODC Inhibition ^a ID ₅₀ (nmol)	Papilloma Regression ID ₅₀ (mmol/kg/day)
	16.4	0.23 ^b
	32.0	
	60.0	
	139.0	
	192.0	
	400.0	0.05 ^{d,e}
	400.0	
	540.0	>0.22 ^d
	540.0	
	Inactive	
	Inactive	

^a ID₅₀: median inhibitory dose.³ ^b Weekly dose.⁸⁰ ^c Weekly dose.⁸⁷ ^d Ref. 89. ^e ID₅₀ (mmol/kg/week) **11**: 0.07; **22**: 0.16; **28**: 0.13.⁸⁷

(**15**) had activity comparable with that of RA, whereas the 13-*cis* acid (**10**) had one half the activity.

Other modifications of the RA skeleton had more significant effects on activity. Replacement of the trimethylcyclohexenyl ring with a 2,3,6-trimethyl-4-methoxyphenyl ring (TMMP, **22**) increased the ID₅₀ value by several orders of magnitude; conversion of the carboxylic acid group of TMMP to the ethyl ester (**11**) had little effect on activity. However, replacement of the vinylic hydrogen at the 8-position of the nonatetraenoate side chain of the ethyl ester of TMMP by an electron-withdrawing fluorine (**17**) decreased the ID₅₀ value to one approaching that of RA, but similar substitutions at the 4- and 6-positions of the ethyl and methyl nonatetraenoate chain (**20** and **21**) enhanced activity by only 2.8- and 1.6-fold, respectively. In contrast, the 2Z- and 6E-isomers of the methyl ester of the 6-fluoro analog of TMMP (**26** and **29**) had negligible inhibitory activity. Replacement of the 8E-double bond of TMMP with a thiomethylene group (**24**) reduced activity 2.5-fold. Conversion of the ethyl ester of TMMP to the corresponding phenol (**12**) increased the mean inhibitory dose by over 28-fold. Removal of the three methyl groups and the methoxy group from the ethyl ester of TMMP (**27**) reduced activity by over an order of magnitude. Replacement of the trimethylcyclohexenyl ring of RA with a 2,4,5-trimethyl-3-thienyl group (**23**) further decreased activity by over two orders of magnitude, but replacement by a 3-furanyl ring (**31**) abolished activity. Replacement of the 3-methyl group on the side chain of the ethyl ester of TMMP with a trifluoromethyl group (**13**) also abolished activity. Both the *N*-(2-hydroxyethyl) amide of RA (**30**) and the *N*-ethyl amide of TMMP (**28**) had very low inhibitory activity; this lack of activity may be due to the absence of the enzymes in the skin required to convert amides to the corresponding acids. The lactone analog (**25**) of 13-*cis*-RA was less active than 13-*cis*-RA by several orders of magnitude.

We were unable to make any significant statistical correlation between these ODC assay ID₅₀ values (nmol) for **5**, **10**, **11**, **16**, **20** to **23**, and **28** with their ID₅₀ values (nmol of retinoid/kg of mouse body weight/day) calculated to cause the regression of chemically induced mouse epidermal papillomas over a 2-week period.^{81,88} The lack of correlation may be caused by the small sample size (N = 9) or by the pharmacokinetic behavior of the retinoids used. In the ODC assay the retinoids were administered topically, whereas in the papilloma regression assay they were given systemically by i.p. injection. In addition, in the latter assay two different protocols were employed: retinoids **11**, **20** to **22**, and **28** were injected i.p. daily on weekdays over a 2-week period whereas the other retinoids were administered i.p. once weekly. Therefore, for purposes of correlation, the latter ID₅₀ doses were divided by 5. This lack of correlation was further supported by the work of Bollag,⁸⁸ who reported that the *N*-ethyl amide of TMMP (**28**) was more effective at causing papilloma regression when administered i.p. than when given orally, whereas doses of 14 μmol/kg given i.p. five times weekly was more effective (54% decrease) at causing regression than a single fivefold dose given i.p. once weekly (48% decrease). In these experiments he also demonstrated that when administered i.p. once weekly, the amide was eight times more effective than RA in reducing the size of chemically induced papillomas and carcinomas in Swiss albino mouse epidermis. However, the amide did not inhibit the growth of subcutaneously transplanted Ehrlich carcinoma and Crocker sarcoma S tumors or Ehrlich carcinoma and L-1210 leukemia grown as ascites.

The effect of oral administration of retinoids on epidermal ODC activity was also determined by Verma and Boutwell and their collaborators.³ Mice were given 17 μmol of four different retinoids by stomach tube 1 h before topical treatment with 17 nmol of TPA and were sacrificed at 4.5 h after TPA application. RA, retinyl acetate, and the ethyl ester of TMMP completely inhibited TPA-induced ODC activity, whereas the *N*-ethyl amide derivative of TMMP inhibited activity by 73%. The activity of the amide indicates that a

metabolic process occurred after oral administration to activate the compound that did not occur after topical administration to the epidermis. An oral dose-response curve was performed for RA. ODC activity was inhibited completely by 1.7 μmol of RA and 50% by 0.17 μmol of RA.

2. Tumor Regression Effects of Retinoids

Scientists at Hoffmann-La Roche⁸⁸ have assessed the effects that a variety of structurally modified retinoids had on the regression of carcinogen-induced epidermal tumors in mice (Tables 4 to 8). Their goal was to identify a retinoid with the optimal therapeutic index — the ratio of the lowest minimum daily i.p. dose required to elicit a defined degree of the toxic hypervitaminosis A syndrome over a 2-week period to the lowest weekly dose of retinoid required to cause 50% regression of papilloma volume in 2 weeks.⁸⁸ The tumor regression protocol used was initially developed by Bollag and his colleagues and was later modified by the same group.⁸⁷⁻⁹¹ Papillomas were produced in random-bred female Swiss albino mice by topical application of two 150- μg doses of DMBA in 0.2 ml of acetone to the shaved backs (5 cm^2) of the animals on days 1 and 15, followed 3 weeks later by twice-weekly applications of 0.5 mg of croton oil in 0.2 ml of acetone. After 3 to 8 months, the animals developed an average of 4 to 10 papillomas. The administration of retinoids was begun when the average diameter of the papillomas reached 3 mm. In the weekly dosage protocol,⁸⁸ retinoids suspended in arachis oil (0.04 to 0.08 ml/20 g of body weight) were generally administered i.p. on days 1 and 8 to groups of 4 mice, while the control group (20 mice) received vehicle alone. In the daily dosage protocol,⁸⁹ retinoids were suspended in a 0.1% aqueous solution of carboxymethylcellulose and administered i.p. at one fifth the weekly dosage five times per week for 2 weeks. Papilloma diameters were measured on days 0 (before retinoid treatment) and 14. The sum of the diameters of the papillomas was determined for each mouse and the average sum was calculated for each group. The increase or decrease of the average sum of diameters per mouse was expressed as the percent of the initial sum determined on day 0. In general, the average sum of the papilloma diameters in the control group increased 22.7% (from 23.4 to 28.7 mm) over the 2-week period.⁸⁸

The extent of chemically induced tumor regression by many retinoids at various dose levels has been reported along with the dosage required to induce the hypervitaminosis A syndrome in the mouse, i.e., the daily i.p. dose required over a 2-week period to give a total symptom grade of 3 for toxicity, which is manifested as weight loss, desquamation of the skin, hair loss, and bone fractures and graded from 0 (none) to 4 (very marked) for each of the symptoms.⁸⁷ The reported data presented problems in interpretation for structure-activity analysis because of (1) the differences in the tumor regression protocols used, (2) the screening of different derivatives in a series, (3) the lack of complete data, and (4) the lack of information on how reported ID₅₀ values were determined. For example, the ethyl ester of RA (**32**), which was more effective than the free acid at causing tumor regression on weekly administration,⁸⁹ was compared with retinoids administered daily. Both TMMP and its ethyl ester were more active on weekly administration than on daily administration.^{87,89} Nevertheless, correlations of activity with structure can be made.

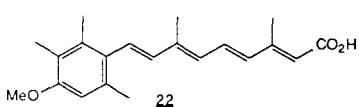
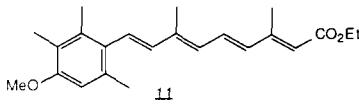
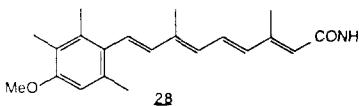
These correlations are especially evident in the extensive work that Pawson and co-workers^{80,89-91} reported on fluorinated retinoic acids and their analogs, presented in Tables 4 and 5. In Table 4 are listed the activities of fluorinated analogs of RA and TMMP and their derivatives in the papilloma regression assay. The order of activity of RA ethyl ester analogs having fluoro group substitutions in the tetraene side chain was: the 10-fluoro analog of the ethyl ester of RA (**33**) < the 12-fluoro retinoid **34** < the 14-fluoro retinoid **35**. Therefore, substitution of fluorine for the vinylic hydrogens on the side chain enhanced antineoplastic activity when the substitution of fluorine was near the polar terminus.

The ethyl ester of TMMP (Tigason®, **11**) is effective in the treatment of psoriasis and

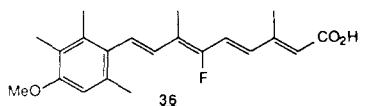
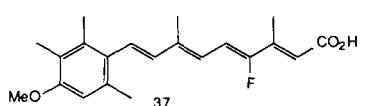
TABLE 4
Effects of Retinoid Fluorine Substitution on Papilloma Regression

Retinoid	Dose (mmol/kg/day)	Papilloma effect % change	Papilloma regression ID ₅₀ (mmol/kg/day)	Ref.
I. Retinoic acid and its ester				
			1.33 ^b	80
	0.12 ^a 0.24 ^a 0.36 ^a 0.61 ^a	-36 -47 -76 -85	1.20 ^c	89
A. Fluorinated analogs of 32				
	0.23	-60	<0.23	89
	0.03 0.06 0.12 0.23	-33 -33 -67 -83	0.07	89
	0.06	-40	>0.06	89

II. Trimethylmethoxyphenyl retinoids

	0.015 0.031 0.062 0.12	-18 -43 -66 -90	0.04	89
	0.028 0.056 0.11 0.23	+9 -29 -33 -47	0.23 ^d	89
	0.014 0.028 0.057 0.11	-11 -23 -54 -74	0.05	89

A. Fluorinated analogs of **22**

	0.007 0.015 0.029 0.058	-28 -30 -54 -87	0.02	89
	0.004 0.007 0.015 0.029	-34 -48 -63 -80	0.008	89

B. Fluorinated analogs of esters of **22**

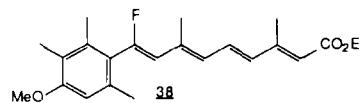
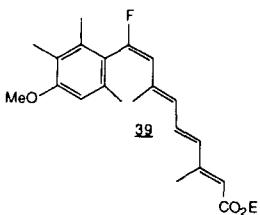
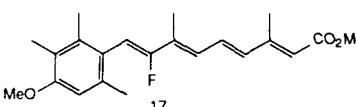
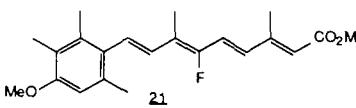
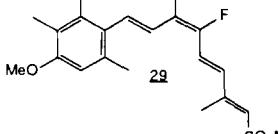
	0.11	-40	>0.11	89
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TABLE 4 (continued)
Effects of Retinoid Fluorine Substitution on Papilloma Regression

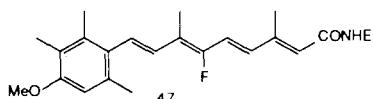
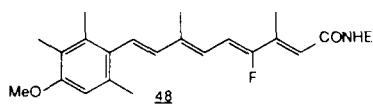
Retinoid	Dose (mmol/kg/day)	Papilloma effect % change	Papilloma regression ID_{50} (mmol/kg/day)	Ref.
	0.11	-26	>0.11	89
	0.22	-3	>0.22	89,91
	0.014 0.028 0.056 0.11	-28 -35 -60 -79	0.04	89
	0.22	-17	>0.22	89

	0.22	-25	>0.22	89
	0.013 0.027 0.054 0.11 0.22	-43 -76 -62 -69 -82	0.01	89
	0.22	-56	~0.22	89
	0.22	-17	>0.22	89
	0.20	-33	>0.20	89
	0.20	-35	>0.20	89
	0.21	-25	>0.21	89
	0.21	-8, +2	>0.21	89

TABLE 4 (continued)
Effects of Retinoid Fluorine Substitution on Papilloma Regression

Retinoid	Dose (mmol/kg/day)	Papilloma effect % change	Papilloma regression ID_{50} (mmol/kg/day)	Ref.
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C. Fluorinated analogs of *N*-ethyl amide of **22**

	0.22	-53	~0.22	89
	0.27 0.54	-73 -67	<0.03	89

^a Five-times indicated daily dose given weekly for 2 weeks.

^b (mmol/kg/week).

^c ID_{50} (mmol/kg/week) calculated from semilog plot using three data points closest to ID_{50} ; however, ID_{50} : 0.15 mmol/kg/day.⁸⁹

^d ID_{50} : 0.65 mmol/kg/week.⁸⁰

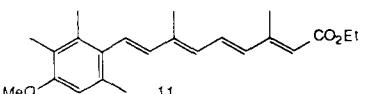
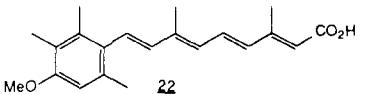
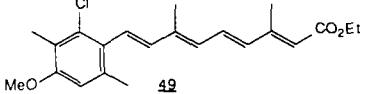
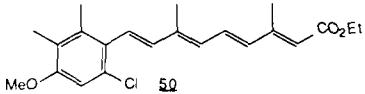
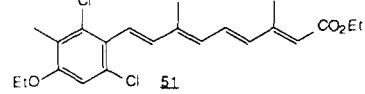
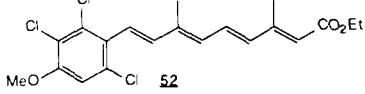
other diseases of keratinization⁹² and is marketed commercially for this purpose. Extensive studies on derivatives and analogs of this compound have been reported. The ester was less active than the acid (**22**) and *N*-ethyl amide (**28**) in causing papilloma regression; its toxicity was comparable with that of the acid in the mouse hypervitaminosis A assay and was one half that of the amide. Therefore, on the basis of therapeutic index calculations — 0.80 (**11**), 0.13 (**22**), and 0.39 (**28**) — the optimal compound would be the acid, indicating that other factors play a decisive role in the selection of a therapeutic drug. Nevertheless, tumor regression studies and toxicity assays in rodents are excellent preliminary tests to screen retinoids.

Fluorinated side-chain analogs of TMMP, its ethyl and methyl esters, and its *N*-ethyl amide have been screened in the papilloma regression assay.^{89,91} In the TMMP series, the 4-fluoro analog of TMMP (**37**) was more active than the 6-fluoro analog (**36**), which was more active than TMMP. If the activity of the ethyl and methyl esters are regarded to be the same for the fluorinated analogs, the activity order was: 4-fluoro ethyl ester (**20**) > 6-fluoro methyl ester (**21**) > the ethyl ester of TMMP (**11**) > 9-fluoro ethyl ester (**38**) > 5-fluoro methyl ester (**40**) > 2-fluoro ethyl ester (**42**) > 8-fluoro methyl ester (**17**). Only substitution at the 4- and 6-positions significantly enhanced activity. As expected and as has been reported for other retinoid double-bond isomers,⁹³⁻⁹⁵ the 4*E*-4-fluoro isomer (**41**) and the 6*E*-6-fluoro isomer (**29**) were less active than the corresponding 4*Z* and 6*Z* isomers **20** and **21**. Although both isomers displayed low activity, the 8*Z*-9-fluoro isomer (**38**) was more active than its 8*E* isomer (**39**). The 2,4-difluoro and 4,6-difluoro isomers (**44** and **43**) had comparable low activity and were less active than the 4- or 6-fluoro analogs. The 2*Z* isomer of **11** having a trifluoromethyl group at the 3-position of the side chain (**46**) was inactive; the 6*E*-7-trifluoromethyl analog (**45**) displayed very low activity. In the *N*-ethyl amide series, the 4-fluoro analog (**48**) was more active than the parent amide (**28**), but the 6-fluoro analog (**47**) was less active. Therefore, substitution of a fluoro group at the 4-position enhanced activity in both TMMP and its ester and amide analogs, but substitution at the 6-position gave variable results depending on the derivative of TMMP assayed.

Ring analogs of the ester of TMMP (**22**) in which the methyl and methoxy groups of the trimethylmethoxyphenyl ring were replaced by chlorine were also screened (Table 5). Mayer and co-workers⁸⁷ reported a weekly-dose tumor regression study on chlorinated analogs of the ethyl ester of TMMP. The 2-chloro, 6-chloro, and 2,6-dichloro aryl ring analogs (**49** to **51**) were more active than **11** but also more toxic. The only compound in the series reported to have low toxicity was the 3-chloro-2,4,6-trimethylphenyl analog (**53**), which also had very low papilloma-regression activity. Limited studies on chlorinated aryl ring analogs of the 4- and 6-fluorinated side-chain analogs of TMMP esters using a daily retinoid-dosage protocol were reported by Pawson et al.,⁸⁹ Chan et al.,⁹⁰ and Lovey and Pawson.⁹¹ In the 4-fluoro series of ethyl esters, the 2-chlorophenyl ring analog (**54**) at a dose of 51 µmol/kg/d was more active than the 6-chlorophenyl ring analog (**55**). Both were more active than **11**. Substitution of two chloro groups at the 2- and 6-positions on the phenyl ring (**56**) appeared to enhance activity. Again, the 3-chloro-2,4,6-trimethylphenyl analog (**57**) was the least active compound in the series. In the 6-fluoro series of methyl esters of TMMP, the 2-chlorophenyl analog (**58**) had activity comparable with that of 6-fluoro TMMP methyl ester **21**, but was more toxic. The 6-chlorophenyl analog (**59**) was less active. It and the other compounds in this series were reported to have therapeutic indices comparable with that of **21**.

Other aromatic retinoic acid analogs were also synthesized. In these cases, instead of replacing the cyclohexenyl ring with a phenyl ring, the double bonds were replaced with aromatic rings to give the conformationally restricted aromatic retinoids.⁹³⁻¹⁰⁰ The first compound of this type reported was 3-{4-[*E*-2-(2,6,6-trimethyl-1-cyclohexenyl)vinyl]phenyl}-2*Z*-butenoic acid (**63**, Figure 4), which was prepared at BASF.^{94,95} The bonds of **63** cor-

TABLE 5
Effects of Chlorinated Trimethylmethoxyphenyl (TMMP) Analogs on Papilloma Regression

Retinoid	Dose (mmol/kg/day)	Papilloma effect % change	Papilloma regression ID_{50} (mmol/kg/day)	Ref.
I. TMMP retinoids				
	0.028 0.057 0.11 0.23	+9 -29 -33 -47	0.23 (0.14) ^a	89
	0.015 0.031 0.062 0.12	-18 -43 -66 -90	0.04 (0.08) ^a	89
II. Chloro TMMP retinoids				
	0.016 ^a			87
	0.034 ^a			87
	0.015 ^a			80
	0.061 ^a			87



A. 4-Fluoro side chain analogs

 54	0.051	-73	<0.05	90
 55	0.013 0.026 0.051	-41 -60 -59	0.03	90
 56	0.012	-58	<0.01	90
 57	0.05 0.11 0.21	-29 -34 -64	0.16	90

B. 6-Fluoro side chain analogs

 58	0.053	-56	~0.05	91
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TABLE 5 (continued)
Effects of Chlorinated Trimethylmethoxyphenyl (TMMP) Analogs on Papilloma Regression

Retinoid	Dose (mmol/kg/day)	Papilloma effect % change	Papilloma regression ID_{50} (mmol/kg/day)	Ref.
	0.11	-41	>0.11	91
	0.20	-64	<0.20	91
	0.19	-58	<0.19	91
	0.22	-48	~0.22	91

^a Weekly dose.⁸⁷

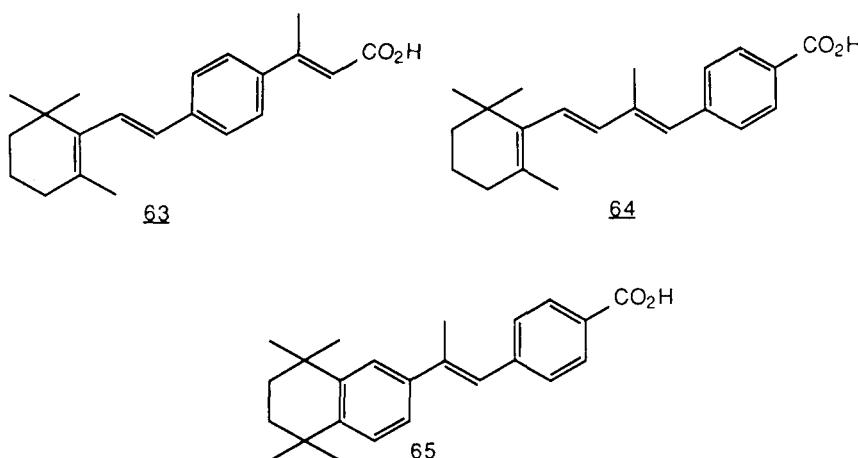


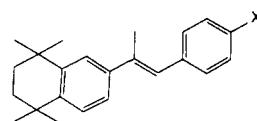
FIGURE 4. Conformationally restricted retinoids **63** to **65**.

responding to the *9E,11E*-double-bond system of the retinoic acid skeleton are restricted to a 10-*s-cis* conformation by inclusion in the phenyl ring. Unfortunately, **63** did not have any significant activity in reversing keratinization in vitamin A-deficient hamster tracheal epithelial cells in organ culture. Therefore, no additional reports on the biological activity of compounds of this type appeared from the retinoid research group at BASF. In 1979, the first conformationally restricted retinoid — 4-[2-methyl-4-(2,6,6-trimethyl-1-cyclohexenyl)-1*E*,3*E*-butadienyl]benzoic acid (**64**) — having significant biological activity in the tracheal organ culture assay (ED_{50} , $2 \times 10^{-10} M$)⁹⁶ was reported by Sporn and co-workers.^{97,98} In benzoic acid **64**, the *11E,13E*-double-bond system of RA has been replaced by a phenyl ring affording a 12-*s-cis*-locked retinoid.

The retinoid research group of Hoffmann-La Roche at Basel investigated conformational restrictions of the retinoid skeleton, which led in 1980 to the report by Loeliger et al.⁹³ of 4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1*E*-propenyl]benzoic acid (**65**) and its analogs. In this 4-substituted benzoic acid, the bonds corresponding to the *5,7E*- and *11E,13E*-double-bond systems of RA are restricted to *s-cis* conformations. In addition, oxidative metabolism leading to compounds of reduced biological activity¹⁰¹ has been blocked at the position corresponding to the 4-position of RA by the geminal dimethyl group at the 5-position of the tetrahydronaphthalene ring. The ethyl ester of **65** (**66**) proved to have the highest antitumorigenic activity of any retinoid reported thus far but, unfortunately, was also correspondingly toxic. This discovery led to further studies on analogs of this new generation of retinoids.

In Table 6 are listed the effects that modifications of the polar terminus (X) of **65** had on tumor regression activity. In these studies reported by Loeliger et al.⁹³ and Klaus et al.,⁹⁹ retinoids were administered by weekly i.p. injection. Therefore, biodistribution and metabolism may have influenced the amount of active retinoid reaching the epidermis. In the benzoic acid series of derivatives, the ethyl ester **66** was the most active at causing reduction in papilloma diameter, followed in order of decreasing activity by the isopropyl ester **67**, the *N*-ethyl amide **69**, the parent carboxylic acid **65**, and the *t*-butyl ester **68**. Retinoids having a carboxylic acid polar terminus are generally reported to have the highest *in vitro* activity at inducing cell differentiation in a variety of cell lines.⁷⁹ Therefore, it has been suggested that retinoids having a polar terminus of a lower oxidation state must first be oxidized to the carboxylic acid form to have activity in controlling cell differentiation. However, 4-substituted benzaldehyde derivative **70** proved to be the most active oxidation state in this series, followed in order of decreasing activity by the related benzyl alcohol

TABLE 6
Effects of 4-Substituted Benzoic Acid 65 and Polar Terminus Analogs on Papilloma Regression



Retinoid	X	Dose ($\mu\text{mol/kg/week}$)	Papilloma effect % change	Papilloma regression ID_{50} ($\mu\text{mol/kg/week}$)	Ref.
65	CO ₂ H	2.3	-38	>2.3	93
66	CO ₂ Et	0.05	-33	0.13	93
		0.13	-48		
		0.27	-56		
		0.53	-75		
67	CO ₂ i-Pr	0.26	-54	~0.26	93
68	CO ₂ t-Bu	3.7	-46	~7.4	93
		7.4	-48		
69	CONHEt	0.26	-7	0.5	93
		0.53	-58		
70	CHO	0.15	-24	0.6	93
		0.30	-37		
71	CH ₂ OH	0.30	-26	>0.3	93
72	CH ₂ OMe	0.14	-40	0.2	93
		0.28	-57		
73	CH ₂ OAc	0.13	-22	0.5	93
		0.26	-25		
		0.53	-61		
74	Me	1.2	-39	>1.2	93
75	SO ₃ Na	0.49	-46	~0.5	99
76	SO ₂ OEt	0.96	-41	>0.96	99
77	SO ₂ NHET	0.95	-23		99
78	SO ₂ Na	0.13	-15		99
79	S(O)OEt	1.00	-51	1.0	99
80	SEt	1.09	-52	1.1	99
81	S(O)Et	0.26	-50	0.26	99
82	S(O) ₂ Et-E	0.03	-50	0.03	99
83	S(O) ₂ Et-Z	1.00	-5		99
84	H	1.30	+1		93

71, the 4-substituted toluene 74, and then the parent benzoic acid 65. The methyl ether of benzyl alcohol 71 (72) was more active than 71, but the benzyl acetate 73 had activity comparable with that of 71. Ethyl ester 66 was reported to have the best therapeutic index, followed by the *N*-ethyl amide 69 and the methyl ether 72.

The sulfur analogs of 65 were generally less active than compounds in the benzoic acid series by at least three orders of magnitude, except for the ethyl sulfone analog 82, which was less active by about two orders of magnitude.⁹⁹ In the case of these compounds, however, activity did correlate with oxidation state or derivatization state, with the more highly oxidized compounds in a series usually having higher biological activity and with the derivatives (ester and amide) having lower activity. Therefore, the sodium sulfonate 75 had higher biological activity than the ethyl sulfonate 76, which was more active than the *N*-ethyl sulfonamide 77. The sodium sulfinate 78 displayed biological activity, but the dose and the percent change in papilloma diameter that were reported made any correlation difficult. The related ethyl sulfinate 79 had activity comparable with that of the ethyl sulfonate 76. The

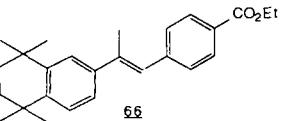
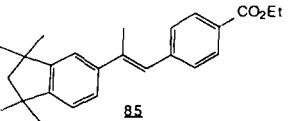
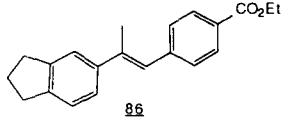
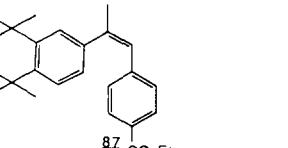
ethyl sulfone **82** was more active than the ethyl sulfoxide **81**, which was more active than the ethyl thioether **80**. As expected for retinoids having a Z-double bond at the position corresponding to the 9-double bond of RA, the Z isomer **83** of **82** had negligible activity. On the basis of ID₅₀ doses reported for this series of analogs, the ethyl sulfinate **79** and the ethyl thioether **80** appeared to have the best therapeutic indices. Removal of the polar substituent (X = H) (**84**) abolished activity at the highest dose tested (1.3 mmol/kg/week).⁹³

Loeliger et al.⁹³ and Klaus¹⁰⁰ also reported the biological activity of a series of analogs of the ethyl ester **65** (**66**) in which other groups replaced either the tetrahydrotetramethyl-naphthalene ring system or 4-(E-propenyl)benzoate moiety (Table 7). The related tetramethylindane (**85**) had less than 30% of the activity of **66**. The indane **86** lacking the four geminal methyl groups displayed negligible activity. In contrast to the 9Z isomers of less active retinoids, the 4-(Z-2-arylpropenyl)benzoate **87** was able to cause the regression of papillomas but was less active than **66** by over two orders of magnitude; interestingly, it was more active than RA. Incorporation of a methyl group *ortho* to the carboxylate group on the phenyl ring (**89**), which would correspond to the 20-methyl group of RA, reduced activity. Replacement of the 4-(E-propenyl)benzoate group with a 3,7-dimethyl-2E,4E,6E-heptatrienoate group (**88**) reduced activity over 30-fold; replacement by a 3-phenyl-2Z-butenoate group (**90**) lowered activity even more. Replacement with a 2-naphthalenecarboxylate group (**91**) was reported to decrease activity. From the data presented, it appears that potency was reduced by 99%. In this series of compounds, **66** had the most favorable therapeutic index (0.04), followed by **85** (1.3).

In Table 8 are listed the tumor regression activities of additional retinoids having modifications in the cyclohexenyl ring and side-chain regions of the retinoid skeleton. Shifting the cyclohexenyl ring double bond from the 1- to the 2-position (**16**) enhanced activity twofold.⁸⁷ The 4,4-dimethyl analog of RA (**92**) displayed biological activity, but activity at only one low concentration was reported.¹⁰⁰ This compound, the oxidative metabolic deactivation at the 4-position of which is blocked, was reported to be six times more toxic than RA.¹⁰⁰ The acyclic analog **93** of RA possessed activity comparable with that of RA. Compared with ethyl retinoate (**32**), the 2-acetyl-5,5-dimethyl-1-cyclopentenyl analog **94** was approximately twice as active, the 3,4-dioxo-2,5,5-trimethyl-1-cyclopentenyl analog **95** was over twice as active, and the ferrocenyl analog **96** and the 2,4,5-trimethylbenzoyl analog **97** were four times more active. Omitting **92**, for which insufficient data was reported, it appears that in this carboxylic acid series of analogs, **16** had a less favorable therapeutic index and the other compounds a more favorable one than that reported for RA. In the case of the ethyl esters, all the compounds except **94** had a higher — and therefore more favorable — therapeutic index (>0.13) than that of ethyl retinoate. The RA analog lacking the 20-methyl group (**98**) had an ID₅₀ value one-half of that of RA and an improved therapeutic index.

Klaus¹⁰⁰ reported limited data on a series of s-cis-conformationally restricted retinoids (**63** and **99** to **101**) that did not permit a meaningful comparison of tumor regression activities and therapeutic indices. Similar data were reported for a series of acetylenic analogs (**102** to **106**) of RA. 7-Substituted 3,7-dimethyl-2E,4E,6E-heptatrienoate analogs (**107** to **110**) of ethyl 4-[2-(5,5,7,7-tetramethyl-2-indanyl)-1E-propenyl]benzoate (**85**) were also examined.⁹³ These compounds proved to be less active. For example, **108** had about 6% of the activity of **85**. In addition, it had 53% of the activity of **88**. From these data, it can be concluded that activity decreased on replacement of the tetrahydronaphthalene ring system with the indane ring system and that replacement of the bond system corresponding to the 11E,13E-double-bond system of ethyl retinoate with a phenyl ring enhanced activity. The parent carboxylic acid (**107**) was less active than the ester **108** by at least an order of magnitude. Removal of the 5,5-dimethyl group (**109**) decreased activity fivefold below that of **108**. Removal of both *gem*-dimethyl groups (**110**) decreased activity by over two orders of magnitude. Indane **108** appeared to have a more favorable therapeutic index⁹³ (1.0) than that (0.5) of tetrahydronaphthalene **88**.

TABLE 7
Effects of 4-Substituted Benzoate 66 and Its Analogs on Papilloma Regression

Retinoid	Dose ($\mu\text{mol/kg/week}$)	Papilloma effect % change	Papilloma regression ID_{50} ($\mu\text{mol/kg/week}$)	Ref.
I. Benzoate (66)				
				
	0.05	-33	0.13	93
	0.13	-48		
	0.27	-56		
	0.53	-75		
II. Tetrahydronaphthalene ring analogs				
				
	0.28	-29	0.44	93
	0.55	-61		
				
	650.0	-34	>650.0	93
III. 4-(E-Propenyl)benzoate analogs				
				
	34.0	-68	<34.0	93

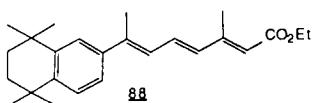
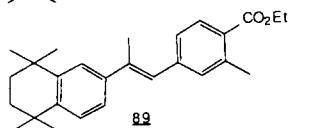
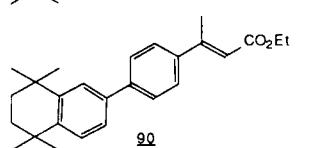
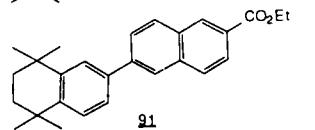
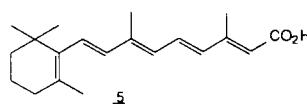
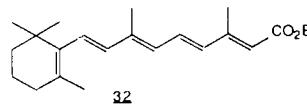
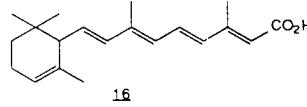
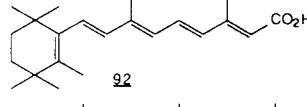
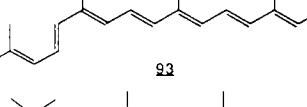
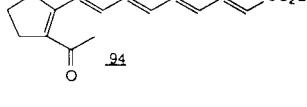
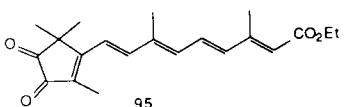
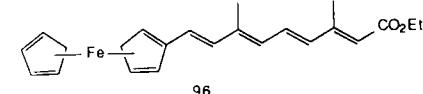
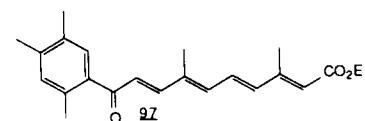
	2.0 4.1 8.2	-44 -47 -59	~4.1	93
	1.0 1.9	-40 -38	>1.9	93
	130.0	-10		100
	32.0	-23		100

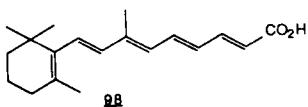
TABLE 8
Effects of Ring and Side Chain Analogs of Retinoic Acid and Its Esters on Papilloma Regression

Retinoid	Dose (mmol/kg/week)	Papilloma effect % change	Papilloma regression ID_{50} (mmol/kg/week)	Ref.
I. Retinoic acid (5) and its ethyl ester (32)				
			1.33	80
			1.20	89
II. Ring analogs				
			0.67	87
	0.08	-7		95
			1.34	87
			0.58	87

	0.44	87
	0.26	87
	0.28	87

III. Chain analogs

A. Chain substituent

	0.70	87
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B. *s-cis* restricted

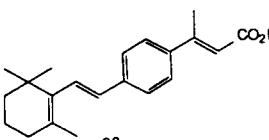
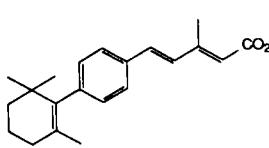
	0.32	-10	100
	0.65	-29	100

TABLE 8 (continued)
Effects of Ring and Side Chain Analogs of Retinoic Acid and Its Esters on Papilloma Regression

Retinoid	Dose (mmol/kg/week)	Papilloma effect % change	Papilloma regression ID_{50} (mmol/kg/week)	Ref.
	0.31	-57	~0.31	100
	0.078	-13		100
	0.34	-24		100
	1.34	-10		100

	0.17	+ 11	100
	0.34	+ 5	100
	1.35	+ 9	100

IV. Chain and ring analogs

	0.077	- 14	93
	0.0021 0.0043 0.0085	- 1 - 15 - 52	0.008
	0.039	- 48	0.039
	0.67	- 20	93

B. SYNTHETIC METHODOLOGY FOR RETINOID STRUCTURAL MODIFICATION

Before describing our studies of the activity of retinoids in the ODC and antipapilloma assays, we digress to present an overview of the methods used to prepare both the retinoids described in previous sections and those retinoids screened in our own program. The polyolefinic retinoids are generally prepared by convergent syntheses using a series of bond-forming reactions, generally to give the 7-, 9-, and 11-double bonds. Isler and Schudel,¹⁰² in their review on classical retinoid methodology, termed these reactions $C_{10} + C_{10}$, $C_{13} + C_7$, and $C_{15} + C_5$, respectively, on the basis of the number of carbons in each of the double-bond precursors. Retinoids containing aromatic-ring systems can be made by these methods if double bonds are present; otherwise, other bond-forming reactions, which are also described below, are used.

Synthetic analogs of RA have been prepared to develop new inducers of cell differentiation that are more effective and less toxic than retinoic acid itself, for potential use in the chemoprevention of cancer and the treatment of proliferative skin diseases.¹ Many different series of closely related analogs have been synthesized to provide compounds for systematic structure-activity studies. These studies and the pharmacologically useful retinoids necessitate rapid, reliable, and adaptable methods of synthesis. Several methods useful for the synthesis of polyenoic acid analogs of RA have been adapted from methods originally developed for the manufacture of RA and retinyl esters.⁹⁴ Other methods have followed from the development of synthetic methodology for the stereoselective synthesis of olefins. The present synthetic emphasis on conformationally restricted and often metabolically stable retinoids has concentrated on compounds in which aromatic groups replace part or all of the polyene structure of RA and yet which display potent cell-differentiating activity.⁹³ Methodology for the synthesis of aromatic retinoids may employ quite different reactions from those used in the synthesis of polyenes, although reactions that were developed for polyene retinoid synthesis are frequently used to introduce olefinic functions.

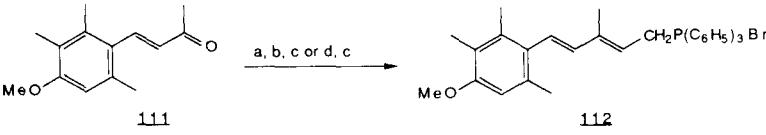
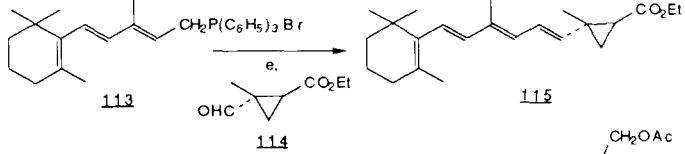
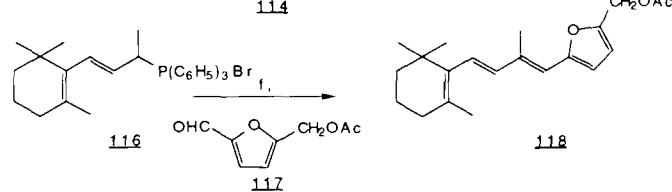
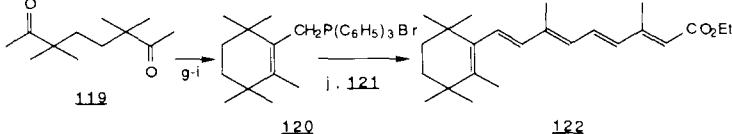
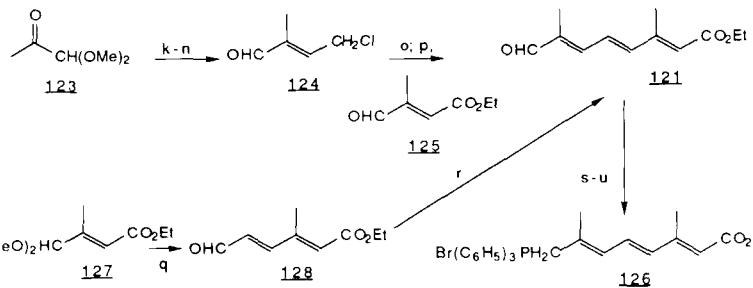
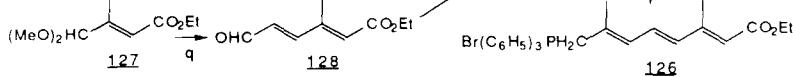
The main objective of this section is to emphasize the newer synthetic approaches as well as the best available methods for all types of carboxylic acid terminated retinoids. Retinoic acid analogs have been conveniently classified⁹⁴ as ring (hydrophobic terminus), side chain (polyene), and polar terminus-modified compounds (Figure 3). With the introduction of aromatic retinoids,^{93,103} compound classification based on conformational restriction is relevant to both structure-activity studies and to the range of useful synthetic options. In this section, polyenoic acid analogs of RA are considered as a class, followed by the aromatic analogs. The synthetic methodology for the preparation of polar terminus-modified retinoids will not be discussed because the synthetic methods employed are often very specific for the polar group introduced and therefore may not have general applicability.

1. Polyenoic Acid Analogs of Retinoic Acid: Principal Synthetic Routes

Synthetic routes to the geometric isomers of RA have been thoroughly reviewed.¹⁰⁴ Only the *E* and 13*Z* isomers of RA are of interest as inducers of cell differentiation.¹⁰⁰ An investigation of 13 of the possible 16 stereoisomers of the aryl tetraenoic acid **22** indicated that only the *E* isomer was active in the papilloma regression assay.¹⁰⁰ Many very similarly related analogs of (*E*)-RA have been prepared. Most of these analogs were designed with steric or electronic modifications of the basic (*E*)-RA skeleton. Several have been designed to block^{100,105} metabolic deactivation by oxidation.¹⁰⁶⁻¹⁰⁸ The tremendous advances made in recent years in the stereoselective synthesis of olefins using organometallic reagents¹⁰⁹ have not had a great impact on retinoid synthetic methodology because of the success of the Wittig and Horner-Wadsworth-Emmons olefination methods, the most widely applied variations of which are summarized in Tables 9 and 10.

The ($C_{15} + C_5$) Wittig synthesis (Example 2, Table 9) frequently uses a phosphonium

TABLE 9
Principal Reactions for the Synthesis of Retinoidal Polyenes: Wittig Reactions

Example	Key Reaction	Ref.
1		111
2		121
3		122
4		118
5		120
6		100

Note: (a) $\text{HC}\equiv\text{CMgBr}$, THF; aq. HCl ; (b) H_2 , Pd/C , quinoline; (c) $(\text{C}_6\text{H}_5)_3\text{P-HBr}$, C_6H_6 ; (d) $\text{H}_2\text{C}=\text{CHMgCl}$, THF; (e) $t\text{-BuOK}$, C_6H_6 ; **114**, C_6H_6 ; (f) NaOEt , EtOH ; **117**, EtOH , CHCl_3 ; (g) $\text{Zn}(\text{Cu})$, TiCl_3 , DME; (h) NBS , DME; (i) $(\text{C}_6\text{H}_5)_3\text{P}$, CH_2Cl_2 (70% from **119**); (j) $n\text{-BuLi}$, THF; **121** (67%); (k) $\text{HC}\equiv\text{CH}$, K , $\text{Fe}(\text{NO}_3)_3$, NH_3 , Et_2O ; NH_4Cl ; (l) H_2 , Pd/C , quinoline, pet. ether; (m) COCl_2 , $\text{C}_5\text{H}_5\text{N}$, CCl_4 ; (n) aq. H_2SO_4 ; (o) $(\text{C}_6\text{H}_5)_3\text{P}$, C_6H_6 ; (p) Et_3N , **125**, $n\text{-BuOH}$; I_2 , hexane; (q) $\text{H}_2\text{C}=\text{CHOEt}$, ZnCl_2 ; (r) $\text{CH}_3\text{CH}=\text{CHOEt}$, ZnCl_2 ; (s) NaBH_4 , EtOH ; aq. HCl ; (t) PBr_3 , hexane, Et_2O ; (u) $(\text{C}_6\text{H}_5)_3\text{P}$, C_6H_6 .

salt that is prepared by rearrangement of a vinyl carbinol^{110,111} (Example 1). This method stereospecifically introduces the 9*E*-double bond, in contrast to the ($\text{C}_{13} + \text{C}_7$) Wittig reaction (Example 3), which affords a mixture of 9-double bond isomers. Palladium(II)-catalyzed equilibration of the mixture gives the 9*E* isomer predominantly,¹¹² thereby improving the latter approach. *n*-Butyllithium is commonly used to generate the ylid from the C_{15} -phosphonium salt, which is then allowed to react with the aldehyde at low temperature to maximize the amount of *E* isomer in the product mixture. A two-phase Wittig reaction can also be employed using aqueous inorganic bases such as NaOH or K_2CO_3 at ambient temperature.¹¹³ 1-Butene epoxide may be used as the acid acceptor in place of the base.^{114,115} Because the

two-phase and acid-acceptor reaction conditions favor the formation of the inactive *Z* isomer, equilibration is necessary to enhance the amount of active *E* isomer. Photochemical¹¹⁶ or palladium(II) salt-catalyzed¹¹² equilibration then gives mainly the all-*E* and 13*Z* isomers.

The (C_{10} + C_{10}) Wittig approach¹¹⁷ (Example 4) has been applied¹¹⁸ when the hydrophobic terminus is modified, and a derivative (**120**) of β -cyclogeranial (**191**), a C_{10} intermediate, rather than 2,2,6-trimethylcyclohexanone (**144**), a C_9 unit, is used to introduce the carbocyclic function. Phosphonium salt **120** was prepared from a hydrocarbon intermediate and not from the more common conversion of a β -cyclogeraniol analog. β -Cyclogeraniol is most easily available by ozonolysis of β -ionone (**149**),¹¹⁹ followed by reduction of aldehyde **191**.

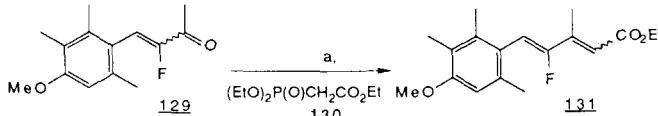
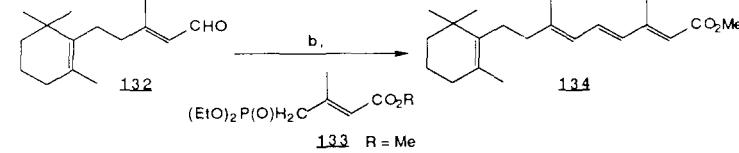
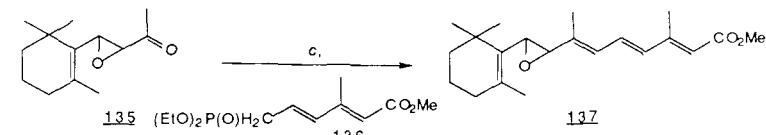
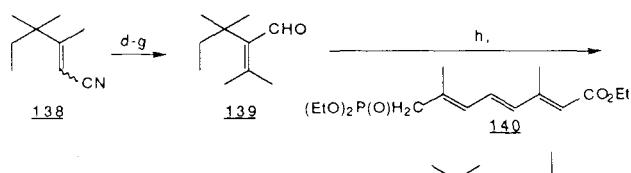
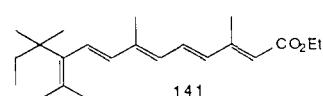
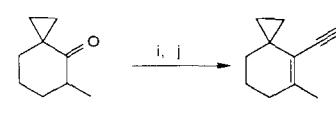
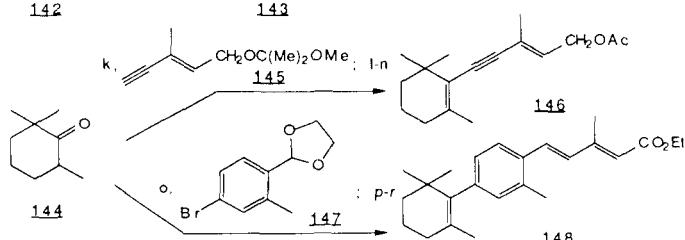
Several efficient routes^{100,120} to trienal **121** in Examples 5 and 6 are available. This aldehyde is a precursor for phosphonium salt **126** that is used in the inverse (C_{10} + C_{10}) Wittig reaction, which has been applied to the synthesis of aryl tetraenoic acid retinoids from aromatic aldehydes.¹¹¹

The Horner-Wadsworth-Emmons reagents in common use (Examples 7 to 10, Table 10) are those in which the derived phosphonate anion is stabilized by conjugation to an electron-withdrawing group. A C_2 -phosphonate unit (Example 7) is used to chain-extend an analog of β -ionone (**129**), giving an isomeric mixture of esters (**131**),⁸⁹ which may be converted to the corresponding aldehydes, which are then equilibrated with a base, especially triethylamine, to optimize the *E*-isomer yield.¹¹⁸ A C_5 -phosphonate reagent (**133**, Example 8) is frequently used to complete the synthesis of a retinoid from this type of precursor.¹²³ This route is highly adaptable because the phosphonates are readily prepared by *N*-bromosuccinimide (NBS) functionalization of the γ -alkyl group of alkoate esters (Example 15, Table 11).¹²⁴ Analogous C_7 -dienoate¹²⁵ and C_{10} -trienoate¹¹¹ dialkyl phosphonate reagents such as **136** (Example 9) and **140** (Example 10), respectively, are occasionally used.

Sterically hindered cycloalkanones have often been used as intermediates in the synthesis of ring-modified retinoids and 7,8-dehydroretinoids.¹⁰⁰ These cyclohexanones are readily functionalized using lithium acetylidyde (Example 11)¹²⁶ or a lithio anion of a terminal alkyne (Example 12).¹⁰⁰ The resultant alkynols are dehydrated under mild conditions ($CuSO_4$,¹¹⁸ $TsNCO$,¹²⁶ or $Et_3NSO_2NCO_2Et$ ¹⁰⁰). The lithio anions of the enyne products are then further functionalized. An example of this type of transformation is shown in Example 28.¹²⁶ The reaction of an aryllithium with a cyclohexanone (Example 13)¹²⁷ has been used in the synthesis of several conformationally restricted retinoids.

One newer approach to retinoid synthesis, introduced by Julia,¹²⁸ has been widely used (Table 11). In this synthon, aryl allyl sulfone anions are alkylated with allylic halides. The polyene products are obtained by treatment of the alkylated sulfones with bases such as $KOt-Bu$,¹²⁸ 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU),¹²⁴ and $NaOH$ in dimethylacetamide.¹²⁹ Because both the allylic halide and sulfone reagents are unstable to base, the alkylation¹²⁹ step must be performed at low temperature ($-65^\circ C$).¹²⁸ The (C_{15} + C_5) route (Examples 14 and 15) has proved to be the most satisfactory for this chain elongation method. In Example 14, the sulfone **151** is prepared from vinyl- β -ionol (**150**)^{130,131} as readily as its corresponding phosphonium salt (**113**) is prepared for use in the (C_{15} + C_5) Wittig reaction. An analogous (C_{10} + C_{10}) route (Example 16) has been reported.¹¹¹ (C_{13} -sulfone + C_7 -halide)¹²⁹ and (C_{15} -halide + C_5 -sulfone)¹³² approaches have been used successfully only to prepare retinol and not RA. Pd(II)-catalyzed isomerization of the reaction mixture from the (C_{13} + C_7) route in which the 9*Z* isomer predominates is necessary. Sulfone **158** (Example 17), which was developed for a general synthesis of carotenoids, has been applied to a lengthy synthesis of RA, employing the alkylation of the C_{10} -sulfone **156** ($R = H$).¹³³ A useful variation of the Julia methodology employs acetal **161** (Example 18)¹³⁴ as the alkylation agent to introduce a protected aldehyde function. A π -allyl-Pd(II) complex (**164**), prepared by oxidation of γ,γ -dimethylallyl acetate with $CuCl_2$ in the presence of $PdCl_2$, is used to

TABLE 10
Principal Reactions for the Synthesis of Retinoidal Polyenes: Horner-Wadsworth-Emmons and Other Olefinations

Example	Key Reaction	Ref.
7		89
8		123
9		125
10		111,118
11		126
12		
13		100 127

Note: (a) 130, NaH, DME; (b) 133, NaH, C₆H₆; (c) 136, NaH, THF; (d) (*i*-Pr)₂NLi, MeI, THF, HMPA; aq. NH₄Cl; (e) MCPBA, Na₂HPO₄, CH₂Cl₂; (f) Na, NH₃, Et₂O; aq. NH₄Cl; (g) (COCl)₂, Me₂SO, Et₃N, CH₂Cl₂ (64% from 138); (h) 140, *n*-BuLi, THF (43%); (i) HCl-Et₃N; (j) 4-CH₃C₆H₄SO₂NCO, MeO(CH₂CH₂O)₂Me; (k) 145, *n*-BuLi; (l) H₃O⁺; (m) Ac₂O, C₅H₅N; (n) Et₃NSO₂NCO₂Et; (o) 147, *n*-BuLi, THF (64%); (p) POCl₃, C₅H₅N; (q) aq. HCl; (r) 133 (R = Et), *n*-BuLi, THF (47%).

alkylate C₁₀-sulfone 165 directly (Example 19).¹³⁵ However, because this reaction is stoichiometric in Pd(II), it is of limited practical application.

2. Less Common Approaches to the Synthesis of Polyenoic Acid Analogs of Retinoic Acid

Diverse C–C bond-forming reactions have been used to prepare close analogs of RA. Some that have general utility are outlined in Table 12. For example, the imine-^{136,137} and

TABLE 11
Principal Reactions for the Synthesis of Retinoidal Polyenes: Sulfone Alkylation
Reactions

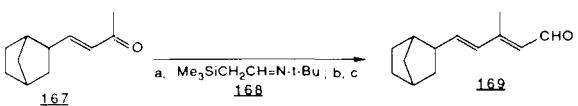
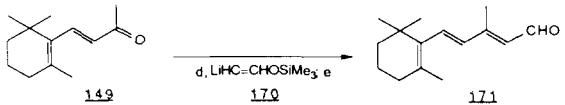
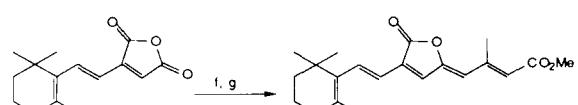
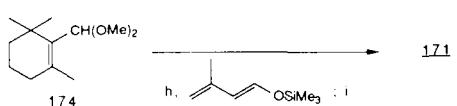
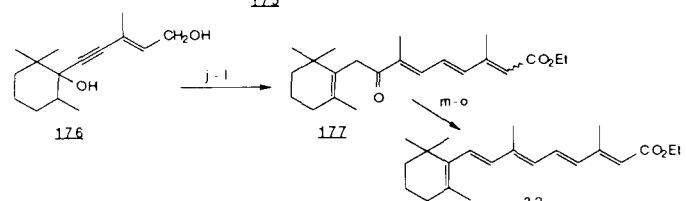
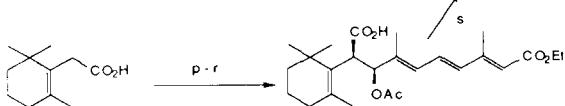
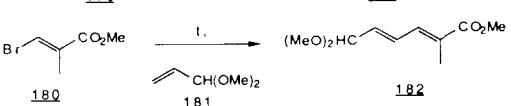
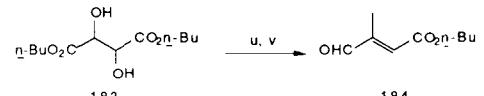
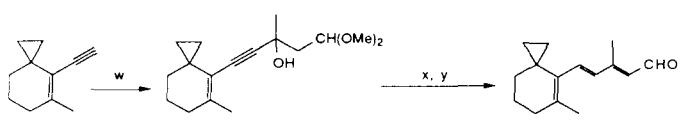
Example	Key Reaction	Ref.
14	 149 → 150 → 151	130,131
15	 151 → 154	124
16	 152 → 153 → 151 → 154	100,111
17	 155 → 156 → 157 → 122 or 122	133
18	 160 → 161 → 162	134
19	 163 → 164 → 165 → 166	135

Note: (a) $\text{H}_2\text{C}=\text{CHMgCl}$, THF; aq. NH_4Cl ; (b) $\text{C}_6\text{H}_5\text{SO}_2\text{Na}$, aq. HOAc ; (c) NBS, AIBN, CCl_4 , hv ; (d) $n\text{-BuLi}$, THF; 153, THF; (e) DBU, Et_2O ; (f) ($\text{R} = \text{Me}$) NBS; (g) $\text{C}_6\text{H}_5\text{SO}_2\text{Na}$; (h) $n\text{-BuLi}$, 157; (i) NaOEt ; (j) 156 ($\text{R} = \text{H}$), $n\text{-BuLi}$, $\text{MeO}(\text{CH}_2\text{CH}_2\text{O}_2\text{Me}$, HMPA; (k) H_2SO_4 , MeOH ; (l) $t\text{-BuOBr}$, pentane; (m) 151, $t\text{-BuONa}$, N-methylpyrrolidone; (n) aq. H_2SO_4 ; (o) PdCl_2 , CuCl_2 , NaOAc , NaCl , HOAc , Ac_2O ; (p) 165, NaH , DMF, $(\text{C}_6\text{H}_5)_3\text{P}$; (q) KOH, $n\text{-BuOH}$; (r) Ac_2O , Et_3N , hexane.

silyl ether-¹³⁸ protected reagents (Examples 20 and 21, respectively) permit “one-pot” two- or three-carbon homologations of β -ionone analogs. A novel butenolide (173) has been prepared by two successive acylmethylene phosphorane condensations (Example 22).¹³⁹ The application of the Ti(IV)-catalyzed reaction of an enol silyl ether (175) with an acetal (174) gave C_{15} -aldehyde intermediate 171 (Example 23).¹⁴⁰

Two novel syntheses of RA from 2,2,6-trimethylcyclohexanone (144) started conventionally by alkylation with an acetylide anion. A V(V)-catalyzed rearrangement was then

TABLE 12
Synthesis of Retinoidal Polyenes: Less Common Reactions of General Utility

Example	Key Reaction	Ref.
20		105
21		138
22		139
23		140
24		141
25		142
26		144
27		150
28		126

Note: (a) $(i\text{-Pr})_2\text{NLi}$, **168**, Et_2O , THF; (b) aq. $(\text{CO}_2\text{H})_2$; (c) I_2 , $\text{C}_6\text{H}_5\text{CH}_3$, Et_2O (40% from **167**); (d) **170**, pentane; (e) aq. HCl ; (f) $(\text{C}_6\text{H}_5)_2\text{P}=\text{CHCOCH}_3$, C_6H_6 ; (g) $(\text{C}_6\text{H}_5)_2\text{P}=\text{CHCO}_2\text{Me}$; (h) **175**, $(i\text{-Pr})_4\text{Ti}$, TiCl_4 , CH_2Cl_2 ; (i) DBU, molecular sieves, CH_2Cl_2 ; (j) MnO_2 , CH_2Cl_2 ; (k) **133** ($R = \text{Et}$), NaH , THF; (l) $[(\text{C}_6\text{H}_5)_2\text{SiO}]_2\text{VO}$, $(\text{C}_6\text{H}_5)_2\text{SiOH}$, $\text{C}_6\text{H}_5\text{CO}_2\text{H}$, xylenes; (m) NaBH_4 , EtOH ; (n) AcCl , $\text{C}_6\text{H}_5\text{N}$, CH_2Cl_2 ; (o) aq. HBr , CH_2Cl_2 ; (p) $(i\text{-Pr})_2\text{NLi}$, THF; **121**, THF; (q) AcCl , $\text{C}_6\text{H}_5\text{N}$, CH_2Cl_2 ; (r) NaHCO_3 , aq. THF; (s) $[(\text{C}_6\text{H}_5)_2\text{P}]_4\text{Pd}$, Et_3N , THF; (t) **181**, $(2\text{-CH}_2\text{C}_6\text{H}_4)_2\text{P}$, $\text{Pd}(\text{OAc})_2$, Et_3N ; (u) $\text{Pb}(\text{OAc})_4$, C_6H_6 ; (v) $\text{CH}_2\text{CH}_2\text{CHO}$, $(n\text{-Bu})_2\text{NH}$; (w) $n\text{-BuLi}$, $\text{CH}_2\text{C}(\text{O})\text{CH}_2\text{CH}(\text{OMe})_2$; (x) LiAlH_4 , THF; (y) aq. HCl , Me_2CO .

used to convert the alkynol product (**176**) to the ketone **177**, which was then converted to RA by reduction and elimination (Example 24).¹⁴¹ This rearrangement was used to prepare¹⁴² the carboxylic acid **178** (Example 25), which had been previously described.¹⁴³ The acid was then alkylated with the C₁₀-aldehyde **121**. A Pd(0)-catalyzed decarboxylative elimination was used to complete the synthesis.

Another example of an organometallic reagent derived from a functionalized vinyl halide (Example 26) used in polyene retinoid synthesis employs the Pd(II)-catalyzed coupling of **180** to acrolein dimethyl acetal (**181**) to afford the methyl-shifted acetal **182**.¹⁴⁴ Although the stereochemistry of the starting vinylic halide is retained in the reaction product, in this example the vinylic halide starting material was used as an *E/Z* mixture of isomers because it was the most easily prepared. Unfortunately, this reaction gives unsatisfactory results with 2-bromo-2-alkenes, and therefore cannot be used for the synthesis of the dimethyl acetal of the RA intermediate aldehyde **128**.

The preparation of C₅- and C₇-aldehyde units from butadiene^{145,146} and isoprene,¹⁴⁷⁻¹⁴⁹ respectively, are described in many patents. These aldehyde units are useful starting materials in retinoid syntheses employing phosphonates. A Hoffmann-La Roche synthesis¹⁵⁰ of C₅-aldehyde **184** by condensation of a glyoxylic ester with propionaldehyde (Example 27) is a convenient laboratory procedure. Aldehydes are often protected during reaction sequences as acetals. In Example 28, terminal alkyne **143**, prepared from a cyclic ketone, is efficiently transformed to acetal **185**, which is then converted to the corresponding aldehyde **186** for reaction with a C₅-phosphonate unit to complete the synthesis of the retinoid.¹²⁶

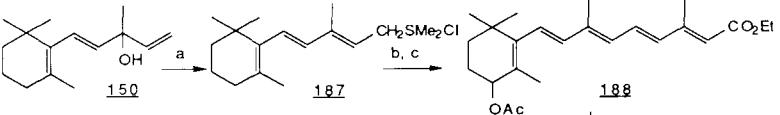
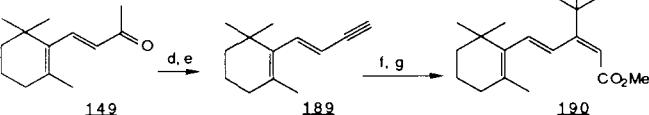
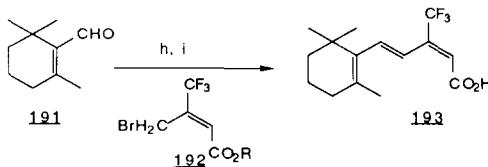
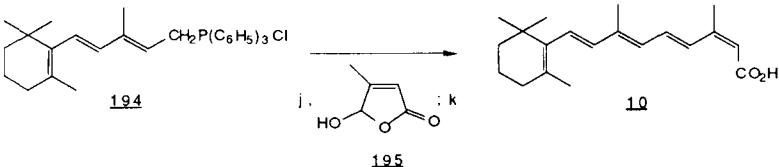
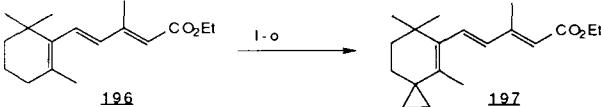
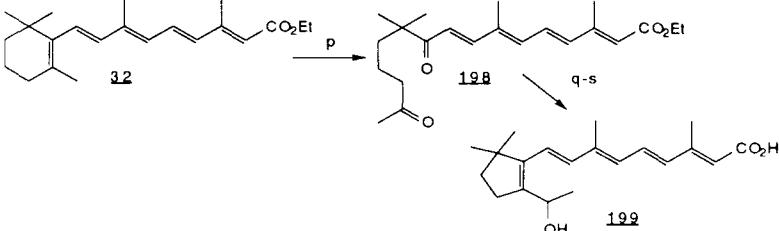
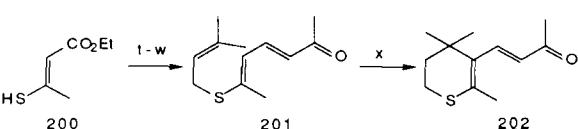
3. Synthesis of Specific Polyenoic Acid Analogs of Retinoic Acid

Specific analogs are occasionally prepared using reactions that have less general application than those described in Tables 9 to 12. Some of these are listed in Table 13. For example, Rosenberger et al.¹⁰⁶ employed a sulfonium ylid in the preparation of the RA metabolite 4-hydroxyretinoic acid (Example 29). Other retinoid metabolites have been synthesized by similar methodology.¹⁰⁶⁻¹⁰⁸ Unfortunately, none of these metabolites appear to be the active compounds involved in the mechanism of cellular differentiation.¹⁰⁰ Hopf and co-workers¹⁵¹ used the conjugate addition of dialkyl cuprates to alkynoate esters to prepare retinoids in which the 19- and 20-methyl groups of RA were replaced by other alkyl groups (Example 30). Dawson and co-workers¹¹⁸ used the same type of stereospecific *cis*-addition reaction at -78°C to prepare a conformationally restricted 13*Z*-retinoate ester analog (Chapter 14). A Reformatsky reaction on β-cyclogeranial (**191**) was used by Liu et al.¹⁵² to introduce a trifluoromethyl group on the side chain in place of the 19-methyl group (Example 31). The 13*Z* isomer of RA and its analogs are best prepared by a Wittig reaction of the ylid derived from phosphonium salt **194** and the lactol **195** (Example 32).¹⁵³ The mixture of 13*Z* and 11*Z*,13*Z* isomers obtained is equilibrated with Pd(II) salts to give almost exclusively 13-*cis*-RA. This procedure has potential as a general method for preparing 13*Z*-retinoid isomers.

A few ring-modified retinoids have been synthesized by manipulation of a retinoate ester or its C₁₅-ester intermediate **196**, e.g., by oxidation at the 4-position of the retinoid skeleton (Example 33).^{105,154} A series of cyclopentenyltetraenoic acids was prepared starting from the Jones oxidation of ethyl retinoate (**32**) to acyclic diketone **198**, which was then cyclized in an aldol reaction (Example 34).¹⁵⁵ 4-Thia-β-ionone (**202**) was made by the sulfuric acid-catalyzed cyclization of a “pseudoionone” analog (**201**) (Example 35).¹⁵⁶ Unlike the parent reaction, which is used to manufacture β-ionone,¹⁵⁷ this reaction is facilitated by the electronic involvement of the heteroatom and not by the loss of a proton from the 4-position.

Dihydro-¹²³ and fluoro-substituted^{89-91,158,159} RA analogs and their 9-aryltetraenoic acid congeners have been systematically investigated. These syntheses involved general Horner-Wadsworth-Emmons and Wittig routes. Dihydromethano-,^{105,121,160} dihy-

TABLE 13
Syntheses of Specific Retinoidal Polyenes

Example	Key Reaction	Ref.
29		106
30		151
31		152
32		153
33		105
34		155
35		156

Note: (a) Me_2S , HCl , Et_2O ; (b) **125**, $\text{C}_6\text{H}_5\text{CH}_2(\text{CH}_3)_3\text{NCl}$, CH_2Cl_2 , aq. NaOH ; (c) HOAc ; (d) $(i\text{-Pr})_2\text{NLi}$, $\text{Cl}-\text{P}(\text{O})(\text{OEt})_2$, THF ; (e) $(i\text{-Pr})_2\text{NLi}$, THF ; (f) $n\text{-BuLi}$, Et_2O ; ClCO_2Me ; (g) $(t\text{-Bu})_2\text{CuLi}$, THF ; MeOH ; (h) **192**, Zn ; (i) NaOMe , MeOH ; (j) KOH , **195**, $i\text{-PrOH}$; (k) $\text{Pd}(\text{NO}_3)_2$, $(\text{C}_6\text{H}_5)_3\text{P}$, Me_3N , THF , MeCN ; (l) NBS , aq. $t\text{-BuOH}$; (m) MnO_2 , CH_2Cl_2 ; (n) $(\text{C}_6\text{H}_5)_3\text{P}=\text{CH}_2$, THF , $t\text{-BuOH}$; (o) $\text{Zn}(\text{Cu})$, CH_2I_2 , Et_2O (15% from **196**); (p) CrO_3 , H_2SO_4 , aq. Me_2CO ; (q) HClO_4 , aq. EtOH ; (r) KOH , EtOH ; aq. H_2SO_4 ; (s) NaBH_4 , aq. MeOH ; aq. H_2SO_4 ; (t) NaOEt , EtOH ; (u) $\text{Me}_2\text{C}=\text{CHCH}_2\text{Br}$, C_6H_6 ; (v) LiAlH_4 , Et_2O ; aq. HCl ; (w) $(i\text{-PrO})_3\text{Al}$, Me_2CO , C_6H_6 ; (x) H_2SO_4 , MeNO_2 .

droepoxy-,^{125,161} and dehydro-^{100,162} retinoic acids have also been made by these standard methods, as have acyclic polyenoic acids.^{111,163} The acyclic polyene ester **141** proved to be far more labile to oxidation on exposure to air than did RA.

9-Aryl-2,4,6,8-tetraenoic acids were introduced for pharmaceutical use as early as 1974¹⁶⁴ by Hoffmann-La Roche and have been investigated intensively.¹⁶⁵ A large number of aromatic and heterocyclic compounds, especially those with alkyl, halogen, and alkoxy substituents, were functionalized by formylation or chloromethylation and then elaborated to the aryltetraenoic acids by standard Wittig, Horner-Wadsworth-Emmons, and sulfone alkylation methods, as illustrated in Table 14.

4. Retinoids in Which Aromatic Groups Replace Part of the Polyene Chain

A 4-substituted benzoic acid can be considered as an electronic analog of a 5-substituted *E,E*-dienoic acid in which the two *trans*-substituted double bonds are restricted to a s-cisoid conformation. Many benzoic acid-terminated retinoids, together with their heterocyclic carboxylic acid analogs, have been synthesized using methods analogous to the general ($C_{13} + C_7$ and $C_{10} + C_{10}$) methods used for the polyenoic retinoids (Table 14).^{96,100,166,167} In the latter method, the all-*E* isomer having two double bonds is best obtained by reaction of a C_{10} -phosphonium salt such as **207** with an aldehyde. The aldehydes may be obtained stereoselectively (Example 36, Table 15) by SeO_2 oxidation of a methyl-substituted olefin⁹⁶ or by an aldol reaction.¹⁰⁰

Once the systematic investigation of the significance of conformational mobility on retinoid activity had gained momentum, aromatic analogs were prepared in which conformational freedom was restricted about each C–C single bond of the polyene chain.⁹⁶⁻¹⁰⁰ Aromatic retinoids, described by Loeliger et al.⁹³ used dimethyl- or tetramethyl-substituted tetrahydronaphthalene or indane as the hydrophobic terminus of the retinoid skeleton in which the 5,7-diene system was confined to coplanarity by the aromatic ring system.

Friedel-Crafts cycloalkylation of aromatic systems is conveniently used to introduce the saturated portion of the aromatic ring system, e.g., the reaction of 2,5-dichloro-2,5-dimethylhexane (**225**) with benzene (Example 37).¹⁶⁸ The trienoic acid side chain was then introduced by standard olefination procedures after suitable functionalization of the cycloalkylation product. 7-Bromo-1,2,3,4-tetrahydro-1,1-dimethylnaphthalene (**229**) is readily accessible by intramolecular alkylation (Example 38) and is used to prepare 7-acyl derivatives because acylation of 1,2,3,4-tetrahydro-1,1-dimethylnaphthalene is not regioselective.¹⁶⁹ The dimethylindane analog (**233**) is prepared from acylcyclopentene **231** by cyclization of a trienol silyl ether (Example 39).¹⁷⁰ Two other types of compounds in which an aromatic group replaces a conjugated diene unit of the side chain are illustrated by Example 13¹²⁷ and structure **63**.¹⁰⁰ Both types are prepared by standard methods. Analogs of the latter series have been reported by the L’Oreal group.¹⁷¹

Further conformational restriction by reducing the number of double bonds in the side chain to a single double bond conjugated to aromatic systems yielded compounds of outstanding activity, as exemplified by the 4-arylvinyI-substituted benzoic acids such as **65**.⁹³ Reaction of the ylid prepared from **204** with 4-carbethoxybenzaldehyde — a procedure analogous to the ($C_{13} + C_7$) Wittig synthesis of RA — gave mainly the ethyl ester of *E* isomer **65**.^{93,172} Many compounds related to **65** have been reported, as shown in Tables 14 and 15 and described in Chapter 14. Several of these were prepared by a route similar to the ($C_{13} + C_7$) Horner-Wadsworth-Emmons synthesis of RA. Dawson et al.¹⁷³ have demonstrated that this method of preparation can be modified to give a very high *E/Z* isomer ratio by the *in situ* equilibration of the ester product mixture with base, especially NaOEt in the reaction solvent dimethylsulfoxide.

Heteroaromatic analogs of **65** proved to have especial interest, particularly the dihydrobenzothiopyran **239** and its analogs. Several groups have investigated the synthesis and

TABLE 14
Syntheses of Aromatic Retinoids: General Methods

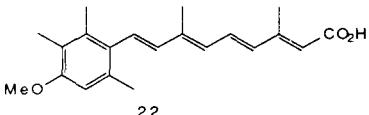
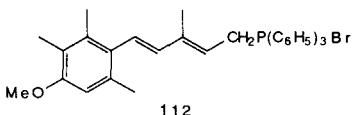
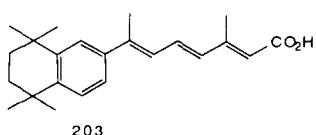
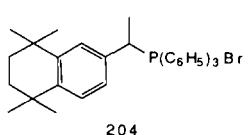
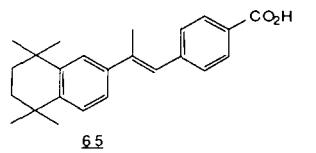
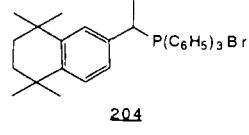
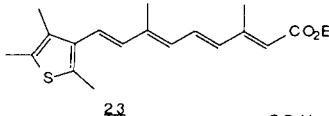
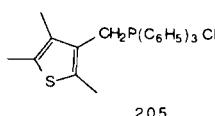
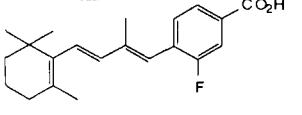
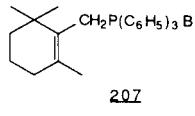
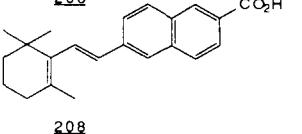
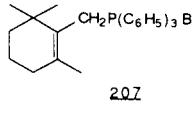
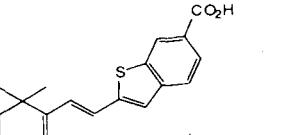
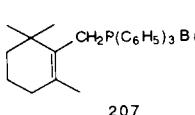
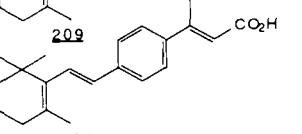
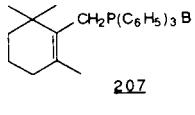
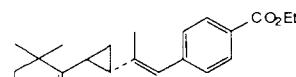
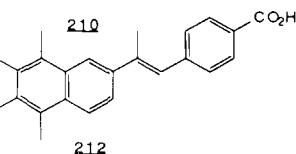
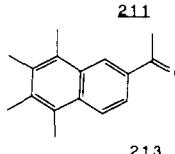
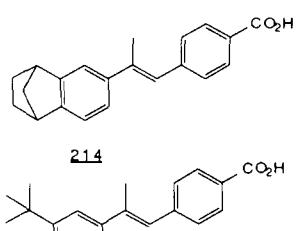
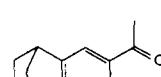
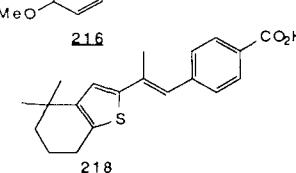
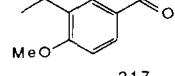
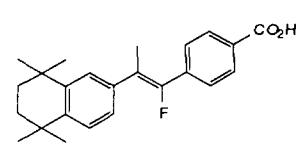
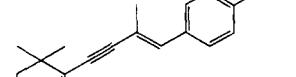
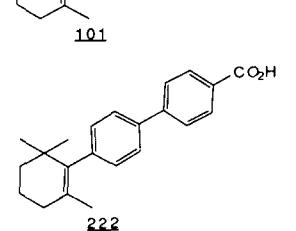
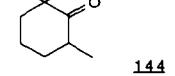
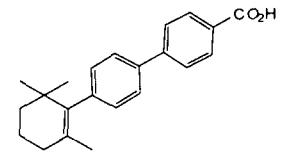
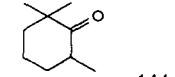
Retinoid	Intermediate	Reaction Example	Ref.
		1,2 4 10 14 16	111,165 111,120,165 111,120 111 111
		3	93
		3	93
		4	166
		4	96
		4	176
		4	118
		4,7	100

TABLE 14 (continued)
Syntheses of Aromatic Retinoids: General Methods

Retinoid	Intermediate	Reaction Example	Ref.
		9	127
		9	173
		9	127
		9	118
		9	118
		9	118
		12,28	100
		13	127

biological activity of **239**,^{127,174,175} which proved to be exceptionally nontoxic in comparison with **65** in animal models. Its heterocyclic precursor (**236**) is prepared by polyphosphoric acid- or Lewis acid-catalyzed cyclization of olefin **234** or tertiary alcohol **235** or by lithium aluminum hydride reduction of the related thiolactone, followed by Friedel-Crafts acetylation (Example 40). Dihydrobenzopyranyl^{127,174,175} and *N*-methyltetrahydroquinolyl¹⁷⁵ analogs were prepared by similar methods.

TABLE 15
Syntheses of Aromatic Retinoids: Specific Methods

Example	Key Reaction	Ref.
36		96
37		168,181
38		118
39		170
40		127,174,175

Note: (a) NaH, DMF; Me₂CO, DMF; aq. HOAc; (b) SeO₂, dioxane (31% from 223); (c) C₆H₅CH₃, AlCl₃; aq. HCl; (d) KMnO₄, NaOH, aq. C₅H₅N; aq. H₂SO₄; (e) NaN₃; 65°C; aq. NaOH; (f) MeMgBr, Et₂O; aq. NH₄Cl; (g) H₂SO₄ (97%); (h) CrO₃, Ac₂O, HOAc; aq. NaHSO₃ (78%); (i) Me₃SiCl, Et₃N, DMF; 150°C; (j) aq. HCl; (k) (MeO)₃CH, H₂SO₄, O₂; (l) SnCl₄, CH₂Cl₂; (m) AcCl, AlCl₃, CS₂ (43% 236 from 234); (n) P₂O₅, H₃PO₄, C₆H₆; (o) AcCl, SnCl₄, C₆H₆; aq. HCl; (p) LiAlH₄, Et₂O; aq. HCl; (q) (C₆H₅)₃P - HBr, MeOH (34% 237 from 235); (r) 4-EtO₂CC₆H₄CH₂P(O)(OEt)₂, NaH, 15-Crown-5, 236, THF; (s) KOH, aq. EtOH; aq. HOAc (47% from 236); (t) 237, 238, 1,2-epoxybutane; (u) KOH, aq. EtOH; aq. H₂SO₄.

In addition, 6-substituted 2-naphthalenecarboxylic acids such as 208 were readily prepared by the C₁₀-ylid route.^{176,177} These compounds can be considered as electronic analogs of 7-substituted 2E,4E,6E-trienoic acids. 2-Substituted 1-benzothiophene-6-carboxylic acids such as 209 can also be prepared by this method.¹¹⁸

5. Retinoids Containing Biaryl Groups

In 222 (Table 14) the tetraene function of RA is replaced by a 4,4'-biphenyl system. Only the endocyclic double bond in the ring is retained. A routine arylation of 2,2,6-

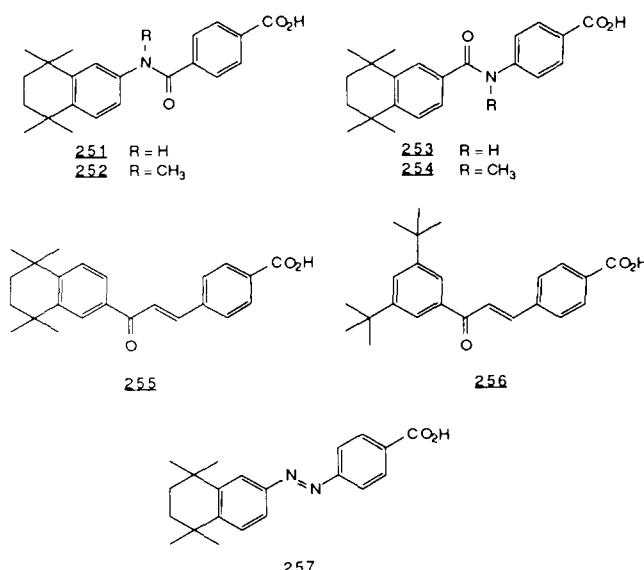
TABLE 16
Syntheses of Biaryl Retinoids

Example	Key Reaction	Ref.
41		176,178
42		118
43		118
44		118

Note: (a) Li(1% Na), Et₂O; ZnCl₂, THF; **241**, [(C₆H₅)₃P]₂Ni, THF; aq. HCl; (b) KOH, aq. EtOH; aq. HOAc (37% from **240**); (c) *n*-BuLi, THF; ZnCl₂, THF; **240**, THF; [(C₆H₅)₃P]₂NiCl₂, (C₆H₅)₃P, (*i*-Bu)₂AlH, THF; aq. HCl; (d) KOH, aq. MeOH; aq. HOAc (40% from **243**); (e) MeMgBr, Et₂O; aq. NH₄Cl; (f) 190°C; (g) DDQ, dioxane (49% from **245**); (h) Mg, THF; ZnCl₂, THF; **241**, THF; [(C₆H₅)₃P]₂NiCl₂, (C₆H₅)₃P, (*i*-Bu)₂AlH, THF; aq. HCl (87%); (i) CrO₃, Ac₂O, HOAc; aq. NaHSO₃ (51%); (j) NaBH₄, Et₂O, Et₂O; (k) EtO₂CN = NCO₂Et, (C₆H₅)₃P, HN₃, C₆H₆; (l) NaOH, aq. MeOCH₂CH₂OH, Et₂O; aq. HCl (54% from **249**).

trimethylcyclohexanone (**144**) can be employed to prepare **222**.¹²⁷ Of much greater biological interest are the 6-aryl-2-naphthalenecarboxylic acids, which are analogs of **208**.¹⁷⁶ Some examples are shown in Table 16. These nonolefinic retinoids required a quite different approach from that used in the synthesis of other conformationally restricted retinoids. A general biaryl synthesis introduced by Negishi et al.¹⁷⁸ (Examples 41 and 42)^{118,176} proved to be the most convenient route because this reaction was tolerant of some reactive functional groups such as carbethoxy, cyano, and bromo that could be converted to a carboxyl group. The Ni(0)- and Pd(0)-triphenylphosphine catalyst complexes that are used to couple the arylzinc chloride and aryl bromide starting materials are conveniently prepared as required by (*i*-Bu)₂AlH reduction of MCl₂[(C₆H₅)₃P]₂ (M = Ni, Pd) in the presence of triphenylphosphine. The parent compound in this series is **242** (Example 41). Heterocyclic biaryl retinoids can also be made by the Negishi procedure (Example 42). A series of methyl-substituted analogs of **242**, the syntheses of which are outlined in Chapter 14, was also prepared using this coupling technique. Analogs of **242** with other substituents on the tetrahydronaphthalene ring can be prepared from the tetralone intermediates such as **230** or **245** (Example 43) or the product of benzylic oxidation of retinoid **248** (Example 44).¹¹⁸

TABLE 17
Amide, Chalcone, and Azo Retinoids



Other types of biaryl retinoids have been reported,¹⁷⁷ some of which are described in Chapter 14.

6. Retinoids in Which the Hydrophobic and Polar Termini Are Linked through Other Nonolefinic Groups

In some very imaginative work, Shudo and co-workers^{179,180} have prepared retinoids in which the double-bond systems are replaced by carboxamide, keto-olefin, and diazo groups with retention of biological activity in many cases (Table 17). This work is covered in Chapter 12 of this volume. Dawson¹¹⁸ prepared the ethyl esters of the tertiary amides **252** and **254**, which were not active in the ODC assay. Shudo prepared the amides from the corresponding aryl amine and aryl carboxylic acid, which are available from 1,2,3,4-tetrahydro-1,1,4,4,6-pentamethylnaphthalene by oxidation to the carboxylic acid followed by the Schmidt reaction.¹⁸¹ They are also available by reduction of the corresponding nitro compound.¹¹⁸ The chalcones (**255** and **256**) containing the enone moiety are readily prepared by an aldol condensation of 4-carbethoxybenzaldehyde with a methyl aryl ketone. The 1,3-di-*t*-butylbenzene group is successfully employed in this series as a substitute for the tetrahydrotetramethylnaphthalene ring system.¹⁸² The azo group of **257** is introduced by condensation of an aryl amine with methyl-4-nitrosobenzoate.¹⁸³

7. Synthetic Methodology Overview

Retinoids can be efficiently synthesized by methods that are often adapted from highly process-developed syntheses of (*E*)-RA. General methods for the synthesis of nonolefinic retinoids are also in general use.

The study of retinoid-protein receptor binding and the probe of the mechanism of retinoid action have encouraged synthetic efforts aimed at increasingly rigid structures to help determine the steric limitations in retinoid activity. Further development in this area should be expected and will require the introduction of new synthetic methods quite unrelated to polyene synthesis. Shudo's introduction of amide, azo, and chalcone-type retinoids will undoubtedly promote interest in other nonolefinic linkages for the hydrophobic and polar

functions. It is expected that strategies for retinoid synthesis will increasingly involve considerations that are appreciably removed from the original challenge of stereocontrolled synthesis of an air- and light-sensitive polyene.

C. ASSAY PROTOCOLS

1. Chemicals

RA was a gift of Dr. Michael B. Sporn, NCI, Bethesda, MD. 13-cis-RA was generously provided by Dr. Y. Fulmer Shealy, Southern Research Institute, Birmingham, AL, and **65** (Ro 13-7410) by Hoffmann-La Roche, Nutley, NJ. The remainder of the compounds reported in Table 18 (Section III.D) were synthesized at SRI International, Menlo Park, CA. Compounds were determined to have greater than 99% purity by high-performance chromatographic analysis (Waters Associates ALC 210 equipped with a RCM-100 column module containing a Radialpak A reverse-phase column, methanol eluant at 1.0 to 2.0 ml/min, detection at 260 nm). New compounds were fully characterized (IR, ¹H and ¹³C NMR, UV, MS, and elemental analyses). Oxygen-sensitive retinoids — those containing more than one double bond in conjugation or sulfur — were sealed under argon in glass ampoules, which were stored at -40°C and opened immediately before use. Light-sensitive retinoids were handled under yellow or subdued light. TPA was purchased from Chemicals for Cancer Research, Eden Prairie, MN; DMBA from Sigma Chemical Co., St. Louis, MO; and reagent-grade acetone from Mallinkrodt, Paris, KN. TPA and DMBA were handled in double-walled containers using butyl-rubber gloves.

2. Animals

Female Charles River CD-1 mice 5 to 7 weeks old were purchased from Charles River Laboratories, Inc., Wilmington, MA. Mice were housed two (ODC assay) or ten (tumor promotion) to a plastic cage with softwood chips as bedding in a controlled-environment room with a light period from 7:00 a.m. to 6:00 p.m. Laboratory chow and water were provided *ad libitum*. Mice were acclimated for two weeks before testing was begun. The dorsal hair of the mice was removed with electric clippers; only those mice exhibiting no hair regrowth after 48 h were used in the ODC and tumor promotion studies.

3. Ornithine Decarboxylase Assay

Mice were randomized in groups of three with two mice in each group for both the control and test groups. Retinoids and controls were assayed in triplicate. Subdued lighting conditions were used to minimize the potential for bond isomerization with the polyolefinic retinoids. Retinoids, at one of five doses (0.017, 0.17, 1.70, 17.0, and 170 nmol), dissolved in 0.2 ml of acetone or 0.2 ml of acetone alone were topically applied to the back of each mouse in a group. After 1 h, 17.0 nmol of TPA in 0.2 ml of acetone was applied to the back of each mouse; the vehicle control group received acetone alone. The mice were killed by cervical dislocation five hours after treatment with TPA.

The epidermis was obtained by the method of Rainieri et al.¹⁸⁴ The epidermal sheets from each treatment group were pooled and homogenized, and the supernatant was collected after centrifugation as described by Chao et al.¹⁸⁵ ODC activity was determined by the microassay described by Verma et al.¹⁸⁶ Enzyme activity was determined in triplicate and expressed as nanomoles of CO₂ released in 30 min/mg of protein. Enzyme activity was linear for the protein concentrations used, which were determined by the Lowry procedure, using BSA as the standard. Data were analyzed statistically using the Student's *t*-test.

4. Tumor Promotion (Antipapilloma) Assay

The two-stage tumor induction-promotion protocol of Verma et al.⁴ was used. Mice were randomized in groups of 30. For the initiation step, the shaved dorsal epidermis of

each mouse was topically treated with 200 nmol of DMBA dissolved in 0.2 ml of acetone. After 14 d, the promotion stage was begun. A solution of 8.5 nmol of TPA in 0.2 ml of acetone was topically applied to the epidermis of each mouse twice each week on days 2 and 5 for a period of 20 weeks. Mice in the test groups received one of four doses — 0.17, 1.70, 17.0, or 170.0 nmol — of retinoid in 0.2 ml of acetone 1 h before promotion with TPA. The mice in the TPA-positive control groups received 0.2 ml of acetone alone.

Starting at the seventh week of promotion, the papillomas appearing on the epidermis of each mouse in a group were counted and recorded weekly. The experiment was terminated at the end of week 20, at which time the numbers of papillomas had reached plateaus. The inhibition of tumor promotion was determined by subtracting from 1 the ratio of the mean number of papillomas per mouse in each retinoid concentration test group to the mean number of papillomas per mouse in the positive control group. Multiplying this fraction by 100 gave the percent inhibition. The inhibition in the test groups relative to that in the control groups was evaluated statistically using the Student's *t*-test.

D. THE EFFECTS OF STRUCTURAL MODIFICATIONS OF THE RETINOIC ACID SKELETON ON ACTIVITY IN THE ORNITHINE DECARBOXYLASE ASSAY

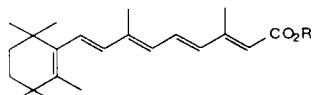
In Table 18 are listed the structures of the retinoids that we have synthesized and the inhibitory doses (ID_{50}) of these retinoids required to inhibit by 50% the induction of ODC in mouse dorsal epidermis treated with the tumor promoter TPA and to prevent the promotion of papillomas in this epidermis pretreated with the tumor initiator DMBA and then treated with TPA. For comparison purposes, the activities of RA, its ethyl ester, 13-*cis*-RA, and the 4-substituted benzoic acid **65** are included. Described above in Section III.C are the procedures used for conducting these assays; in this section are presented the rationale for the design of these modified retinoids and the effects that these modifications of the retinoid skeleton have on biological activity in the assays. In describing the effects of structure on activity, retinoid numbering as shown in Figure 3 is used. ID_{50} values for RA and 13-*cis*-RA given in Table 18 differ from those reported by Verma et al.³ (see Table 3). These differences may reflect the differences in the assay protocols used or the purity of the retinoids tested.

Structural modifications were made in the ring, polyolefinic chain, and polar terminus regions of the retinoid skeleton. Generally, these modifications were systematic in nature to provide data for future quantitative structure-activity correlations. Modifications were made (1) to prevent metabolism to less active species, (2) to restrict conformational mobility in order to enhance activity by obtaining a structure having the optimum conformation — that which the retinoid skeleton assumes on binding to its receptor, (3) to reduce toxicity by altering lipophilicity, and (4) to provide prodrugs that could be metabolized to active species. Compound activities in Table 18 are reported as ID_{50} values except in those cases where this information was not obtained; then the dose closest to the ID_{50} is listed along with the percent inhibition, which is given in parentheses.

Examination of retinoid activity in inhibiting the induction of ornithine decarboxylase indicates several trends. In the polyolefinic retinoids as exemplified by the basic RA structure, esters, which would serve as more lipophilic prodrugs that would either be hydrolyzed or cleaved by esterases to the carboxylic acids, were less active than the acids, generally by at least an order of magnitude. In the benzoic acid series of retinoids, of which structure **65** is the parent compound,⁹³ the esters were generally less active, whereas in the naphthalenecarboxylic acid series, of which structure **242** is the parent retinoid,¹²⁷ activity ranking between the ester and the acid varied. With one exception (ethyl ester **293** of thiophene-carboxylic acid **292**),¹⁰⁵ the 9*Z* isomer was less active than the 9*E* isomer or was inactive. The 13*Z* isomer was less active than the all-*E* by about an order of magnitude. The 7*E*,9*E*-

TABLE 18
Retinoid Inhibition of Mouse Epidermal ODC Induction and Tumor Promotion by TPA

Retinoid	Code No.	ODC assay ID ₅₀ (nmol)	Antipapilloma assay ID ₅₀ (nmol)
I. Control compounds			
 <u>5</u> R = H		0.04 1.7	3.5
 <u>32</u> R = Et			
 <u>10</u>		1.4	64
II. Ring modifications			
 <u>258</u> R = H	<u>259</u> R = Et	SRI 5387-96B SRI 5631-2E	0.26 6.3
 <u>260</u>		SRI 5631-2Z	38



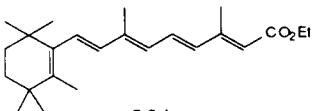
92
122

R = H
R = Et

SRI 5397-41
SRI 5397-32

0.19
2.8

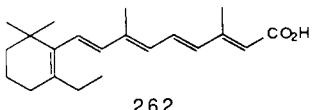
2.5



261

SRI 2285-78E

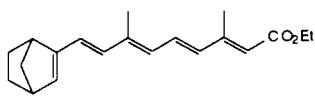
<17 (72)



262

SRI 2712-24

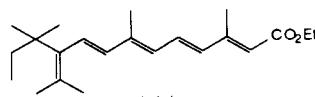
<1.7 (66)



263

SRI 2840-67

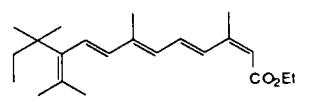
2.9



141

SRI 3920-90D

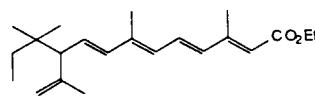
7.2



264

SRI 3920-90A

>17 (20)

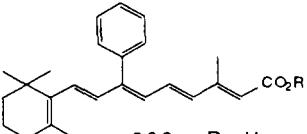
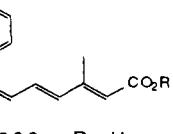
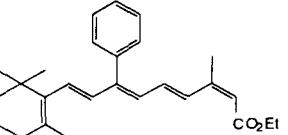
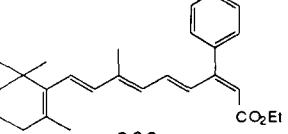
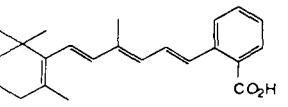


265

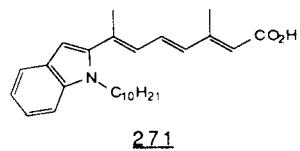
SRI 3920-90E

14

TABLE 18 (continued)
Retinoid Inhibition of Mouse Epidermal ODC Induction and Tumor Promotion by TPA

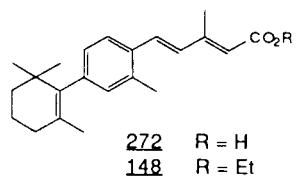
Retinoid	Code No.	ODC assay ID ₅₀ (nmol)	Antipapilloma assay ID ₅₀ (nmol)
III. Chain substituent modifications			
 266	SRI 4875-20	54	
 267	SRI 4875-17C	>17 (10)	
 268	SRI 4875-17A	>17 (0)	
 269	SRI 4092-75	>17 (14)	
IV. Conformational restrictions of the side chain			
A. 13-cisoid			
 270	SRI 3204-91	>17 (2)	

B. 5,7*E*-cisoid

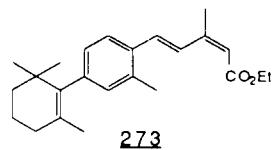


SRI 5397-88E >17 (0)

C. 7*E*,9*E*-cisoid



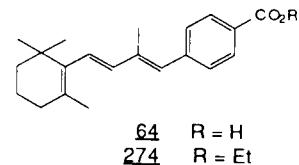
SRI 4657-47 >17 (23)
SRI 4657-46B -17 (22)



SRI 4657-46A >17 (10)

D. 11*E*,13*E*-cisoid

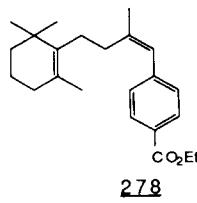
1. Compounds **64** and **274**



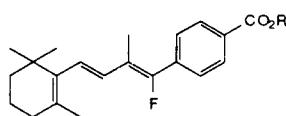
SRI 2965-38 4.0
SRI 3498-95 <17 (80)

TABLE 18 (continued)
Retinoid Inhibition of Mouse Epidermal ODC Induction and Tumor Promotion by TPA

Retinoid	Code No.	ODC assay ID₅₀ (nmol)	Antipapilloma assay ID₅₀ (nmol)
2. Trimethyl cyclohexenyl ring analogs			
 <u>275</u>	SRI 3618-93B	<17 (80)	
3. Side chain analogs			
 <u>210</u>	SRI 4657-55B	0.4	
 <u>276</u> R = H	SRI 5193-27	2.3	34
 <u>277</u> R = Et	SRI 4875-43B	2.2	



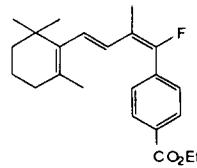
SRI 4875-43A >17 (32)



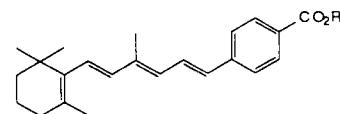
SRI 3809-83 2.1 43

280 R = H
 R = Et

SRI 3809-79B 4.5



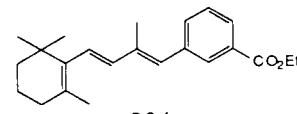
SRI 3809-79A >17 (27)



SRI 4092-68 7.3
SRI 4092-66 38

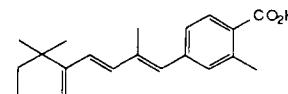
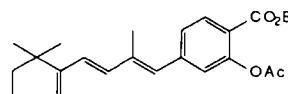
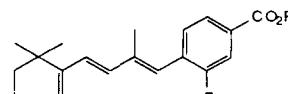
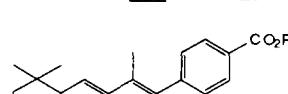
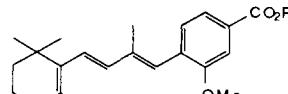
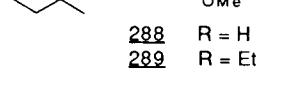
283 R = H
 R = Et

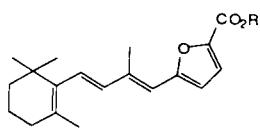
4. Substitutional variations of the aromatic ring



SRI 4092-52 >17 (6)

TABLE 18 (continued)
Retinoid Inhibition of Mouse Epidermal ODC Induction and Tumor Promotion by TPA

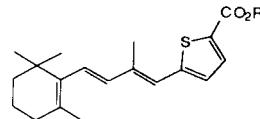
Retinoid	Code No.	ODC assay ID ₅₀ (nmol)	Antipapilloma assay ID ₅₀ (nmol)
 285	SRI 3204-87	>17 (9)	
 286	SRI 5193-7	22	
 286 R = H	SRI 4657-10	3.5	
 286 R = Et	SRI 4657-4	22	
 288 R = H	SRI 4592-48	9.1	
 289 R = Et	SRI 4092-44	>17 (25)	



290 R = H
291 R = Et

SRI 4445-40
SRI 4445-39

252
>17 (9)

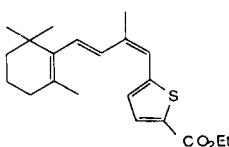


292 R = H
293 R = Et

SRI 3920-59
SRI 3920-25E

3.7
0.6

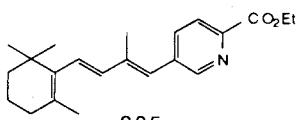
57



294

SRI 3920-25Z

3.9



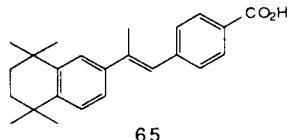
295

SRI 4445-55

5.9

E. 5,7*E*- and 11*E*,13*E*-cisoid

1. Compound **65**



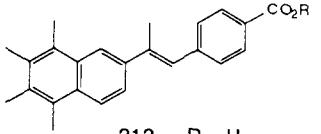
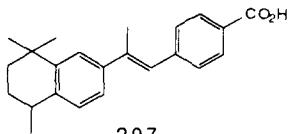
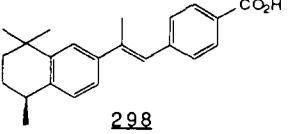
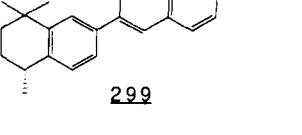
65

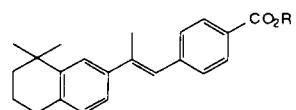
Ro 13-7410

0.03

0.14

TABLE 18 (continued)
Retinoid Inhibition of Mouse Epidermal ODC Induction and Tumor Promotion by TPA

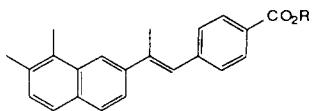
Retinoid	Code No.	ODC assay ID ₅₀ (nmol)	Antipapilloma assay ID ₅₀ (nmol)
2. Modifications of the tetrahydronaphthalene ring			
	<u>212</u> R = H	SRI 5193-55	1.6
	<u>296</u> R = Et	SRI 5193-52	1.6
	<u>297</u>	SRI 6124-47	0.07
	<u>298</u>	SRI 6910-50	0.05
	<u>299</u>	SRI 6910-29	0.06
			0.2



300 R = H
301 R = Et

SRI 5639-27
SRI 5639-17.6

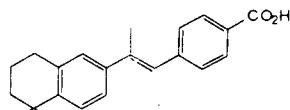
1.1
0.9



302 R = H
303 R = Et

SRI 5387-34
SRI 5387-23B

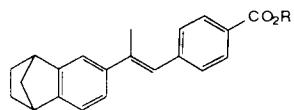
>170 (9)
121



304

SRI 5942-64

0.6



305 R = Et

SRI 4445-86B
SRI 4445-84

3.1
<1.7 (60)

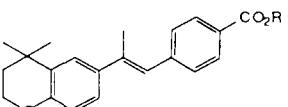
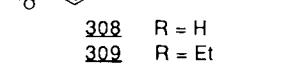
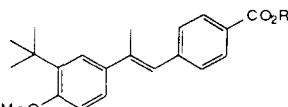
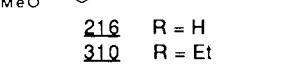
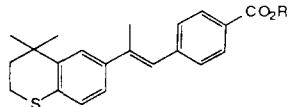
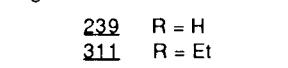
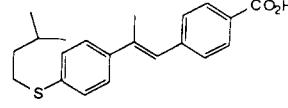
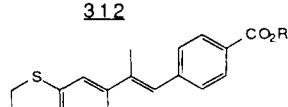
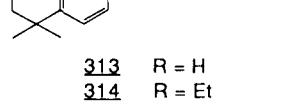


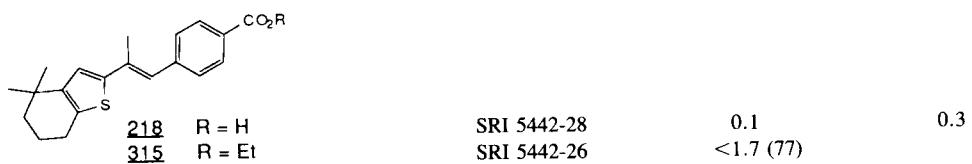
306 R = H
307 R = Et

SRI 4445-86A
SRI 4445-83A

22
13

TABLE 18 (continued)
Retinoid Inhibition of Mouse Epidermal ODC Induction and Tumor Promotion by TPA

Retinoid	Code No.	ODC assay ID ₅₀ (nmol)	Antipapilloma assay ID ₅₀ (nmol)
	SRI 5387-12	2.7	3.5
	SRI 5896-52	0.7	
	SRI 6575-38	0.4	
	SRI 6575-25	<1.7 (84)	
	SRI 5896-39	0.5	3.0
	SRI 5896-34	2.4	
	SRI 7323-85	170	
	SRI 6575-26	0.6	1.5
	SRI 6575-10	<1.7 (63)	



3. Modifications of the side chain

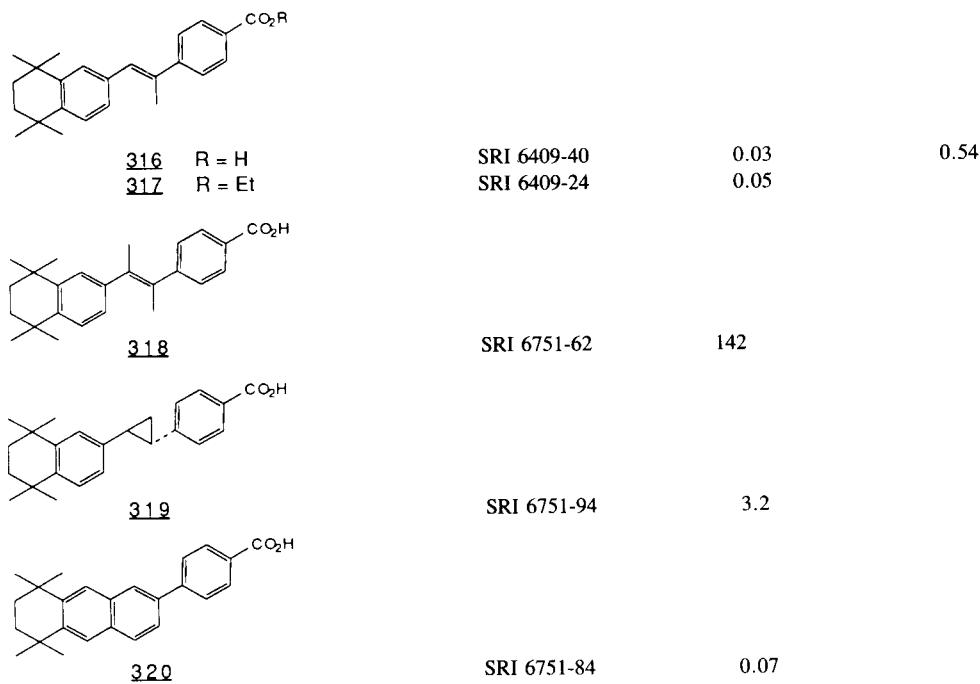
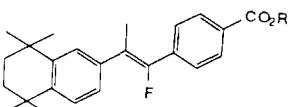
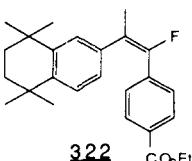
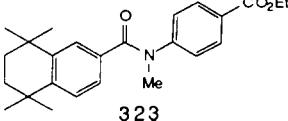
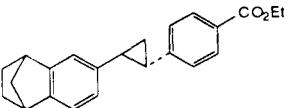


TABLE 18 (continued)
Retinoid Inhibition of Mouse Epidermal ODC Induction and Tumor Promotion by TPA

Retinoid	Code No.	ODC assay ID ₅₀ (nmol)	Antipapilloma assay ID ₅₀ (nmol)
	<u>220</u> R = H	SRI 5631-96	0.08
	<u>321</u> R = Et	SRI 5631-93B	0.2
	<u>322</u>	SRI 5631-93A	8.5
	<u>323</u>	SRI 6409-50	128
	<u>324</u>	SRI 4875-54A	>17 (12)

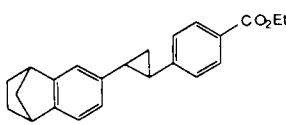
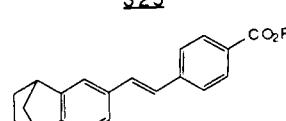
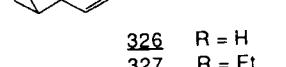
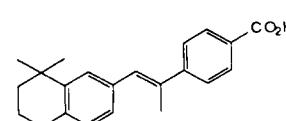
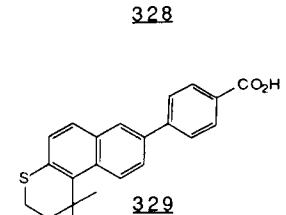
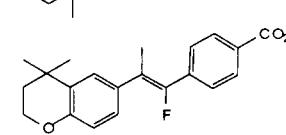
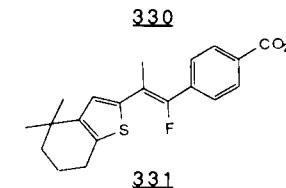
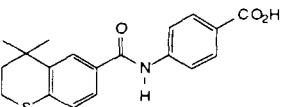
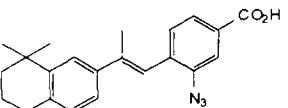
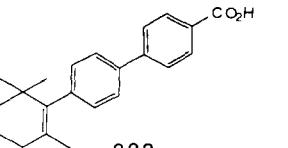
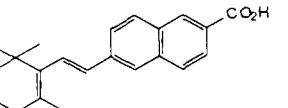
	325	SRI 4875-54B	>17 (4)
	326	SRI 4875-28	48
	327 R = Et	SRI 4875-29	>17 (39)
	328	SRI 7323-78	0.6
	329	SRI 5442-80	22
	330	SRI 5631-96	7.3
	331	SRI 5442-41	0.6

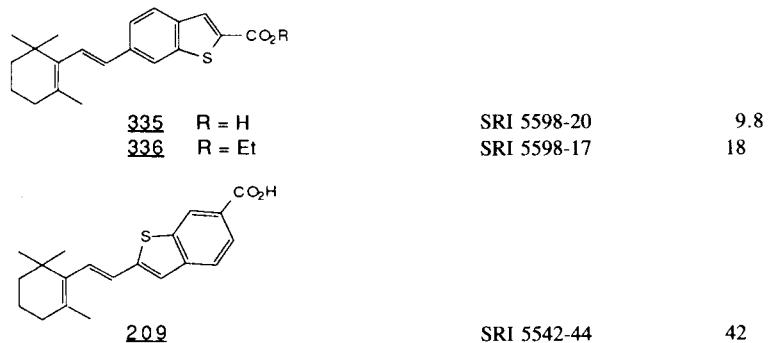
TABLE 18 (continued)
Retinoid Inhibition of Mouse Epidermal ODC Induction and Tumor Promotion by TPA

Retinoid	Code No.	ODC assay ID ₅₀ (nmol)	Antipapilloma assay ID ₅₀ (nmol)
 <u>332</u>	SRI 5442-84	>170 (36)	
5. Modification of the phenyl ring			
 <u>333</u>	SRI 7167-55	8.0	
F. 7E,9E- and 11E,13E-cisoid			
 <u>222</u>	SRI 4529-19	23	170 (29)
G. 9E,11E,13E-cisoid			
1. Compound 208	 <u>208</u>	SRI 4445-75	22
			79

2. Trimethylcyclohexenyl ring modification



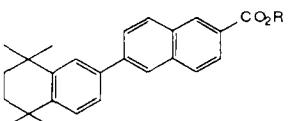
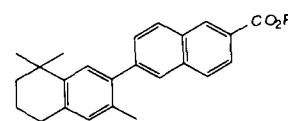
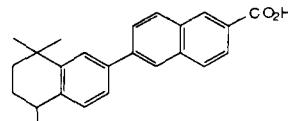
3. Naphthalene ring modifications

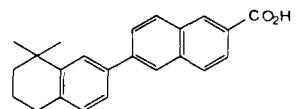


4. Polar terminus modification



TABLE 18 (continued)
Retinoid Inhibition of Mouse Epidermal ODC Induction and Tumor Promotion by TPA

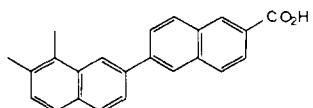
Retinoid	Code No.	ODC assay ID ₅₀ (nmol)	Antipapilloma assay ID ₅₀ (nmol)
H. 5,7E- and 9E,11E,13E-cisoid			
1. Compounds 242 and 91			
 242 R = H 91 R = Et	SRI 5898-52 SRI 5898-50	2.2 1.7	21
2. Modifications of the tetrahydronaphthalene ring			
 338 R = H 339 R = Et	SRI 5193-43 SRI 5193-41	0.4 5.0	53
 340	SRI 6387-62	2.1	



341

SRI 6153-40

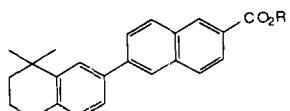
6.8



342

SRI 5387-29

>17 (23)



343 R = H
344 R = Et

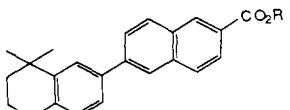
SRI 6088-20

29

>170 (15)

SRI 5387-20

50



345 R = H
346 R = Et

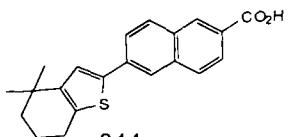
SRI 5387-31

25

>170 (9)

SRI 5387-5

14

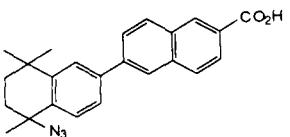


244

SRI 5442-30

15

312



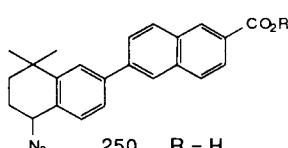
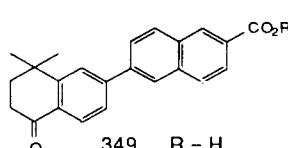
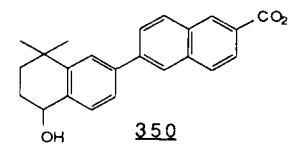
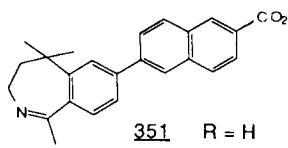
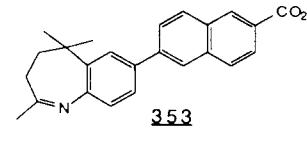
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SRI 6387-7

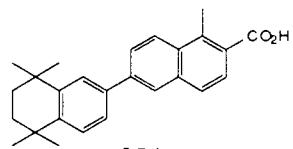
0.8

8.3

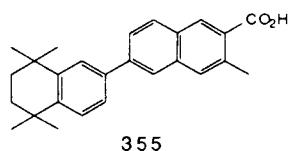
TABLE 18 (continued)
Retinoid Inhibition of Mouse Epidermal ODC Induction and Tumor Promotion by TPA

Retinoid	Code No.	ODC assay ID ₅₀ (nmol)	Antipapilloma assay ID ₅₀ (nmol)
	250	SRI 6387-20	0.5
	<u>348</u>	SRI 6387-16	2.6
	349	SRI 6088-84	27
	<u>249</u>	SRI 6153-87	3.6
	350	SRI 6387-12	11
	351	SRI 6088-80	>170 (0)
	<u>352</u>	SRI 6088-29	42
	353	SRI 6088-81	>170 (0)

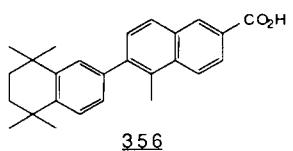
3. Modifications of the naphthalene ring



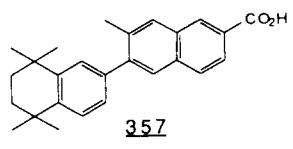
SRI 6787-88 1.7



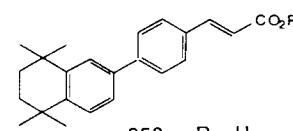
SRI 7567-65 3.3



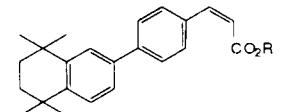
SRI 6787-81 66



SRI 7567-53 10

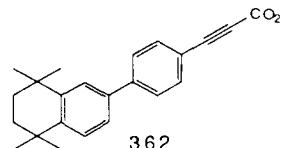
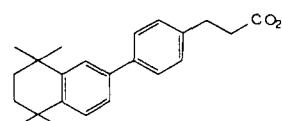
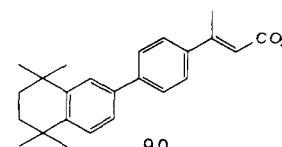
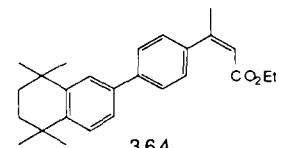
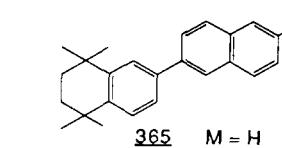
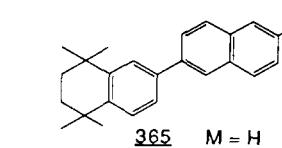


SRI 7101-27 7.5
SRI 7101-21 5.0

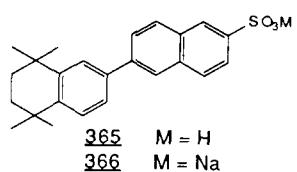


SRI 7101-53 9.5
SRI 7101-80 99

TABLE 18 (continued)
Retinoid Inhibition of Mouse Epidermal ODC Induction and Tumor Promotion by TPA

Retinoid	Code No.	ODC assay ID ₅₀ (nmol)	Antipapilloma assay ID ₅₀ (nmol)
 362	SRI 7101-94	7.7	
 363	SRI 7101-96	5.5	
 9.0	SRI 7101-94A	5.9	
 364	SRI 7101-94B	>170 (18)	
 365	SRI 5942-92X	>17 (0)	
 366	SRI 5942-92S	>17 (0)	

4. Polar terminus modifications



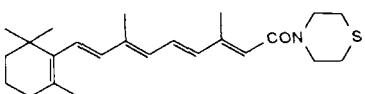
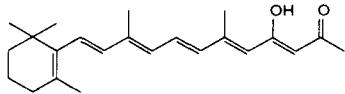
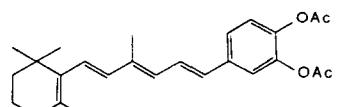
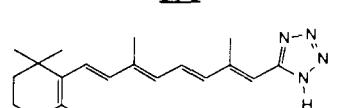
	<u>367</u>	R = CH ₂ O H	SRI 5442-11	26
	<u>368</u>	R = CH ₂ O Me	SRI 5442-12	2.9
	<u>369</u>	R = CH ₃	SRI 5193-67	>170 (34)
	<u>370</u>	R = H	SRI 5193-71	>170 (0)
	<u>371</u>	R = O H	SRI 5942-82	>170 (0)
	<u>372</u>		SRI 5442-60	>170 (39)

V. Polar terminus modifications

A. Derivatives

	<u>373</u>		SRI 4092-16	7.0
	<u>374</u>		SRI 4092-13B	23
	<u>375</u>		SRI 4092-13A	23

TABLE 18 (continued)
Retinoid Inhibition of Mouse Epidermal ODC Induction and Tumor Promotion by TPA

Retinoid	Code No.	ODC assay ID_{50} (nmol)	Antipapilloma assay ID_{50} (nmol)
	<u>376</u>	SRI 4092-45	>17 (16)
B. Carboxylic acid equivalents			
	<u>377</u>	SRI 6567-60	22
	<u>378</u>	SRI 4445-11D	20
	<u>379</u>	SRI 2712-79	>170 (2)

cisoid conformational restriction decreased activity appreciably; the 11*E*,13*E*- and 9*E*,11*E*,13*E*-cisoid conformations also decreased activity but not to such an extent. Only the 5,7*E*-cisoid conformation enhanced activity in the retinoids screened in Table 18.

Ring modifications in the RA series of analogs were made (1) to increase the lipophilic bulk of the ring in the attempt to enhance retinoid activity as was found for **65** and (2) to prevent metabolism at the 4-position of the retinoid skeleton¹⁰¹ by blocking hydrogen abstraction either by replacement of a proton at this position with an alkyl group (**92** and **261**¹⁰⁵) or by sterically hindering this position (**258** and **262**¹⁸⁷). Neither modification enhanced activity although replacement of the hydrogens at C₃ or C₄ by methyl groups appeared to have the least deleterious effect on activity. Decreasing the volume of the ring by replacing the trimethylcyclohexenyl ring with a norbornenyl ring (**263**)^{127,187} or increasing flexibility by cleaving the retinoid C₃–C₄ bond (**141**) decreased activity. Replacement of the retinoid C₁₉ and C₂₀ methyl groups on the side chain by phenyl rings (**266** and **269**,¹⁸⁸ respectively) also decreased activity.

Restricting the flexibility of the side chain by incorporation of selected bonds into aromatic ring systems had profound effects on activity. For example, in the 2-substituted benzoic acid **270**⁹⁶ the bond corresponding to the 13-double bond of 13-*cis*-RA is restricted to a cisoid conformation by inclusion in a phenyl ring, leading to loss of activity at the 17-nmol dose relative to 13-*cis*-RA. The 2-substituted *N*-decyl indole **271**,¹⁸⁷ which has a cisoid conformational restriction in the 5,7*E*-region of the skeleton, could be considered an analog of the active retinoid **88**.⁹³ The decyl group was introduced to provide the lipophilic bulk given by the methyl substituents on the tetrahydronaphthalene ring of **88**. Unfortunately, **271** was devoid of activity. The 7*E*,9*E*-cisoid conformational restriction (**272**)¹²⁷ abolished activity. In contrast, compounds having 11*E*,13*E*-cisoid geometry generally had good activity although reduced by at least an order of magnitude relative to that of RA. The activity of the parent 4-trienylbenzoic acid **64**, which was the first compound in this series reported,^{96–98} prompted the synthesis of analogs having modifications in the ring, dienyl side chain, and benzoic acid ring. At the 17-nmol dose, replacement of the ring double bond of **64** by a cyclopropane ring (**275**)¹⁰⁵ did not reduce activity in the ODC assay. Substitution of the 7*E*-double bond by a *trans*-substituted cyclopropane ring (**210**)¹²⁷ enhanced activity, as did saturation of the bond (**276**).¹²⁷ Incorporation of an electron-withdrawing fluorine on the side chain at the retinoid 10-position (**279**)¹⁶⁷ enhanced activity approximately twofold. Extending the conjugation of the side chain by the addition of another double bond (**282**)¹²⁷ decreased activity. Shifting the polar terminus on the aromatic ring from the position *para* to the side chain to one that was *meta* abolished activity (**284**),¹²⁷ as did placement of a methyl group on the ring *ortho* to the carboxylic acid group (**285**),¹²⁷ which corresponded to the C₂₀-methyl of RA. Activity was decreased fourfold when an acetoxy group was placed *ortho* to the polar terminus (**286**).¹⁸⁸ A *meta*-fluoro group on the ring had little effect on activity (**206**),¹⁶⁷ whereas the larger and more hydrophilic methoxy group decreased activity over twofold (**288**).¹⁶⁷ The phenyl ring of **64** was replaced by heterocyclic rings. The 5-substituted 2-furic acid **290**¹⁶⁷ was essentially inactive perhaps because of the instability of this compound in the epidermis. The related thiophenecarboxylic acid **292**¹⁶⁷ had activity comparable with that of **64**, whereas the 5-substituted pyridine-2-carboxylate **295**¹⁶⁷ was slightly less active.

Retinoid **65**, which has 5,7*E*- and 11*E*,13*E*-cisoid geometry, had activity comparable with that of RA in the ODC assay but was 25-fold more active at inhibiting tumor promotion. The enhanced activity of **65** in the latter assay may reflect its stability to catabolism by the retinoid pathway. Analogs of **65**, which is the most active retinoid of those tested in the hamster tracheal organ culture reversal of keratinization assay (Chapter 14), were synthesized (1) to determine the structural requirements for this high activity and (2) to determine the modifications necessary to reduce toxicity because we found that **65** was the most toxic

retinoid of those we have screened by i.p. injection in the mouse.¹¹⁸ Compounds in these series have 5,7*E*- and 11*E*,13*E*-cisoid restrictions of the side chain. Modifications of the tetrahydronaphthalene ring, propenyl side chain, and benzoic acid terminus of **65** were made. Aromatization of the tetrahydronaphthalene ring of **65** (**212**) decreased activity more than 50-fold, indicating that the four methyl groups on the ring must reside out of the plane of the ring to confer activity.

We determined the importance of these methyl groups on activity. Removal of one of the methyl groups at the 5-position of the tetrahydronaphthalene ring system (**297**) — corresponding to C₄ of the retinoid skeleton — decreased activity by one half. Testing of the enantiomers of **297** (**298** and **299**) indicated that both methyl groups had equivalent effects on ODC activity. This result contrasts with the tumor promotion study, in which the (S)-retinoid (**298**) was the more active. The difference in activity in the two assays may reflect differences in metabolic deactivation of the enantiomeric retinoids. Removal of both methyl groups at the 5-position (**300**)¹⁸⁹ decreased activity 36-fold. This result differs from the results in the RA series of compounds, in which substitution of methyl groups at the corresponding position on the cyclohexenyl ring decreased activity. Similar results were found in the activity of these retinoids in the reversal of keratinization assay (Chapter 14). These studies indicate that modifications in one region of the skeleton influence the effects of modifications in other regions, and therefore structure-activity correlations should be made with caution. Aromatization of the ring system of **300** (**302**) abolished activity, again supporting the idea that the methyl groups must reside out of the plane of the ring system to enhance activity. Removal of the methyl groups at the 8-position of the tetrahydronaphthalene ring of **65** (**304**) decreased activity 20-fold. A further reduction in lipophilic bulk is found in the benzonorbornene **214**,¹²⁷ which had 35% of the activity of **300** and was less active than **65** by two orders of magnitude.

Heterocyclic analogs of **65** were prepared beginning with the 6-substituted dihydro-2*H*-1-benzopyran **308**.¹²⁷ This compound and the corresponding dihydro-2*H*-1-benzothiopyran (**239**)¹²⁷ have a hetero atom at the retinoid 4-position, at which oxidative metabolism occurs.¹⁰¹ The more polar dihydrobenzopyran was less active than **300**, whereas the dihydrobenzothiopyran was 2.2 times more active. Although less active than **65**, dihydrobenzothiopyran **239** has a more favorable therapeutic index, being one of the least toxic retinoids on intraperitoneal injection in the mouse that we have found. Acyclic versions of these compounds were prepared. The methyl phenyl ether **216**, in which the retinoid C₂-C₃ bond is omitted, was over six times as active as **308** but had far higher toxicity. The 3-methylbutyl phenyl sulfide **312** had very low activity. The lack of activity could be due to the greater flexibility of the molecule in the ring region of the skeleton or to the higher rate of metabolic deactivation to the less active sulfoxide and sulfone. Molecular mechanics energy minimization established that the (3-methylbutyl)thio group adopted a chain-extended conformation and not the conformation illustrated in Table 18 because of steric interactions with the *ortho*-hydrogen of the aromatic ring. Therefore, this group cannot occupy the receptor site for the β-cyclogeranylidene ring of RA. The 2-substituted tetrahydrobenzothiophene **218**¹⁸⁵ had one third the activity of **65** but was eleven times more active than **300**, even though it lacks the geminal methyl groups on the ring in the position corresponding to the retinoid C₄. If a plane is drawn through **218** and **300** that passes through C₁ and C₄ of the benzoic acid ring and is perpendicular to that ring, the two methyl groups on the aromatic ring system of **218** occupy a different environment with respect to this plane than do the methyl groups of **300** — a fact that may account for the difference in ODC activity. Replacement of the C₈-methylene group of **304** with sulfur gave the 7-substituted dihydro-2*H*-1-benzothiopyran **313**, which had activity comparable with that of the 6-substituted dihydrobenzothiopyran **239**.

Testing results on side chain-modified analogs of **65** indicate that the effects of structure

on the orientation of the two aromatic ring systems may have a more profound influence on activity than the substituents themselves have. For example, shifting the position of the methyl group of the side chain from the retinoid C₉ to C₁₀ (**316**) did not affect activity. Similar results were found on comparison of the activities of dihydrobenzothiopyran **239** and its methyl-shifted analog **328**. In contrast, two *trans*-methyl groups on the double bond joining the two aromatic rings (**318**), which restrict rotation about both the retinoid C₈–C₉ and C₁₀–C₁₁ bonds, decreased activity by over three orders of magnitude. Replacement of the propenyl group by a *trans*-1,2-disubstituted cyclopropane ring (**319**) decreased activity by two orders of magnitude. In contrast, the 5- and 7*E*-double bonds can be replaced by cyclopropane rings without loss of activity. Evidently, the retinoid C₈–C₁₁ bond system must have a planar orientation to confer high activity. These results are supported by the lack of activity of the benzonorbornenes **324** and **325**,¹²⁷ which also have a cyclopropane ring joining the aromatic systems. In the benzonorbornene series, it was also found that replacement of the propenyl side chain by a 1,2-*trans*-substituted vinyl group (**326**)¹²⁷ decreased activity by over an order of magnitude, indicating that either a lipophilic substituent on the double bond is required for activity or that enhanced rotation of the ring systems relative to one another decreases activity. In the 2-substituted 5,6,7,8-tetrahydro-5,5,8,8-tetramethylanthracene **320** only rotation about the retinoid C₁₀–C₁₁ bond is possible. This compound has the bonds corresponding to the 7*E*- and 9*E*-double bonds of the retinoid skeleton held in a transoid conformation by the tetrahydroanthracene ring. It had one half the activity of **65**. For the related 1*H*-naphtho[2,1-*b*]thiopyran **329** activity was reduced almost three orders of magnitude. Fluorine substitution at the retinoid 10-position led to loss of activity (**220**, **330**, and **331**). In contrast, Pawson et al.⁸⁹ reported that retinoids having fluorine at the retinoid 10-position had enhanced activity in the papilloma regression assay. Replacement of the propenyl groups of **239** by a carboxamide (**332**) resulted in a large drop (>340-fold) in activity. The 4-substituted 3-azidobenzoic acid **333** was designed as a photoaffinity label to study the putative retinoid receptor. Unfortunately, the azido group on the phenyl ring *meta* to the carboxylic acid group decreased activity 270-fold compared with that of **65**.

The 4'-substituted 4-carboxybiphenyl retinoid **222**¹²⁷ has the 7*E*,9*E*- and 11*E*,13*E*-cisoid geometry that decreased activity almost 600-fold relative to RA and sixfold relative to **64**. The 9*E*,11*E*,13*E*-cisoid conformational restriction (**208**)¹²⁷ also reduced activity (over 500-fold relative to RA). Incorporation of a *gem*-dimethyl group on the ring at the retinoid 4-position (**334**) enhanced activity eightfold. The naphthalene ring of **208** was replaced by benzothiophene rings to optimize the geometry between the carboxyl group and the trimethylcyclohexenylvinyl group. Replacement by a 6-substituted 2-benzothiophenecarboxylic acid (**335**) enhanced activity about twofold, whereas replacement by a 2-substituted 6-benzothiophenecarboxylic acid (**209**) decreased activity twofold, implying that activity is enhanced when the *gem*-dimethyl group lies close to the plane through C₂ and C₆ of the aromatic ring system. The analog of **208** having a tetramethyltetrahydronaphthalene ring in place of the trimethylcyclohexenylvinyl group (**242**)^{127,176,190} was an order of magnitude more active than **208**. Because this 6-substituted 2-naphthalenecarboxylic acid showed high activity that was comparable with those of RA and **65** in the tracheal organ culture reversal of keratinization assay (see Chapter 14), analogs having modifications of the tetrahydronaphthalene and naphthalene rings and polar terminus were synthesized to determine what structural parameters enhanced activity. Incorporation of a methyl group at the 3-position of the tetrahydronaphthalene ring (**338**)¹⁸⁹ enhanced activity fivefold. A similar enhancement of activity in the HL-60 cell differentiation assay was found in the corresponding analog of **65**.¹⁹⁰ We have not established whether this activity enhancement is due to enhanced binding of the retinoid to its receptor because of restricted rotation about the retinoid C₈–C₉ bond or is the result of its increased solubility in the assay medium.

As in the benzoic acid retinoid series, we studied the influence of the tetrahydro-

naphthalene ring methyl groups on activity. Removal of one methyl group at C₅ (**340**) did not decrease activity, whereas removal of both methyl groups (**341**)¹⁸⁸ decreased activity threefold and aromatization of the ring system of **341** (**342**) abolished activity, again supporting the concept that the ring methyl groups must reside out of the plane of the ring system in a region close to the plane through C₂ and C₆ of the naphthalene ring. The 6-substituted dihydrobenzopyran and -thiopyran and 2-substituted dimethyltetrahydrobenzo-thiophene analogs (**343**,¹⁸⁸ **345**,¹⁸⁸ and **244**, respectively) were also screened. These heterocyclic naphthalenecarboxylic acids were less active than their analogs in the benzoic acid series. In fact, all the 6-substituted naphthalenecarboxylic acids were less active than the corresponding 4-substituted benzoic acids; however, the relative decrease in activity was not consistent throughout the series of compounds examined.

The tertiary and secondary azido retinoids **347** and **250**, respectively, were prepared as photoaffinity probes. Although these compounds are alkyl azides, which generally are poor photoaffinity labels, their high biological activity, which was above that of **242**, and *in vitro* trapping experiments of the nitrenes generated on photolysis indicated that they might have potential for this use. On photolysis, in addition to reacting with the solvent, the azides underwent intramolecular reactions to give benzazepines and the tetralone **349**. These compounds were also screened to determine their effects on the bioassays; if they were more active than the azide, they could interfere with binding by the azide to the retinoid receptor. Benzazepines **351** and **353** were inactive; **349**, the related ethyl ester (**249**), and the reduction product tetralol **350** had low activity. Unfortunately, tritiated azides **250** and **347** did not covalently label cellular retinoic acid-binding protein on irradiation of the retinoid-protein complex.

Methyl groups were placed on the naphthalene ring of **242** in positions corresponding to those that the retinoid C₂₀ methyl occupies in the two 11*E*,13*E*-cisoid conformations of RA. The 6-substituted 1-methyl-2-naphthalenecarboxylic acid **354** was more active than **242**, whereas the 3-methyl analog (**355**) was less active. However, the activity difference was so small that no conclusion can be drawn about the optimum conformation at the receptor. Methyl groups were also substituted at the 5- and 7-positions of the naphthalene ring (**356** and **357**, respectively) — positions that would restrict rotation about the retinoid C₈–C₉ bond joining the rings. Both substitutions decreased activity, with that at the C₅ having the larger effect. A similar retinoid 10-substitution in the benzoic acid series (**318**) also decreased activity.

The effect of increasing the flexibility of the ring system adjacent to the polar terminus was assessed by removing C₃ and C₄ from the naphthalene ring (**90**, and **358** to **364**). This modification decreased activity even when a methyl group corresponding to the retinoid C₂₀ methyl was incorporated onto the propenyl side chain (**90**). The 3-substituted propynoic and propanoic acid analogs **362** and **363**, respectively) had activity comparable with that of the 3-substituted propenoic acid **358**.

Polar terminus-modified analogs of **242** were also prepared. The 6-substituted naphthalenesulfonic acid (**365**) and its sodium salt (**366**) at the 17-nmol dose were inactive in the ODC assay. This lack of activity was also found with the 4-substituted benzenesulfonic acid sodium salt **75**.⁹⁹ Other 2,6-disubstituted naphthalenes (**367** to **369**)¹⁸⁸ were synthesized as prodrugs that could be metabolized to **242**. The 6-substituted 2-naphthalenemethanol **367** had 0.08 the activity of **242**, whereas its more lipophilic methyl ether **368**, which first would be cleaved to the alcohol that would be oxidized to the carboxylic acid, had activity approaching that of **242**. The 2-methylnaphthalene **369** had very low activity. The decarboxylated analog of **65** (**84**), which is patented for the treatment of acne,¹⁹¹ is a prodrug that could undergo oxidative metabolism to the corresponding 4-substituted phenol. The related naphthalene **370**¹⁸⁰ and 2-naphthalenol **371** were inactive. Shifting of the carboxylic acid group of **242** from the 2- to the 3-position of the naphthalene ring (**372**) eliminated activity,

indicating that both the geometry and the distance between the lipophilic and polar termini are important.

The polar terminus of RA was modified to afford prodrugs that could be cleaved to release the active retinoid *in vivo* (373 to 376) and to provide compounds that had other acidic groups in place of the carboxylic acid group (377 to 379). The pentaerythritol ester of RA (373) was prepared as a polar derivative and proved to be less active than the ethyl ester of RA (32). Its less-polar benzal acetals (374 and 375) were three times less active. The thiomorpholinyl amide of RA (376) had low activity in the ODC assay. This result was expected because the epidermis lacks the enzymes required to cleave amides. The keto-enol 377 and the 4-substituted catechol diacetate 378 were far less active than RA.¹⁸⁸ Both of these compounds were less acidic than RA. The 5-substituted tetrazole 379¹⁸⁸ has a pK_a comparable with that of RA but was inactive.

E. CORRELATION OF RETINOID INHIBITORY ACTIVITIES IN THE ORNITHINE DECARBOXYLASE AND TUMOR PROMOTION ASSAYS

Twenty-five of the retinoids in Table 18 were screened for their ability to inhibit tumor promotion in CD-1 mouse epidermis initiated with DMBA and promoted over a 20-week period with TPA. These retinoids had a range of activities in the ODC assay and were members of the polyenoic, benzoic acid, and naphthalenecarboxylic acid polar-terminated classes. Compounds that were effective inhibitors of the induction of ODC also proved to be effective inhibitors of tumor promotion; however, in all cases, higher doses of retinoids were required to cause a 50% response in the tumor-promotion inhibition assay. Linear regression analysis of the ID_{50} values for these retinoids at inhibiting ODC and tumor promotion using a 8.5-nmol dose of TPA gave an excellent correlation ($y = 0.05342x + 0.69002$, $r = 0.67277$, $p < 0.001$, $n = 22$), indicating that the ODC assay can be used as a rapid *in vivo* assay to predict the potential ability of retinoids to inhibit tumor promotion. These results support and extend the work of Verma et al.⁴ on the correlation of retinoid activities in the two assays (see Table 2) by employing ID_{50} values rather than single concentrations, a larger sample of retinoids, and retinoids having high, moderate, and low activities.

Retinoids that were screened in the tumor promotion assay were also screened for toxicity in adult female Swiss-Webster mice (20 to 22 g) by intraperitoneal injection of a homogenized mixture of retinoid in 0.2 ml of an aqueous solution of 8% Cremophor EL® (Sigma) and 10% propylene glycol (Sigma) on weekdays over the course of 2 weeks. Animals were scored according to the hypervitaminosis A toxicity protocol⁸⁸ on days 5, 7, 10, and 15. Retinoids that were highly active at inhibiting tumor promotion ($ID_{50} < 1.0$ nmol) also caused toxic side effects, including weight loss, skin redness and scaling, fur loss, and decalcification of bones. Unfortunately, we were unable to make a quantitative assessment of toxicity using this protocol on this strain of mice because of the variability of response of the animals to each of the toxicity parameters with different retinoids. Therefore, toxicity was assessed by weight loss and by determining LD_{50} levels. Active retinoids having a carboxylic acid terminus were correspondingly toxic — a finding that is understandable because retinoids do control cell differentiation and the toxicity symptoms are manifestations of the effects of retinoids on cell differentiation. Retinoids that are highly active on one cell system may also be active on others. These results underscore the need for the development of retinoid prodrugs that could be metabolized or converted *in vivo* to the active form, which then could be rapidly catabolized to less active, less toxic compounds.

IV. SUMMARY AND CONCLUSIONS

Both the naphthalenecarboxylic acid and benzoic acid-terminated retinoids have advantages over the polyenoic retinoids in that, lacking the highly conjugated double-bond system,

they are more stable to both light and oxygen. Therefore, they are far more easy to manipulate synthetically and to use in biological studies. Consequently, our work has concentrated on the effects that structural modifications in these two series have on biological activity.

The *in vivo* ODC assay is a reliable, rapid method for predicting the ability of retinoids to inhibit the promotion of tumors by both potent promoters such as TPA and weak promoters such as anthralin.¹⁹² Our work and that of Verma et al.⁴ have established that the ability of retinoids to inhibit the induction of ODC correlates well with their ability to inhibit tumor promotion. Test results indicate that portions of the retinoid skeleton can be restricted to particular conformations by inclusion in restrained systems, with either loss or enhancement of activity. This information provides evidence that retinoic acid adopts a particular conformation at its active site, in which the hydrophobic head and polar terminus have a definite spatial orientation. Optimum interaction with the receptor requires (1) a hydrophobic head having three-dimensional geometry rather than lipophilic groups oriented in a plane and (2) a spacer or side chain joining the hydrophobic head with the polar terminus having that portion of the bond system corresponding to the retinoid C₈—C₁₁ bonds that is able to adopt a planar conformation. Modifications of the skeleton are interrelated rather than additive because modifications in one region may influence the effect that modifications have on other regions of the skeleton, making predictive structure-activity analysis difficult. Test results indicate both the potential of retinoids for controlling diseases of cell differentiation and the need for the development of new compounds that, instead of having high inherent activity and accompanying toxicity, are prodrugs that are converted to the active form *in vivo*. New, conformationally restricted and photoaffinity label-bearing retinoids will be valuable probes for determining the geometry of the retinoid receptor and elucidating the mechanism of retinoid action.

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Chapter 17

ROLE OF RETINOIDS IN PREVENTION OF CUTANEOUS CANCER**Helen L. Gensler and Frank L. Meyskens, Jr.****TABLE OF CONTENTS**

I.	Introduction	468
II.	Possible Mechanisms of Anticarcinogenesis	468
III.	Cellular Responses to Retinoids.....	470
	A. Prevention of Transformation.....	470
	B. Antiproliferative Effect upon Malignant Cutaneous Cells.....	472
	C. Molecular Mechanisms of Growth Control by Retinoids	472
IV.	The Mouse Skin Model of Tumorigenesis	473
	A. Effects of Retinoids on Tumor Initiation or Promotion	474
	B. Influence of the Mode of Administration on Retinoid Tumor Prevention	474
	C. Duration of Tumor Prevention.....	475
V.	Clinical Investigations	475
	A. Actinic Keratosis	475
	B. Keratoacanthoma	476
	C. Basal Cell Carcinomas	476
	D. <i>Epidermolympiasis verruciformis</i>	476
	E. Dysplastic Nevi	476
VI.	Side Effects and Toxicity	477
VII.	Summary	478
	Addendum.....	478
	Acknowledgments	479
	References.....	479

I. INTRODUCTION

The prevention of chemically induced skin tumors in mice by vitamin A was reported as early as 1943.¹ However, its use as an anticarcinogenic agent was curtailed by toxic side effects. The need for effective antitumor agents has stimulated efforts to synthesize analogs with (1) inhibitory effects on neoplasia, (2) reduced side effects, and (3) targeted tissue affinity.

The basic structure of the retinoid molecule includes a cyclic end group, a polyene chain, and a polar end group, as shown in Figure 1. Modifications of different components of the basic structure have led to changes in the activities of the resultant molecules.² The retinoids that have been most extensively characterized for antitumor activity include vitamin A (retinol) and its esters, retinoic acid (vitamin A acid, or Tretinoin), 13-*cis*-retinoic acid (Isotretinoin, or Accutane), and an aromatic ethyl ester derivative of retinoic acid (Etretinate, or Tigason). Recently, two additional classes of synthetic retinoids, retinamides and retinoidal benzoic acid derivatives, have entered clinical trials.^{2,3}

The natural dietary sources of vitamin A occur in two forms, namely, (1) the provitamin β -carotene, which is found chiefly in vegetables, and (2) vitamin A alcohols, aldehydes, and esters, which are found in meats and dairy products or as pharmaceutical preparations. Ingested β -carotene is largely converted to retinol upon absorption through the intestinal mucosa. Retinol is transported in the plasma by lipoproteins, and the majority is stored in the liver as retinyl esters. Retinoic acid, however, is absorbed primarily through the venous portal route, metabolized, and eliminated without hepatic storage. Mobilization of retinyl esters from the liver involves hydrolysis to retinol and transportation in the blood in a complex consisting of retinol-binding protein, transthyretin and thyroxine.⁴ Retinol is metabolized to functional forms in the target tissues. Oxidation can lead to formation of retinoic acid, which may be further metabolized to both known and unknown metabolites.⁵ One of the known metabolites is 13-*cis*-retinoic acid⁶ and this retinoid can be isomerized to all-*trans*-retinoic acid in tissues of rats.⁶ We have found that retinol and retinyl palmitate accumulated in a dose-dependent manner in liver, skin, subdermal fat, and in chemically induced skin tumors of mice fed with increased levels of dietary retinyl palmitate for 25 weeks.⁷ There was tissue specificity in the extent of retinoid accumulation; for example, serum did not accumulate significant levels of retinoids, but appeared to be under homeostatic regulation.

II. POSSIBLE MECHANISMS OF ANTICARCINOGENESIS

Current information suggests that chemical carcinogenesis is a multistage process.⁸⁻¹⁰ The events in carcinogenesis are depicted in Figure 2. The first step, initiation, is an irreversible change resulting from a subthreshold dose of a carcinogen and is characterized by single hit kinetics. Evidence in the mouse skin system that this step is a mutation will be presented below. There is no morphological distinction between initiated and normal cells. The promotion stage of tumorigenesis requires repetitive treatment with an agent that does not induce tumors in uninitiated tissue. Promotion is reversible for a period of time, later becoming irreversible. The molecular mechanism underlying promotion has not yet been defined, although sustained hyperplasia appears to be a necessary but not sufficient requirement. If terminal differentiation and programmed cell death is inhibited in the initiated cells, as is suggested by the growth characteristics of benign papilloma cells in culture,¹¹ then clonal expansion of initiated cells will result from promotion. Such clonal expansions form benign tumors, or preneoplastic lesions. Progression of preneoplastic lesions to irreversible malignant lesions is yet another step in the carcinogenic pathway. Based on the finding that malignant conversion of benign mouse skin tumors is increased by tumor initiators

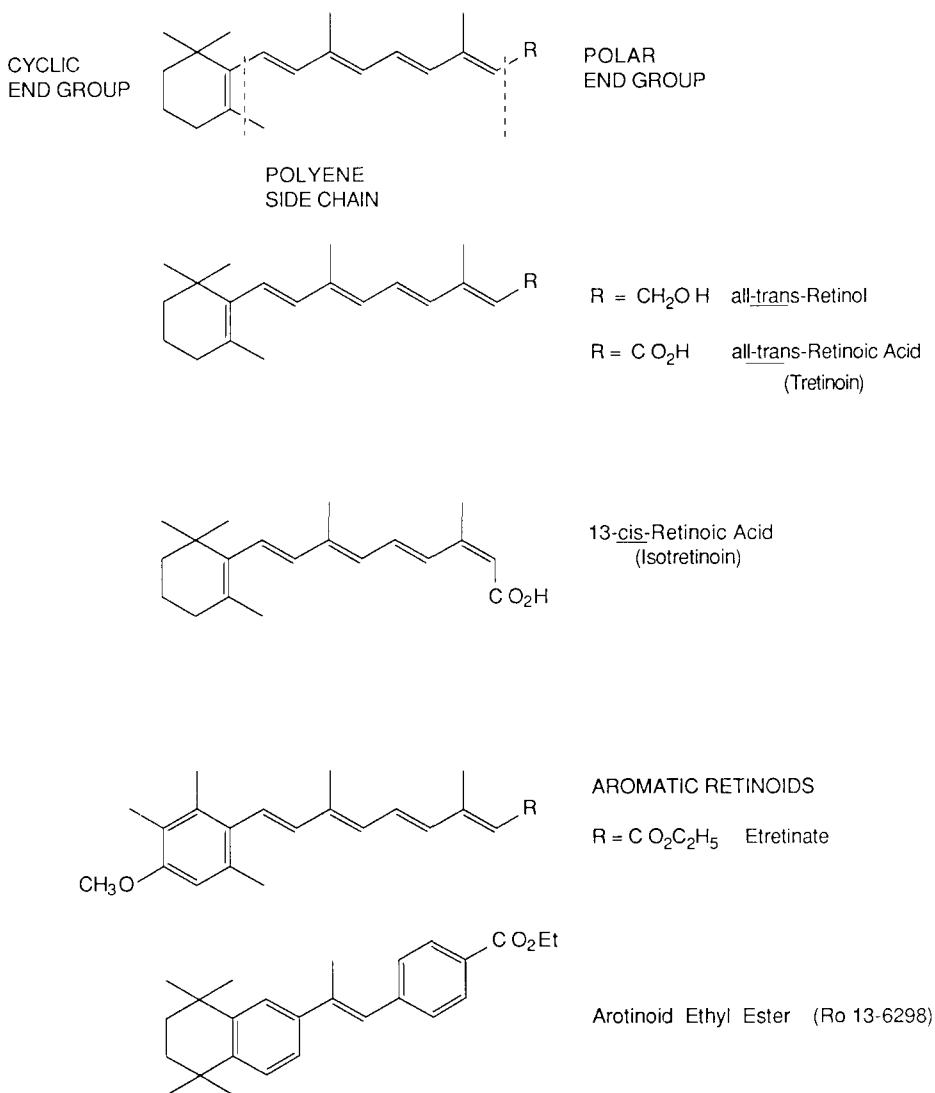


FIGURE 1. Chemical structures of retinoids.

and unaffected by tumor promoters, this step is thought to involve a mutation.¹² Acquisition of the capacity for metastasis is a further change that occurs in some malignant cells. This step involves activation of genes that encode adherence proteins, proteases and locomotion factors. The frequency of metastatic conversion is tumor-type dependent, e.g., melanoma has a high incidence of metastasis, whereas basal cell carcinomas rarely metastasize. Interference with any requisite step in the carcinogenic pathway can effectively achieve prevention of cancer. As we have discussed in detail previously,¹³ primary prevention interferes with the initiation step, secondary prevention interferes with a step after initiation, but before preneoplasia, and tertiary prevention represents treatment of preneoplastic or premalignant lesions with production of regression or curtailment of progression.

The natural retinoids are pleiotropic in their action so that it is likely that more than one of their effects are involved in the total chemopreventive capacity. Retinoid activities that may be anticarcinogenic include (1) modulation of genetic expression,¹⁴⁻¹⁶ (2) induction of differentiation,^{17,18} (3) reduction of cell proliferation,¹⁹⁻²¹ (4) inhibition of the release of

Event

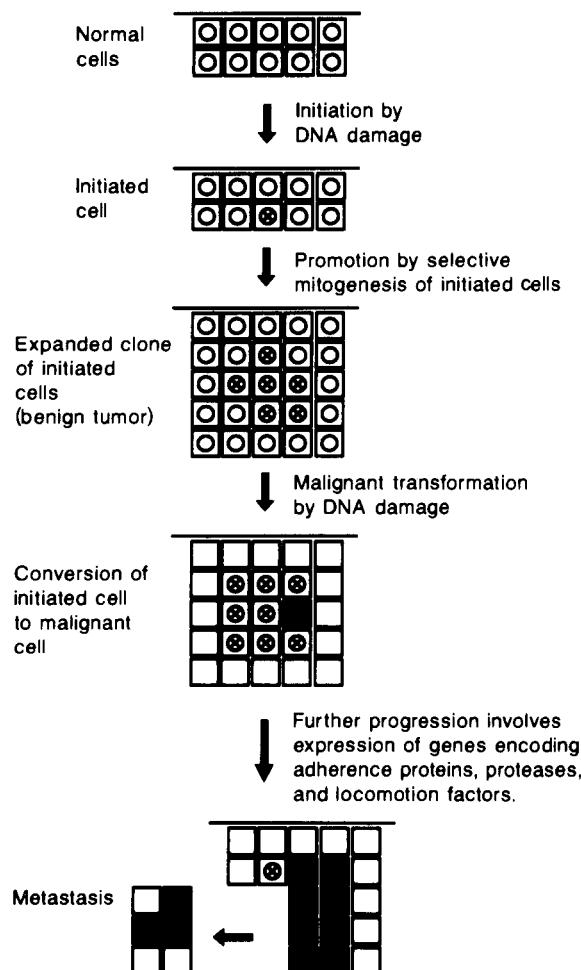


FIGURE 2. Diagram of events in multistep carcinogenesis.

reactive oxygen species by leukocytes stimulated with tumor promoters,²² and (5) enhancement of immunological surveillance.^{23,24} More than 2000 synthetic retinoids have been made, with selected structural modifications, and are being tested for correlation of these activities with anticarcinogenic potential and low toxicity.

III. CELLULAR RESPONSES TO RETINOID

A. PREVENTION OF TRANSFORMATION

Using the mouse skin model of initiation and promotion, Verma et al.²⁵ demonstrated that retinoic acid, 13-*cis*-retinoic acid and analogs of ethyl retinoate prevented phorbol ester-induced tumor promotion, but did not influence tumor initiation by 7,12-dimethylbenz[a]anthracene. To study the mechanism by which retinoic acid (β -all-*trans*) prevents tumor promotion, we have used an *in vitro* cell model (JB6 Cl 41 mouse epidermal cell line). This line was developed by Colburn as a cellular model for tumor promotion.^{26,27} These cells, derived from neonatal BALB/c primary epidermal cultures, acquired the heritable capacity to respond to phorbol esters and late tumor promoters with induction of anchorage

independence and tumorigenicity, i.e., "transformation". This transformation was sensitive to inhibition by retinoids.^{19,28}

To determine if retinoic acid was preferentially cytostatic for transformed cells, we measured the effect of a 7-d exposure to the retinoid on anchorage-dependent colony formation by untransformed and previously transformed JB6 cells. The extent of suppression of proliferation was similar, therefore retinoic acid was not preferentially cytostatic for transformed cells.¹⁹ The next potential mechanism of promotion-prevention considered was selective cytotoxicity for transformed cells. JB6 cells that had been previously transformed were cultured in the presence or absence of retinoic acid for 3 d, and were then assayed for growth in soft agar without retinoic acid. At concentrations of retinoic acid known to prevent transformation of JB6 Cl 41 cells, no inhibition of anchorage-independent colony formation was evident.¹⁹ Thus, prevention of *in vitro* "promotion" by retinoic acid was not mediated via preferential cytotoxicity for transformed cells.

Another potential means to prevent promotion is inhibition of transformation. To determine the capacity of retinoic acid to prevent the acquisition of the transformed phenotype, JB6 Cl 41 cells that had been previously transformed, or untransformed JB6 Cl 41 cells plus a promoter, were plated in soft agar containing retinoic acid.¹⁹ Our rationale for this experiment was that prevention of transformation should be manifested by a reduction in soft-agar-colony yield. Inhibition of proliferation should also reduce colony formation. If retinoic acid acted to prevent transformation as well as to reduce proliferation, JB6 cells undergoing both transformation and growth in the presence of retinoic acid should have a greater inhibition of colony formation than previously transformed JB6 cells undergoing only growth in the presence of retinoic acid. However, the inhibition of colony formation was similar in these two cases. Thus, no evidence for an antitransformation effect by retinoic acid was found in JB6 cells. The antiproliferative activity of retinoic acid was sufficient to explain its antipromoter effect in this system.

Retinoids have also been shown to inhibit neoplastic transformation in other cell types, such as a mouse fibroblast cell line (C3H/10T 1/2). In this system, cells from log-phase cultures were plated at a low density in growth medium, and treated after 24 h with a carcinogen during 24 h of proliferation. Cultures became confluent and growth arrested by day 10.²⁸ At this time treated cultures looked indistinguishable from control cultures. At 4 to 5 weeks after passage, small foci of morphologically transformed cells appeared. When cells from these foci were cloned and injected into immunosuppressed syngeneic animals, about 90% developed into sarcomas. Merriman and Bertram found that all-*trans*-retinyl acetate, all-*trans*-retinol, and all-*trans*-retinal were essentially equipotent in inhibiting 3-methylcholanthrene-induced transformation of C3H/10T1/2 clone 8 cells.²⁹ This inhibition was reversible as cell growth resumed after removal of the retinoids from the medium. All-*trans*- and 13-*cis*-retinoic acids enhanced rather than decreased transformation in this 10T1/2 system.³⁰

Certain retinoids are also able to prevent cell transformation induced by sarcoma growth factors (SGFs).³¹ These molecules are low-molecular mass, acid- and heat-stable polypeptides isolated from the conditioned medium of Maloney sarcoma virus-infected mouse 3T3 cells.³² Growth factors from such conditioned medium compete with epidermal growth factor for binding to membrane receptors and reversibly confer the ability for anchorage-independent growth on normal rat kidney cells. Growth in soft agar, i.e., anchorage-independent growth, is a phenotype frequently associated with tumorigenicity,³³ and constitutes a major *in vitro* assay for transformation. Todaro et al.³¹ plated logarithmically growing normal rat kidney fibroblasts at low density in 0.3% soft agar containing SGF with and without retinyl acetate, retinylidene dimedone, or retinyl methyl ether. Each of these retinoids was effective at blocking the SGF-mediated transformation.

B. ANTIPIROLIFERATIVE EFFECT UPON MALIGNANT CUTANEOUS CELLS

The nature of retinoid inhibition of growth of malignant cutaneous cells has been studied in detail in murine melanoma Cloudman S91 cells, which were among the first cell lines found to be growth-inhibited by retinyl acetate and retinoic acid.²¹ Treatment of S91 cells with retinoic acid inhibited proliferation, within 48 to 72 h, in a dose-dependent fashion. Analysis of the DNA distribution by flow cytometry indicated that a 5-d treatment with retinoic acid resulted in an increase in the proportion of cells in the G₁ phase of the cell cycle.³⁴ A general decrease in the rate of RNA and protein synthesis may have caused the G₁ block.³⁵

Cells from human tumors, cloned in soft agar, have been used for growth inhibition studies. Anchorage-independent growth of cells from biopsies of human melanoma tissue was generally inhibited by 13-*cis*-retinoic acid, retinoic acid, retinol, or etretinate.³⁶ However, the growth responses of melanoma biopsy cells to noncytotoxic concentrations of retinoids (<10 μM) were varied, and appeared to be donor- and retinoid dependent.^{36,37} Heterogeneity of cells from human breast, lung, and ovarian carcinoma biopsies in response to 13-*cis*-retinoic acid or *N*-(4-hydroxyphenyl)-all-*trans*-retinamide was also demonstrated.³⁸ Thus, because of the heterogeneous response of tumor cells to retinoids, each tumor had to be assayed individually for its susceptibility to retinoid growth inhibition. A means of overcoming cellular differences in susceptibility to single agents would be to develop a combination of therapeutic drugs that would override individual variations. A study in our laboratory to test this possibility was successful.³⁹ Human melanoma cell lines that were resistant to single-agent treatment with dexamethasone, α-interferon, or retinoic acid were treated with a combination of these agents plus α-difluoromethylornithine. Suppression of colony formation averaged 90%, indicating a synergistic effect. After this treatment, the cells were able to metabolize tetrazolium suggesting a cytostatic rather than a cytotoxic arrest. In a further study of drug combinations, the anchorage-independent growth of murine melanoma S91 cells and a human melanoma cell strain was inhibited by dexamethasone (DEX), prostaglandin A (PGA), or retinoic acid (RA), in a dose-dependent fashion.²⁰ Combinations of dexamethasone and retinoic acid yielded a synergistic reduction of colony formation. Addition of PGA to the DEX-RA combination resulted in further reduction of colony yield. Thus, the proliferative capacity of human and murine melanoma cells may be simultaneously controlled by dexamethasone, prostaglandin A, and retinoic acid.

The influence of retinoids on growth of cutaneous tumors *in vivo* will be considered below.

C. MOLECULAR MECHANISMS OF GROWTH CONTROL BY RETINOID

The means by which retinoids affect cells has been under active investigation for a decade. Chytil and Ong⁴⁰ first identified specific cellular retinol- and retinoic acid-binding proteins, designated CRBP and CRABP, respectively, in cytosolic preparations from a number of organs. These binding proteins have been purified and are not tissue specific.⁴⁰ Chytil and Ong⁴⁰ proposed that these binding proteins act like steroid receptors in that they transport retinoids into the nucleus, where they bind to the chromatin and modulate gene transcription. Purified [³H] retinol-CRBP has been found to bind specifically to isolated rat liver nuclei,⁴¹ and, further, can lead to specific binding of the retinol to isolated chromatin.⁴² These results are consistent with the hypothesis that the binding of retinol to sites in chromatin is important or related to the mechanism for retinoid modulation of gene transcription.

Since retinoid-binding proteins may mediate retinoid-dependent anti-tumor and antiproliferative effects, Lotan developed an *in vitro* system to study the mechanism of retinoid action on S91 melanoma cells.⁴³ He found that the cells contained CRBP and CRABP at levels of 720 and 430 pmol/10⁹ cells, respectively. Among the retinoic acid analogs that had a free carboxyl group at carbon 15, only those which bound to CRABP inhibited S91

cell proliferation. Retinoic acid-resistant mutants, induced by ethyl methanesulfonate, were found to produce no significant reduction in retinoic acid uptake. Some of the resistant clones contained CRABP at similar or higher concentrations than control S91 cells, indicating that the presence of this protein was not sufficient to make cells responsive to retinoic acid.

Another potential mechanism for retinoid control of cell proliferation is retinoid prevention of promoter-induced cellular activities. 12-O-Tetradecanoylphorbol-13-acetate (TPA) and other promoters, such as teleocidin and aplysiatoxin, activate protein kinase C.⁴⁵⁻⁴⁸ This activation is similar to that which ensues upon binding of platelet-derived growth factor (PDGF) to its receptor in the cell membrane.⁴⁹ PDGF binding leads to induction of phospholipase, which breaks down phosphatidyl inositol bisphosphate to release diacylglycerol, which directly activates protein kinase C.⁵⁰ TPA can compete with diacylglycerol for activation of protein kinase C.⁵¹ Among the cellular genes whose expression is enhanced by TPA are a group of "competence" genes that are also induced by PDGF.⁵² These include *c-myc*, *c-fos*, β -actin, and ornithine decarboxylase (ODC). Exposure of quiescent fibroblasts to PDGF moves the cells out of the G_0 phase of the cell cycle and into G_1 .⁵³ Further stimulation with epidermal growth factor and somatomedins is necessary for completion of the G_1 phase. Therefore, tumor promoters induce the same gene(s) as does PDGF, which keep(s) fibroblasts in the division cycle rather than in a quiescent state.

It is significant that ODC is one of the "competence" genes. α -Difluoromethylornithine (DFMO) is a specific inhibitor of ODC activity and prevents tumor promotion by TPA.⁵⁴ Thus, prevention of TPA-induced ODC activity by DFMO is sufficient to prevent promotion by TPA. A correlation has been found between the ability of selected retinoids (retinoic acid, 13-cis-retinoic acid, and analogs of ethyl retinoate) to prevent phorbol ester-induced ODC activity and to inhibit tumor promotion.²⁵ A number of reports have presented convincing evidence suggesting that retinoic acid can block TPA-induced activation of protein kinase C.^{55,56}

The mechanism whereby TPA can regulate gene expression is under investigation. TPA has recently been found to activate the SV40 enhancer.⁵⁷ Enhancers are regulatory DNA elements, usually about 200 base pairs long, which are able to stimulate transcription of linked genes in a manner largely independent of distance and orientation.⁵⁸ Specific protein binding is responsible for the SV40 enhancer activity in an *in vitro* model. The binding proteins are cell-type specific and are thought to be involved in enhancement of transcription.⁵⁹ The model of tumor promoter control of gene expression suggested by the current findings is (1) the binding of a tumor promoter, (2) activation of protein kinase C, (3) phosphorylation of a *trans*-acting transcription factor, (4) binding of the phosphorylated factor to specific *cis*-acting regulatory regions of DNA, and (5) increased genetic expression of the specific genes so regulated.

IV. THE MOUSE SKIN MODEL OF TUMORIGENESIS

Tumor formation in mouse skin after exposure to chemical agents has been the primary model system leading to the biological principles of multistage carcinogenesis. The events in multistage carcinogenesis are depicted in Figure 2. Skin tumors can be induced by the sequential application of a subthreshold dose of a carcinogen (initiation) followed by repetitive treatments with a noncarcinogenic promoter. The initiation phase requires only a single application of a genotoxic agent, and is essentially irreversible, while promotion involves many treatments after initiation and is reversible for a period of time. Initiation is likely to be a somatic mutation, that is, an irreversible change in the nucleotide sequence of the DNA. Recent studies in Balmain's laboratory have shown that over 90% of mouse skin tumors induced by initiation with 7,12-dimethylbenz[a]anthracene (DMBA) and promoted with TPA, have an A→T transversion in the 61st codon of the *c-Ha-ras* protoon-

cogene.⁶⁰ The frequency of this mutation is dependent on the initiating, but not on the promoting, agent used. Balmain has also shown that introduction of an activated *Ha-ras* gene via Harvey sarcoma viral infection into mouse skin cells *in vivo* led to an initiated phenotype.⁶¹ Tumors developed only in those mice that subsequently received promotion by topical TPA treatments.

Promotion is the most reversible stage in the carcinogenic pathway, and is, therefore, of interest for potential clinical manipulation. Current hypotheses concerning the role of promotion in tumorigenesis include: (1) selective expansion of initiated cells,⁶² perhaps by induction of differentiation of uninitiated cells,^{63,64} (2) relatively slow induction of DNA damage,⁶⁵⁻⁶⁷ or (3) gene amplification.⁶⁸ Obviously, these roles are not mutually exclusive.

A. EFFECTS OF RETINOIDS ON TUMOR INITIATION OR PROMOTION

We have been interested in the efficacy and mechanism of retinoid prevention of mouse skin tumorigenesis. 13-*cis*-Retinoic acid was found to have no influence upon mouse skin initiation by benzo[a]pyrene, a polycyclic aromatic hydrocarbon that requires activation by enzymatic means or by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, an alkylating agent, which does not require enzymatic activation.⁶⁹ These findings extended the results of Verma et al.²⁵ who found that retinoic acid did not influence initiation by DMBA. In contrast, a number of retinoids, including retinoic acid, 13-*cis*-retinoic acid, and analogs of ethyl retinoate, have been found to be effective in the prevention of mouse skin tumor promotion by TPA.^{25,44} This activity correlated with the inhibition of TPA-induced ornithine decarboxylase in the mouse epidermis.²⁵ For example, topically applied 13-*cis*-retinoic acid was less effective than retinoic acid in prevention of TPA-induced tumor promotion and ODC activity. The effectiveness of retinoid chemoprevention is promoter-dependent as well as analog-dependent. Benzoyl peroxide has been found to be an effective tumor promoter in SENCAR and in C57BL/6 mice.⁷⁰ In contrast to TPA, benzoyl peroxide does not induce ODC⁷¹ and promotion by benzoyl peroxide is not inhibited by retinoic acid.⁷⁰ 13-*cis*-Retinoic acid at 17 nmol per treatment, prevented TPA-induced promotion,⁴⁴ but did not prevent anthralin-induced promotion.⁶⁹ However, Dawson et al. have shown that a tenfold higher dose of 13-*cis*-retinoic acid (170 nmol) is effective at preventing promotion by anthralin (444 nmol applied twice weekly for 32 consecutive weeks).⁷² These results are in agreement with the hypothesis that retinoic acid prevents tumor promotion by preventing promoter-induced ODC, or perhaps a gene product coordinately regulated with ODC.

B. INFLUENCE OF THE MODE OF ADMINISTRATION ON RETINOID TUMOR PREVENTION

Chemoprevention by some retinoids is dependent upon the mode of administration. Topical application of 13-*cis*-retinoic acid was found to be effective against promotion by TPA.^{25,44} However, dietary administration of 13-*cis*-retinoic acid slowed the growth rate of TPA-promoted papillomas, but did not reduce the number of papillomas.⁷³ Similar results have been reported by Verma.⁷⁴ In contrast, dietary administration of retinyl palmitate was effective at preventing the appearance of TPA-promoted tumors as well as the growth of tumors.⁷³ The lack of accumulation of 13-*cis*-retinoic acid in subdermal fat,^{74a} which may result from the interconversion of 13-*cis*-retinoic acid with retinoic acid,⁶ and the rapid excretion of retinoic acid⁷⁵ may account for the low level of skin tumor prevention by dietary 13-*cis*-retinoic acid.

The efficacy of tumor inhibition when the chemopreventive agent is applied at various times after the beginning of promotion has clinical implications. Therefore, we compared the levels of inhibition achieved when 13-*cis*-retinoic acid was applied (1) at the beginning of tumor promotion, (2) at the time of the initial appearance of tumors, or (3) at the time of initial conversion of papillomas to carcinomas.⁴⁴ All mice (CD-1) had been initiated with

400 nmol of benzo[a]pyrene and promoted with TPA (3.2 nmol twice weekly for 2 weeks, and 8 nmol twice weekly for the subsequent 23 weeks). We found a correlation between the promptness, relative to promotion, with which prophylaxis was begun, and the extent of tumor inhibition. Thus, 13-cis-retinoic acid (17 nmol, 30 min before each TPA treatment) applied at the times listed above yielded 47, 28, or 19% inhibition, respectively. It is noteworthy that some tumor inhibition occurred even with late administration of 13-cis-retinoic acid, e.g., at the time when the first tumors had converted to carcinomas. These results performed *in vivo* could not distinguish whether a single or multiple steps in the carcinogenic pathway were blocked by 13-cis-retinoic acid. However, retinoid inhibition of induced expression of ODC¹⁶ or retinoid induction of differentiation of epidermal cells⁷⁶ are potential chemopreventive mechanisms that could occur throughout the carcinogenic pathway, and even after early carcinoma development. Thus, these mechanisms could underlie both preventive and therapeutic activity of retinoids.

C. DURATION OF TUMOR PREVENTION

The duration of the tumor prevention induced by retinoids is of obvious clinical interest. To study the duration of retinoid chemoprevention, we applied 13-cis-retinoic acid (17 nmol) 0.5 h before TPA treatments (8 nmol) to mice that had been initiated with 400 nmol of benzo[a]pyrene.⁴⁴ The 13-cis-retinoic acid mediated-inhibition of TPA-induced tumor promotion was found to remain stable during a 15-week observation period after termination of both retinoid and promoter treatments.⁴⁴ This result suggests that the initial step in tumor progression that was blocked by 13-cis-retinoic acid preceded the irreversible step of conversion of papilloma cells from promoter dependence to independence. The clinical implication of these results is that prevention by retinoids at an early stage of promotion may be irreversible and not require maintenance therapy to prevent relapse.

V. CLINICAL INVESTIGATIONS

Retinoid induction of differentiation and growth regulation in epidermal cells,^{17,76} as well as the accumulation of selected retinoids in the skin^{7,74a} has led to clinical tests for the prevention and therapy of cutaneous cancers. Many types of skin cancer show a tendency for multiple primary lesions, so that therapy for one lesion may be prevention for potential subsequent lesions.

A. ACTINIC KERATOSIS

Actinic keratoses are epidermal lesions that have a 3 to 5% probability for converting to nonmelanomatous skin cancer. They occur in caucasians exposed to high levels of ultraviolet radiation.

Belisario⁷⁷ and Bollag and Ott⁷⁸ have independently treated actinic keratosis patients with topical retinoic acid cream and obtained 40 to 50% complete regression, and 45 to 50% partial response in a total of 153 cases. Three clinical trials have involved the aromatic retinoid, etretinate, administered orally to treat actinic keratosis.⁷⁹⁻⁸¹ Grupper and Beretti⁷⁹ used 1 mg/kg/d for 3 months, with subsequent dosage reduced by one half or less for 4 or 5 months. Complete regression occurred in 75% and partial regression in 20% of the 46 patients. However, 76% of 34 patients had recurrences within 1 year of treatment termination. Most patients remained lesion-free if they took 1 mg/kg/d for 2 out of 12 months. Moriarty et al.⁸⁰ used etretinate at a dose of 75 mg/d for 2 months in a double-blind, placebo-controlled crossover trial involving 50 patients. Responses occurred within 1 month. Eighty-four percent^{5,46} showed complete or partial remission, even in the 17 cases in which dose reductions were necessary. In another double-blind study, Watson⁸¹ randomly placed 15 patients on etretinate at a dose of 1 mg/kg/d or placebo for 4 months before treatment crossover.

Complete or partial regression occurred in 14 of 15 patients. Of nine patients who then received placebo, one improved, one progressed and seven remained stable. Oral administration of the arabinoid ethyl ester Ro 13-6298 at a dose of 1 mg/kg/ for 28 d led to regression in 10 of 16 cases.⁸²

Thus, each of these retinoids were efficacious for therapy of these "preneoplastic" cutaneous keratoses. Phase III randomized trials have been begun at the University of Arizona and the Memorial Sloan-Kettering Cancer Center to determine whether retinol or 13-cis-retinoic acid can function as true chemopreventive agents over the long term.⁸³

B. KERATOACANTHOMA

Keratoacanthomas are hyperkeratotic lesions that resemble cutaneous squamous cell carcinoma. They can regress or become malignant.^{84,85} Treatment of four patients bearing multiple keratoacanthomas with 13-cis-retinoic acid (2 mg/kg/d) orally resulted in impressive sustained regression.⁸⁶⁻⁸⁸ Keratoacanthomas in 6 patients underwent complete regression after 2 to 3 months of oral etretinate at 1 mg/kg/⁷⁹ and have not returned at 1 year after treatment was terminated. These keratoacanthomas had persisted for a number of years prior to therapy.

C. BASAL CELL CARCINOMAS

Therapy of basal cell carcinomas has been reported with topical retinoic acid, oral 13-cis-retinoic acid and oral etretinate. Many patients with basal cell carcinomas tend to develop multiple primary tumors, so that prevention would be clinically important. Of 49 patients treated with topical retinoic acid in 4 independent studies, 33% showed complete and 67% showed partial regressions.^{77,78,89,90}

The use of oral 13-cis-retinoic acid (4.5 mg/kg/d) resulted in complete regression in 10% and partial regression in 67% of 248 tumors in 11 patients.^{91,92} Tumors less than 10 mm in diameter were more responsive to regression than were larger tumors. Termination of treatments results in recurrences within 8 to 18 months. We have administered oral 13-cis-retinoic acid to 5 patients with basal cell carcinoma.⁹³ Partial regression has been achieved in two of these patients, lasting more than 6 or 16 months. Oral etretinate has also been used successfully in patients with multiple or isolated basal cell cancers.⁷⁹ Although there were seven partial regressions in the multiple as well as seven in the single basal cell cancers, the recurrence rate was significantly higher in those patients who had had multiple basal cell carcinomas. Maintenance etretinate was necessary to sustain response. Peck has reported that three patients who had had multiple basal cell carcinomas, have had no new tumors to 2.5 years after beginning treatment with oral 13-cis-retinoic acid.⁹⁴ The maintenance dose was 1.5 mg/kg/d.

Several phase III randomized trials are in progress to determine whether retinol, 13-cis-retinoic acid, or β -carotene can prevent the development of basal or squamous cell cancers of the skin in patients with varying risks.

D. EPIDERMODYSPLASIA VERRUCIFORMIS

Patients with *Epidermodyplasia verruciformis* develop multiple squamous cell carcinomas and multiple flat, wart-like lesions during childhood. The lesions usually contain human papilloma virus type 5, which is oncogenic.⁹⁵ Oral etretinate (1 mg/kg/) has led to a complete regression of *E. verruciformis* within 2 months in a man who had had the disease for 30 years.⁹⁶ His lesion sites had become HPV-5 negative. In some cases oral etretinate has resulted in lesions becoming viral negative;^{97,98} in other cases, the lesions remained viral positive. Relapse has followed retinoid reduction.

E. DYSPLASTIC NEVI

The dysplastic nevus syndrome involves the presence of 1 to over 100 pigmented macules

or flat papules containing dysplastic melanocytes.⁹⁹ These nevi, which may progress to malignant melanoma, can occur sporadically or exhibit a familial pattern.¹⁰⁰ Treatment is generally surgical, however, in cases of many nevi, excision of all lesions is not practical. In such cases, nevi are removed after a visible change occurs in the lesion morphology, as detected by repeated photography.¹⁰¹ We have treated three patients bearing multiple dysplastic nevi with 0.05% retinoic acid cream applied daily.¹⁰² All lesions biopsied after the 10- to 12-week treatment period was over showed marked reduction in dysplasia. After 7 or 8 months, no recurrence of dysplasia had become evident. Further investigation is needed to determine the role of retinoids in preventing progression of nevi to malignant melanoma.

VI. SIDE EFFECTS AND TOXICITY

The prolonged use of retinoids as chemopreventive or therapeutic agents is limited by their toxic side affects. The observation that differences in metabolism, distribution, and storage of various retinoids can lead to differences in toxicity is one of the important bases for synthesizing new analogs.^{103,104} The main signs and symptoms of hypervitaminosis A syndrome in man involve the following changes in the skin: cheilitis, desquamation, pruritis, dry mucous membranes, pigmentation, and hair loss.¹⁰⁴ Central nervous system symptoms include high spinal fluid pressure, which leads to exophthalmia, dizziness, nausea, and lethargy. Other side effects include pains in the bones, joints, and muscles, as well as hepatic dysfunction. In animals the hypervitaminosis A syndrome is similar, with manifestations of body weight loss, muscle weakness, desquamation of the skin, hair loss, inflammation in mucous membranes, osteoporosis, calcification of internal organs, bone fractures, and teratogenic effects.¹⁰⁵⁻¹⁰⁷

These severe side effects have led to a search for retinoids with diminished toxicity. One vitamin A analog, 13-*cis*-retinoic acid, was less lethal than retinoic acid after a 15-week treatment period in rats.¹⁰⁸ We have found that systemic 13-*cis*-retinoic acid was less lethal than retinyl palmitate in mice, based upon the death of 0 out of 35 mice and 12 out of 70 mice fed 700 IU of the respective retinoids/g diet for 6-weeks.⁷³ Thus, 13-*cis*-retinoic acid has promise as a retinoid with low toxicity.

The only synthetic retinoid commercially available for systemic use in the U.S. now, is Accutane® or 13-*cis*-retinoic acid.¹⁰⁹⁻¹¹¹ The short term side effects of Accutane in 150 acne patients have recently been reviewed,¹¹² and we have reported the effects of Accutane at low doses (10 mg/d) in normal subjects.¹¹³ The skin effects listed in the general syndrome occurred in about one third of the subjects. Conjunctivitis in 40%, gastrointestinal disturbances in 20%, musculoskeletal problems in 15%, and headaches or lethargy in 10% of subjects were additional side effects.

The occurrence of bone toxicity after chronic retinoid treatment has been reported in animal and human studies. Hixson and Denine¹¹⁴ found a correlation between the oral dose of 13-*cis*-retinoic acid and the number of Swiss mice developing more than one bone fracture. In the investigation, retinoic acid resulted in approximately four times as many fractures as did 13-*cis*-retinoic acid. Turton et al.¹¹⁵ have measured femur weight in female B6D2F₁ mice fed diets containing 0.19, 0.38, or 0.75 mmol of 13-*cis*-retinoic acid per kg diet for 12.5 weeks. Although body weight was not affected by this diet, femur weight was significantly reduced at all doses. Based upon radiographic analysis, we have found a dose-related increase in the mean number of osteoporotic bones in CD-1 mice after 23 weeks of dietary 13-*cis*-retinoic acid. Doses of 4, 200, or 700 IU of 13-*cis*-retinoic acid per g diet resulted in 0.3, 1.1, or 1.5 osteoporotic bones per mouse, respectively (Forsyth, K. S., Gensler, H. L., Watson, R., and Bowden, G. T., unpublished results).

Therapy of human skin disorders, such as acne or keratinizing dermatoses, with 13-*cis*-retinoic acid has been found to lead to bone abnormalities.^{111,116-118} At doses ranging from

1.4 to 3.4 mg/kg/d for 34 to 81 d, McGuire et al.¹¹¹ found six major alterations of ossification in man that may be attributable to 13-*cis*-retinoic acid: (1) osteoporosis, (2) cortical hyperostosis, (3) accelerated maturation of the growth plate, (4) flaring of the ends of long bones, (5) decreased shaft width of long bones, and (6) calcification of ligaments. Pittsley¹¹⁶ found that four of nine consecutive patients receiving 13-*cis*-retinoic acid at 3 to 4 mg/kg/d for two or more years had skeletal hyperostosis. In a prospective study, 75% of the patients developed vertebral osteophytes in 12 months at 2 mg/kg/d.¹¹⁹ These changes have not been reported at doses smaller than 1 mg/kg/d and, therefore, are dose-dependent.

Teratogenicity has been observed in animals and, recently, in humans exposed to 13-*cis*-retinoic acid.¹²⁰⁻¹²² Lammer has found a 25-fold increase in the risk for major embryopathy in 154 pregnancies with fetal exposure to Accutane.¹²³ Spontaneous abortions and a characteristic pattern of craniofacial, cardiac, thymic, and central nervous system malformations were found in infants exposed to Accutane before day 28 of gestation. At subteratogenic doses, retinoids can lead to behavioral, cognitive and emotional abnormalities in the offspring of rats. There teratogenic risks of 13-*cis*-retinoic acid necessitate that women of childbearing age should receive pregnancy testing, use contraceptive protection before entering clinical trials, and should be at risk for a serious cancer.

VII. SUMMARY

A number of retinoids can prevent mouse skin tumor promotion, both *in vivo* and in an epidermal cell culture model. The mechanism underlying this prevention appears to be a block in proliferation in the cell culture assay. Tumor prevention *in vivo* may be more complex in that retinoids provoke a burst of DNA synthesis in the intact animal,¹²⁴ whereas they generally inhibit keratinocyte proliferation *in vitro*.^{19,125} An indirect action, such as the inhibition of a promoter-induced effect, may be involved in skin tumor prevention *in vivo*.

A correlation has been found between the ability of retinoids to prevent TPA-induction of ODC and tumor formation. The regulation of ODC, itself, may be important, or it may be that another gene(s), also regulated by protein kinase C, is required for tumor promotion. Hence, retinoid prevention of TPA-activation of protein kinase C may be the central mechanism underlying retinoid antipromotion. This possibility is attractive because there is precedence for protein kinase C regulation of gene expression in its activation of the SV40 enhancer.

Clinically, retinoids have exhibited cancer-preventing properties in several cutaneous premalignancies and malignancies. Advances in the development of more potent and less toxic synthetic derivatives have led to consideration of selected retinoids for prevention of human malignancies. Since most retinoid-responsive neoplasias require maintenance therapy to prevent relapse, there is a need for drugs with high therapeutic indices for prolonged low-dose prevention or therapy studies. Additive and synergistic activity of retinoids with hormones, DNA synthesis inhibitors, radiation therapy, differentiation factors, and DFMO have been found with tumor cells in culture.^{20,39} The use of specific combinations for specific tumor types is an exciting area for future development.

ADDENDUM

Considering the critical role of UV-induced immunosuppression in photocarcinogenesis,¹²⁵ and that vitamin A administration has been found to prevent immunosuppression induced by burns, surgery, and neonatal tolerizing procedures,¹²⁶ we hypothesized that retinyl palmitate, with or without concurrent carotenoid treatment, could prevent UV-induced immunosuppression and prevent photocarcinogenesis in the mouse. To test this hypothesis, pigmented C3H/HeN mice were fed the basal American Institute of Nutrition diet 76A,

supplemented with 120 IU retinyl palmitate per g diet, with or without concurrent 1% canthaxanthin, for 18 weeks before UVB irradiation (280 to 320 nm), and throughout the study. Mice were UV-irradiated for 30 min, five times weekly for 12 or 24 weeks in groups studied for immunosuppression or photocarcinogenesis, respectively. The UV source was a bank of 6 Westinghouse FS40 lamps, which delivered to the mice a total of 4.5×10^5 or $9.9 \times 10^5 \text{ J m}^{-2}$, respectively for 12 or 24 weeks of treatment. The mean tumor burden per mouse was 110.2 ± 28.2 , 57.7 ± 12.4 , and 36.2 ± 10.1 per mm^2 in UV-irradiated mice fed the basal diet supplemented with placebo beadlets, retinyl palmitate, or retinyl palmitate plus canthaxanthin, respectively, at 29 weeks after the first UV-radiation treatment. Based on analysis of variance, retinyl palmitate, with or without canthaxanthin, significantly reduced the primary tumor growth of UV-irradiated mice.

The level of immunosuppression in UV-irradiated mice was measured by a passive transfer assay. The inability to reject immunogenic UV-induced tumors can be passed to a naive recipient with splenocytes from a UV-irradiated donor.¹²⁷ Transfer of splenocytes from UV-irradiated mice into naive recipients resulted in enhanced growth of subsequent syngeneic, immunogenic, UV-induced tumor implants in the recipients. However, those recipients of splenocytes from UV-irradiated donors fed the basal diet supplemented with retinyl palmitate plus canthaxanthin or retinyl palmitate alone had 0 or 25% of this enhanced growth, or immunosuppression, respectively. Based on the Wilcoxon rank sum test, the difference was significant ($p = 0.02$) when the donors were fed the combination diet, and marginally significant ($p = 0.054$) when the donors were fed retinyl palmitate as a single supplement. Thus, dietary retinyl palmitate administration resulted in reduced tumor growth and decreased immunosuppression in UV-irradiated mice, and these activities were enhanced by dietary canthaxanthin. These results indicate that retinyl palmitate can augment the immunological response to an antigenic tumor in UV-irradiated mice and is consistent with the hypothesis that retinyl palmitate reduced photocarcinogenesis by preventing induction of immunosuppression by UV irradiation.

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Chapter 18

EPIDERMAL RESPONSES TO RETINOIDS *IN VIVO***Michael J. Connor****TABLE OF CONTENTS**

I.	Introduction	486
II.	Vitamin A and the Epidermis	486
III.	Effects of Vitamin A and Retinoid Supplementation on Hairless Mouse Epidermis	488
	A. Response to Subtoxic Doses of Retinoids	488
	B. Response to Toxic Doses of Retinoids	488
	C. Mechanism of Induction of the Hyperplastic Response	489
IV.	Effects of Vitamin A and Retinoid Supplementation on Rhino Mouse Epidermis	491
V.	Effects of Variation in Retinoid Structure on the Induction of Epidermal Hyperplasia	492
VI.	Conclusions and Implications for Retinoid Action	496
	Acknowledgments	497
	Abbreviations	497
	References	497

I. INTRODUCTION

The molecular basis for vitamin A and retinoid activity in growth and epithelial function is elusive, and (perhaps consequently) there is considerable debate and controversy even over the relative importance of the plethora of biological effects mediated or induced by retinoids. Lack of fundamental understanding in these areas has not prevented the now extensive use of natural and synthetic retinoids in the treatment of a variety of skin disorders,¹ including acne, ichthyosis, dyskeratinizing disorders, and psoriasis, and they are even being advocated for somewhat more mundane "pharmaco-cosmetic" uses as medicated skin repair or renewal agents. The major target organ in these therapeutic uses of retinoids is the epidermis. Retinoid supplementation can dramatically alter epidermal morphology, one of the more obvious changes induced being the dose-dependent induction of epidermal hyperplasia. Despite the marked potency with which retinoids induce this response, and the implications such a response may have to understanding retinoid action in skin disease, it is frequently attributed to toxicity or secondary to inhibition of epidermal "keratinization" or differentiation. To more clearly define the nature of the hyperplastic response, and to obtain information that may indicate its relation to the physiological and pharmacological roles of retinoids in the epidermis, we have performed extensive investigations of the hyperplastic response of mouse epidermis, a particularly sensitive tissue, to retinoid treatment *in vivo*. In this chapter the *in vivo* impact of natural and synthetic retinoids on epidermal morphology and differentiation, and the mechanisms involved are described, and the effects of variation in retinoid structure on these responses are discussed. A brief review of vitamin A and the epidermis is provided to lay the foundation for the discussion of the effects of synthetic retinoids. Detailed reviews of the epidermis² and the effects of retinoids on epithelial cells grown *in vitro*³ have recently been published.

II. VITAMIN A AND THE EPIDERMIS

Postpartum mammalian epidermis is a stratified keratinizing epithelium forming the outermost cell layers of the body, and is in a continuous state of self renewal. The epidermis is comprised largely of four morphologically distinct strata, each exhibiting a progressive level of differentiation over their inferior strata.² Sections of mouse skin are illustrated in Figure 1. The innermost layer, the stratum basale (basal cell layer), lies immediately above the dermis and contains most of the actively dividing cells of the epidermis; in the mouse it consists of a single cell layer (Figure 1a). Above the basal cell layer lies the stratum spinosum (prickle cell layer), comprised of large nucleated cells with prominent tonofibrils and desmosomes, and this is overlaid by one or two layers of the flattened elongated cells containing conspicuous darkly-staining keratohyalin granules typical of the stratum granulosum. The outermost layers, the stratum corneum, are comprised of fully differentiated enucleated "keratinized" cells (corneocytes), and is an organized, compact, stacked structure containing 6 to 7 cell layers. Cells formed in the basal layer continually feed into the superficial layers where they complete their program of maturation and differentiation, and are ultimately sloughed off into the environment. The total number of epidermal cell layers present at any one time reflects the dynamic equilibrium between cell formation through cell division in the basal cell layer and cell loss through desquamation of the corneocytes of the stratum corneum.

Epithelial differentiation is impaired in vitamin A deficiency. In their classic descriptive study Wolbach and Howe observed that various mucus-secreting epithelia appeared abnormal on histological evaluation in rats fed vitamin A-deficient diets, being replaced by keratinized squamous epithelia.⁴ Although they did not examine the epidermis directly, they commented in passing that epidermis present in the margins of sections derived from other tissues

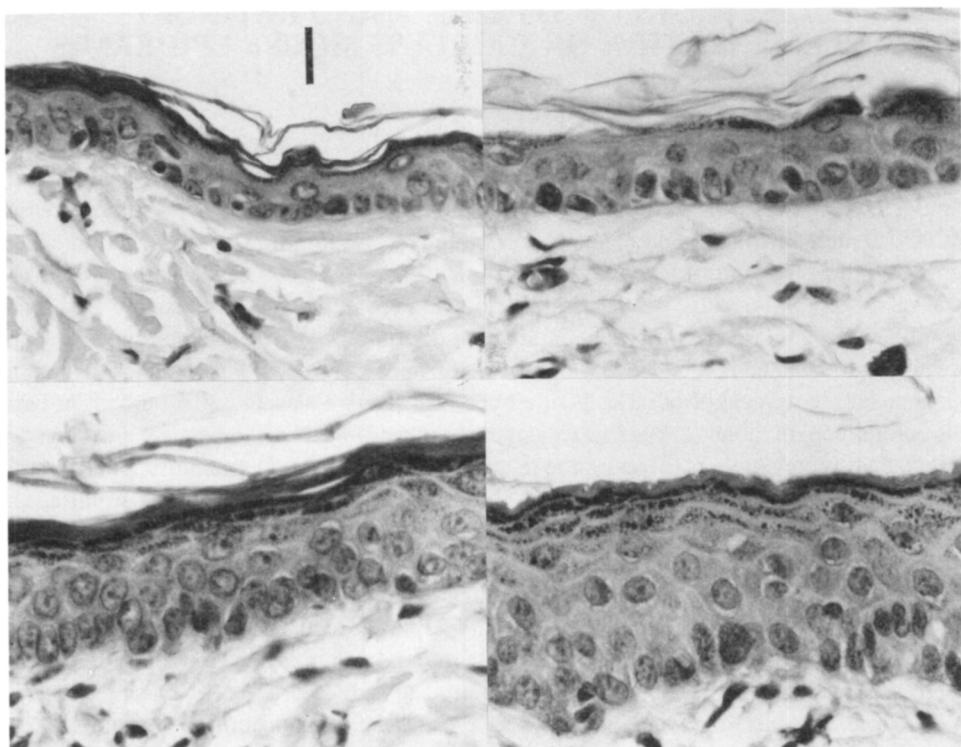


FIGURE 1. Dose-dependent changes in epidermal morphology in hairless mice treated with single retinoid doses: (a) 0.1 ml of acetone (control); (b) 0.1 nmol of TTNPB in 0.1 ml acetone; (c) 1.0 nmol of TTNPB in 0.1 ml acetone; (d) 10 nmol of TTNPB in 0.1 ml acetone. The biopsies were recovered 4 d after treatment. All micrographs were obtained at $\times 400$ magnification. The bar on (a) is 20 μ in length.¹³

appeared normal, a not surprising fact since this tissue is *de facto* a keratinizing epithelium. In later studies epidermal basal cell proliferation, determined from the mitotic rate⁵ and [3 H]thymidine-labeling indices,⁶ was found to be depressed in vitamin A-deficient rodents, establishing that in vitamin A deficiency the process of epidermal cell renewal is impaired. In correlation to this, supplementation of the epidermis of vitamin A-replete or -deprived animals with retinol or other retinoids has been shown to stimulate the basal cell mitotic rate,⁵ epidermal DNA synthesis,^{7,8} and cell production.⁹ The consequence of this stimulated proliferation is the induction of a marked hyperplasia in mouse epidermis (see Figure 1).^{5,7-13} Similar responses have been described in a variety of mammals including the human,¹⁴ the mouse,¹⁵ the rat,⁶ and the guinea pig,¹⁶ and in the chicken.¹⁵ Related stimulatory effects of retinoids on cell growth have been observed in epidermal cells grown *in vitro*, although contrary effects have also been reported.³

In contrast to the effects observed in postpartum skin, fetal epidermis may respond somewhat differently, depending on the state of morphogenic development. As demonstrated in the landmark studies of Fell¹⁷ using explants of embryonic chick skin, excess vitamin A can cause the otherwise keratinizing epidermis to reform into a mucus-secreting epithelium akin to that of the nasal mucosa. This effect of retinoids on the epidermis appears to be restricted to early stages of embryonic development,¹⁷ probably prior to the final commitment of the tissue to a truly keratinizing epithelium. The morphological transformation does not occur in adult chick or mammalian skin,¹⁸ although analogous events have been observed in certain skin neoplasias such as keratoacanthomas.¹⁹

III. EFFECTS OF VITAMIN A AND RETINOID SUPPLEMENTATION ON HAIRLESS MOUSE EPIDERMIS

A. RESPONSE TO SUBTOXIC DOSES OF RETINOIDS

Administration of a single dose of retinoid to hairless mice induces a transient dose-dependent expansion of the numbers of cell layers of the stratum spinosum and stratum granulosum (Figure 1a-d).^{7,8,11,12} Generally the number of cell layers is noticeably increased by 2 to 3 d after treatment, and reaches a maximum after 4 to 5 d. The epidermal thickness gradually returns to control levels by 10 to 14 d. Similar time courses have been observed for a variety of biologically active natural and synthetic retinoids, administered by topical or oral routes.^{12,13} Provided the dose is subtoxic the number of cell layers of the stratum corneum is unchanged.^{11,12} The basal cells may appear more elongated than usual, an appearance more typical of basal cells in a hyperproliferative state, but the number of basal cells per unit length (about 1.4 per 10 μ) and the number of basal cell layers are unchanged.¹² While the absolute numbers increase, the relative number of stratum granulosum and stratum spinosum cell layers remain fairly constant during the development of the hyperplasia, and epidermal differentiation appears to be proceeding normally on histological evaluation. The increases in thickness of the individual strata parallel the increase in the number of cell layers in all cases, and the growth or thickening of the epidermis is due to increased numbers of cells, rather than increased cell size. The hyperplasia occurs in both the interfollicular and the contiguous follicular epidermis. Multiple retinoid treatments induce a dose-dependent hyperplastic response.^{10,11} The daily dose required to induce a given level of response is lower than is required for induction by a single dose: daily application of 3.4 nmol of *all-trans*-retinoic acid, a typical dose used in skin carcinogenesis studies, induces a marked epidermal hyperplasia by 20 d.¹⁰ Retinoids induce epidermal hyperplasia after systemic administration,¹¹ although higher dosing levels are needed to achieve delivery of sufficient retinoid to the epidermis.

Since the induction of the hyperplasia reflects an expansion of the cell numbers of the epidermis, increases in nucleic acid and protein synthesis, concomitant with the increased cell division and growth, accompany the development of the hyperplasia.^{7,8} Changes in the expression of specific macromolecules associated with proliferation or with particular cell populations may occur. The ability of low doses of retinoids to enhance epidermal ornithine decarboxylase activity,⁷ a sensitive marker of proliferation, reflects the induction of basal cell proliferation that underlies the hyperplastic response. The relative increase in the number of cell layers of the stratum spinosum and stratum granulosum (see Figure 1), but little or no change in the number of basal or stratum corneum cell layers, may also lead to alterations in the relative amounts of stratum-specific differentiation products during retinoid treatment. Since the hyperplastic expansion leads to increased epidermal thickness and total cell number, the absolute amounts of functionally significant epidermal enzymes may also change, including stratum granulosum-associated transglutaminase.⁹

B. RESPONSE OF THE EPIDERMIS TO TOXIC DOSES OF RETINOIDS

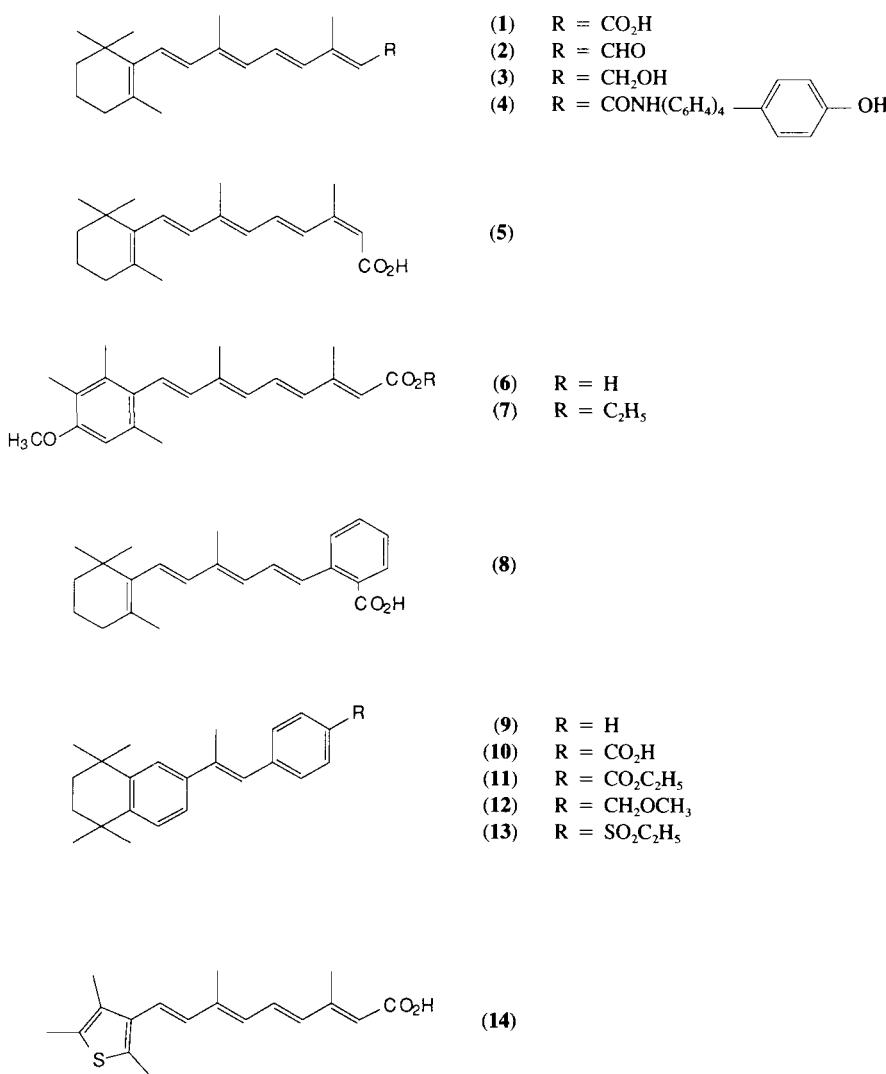
Because of the tremendous range of potencies found among retinoid analogs, the doses inducing toxicity vary from retinoid to retinoid. The dose ranges used to induce a hyperplastic response are such that toxicity, if it does occur, is usually limited to mild scaling of the skin. Mild scaling occurs at retinoid doses below those inducing other cutaneous symptoms of toxicity,¹² and is a sensitive indicator that higher doses may induce toxicity. The occurrence of mild scaling presumably reflects the overwhelming of the normal desquamation process due to overly high production of corneocytes. In contrast to the appearance of the epidermis after low to moderate doses of retinoids, higher doses are progressively associated with distinct evidence of cutaneous toxicity. For example, treatment with moderate to severe

toxic doses of TTNPB ethyl ester¹¹ led to a decrease in the number of stratum granulosum cell layers, a thinning of the interfollicular epidermis, some acantholysis, and general tissue fragility. At doses sufficient to induce weight loss, the epidermis was eroded in some areas, with the pilar units protruding, while other sites showed subcorneal pustules with a parakeratotic stratum corneum above. The cells of the sebaceous glands were granular and shrunken, with loss of lipid.¹¹ This is in contrast with the almost normal appearance of the sebaceous glands seen after lower concentrations of retinoids. Chronic exposure to high doses may be associated with changes in epidermal cell morphology, loss of body weight, and enhanced trans-epidermal water loss leading to dehydration as the integrity of the epidermis is damaged.²⁰

C. MECHANISM OF INDUCTION OF THE HYPERPLASTIC RESPONSE

Since the number of epidermal cell layers reflects the equilibrium between cell formation in the basal layer and cell loss through desquamation from the stratum corneum, increased cell production due to stimulation of basal cell proliferation provides a ready explanation for the induction of epidermal hyperplasia by retinoids. However, if retinoids inhibit epidermal desquamation or a late step in cell differentiation or maturation, then the hyperplasia could partly reflect accumulation of incompletely differentiated or immature cells. In this respect all-*trans*-retinoic acid (**1**, Table 1) has been found to decrease "cornified envelope" formation, a possible marker for keratinocyte terminal differentiation, from mouse epidermal cells cultured under certain conditions.²¹ Although results of our histological studies were incompatible with abnormal maturation because there was no change in the number of stratum corneum cell layers and there was no evidence for a marked imbalance in the relative numbers of stratum granulosum and stratum spinosum cell layers following single¹² or multiple retinoid treatments^{10,12} we sought more direct evidence to assess the possible contribution of inhibition of differentiation in the development of retinoid-induced epidermal hyperplasia. Epidermal transglutaminase (soluble and particulate), an enzyme that is involved in the protein cross-linking that occurs during epidermal differentiation and that is associated with the stratum granulosum,²² and the turnover of the stratum corneum were measured in hairless mice after hyperplasia-inducing doses of all-*trans*-retinoic acid or ethyl (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propen-1-yl]benzoate (**11**, TTNPB ethyl ester).⁹ Transglutaminase, detected histochemically, was localized about the stratum granulosum, as was observed by others in pig snout epidermis.²² The histochemical localization was similar in retinoid-induced hyperplastic epidermis but the band of staining was more marked and broader reflecting the increased thickness of the stratum granulosum. The specific activity (units per milligram of protein) of the transglutaminase activity found in epidermal extracts was not changed by retinoid treatment, but the absolute amount (activity per unit area of skin surface) markedly increased with time to a maximum at 4 to 5 d, corresponding to the time of maximum epidermal hyperplasia. This difference in relative and absolute amounts reflects the retinoid induced hyperplastic state, i.e., the net increase in the number of layers of differentiating cells per unit area of skin. The total transglutaminase activity per square centimeter that was found in epidermal extracts after various doses of all-*trans*-retinoic acid was directly proportional to the number of cell layers of the stratum granulosum.⁹ The individual cells of the stratum granulosum thus appear to express similar levels of transglutaminase in both unstimulated and retinoid-induced hyperplastic epidermis. Stratum corneum turnover was more rapid in the retinoid-treated mice.⁹ Since both the formation of epidermal cells and the loss of differentiated cells are increased by retinoid treatment, reduction of cell loss through inhibition of epidermal cell differentiation or delayed maturation cannot have a role in the mechanism by which retinoids induce the hyperplasia. Rather, retinoid-induced epidermal hyperplasia is a consequence of an increased production of cells that show the histological, biochemical and physiological behaviour of cells competent for

TABLE 1
Structures of the Retinoids used in Tables 2 and 3



normal differentiation. It is not yet clear if the increase in cell production is caused by an increase in the number of dividing cells (i.e., recruitment of basal cells that are not cycling into the dividing cell pool) or due to a decrease in the average cell-cycle time. Unfortunately, it is difficult to assess this because even the size of the dividing-cell fraction in unstimulated epidermis is controversial.

Although the molecular basis for any retinoid activity in the epidermis is unknown, it is noteworthy that the epidermis is relatively rich in cellular retinoic acid-binding protein (CRABP),^{23,24} which is the only well characterized protein implicated in mediating the activity of retinoids having a carboxyl group at the polar terminus.²⁵ Because the endogenous retinoic acid level is less than 10 nmol/kg^{26,27} (a concentration approaching the dissociation constant for binding to CRABP²⁵) and if it is assumed that CRABP does not have non-retinoid physiological ligands, the bulk of the epidermal CRABP is present in an apo form (i.e., ligand free) and is thus available as a target even for retinoids having low binding affinities. Why CRABP should be present in the epidermis in such a large excess over its

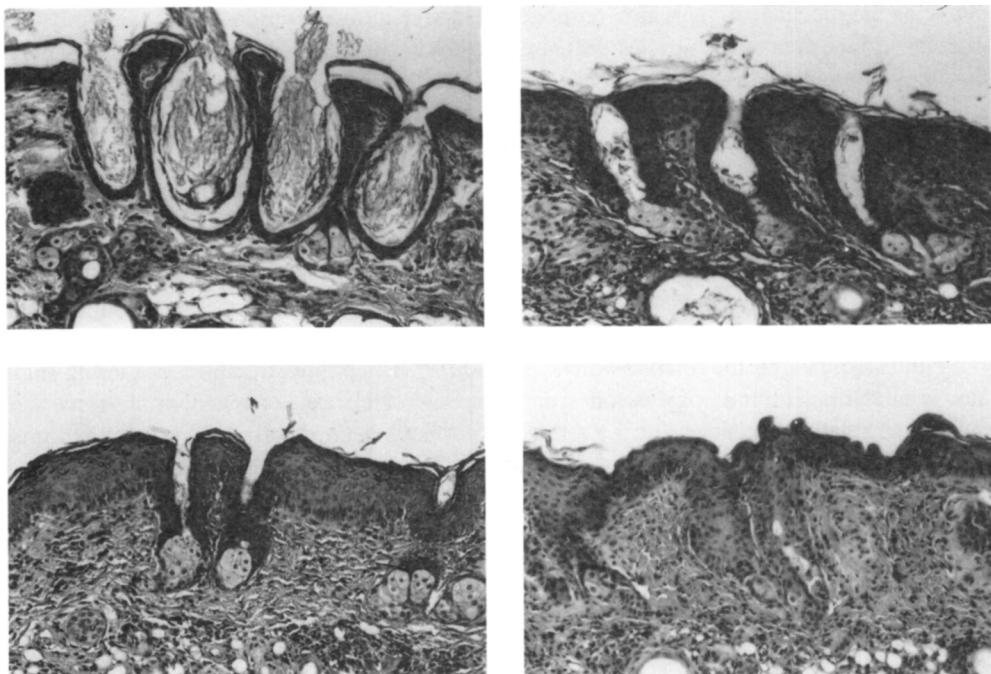


FIGURE 2. Dose-dependent changes in the epidermal morphology of rhino mouse skin in response to multiple retinoid treatments: (a) 0.1 ml of acetone daily (vehicle); (b) 3 pmol of TTNPB daily; (c) 30 pmol of TTNPB daily; (d) 3 nmol of TTNPB daily, showing marked toxicity. Biopsies were taken 2 d after the last of a series of 10 daily applications of the stated doses of TTNPB. All micrographs were obtained at $\times 100$ magnification.

putative physiological ligand is puzzling. It seems unlikely that its role is to act merely as a "sink" for toxic retinoids. Retinol levels are 100 to 1000 times higher than those of retinoic acid, yet the levels of cellular retinol-binding protein are lower than those of CRABP.²⁴ As will be discussed later, structure-activity studies for the induction of epidermal hyperplasia are not inconsistent with CRABP mediation.

IV. EFFECTS OF VITAMIN A AND RETINOID SUPPLEMENTATION ON RHINO MOUSE EPIDERMIS

The "rhino" allelic variant of the hairless mouse has characteristic follicle-derived horn-filled utricles present in the epidermis (Figure 2a), the morphology and size of which alter in response to retinoids.^{11,28-30} Multiple applications of low doses of retinoids induce a dose-dependent hyperplasia of the interfollicular and utricular epidermis¹¹ and a reduction in utricle diameter.^{11,30} The utricle walls thicken concomitantly with a change in the shape of the utricles to that resembling the normal follicles of hairless mice (Figure 2a to c). This "redifferentiation" or "remodeling" of the utricles to more normal-looking follicles occurs only at retinoid doses above those that induce epidermal hyperplasia,¹¹ using a variety of retinoids of different potencies. Hyperplasia in the follicles is often more evident at the base around the opening of the sebaceous glands. This differential response may be involved in the straightening of the otherwise rounded utricles. The response found at higher toxic doses is clearly distinguishable (Figure 2d); the epidermal architecture is abnormal, and the stratum granulosum and sebaceous glands are less evident. Although the rhino mouse model is occasionally referred to as a model for assaying "anti-keratinizing agents",³⁰ as with its hairless cousin there is no histological basis to support inhibition of epidermal keratinization

per se as the mechanism by which retinoids are active in this mouse strain.¹¹ Certainly, the internal surface area of the utricles decreases as they shrink and remodel and the horny plugs that they contain are lost, effectively leading to a decrease in the area of the contiguous stratum corneum and the absolute amount of keratin. But, this process is obviously secondary to the morphological changes. The histology data¹¹ suggest that enhanced production of differentiation competent cells occurs in response to retinoids. This stimulation is the driving force for the changes in epidermal and follicular morphology. It is not yet clear if the decrease in the characteristic wrinkling of the skin or rugosity (the attribute responsible for the name of this mouse strain) that occurs in response to retinoids, which was first reported by Frazer in 1949,²⁸ reflects the thickening of the epidermis, the remodeling of the utricles, or a more subtle dermal effect.

Published data on the relative potencies of different retinoid structures at altering rhino mouse epidermal morphology is too scanty to draw significant conclusions, however the ranking of relative potencies for the retinoids that have been reported tend to follow those found for the induction of epidermal hyperplasia. The relative rankings reported for effects on utricular size are TTNPB ethyl ester >> all-*trans*-retinoic acid > etretinate (7) > 13-*cis*-retinoic acid (8) (delivered in acetone),¹¹ and all-*trans*-retinoic acid > 13-*cis*-retinoic acid > etretinate > motretinid (delivered in ethanol:propylene glycol).³⁰

V. EFFECTS OF VARIATION IN RETINOID STRUCTURE ON THE INDUCTION OF EPIDERMAL HYPERPLASIA

A variety of natural and synthetic retinoids has been found to induce hyperplasia of the suprabasal layers of mouse epidermis after local application.^{8,10-12,20} The thickness and number of cell layers of the viable epidermis (i.e., all the cell layers except for the stratum corneum) and the component strata can be readily measured under light microscopy (see Figure 1). We have used such measurements to examine the dose responses for the induction of hyperplasia in skin biopsies taken from mice treated with various retinoids.^{11,12} During the studies the mice were examined daily for signs of toxicity such as unusual scaling, erythema or edema, and weight loss, and the skin biopsies were routinely examined for more subtle histological indications of toxicity such as the presence of inflammatory-cell infiltration, spongiosis, keratinocyte necrosis, or epidermal erosion. Dose response curves for the induction of hyperplasia by TTNPB ethyl ester and all-*trans*-retinoic acid are shown in Figure 3. Above threshold doses, the number of cell layers increases in proportion to the log of the dose up to a maximum, after which the response plateaus and may decline. The initial increase is sufficiently linear that kinetic parameters can be derived by regression analysis. In practice, the slopes obtained are generally similar for active retinoids, ranging from 1.2 to 2.0 cell layers per log increase in dose. The distance between the parallel regression lines is a measure of relative potency. Potency values can be assigned by determining the dose inducing a fixed response. We arbitrarily used the dose corresponding to an increase in the number of cell layers by 50% (ED_{50}) because less potent retinoids, which may require dosing at high levels, can be assigned values. The super maximal region of the dose response curve is increasingly associated with histological and gross signs of toxicity, as described above.

Potencies relative to all-*trans*-retinoic acid and ED_{50} values for 14 retinoids after local application are shown in Table 2. Changes in any of three major structural components—the cyclohexene ring, the polyene chain, and the polar terminal group can have dramatic effects on potency and activity. The ED_{50} values found ranged over four orders of magnitude. Alterations in the cyclohexene ring generally lowered potency but with less striking effects than changing the stereochemistry of the polyene side chain or changing the polar terminal group (Table 2). Aromatization of the cyclohexene ring (as in the etretin analogs) reduced

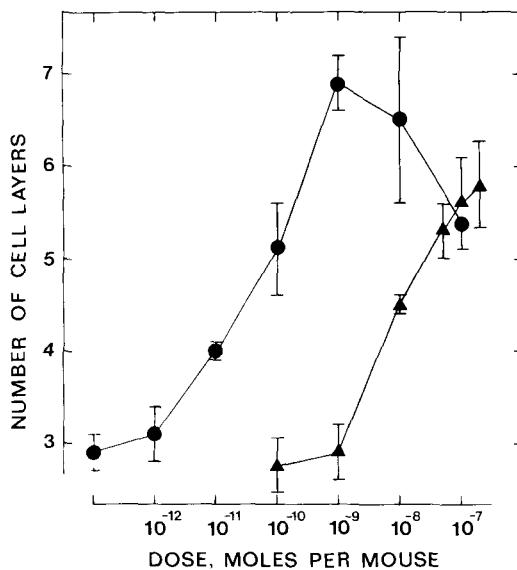


FIGURE 3. Dose response curves for the induction of epidermal hyperplasia by TTNPB ethyl ester (dots) and all-*trans*-retinoic acid (triangles). Biopsies were taken 4 d after a single topical treatment. Values for the number of cell layers are the mean \pm standard deviations for groups of 3 to 6 mice.

TABLE 2
The Induction of Epidermal Hyperplasia by Topically Applied Retinoids

Retinoid	ED ₅₀ (nmol)	Slope*	Relative potency
TTNPB ethyl ester (11)	0.034	1.45 \pm 0.20	559
TTNPB methyl (12)	0.11	1.45 \pm 0.20	173
TTNPB free acid (10)	0.51	1.90 \pm 0.21	37
TTNPB ethyl sulfone (13)	1.15	1.65 \pm 0.25	16
Decarboxylated TTNPB (9)	>1000	NS	—
All- <i>trans</i> -retinoic acid (1)	19.0	1.28 \pm 0.18	1
Retinol (3)	40.5	4.61 \pm 1.13	0.47
Retinal (2)	35.7	3.97 \pm 1.14	0.53
Etretinate (7)	36.4	1.77 \pm 0.68	0.52
Etretin (6)	88.3	1.85 \pm 0.38	0.22
Trimethylthiophene analog of all- <i>trans</i> -retinoic acid (14)	50.0	1.28 \pm 0.41	0.38
N-(4-hydroxyphenyl)retinamide (4)	181	2.26 \pm 0.14	0.10
13-cis-Retinoic acid (5)	233	2.25 \pm 1.14	0.08
cis-Locked aryl triene analog of 13-cis-retinoic acid (8)	>1000	NS	—

* Slope \pm standard error. Slopes are significantly different from zero ($p < 0.05$) except where marked NS (not significant).

the potency of the retinoids to 30 to 50%. The trimethyl thiophene analog (**14**) of retinoic acid was as potent as etretin (**6**). Neither ring modification impaired activity. The potent TTNPB analogs, which contain an aromatic ring adjacent to the cyclohexene ring, are discussed later.

In contrast to the minor effects on potency afforded by alterations in the ring system,

TABLE 3
Comparison of the Relative Potencies of Retinoids to All-*Trans*-Retinoic Acid in
Four *In Vivo* Assays

Retinoid	Epidermal hyperplasia	ODC induction	Antipapilloma effect	Rat growth assay
TTNPB (10)	37	5-10 ³⁸	500 ³⁶	35 ³⁵
All- <i>trans</i> -retinoic acid (1)	1	1	1	1
Retinol (3)	0.47	0.13 ²⁶	—	1
Etretin (6)	0.22	0.01 ³⁷	8 ⁴²	—
13- <i>cis</i> -retinoic acid (5)	0.08	0.14 ³⁸	0.5 ⁴³ inactive ⁴⁴	(0.85) ⁴⁵

Note: The data presented is compiled from reports in which dose response studies were performed, except where given in parenthesis. The retinoids were applied topically for the induction of epidermal hyperplasia and the inhibition of ornithine decarboxylase induction, and systemically for the antipapilloma effect and rat growth assays.

striking increases and decreases in activity and potency are induced by changes in the polyene side chain. Other than the decarboxylated TTNPB derivative (**9**), which lacks a polar terminal group, the TTNPB derivatives were much more potent than retinoic acid (Table 2). A single topical dose of 0.01 nmol of TTNPB ethyl ester was effective (Figure 3). The exotic molecular structure of these retinoid analogs, particularly the virtual replacement of the polyene side chain, makes their great potencies even more remarkable. Their activity as retinoids is ascribed to their structural analogy to a rigidly locked polyene chain.³⁶ Based on X-ray diffraction studies Strickland et al.³⁹ have shown that the three-dimensional shapes of TTNPB and all-*trans*-retinoic acid are almost superimposable. Although the topological similarity is based on analysis of crystal structures, and may or may not reflect the actual steric configurations occurring in biological systems, these workers also showed that the biological activity of the *meta*-carboxy isomer of TTNPB, which is not superimposable on the all-*trans*-configuration, was 1000-fold lower than that of TTNPB. Consequently, the molecular topography of the polyene side chain and not just its chemical nature may be an important determinant of biological activity. Extensions of this line of argument are supported by the relative activities of all-*trans* and *cis* isomers of the more traditionally structured retinoids. All-*trans*-retinoic acid is more potent than 13-*cis*-retinoic acid (**5**) at inducing epidermal hyperplasia (Table 2), as is true in other *in vivo* assays (Table 3). Because 13-*cis*-retinoic acid and all-*trans*-retinoic acid exhibit similar pharmacokinetic behavior after topical application,³³ gross differences in epidermal uptake or loss do not appear to be a factor in the difference in potencies in the epidermis. The dose of 13-*cis*-retinoic acid required to inhibit the induction of ornithine decarboxylase activity is seven times higher than that of all-*trans*-retinoic acid.³⁸ In addition 13-*cis*-retinoic acid shows little activity in the antipapilloma assay.^{43,44} A 13-*cis*-retinoic acid analog locked in the *cis* configuration by the introduction of a benzene ring in the side chain (the *cis*-locked aryl triene analog, **8**) is inactive at inducing epidermal hyperplasia (Table 2). This *cis*-locked analog also shows little ability to inhibit the induction of epidermal ornithine decarboxylase activity although the equivalent all-*trans* form is active.⁴⁰ Therefore, it seems likely that these biological activities of 13-*cis*-retinoic acid are due to chemical, light, or enzymic isomerization to the *trans* form *in situ*, and this may, in turn, explain its diminished toxicity. In addition to exerting effects on the follicular epidermis in acne therapy similar to those seen with all-*trans*-retinoic acid, 13-*cis*-retinoic acid exhibits an unusual property in man. After systemic administration of therapeutic doses of 13-*cis*-retinoic acid there is a dose-dependent reduction in sebum secretion from the sebaceous glands that may have a pivotal role in the therapeutic action of this retinoid against severe cystic acne.¹ A comparable response has yet to be observed after administration of

other retinoids to humans, with the caveat that relatively few retinoids have been thoroughly evaluated in the systemic therapy of acne. Thus, it is not clear (1) if the anti-sebaceous action of 13-*cis*-retinoic acid can be regarded as a true "retinoid" effect, and (2) in view of the lack of a comparable response after topical administration, if the effect on the sebaceous glands is direct, or if the response is mediated by a systemically formed (presumptively *cis*) metabolite.

Modifications of the polar terminal group also lead to markedly altered activity and potency at inducing epidermal hyperplasia. Generally the differences can be attributed to the need for metabolic activation in the case of diminished activity, or to more favorable changes in lipophilicity imparted by abrogating the charge of the polar group in the case of increased potency. The governing factor in these areas is, of course, the metabolic pathways in the epidermis. Thus, despite their potent effects in some tissues, locally applied retinoic acid amides tend to show low biological activity in the epidermis. For example they are inactive or have low potency in inhibiting the induction of epidermal ornithine decarboxylase activity.³⁷ *N*-(4-Hydroxyphenyl)retinamide (**4**) has both low potency (0.1 relative to all-*trans*-retinoic acid) and diminished activity at inducing epidermal hyperplasia, inducing an increase in epidermal thickness that plateaus at about 50% above control levels.¹¹ The *N*-ethylretinamide analog of etretin (motretinid) has a relative potency of 0.02 compared to that of all-*trans*-retinoic acid at decreasing utricle diameter in the rhino mouse.³⁰ In contrast, motretinid administered systemically has been reported to be four-fold more potent than retinoic acid at causing regression of chemically induced skin papillomas in the mouse.⁴² It has yet to be established if the latter observation reflects activation by systemic metabolism or a difference in the susceptibility of papillomas compared to the epidermis. It is apparent, however, that mouse epidermis is unable to utilize these amides efficiently. Therefore, it is possible that the diminished activity of *N*-(4-hydroxyphenyl)retinamide at inducing epidermal hyperplasia, even at relatively high doses, may reflect saturation of a crucial metabolic step.

Analysis of the dose response curves for the naturally occurring retinoids, retinol (**3**) and retinal (**2**), reveals that the slopes are notably steeper than those for retinoic acid and its analogs (Table 2). The anomalous behavior of retinol and retinal reflect several factors, including the relatively high endogenous retinol level (1 nmol/g of epidermis),²⁶ the lower tissue uptake of retinol,²⁶ and the need for metabolism for activity. Topically applied retinol is oxidized to retinoic acid in the epidermis,²⁶ and the enzymic pathway involved has been partly characterized.³¹ The reaction is catalyzed in the epidermis by an alcohol dehydrogenase isozyme, absent from the liver but common to other retinoid target tissues, and by an aldehyde dehydrogenase. If the oxidation is inhibited,³¹ the potency of retinol at inducing epidermal hyperplasia is reduced,³² implying that retinoic acid formation is required for this activity. The need for metabolism establishes a biochemical mechanism to explain the differences in the slopes of the dose response curves for retinol and retinoic acid. The observation also has important implications for structure-activity studies. The amount of retinoic acid found in the epidermis after topical retinol application is small, amounting to 2 to 3% of the retinol found,^{26,31} and is much lower than the epidermal retinoic acid level found after comparable doses of retinoic acid, itself. Because the relative potencies of retinol and retinoic acid differ by only about twofold (an approximation because the relative potency is dose dependent since the regression lines for the dose-response curves are not parallel), it follows that only a small fraction of the retinoic acid present in the epidermis after retinoic acid application is actively involved in evoking the biological response. Consequently, the majority of the retinoic acid in the epidermis found after topical application must be sequestered or quartered in a biologically refractory pool. The subsequent metabolism and handling of this sequestered pool may differ substantially from that of the retinoic acid involved in evoking the biological response.

Although the ethyl esters of TTNPB and etretin are hydrolyzed in the epidermis to the

free acids in small amounts,³³ it is not clear if hydrolysis of the ester bond is obligatory for activity, because the esters tend to be more potent than the free acids (Table 2). In view of the role of the small amount of retinoic acid formed from retinol in inducing the hyperplastic response, the obligatory hydrolysis of the esters for activity cannot be ruled out. It is possible that the greater potency of the esters reflects facilitated entry into critical cell compartments or cell subpopulations that are less accessible to retinoids bearing charged carboxylic acid groups. Although 33-fold less potent than the ethyl ester of TTNPB, the ethyl sulfone analog (**13**) is still 16-fold more potent than retinoic acid. The potency of the TTNPB ethyl sulfone may indicate that it is the possession of a terminal acid function, rather than a carboxyl group *per se*, that is the determinant for activity. More extensive metabolism other than simple hydrolysis is needed to explain the activity of the methyl ether analog of TTNPB (**12**), which is less potent on topical application than the ethyl ester is but more potent than the free acid. A possible metabolic route could be by conversion to the alcohol, as is known to occur for retinyl methyl ether,³⁴ followed by oxidation to the acid. The advantage of the ester over the ether appears to be lost after systemic dosing. On oral administration the ethyl ester and methyl ether analogs of TTNPB (0.3 $\mu\text{mol}/\text{kg}$) induced identical submaximal hyperplastic responses.¹² Further studies are needed to establish the metabolic routes and subcellular compartments available to these interesting compounds before definitive answers can be gained.

Three commonly used *in vivo* assays for retinoids are (1) the ability to support the growth of rats on vitamin A-deficient diets,³⁵ (2) the ability to cause the regression of chemically induced skin papillomas,³⁶ and (3) the ability to inhibit the induction of epidermal ornithine decarboxylase.^{37,38} In Table 3 the relative potencies of several parent retinoid structures are compared for these assays using, wherever possible, data from published studies in which dose dependencies were evaluated. While direct comparisons of results obtained in these assays with results obtained in the epidermal hyperplasia assay are complicated by methodological differences in the derivation and calculation of the results, by pharmacological variables such as variations in dosing routes, and by the species used, the rank orders of potencies obtained for different retinoids are similar for the four activities. The TTNPB analogs are the most potent retinoid analogs in all of the *in vivo* assays. TTNPB (**10**) was recently reported to support the growth of vitamin A-deficient rats,³⁵ thus fulfilling the nutritional definition of a retinoid. Loeliger et al.³⁶ compared various TTNPB analogs in the regression of skin papillomas assay obtaining results that parallel those presented in Table 2. The ethyl ester of TTNPB was more potent than the methyl ether and both were considerably more potent than the free acid. The decarboxylated analog (**9**) was inactive. The dose of TTNPB (free acid) needed to inhibit the induction of epidermal ornithine decarboxylase activity by 50% was 5 to 10 times lower than that of all-*trans*-retinoic acid.³⁸ The similarity in the ranking of these parent structures and their derivatives may bespeak of a uniform underlying molecular mechanism. In so far as the data suggests, the presence of a free acid group or the metabolic potential for the formation of a free acid group is required to induce the hyperplastic response. A role for CRABP in mediating this activity is possible. However the lack of substrate stereospecificity for binding to CRABP, in contrast to the biological responses in the epidermis, indicates that CRABP binding is not the ultimate factor in controlling activity.

VI. CONCLUSIONS AND IMPLICATIONS FOR RETINOID ACTION

Although extrapolation of data derived from animals to the human must be treated with caution, ample evidence supports the occurrence of similar stimulatory processes in human epidermis to those observed in the mouse. Thus, topical retinoid treatment has been found

to increase desquamation and the epidermal mitotic index in ichthyosis patients⁴¹ and to increase proliferative activity and cell renewal in the follicular epidermis of patients with acne.¹⁴ The stimulation of the formation of differentiation competent cells may explain the reappearance of the granular cell layer, an early event in the clearing of psoriatic plaques. The ability of retinoids to stimulate the production of differentiating cells through enhanced epidermal proliferation forms a potentially useful working hypothesis to explain their therapeutic efficacy in such a diverse group of skin diseases.

The structural requirements for retinoids to induce epidermal hyperplasia fulfill the general topological needs observed for retinoid activity in the areas of growth and epithelial function. The requisite structural attributes may be summarized as the presence of a hydrophobic end group, a hydrophobic linkage group with a stereochemical configuration analogous to an all-*trans* polyene chain, and the presence of, or metabolic potential for the formation of, a terminal acid function. The unique accessibility of the epidermis facilitates investigations of the disposition, metabolism, and handling of retinoids in relation to their activity *in vivo*. Further studies in this system offer good opportunities for additional refinements of the structural needs of retinoids for activity.

ACKNOWLEDGMENTS

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ABBREVIATIONS

Unless stated otherwise the retinoids discussed are in the all-*trans*-configuration. The *cis*-locked aryl triene analog of 13-*cis*-retinoic acid, (*E*)-1-(2-carboxyphenyl)-4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1,3,5-hexatriene (**8**); etretin, (*E*)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid (**6**); etretinate, etretin ethyl ester (**7**); motretinid, *N*-ethyl-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nona-tetraenamide; retinoic acid, (*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid (**1**); 13-*cis*-retinoic acid, (13*Z*)-retinoic acid (**5**); trimethylthiophene analog of retinoic acid, (*E*)-3,7-dimethyl-9-[3-(2,4,5-trimethyl)thienyl]-2,4,6,8-nonatetraenoic acid (**14**); TTNPB, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propen-1-yl]benzoic acid (**10**).

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Chapter 19

RETINOID INHIBITION OF EXPERIMENTAL CARCINOGENESIS**Richard C. Moon and Rajendra G. Mehta****TABLE OF CONTENTS**

I.	Introduction	502
II.	Retinoids and Urinary Bladder Carcinogenesis.....	502
III.	Retinoids and Mammary Carcinogenesis.....	504
A.	Carcinogenesis Models	504
B.	Chemoprevention of Mammary Cancer by Retinoids	505
1.	Are Retinoids Selectively Effective Against Initiation or Promotion Phases of Carcinogenesis?.....	507
2.	Can Retinoid Treatment Be Delayed? If So, How Long?	507
3.	Can Retinoids Influence the Growth of the Existing Tumors?	507
4.	Are Retinoids Effective After the Surgical Removal of the First Tumor?	508
5.	Can Retinoid Treatment be Improved by Combination with Other Modifiers of Carcinogenesis?	509
IV.	Effect of Retinoids in Mammary Gland Organ Culture	510
V.	Mechanism of Retinoid Action in the Mammary Gland.....	513
	References.....	516

I. INTRODUCTION

Several epidemiological investigations have indicated an inverse relationship between vitamin A intake and risk for developing cancer.^{1,2} During the past 15 years, extensive effort has been directed towards understanding the role of vitamin A and retinoids in the prevention of cancer as well as the mechanism of action of such compounds. A retinoid by definition includes any substance, natural or synthetic, with vitamin A-like activity. Since vitamin A cannot be synthesized in the body, it must be ingested as either a provitamin (e.g., β -carotene), in food, or as preformed retinol or its esters. Lack of vitamin A in experimental animals as well as in human beings results in deficiency syndromes leading to several diseases, many of which may be fatal.^{3,4}

Experimentally, the role of vitamin A in regulating epithelial cell differentiation and maintenance was first demonstrated by Mori⁵ and subsequently by Wolbach and Howe⁶ over 60 years ago. They showed that feeding animals a diet deficient in retinoids resulted in the appearance of hyperkeratinization, squamous metaplasia, and gross tumors in a variety of epithelial tissues in these experimental animals, a process very similar to that induced by certain chemical carcinogens.⁷ Furthermore, it has also been reported that exposure of vitamin-A deficient animals to chemical carcinogens results in an increased incidence of preneoplastic lesions and cancer. Although the response of various epithelia to the deficient state varies significantly, the systemic administration of retinoids reverses the process and restores the epithelium to a normal functional capacity.

Since carcinogen-induced metaplasia appears similar to that resulting from retinoid deficiency and retinoids are known to reverse such conditions in many epithelial tissues, attempts have been made to study the effects of exogenous retinoids on the inhibition of induction and progression of cancers at various organ sites, including skin, esophagus, stomach, intestine, liver, nervous system, pancreas, urinary bladder, lung, and mammary gland.⁸ However, the majority of evidence has accumulated from studies of retinoids as chemopreventive agents for carcinogenesis of the skin, urinary bladder, and breast. Since the major emphasis of retinoid research in our laboratory is focused on the chemoprevention of urinary bladder and mammary carcinogenesis, this report will summarize the salient features of retinoid inhibition of cancer in these two organs and address the mechanism of inhibition in mammary gland.

II. RETINOIDS AND URINARY BLADDER CARCINOGENESIS

Chemically induced carcinogenesis of the urinary bladder in experimental animals is a multistage process involving initiation and promotion.⁹ Earlier studies have shown that vitamin A deficiency resulted in the development of squamous metaplasia of the normal transitional epithelium of the bladder. Furthermore, the vitamin A-deficient animals were more sensitive to the bladder carcinogen *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) than were animals receiving a physiological concentration of vitamin A.¹⁰ Also, epidemiological studies have indicated an inverse relationship between vitamin A intake or serum vitamin A level and bladder cancer.¹¹ The results of these studies provided a sound rationale for studying the effects of dietary retinoids on urinary bladder carcinogenesis.

Inhibition of bladder carcinogenesis by retinoids was first reported by Sporn and colleagues.¹² They showed that dietary 13-*cis*-retinoic acid inhibited the incidence and reduced the severity of the squamous cell carcinomas of the bladder in Wistar rats induced by intravesicle administration of *N*-methyl-*N*-nitrosourea (MNU). Several animal model systems have been utilized to study bladder carcinogenesis in rodents (Table 1). Since the majority of the urinary bladder cancers that develop in humans are of the transitional cell rather than squamous cell type, a model system for development of transitional cell carcinomas both

TABLE 1
**Experimental Animal Models for Evaluating Effects of Retinoids in Urinary
 Bladder Carcinogenesis**

Strains	Carcinogen	Predominant tumor type
Sprague-Dawley rats	FANFT	Transitional cell carcinoma (TCC)
Wistar Lewis rats	MNU	TCC, squamous cell carcinoma (SCC) and papilloma
	OH-BBN	
Fisher F344 rats	OH-BBN	TCC (Low grade), papilloma
	FANFT	SCC
BDF mice	OH-BBN	TCC (Highly invasive)
C57BL/6 mice	OH-BBN	SCC

Note: OH-BBN: *N*-butyl-*N*-4-hydroxybutylnitrosamine; FANFT: *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; MNU: *N*-methyl-*N*-nitrosourea.

in rats and mice was established in our laboratory to evaluate the efficacy of various retinoids on the lesions. The majority of tumors in this model induced by 60-90 mg total dose of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (OH-BBN) were principally transitional cell carcinomas.¹³ In Fischer F344 female rats, oral administration of 150 mg of OH-BBN twice weekly for 6 weeks resulted in nearly 60% transitional cell carcinomas within 6 months,¹³ whereas in C57BL/6 × DBA/2-F1 (BDF) male mice 10 weekly intragastric administrations of 6 mg of OH-BBN resulted in 30 to 40% transitional cell carcinomas.¹⁴

In these studies utilizing the OH-BBN model, 13-*cis*-retinoic acid supplementation in the diet initiated 1 week after the last carcinogen dose significantly reduced the incidence and severity of transitional cell carcinoma. Since in the clinical situation bladder cancer is preceded for many years by dysplasia or preneoplasia, it was deemed necessary to determine whether it was possible to delay the initiation of retinoid treatment for some time after the carcinogenic insult without compromising retinoid efficacy. In a study where retinoid treatment was initiated at 0, 1, 3, 5, and 9 weeks postcarcinogen exposure, as shown in Table 2, it was observed that the administration of 13-*cis*-retinoic acid could be delayed up to 9 weeks following carcinogen exposure without losing its activity as a chemopreventive agent.¹⁵

Since these early reports utilizing the natural retinoids and 13-*cis*-retinoic acid, several synthetic retinoids have been evaluated for their anticancer activity in mouse and rat bladder carcinogenesis. Extensive work along these lines has been completed in our laboratory; in particular, numerous amide analogs of retinoic acid have been evaluated because of their low toxicity and high potency in the hamster tracheal organ culture assay.¹⁶ The activity of these various retinamides is summarized in Table 3. The results indicated that although *N*-(2-hydroxypropyl) retinamide was effective against OH-BBN-induced bladder cancers, the propyl derivatives hydroxylated at the 3-position [*N*-(3-hydroxy- or *N*-2,3-dihydroxy)propyl-retinamides] were inactive. *N*-(4-hydroxyphenyl) retinamide was found to be the most active when both toxicity and efficacy were considered. A comparison of the all-*trans* and 13-*cis* isomers of retinamides indicated that the 13-*cis* isomers were just as active as the all-*trans* isomers in inhibiting bladder carcinogenesis.¹⁷

A definite pattern of structure-activity relationships was not apparent for the ability of retinoids to prevent experimental bladder cancer, despite the fact that all of the retinoids that have been tested for prevention of bladder cancer possess significant *in vitro* activity in controlling epithelial cell differentiation in the hamster tracheal organ culture bioassay. Thus, in addition to the intrinsic biological activity of retinoids, other factors such as their bioavailability or their metabolism *in vivo* play a key role in determining cancer prevention in the whole animal.

TABLE 2
Effect of 13-cis-Retinoic Acid: Incidence and Average Number of TCC

Carcinogen dose level	TCC with atypia score equal to or greater than	Avg. no. of TCC/animal					
		Retinoid treatment delay period ^a			Combined data ^b		
		Wk 1	Wk 5	Wk 9	Retinoid-treated	Placebo-treated	
Low dose (1200 mg)	5	0.0	0.0	0.0	0.0 ^c	0.11	
	3	0.10	0.20	0.30	0.20 ^d	0.62	
	1	0.43	0.57	0.60	0.53 ^c	0.89	
Middle dose (1800 mg)	5	0.0	0.0	0.0	0.0 ^c	0.10	
	3	0.85	0.71	0.89	0.57 ^d	1.24	
	1	1.31	0.96	1.32	1.20 ^d	1.84	
High dose (2400 mg)	5	0.0	0.0	0.0	0.0 ^d	0.17	
	3	0.96	0.73	0.93	0.88 ^d	1.95	
	1	1.68	1.40	1.37	1.48 ^d	2.69	

^a At 1, 5, and 9 weeks after the last OH-BBN intubation, animals were fed diet supplemented with 240 mg of 13-cis-retinoic acid per kg for the duration of the study.

^b Data from the 1-, 5-, and 9-week delay periods were analyzed for statistical homogeneity; no statistically significant differences were found. Therefore, data were combined into either a retinoid- or placebo-treated group.

^c Different from respective control group, $p < 0.01$ (one sided).

^d Different from respective control group, $p < 0.001$ (one sided).

TABLE 3
Retinoids Evaluated for Cancer Chemopreventive Activity Against Experimental Urinary Bladder Cancer in Rats and/or Mice

Active	Inactive
Retinoid acid	Retinyl palmitate
13-cis-Retinoic acid	<i>N</i> -(4-Carboxyphenyl)retinamide
Retinyl acetate	<i>N</i> -(3-Carboxypropyl)retinamide
<i>N</i> -Ethylretinamide	<i>N</i> -(3-Hydroxypropyl)retinamide
<i>N</i> -Ethyl-13-cis-retinamide	<i>N</i> -(2,3-Dihydroxypropyl)retinamide
<i>N</i> -(2-Hydroxyethyl)retinamide	<i>N</i> -(n-Butyl)retinamide
<i>N</i> -(2-Hydroxyethyl)-13-cis-retinamide	<i>N</i> -(n-Butyl)-13-cis-retinamide
<i>N</i> -(4-Hydroxyphenyl)retinamide	<i>N</i> -(4-Hydroxybutyl)retinamide
<i>N</i> -(4-Hydroxyphenyl)-13-cis-retinamide	<i>N</i> -(4-Hydroxybutyl)-13-cis-retinamide
<i>N</i> -(2-Hydroxypropyl)retinamide	<i>N</i> -(5-Tetrazolyl)-13-cis-retinamide
<i>N</i> -5-Tetrazolylretinamide	TMMP ^a analog of <i>N</i> -ethylretinamide
TMMP ^a analog of retinoic acid ethyl ester	

^a TMMP = 4-Methoxy-2,3,6-trimethylphenyl.

III. RETINOIDS AND MAMMARY CARCINOGENESIS

A. CARCINOGENESIS MODELS

Numerous reports have indicated that mammary cancers can be selectively induced in rats by either 7,12-dimethylbenz[a]anthracene (DMBA) or *N*-methyl-*N*-nitroso-urea (MNU). Both MNBA- and MNU-induced mammary tumor models have been successfully utilized for chemoprevention studies. The MNU-induced tumor model was originally described by Gullino et al.¹⁸ and subsequently modified in our laboratory.¹⁹ When DMBA was administered in a single intragastric dose at a concentration of 15 mg/ml of sesame oil/rat, a 90

TABLE 4
Effect of Retinyl Acetate on Mammary Carcinogenesis

Group	NMU	Retinoid	No. of rats with cancer/total	Incidence of mammary cancer (%)	Total no. of tumors	
					Cancer	Benign
1	None	Placebo	0/30	0	0	0
2	None	Retinyl acetate	0/30	0	0	0
3	High dose	Placebo	25/30	83	61	17
4	High dose	Retinyl acetate	15/30	50 ^a	25 ^a	4 ^a
5	Intermediate dose	Placebo	12/29	41	16	3
6	Intermediate dose	Retinyl acetate	7/30	23 ^a	11 ^a	1
7	Low dose	Placebo	6/30	20	7	2
8	Low dose	Retinyl acetate	0/30	0 ^a	0 ^a	0

Note: Female Sprague-Dawley rats received either saline or MNU in saline (5.0, 2.5, or 1.25 mg per 100 g of body weight) by i.v. injection at 50 d of age. A second injection was administered one week later. The animals were placed on either retinyl acetate (250 mg per kg of diet) or placebo beadlets 3 d after the second injection and sacrificed 175 d after the initial MNU injection.

^a Significantly different from respective placebo control group, *p* < 0.05 or less.

to 100% incidence of mammary tumors was obtained at 180 days postcarcinogen whereas a single intravenous injection of 50 mg of MNU/kg of body weight (pH 5.0) also induced a 100% mammary tumor incidence in rats during the six-month period. The majority of the cancers induced by these carcinogens are ovarian hormone dependent with a small percentage of tumors remaining as hormone independent. Earlier studies on chemoprevention by retinoids were conducted with the DMBA-induced cancer model; however, the MNU-induced mammary cancer model has remained a model of choice in our studies for several reasons: (1) DMBA-induced tumors are encapsulated and do not metastasize; (2) DMBA must be metabolized to an active form; and (3) DMBA induces a high incidence of adenomas and fibroadenomas. These complications do not arise in the MNU-induced cancer model. Retinoid chemoprevention studies on spontaneous mammary cancer in mice have been conducted with virus-positive mice, except for the study in which C3H mice negative for mammary tumor virus (MTV) were used (unpublished). Other studies in mice have involved mammary tumor induction in the BDF₁ and GR strains with DMBA and the ovarian steroids, respectively.

B. CHEMOPREVENTION OF MAMMARY CANCER BY RETINOID

The first report relative to retinoid chemoprevention of mammary carcinogenesis was by Schmahl et al.²⁰ They observed that retinyl palmitate afforded little protection against DMBA-induced mammary carcinogenesis. The first evidence that retinoids may be effective chemopreventive agents against breast cancer was that reported by Moon et al.,²¹ who showed that dietary supplementation of retinyl acetate resulted in a 52% inhibition of MNU-induced mammary carcinogenesis (Table 4). The criteria used for determination of chemopreventive activity of the retinoid were an increase in latency of first tumor appearance, decrease in number of animals with cancer, and a reduction in the number of carcinomas per rat. Since the original studies with retinyl acetate, a series of synthetic retinoids have been evaluated for efficacy against chemically-induced mammary cancer. The relative efficacy of all the retinoids evaluated for mammary carcinogenesis is listed in Table 5. When both the toxicity and chemopreventive activity of various retinoids were compared, it was observed that *N*-(4-hydroxyphenyl)retinamide (4-HPR) was the most efficacious.²²

Recently, the effects of retinoidal benzoic acid derivatives (arotinoids) have been eval-

TABLE 5
Effect of Retinoids on Mammary Carcinogenesis of Rats *In Vivo*

Host/Strain	Carcinogen	Retinoid	Effect
Rat (S/D) ^a	DMBA	Retinyl palmitate	No effect
Rat (S/D)	DMBA	Retinyl acetate	Inhibition
Rat (Lewis)	DMBA	Retinyl acetate	of
Rat (S/D)	DMBA	<i>N</i> -(4-Hydroxyphenyl)retinamide	carcinogenesis
Rat (S/D)	MNU	Retinyl acetate	
		Retinyl methyl ether	
		<i>N</i> -(4-Hydroxyphenyl)retinamide	
		Axerophthene	
		All- <i>trans</i> -Retinoic acid	
		<i>N</i> -(4-Hydroxyphenyl)-13- <i>cis</i> -retinamide	
		13- <i>cis</i> -Retinoic acid	
		Retinyl butyl ether	
		<i>N</i> -Ethylretinamide	
		Retinylidene dimedone	
		Retinylidene acetylacetone	
		TMMP analog of retinyl ethyl ester	
		TMMP analog of retinyl methyl ether	
		Retinyl acetate	No inhibition
Rat (Lew/Mai)	Benzo[a]pyrene	Retinyl acetate	Inhibition of carcinogenesis

^a S/D: Sprague-Dawley.

uated against rat mammary cancer induced by DMBA. It was observed that oral administration of arabinoid ethyl ester and arabinoid ethyl sulfone inhibited the tumor multiplicity and volume of mammary tumors; however, a reduction in the body weight accompanied the administration of these compounds.²³ More recently, Bollag and Hartmann²⁴ have studied the effects of Ro 15-0778, an arabinoid without a polar end group; this compound apparently exhibits little, if any, toxicity. The results indicated that the retinoid inhibited carcinogenesis in a dose-dependent manner; at a 270 mg/kg of body weight/day-dose level, tumor number was reduced by 70%. Experiments are in progress in our laboratory to determine the efficacy of this retinoid against both OH-BBN-induced urinary bladder and MNU-induced mammary carcinogenesis.

The evidence supporting the chemopreventive activity of retinyl acetate in rat mammary tumor models is substantial; however, only three studies describing the use of this retinoid in mammary carcinogenesis in mice have been reported. In two of these experiments, the effect of retinyl acetate was determined on either the virally induced (C3H/A^v mice) or ovarian hormone-induced mammary tumors in the virus-positive GR strain of mice.^{25,26} Retinyl acetate did not have any effect on the tumor incidence or latency in C3H mice,²⁵ whereas in GR mice tumor development appeared to be enhanced.²⁶ In a third study using virus-free C3H mice, however, retinyl acetate decreased tumor incidence.²⁷ These results indicate that there appears to be a degree of species as well as strain specificity for retinoid chemoprevention in rodents.

The studies described above clearly indicate that certain retinoids are effective chemopreventive agents against chemically induced rodent mammary cancers when administered shortly after carcinogenic insult. However, it is not possible to determine the exact period of carcinogen exposure in the human population. For retinoids to be clinically useful, several questions need to be answered.

1. Are retinoids effective against initiation, promotion, or both phases of carcinogenesis?
2. Can retinoid treatment be delayed? If so, how long?

3. Can retinoids influence the growth of existing tumors?
4. Are retinoids effective after surgical removal of the first tumor?
5. Can the retinoid effect be improved by combination therapy with other modifiers of carcinogenesis?

We have attempted to address some of these questions, and the results from such experiments are summarized below.

1. **Are retinoids selectively effective against the initiation or promotion phase of carcinogenesis?** McCormick et al.²⁸ studied the temporal relationships of carcinogen and retinoid administration. They reported significant inhibition of tumorigenesis when retinyl acetate treatment was begun 1 week after the administration of DMBA and continued until the termination of the experiment; they also noted that the administration of retinyl acetate during a restricted period (2 weeks before to 1 week after DMBA) during the early phase of mammary carcinogenesis inhibited the development of mammary tumors. The treatment with retinyl acetate during this early phase appears to impart a permanent inhibition upon tumorigenesis, while treatment with retinyl acetate beginning 1 to 12 weeks after DMBA appears to require continued treatment with the retinoid since the mammary tumor response approaches that of the control animals after cessation of retinoid treatment. Subsequent studies confirmed these earlier findings and showed that a continuous intake of retinoid was necessary to sustain the chemopreventive effect during the progression stage of mammary carcinogenesis.²⁹
2. **Can retinoid treatment be delayed? If so, how long?** In a majority of chemoprevention studies, retinoid treatment is initiated shortly after carcinogen administration. However, for clinical trials it is beneficial to know whether the initiation of retinoid treatment can be delayed for a period of time after carcinogenic insult without losing its chemopreventive activity. McCormick and Moon³⁰ conducted a detailed study in which mammary tumors were induced with two dose levels (25 mg and 50 mg/kg of body weight) of MNU. At the high MNU dose level, retinoid treatment was begun at 1, 4, or 8 weeks postcarcinogen treatment, whereas for the 25 mg/kg-MNV dose level, dietary retinoid (328 mg of retinyl acetate/kg of diet) administration was initiated at 1, 4, 8, 12, 16, and 20 weeks postcarcinogen. The results of this study are shown in Table 6. It was observed that at the low-carcinogen-dose level, the retinyl acetate treatment could be delayed for up to 12 weeks without any loss of chemopreventive activity, whereas delaying the retinoid treatment for 20 weeks resulted in little protection against cancer development. At the higher concentration of MNU, on the other hand, the retinoid treatment could be delayed only up to 4 weeks without causing any loss in activity. These results indicate that during the process of mammary carcinogenesis there is apparently a critical phase or point at which the cells are responsive to retinoid inhibition; after this time, retinoid treatment becomes less effective. It is also possible that after a certain period of time, both retinoid-resistant and -sensitive cells may be present in the lesion. Thus, even if retinoid-sensitive cells respond to active retinoid, the effect may not be observed unless critical measurements of tumor size with respect to time are made. In earlier studies, McCormick and Burns²⁸ showed that retinyl acetate treatment can be delayed for 12 weeks in the DMBA-induced carcinogenesis model with a slight protection against cancer development. However, delay of 20 weeks with the same retinoid in the benzo[a]pyrene-induced mammary cancer model offered no protection against tumorigenesis.³¹
3. **Can retinoids influence the growth of the existing tumors?** Retinoids have always been used as chemopreventive agents under experimental conditions. As described in the previous section, treatment with retinoids normally begins after carcinogen admin-

TABLE 6
Effect of Delay in Retinyl Acetate (ROAc) Administration on Mammary Carcinogenesis in Rats Given 25 mg of MNU per kg of Body Weight³⁰

Group	Diet	T ₅₀ (days)	Body weight (g)	Cancer incidence (%)
1	Placebo	127	302 ± 4 ^a (100) ^b	80
2	ROAc + 1	160	291 ± 5(96)	78 ^c
3	ROAc + 4	170 ^c	281 ± 6 ^c (93)	69 ^d
4	ROAc + 8	205 ^c	281 ± 3 ^c (93)	64 ^d
5	ROAc + 12	163 ^c	277 ± 7 ^c (92)	70 ^d
6	ROAc + 16	134	288 ± 3 ^c (95)	70 ^c
7	ROAc + 20	131	285 ± 3 ^c (94)	74

^a Mean ± S.E.^b Numbers in parentheses, percentage of placebo value.^c p < 0.05 vs. placebo.^d p < 0.01 vs. placebo.

Note: Female Sprague-Dawley rats received dietary retinyl acetate (328 mg/kg of diet) beginning at various periods from 1 week (ROAc + 1) to 20 weeks (ROAc + 20) after administration of MNU. T₅₀ represents time to a 50% cancer incidence.

istration and continues until the end of the experiment. If the retinoid treatment is discontinued during a carcinogenesis experiment, the incidence and multiplicity of cancers in retinoid-treated animals subsequently approach those of the placebo-treated control group of animals.²⁹ Second, the delay of retinoid administration beyond a critical point renders retinoid treatment ineffective. These results suggest that in order for retinoids to function as chemopreventive agents, uninterrupted treatment is required. Until recently, no reports were available in which retinoid treatment was initiated after mammary tumors had appeared. Preliminary unpublished data from our laboratory suggest that certain retinoids may exert a chemotherapeutic effect as well as a chemopreventive effect on mammary cancer. In this study, rats were treated with a single injection of MNU and were then assigned to either a placebo or 4-HPR treatment group after the appearance of the first palpable tumor. Tumor size was monitored once each week, and the first tumor in 6 animals from the 4-HPR-treatment group of 30 rats regressed to a nonpalpable size and failed to reappear during the duration of the experimental period (in press). This observed 20% responsiveness to 4-HPR suggests that retinoids may provide efficacy as a chemotherapeutic agent in addition to their chemopreventive role against mammary carcinogenesis induced by MNU.

4. **Are retinoids effective after the surgical removal of the first tumor?** For retinoids to be clinically acceptable for women at high risk for breast cancer, they must either have chemotherapeutic activity, must influence the tumor recurrence after the surgical removal of an existing cancer, or must prevent the development of additional tumors. The effect of retinoids as a chemotherapeutic agent was described in the previous section. The efficacy of retinoids as protective agents against the recurrence of mammary tumors or the occurrence of subsequent tumors at other sites has also been evaluated in experimental animals. Two experiments, one with retinyl acetate and the other with 4-HPR (unpublished), have been conducted. The animals were treated with the carcinogen and allowed to consume a control diet. When the first palpable tumor reached one centimeter in size, the tumor was surgically removed and the animals were assigned to respective treatment groups. The results indicated that during the first 2 months after tumor excision very little quantitative tumor inhibition by the retinoid was evident, but a striking reduction in the appearance of subsequent tumors

TABLE 7
Influence of *N*-(4-hydroxyphenyl)retinamide (4-HPR) in Combination with Ovariectomy and Tamoxifen on Mammary Cancer Induction by MNU^a

Expt. No.	Groups	No. of animals	Cancer incidence (%)	Cumulative cancers per rat	T ₅₀ (days)
1	Intact-Placebo	25	100	4.50	55
	Intact-4-HPR	25	92 ^c	3.29 ^c	85
	Ovex ^b -Placebo	50	18 ^c	0.24 ^c	— ^e
	Ovex-4-HPR	50	2 ^{c,d}	0.02 ^{c,d}	— ^e
2	Placebo	30	100	6.64	63
	4-HPR	30	97	4.93 ^f	90
	Tamoxifen	30	73 ^f	1.16 ^f	165 ^f
	4-HPR + Tamoxifen	30	67 ^f	0.73 ^{f,g}	183 ^f

^a Animals sacrificed at 225 days postcarcinogen.

^b Ovex: ovariectomy. Tamoxifen (100 µg/rat) 3 times per week, reduced to 5 µg per rat at 16 weeks post-MNU.

^c Significantly different from intact-placebo group, *p* <0.05 or less.

^d Significantly different from ovex-placebo group, *p* <0.01 or less.

^e Group never reached 50% cancer incidence.

^f *p* <0.01 vs. vehicle control.

^g *p* <0.05 vs. 4-HPR only and tamoxifen only groups.

was noted in the retinyl acetate-treated rats.³² These results indicate that the inhibition of mammary cancer by the retinoid may result from suppressing the progression of an early lesion in the course of tumor development. These results also suggest that, if the retinoid protected against established tumors, the observed effect of retinoids on tumor development would not have been delayed for approximately 2 months. However, as noted in the previous section, 4-HPR treatment resulted in the regression of approximately 20% of established first tumors. The apparent paradox of these two sets of data is difficult to explain although in the previous experiment the size of the first tumor was monitored, whereas in this experiment the first tumor was excised. However, the data on the appearance of subsequent tumors in the 4-HPR-treated groups in both of these experiments were comparable.

5. **Can retinoid treatment be improved by combination therapy with other modifiers of carcinogenesis?** It has been well documented that hormonal alteration such as ovariectomy or use of antiestrogens inhibits mammary carcinogenesis in rodents and protects from recurrence in the human.³³ Similarly, treatment of carcinogen-exposed rats with an inhibitor of prolactin secretion, 2-bromo- α -ergocryptine, also reduced mammary cancer incidence and multiplicity.³⁴ Thus, experiments were conducted to determine if a combination of retinoid with hormonal alteration would provide enhanced protection against mammary carcinogenesis.

Earlier studies showed that both retinyl acetate and ovariectomy were effective against MNU-induced mammary carcinogenesis. The combined treatment synergistically enhanced the suppression of mammary tumor occurrence.³⁵ These studies were extended to evaluate the interaction of 4-HPR with either ovariectomy or tamoxifen,^{36,37} and the results again provided evidence for enhanced inhibition with the combination treatment of retinoid and hormonal deprivation. Better protection was afforded by the combination of retinoid and ovariectomy than by tamoxifen and retinoid (Table 7).

A similar interaction of hormone modulation with a retinoid has been studied using 2-bromo- α -ergocryptine and retinyl acetate.³⁸ The results were comparable to studies

described above in that the combination treatment was more effective than either treatment alone. Blood prolactin levels in rats treated with the retinoid were comparable to those of the control rats indicating that the retinoid effect was not mediated by a further suppression of prolactin secretion.

The effects of a combination of either ovariectomy³² or tamoxifen (unpublished) and retinoids have also been studied on the appearance of subsequent tumors following the surgical excision of the first palpable tumor. Each modality — ovariectomy, tamoxifen, or retinoid treatment — significantly inhibited the development of subsequent mammary tumors. A combination of either ovariectomy or tamoxifen with retinoid administration synergistically increased the inhibitory activity compared with treatment of each alone.

The results of the studies discussed above and in the previous sections provide evidence for the existence of both retinoid-sensitive and retinoid-resistant cell populations. It is also possible that a majority of the retinoid-sensitive cells may be hormone independent, thus giving rise to the synergistic interaction of retinoid treatment and hormonal deprivation. However, these data do not provide sufficient evidence to conclude that the majority of the hormone-dependent cells are retinoid resistant.

In addition to hormonal deprivation, several other modifiers of carcinogenesis have been evaluated in combination with retinoids against mammary carcinogenesis. Selenium in combination with retinyl acetate has been shown to offer enhanced protection against both MNU- and DMBA-induced mammary cancers in rats.^{39,40} However, immunostimulants such as maleic anhydride-divinyl ether copolymer (MVE-2) or the residue of a methanol extract of *Bacillus Calmette-Guerin*, cell wall skeleton of *Noncardia*, or the mammary tumor cell particulate in Freund's adjuvant in combination with retinyl acetate did not enhance the inhibition of chemically induced mammary carcinogenesis provided by the retinoid alone.^{41,42}

IV. EFFECT OF RETINOIDS IN MAMMARY GLAND ORGAN CULTURE

In addition to cell culture systems for elucidation of retinoid action, organ cultures have also been utilized as *in vitro* models. The direct effect of retinoids on an organ without the physiological complexities inherent in whole animal studies was first shown for embryonic chick skin, in which both retinol and retinyl acetate inhibited keratinization of the skin and also influenced ectodermal differentiation into secretory epithelium. Another study of historic importance, which provided clear evidence that retinoids can suppress carcinogen induced changes *in vitro*, is that of Lasnitzki,⁴³ who showed that retinol inhibited methylcholanthrene-induced squamous metaplasia and decreased the incidence of hyperplasia in mouse prostate organ cultures. Subsequently, Sporn and his colleagues introduced the hamster tracheal organ culture system as a bioassay for determining the biological activity of newly synthesized retinoids,⁴⁴ and it has now become a standardized test for evaluating structure-activity relationships for hundreds of retinoids.

In the past few years we have utilized the mouse mammary gland organ culture system to study not only the effectiveness of retinoids from the screening point of view but also for studying the mechanism of retinoid action.

Mammary glands excised from the young virgin female mice can be hormonally-induced to undergo differentiation. Glands treated with insulin and prolactin exhibited extensive end-bud development, however in the absence of prolactin only ductal structures were observed.⁴⁵ Earlier, we found that certain retinoids such as all-*trans*-retinoic acid and *N*-(4-hydroxy-phenyl)retinamide inhibited the prolactin-induced differentiation of the mammary gland (Table 8). This arrest of growth was accompanied by inhibition of DNA synthesis in these

TABLE 8
Effects of Retinoids on the Development of
Mammary Gland in Organ Culture

Retinoids ($1 \times 10^{-6} M$)	Effectiveness
all-trans-Retinoic acid	+++
13-cis-Retinoic acid	±
N-(4-hydroxyphenyl)retinamide	+++
Retinyl methyl ether	—
Retinyl acetate	—
Retinylidene dimedone	—
N-(4-methoxyphenyl)retinamide	Stimulatory
Axerophthene	±

Note: Effectiveness was measured as inhibition of prolactin-induced differentiation of the glands.

glands. It has also been observed that this inhibition of proliferation was not due to toxic effects of the retinoids since removal of the retinoid from the culture medium resulted in the resumption of DNA synthesis and mammary growth. In contrast, retinyl acetate, which is an effective chemopreventive agent for rat mammary carcinogenesis but ineffective in mice, was also ineffective in this *in vitro* system.⁴⁶

4-HPR was also effective in inhibiting the *in vitro* development of carcinogen induced alveolar lesions in mammary glands of RIII or C57/BL mice.⁴⁷ In this system, 4-HPR preferentially induced the regression of the abnormal alveolar lesions without affecting the normal alveolar structures. A similar lack of inhibition of the steroid induced alveolar structures by retinoids was also evident in our *in vitro* studies. These results indicate the effect of a retinoid may be dependent upon the stage of differentiation of the gland at the time the retinoid is introduced into the culture medium.

The mammary gland organ culture system has been further extended to mimic physiological stages observed *in vivo*. Mammary glands of certain mouse strains undergo *in vivo* a distinct preneoplastic phase during which the histology of the lesion resembles that of normal proliferated mammary gland. However, the majority of these lesions, hyperplastic alveolar nodules (HAN), develop into tumors if allowed to progress.⁴⁸ This condition can also be induced in mammary glands in organ culture by exposing the gland to DMBA during the growth phase induced by appropriate hormonal conditions.⁴⁹ The lesions induced by DMBA in culture have been shown to be preneoplastic because transplantation of the cells prepared from these unregressed areas to syngeneic mice resulted in mammary adenocarcinomas. It has also been reported from our laboratory, as well as by others, that treatment of such glands with certain retinoids during the hormone-induced proliferative phase significantly decreased the *in vitro* incidence of nodulogenesis.^{50,51} Similar inhibition of hyperplastic alveolar nodule-like lesions in C3H mice has also been observed (Table 9). Although more established organ culture systems, such as the tracheal organ culture, have been used to predict the effectiveness of retinoids in the inhibition of carcinogenesis, at times these results could well be misleading. The retinoid literature is replete with studies indicating that the effects of retinoids are most likely target-organ specific. For example, 13-cis-retinoic acid is very effective in reversing keratinization of tracheal epithelium and in inhibiting bladder carcinogenesis but is ineffective against mammary carcinogenesis. Retinyl acetate, on the other hand, is effective against mammary carcinogenesis but ineffective against skin carcinogenesis.⁵² Thus, the mammary gland organ culture system, when used as a quantitative and precise assay system, could be invaluable in screening retinoids and other chemopreventive agents for their effectiveness against breast cancer.

TABLE 9
Effect of Retinoids on the Number of Hyperplastic Alveolar Nodules in Abdominal-inquinal Mammary Glands of Virgin Female C3H/He Mice

Group	Diet*	No. of mice	No. of hyperplastic alveolar nodules >0.5 mm (mean \pm SE)	Mean initial body weight (g)	Mean final body weight (g)	Mice surviving at 20 months (%)
1	Basal ^b	35	4.06 \pm 8.87	20.6	31.2	70
2	Basal + retinoid solvent	37	3.64 \pm 0.60	20.5	31.0	74
3	Basal + 4-HPR (194 mg/kg diet)	35	2.43 \pm 0.46	20.3	30.0	70
4	Basal + 4-HPR (391 mg/kg diet)	31	1.84 \pm 0.61*	19.0	31.2	62
5	Basal + retinyl acetate (82 mg/kg diet)	15	3.86 \pm 0.46	21.1	27.3	60

Note: 4-HPR, N-(4-hydroxyphenyl) retinamide.

* $p < 0.05$ compared with Group 1 (basal diet) and Group 2 (basal diet plus retinoid solvent).

^a Animals received diets from 6 weeks of age to 20 to 24 months of age.

^b Basal diet, Wayne Lab Meal.

V. MECHANISM OF RETINOID ACTION IN THE MAMMARY GLAND

The mechanism by which retinoids provide a chemopreventive effect against carcinogenesis is largely unresolved. Several inferences can be drawn from previous studies relative to the influence of retinoids on mammary glands that may provide some understanding of retinoid action. Retinoids effective against mammary carcinogenesis inhibit end bud proliferation and reduce ductal branching of mammary glands both *in vivo* and *in vitro*.^{22,46,51} Hyperplastic alveolar nodule formation in C3H mice as well as DMBA-induced formation of nodule-like lesions *in vitro* are also inhibited by 4-HPR. These inhibitory activities of retinoids are accompanied by reduced DNA synthesis.⁴⁶

Since the recent studies are suggestive of the fact that the effect of retinoids may be at a genomic level, one of the most attractive hypotheses has been the mediation of retinoid action via a receptor mechanism analogous to that observed for steroid receptors. It was originally proposed by Bashor et al.⁵³ that the action of retinoids on the cell may be mediated in a manner similar to that of the steroid hormones, in which there is an association with a specific cytosolic receptor protein, followed by translocation of steroid-receptor complex to the nucleus, interaction with chromatin, and alteration of the cellular response. Since then, investigators from several laboratories have found both retinol- and retinoic acid-binding proteins in many normal and neoplastic tissues.⁵⁴

In order to determine whether retinoid action is mediated by the retinoid receptors in mammary carcinogenesis, the properties of retinoic acid-binding proteins (CRABP) were studied during various physiological states of the mammary gland. As shown in Table 10, there appears to be a hormonal regulation of CRABP in mammary tissues. Mammary glands from pregnant rats contained a higher concentration of CRABP compared with those of virgin and lactating rats. Moreover, treatment of lactating animals with 17 β -estradiol enhanced the level of CRABP. Similar hormonal regulation was also evident in mammary tumors in relation to CRABP content. In human mammary cancer biopsies no apparent difference was observed between steroid receptor-positive or -negative tumors.

The cytosolic retinoic acid-binding protein complex of carcinogen-induced mammary cancer sediments as a 2S component. Unlabeled all-*trans*-retinoic acid as well as some other retinoids with a free carboxyl group competes effectively with radiolabeled all-*trans*-retinoic acid for the binding sites. However, certain retinoids that are effective against mammary carcinogenesis (4-HPR, retinyl acetate) failed to compete for retinoic acid-binding sites. This finding is supported by the evidence that radioactive 4-HPR did not bind with any retinoid-binding protein of tumor cytosol.^{55,56} These results suggest that 4-HPR requires metabolism to an active component within the mammary cell, which then effectively binds to cytoplasmic retinoic acid-binding protein (CRABP). Recently, we have studied the metabolism of 4-HPR in mammary gland *in vitro*. The results indicate that 4-HPR was indeed metabolized by the mammary cells yielding various metabolites. The ability of these metabolites to compete for retinoic acid-binding protein was also evaluated. Although 4-HPR did not compete for the binding, one of the metabolites effectively competed for the retinoic acid-binding proteins (unpublished). The structure of this metabolite is currently unknown.⁵⁷ Although some of the earlier results from our laboratory suggested a possible correlation between the ability of retinoids to suppress mammary carcinogenesis and the level of CRABP in the cytoplasm of mammary cancers,⁵⁸ more recent data indicate that the action of retinoids may depend on both the presence of CRABP and the ability of the tissue to metabolize the retinoids. The absolute concentration of CRABP may not be a true indicator of the responsiveness of the parenchymal cell to retinoids.⁵⁹

A few investigators have studied the role of the retinoid-binding proteins on the interaction of the retinoid with the nucleus of target cells. Studies on the role of retinol-binding

TABLE 10
Distribution of Cellular Retinoic Acid Binding Proteins
in Mammary Tissue During Various Physiological Stages

Physiological stage	No. of experiments	Retinoic acid-binding proteins (pmol/mg of protein) (mean \pm SD)
Normal mammary gland		
Virgin	4	1.02 \pm 0.23
Pregnancy	24	2.84 \pm 0.76
Lactation	19	0.72 \pm 0.11
Lactation + E	5	2.43 \pm 0.30
MNU-induced mammary tumors		
Intact	18	3.24 \pm 0.51
Ovariectomized	15	4.62 \pm 0.42
Regressing after ovariectomy	5	0.65 \pm 0.16
Regressing + E	10	4.95 \pm 0.30
Growing after ovariectomy	6	4.02 \pm 0.37
Human breast cancer		
ER ⁺ PR ⁺	15	5.5 \pm 0.9
ER ⁻ PR ⁻	13	4.3 \pm 1.2
Poorly differentiated	6	1.55 \pm 0.59
Moderately differentiated	15	3.89 \pm 0.64
Well differentiated	6	8.0 \pm 1.7

Note: ER: estrogen receptors; PR: progesterone receptors. The levels of estradiol (E) and progesterone (P) receptors (R) were measured using sucrose-density-gradient analysis; less than 10 fmol/mg protein of ER and PR were considered negative. Mean CRABP \pm standard error. Retinoic acid-binding protein (RABP) were measured using sucrose-density-gradient analysis. Area under the 2S region was calculated, specific binding was determined as the difference between the total and nonspecific binding in the 2S region.

proteins in the interaction of retinoids with the nucleus,^{54,60} as well as the presence of retinoic acid-binding proteins in the nuclei of retinoblastoma cells, Lewis lung tumors and embryonal carcinoma cells, have been reported.⁶¹⁻⁶³ Similar retinoid interaction with mammary cell nuclei have been investigated in our laboratory.⁶⁴ It was concluded that the formation of a retinoic acid-receptor complex in the mammary tumor cytoplasm is essential for the interaction of retinoic acid with the nucleus. Retinoic acid per se does not bind to nuclei or to nuclear components. However, following incubation of purified nuclei with the cytoplasmic retinoic acid-receptor complex, nuclear retinoic acid-binding protein (nRABP), which sediments as a 2S component, can be extracted from these nuclei (Table 11). Furthermore, incubation of a constant amount of nuclei (constant DNA concentration, \sim 100 μ g) with an increasing concentration of cytoplasmic retinoic acid-receptor complex resulted in saturable nuclear binding. The data, when analyzed by a Scatchard plot, indicated that the mammary nuclei bound retinoic acid with a high affinity ($K_D = 1.7 \times 10^{-9}$ M).

At present, it is speculative to suggest that the interaction of retinoic acid with the nucleus results in altered genomic expression. However, there are numerous reports which indirectly support such a view. For example, retinoids inhibit tumor promoter induced ornithine decarboxylase activity,⁶⁵ carcinogen induced DNA synthesis,⁶⁶ and growth factor-induced transformation.⁶⁷ Our studies of RNA polymerase activity of mammary tumor nuclei are also suggestive of such an effect; nuclei isolated from mammary cancers preincubated with retinoic acid exhibited reduced RNA polymerase activity compared with tissues incubated under similar conditions without the retinoid. Furthermore, the nuclei that were

TABLE 11
Nuclear Uptake of [³H]Retinoic Acid Under Various Experimental Conditions

Expt. No.	Reaction	Time and temperature of activation	Time and temperature of incubation for nuclear binding	(CRABP) ^a (pmol/100 µg of DNA)
1	[³ H]RA + buffer + nuclei	—	0°C, 30 min	<1
			25°C, 30 min	<1
	[³ H]RA + 0.4 M KCl + extract of nuclei	—	0°C, 30 min	<1
			25°C, 30 min	<1
	[³ H]RA-CRABP ^b	0°C, 30 min	25°C, 30 min	7.49
		25°C, 30 min	25°C, 30 min	7.68
		30°C, 30 min	25°C, 30 min	6.89
	[³ H]RA-bovine serum albumin		25°C, 30 min	<1
	[³ H]RA-CRABP	0°C, 30 min	25°C, 30 min	2.45
		25°C, 30 min	25°C, 30 min	2.49
2		37°C, 5 min	25°C, 30 min	2.00
		37°C, 30 min	25°C, 30 min	0.85

^a Concentrations of CRABP in the nuclei were determined by using sucrose-density-gradient analysis.

^b [³H]RA-CRABP complex was prewarmed at the temperatures indicated before incubation with nuclei at 25°C for 30 min.

preincubated with mammary cytosol containing retinoic acid-receptor complex also showed reduced RNA polymerase activity, as compared with that of nuclei incubated with either buffer or with free retinoic acid (unpublished). Activity of both RNA polymerase I and II was reduced as a result of retinoid treatment. These results indicate that retinoids may be active at the chromatin level, and that the retinoic acid-retinoic acid receptor complexing may be an important step in the mediation of retinoid action.⁵⁴

Based on the data presented above, a proposed mechanism of action for retinoids at the cellular level can be summarized briefly.

The active retinoid may enter the target cell as an authentic retinoid or as a metabolite; some membrane factors may influence the entry of these compounds into the cell. Once in the cell, the retinoid may require further metabolism to an active component that may bind to a 2S cytoplasmic binding protein that specifically binds retinoic acid. The actual concentration of CRABP in the cell may not be of any significance so long as active metabolite and the retinoid receptors are present in the cell. The retinoic acid-receptor complex then may translocate to the nucleus. Unlike steroid receptors, this step does not require temperature activation. The retinoic acid or the retinoic acid-receptor complex in the nucleus may interact with the chromatin and alter the synthesis of specific messenger RNA which, in turn, may influence translation of an enzyme or a protein responsible for the chemoprotective effect of the retinoid. The entire sequence of events, however, is poorly understood and is highly speculative at this stage.

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Chapter 20

RETINOIDS AS POTENTIAL ANTIRHEUMATIC AGENTS

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TABLE OF CONTENTS

I.	Introduction and Background	520
II.	Activity of Retinoids in Rat Models of Arthritis	520
	A. Adjuvant-Induced Arthritis	520
	B. Collagen-Induced Arthritis and Streptococcal Cell Wall-Induced Arthritis	522
III.	Mechanism of Action	526
	A. General	526
	B. Passively Transferred Adjuvant-Induced Arthritis	527
	C. Retinoids and Arachidonic Acid Metabolism	527
IV.	Clinical Experience with Retinoid Treatment in Human Rheumatic Diseases	529
	A. General	529
	B. Psoriatic Arthropathy	529
	C. Reiter's Disease	532
	D. Cutaneous Lupus Erythematosus	532
	E. Rheumatoid Arthritis	533
V.	Summary and Perspective	533
	Acknowledgments	534
	References	534

I. INTRODUCTION

Chemical efforts to improve the therapeutic profile of retinoids have resulted in the synthesis of a large number of second and third generation compounds. A number of these synthetic retinoids have reduced hypervitaminosis A toxicity. As a result of the striking clinical effects which have been achieved with certain of these compounds (e.g., etretinate and 13-*cis*-retinoic acid) in the treatment of hyperproliferative inflammatory skin disease,¹ interest has developed in the possible therapeutic benefit of second and third generation retinoids in the treatment of a variety of other inflammatory disorders. Recent clinical studies have demonstrated the efficacy of etretinate in the treatment of psoriatic arthritis.^{2,3} These clinical findings, along with data from preclinical studies with retinoids in animal models of arthritis (see Section II), suggest that trials with retinoids in other inflammatory arthropathies may be warranted. This report highlights the preclinical and clinical evidence supporting the possible utility of retinoids in the treatment of inflammatory joint disease, in particular, rheumatoid arthritis.

In rheumatoid arthritis, the synovial membrane is infiltrated by activated macrophages and lymphocytes that release a number of mediators of inflammation including a variety of monokines and lymphokines. As a result of complex interactions involving these mediators, the synovial cells are activated and induced to proliferate, leading to the formation of the rheumatoid pannus. The proliferative pannus invades the joint space and produces a number of effector molecules, e.g., prostaglandin E₂ (PGE₂) and collagenase, which are responsible for tissue destruction in the rheumatoid joint.⁴ Polymorphonuclear leukocytes (PMN) also accumulate in the rheumatoid joint, but they localize mainly in the synovial fluid rather than in synovial tissue. While the etiology of rheumatoid arthritis remains unknown, a large body of data suggests that it is fundamentally immunologic in nature. Current theory suggests that an immune response to some unknown, but persistent, antigen of exogenous or endogenous origin may underlie the chronic synovitis of rheumatoid arthritis.

In this chapter, the data from our studies with retinoids in adjuvant-induced arthritis are presented and these are compared with data, collected in other laboratories, regarding the activity of retinoids in collagen- and streptococcal cell wall-induced arthritis. The status of studies to explore possible mechanisms of action of the antiinflammatory retinoids in adjuvant-induced arthritis is also described. These studies were conducted using the retinoids whose structures are presented in Figure 1. Finally, a discussion of clinical data relating to the antirheumatic activity of retinoids is included.

II. ACTIVITY OF RETINOID IN RAT MODELS OF ARTHRITIS

A. ADJUVANT-INDUCED ARTHRITIS

Adjuvant-induced arthritis, a widely studied model of arthritis, can be induced by the injection of complete Freund's adjuvant into a hind foot pad of susceptible rats. When injected into the hind foot pad, the adjuvant induces an acute inflammatory response, which reaches its maximum at 3 to 4 d and then either declines or remains steady over the next several days (Figure 2). Starting on approximately day 10, chronic lesions develop progressively in the adjuvant-injected hind paw, the contralateral paw, and sometimes the forepaw. These secondary (chronic) lesions develop as the result of a cell-mediated immune response either to some disseminated antigen from the adjuvant, to some endogenous autoantigen, or perhaps to some antigen of viral origin.⁶ These secondary lesions share a number of histopathological characteristics with the lesion of the rheumatoid joint.⁷ Treatment with 13-*cis*-retinoic acid suppressed the secondary lesions that developed in both the adjuvant-injected and the noninjected hind paw between days 10 and 22 but had no significant effect on the acute lesion that developed in the injected paw between days 0 and 4 (see Figure 2).

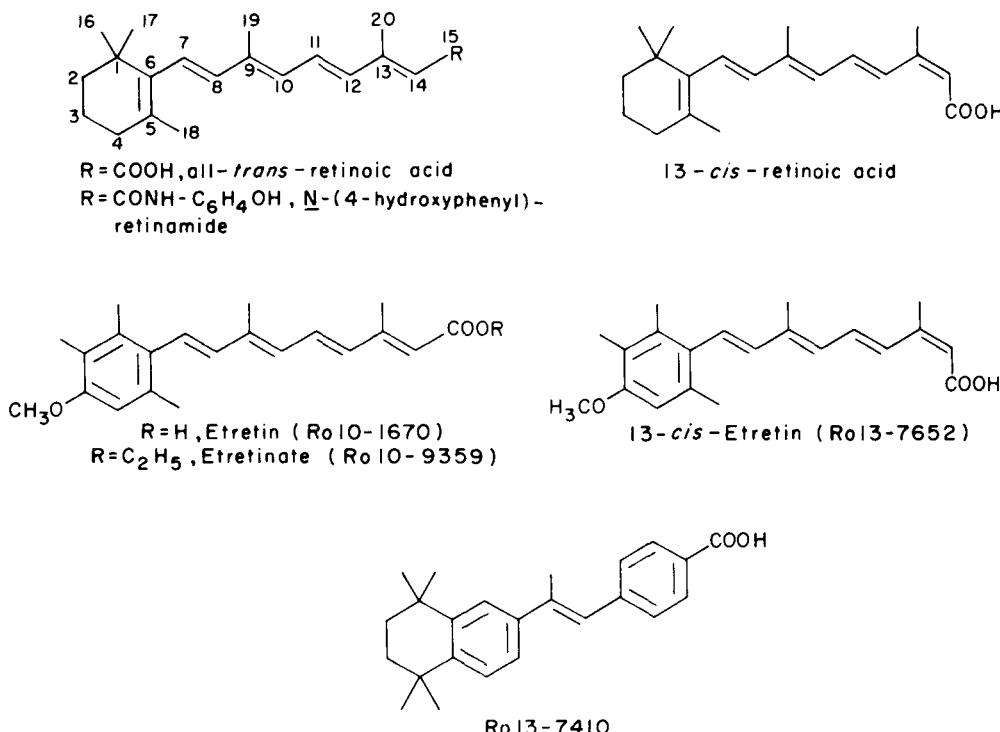


FIGURE 1. Structures of retinoids.

and Reference 5). Indomethacin and cyclosporin A were included in this experiment for comparison. These drugs were similar to 13-cis-retinoic acid in that they also suppressed the lesions that developed in the hind paws during the chronic stage of adjuvant-induced arthritis; however, unlike the retinoids, these drugs also inhibited the acute lesion in the adjuvant-injected paw.

Table 1 contains data comparing the antiinflammatory activity of 13-cis-retinoic acid in the adjuvant-induced arthritis model with that of several other widely studied retinoids. The pattern of activity was similar for all of these compounds, i.e., little or no effect on the acute (day 4) paw lesion, but dose-dependent suppression of the chronic (day 23) lesion. Of the retinoids evaluated as antiinflammatory agents in this study, all-trans-retinoic acid was the most potent; however it was also the most toxic as reflected by decreased body weight gain. A number of other retinoids with antiinflammatory activity in the adjuvant model have now been identified. The pattern of activity described appears to be characteristic of this entire group of compounds; however, a discussion of structure-activity relationships within the series of antiinflammatory retinoids is beyond the scope of this chapter.

In addition to the effect on the paw lesions, treatment with the antiinflammatory retinoids also lowered the level of the acute phase protein, fibrinogen, in the adjuvant rat. This is another indication of their effectiveness as therapeutic agents in this model of arthritis. Body weight gain in arthritic rats treated with therapeutically effective doses of the retinoids was either not changed or decreased slightly as compared with that seen in vehicle-treated arthritic animals. This finding also differentiates the antiinflammatory retinoids from nonsteroidal antiinflammatory drugs (NSAIDs) such as indomethacin. Body weight gain is increased in NSAID-treated arthritic rats as compared with that seen in vehicle-treated arthritic rats. The antiinflammatory retinoids are also effective as therapeutic agents in the adjuvant-induced arthritis model when administered to animals with established disease. Representative data for *N*-(4-hydroxyphenyl)retinamide as an antiinflammatory agent in the established adjuvant-induced arthritis model are presented in Table 2.

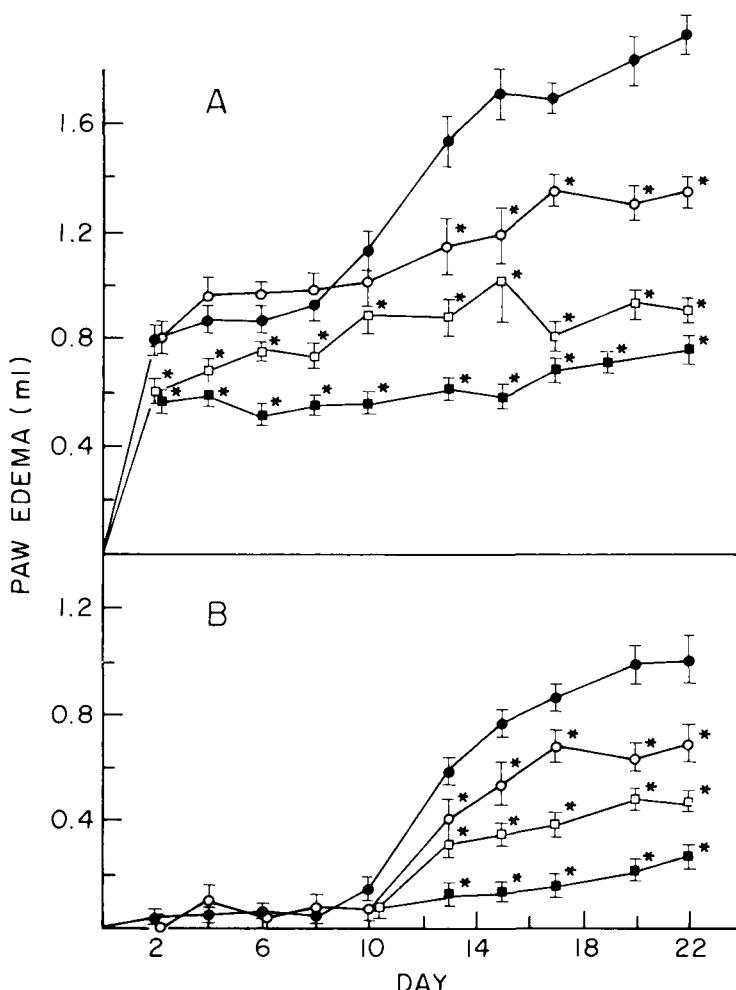


FIGURE 2. Effect of treatment with 13-cis-retinoic, indomethacin, or cyclosporin A on the course of developing adjuvant-induced arthritis. The arthritis was induced by the injection of adjuvant (suspension of heat-killed *Mycobacterium butyricum* in mineral oil) into the right hind paw of Lewis rats. Paw volumes were measured as previously described.⁵ The drugs were administered daily by intubation from day 0 with Tween 80 as vehicle. Paw edema equals paw volume on day of measurement minus paw volume on day 0. Means \pm SE are presented. A, right hind paw; B, left hind paw; ●—●, vehicle; ○—○, 13-cis-retinoic acid (18 mg/kg; 60 μ mol/kg); □—□, indomethacin (1 mg/kg); ■—■, cyclosporin A (10 mg/kg); * p < 0.05 vs. value for vehicle-treated animals (Student's *t*-test).

B. COLLAGEN-INDUCED ARTHRITIS AND STREPTOCOCCAL CELL WALL-INDUCED ARTHRITIS

Collagen arthritis is induced in the rat following immunization with type II (cartilage) collagen in Freund's incomplete adjuvant.⁸ The animals develop cellular and humoral immune responses to the collagen together with inflammatory lesions of the paws. A study by Trentham and Brinkerhoff demonstrated that the retinoids *N*-(4-hydroxyphenyl)retinamide and 13-cis-retinoic acid exacerbate the paw lesions in rats with developing collagen-induced arthritis despite the fact that these compounds have no effect on cellular or humoral immune responses to type II collagen⁹ (Table 3). More recent studies by Bradshaw et al.¹⁰ have extended these findings to etretinate and to the established collagen-induced arthritis model.

TABLE 1

Antiinflammatory Activity of Retinoids in the Developing Adjuvant-Induced Arthritis Model in the Lewis Rat^a

Treatment	Dose (μ moles/kg)	Antiinflammatory Activity increase in paw volume (ml) ^b			Plasma fibrinogen (ml/dl)	Body weight gain ^b (g)	
		Day 4 right	Day 23				
			right	left			
Vehicle	(20) ^c	—	1.20 \pm 0.05	2.33 \pm 0.11	1.60 \pm 0.09	1600 \pm 92	40 \pm 3
13-cis-Retinoic acid	(10)	25	0.96 \pm 0.02*	1.61 \pm 0.09*	1.09 \pm 0.06*	1258 \pm 74*	38 \pm 3
			(-20.0) ^d	(-30.9)	(-32.0)	(-21.4)	
		75	1.06 \pm 0.07	1.11 \pm 0.05*	0.64 \pm 0.04*	739 \pm 47*	26 \pm 3*
all-trans-Retinoic acid	(10)	8.3	1.16 \pm 0.06	1.53 \pm 0.10*	1.03 \pm 0.07*	942 \pm 72*	32 \pm 3
			(-3.4)	(-34.3)	(-35.7)	(-41.1)	
		25	1.14 \pm 0.05	1.37 \pm 0.08*	0.81 \pm 0.05*	725 \pm 65*	28 \pm 4*
Etretinate	(10)	25	1.13 \pm 0.06	1.49 \pm 0.10*	1.19 \pm 0.08*	540 \pm 50*	38 \pm 2
			(-5.2)	(-35.9)	(-25.6)	(-66.3)	
		75	1.08 \pm 0.07	1.17 \pm 0.09*	0.83 \pm 0.04*	434 \pm 64*	31 \pm 2
Indomethacin	(5)	2.8	0.64 \pm 0.09*	0.66 \pm 0.04*	0.38 \pm 0.04*	1122 \pm 70*	59 \pm 3*
			(-46.2)	(-71.8)	(-76.0)	(-29.9)	
Vehicle ^e	(10)	—	1.30 \pm 0.06	2.43 \pm 0.08	1.35 \pm 0.12	1493 \pm 42	37 \pm 3
<i>N</i> -(4-Hydroxyphenyl)retinamide	(10)	75	1.08 \pm 0.04*	1.24 \pm 0.06*	0.66 \pm 0.06*	860 \pm 79*	39 \pm 2
			(-17.2)	(-48.7)	(-51.2)	(-42.4)	

^a The retinoids were administered daily by intubation for 23 d starting on day 0 with Tween 80 as vehicle. The data for *N*-(4-hydroxyphenyl)retinamide are from a separate experiment. Means \pm SE are reported.

^b Increase in paw volume equals paw volume on day 4 or day 23 minus paw volume on day 0. Body weight gain equals body weight on day 23 minus body weight and day 0.

^c Number of animals per group.

^d Percent change as compared with value for vehicle-treated arthritic rats.

^e The data for *N*-(4-hydroxyphenyl)retinamide are from a separate experiment. Therefore data for the control group are included.

* These values are significantly different from the values of the vehicle-treated arthritic rats ($p < 0.05$; Student's *t*-test).

TABLE 2
Effect of Retinoid Treatment on the Progression of Established Adjuvant-Induced Arthritis^a

Treatment	Dose (μ mol/kg)	Change in paw volume ^b (ml)	Plasma fibrinogen (mg/dl)	Body weight gain (g)
Vehicle	(8) ^c	—	0.64 \pm 0.12	1567 \pm 86
<i>N</i> (4-Hydroxyphenyl)retinamide	(8)	75	-0.27 \pm 0.06 ^d	1489 \pm 35
Vehicle	(12)	—	0.69 \pm 0.12	1718 \pm 28
Indomethacin	(6)	2.8	-0.50 \pm 0.08 ^d	909 \pm 44 ^d
				32 \pm 2 ^d

^a The arthritis was allowed to develop untreated for 21 d and then the test drugs were administered daily by intubation for 7 d with Tween 80 as vehicle. The data for indomethacin that are included for comparison are from a separate experiment.

^b Change in paw volume or body weight gain equals paw volume or body weight on day 28 minus paw volume or body weight on day 21.

^c Number of animals per group.

^d These values are significantly different from the value for the vehicle-treated arthritic animals ($p < 0.05$, Student's *t*-test).

On the other hand, Haraoui et al.¹¹ have shown that *N*-(4-hydroxyphenyl)retinamide is an effective therapeutic agent in both the developing and established models of streptococcal cell wall-induced arthritis, an arthritis induced by the intraperitoneal injection of an aqueous suspension of streptococcal cell wall fragments. While other retinoids have not been studied in the streptococcal cell wall-induced arthritis model, it seems likely that the antiinflammatory retinoids as a class will be effective in this model, just as they are in the adjuvant-induced arthritis model. The reasons for the disparate findings with retinoids in the collagen-induced arthritis model are not understood, however the retinoids may be useful probes to explore the pathogenesis of collagen-induced arthritis as compared with that of adjuvant- and streptococcal cell wall-induced arthritis.

Table 3 also compares the effects of the antiinflammatory retinoids in the three models of arthritis with those of NSAIDs, steroids, and immunomodulators (i.e., cyclosporin A and cyclophosphamide). The NSAIDs and steroids are effective as antiinflammatory agents under all experimental conditions in the adjuvant- and collagen-induced arthritis models. To our knowledge, no published information is available regarding the antiinflammatory activity of NSAIDs and steroids in streptococcal cell wall-induced arthritis, but it is likely that these agents would also be effective in this model. A comparison of the profiles of retinoids and cyclosporin A in the arthritis models reveals a number of similarities and several differences. Like the antiinflammatory retinoids, cyclosporin A is an effective therapeutic agent during the chronic stage of developing adjuvant- and streptococcal cell wall-induced arthritis and in established disease in both models. Unlike the retinoids, cyclosporin A exhibits antiinflammatory activity during the acute stage of adjuvant-induced arthritis and also in animals with developing collagen-induced arthritis. The profile of cyclophosphamide also clearly differs from that of antiinflammatory retinoids.

Finally, Table 3 contains data from Brinckerhoff and associates regarding the effect of treatment with an antiinflammatory retinoid [i.e., 13-*cis*-retinoic acid or *N*-(4-hydroxyphenyl)retinamide] on the *ex vivo* production of collagenase and PGE₂, two important mediators of tissue damage in inflammatory joint disease, by synovial cells from arthritic animals.^{5,9,11} Collagenase and PGE₂ production by cultured synovial cells from arthritic rats is clearly elevated in comparison with that seen with cells from normal rats.⁹ When the synovial cells are harvested from retinoid-treated arthritic rats, collagenase production is decreased in all three animal models of arthritis. The effects on PGE₂ production are more

TABLE 3
Pharmacologic Activities of Retinoids and Other Antiinflammatory/Antiarthritic Agents in Rat Models of Arthritis^a

Model of Arthritis	Treatment	Paw inflammation ^b			Ex vivo production of collagenase and PGE ₂ by synovial cells ^c		Ref.	
		Developing disease		Established disease	Collagenase	PGE ₂		
		Acute	Chronic					
Adjuvant-induced	Retinoids	↔ ^d	↓	↓	↓	↔	5	
	NSAIDs	↓	↓	↓	ND ^f	↓	6	
	Steroids	↓	↓	↓	ND	↓	6	
	Immunomodulators							
	Cyclosporin A	↓	↓	↓			12,13	
	Cyclophosphamide	↓	↓	↓			14	
	Retinoids		↑	↑	↓	↓	9, 10	
Collagen-induced	NSAIDs		↓	↓		↓	15, 16	
	Steroids	NA ^e	↓	↓	ND	ND	15, 16	
	Immunomodulators							
Streptococcal cell wall-induced	Cyclosporin A		↓	↑			17	
	Cyclophosphamide		↓	↓			15, 18	
	Retinoids	↓, ↔	↓	↓	↓	↓	11	
	NSAIDs				ND			
	Steroids			ND				
	Immunomodulators							
	Cyclosporin A	↔	↓	↓			19	
	Cyclophosphamide		ND					

^a Retinoids, NSAIDs (i.e., cyclooxygenase inhibitors) and steroids are used as general terms and the findings reported indicate the characteristic effects, to date, of test agents from these classes in the models of arthritis.

^b Developing disease indicates that drug therapy was initiated at the time that the eliciting agent was administered to induce the arthritis. Acute refers to the inflammation that develops during the first few days after administration of the eliciting agent, while chronic refers to the arthritis that develops over a period of weeks. Established disease indicated that the arthritis was allowed to develop untreated for a selected period of time prior to initiation of drug therapy.

^c Synovial cells were isolated from the ankles of drug-treated arthritic rats and maintained in drug-free culture medium as previously described.⁹ The spontaneous production of collagenase and PGE₂ was then quantified.

^d ↓, inhibited; ↑, potentiated; ↔, no effect; ND, not determined.

^e Acute as defined above is not applicable to collagen-induced arthritis.

^f The effect of drug treatment on collagenase and PGE₂ production by synovial cells *ex vivo* has not been studied with drugs other than retinoids.

variable, i.e., treatment with 13-cis-retinoic acid has no effect on PGE₂ production by synovial cells from animals with adjuvant-induced arthritis; however, treatment with *N*-(4-hydroxyphenyl)retinamide suppresses PGE₂ production in cells from animals with streptococcal cell wall- or collagen-induced arthritis.

III. MECHANISM OF ACTION

A. GENERAL

The mechanisms by which retinoids exert their antiinflammatory effects in the developing and established models of adjuvant- and streptococcal cell wall-induced arthritis are not understood. However, retinoids have multiple actions on immune, inflammatory, and synovial cells. A combination of immunomodulatory, antiinflammatory, and antiproliferative effects may be responsible for their activity in these models of arthritis. A body of sometimes conflicting data exists regarding the effects of retinoids on the immune system. Compounds from this class often have a stimulatory effect on both the cellular and humoral arms of the immune system,²⁰⁻²³ but retinoids have also been associated with inhibition of the delayed-type hypersensitivity (DTH) response to methylated bovine serum albumin,²⁴ mitogen responses,²⁵ natural killer cell activity,^{26,27} interferon production,²⁸ and IgG synthesis.²⁹ In addition, studies with a new series of synthetic retinoids have clearly shown that these molecules can be potent suppressors of both cellular and humoral immune responses *in vivo*.³⁰ The immunoregulatory effects of retinoids are only beginning to be appreciated and our understanding is far from satisfactory. Results obtained in immunological studies with retinoids depend not only upon the test compound, but also upon the experimental conditions.

The cells involved in the generation and regulation of an immune reaction respond differently to retinoids and thus the effect seen in a given experiment may depend upon the relative number, the source, and activation state of the cells participating in the response under investigation. For example, recent studies have shown that, while retinoids potentiate the response of T-cells to mitogens, they either inhibit or have no effect on B-cell responses to mitogens.^{31,32} No data are available regarding the effects of retinoids on the antigen-presenting activity of macrophages, but retinoids do suppress the secretory and endocytic functions of macrophages. Effects seen *in vitro* include stimulation of interleukin 1 (IL-1) release,³³ suppression of Fc receptor-mediated binding and phagocytosis,³⁴ and inhibition of arachidonic acid metabolism.³⁵ Recent studies suggest that retinoids are also able to induce gene expression in the macrophage.³⁶

In addition to their effects on cells of the immune system, retinoids also modulate the activity of PMN and synovial cells, two other cell types playing roles in the development of inflammatory joint disease. Retinoids have been reported to inhibit the generation of chemotactic factors for PMN and perhaps the diapedetic activity of PMN,^{37,38} to inhibit the accumulation of PMN at inflammatory sites,³⁹ to interact with neutrophil receptors to increase cell adhesiveness,⁴⁰ and to inhibit the generation of reactive oxygen species by PMN.^{40,41} In addition, *in vitro* studies with synovial cells have demonstrated that retinoids suppress cell proliferation and antagonize the mitogenic effect of growth factors.⁴² These latter data suggest that retinoids might have the capacity to inhibit the development of the proliferative pannus. Furthermore, retinoids also inhibit the production of collagenase by activated synovial cells.^{5,9,11,43}

The mechanism(s) by which retinoids exacerbate the development of collagen-induced arthritis is equally elusive. In the three models of arthritis discussed in this chapter, the balance between the activities of T-cell populations, i.e., T-helper and T-suppressor cells, appears to control the course of the disease.^{19,44,45} The studies with retinoids and also cyclosporin A suggest that these drugs may differentially affect the T-cell regulatory mechanisms controlling the development of collagen-induced arthritis as compared with those

controlling adjuvant- and streptococcal cell wall-induced arthritis. Since humoral immunity is a pathogenic factor in collagen-induced arthritis but not in the other models of arthritis, direct effects on the humoral immune system could also contribute to the exacerbatory effect of retinoids in collagen-induced arthritis. However this seems unlikely since retinoid treatment has no effect on antibody titers to type II collagen in the arthritic animals.^{9,10} Finally, recent studies have shown that macrophages from rats with collagen-induced arthritis spontaneously release more IL-1 than do macrophages from control rats and that the opposite is true with macrophages from rats with adjuvant-induced arthritis.⁴⁶ Since retinoids have been shown to stimulate the production of IL-1 by macrophages,³³ effects on the IL-1 system could possibly also contribute to the diverse effects of retinoids in adjuvant- and collagen-induced arthritis.

B. PASSIVELY TRANSFERRED ADJUVANT-INDUCED ARTHRITIS

Passively transferred adjuvant-induced arthritis has been used in our laboratory as a model system in which to investigate the effects of retinoids on the cellular systems involved in the development of this arthritis. Adjuvant-induced arthritis can be passively transferred to intact naive rats by the intravenous injection of sensitized spleen cells from arthritic animals, provided the spleen cells are further activated *in vitro* by treatment with concanavalin A.⁴⁷ When sensitized spleen cells from drug-free arthritic rats were transferred to normal rats that had been treated with 13-*cis*-retinoic acid (15 mg/kg, starting 3 d prior to cell transfer and continuing for 19 d), the development of the passive arthritis, as assessed by paw swelling and plasma fibrinogen, was suppressed.⁴⁸ Likewise, when spleen cells from 13-*cis*-retinoic acid-treated arthritic (donor) rats were transferred to drug-free recipients, the passive arthritis was again suppressed. These results suggest that retinoids may affect both induction and effector mechanisms involved in the development of adjuvant-induced arthritis.

In principle, there are a number of mechanisms by which 13-*cis*-retinoic acid might suppress the development of passively transferred adjuvant-induced arthritis. These include the elimination of sensitized and immunologically committed lymphoid cells, alterations in the subsets of lymphoid cells, suppression of the functional capabilities of lymphoid cells, and elimination or suppression of nonlymphoid accessory cells. As a first attempt to explore these possibilities, splenocytes from arthritic animals were examined using monoclonal antibodies to rat T-cells and T-cell subsets. In the adjuvant rat, treatment with 13-*cis*-retinoic acid, at an effective antiinflammatory dose, was not associated with alterations in T-cell number or in the ratio of helper to nonhelper T-cells in the spleen.⁴⁸ Such findings suggest that modulation of accessory cell function may contribute to the antiinflammatory activity of retinoids in adjuvant-induced arthritis. Studies to investigate this possibility are underway.

C. RETINOIDS AND ARACHIDONIC ACID METABOLISM

At present, the mechanism of action of the antiinflammatory retinoids at the molecular level is also unknown. Since retinoids do inhibit the production of arachidonic acid (AA) metabolites in several cell systems, such effects could conceivably contribute to their antiinflammatory activity. Most of the literature in this area describes the effect of retinoids on PGE₂ production, and few attempts have been made to relate the findings to potential antiinflammatory activity. Retinoids, i.e., all-*trans*-retinoic acid, 13-*cis*-retinoic acid, and *N*-(4-hydroxyphenyl)retinamide, have been reported to inhibit the production of PGE₂ by rheumatoid synovial cells,⁴³ chick embryo fibroblasts,⁴⁹ bovine aorta smooth muscle cells,⁵⁰ Madin-Darby canine kidney (MDCK) cells,⁵¹ and rabbit synovial fibroblasts.^{52,53} Few reports are available regarding the effects of retinoids on AA metabolism by inflammatory cells.

To investigate further the possible involvement of AA metabolites in the antiinflammatory activity of retinoids, we evaluated the effect of several antiinflammatory retinoids on the release and metabolism of AA in rat peritoneal macrophages (Table 4). All-*trans*-

TABLE 4
Activity of Selected Retinoids as Inhibitors of the Release and Metabolism of
Arachidonic Acid by Rat Peritoneal Macrophages Challenged with Ca²⁺ Ionophore
A23187

Retinoid	(μM)	Ionophore A23187 (percent inhibition)				
		¹⁴ C-release	LTB ₄	PGE ₂	TXB ₂	6-Keto-PGF _{1α}
all-trans-Retinoic acid	1	n.s.	n.s.	22 ± 9	21 ± 0	38 ± 50
	3	45 ± 0	53 ± 1	47 ± 9	35 ± 11	54 ± 11
	10	70 ± 0	89 ± 1	71 ± 3	62 ± 0	70 ± 4
	33	80 ± 1	100 ± 0	71 ± 7	79 ± 3	88 ± 4
13-cis-Retinoic acid	1	n.s.	n.s.	n.s.	n.s.	n.s.
	3	37 ± 5	n.s.	50 ± 12	n.s.	56 ± 11
	10	78 ± 5	100 ± 0	84 ± 7	76 ± 11	88 ± 7
	33	77 ± 3	100 ± 0	91 ± 1	91 ± 3	95 ± 1
Etretinate	10	n.s.	24 ± 4	Stim.	Stim.	Stim.
	33	n.s.	42 ± 1	Stim.	Stim.	n.s.
Etretin	10	Stim.	Stim.	Stim.	Stim.	Stim.
	33	Stim.	Stim.	Stim.	Stim.	Stim.
13-cis-Etretin	1	n.s.	n.s.	23 ± 21	24 ± 0	n.s.
	3	44 ± 8	64 ± 7	39 ± 9	45 ± 19	43 ± 2
	10	76 ± 4	100 ± 0	73 ± 13	84 ± 1	79 ± 3
	33	84 ± 1	100 ± 0	91 ± 3	100 ± 0	100 ± 1
Ro 13-7410	0.1	n.s.	n.s.	n.s.	n.d.	n.d.
	1	59 ± 2	69 ± 5	69 ± 3	n.d.	n.d.
	3	76 ± 4	81 ± 2	100 ± 0	n.d.	n.d.
	10	94 ± 3	87 ± 7	100 ± 0	n.d.	n.d.

Note: Resident peritoneal cells were harvested by lavage from male Sprague-Dawley rats. The adherent cells (macrophages) in this population were labeled with [¹⁴C]arachidonic acid and stimulated with ionophore A23187 (0.5 μM) as described previously.³⁵ Prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄) release was quantified by RIA and HPLC, respectively.

Percent inhibition is expressed as the mean of duplicate experiments ± standard deviation. n.d. = not determined; n.s. = less than 20% inhibition; stim. = consistent stimulation.

and 13-cis-retinoic acid inhibited the release of the AA metabolites, leukotriene B₄ (LTB₄), PGE₂, 6-keto-PGF_{1α}, and thromboxane B₂ (TXB₂), from ionophore-stimulated cells in a dose-dependent manner at concentrations from 3 to 33 μM. In contrast, etretinate and etretin, the primary blood metabolite of etretinate, were either inactive or stimulated the production of the AA metabolites. Since the latter two compounds are active as antiinflammatory agents in the adjuvant-induced arthritis model, these data appear incompatible with the hypothesis that inhibitory effects on AA metabolism contribute to the antiinflammatory activity of retinoids in this model. However, a quantitatively important blood metabolite of both etretinate and etretin, i.e., 13-cis-etretin,⁵⁴ did inhibit the production of AA metabolites in ionophore-stimulated cells with a potency similar to those of all-trans- and 13-cis-retinoic acid. This metabolite could possibly be responsible for the antiinflammatory activity of etretinate and etretin in the adjuvant model. Of the retinoids evaluated in this study, the arotinoid, Ro 13-7410, was the most potent inhibitor of AA metabolism. Unfortunately, it is difficult to establish a correlation between the potencies of test retinoids as inhibitors of AA metabolism *in vitro* and their potencies as antiinflammatory agents in the adjuvant model since the bioavailability, metabolism, and pharmacokinetics of each retinoid differ significantly. Earlier studies have demonstrated that retinoids inhibit the production of LTB₄-like activity in ionophore-stimulated rat PMN,⁵⁵ however the structure-activity profile reported for retinoids as inhibitors of LTB₄ production in the rat PMN study differs somewhat from

that obtained here (Table 4), e.g., etretin inhibited LTB₄ production in the PMN but not in the macrophage.

The active retinoids in Table 4 were also inhibitors of AA metabolism in rat peritoneal macrophages stimulated with opsonized zymosan.³⁵ However, with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) as the stimulus, Ro 13-7410 was the only potent inhibitor among these retinoids.³⁵ TPA activates the cyclooxygenase (CO), but not the lipoxygenase (LO), pathway of AA metabolism in several cell systems while ionophore and zymosan activate both pathways.^{35,56,57} The mechanism(s) by which retinoids inhibit the formation of AA metabolites in ionophore- and zymosan-stimulated rat peritoneal macrophages is uncertain. Retinoids, with a few exceptions, do not appear to function as inhibitors of either CO or Δ⁵-LO.³⁵ The retinoids evaluated in these studies did inhibit snake venom phospholipase A₂ (PLA₂) in an *in vitro* enzyme assay using multilayer liposomes of dipalmitoyl-L-α-phosphatidyl choline as substrate.³⁵ However, whether the retinoids function as direct inhibitors of PLA₂ in the rat peritoneal macrophage system remains an open question, especially in view of the data from the studies in TPA-stimulated macrophages. An alternate hypothesis is that the retinoids might interfere with the activation of PLA₂ by ionophore and zymosan. The hypothesis that retinoids may interfere with the mobilization of Ca²⁺ appears attractive.³⁵

The data discussed above demonstrate that retinoids can inhibit the production of AA metabolites in a variety of cell types *in vitro*. However, in several other studies, retinoids were shown to stimulate the production of prostaglandins by cultured cells.⁵⁸⁻⁶⁰ The concentrations of retinoid required to affect AA metabolism in these *in vitro* systems often appear relatively high, i.e., μM concentrations. However these studies usually involve short term exposure of the cells to test compound and it is possible that lower concentrations might be effective after long term exposure under *in vivo* conditions. Indeed several studies suggest that systemic retinoids may lower the production of eicosanoids in experimental animals^{61,62} and in humans.⁶³ Whether effects on AA metabolism play an important role in the antiinflammatory actions of retinoids in animal models of arthritis is not known. However, in view of the important roles (both stimulatory and inhibitory) which eicosanoids play as mediators and regulators of the inflammatory response^{64,65} and as regulators of the immune response,⁶⁶ further studies in this area appear warranted.

IV. CLINICAL EXPERIENCE WITH RETINOID TREATMENT IN HUMAN RHEUMATIC DISEASES

A. GENERAL

Current reported clinical experience with retinoid treatment in rheumatology includes studies evaluating etretinate, Ro 13-6298 (the ethyl ester of Ro 13-7410), and 13-*cis*-retinoic acid. Psoriatic arthropathy,^{2,3,67-71} Reiter's disease,⁷² cutaneous lupus erythematosus,⁷³ and rheumatoid arthritis⁷⁴ have been studied. This clinical experience is summarized in Table 5 and includes observations regarding adverse drug effects (ADE), laboratory findings, and, in some cases, immune function.

B. PSORIATIC ARTHROPATHY

Clinical trials evaluating etretinate treatment of psoriatic arthropathy, involving about 100 patients, have demonstrated evidence of improvement in symptoms and signs, as well as in some parameters of inflammatory activity. The symptomatic improvements were similar to those attained with NSAIDs.

In a 24-week double-blind study comparing etretinate with ibuprofen, Hopkins et al.² evaluated 40 patients with psoriatic arthritis, 20 of whom received etretinate (<0.5 mg/kg/d). Eleven of 20 patients completed 24 weeks of therapy with etretinate, while only 1 of 20 patients completed 24 weeks of therapy with ibuprofen alone. Statistically significant

TABLE 5
Results of Human Trials with Retinoid Compounds in Rheumatic Diseases

Condition	Drug	Dose/duration	Design	Number of Patients	Clinical	Outcome lab	ADEs	Ref.
Psoriatic arthropathy	Etretinate	0.5 mg/kg/day 24 wk	double-blind vs. ibuprofen	20	11/20 completed, imp. joints	↓ ESR, CRP ↑ Hgb	cheilitis, dry skin	2
	Etretinate	~30 mg. qd (20—45 mg) 13—24 wk	open	13	11/13 completed ↓ a.m. stiffness ↓ Ritchie index	↑ total T-cells ↑ active T-cells ↑ ConA blasts ↓ B-cells ↓ ESR	mucocutaneous dryness	67
	Etretinate	1 mg/kg/day × 1 mo. ~25 mg/day maint.	open	20	"improved"	N.R.	"acceptable"	68
	Etretinate	50 mg/day 24 wk	open	40	26/40 completed ↓ a.m. stiffness ↓ no. of painful joints ↓ no. of swollen joints	↓ ESR	mucocutaneous dryness	3
	Ro 13-6298	0.5 to 1 µg/kg	open (pilot)	12	↑ joint mobility ↓ swelling ↓ a.m. stiffness	↓ ESR	pruritus-1/12 cheilitis-3/12	69
	Ro 13-6298	60 µg/day 12 wk	open crossover to etretinate 50 mg/day	10	↑ joint mobility ↓ erythema ↑ joint flexibility	↓ ESR imp. x-ray 2/10	↑ transaminase-2/10 ↑ triglyceride-1/10	70
Rheumatoid	Etretinate	1 mg/kg/day × 4 wk 0.5 mg/kg/day × 23 wk	open-parallel group all on ibuprofen	15	6/15 completed, imp. joint circumference trend, towards imp. joint index and grip strength, lack of efficacy 4/15	↓ IgM ↓ ESR ↑ Hgb	mucocutaneous dryness plus pruritis 6/15 hair loss 2/15	71
Reiter's	Etretinate	0.75 mg/kg/day × 2 wks then 0.5—0.65 mg/kg × 10 wk	Double-blind, placebo, with crossover to etretinate	60	53/60 completed skin le- sions, imp. "trend" to global point improve- ment for etretinate	N.R.	mucocutaneous dryness 70—100% headache - 7%	72

Cutaneous lu-	13-cis-Reti-	80 mg/day	open-pilot	10	8/10 excellent	↓ skin inflammatory infiltrate	mucocutaneous dryness	73
pus erythema-	noic acid	16 wk			↓ erythema		sl. ↑ triglyceride	
tous					↓ scaling, ↓ no. of lesions,	↓ T-lymphocytes in skin		

↓ scarring

Note: imp., improved; ↓, decreased; ↑, increased; n.r., no response; sl., slight.

clinical improvement was seen for articular index at 16 weeks and was similar in both groups. Laboratory parameters reflecting disease activity also improved significantly. In the ibuprofen group, only serum histidine increased whereas in the etretinate-treated group, the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) decreased, while hemoglobin (Hgb) and histidine increased. Modest adverse effects of etretinate included mucocutaneous dryness and slight elevations of cholesterol and triglycerides.

Additional open studies with etretinate (0.5 to 1.0 mg/kg/d) in psoriatic arthritis have also suggested efficacy. Improvement in morning stiffness, decreased numbers of painful and swollen joints, and decreases in ESR and CRP were observed. In one of these trials,⁶⁷ immunologic studies showed significant changes in lymphocyte populations, characterized by a relative decrease in T-lymphocytes, a relative increase in B-lymphocytes, and enhanced concanavalin A activation. Adverse effects were confined to mucocutaneous dryness. In addition, it is also important to note that etretinate, like vitamin A, is teratogenic.

A potent arabinoid, Ro 13-6298 (0.5 to 1.0 µg/kg/d), has also been evaluated in a pilot study in more than 20 patients with severe psoriatic arthropathy, unresponsive to NSAIDs.^{69,71} Improvement occurred within 2 to 4 weeks, demonstrated by a reduction in swelling and redness of the affected joints with improved joint mobility, decreased morning stiffness, and a substantial decrease in the daily dosage of analgesics. Two patients experienced a complete remission at 45 and 60 d, respectively. In one patient, relapse did not occur over 6.5 months following therapy. X-ray improvement in the number of erosive and osteolytic bone lesions was observed in two of ten patients, with a decrease in the number and intensity of bone scan foci in four of ten patients. The ESR decreased significantly in all patients. Adverse effects were confined to modest mucocutaneous symptoms, without hair loss or skin fragility. No abnormalities of blood lipids, liver function, or renal function occurred.

C. REITER'S DISEASE

The clinical symptoms of Reiter's disease, which include an arthropathy and a psoriatic-like dermatitis, prompted Lassus et al.⁷² to investigate the potential efficacy of etretinate for the treatment of this condition. In a 12-week randomized study of etretinate vs. placebo in 60 patients with documented Reiter's disease, patients were treated with etretinate, 0.75 mg/kg/d, during the first 2 weeks, with subsequent adjustments to 0.5 to 0.65 mg/kg/d. After 6 weeks of treatment with either placebo or drug, all patients received etretinate. Since all patients improved during the study period, no statistically significant differences were seen, but the trend was in favor of etretinate, i.e., improvement in 50% of placebo-treated patients, 76% of patients treated with placebo-etretinate, and 86% of patients treated with etretinate alone.

D. CUTANEOUS LUPUS ERYTHEMATOSUS

Cutaneous lupus erythematosus is often resistant to treatment with current therapies, such as avoidance of sunlight exposure and intralesional or topical corticosteroids. Recently, Newton et al.⁷³ described treatment of this disease with 13-*cis*-retinoic acid (80 mg/day) for 16 weeks. Eight of ten patients experienced an excellent clinical response, without significant side effects. Erythema and scaling decreased dramatically, as did the size and extent of the skin lesions. In some instances, total resolution of skin lesions occurred, with minimal scarring. The only adverse experiences were mucocutaneous dryness and very slight elevations of the serum triglycerides. Antinuclear antibody titers, if present before therapy, did not change. However, there were dramatic changes in the microscopic appearance of the skin lesions, characterized by decreased mononuclear inflammatory cell infiltration, decreased hyperkeratosis, and a loss of characteristic vacuolar changes at the junction of the dermis and epidermis. Immunologically, no significant changes in mononuclear cell counts or in T4/T8 lymphocyte ratios were noted, but there was a striking decrease in the numbers of

T-lymphocytes detectable at the dermoepidermal junction. These investigators considered 13-*cis*-retinoic acid to be an effective short-term therapy for establishing rapid control in treatment-resistant lesions of cutaneous lupus erythematosus.

E. RHEUMATOID ARTHRITIS

Only anecdotal and unpublished observations are available for clinical experience with retinoids and their derivatives in rheumatoid arthritis.⁷⁴⁻⁷⁶ As discussed previously, studies in animal models would suggest that 13-*cis*-retinoic acid and other retinoids might be effective in rheumatoid arthritis. Improvement in acne fulminans-associated arthritis following treatment with 13-*cis*-retinoic acid is also suggestive.⁷⁷ However the occasional occurrence of diseases with immunologic abnormalities, such as inflammatory bowel disease,⁷⁸ arthritis,⁷⁹ and rarely, vasculitis,⁸⁰ in patients treated with 13-*cis*-retinoic acid has discouraged the clinical evaluation of this agent in rheumatoid arthritis. Several clinicians have observed modest indications of efficacy during treatment with etretinate^{74,75} and Ro 13-6298.⁷⁶

Phase I clinical trials in normal volunteers have been performed with *N*-(4-hydroxyphenyl)retinamide.⁸¹ Because of demonstrated efficacy in animal models of arthritis,^{11,82} clinical studies were considered to evaluate this retinoid as a therapy for rheumatoid arthritis. It appears that this program was discontinued because of the development of skin toxicity, and thus clinicians continue to await the synthesis and preclinical study of retinoid derivatives with potential efficacy in rheumatoid arthritis and better mucocutaneous tolerability. Nevertheless, substantial enthusiasm remains.⁸³

V. SUMMARY AND PERSPECTIVE

A number of retinoids exhibit antiinflammatory activity in chronic adjuvant-induced arthritis in the rat and a few studies in streptococcal cell wall-induced arthritis suggest that the antiinflammatory retinoids, as a class, will also be effective in this model. However, several of the same retinoids exacerbate the development of collagen-induced arthritis and the reasons for this are not understood. The mechanism(s) of action of the antiinflammatory retinoids in adjuvant- and streptococcal cell wall-induced arthritis is not clear. However retinoids have a number of biological effects that could contribute to their antiinflammatory activity in these models, e.g., immunoregulatory effects, antiproliferative effects, inhibitory effects on collagenase production by synovial cells, and inhibitory effects on arachidonic acid metabolite production in macrophages. Further studies to define the mechanism of action of the retinoids in these models of arthritis are necessary. Such studies may also help to establish which of these models is most likely to select compounds that will be therapeutically effective in human inflammatory arthropathies.

Etretinate and 13-*cis*-retinoic acid, drugs developed primarily for their efficacy in skin diseases, are the only retinoids currently available for clinical use. However, since these and related retinoids demonstrate antiinflammatory and immunoregulatory activity in pre-clinical studies, a limited number of clinical trials have been conducted with retinoids in several rheumatic diseases. The results from these investigations suggest that appropriate retinoids might be novel therapeutic agents for the treatment of several rheumatologic disorders including rheumatoid arthritis.

Several adverse side effects are associated with the use of the clinically available retinoids. These include: (1) mucocutaneous dryness and thinning of the skin, (2) occasional hyperostoses of the spine, (3) occasional elevations of liver enzymes and blood lipids, (4) changes in night vision, and (5) teratogenicity. Therefore, additional preclinical studies (chemical and biological) are needed to identify potential new antirheumatic retinoids with fewer and less severe adverse side effects. Such studies are being actively pursued at this time in several laboratories and the hope is that, at least, one improved antirheumatic retinoid will become available for clinical testing within the next few years.

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Chapter 21

**MOLECULAR CORRELATES IN RETINOID PHARMACOLOGY
AND TOXICOLOGY****Calvin C. Willhite****TABLE OF CONTENTS**

I.	Introduction	540
II.	Human and Animal Teratogenicity	540
A.	Natural Retinoids	540
B.	Synthetic Retinoids	551
1.	Humans	551
2.	Animals	551
III.	Placental Permeability and Mechanisms of Action	556
IV.	Therapeutic and Chemopreventive Activities	560
V.	Summary and Conclusions	564
	Acknowledgments	565
	References	565

I. INTRODUCTION

The retinoids comprise a structurally diverse series of compounds that possess pharmacologic activities generally attributable to the naturally occurring congeners, retinol and retinoic acid. The toxicologic activities of the synthetic retinoids are similar to those of their natural congeners as well. That deficiencies or excesses of vitamin A can have profound consequences for mammalian embryonic development have been known for at least 50 years. However, because of the often extraordinarily large doses of retinol or its esters associated with animal terata, workers in this area have often considered retinoids as unlikely agents in the etiology of human malformations. With the advent of synthetic retinoids and their widespread prescription in dermatologic conditions affecting women of childbearing age, numerous reports have appeared confirming human susceptibility to retinoid teratogenesis. Moreover, the human malformation syndrome is remarkably similar to that observed in laboratory animals. In general, the doses of synthetic retinoids associated with human terata are often considerably less than the doses associated with terata in rodents.

The present report reviews briefly the dysmorphogenicity induced by prenatal retinoid exposure in animals and humans and summarizes the concepts put forward to explain the morphogenetic basis of these malformations. This chapter then compares and contrasts structure-activity relationships from *in vitro* and *in vivo* techniques to assess the therapeutic and chemopreventive properties and the potential developmental toxicities of the retinoids. The goal of the present communication is to point out those features of the retinoid skeleton associated with improved therapeutic ratios.

For purposes of this discussion, it is useful to separate consideration of retinoid status and the risk of human and animal developmental toxicity into "natural" and "synthetic" retinoids although certain compounds like all-*trans*-retinoic acid (all-*trans*-RA) and 13-*cis*-retinoic acid (13-*cis*-RA) can be classified in either group. As there are no data at present to implicate carotenoids as anything but practically nontoxic, no overview of their activities is given here. The general toxicology of retinoids in humans and animals has been reviewed.¹

II. HUMAN AND ANIMAL TERATOGENICITY

A. NATURAL RETINOIDs

Apparently, a range of dietary retinol and/or its esters will suffice for normal mammalian development. The exact vitamin A status associated with normal embryogenesis has not been determined primarily because the maternal circulating concentrations do not necessarily indicate deficiency.²

The first reports of vitamin A in relation to abnormal development arose from anecdotal incidents and controlled nutritional deprivation. Hale³⁻⁵ found that vitamin A deficiency induced embryonic death, microphthalmia, anophthalmia, ectopic ureters, cleft lip, cleft palate, abnormal ears, and limb malformations in pigs. Congenital microphthalmia in pigs also occurred under drought conditions on farms when no green forage was available.⁵ Warkany and coworkers found skeletal,⁶ eye,⁷ cardiac,⁸ and genitourinary tract^{9,10} malformations in fetal rats after maternal vitamin A deficiency. Lamming¹¹ subsequently found hydrocephalus in rabbits secondary to maternal vitamin A deficiency, and Millen et al.¹² found hydrocephalus in mice after maternal vitamin A deprivation. Studies on vitamin A deficiency in fetal rats have been extended to *in vitro* work where this condition retarded dentinogenesis and induced atrophic amenoblasts, squamous metaplasia, and abnormal keratinization in cultured molars.¹³ There are reports of human microcephaly,¹⁴ anophthalmia,^{14,15} and other teratism¹⁶ associated with severe maternal vitamin A deficiency. Hurley¹⁷ cited observations which "suggest that prenatal vitamin A deficiency may be partially responsible for eye abnormalities and impaired vision in children". Among offspring of

indigent mothers during World War I, Bloch^{18,19} demonstrated the reversibility of xerophthalmia in newborns by feeding vitamin A-rich foods. More than 30% of these children died prior to age 8 years,²⁰ but the contribution of congenital malformations to the elevated mortality could not be ascertained with certainty.²¹

Retinoic acid can fulfill many of the physiological functions of retinol, but animals given the acid in lieu of normal levels of dietary retinol or retinyl esters can conceive normally, but the offspring die.^{22,23} Resorptions occurred rather late in gestation, and of the surviving rats, renal malformations were prevalent.²³ Fetal deaths were accompanied by placental necrosis.²⁴ When retinol was given to vitamin A-deficient rats, pregnancy was maintained and Wilson et al.²⁵ concluded that maternal vitamin A deficiency led to terata. Pregnant rats given only a marginal retinol diet (but supplemented with retinoic acid) had litters with fetal resorptions and gross terata; pregnant normal dams had normal litters, but completely deficient dams failed to carry their litters. The suggestion that vitamin A is required by the mammalian embryo for normal organogenesis rather than placental toxicity per se being held responsible for the developmental abnormalities is supported by the observation that vitamin A deficiency stops growth during chick embryogenesis. Hens given vitamin A-deficient diets lay fertile eggs at a normal rate, but embryonic arrest occurred after 48 h of incubation.^{26,27} Embryonic arrest was accompanied by failure to develop the *area vasculosa* and subsequent failure to develop an active circulatory system.²⁸ Thompson²⁸ reported that retinol, retinal, methyl retinoate, or retinyl acetate could stimulate embryogenesis in retinol-deficient quail eggs, but 0.2 to 2 µg/egg of retinoic acid failed to stimulate embryogenesis and quantities greater than 2 µg/egg were toxic. Supporting the notion that carotenoids are relatively nontoxic, β-carotene was inactive in quail eggs at up to 100 µg/egg.²⁸ The fact that methyl retinoate was not as toxic for the quail embryo as all-*trans*-RA may be attributable to only limited conversion of the parent to the acid congener in quail eggs.

The role of steroid hormones in retinol deficiency-induced reproductive failure remains a mystery. Juneja et al.²⁹ reported that the steroid hormones pregnenolone, 17β-estradiol, or progesterone, or pituitary homografts could prevent resorption in retinol-deficient rats supplemented with all-*trans*-RA or methyl retinoate; Hays and Kendall³⁰ showed that progesterone maintained pregnancy in vitamin A-deficient rabbits. But whether the influence of progesterone or pregnenolone on pregnancy during vitamin A deficiency is due to mobilization of hepatic retinoid reserves or due to participation of retinol in steroid biosynthesis or due to a direct effect on the placenta or embryo is not clear.

The placental permeability of retinol, its esters, and its binding protein (RBP) has been studied. Matsumoto et al.³¹ found accumulation of retinyl palmitate in fetal mouse liver following oral administration of retinyl acetate to the dam. The ester accumulated specifically in fetal fibroblasts adjacent to hepatic blood vessels and islands. Serum vitamin A concentrations in retinol-deficient dams and neonates were reduced, but the serum concentrations in vitamin A-deficient neonates were greater than those in the dam.³² Hepatic concentrations in vitamin A-deficient dams were reduced, but neonatal hepatic vitamin A concentrations were not as severely depressed as those of the dam.³² Total vitamin A concentrations in rat embryonic or fetal tissues varied with gestational age, and the embryonic RBP concentrations tended to parallel the vitamin concentrations. As RBP is not released from and accumulates in the adult liver during vitamin A deficiency (even when animals are supplemented with all-*trans*-RA) and RBP is secreted into circulation when exogenous retinol is supplied,³³ an intravenous bolus of retinol in deficient dams caused a doubling of placental RBP and a marked increase in placental vitamin A.³² Concentrations of fetal RBP in vitamin A deficiency were approximately 50% that in normal fetuses, and the normal age-dependent increase in RBP was delayed in vitamin A deficiency. Retinol deficiency was associated with RBP accumulation in fetal livers, suggesting that during the latter stages of fetal development, RBP is synthesized in the fetal liver. The rat data also suggested that plasma holoRBP was

released from maternal hepatocytes during retinol supplementation, where circulating holoRBP crosses the placenta to reach the conceptus.

Cohlan³⁴ was the first to describe excess retinoid-induced teratogenesis in mammals using single or multiple oral doses of 15,000 to 75,000 I.U. of "natural vitamin A" in pregnant Wistar rats. The developmental toxicity of retinol, retinyl esters, retinyl acetate, and all-*trans*-RA has been studied in nearly every species of laboratory animal.^{35,36} Geelen³⁵ categorized the data by congenital defect: exencephaly, meningocele, meningoencephalocele, spina bifida aperta or cystica, hydrocephalus, microcephaly, cyclocephaly, anophthalmia, microphthalmia, congenital cataract, coloboma, exophthalmos, hypoplastic tympanic cavity with auricular aplasia, micrognathia, median cleft mandible, mandibular ankylosis, malformed tongue and teeth, cleft palate, ventricular septal defect, overriding aorta, transposition of the great vessels, aortic stenosis, pulmonary hypoplasia, anal atresia, umbilical hernia, hydroureter, hydronephrosis, unilateral and bilateral renal or ureter agenesis, hypoplastic genital papilla, syndactyly, shortened long bones, oligodactyly, and miscellaneous skeletal malformations (rib fusions, absent vertebrae, kyphosis, kyphoscoliosis). Retinol-induced malformations in fetal hamsters after a single oral 15 to 90-mg/kg (5000 to 30,000-I.U.) dose³⁷ were not unlike those in other rodents, lagomorphs, or nonhuman primates. Malformations of the skull and brain (microcephaly, exencephaly, encephalocele, hydrocephalus), face (clefts, macrostomia, microstomia, micrognathia, agnathia, maxillary retrocession), spine (rachischisis occulta or aperta), mouth (cleft palate, cheeks, hypoplastic tongue), eyes (anophthalmia, exophthalmos), ears (imperforate auditory meatus, hypoplastic and displaced pinnae), abdomen (gastroschisis, omphalocele, umbilical hernia), urinary tract (renal agenesis, absent ureter, exostrophic bladder), and a "caudal regression syndrome" occurred in a dose-dependent manner. Remnants of the first branchial groove could also be found, located at the ventral neck region posterior to the lower jaw should the mandible be present.³⁷

Case reports of human terata associated with ingestion of excessive doses of retinol or the esters have also appeared. Limb,³⁸ renal,³⁹⁻⁴¹ heart,⁴² palate,⁴² jaw,⁴³ facial,⁴³ eye,⁴⁴ ear,⁴⁴ skull,⁴⁴ central nervous system (CNS),⁴⁴ and skeletal malformations⁴⁴ associated with megadose vitamin A ingestion have appeared. The uncontrolled nature of the case reports makes critical evaluation of retinol or retinyl ester-induced human teratogenesis difficult and rigorous epidemiological data do not exist. However, the retinoid malformation syndrome in hamsters³⁷ shows concordance with the human data and Rosa et al.⁴⁴ concluded that the retinoid dose and the distribution of congenital malformations in hamsters more closely paralleled the situation in humans than even the monkey findings.

A number of *in vivo* and *in vitro* approaches have been taken to study the teratogenic activity of naturally occurring retinoids. First, the teratogenic response depends upon the animal's genotype,⁴⁵ environmental factors,⁴⁶ and gestational age.⁴⁷ Studies of early embryos or near-term fetuses from dams treated with vitamin A have documented the extent and pathogenesis of the malformations. Anomalies of the styloid process, the incus and stapes occurred in rats⁴⁸ and ddN mice⁴⁹ after intraperitoneal injection of "water-miscible" vitamin A. Heterotrophic tooth germ and hypoplastic or disarranged molar germ occurred in Slc-ICR mouse fetuses after a single maternal 200,000-I.U. retinyl palmitate injection.⁵⁰ Parenteral retinyl palmitate at 75,000 to 150,000 I.U. on day 12 in pregnant Wistar rats delayed the development of circumvallate papillae and induced abnormal development of the tastebuds in their offspring. Nerve fiber development and distribution to the tastebuds were decreased with increased retinoid dose.⁵¹ The animal data suggest that followup studies of human infants born to mothers ingesting synthetic retinoids focus not only on conditions obvious at birth, but on subtle changes in auditory function, dentition, and functional deficits. Marin-Padilla⁵² found a short, lordotic basioccipital and basisphenoid with a foreshortened and folded notochord in fetal hamsters after a single oral 20,000-I.U. vitamin A dose in the dam. Vitamin A-induced encephaloceles and myeloceles in hamsters were considered the

consequence of partial failure of neural fold closure where surface ectoderm and mesoderm closed, but the neuroectoderm exhibited a partial schisis.⁵³ On the other hand, primary failure of closure of the anterior neural tube (including failure of opposing epithelial lamelopodia and filopodia to contact) underlies pathogenesis of vitamin A-induced exencephaly in rats.⁵⁴ Subsequent spontaneous disintegration of everted neural tissue accounted for anencephalia.⁵⁴ Arnold-Chiari malformation, characterized in fetal hamsters by downward displacement of the cerebellum, medullary compression, lordosis, and protrusion of the odontoid process into the cranial cavity can also be induced by retinol excess,^{37,55} but the exact nature of the skull defects induced by retinol excess depends upon gestational age at treatment. In mammals, all-*trans*-RA is a more potent teratogen than retinol or retinyl esters^{56,57} (Table 1). Hypervitaminosis A (as retinyl acetate or all-*trans*-RA) also induces cardiac,⁵⁸ face,^{58,59} limb,^{58,59} trunk,⁵⁸ and skin⁶⁰ abnormalities in chick embryos. The susceptible organ systems in avian species parallel those in mammals, including humans.

Jacobson⁶¹ reviewed the older literature around *in vitro* teratogenesis induced by retinol, retinyl esters or all-*trans*-RA with emphasis on palate and limb morphogenesis. Steele et al.⁶² found that human sera taken from men at up to 5 h after ingestion of 100,000 U.S.P. units (30 mg) of "natural vitamin A" was teratogenic for cultured CD rat embryos. In fetal rat bone culture,⁶³ all-*trans*-RA-induced teratogenesis depended upon RNA, protein, and glycoprotein synthesis; altered protein synthesis patterns and altered protein glycosylation were independent of DNA synthesis. Kistler⁶³ concluded that all-*trans*-RA "may exert its action by altering gene expression" in the fetal cells. In cultured chick embryo chondrocytes, all-*trans*-RA inhibited membrane bone formation and chondrogenesis.⁶⁴ In isolated chick mesenchymal cells cultured with all-*trans*-RA, differentiation was inhibited.⁶⁴ Polydactyly resulted from local application of 3 to 25 pg of all-*trans*-RA to cultured chick limb buds, slightly elevated concentrations caused oligodactyly and higher concentrations caused truncations. The local retinoid concentration apparently determined apical ectodermal ridge morphology, the effect being similar to the graded effect achieved by grafting polarizing region mesenchyme beneath the apical ridge. Lee and Tickle⁶⁵ concluded that either all-*trans*-RA acted directly on ridge cells or that it converted the tissue adjacent to implanted all-*trans*-RA into polarizing tissue. Cultured mouse limb buds treated with 10 I.U./ml of retinol⁶⁶ or limb buds taken from embryos of mice treated with all-*trans*-RA⁶⁷ exhibited gestation age-dependent polydactyly, ectrodactyly, syndactyly, or micromelia. Others⁶⁸ noted obliterations of the cultured mouse limb bud apical ridge with vitamin A concentrations associated with limb truncation; thus, cultured mammalian and avian limb buds respond in a qualitatively similar fashion to exogenous retinoids. Whether retinoid effects were targeted towards the limb bud mesoderm or ectoderm remains controversial (just as in the instance of retinoid-induced neural tube defects⁶⁹), but the net result appears to depend upon interaction of mesenchymal and ectodermal cell populations. Apical limb bud morphogenesis depends upon an "apical ridge maintenance factor"; however, synergism between exogenous retinoid and the limb bud polarizing region suggested that all-*trans*-RA (or a metabolite) influenced the behavior of neighboring cell populations, perhaps via gap junction-mediated communication.⁶⁵ From the *in vitro* work, one can hypothesize that all-*trans*-RA and/or metabolite(s) act as localized molecular signals or perhaps diffusible morphogens capable of acting over only limited distances in a concentration or dose-dependent manner to control cellular embryogenesis. Of course, since retinoids cannot be synthesized *de novo* by mammalian cells, retinoids required *in situ* under this paradigm must arise from maternal circulating levels.

Also of interest is the demonstration that all-*trans*-RA treatment can materially reduce the incidence of spontaneous or chemically-induced terata. A single oral dose of 5 mg/kg on day 9 of pregnancy reduced spontaneous neural tube defects in curly tail⁷⁰ and splotch⁷¹ mice. Intraperitoneal injection of 0.5 µg/kg of all-*trans*-RA on day 10 in pregnant mice suppressed significantly the numbers of abnormal fetuses associated with a teratogenic dose

TABLE 1
Retinoid Nomenclature, Structure and Teratogenicity

Common name or code no.	Name	Structure	TD ₅₀ ^a (mg/kg)	Lowest dose ^b (μg)
all- <i>trans</i> -Retinol	(<i>E</i>)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenol		22.9	125
all- <i>trans</i> -Retinoic acid (Tretinoin) Ro 1-5488	(<i>E</i>)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid		10.5	12.5
15-Fluororetinone	all- <i>trans</i> -retinoyl fluoride		53.8	—
Retinylidene acetylacetone	all- <i>trans</i> -3-retinylidene-2,4-pentanedione		>190	—
—	all- <i>trans</i> -2-retinylidene-1,3-cyclopentanedicone		>190	—
Retinylidene dimedone	all- <i>trans</i> -2-retinylidene-5,5-dimethyl-1,3-cyclohexanedicone		>210	—

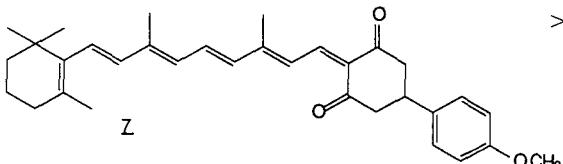
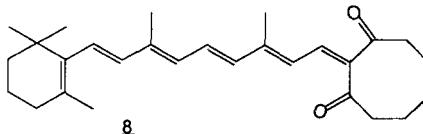
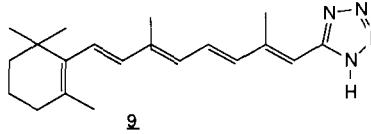
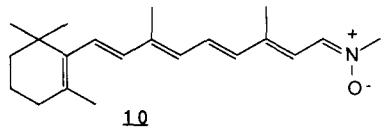
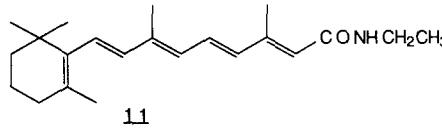
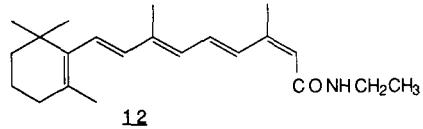
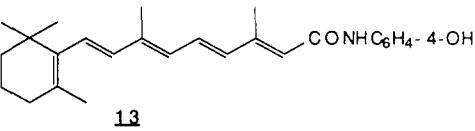
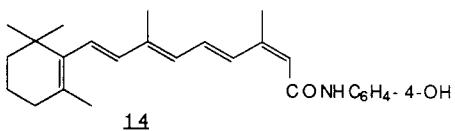
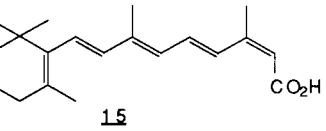
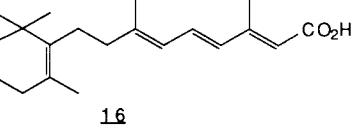
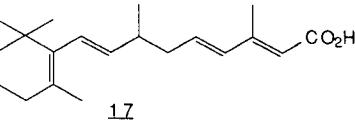
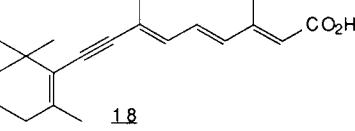
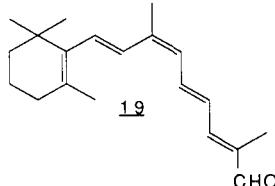
—	all- <i>trans</i> -2-retinylidene-5- <i>p</i> -methoxyphenyl-1,3-cyclohexane-dione		>250	—
—	all- <i>trans</i> -2-retinylidene-1,3-cyclooctanedione		>210	—
—	(<i>E</i>)-5-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1,3,5,7-octatetraen-1-yl]tetrazole		<81	—
all- <i>trans</i> -Retinylidene methyl nitronate	<i>N</i> -(<i>trans</i> -retinylidene)methylamine- <i>N</i> -oxide		39.1	—
Ro 8-4968	all- <i>trans</i> - <i>N</i> -ethylretinamide		>163	—
Ro 13-3987	13- <i>cis</i> - <i>N</i> -ethylretinamide		>163	—

TABLE 1 (continued)
Retinoid Nomenclature, Structure and Teratogenicity

Common name code no.	Name	Structure	TD ₅₀ ^a (mg/kg)	Lowest dose ^b (μg)
—	13-cis-N-(4-hydroxyphenyl)retinamide		138.6	—
—	13-cis-N-(2-hydroxyethyl)retinamide		>173	—
13-cis-Retinoic acid (Isotretinoin), Accutane® (Ro 4-3780)	(2Z,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid		22.3	125
7,8-Dihydroretinoic acid	(E)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6-nonatrienoic acid		<38	—
9,10-Dihydroretinoic acid	(E)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,8-nonatrienoic acid		>76	—
7,8-Dehydroretinoic acid	(E)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-nona-2,4,6-trien-8-ynoic acid		<37	—

9-cis-Retinal

(2E,4E,6Z)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenal

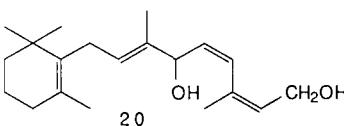


23.2

—

Hydroxenin

(2Z,4Z,6Z)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)nona-2,4,6-triene-1,6-diol

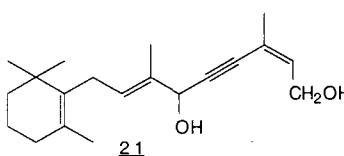


>510

—

Oxenin

(2Z,7E)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)nona-2,7-dien-4-yne-1,6-diol

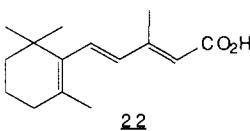


>750

—

C₁₅ acid

(E)-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-methylpenta-2,4-dienoic acid

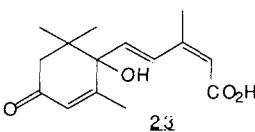


>220

—

Abscisic acid

R-(2Z,4E)-(+)-5-(1-hydroxy-2,6,6-trimethyl-4-oxo-2-cyclohexen-1-yl)-3-methyl-2,4-pentadienoic acid

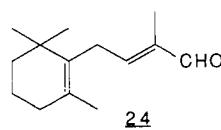


>250

—

β-C₁₄ aldehyde

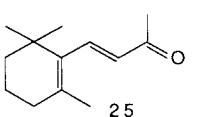
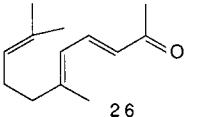
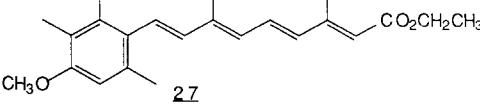
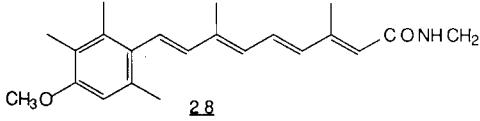
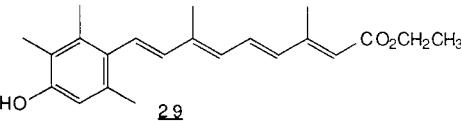
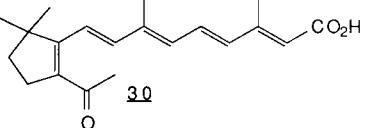
(E)-2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-butenal



>980

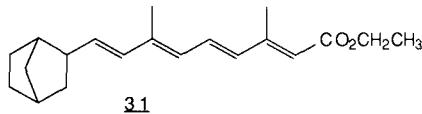
—

TABLE 1 (continued)
Retinoid Nomenclature, Structure and Teratogenicity

Common name code no.	Name	Structure	TD ₅₀ ^a (mg/kg)	Lowest dose ^b (μg)
β-Ionone	(E)-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-but-en-2-one		>480	—
Pseudoionone	(E)-6,10-dimethyl-3,5,9-undecatrien-2-one		>960	—
Etretinate Tigason® (Ro 10-9359)	ethyl (E)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate		5.7	125
Motretinid Tasmaderm® (Ro 11-1430)	N-ethyl (E)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenamide		>330	—
Ro 11-4768	ethyl (E)-9-(4-hydroxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate		87.3	—
Ro 8-7699	(E)-9-(2-acetyl-5,5-dimethyl-1-cyclopenten-1-yl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid		10.9	—

2-Norbornyl ethyl ester

ethyl (*E*)-9-(*exo*-2-bicyclo[2.2.1]heptyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate

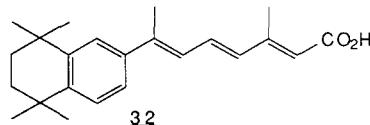


<75

—

Ro 13-6307

(*E*)-7-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-3-methyl-2,4,6-octatrienoic acid

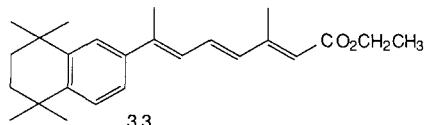


0.66

12.5

Ro 13-2389

ethyl (*E*)-7-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-3-methyl-2,4,6-octatrienoate

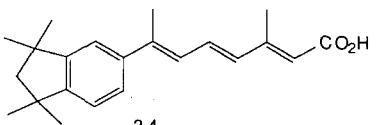


0.70

12.5

Ro 13-4306

(*E*)-3-methyl-7-(1,1,3,3-tetramethylindan-5-yl)-2,4,6-octatrienoic acid

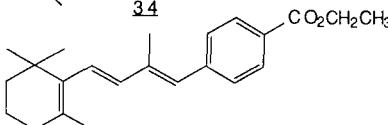


4.7

—

trans-Aryltriene analog of retinoic acid ethyl ester

ethyl (*E*)-4-[2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1,3-butadien-1-yl]benzoate

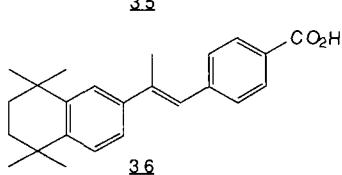


<84

—

Ro 13-7410 (TTNPB)

(*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propen-1-yl]benzoic acid



<0.019

1.25

TABLE 1 (continued)
Retinoid Nomenclature, Structure and Teratogenicity

Common name code no.	Name	Structure	TD ₅₀ ^a (mg/kg)	Lowest dose ^b (μg)
Ro 13-6298	ethyl (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propen-1-yl]benzoate		<0.023	1.25 (inactive)
Ro 13-8320	(E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propen-1-yl]phenylmethanol		<0.036	—
Ro 13-9272	(E)-1,2,3,4-tetrahydro-1,1,4,4-tetramethyl-6-[1-(4-methylphenyl)-1-propen-2-yl]naphthalene		<0.032	—

^a TD₅₀ = Teratogenic dose 50 in hamsters.

^b Dose/egg summarized from Cadi et al.⁹⁵

of urethane.⁷² In each case, however, the antagonistic effect was dependent upon retinoid dose as larger doses induced typical retinoid embryotoxicity.

B. SYNTHETIC RETINOIDS

1. Humans

Over 500,000 patients with nodulocystic and conglobate acne were treated with 0.2 to 2.0 mg/kg/d of 13-*cis*-RA from 1982 to 84 in the U.S. alone. Among those, some 120,000 women of childbearing age were treated and among 35 registered pregnancies, 29 or 83% experienced reproductive failure (spontaneous abortion, congenital malformation).⁷³ The human Accutane® dysmorphic syndrome affecting primarily cardiac, craniofacial, and CNS morphogenesis has been described in detail.^{74,75} Briefly, malformations of the fourth ventricle, medullary velum, and cerebellum can be traced to abnormalities of the embryonic rhombencephalon and were accompanied by hydrocephalus or microcephaly. The conotruncal cardiac defects, the thymic defects (DiGeorge sequence), and the facial dysmorphia can be attributed to abnormal migration and malfunction of cranial neural crest cells. The related retinoid etretinate (Tegason®) (Table 1) was recently approved for treatment of recalcitrant pustular or erythrodermic psoriasis by the U.S. Food and Drug Administration.⁷⁶ Approximately 10,000 to 20,000 Europeans have received oral etretinate at doses of 25 to 50 mg/day for generalized pustular psoriasis, ichthyosis, Darier's disease, and other, often rare, skin disorders (psoriatic erythroderma, Sezary's syndrome, rhagadiform eczema, arthropathy). Thirty-seven pregnancies have been associated with oral etretinate exposure, 19 of which were associated with direct exposure and 18 of which occurred within 2 years of discontinuation. It is notable that etretinate has been detected in patient serum at up to 3 years after cessation of treatment.⁷⁶ Malformations occurred in at least four cases and three induced abortions were reported.⁷⁷⁻⁷⁹ The three aborted fetuses showed CNS terata⁸⁰ and the newborns were afflicted with skeletal malformations^{79,80} and/or craniofacial defects.⁸¹

Isotretinoin or etretinate-induced human terata have features in common, but based on the rather limited cases in the open literature, etretinate exposure appears to induce malformations of the limbs and digits more often than does isotretinoin exposure. The limb reduction defects could be more a consequence of prolonged maintenance of high circulating concentrations of etretinate and its major metabolite, etretin (Ro 10-1670), through the period of limb formation even after discontinuation of therapy because of the prolonged elimination half-life, rather than to a particular xenobiotic affinity for limb bud tissues. In addition, retinoid-induced syndromes of terata have features in common with symptomatology of previously described syndromes of "spontaneous" human dysmorphia. The saddle nose, low-set ears, micropinnae, microtia with stenotic auditory canal, coronal suture alterations and synostoses, basichondrocranial alterations with Dandy Walker and posterior fossa cysts, cerebral defects, and depressed midface induced by etretinate⁸² or isotretinoin^{74,75} are reminiscent of Treacher Collins syndrome.⁸³ The trigonocephaly, abnormal facies with the broad flat nose, micrognathia, prominent occiput, apparent maxillary retrocession and hypoplasia, syndactylies, malformations of the hip, ankle, forearm, and digits, muscular hypotonia, renal malformations, and neurologic deficits found in etretinate⁷⁹⁻⁸¹ or isotretinoin^{74,75} dysmorphia are also characteristic of the Opitz trigonocephaly syndrome.⁸⁴ These rather striking similarities suggest that similar populations of neural crest and mesenchymal cells are affected in synthetic retinoid-induced terata as in Treacher Collins and Opitz trigonocephaly. These correlations and Rosa's case reports⁴⁴ raise concerns about retinoid status as a possible contributory agent to the background load of human congenital malformations of unknown etiology.

2. Animals

The comparative gestational age, pharmacokinetics, metabolic fate, and syndromes of terata induced by oral 13-*cis*-RA exposure in rodents and humans have been summarized.⁷⁵

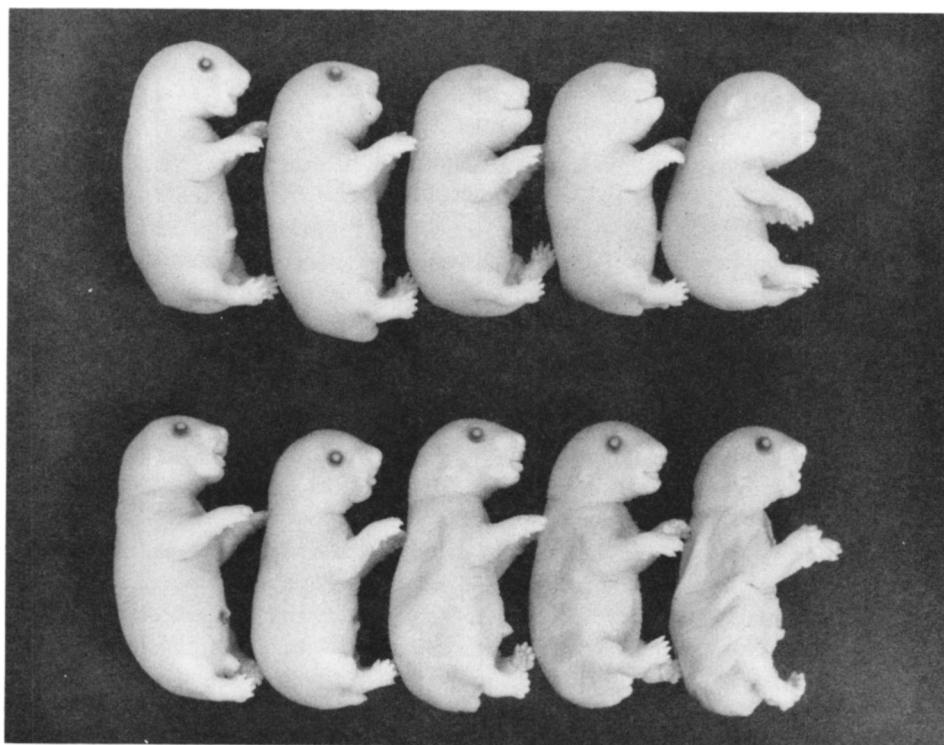
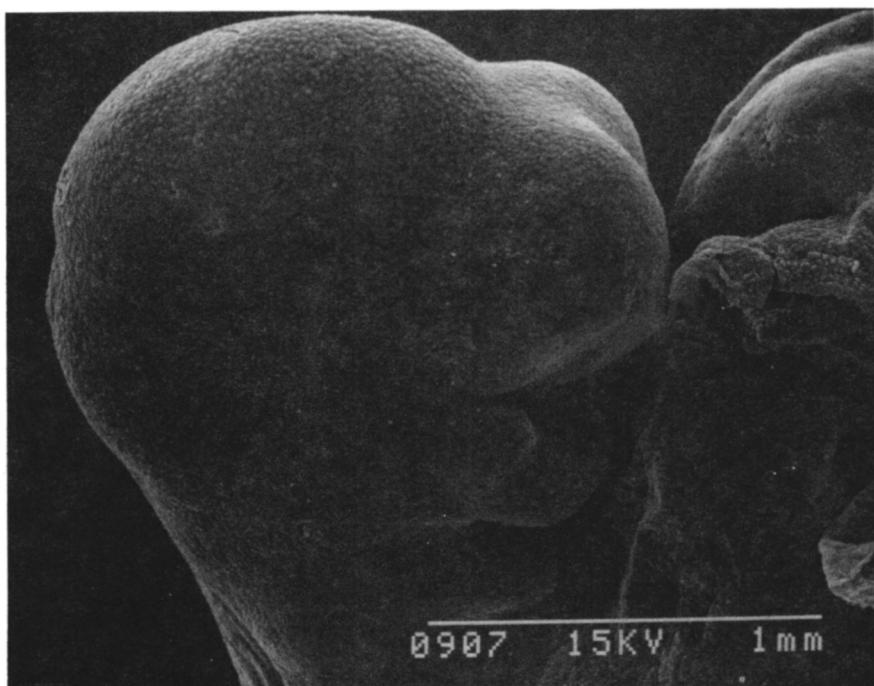


FIGURE 1. Golden Syrian hamster fetuses recovered on day 14 of gestation. The animal at the upper right is a control and the remaining are littermates from a dam given a single oral dose of 50 mg/kg of 13-cis-retinoic acid (isotretinoin, Accutane[®]) on day 8 of pregnancy. Note the absent eyelids with exophthalmia, the rudimentary ears, the stunted tails, and the dome-shaped contour of the skull. The malformations of the mouth range from macrostomia with apparent retrocession of the maxilla to microstomia with a protruding tongue.⁸⁵

Oral isotretinoin⁸⁵ and etretinate⁸⁶ are teratogenic in hamsters where the aromatic congener was approximately four times as potent as isotretinoin (Table 1). The terata in hamsters (Figure 1) was essentially identical to that induced by similar administration of retinol, retinyl esters,^{37,52,53,55} or all-trans-RA.^{85,87} The malformations of the face (Figure 1) appear due to primary failure of growth of the first branchial arch (Figure 2)⁸⁸ and are most likely the result of delay, disorganization, and/or death of cranial neural crest cells.⁸⁷ The malformations of the hamster skull, however, have been attributed to disturbances within the mesoderm.⁸⁹ Cardiac malformations occurred in about 75% of fetal hamsters after maternal oral dosing with all-trans-RA on day 9 of gestation, and consisted primarily of ventriculo-bulbar deformities (double outlet right ventricle, transposition of the great vessels, ventricular septal defect, overriding aorta).⁹⁰

Structure-teratogenicity relationships can be constructed (Table 1) from studies in hamsters^{37,85,86,91-94} treated with a single oral retinoid dose on day 8 of gestation and in chicken eggs treated at day 10 of incubation.⁹⁵ These data are useful to rank retinoids and to study the characteristics of retinoid structure that confer embryotoxicity. Of course, the retinoid teratogenic profile and effective dose depend upon gestational age⁹⁶ and species^{97,98} studied. In hamsters, malformations induced by synthetic retinoids are essentially identical to those induced by treatment with retinol³⁷ or all-trans-RA.^{85,96} The retinoids differ quantitatively in teratogenic activity rather than qualitatively. For purposes here, retinoids with TD₅₀ values greater than 100 mg/kg can be considered nontoxic; doses larger than those given in Table 1 could not be investigated either because of the physical limits of solubility



A



B

FIGURE 2. (A) Control day-9 hamster embryo. Note the size and location of the branchial arches which give rise to, among other structures, the maxilla and the mandible. (B) Day 9 hamster embryo recovered from a dam 24 h after a single oral dose of 13-cis-retinoic acid. Note the rudimentary branchial arches and the rudimentary first branchial groove.^{75,88}

for suspension in the vehicle or because of the limited quantities of retinoid available for study. From Table 1 and from the studies outlined below, the following generalizations about retinoid teratogenicity can be made:

1. Retinoid teratogenicity depends upon the presence of or biotransformation to a retinoid containing a polar terminus with an acidic pK_a , and the acidic polar terminus need not necessarily be a carboxyl residue.
2. The polyene or substituted side chain must be of sufficient length, must maintain lipophilicity, and must maintain uninterrupted π electron delocalization across the retinoid; *cis* isomerization diminishes embryotoxicity.
3. A ring structure (or possibly another conjugated hydrophobic moiety) is required at the end of the molecule opposite the terminus: but the ring need not necessarily contain six carbon atoms nor must it maintain a high degree of lipophilicity.
4. While increasing conformational restriction *per se* failed to increase teratogenic potency, increasing the degree of conformational restriction of acidic retinoids increased embryotoxicity.

Nonetheless, each characteristic listed above is not in and of itself sufficient to confer embryolethality or teratogenicity.

Examples to substantiate the concept that an acidic polar terminus was required include the facts that all of the retinylidene 1,3-diketones (e.g., retinylidene acetylacetone, retinylidene dimedone) and the retinamides (e.g., Ro 8-4968, Ro 13-3987, Ro 11-1430⁹⁹) were devoid of teratogenic activity in hamsters. It is noteworthy here to point out that Ro 11-1430 was also inactive in fetal rat bone culture,¹⁰⁰ an assay that measures intrinsic induction of proteoglycan release and cartilage resorption; likewise, it was the least effective retinoid tested for inhibition of chondrogenic differentiation of cultured NMRI mouse limb bud mesenchymal cells.¹⁰¹ In contrast, Kochhar et al.¹⁰² reported that a single oral dose of 100 mg/kg of motretinid (Ro 11-1430) on day 12 of pregnancy in ICR or NMRI mice induced a 35 and 70% frequency, respectively, of cleft palate. Most studies conclude that the retinamides^{103,104} and the retinylidene 1,3-diketones¹⁰⁵ were not biotransformed to pharmacologically significant concentrations of all-*trans*-RA, but Kochhar and associates¹⁰² suggested that perhaps in mice motretinid was partially hydrolyzed to the free acid, etretin, to account for teratogenic activity. Biotransformation of retinol, all-*trans*-retinoyl fluoride,¹⁰⁶ all-*trans*-retinylidene methyl nitrone,^{92,98} 9-*cis*-retinal,⁹³ and of esters like etretinate,¹⁰⁷ Ro 13-6298,¹⁰⁸ the 2-norbornenyl analog,^{91,109} and the aryl triene^{91,110} to their acidic congeners can account for their teratogenic activities. Other retinoids like the tetrazole^{91,112} or the arotinoid sulfinic acid^{113,114} have pK_a values similar to the carboxyl and are also potent teratogens.

In spite of the presence of the natural β -cyclogeranylidene ring and a free acid moiety at C₁₅, 9,10-dihydroretinoic acid was teratogenically inactive⁹³ (Table 1). Other retinoids like oxenin and hydroxenin with the natural ring and a free alcohol at C₁₅ were also inactive as were compounds like abscisic acid, the C₁₅ acid, and the other short chain analogs.⁹³ In each case, failure to maintain sufficient chain length, conjugation or lipophilicity diminished teratogenic potency. Major modifications in the side chain as in the aryl triene^{91,110} or multiring Ro 13-6307¹¹¹ failed to diminish teratogenic activity; in these latter cases, both conjugation and the acidic polar terminus were maintained. Intubation of the 13-*cis* or 9-*cis* isomers was associated with diminished teratogenic potency as compared to all-*trans*-RA (Table 1).

Modification of the retinoid β -cyclogeranylidene ring alone influenced embryotoxicity the least of any region studied. Introduction of a substituted aromatic ring as in etretinate (Ro 10-9359)⁸⁶ (Table 1) or its free acid congener, etretin (Ro 10-1670),¹⁰⁷ was associated

with teratogenic potency equal to or greater than that of all-*trans*-RA. However, substitution of the 4-hydroxytrimethylphenyl ring (Ro 11-4768) in place of the 4-methoxy congener (Ro 10-9359) reduced teratogenic activity 15-fold. Substitution of the 7-carbon norbornenyl ring system with elimination of the dimethyl substituents at C₁ and elimination of the 5,6-double bond failed to reduce embryotoxicity in hamsters compared with an equimolar dose of all-*trans*-RA.⁹¹ Substitution of the cyclopentenyl ring (Ro 8-7699) enhanced potency compared to all-*trans*-RA (Table 1). Intubation of the 4-oxo metabolite of all-*trans*-RA in pregnant hamsters¹¹⁵ resulted in teratogenic activity equal to that of the parent; intubation of the 4-oxo metabolite of 13-*cis*-RA in pregnant mice was also associated with teratogenic activity equal to or somewhat greater than that of the parent.¹¹⁶

Increasing conformational restriction of acidic retinoids or retinoids, which gives rise to acidic metabolites *in vivo*, enhanced teratogenic potency in hamsters. Introduction of a supplementary aromatic ring with concomitant diminished side-chain flexibility of acidic (Ro 13-6307) or esterified (Ro 13-2389) retinoids increased potency 15-fold compared to all-*trans*-RA.¹¹¹ Substitution of a supplementary aromatic ring gave the indanyl retinoid (Ro 13-4306) and doubled teratogenic potency compared to its cyclopentenyl congener (Ro 8-7699). Teratogenic potency increased at least 35-fold through the introduction of yet another aromatic ring with the additional constraint of a planar cisoid three-dimensional configuration at the C₁₁-C₁₃ region of all-*trans*-RA when comparing the free acid Ro 13-6307 and Ro 13-7410 retinoids.⁹⁴ Comparing the esterified congeners Ro 13-2389 and Ro 13-6298 found teratogenic potency increased at least 30-fold with the planar cisoid conformation. Retinoids like the hydroxymethylphenyl Ro 13-8320 and the methylphenyl Ro 13-9272 can be oxidized or undergo aliphatic hydroxylation and subsequent oxidation, respectively, to their common acidic congener Ro 13-7410 to account for their similar TD₅₀ values (Table 1). These retinoidal benzoic acid congeners were at least 750 times as potent teratogens as all-*trans*-RA in hamsters.⁹⁴

Studies with retinoids in chicken eggs or in mammalian embryonic cell or fetal organ cultures parallel, in the main, the structure-toxicity relationships observed in intact hamsters—provided metabolism and *in vivo* distribution are taken into account. Cadi et al.⁹⁵ examined Wyandotte × Rhode Island Red chick embryos after retinoid treatment and recorded the incidence of crossed beak, acropodial truncature, club-shaped feather filaments, and ptilopody. The terata were associated with significant reduction in mean body weight and the larger doses killed the embryos. The lowest doses associated with ptilopody can be compared using all-*trans*-RA as a reference (Table 1). Retinol was less potent than all-*trans*-RA and retinal was judged inactive. The 13-*cis*-RA was approximately 1/60 as effective as all-*trans*-RA. The free acid metabolite of etretinate, Ro 10-1670, was somewhat less potent than all-*trans*-RA and the parent ester, Ro 10-9359 was less active yet. The acetyltrimethylcyclopentenyl analog of retinoic acid (Ro 8-7699) was approximately of equal potency to the free acid, etretin. Introduction of a supplementary aromatic ring in the side chain and addition of *gem*-dimethyl groups in the tetramethylated tetralin acid (Ro 13-6307) and ester (Ro 13-2389) increased teratogenic activity as compared to all-*trans*-RA. The arotinoid Ro 13-7410 was at least 10 times as potent as the tetramethylated tetralins in chicken eggs and at least 100 times as potent as all-*trans*-RA. In contrast, the ester Ro 13-6298 was inactive at the dose studied.⁹⁵ Controlled release studies confirmed that Ro 13-7410 was at least 30 times more potent a teratogen than was all-*trans*-RA in chick embryos.¹¹⁷ In cultured chick embryo tarsometatarsal skin explants,¹¹⁸ the relative biological activities paralleled those in intact chick embryos and in intact hamsters. Comparing the concentrations required to inhibit completely scale differentiation, all-*trans*-RA achieved the effect at 10⁻⁵ M, whereas 3 × 10⁻⁵ M 13-*cis*-RA was required.¹¹⁸ Etretinate was inactive in cultured chick embryo skin, but addition of an esterase resulted in complete inhibition of epidermal differentiation at 10 μM, but the free acid etretin (Ro 10-1670) achieved complete inhibition at doses equipotent

to all-*trans*-RA. The amide derivative Ro 11-1430 was inactive in the cultured chick embryo skin assay,¹¹⁸ just as it was essentially inactive as far as inducing terata in hamsters (Table 1). Conversely, the free acid metabolite of the hydroxyl congener Ro 11-4768, which was teratogenic in hamsters at doses nine times that of all-*trans*-RA and is metabolized to its free acid *in vivo*, was inactive in cultured chick embryo skin. The tetramethylindane (Ro 13-4306) and tetralin (Ro 13-6307) were 100 and 1000 times more potent, respectively, than all-*trans*-RA in chick embryo skin, whereas these congeners were only 2 to 15 times more potent teratogens than all-*trans*-RA in intact hamsters.¹¹¹ The retinoidal benzoic acid derivatives Ro 13-6298 and Ro 13-8320 were 10 times more potent in chick embryo skin and the free acid Ro 13-7410 was 1000 times more potent than all-*trans*-RA.¹¹⁸ These latter retinoids were 750 to 1000 times more potent teratogens than all-*trans*-RA in hamsters⁹⁴ (Table 1).

Goulding and Pratt¹¹⁹ confirmed the teratogenicity of 13-*cis*-RA in cultured mouse embryos. In cultured whole rat embryos, neither all-*trans*-*N*-ethylretinamide (Ro 8-4968), 13-*cis*-*N*-ethylretinamide (Ro 13-3987), all-*trans*-*N*-(2-hydroxyethyl)retinamide, nor etretinate (Ro 10-9359) induced terata.¹²⁰ Again, the failure to elicit a response *in vitro* with etretinate can be attributed to only limited conversion of the parent ester to its free acid metabolite, etretin. At higher concentrations *in vitro*, all-*trans*-*N*-(4-hydroxyphenyl)retinamide was teratogenic for rat embryos,¹²⁰ similar to the relative response observed with its 13-*cis* congener in hamsters (Table 1). Kochhar and Penner¹¹⁶ reported that 13-*cis*-RA and its 4-oxo metabolite were equipotent with all-*trans*-RA toward inhibition of chondrogenesis in embryonic mouse limb cell and limb bud cultures. Motretinid or etretinate added to cultured ICR mouse limb buds failed to produce any suppression of chondrogenesis whereas addition of all-*trans*-RA, 13-*cis*-RA, or etretin produced marked inhibition at 50 ng/ml.¹⁰² The frequency of morphological transformation for all-*trans*-RA > 13-*cis*-RA > all-*trans*-retinol in cultured primary hamster fetal cells¹²¹ also mirrored their relative teratogenic potencies in intact hamsters (Table 1). There was an increase in "apparent" microviscosity of embryonal carcinoma cells that accompanied differentiation after addition of all-*trans*-RA, 13-*cis*-RA, or etretin, but addition of the phenyl analog of retinoic acid failed to enhance microviscosity and it was a poor inducer of differentiation.¹²² The phenyl analog was embryolethal at high doses in hamsters, but it was a relatively poor teratogen *in vivo*,¹²³ just as it showed only limited activity in cultured embryonal cells.¹²²

III. PLACENTAL PERMEABILITY AND MECHANISMS OF ACTION

One of the major advantages to knowledge about transplacental xenobiotic disposition is that it allows determination of possible anatomic locations of teratogen action, be it secondary to disturbance of maternal homeostasis, disruption of placental function, or toxicity within the conceptus itself. The following discussion summarizes those data pertaining to "natural" and "synthetic" retinoids and supports the view that not only do retinoids cross the rodent placenta, but the data point toward the embryo as the site of retinoid-induced developmental toxicity. The data published to date also suggest that it is the retinoid molecular structure that determines interaction with specific receptors in specific embryonic cell populations to account for retinoid-induced teratogenicity.

Kochhar¹²⁴ studied the accumulation of total radioactivity in fetal ICR/DUB mice after oral intubation of 3.85 or 100 mg/kg of (11,12-³H₂) all-*trans*-RA on day 12 of gestation. Peak concentrations of radioactivity in the placenta occurred 2 h after dosing with 3.85 mg/kg and fetuses and the visceral yolk sac accumulated peak concentrations at 6 h after dosing. At 6 h, the concentration of radioactivity in the embryo was twice that in the placenta. After a teratogenic dose of 100 mg/kg, peak concentrations in fetuses occurred at 12 h after dosing,

but peak concentrations in the placenta occurred at 6 h. At the higher dose, fetuses contained 2 to 3 times the radioactivity found in the visceral yolk sac, and total radioactivity in the embryos was 26 times more after a teratogenic dose than after the lower, nonteratogenic dose.¹²⁴ Dencker¹²⁶ extended the observation in mice to label localization within the neuroepithelium. Intravenous injection of [15-¹⁴C]all-*trans*-RA on day 10 of gestation in mice resulted in label incorporation into the outer layers of the neuroepithelium. After injection on day 14, label accumulated in the myelencephalon and spinal cord. In comparison, the label accumulated primarily in the extraembryonic endoderm after an equivalent injection of [15-¹⁴C]all-*trans*-retinylacetate. When retinyl acetate was given during mouse organogenesis, the label was incorporated primarily in neuroepithelium, limb buds, and branchial arches, but injection during late pregnancy resulted in label accumulation in liver, lungs, pleura, heart, and intestine. Intravenous injection of [11-³H]all-*trans*-RA or [11,12³H]all-*trans*-retinylacetate showed retinoid-specific concentration profiles; retinylactate levels remained elevated over days 11 to 14 then decreased as term approached, but the label concentrations associated with all-*trans*-RA decreased rapidly over days 11 to 14. The label decrease was primarily due to decreasing label concentrations in the CNS.¹²⁵

Creech-Kraft et al.¹²⁶ studied the comparative placental permeability of all-*trans*- and 13-*cis*-RA in NMRI mice on day 9 or 11 of pregnancy. Following an oral dose of 100 mg/kg of all-*trans*-RA on day 9, 4 to 5% of the total dose appeared in maternal plasma as 13-*cis*-RA and in the embryo, 4 to 8% consisted of 13-*cis*-RA. The embryonic all-*trans*-RA concentration reached values 58% of that of the circulating maternal concentrations. After a dose of 100 mg/kg of 13-*cis*-RA, 10 to 20% of the parent drug appeared as the all-*trans* isomer in maternal plasma and, although both 13-*cis*-RA and its 4-oxo metabolite appeared in the embryo, the concentrations of the parent 13-*cis*-RA were considerably less than were the concentrations of the parent all-*trans*-RA after similar treatment. Creech-Kraft et al.¹²⁶ concluded that lower embryonic 13-*cis*-RA concentrations than all-*trans*-RA concentrations accounted for the diminished teratogenic activity of the 13-*cis* isomer.

A single oral dose of [10,11-³H₂]all-*trans*-RA, [11-³H]13-*cis*-RA, [11-³H]13-*cis*-N-ethyl-retinamide, [11-³H]all-*trans*-N-ethylretinamide or [³H₂]TTNPB (Ro 13-7410) at the same gestational age that in previous studies was used to evaluate their relative teratogenic potencies in hamsters (Table 1) resulted in label deposition within the embryo at approximately the same concentrations as those found in the placenta.¹²⁷ In the case of all-*trans*-RA and 13-*cis*-RA, the activity in maternal plasma peaked at 1 to 2 h after intubation, but the levels of 13-*cis*-RA were five times those of all-*trans*-RA after an equimolar oral dose. For the two retinamides, peak activity in maternal blood occurred between 4-8 h after treatment. Radioactivity tended to accumulate in the maternal liver and adipose tissue.¹²⁷ The data with all-*trans*-RA in hamsters were consistent with the earlier studies of [³H]all-*trans*-RA,¹²⁴ and 13-*cis*-RA¹²⁰ in mice. Creech-Kraft et al.¹²⁸ mentioned that etretinate and motretinid were metabolized in pregnant NMRI mice to their free acid congener, etretin, and that etretin was found in their embryos after a single oral dose of the parent retinoid in the dam at 100 mg/kg on day 11. Others¹²⁹ have determined the embryonic concentrations of the all-*trans* and 13-*cis* isomers of etretin after a single oral etretin dose in pregnant mice.

Since the concordance between the malformations produced by retinoid exposure in rodents and humans is so close, studies on the mechanism of retinoid teratogenicity in animals are likely to explain the situation in humans as well. That the facial dysmorphia is due to rudimentary brachial arch development has been established,⁸⁸ and it is likely that this effect is due to inhibition of proliferation of (defective) mesenchymal cells^{47,120,130} that depend, in turn, upon migration of cephalic neural crest cells from areas adjacent to the neuroepithelium.⁸⁷ Retinoid-induced delay or disorganization of neural crest migration and death and damage in the crest cells occurs in avian¹³⁰⁻¹³² and mammalian^{83,87} embryos. Dencker et al.¹³³ reported gestation stage-dependent localization of label in the mouse neu-

roepithelium, the developing CNS and cranial neural crest after [^{14}C]all-*trans*-RA treatment. The cytosolic binding protein for all-*trans*-RA is apparently expressed in neural crest cells only during selected developmental stages.¹³³ Since retinoid treatment causes specific developmental lesions rather than generalized embryotoxicity, the existence of specific receptors at critical periods in particular cell populations could account for the gestational age-dependent teratogenic profile. In cultured mouse limb buds treated with all-*trans*-RA, mesenchymal cells assumed a stellate rather than a bipolar shape and they exhibited decreased migration.¹³⁴ Transposition of the great arteries, ventricular septal defects and double outlet right ventricle induced by a single oral 78-mg/kg all-*trans*-RA dose in pregnant ICR mice was attributed to "maldevelopment of endocardial cushion material", a consequence of decreased mesenchymal cell proliferation and decreased extracellular matrix (cardiac jelly) production.¹³⁵ At least three reports¹³⁶⁻¹³⁸ have concentrated on craniofacial dysmorphia induced by 7.5 to 10 mg/kg of all-*trans*-RA in non-human primates, and the malformed ears, hypertelorism, retrognathia, exophthalmos (attributed to small eye sockets), and hypoplastic viscerocranum were a consequence of "defective neural crest migration in the first and second branchial arches".¹³⁸ Lammer¹³⁹ reviewed and commented on the evidence supporting cranial neural crest dysfunction in the pathogenesis of retinoid-induced terata.

The biochemical basis of retinoid developmental toxicity is not clear, but the hypothesis that retinoid-induced terata could be attributed to non-specific membrane disruption due to their amphipathic properties cannot be supported by the available data. For example, retinoids like 9,10-dihydroretinoic acid (Table 1) contain the lipophilic β -cyclogeranylidene ring and the terminal polar carboxyl group but are teratogenically inactive.⁹³ Retinoic acid is a known inhibitor of chondrogenesis, preventing embryonic biosynthesis of chondroitin sulfate proteoglycans¹⁴⁰ and glycosaminoglycans¹⁴¹ and causing disturbances in hyaluronate metabolism.¹⁴¹ There is evidence that all-*trans*-RA may alter phenotypic expression in developing muscle,¹⁴² but the present weight of evidence indicates that retinoids produce mesenchymal cell degeneration⁴⁷ and alter protein synthesis in developing bone.^{143,144} Retinoids apparently induce differentiation of pluripotent embryonic cells¹⁴⁵ without directly affecting DNA or RNA synthesis.^{143,144} Others have postulated that retinoids affect embryogenesis by changing cell-surface structure or composition¹⁴⁶ or by changing migratory patterns as a consequence of altered internal viscosity.¹⁴⁷ In turn, those physiochemical changes may be due to retinoid-induced disturbances in glycoprotein and glycosaminoglycan biosynthesis and secretion^{148,149} perhaps by altering posttranslational modification of glycoprotein.¹⁵⁰

The specific structure-toxicity relationships for retinoids in hamsters and in chicken eggs (Table 1) argue for the existence of specific cellular receptors in retinoid teratogenesis. Cellular retinoic acid-binding protein (CRABP) has been implicated in other systems^{151,152} as a holoprotein acting in cytosolic transport of the retinoid. The CRABP has been located in embryonic mouse,¹⁵³ embryonic chick,^{154,155} fetal rat,¹⁵⁶ and fetal rabbit.¹⁵⁷ The pharmacologic activity of retinoids in adult hamster tissue correlates with their binding efficiency to CRABP.¹⁵⁸ In chick embryos, the relative teratogenic potencies (Table 1)^{95,117,118} also correlate with retinoid binding affinity for chick limb bud CRABP.¹⁵⁵ Chick limb bud CRABP is a specific, saturable cytoplasmic protein for all-*trans*-RA (present at pmol/mg of cytosolic protein from embryonic stages 20 to 35) with a dissociation constant (Kd) of 140 to 280 nM.¹⁵⁵ Binding to embryonic CRABP is specific for retinoids with an acidic polar terminus although some affinity was demonstrated for retinal. Retinol, retinyl palmitate, retinyl acetate, and the esters Ro 10-9359 and Ro 13-6298 failed to compete with all-*trans*-RA for binding to CRABP. 13-*cis*-RA was three to four times less effective at competing for CRABP binding than all-*trans*-RA, just as 13-*cis*-RA was less active than all-*trans*-RA in chick limb bud,¹⁶⁰ chick skin,⁹⁵ or in hamster⁸⁵ or mouse⁹⁷ embryos. Ro 13-7410, which is the most potent retinoidal teratogen yet reported in mammals⁹⁴ and is more effective than all-*trans*-RA in chick limb bud¹⁶¹ and skin⁹⁵ (Table 1), had a greater affinity for embryonic CRABP

than did all-*trans*-RA.¹⁵⁵ Maden and Summerbell¹⁵⁵ ranked retinoid analogs in order of binding affinity for embryonic chick CRABP as Ro 13-7410 > all-*trans*-RA > 13-*cis*-RA > Ro 10-1670 > retinal with no appreciable affinity for alcoholic or esterified congeners. These data implicate retinoid binding with CRABP as a step in the biochemical mechanism of retinoid teratogenesis. There appear to be multiple forms of CRABP which may be plasma membrane bound or located in the cytosol and whose activities may be controlled by enzymatic phosphorylation. When bound to all-*trans*-RA, phosphorylation of CRABP by plasma membrane, calcium phosphatidylserine-dependent protein kinase C (the phorbol ester receptor) is inhibited.¹⁵⁹ The interrelations between retinoid antagonism of tumor promotion and retinoid developmental toxicology, retinoid concentrative endocytosis, the stage-specific expression of CRABP in neural crest cells, the nuclear binding, and retinoid interaction in the control of gene expression can possibly be deduced from biochemical studies of retinoids, including determination of *in vitro* binding affinities for CRABP in avian¹⁵⁵ and mammalian embryonic cell populations.

How the interaction between teratogenic retinoids, CRABP, and the family of retinoid nuclear receptors leads to pathologic changes in germ layers and their derivatives is not at all clear. Pharmacologic actions in embryonal carcinoma cells and embryonic fibroblasts, while not necessarily indicative of retinoid effects in normal early undifferentiated embryonic cells, have provided some insight on the cellular mechanisms of retinoid action. Not only does all-*trans*-RA induce neural,¹⁶² muscle,¹⁶³ or endodermal¹⁶⁴ differentiation of pluripotent embryonal cells, retinoid concentration gradients appear to determine the type of cell differentiation achieved.¹⁶⁵ Embryonic carcinoma cells with reduced CRABP levels are refractory to all-*trans*-RA;¹⁶⁶ retinoid activity via CRABP binding appears to induce maturation of embryonal and fetal cells.¹⁶⁷ In virus-infected mouse embryo fibroblasts, all-*trans*-RA acts at the level of transcription such that there is *de novo* synthesis of a novel protein, which, in turn, acts to control expression of the inserted viral genome.¹⁶⁸ In mouse teratocarcinoma cells, all-*trans*-RA controls the induction and rate of gene transcription and the expression of those DNA sequences corresponding to low-abundance mRNAs.¹⁶⁴ Retinoic acid treatment of embryonal carcinoma cells also increases cytosolic and plasma membrane protein kinase (cAMP-PK) such that the increased activity sensitizes cells to elevated cyclic nucleotide concentrations resulting in decreased rates of growth.¹⁶⁹ Anderson et al.¹⁶⁹ suggested that it is rapidly growing cells which "may require prior treatment with retinoids to sensitize the cells to cyclic AMP modulation of growth and development". Changes in the activity and subcellular distribution of cAMP-dependent protein kinases and Ca²⁺, phospholipid-dependent protein kinase C (PK-C) in mouse embryonal carcinoma cells from plasma membrane to cytosol by holo-CRABP¹⁶⁹ and/or all-*trans*-RA^{169,170} have been reported. Changes in the relative ratios of cAMP-PK regulatory subunits may be one of the early events induced by retinoids that sensitize cells to cAMP. Ligand-free CRABP is phosphorylated by PK-C, whereas holo-cRABP reportedly inhibits its own phosphorylation; control of protein phosphorylation and inhibition of PK-C activity appear to be early and related events in retinoid control of cell differentiation.¹⁷¹ Retinoids also induce ornithine decarboxylase (ODC) activity and enhance binding of epidermal growth factor (EGF) via increased numbers of EGF receptors (without altering receptor affinity) in rat embryo¹⁷² and other¹⁷³ cells, but the presence of CRABP is apparently unrelated to these aspects of retinoid activity.^{172,173} Therefore, retinoid effects on EGF and ODC activities presumably are not initial changes integral to retinoid-induced teratogenesis as data published to date point towards retinoid interaction with CRABP as a step in retinoid-induced terata. Others^{174,176} have suggested that retinoid modulation of cellular, surface and secreted glycoconjugates is related to differentiation of embryonal carcinoma and other cells, but the conformationally restricted retinoids (e.g., Ro 13-7410, Ro 13-6298), which are among the most potent retinoidal teratogens yet reported,⁹⁴ do not participate in retinyl phosphate mannose control

of differentiation.¹⁷⁷ Thus, retinoid participation in sugar transfer reactions cannot be counted among the first events in retinoid teratogenesis.⁹⁴

IV. THERAPEUTIC AND CHEMOPREVENTIVE ACTIVITIES

As with any pharmacologic intervention, the objective of retinoid therapy assumes that the therapeutic ratio is such that toxicity will be either of only minor concern or that the benefits outweigh the risks. The nature of the conditions amenable to retinoid treatment dictate that protracted administration is necessary. The retinoids presently available for clinical use possess such narrow therapeutic ratios, especially regarding developmental toxicity, that their use in dermatology and cancer chemoprevention is limited and patients must be informed of and carefully monitored for untoward side effects. Many of these limitations can potentially be overcome by synthesis of congeners with decreased chronic toxicities and reduced teratogenic potential, while maintaining therapeutic efficacy. Depending upon individual compound structure, at least two classes of experimental retinoids, the retinylidene 1,3-diketones and the retinamides, have shown progress in this direction. By comparing the retinoids tested for teratogenic activity (Table 1) with their activities in three different *in vitro* systems designed to assess their intrinsic potential as chemopreventive agents (Table 2) and in light of the animal chemoprevention bioassay results, it is possible to draw preliminary conclusions about promising directions in retinoid pharmacology.

In addition to clinical trials and cancer chemoprevention studies in animals, a number of *in vitro* assays including the hamster tracheal organ culture,^{178,179} mouse mammary gland culture,¹⁸⁰ inhibition of phorbol ester-induced epidermal ODC activity,¹⁰⁹ and induction of differentiation in HL-60 (promyelocytic leukemia) cells¹⁸¹ have been used to identify retinoids with potential antineoplastic activity. Retinoid inhibitory activity in lactogenic hormone-induced lobuloaveolar differentiation of cultured mouse mammary tissue¹⁸⁰ and the published EC₅₀ values for retinoid-induced differentiation in promyelocytic leukemia cells¹⁸¹ follow the general trends of retinoid intrinsic activity in control of epidermal differentiation as measured in the hamster tracheal organ culture.^{178,179} Furthermore, Kistler¹⁸⁰ observed that the structure-activity relationships of retinoids in mouse mammary gland culture paralleled those in fetal rat bone culture,¹⁰⁰ cultured chick foot skin,¹¹⁸ and in the mouse papilloma assay.^{182,183} The retinoid ED₅₀ values in hamster tracheal organ culture (Table 2) can be compared with their TD₅₀ values in hamster teratology studies (Table 1). Leavitt and Mass¹⁸⁴ classified the retinoids (which have a potency range over five orders of magnitude) in the hamster tracheal organ culture into 40 "more active" and 27 "less active" compounds. Except in the cases of selected retinamides and dimedones, retinoids that were highly active in the hamster tracheal organ culture (Table 2) were also potent teratogens in the intact animal (Table 1). In each case, it is useful to compare the potency of novel retinoids to that of a standard compound, in the present case all-*trans*-RA, where it is of intermediate activity in each system. There are marked differences in activity depending on retinoid structure using *in vitro* test systems; for instance, the (*E*)-norbornenyl retinoid showed only moderate inhibition of phorbol ester-induced ODC activity in mouse epidermis as compared to all-*trans*-RA, but it was much less active than all-*trans*-RA in reversal of keratinization in the hamster tracheal organ culture, having only 9% of the activity of all-*trans*-RA at 10⁻⁹ M.¹⁸¹ The norbornenyl retinoid was, however, of approximately the same teratogenic potency as all-*trans*-RA (Table 1). In addition, retinoids that showed high intrinsic activity in the hamster tracheal organ culture also possessed high binding affinity for CRABP,¹⁵⁸ just as similar correlations could be drawn for embryonic CRABP affinity¹⁵⁵ and the activity of retinoids in chick tissue (Table 1). Leavitt and Mass¹⁸⁴ commented that in the hamster tracheal organ culture, an intact, conjugated alkene, alkyne, or aromatic system extending from the retinoid polar terminus to the β-cyclogeranylidene ring was associated with high intrinsic activity.

TABLE 2
Retinoid Activity *in vitro*

Common name	Polar terminus	Effective dose		
		Hamster tracheal organ culture ^a (nM)	Fetal rat bone culture ^b (nM)	Mouse mammary gland culture ^c (nM)
all-trans-Retinoic acid	COOH	0.03	1200	1000
all-trans-Retinol	CH ₂ OH	0.70	3000	1000
all-trans-Retinal	CHO	0.30	3000	—
Retinylidene acetylacetone	C ₅ H ₆ O ₂	2.0	—	—
Retinylidene cyclopentane-dione	C ₅ H ₄ O ₂	>10 (Inactive)	—	—
Retinylidene dimedone	C ₆ H ₂ O ₂ (CH ₃) ₂	0.2	—	—
Retinylidene methoxyphenylcyclohexanediene	C ₆ H ₅ O ₂ (C ₆ H ₄)OCH ₃	0.1	—	—
Retinylidene cyclooctane-dione	C ₈ H ₁₀ O ₂	0.2	—	—
Retinylidene methylnitronate	CH ₃ NO	<1.0	—	—
all-trans-N-Ethylretinamide	CONHC ₂ H ₅	<1.0	>20,000	—
13-cis-N-Ethylretinamide	CONHC ₂ H ₅	0.3	>20,000	—
13-cis-N-(4-Hydroxy)phenylretinamide	CONHC ₆ H ₄ OH	<1.0	—	—
13-cis-N-2-(Hydroxy)ethylretinamide	CONHC ₂ H ₄ OH	0.3	—	—
13-cis-Retinoic acid	COOH	0.03	2000	100
7,8-Dihydroretinoic acid	COOH	10	—	—
9,10-Dihydroretinoic acid	COOH	100	—	—
7,8-Dehydroretinoic acid	COOH	0.5	—	—
C ₁₅ Acid	COOH	>1000 (Inactive)	—	—
Abscisic acid	COOH	>10 (Inactive)	—	—
Etretinate (Ro 10-9359)	COOC ₂ H ₅	20	(Inactive)	—
Etretin (Ro 10-1670)	COOH	5.0	2000	1000
Motretinid (Ro 11-1430)	CONHC ₂ H ₅	—	(Inactive)	>10,000
Ro 11-4768	CO ₂ C ₂ H ₅	>1000 (Inactive)	—	—
Ro 8-7699	COOH	—	500	—
2-Norbornenyl ethyl ester	CO ₂ C ₂ H ₅	>10 (Inactive)	—	—
Ro 13-6307	COOH	—	35	1.0
Ro 13-2389	CO ₂ C ₂ H ₅	—	400	—
Ro 13-4306	COOH	—	200	—
Aryltreine ester	CO ₂ C ₂ H ₅	>20	—	—
Ro 13-7410	COOH	0.01	6	0.1
Ro 13-6298	CO ₂ C ₂ H ₅	0.01	7	0.1
Ro 13-8320	CH ₂ OH	—	600	—
Ro 13-9272	CH ₃	—	(Inactive)	—

^a ED₅₀ data summarized from Newton et al.^{178,179}

^b ED₄₀ data summarized from Kistler.¹⁰⁰

^c Lowest inhibiting concentration data summarized from Kistler¹⁸⁰

By extension, it is likely not only that the conjugated system is associated with high binding affinity for adult hamster tracheal CRABP, but since retinoids without the conjugated system possess only limited, if any, teratogenic activity in intact hamsters,⁹³ a conjugated system may also confer increased binding affinity for hamster embryonic CRABP as well. Leavitt and Mass¹⁸⁴ suggested that the conjugated system may be related to charge transport through the retinoid if it were embedded in a membrane.

There are numerous exceptions to the above paradigm when one considers the retinoid amide, ester, or ether congeners. For example, retinylidene acetylacetone, the cyclohexane-

1,3-dione derivatives, and the cyclooctane diketone were all active in the *in vitro* control of epidermal differentiation in the hamster tracheal organ culture, but the all-*trans*-2-retinylidene-1,3-cyclopentanedione was essentially inactive (Table 2). All of the retinylidene 1,3-diketones were essentially without teratogenic activity in intact hamsters (Table 1). The classification of retinoids as more or less active may actually be more a reflection of their relative susceptibility to hydrolysis, oxidation, and other pathways of biotransformation and permeability rather than an indication of their direct interaction with CRABP or other receptors in each test system.

Hill and Grubbs¹⁸⁵ summarized retinoid efficacy determination efforts to 1982 in experimental mammalian carcinogenesis. The retinamides and the retinylidene 1,3-diketones have also shown reduced acute and chronic toxicities and reduced teratogenic potential (Table 1) as compared to their free acid congeners. For example, all-*trans*-*N*-(4-hydroxyphenyl)retinamide (4-HPR) antagonized the proliferation of rat mammary gland cells and inhibited mammary carcinogenesis induced by *N*-nitroso-*N*-methylurea.¹⁸⁶ McCormick et al.¹⁸⁷ confirmed the inhibition of experimental mammary and urinary tract carcinogenesis by 4-HPR. Dietary 4-HPR was associated with decreased ductal branching and decreased end bud proliferation in rat mammary gland relative to control,¹⁸⁸ and treatment with 4-HPR inhibited prolactin-induced differentiation of cultured mammary tissue.¹⁸⁹ Welsch et al.¹⁹⁰ observed a significant reduction in the incidence of spontaneous mammary carcinomas in C3H mice after dietary 4-HPR. Dietary 4-HPR also had significant chemopreventive activity against *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced mouse urinary bladder carcinogenesis¹⁹¹ and it reduced the incidence of pancreatic adenoma in female (but not in male) Golden Syrian hamsters.¹⁹² The 13-*cis* isomer of 4-HPR was of equal activity toward inhibition of mouse urinary bladder carcinogenesis, but the chronic toxicity of 13-*cis*-4-HPR was reduced as compared to the all-*trans* isomer.¹⁹¹ Ovariectomy enhanced the chemopreventive response associated with 4-HPR in mammary carcinogenesis,^{187,193} and dietary 4-HPR inhibited dimethybenzanthracene-induced and phorbol ester-promoted mouse skin carcinogenesis.¹⁹⁴ Additionally, 4-HPR failed to show the promoting activity ascribed to all-*trans*-RA,¹⁹⁴ and it was not metabolized *in vivo* to detectable concentrations of all-*trans*-RA.¹⁹⁵

Other retinamides have also shown evidence for *in situ* chemopreventive activity. Thompson et al.¹⁹⁶ found that all-*trans*-*N*-ethylretinamide and all-*trans*-(2-hydroxyethyl)retinamide were as effective as 13-*cis*-RA towards inhibition of rat and mouse urinary bladder carcinogenesis. Moon et al.¹⁹¹ confirmed that observation for *N*-(2-hydroxyethyl)retinamide and extended it to the *N*-(2-hydroxypropyl)retinamide, the 13-*cis*-*N*-(2-hydroxyethyl)retinamide and the 13-*cis*-*N*-(1*H*-tetrazol-5-yl)retinamide. Substitution of the hydroxyl at position three rather than position two or substitution of the hydroxyl groups at positions two and three eliminated chemopreventive activity. Likewise, *N*-butylretinamides were without chemopreventive activity. Hicks et al.¹⁹⁷ reported a similar association in rats for dietary all-*trans*-*N*-ethylretinamide in addition to increased survival from urinary bladder carcinogenesis. However, potentiation of respiratory tract carcinogenesis occurred after retinamide treatment occurred; intratracheal instillation of the direct-acting carcinogen, *N*-methyl-*N*-nitrosourea, in male hamsters given dietary all-*trans*-*N*-ethylretinamide or *N*-(2-hydroxyethyl)retinamide increased the incidence of tracheal epithelial neoplasms and carcinomas as contrasted with carcinogen treatment alone.¹⁹⁸ Longnecker et al.¹⁹⁹ reported a significant reduction in the incidence of azaserine-induced pancreatic carcinoma in male and female Wistar/Lewis rats after dietary *N*-2-(hydroxyethyl)retinamide. Dietary *N*-(4-pivaloyloxyphenyl)retinamide (PVPR) or *N*-(2,3-dihydroxypropyl)retinamide also reduced the incidence of azaserine-induced pancreatic carcinoma in male and female Lewis rats, but their *N*-(2-hydroxypropyl) and *N*-(3-hydroxypropyl) congeners were less effective.²⁰⁰ Nevertheless, the PVPR and *N*-(2-hydroxypropyl)retinamide also apparently served as promoters of female rat hepatocar-

cinogenesis and displayed marked testicular toxicities.²⁰⁰ Inhibition of chemical carcinogen-induced forestomach dysplasia in Swiss-Webster mice given all-*trans*-4-(aminosulphonylphenyl)retinamide has been observed.²⁰¹ The pivaloyl ester derivative of 4-HPR was relatively resistant to hydrolysis and PVPR and 4-HPR were of approximately equivalent activity in the hamster tracheal organ culture;¹⁹⁵ PVPR may act as a parent form of 4-HPR in the intact animal, but in any case, direct interaction with CRABP cannot be held responsible for retinamide pharmacologic activities.

Birt et al.¹⁹² found that all-*trans*-N-(2-hydroxyethyl)retinamide inhibited the development of *N*-nitrosobis(2-oxopropyl)amine (BOP)-induced liver adenomas in female hamsters, but Longnecker et al.²⁰³ failed to observe significant reductions in the incidence of BOP-induced pancreatic carcinomas after retinamide feeding. The incidence of BOP-induced pancreatic carcinoma in Syrian hamsters was not influenced by dietary all-*trans*-N-ethylretinamide or all-*trans*-N-(2-hydroxyethyl)retinamide, and dietary *N*-(2-hydroxyethyl)retinamide actually increased the numbers of pancreatic adenomas in males.¹⁹² In BOP-initiated male hamsters, however, dietary PVPR reduced the incidence of pancreatic carcinoma.²⁰³ In summary, the size of the *N*-alkyl residue determined, in the main, the potential chemopreventive activity of retinamides in bladder carcinogenesis: the larger the terminal group, the less the chemopreventive potential.²⁰⁴ The hydroxypropyl moiety of the retinamide was associated with enhanced testicular toxicity.²⁰⁰ It is not clear at present whether inhibition of carcinogenesis and the enhanced testicular toxicities are due to direct effects of the retinamides or to indirect effects including disturbances of endogenous hormonal or metabolic processes. Arguments for a direct effect come from studies where there was no effect of 4-HPR on insulin, prolactin, aldosterone, or hydrocortisone-induced mouse mammary alveolar proliferation, while, at the same time suppressing, spontaneous preneoplastic alveolar lesions.²⁰³ Hicks and Turton²⁰⁴ discussed the extenuating factors in 4-HPR and 13-*cis*-*N*-ethyl retinamide chemoprevention studies.

Another retinamide, motretinid (Ro 11-1430) (Table 1), possessed a tenfold more favorable therapeutic ratio in the mouse papilloma assay than all-*trans*-RA,²⁰⁵ and it was active at the same concentrations as its ethyl ester congener, etretinate, in the control of epithelial cell differentiation as measured in the hamster tracheal organ culture assay.¹⁷⁹ Motretinid is available clinically in Europe²⁰⁸ and topical application of a 0.1% solution in acne vulgaris gave positive results with less skin irritation than with topical all-*trans*-RA.²⁰⁹ Although motretinid inhibited radiation-induced oncogenic transformation in cultured C3H mouse fibroblasts,²¹⁰ oral motretinid failed to inhibit mammary or bladder cancer in rats.²⁰⁴ Motretinid treatment induced regression of carcinomas and papillomas in mice²¹¹ and dietary or parenteral (10 to 80 mg/kg/d) motretinid was less toxic than etretin (Ro 10-1670) or etretinate.²¹² Motretinid inhibited the growth of transplantable chondrosarcoma and caused regression of established tumors in F344 female rats.²¹² Since motretinid binding to CRABP cannot be responsible for the antineoplastic and dermatologic actions, studies on other possible mechanisms including retinamide-induced immunologic effects^{213,214} have been carried out.

Certain retinylidene diketones have also been associated with significant chemopreventive activities. Longnecker and associates¹⁹⁹ found significant inhibition of pancreatic carcinogenesis by dietary retinylidene dimedone in azaserine-treated male Wistar/Lewis rats; since the retinoid was given after azaserine initiation, and the ratio of invasive to localized pancreatic carcinomas was decreased after retinoid treatment, the retinoid apparently retarded the progression of neoplasia. The chemopreventive activity of dietary retinylidene dimedone in the pancreas of BOP-treated male hamsters was subsequently confirmed.²⁰³ Retinylidene dimedone was also associated with significant suppression of 7,12-dimethylbenz[a]anthracene (DMBA)-induced neoplastic-transformation in cultured mouse mammary glands.²¹⁵ However, retinylidene dimedone was ineffective in suppressing mammary neo-

plasia when direct-acting carcinogens like *N*-hydroxy-2-fluorenylacetamide or benzpyrene-diol epoxide were studied,²¹⁶ and it was effective only when administered after DMBA.^{215,216} When Leavitt and Mass¹⁸⁴ categorized 67 retinoids for the ability to reverse squamous metaplasia and keratinization in hamster tracheal epithelium, among those classified as "more active" were retinylidene dimedone, all-*trans*-2-retinylidene-1,3-cyclooctanedione, all-*trans*-2-retinylidene-1,3-cycloheptanediol, and all-*trans*-2-retinylidene-1,3-cyclononanediol.

Certain retinamides and retinylidene diketones have, under specific circumstances, demonstrated significant potential for cancer chemoprevention. It is not clear at present whether the specific carcinogens tested, the dosages utilized, or the protocols studied were appropriate for a rigorous evaluation of their true chemopreventive potential. Only after studies on species-related retinoid pharmacokinetics, tissue distribution, and metabolic fate have come to light can experiments be designed to test clearly the *in vivo* potential of retinoids in cancer chemoprevention. The separation of desirable dermatotherapeutic and chemopreventive activities from toxicity represents a desirable goal in retinoid pharmacology and the decreased teratogenic and toxicologic properties of the retinamides and retinylidene diketones represent a step in that direction. Of course, the retinamides and retinylidene diketones are not without their own dose-dependent toxicities; dietary all-*trans*-*N*-ethylretinamide or all-*trans*-*N*-(2-hydroxyethyl)retinamide induced atrophy of the testicular germinal epithelium in hamsters, but the levels of the retinamides associated with testicular toxicity were much greater than that of dietary 13-*cis*-RA associated with a similar response.²¹⁷ A useful approach may be to combine the conformational restriction characteristic of Ro 13-7410 and congeners, which is associated with marked biologic activity, with a selected diketone or retinamide polar terminus associated with decreased toxicity, yet which retains the chemopreventive potential.

V. SUMMARY AND CONCLUSIONS

Vitamin A deficiency induces congenital malformations in many of the same organ systems as that observed after megadose vitamin A exposure or after synthetic retinoid treatment. Retinoids readily cross the placenta and there are data that suggest that differential placental permeability plays an important role in differential teratogenic potencies, whereas other data indicate it is primarily retinoid affinity for embryonic CRABP which determines teratogenic potency. Retinoids apparently act as diffusible morphogens capable of altering embryonic cell migration, proliferation, and differentiation, functioning in control of normal as well as abnormal embryogenesis.

The main stumbling block to practical clinical utility of presently available retinoids is their toxicity.¹ Retinoids can be potent human and animal embryotoxins, but as with other aspects of retinoid pharmacologic activities, there are definite structure-activity relationships that determine retinoid teratogenic potential. The contribution of naturally occurring retinoids, even when megadose vitamin A supplements are considered, to the load of human embryonic morbidity and mortality is far from clear. Retinoids do not interfere with the actions of oral contraceptive steroids²¹⁸ and, in principle, human retinoid teratogenesis should not occur as long as patients adhere with strict contraception. But as was the case with Accutane® where approximately one third of the affected pregnancies occurred in spite of contraception,⁷⁴ it is probable that instances of human reproductive failure will occur in the U.S. as a result of the recent approval of etretinate,⁷⁶ if for no other reasons than steroids are not 100% reliable and patients are otherwise far from reliable in avoiding pregnancy. Etretinate treatment carries with it the unique risk of prolonged high circulating concentrations, even after discontinuation of therapy because of the extended elimination half-life,⁷⁶ and reliable patient contraception from one to several years after cessation of etretinate therapy is problematic.¹

Many of the toxicological problems associated with the retinoid protocol necessary for

dermatologic and chemoprevention studies can be addressed by synthesis of retinoids with enhanced activity and reduced toxicity, as exemplified by the decreased bone toxicity of the sulfur-containing analogs.¹¹⁴ Unfortunately, to date there has been considerably more success with identification of the retinoid structural features responsible for increased pharmacologic efficacy¹⁸⁴ than with identification of features associated with decreased toxicity. Among retinoids with reduced toxicities, the retinamides²¹⁹⁻²²¹ and retinylidene 1,3-diketones²²⁰ show promise (possibly acting as antipromoters²¹⁹), but these retinoids are not consistently efficacious in cancer chemoprevention.²²²⁻²²⁵ The development of effective yet nonteratogenic retinoids remains a priority in retinoid pharmacology. It is possible to mitigate the teratogenic activity to some extent by adjusting the dose and/or route of administration (e.g., topical) such that a toxicologically significant concentration of the parent retinoid or teratogenic metabolite does not reach the conceptus. Should retinoids prove to operate by the same molecular mechanism in all cell systems, it will be extremely difficult to design systemic retinoids with the desired profile of activities. Only acidic retinoids bind to CRABP and it is only those forms that have embryotoxic potential. Other studies²²⁶ show that cellular differentiation can be correlated with stimulation of cAMP-dependent or phospholipid-sensitive protein kinase activities. As the role of these enzymes in retinoid modulation of differentiation becomes clear, particularly with regard to their interaction with CRABP,^{159,169,170} additional opportunities to separate retinoid toxicity from efficacy may occur. The true separation of these activities and the rational design of chemoprevention studies to explain the apparent inconsistencies in the experimental mammalian carcinogenesis results await elucidation of the molecular mechanisms of retinoid action.

Perhaps the major lesson to be learned from the discussion of retinoid teratogenesis is that no matter how oblique the types of animal terata or changes in fetal development first appear when attempting to extrapolate those data to humans, there is little basis for including or excluding FDA Phase II animal reproduction data in the estimation of the human response to xenobiotics without companion studies on the metabolic fate and pharmacokinetic parameters of the agent in both the test species and humans.^{75,227} It is common practice in regulatory toxicology to apply a safety factor to the species (usually rat, mouse, or rabbit) associated with the lowest observed effect level to calculate acceptable human daily intakes. Without data on the uptake and disposition of the agent administered to animals (usually by the same route as that intended for humans), it is not possible to decide with any precision which, if any, of the test species more closely resembles the human.

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Chapter 22

**CARCINOGENESIS INHIBITORY PROPERTIES OF RETINOIDS:
A QSAR-MTD ANALYSIS****I. Niculescu-Duvăz, Z. Simon, and N. Voiculețz****TABLE OF CONTENTS**

I.	Introduction	576
II.	Methodology of the QSAR-MTD Approach	576
III.	QSAR-MTD Analysis of Retinoids	579
	A. Construction of the Hypermolecule	583
	B. Mapping of the Receptor Site	587
	1. Series A	587
	2. Series B	588
	3. Series C	589
	4. Final Receptor Map	590
IV.	Analysis of Conformationally Restricted Retinoids.....	591
V.	Analysis of the Unified Set of Retinoids.....	594
	A. The Cyclic Moiety Series	596
	B. The Polyenic Chain Series	596
	C. The Terminal Group Series	597
	D. Unified Set Receptor Map	597
VI.	Correlation of Our Data with the Possible Mechanisms of Action of Retinoids.....	598
VII.	Summary and Conclusions	603
	Acknowledgments	604
	References	604

I. INTRODUCTION

Chemoprevention is a recent and rapidly expanding area in contemporary cancer research that arose from chemical carcinogenesis studies indicating the ability of several classes of compounds to suppress the *in vivo* development of chemically induced tumors in experimental animals.^{1,2} These compounds were defined as carcinogenesis inhibitors and were classified according to their possible mechanism of action into either blocking or suppressive agents^{3,4} (see Table 1). However, in spite of recent advances in the study of various types of such inhibitors, the molecular mechanisms involved in carcinogenesis suppression remain largely unknown.

Both types of inhibitors have been studied in our laboratory in order to elucidate some aspects of their mechanism of action.⁵⁻⁸ In this respect, the retinoids appear as a particularly interesting family of compounds because they are able to suppress the carcinogenesis process both *in vitro*^{9,10} and *in vivo*,^{11,12} exerting effects on the differentiation of certain fully transformed, invasive, neoplastic cells.^{13,14} Several reviews summarize the extensive work carried out in this area.^{12,15,16} It was demonstrated that retinoic acid (RA) failed to protect DNA *in vitro* against carcinogens^{17,18} but acted as a typical suppressive agent by inhibiting the transformation of several types of initiated cells.^{12,15,16} The mechanism(s) by which retinoids promote differentiation or act as suppressive agents is not clear (see Section VI). Therefore, it is certainly of interest to develop structure-activity relationships for large series of retinoids, based on a standard reproducible biological test such as the reversal of keratinization of hamster tracheal cells in organ culture (TOC assay) in order to compare the results thus obtained with experimental data reported for other cell systems or with theoretical models of differentiation. This information is also useful for the design of more effective and less toxic retinoids.

We undertook the theoretical analysis of this class of compounds for the following reasons:

1. The great number of available structures, as well as a reproducible and sensitive *in vitro* biological standard test that measures the intrinsic ability of retinoids to control epithelial cell differentiation (TOC assay, Section VI).
2. The very few similar approaches for retinoids reported in the literature.¹⁹⁻²³
3. The potential of natural and synthetic retinoids for cancer prevention, and perhaps also for therapy.^{14,24,25}

II. METHODOLOGY OF THE QSAR-MTD APPROACH

Among the procedures used for quantitative structure-activity relationship (QSAR) studies (for example, the Free-Wilson method, quantum mechanical methods, pattern recognition, etc.), we chose the linear free energy procedure of Hansch^{26,27} to investigate retinoids. This technique has generated the most widespread interest in this area because it is relatively easy to perform and because statistically based methods can be used to evaluate the significance of the results obtained. The advantage offered by three-dimensional computer modeling of molecules was also taken into account²⁸ in our work.

Biological activity often arises as a consequence of interaction with a biological receptor. The parameters that govern the transport and the receptor-site interaction for a given compound are factored into structural, electronic and physicochemical (i.e., pK_a, molar refractivity, hydrophobicity) components. Each contribution is modeled with a substituent constant, and the activities of a set of congeneric compounds are fitted with a multilinear regression equation of the form:

$$\log (1/C) = A + B \log P \text{ (or } \pi \text{)} + C\sigma + DE_s \quad (1)$$

TABLE 1
Inhibitors of Chemical Carcinogenesis

No.	Mechanism of action	Effect	Type of inhibitors (classes of compounds)
1	Inhibition of the initiation step	Decrease the concentration of carcinogen-DNA adducts	Blocking agents, anti-initiators (phenolic antioxidants, indoles, flavones, coumarins, SH-containing compounds, etc.)
2	Inhibition of the promotion step	Prevent the malignant transformation of initiated cells	Suppressive agents, antipromotors (retinoids, prostaglandin inhibitors, etc.)

where C = the concentration required to produce a standard biological response (moles per liter, M); P = the octanol/water partition coefficient; π = substituent constants for lipophilicity; σ = the electronic parameter or Hammett constants; E_s = the steric substituent constants of Taft; A, B, C, D = coefficients of regression.

Several factors prompted us to choose the Hansch methodology for the QSAR analysis of retinoids. The first one is that our set of retinoid congeners fulfilled the requirements needed by this procedure to obtain significant correlations, namely:

1. The compounds were structural analogs, probably acting by the same mechanism (see Section VI).
2. Reliable physicochemical substituent parameters were available for all compounds.
3. The number of compounds was large enough so that statistical methods were applicable.
4. The biological assay was reproducible.

As we previously mentioned, requirements (2) to (4) were fulfilled. Whether or not all analyzed retinoids act by the same mechanism remains an unanswered question. However, this assumption appears reasonable in the case of the TOC assay (see Section VI).

The second reason for our choice of the Hansch procedure was that a steric parameter, namely Minimal Topological Differences (MTD), was developed by one of us,^{29,30} that allowed a much better estimation of the shape and the specific structural features of a molecule with respect to the originally proposed E_s parameter (see Equation 1). The former parameters represent the approximate measure of the steric misfit between a given receptor site and the effector molecule. The space occupied within a receptor site by a series of molecules is represented by the hypermolecule, a topological network, whose vertices correspond to the approximate position of nonhydrogen atoms in the molecules. MTD is defined, for every molecule (M_i) in a series of N related compounds, with respect to a receptor map (S) describing the receptor cavity, walls and external region, as the number of cavity vertices not occupied by the considered molecule (M_i) plus the number of wall vertices occupied by this molecule (see Equation 2 and footnote).

This method is comprised of the following steps:

1. All N molecules in the series are superimposed atom per atom (neglecting hydrogens), thus obtaining an atomic lattice having n vertices called a Hypermolecule (H)*
2. The constitution of each molecule (M_i) is described by an occupancy (x_{ij}) vector (Kronecker delta) with x_{ij} being 1 (1.5 or 2.0) if the vertex j is occupied in M_i by a second, third, or fourth period atom, ** otherwise x_{ij} is 0.
3. By inspection of biological activities, an initial standard vector (corresponding to the S^o receptor map) is proposed; it is an n -dimensional vector in which each component

* Usually the most effective compound is chosen as the standard molecule.

** Elements Li to Ne constitute the second period of Mendeleev's table, Na to Ar the third, and K to Kr the fourth period.

has one of the three values -1 , 0 , or 1 . If the vertex j belongs to the receptor cavity (beneficial or effective vertex), $E_j = -1$; if the vertex j falls on the receptor wall (detrimental or noneffective vertex), $E_j = +1$; and, if it is situated in a sterically irrelevant region (irrelevant vertex), the $E_j = 0$. To obtain a reasonable map (without isolated effective vertices or corresponding to a fragmented receptor cavity—a very improbable situation), the connectivity condition for the cavity vertices, as in the SIBIS method,³² was introduced.

4. The method allows, by the variational procedure, the optimization of the initial guess standard vector (receptor map S^o) until a maximum correlation coefficient is obtained for Equation 3. In the optimization procedure one starts from the initial set, S^o of E_j -assignments and changes them, one by one, aiming at minimizing the sum Y of the squares of differences between the experimental (A_i) and calculated ($A_{i,calc}$) activities.

$$Y = \sum_{i=1}^N (A_i - A_{i,calc})^2 \quad (2)$$

The receptor map is considered to be optimized (S^*) if other singular E_j -changes do not further reduce Y .

5. The MTD for molecules M_i is calculated as:

$$MTD_i = s + \sum_{j=1}^M \epsilon_j x_{ij} \quad (3)$$

where s is the number of vertices with E_j equal to -1 in the optimized receptor map S^* and $\sum_{j=1}^M \epsilon_j x_{ij}$ represents the sum of effective and noneffective ($E_j = +1$) vertices occupied by the atoms (neglecting hydrogens) of the molecule M_j .* The correlation equation between the computed activity (A_{calc}) and the structure of molecule in the MTD-method is of the form:

$$A_{calc} = a - b \cdot MTD \quad (4)$$

where a, b are regression coefficients.

The optimized standard (receptor map) S^* enables the mapping of the receptor site of an enzyme or of a receptor protein, indicating the best molecular shape of the inhibitor.** A computer program has been written for this approach.³²

* For instance, let us assume that the optimized map, S^* , for a set of 50 molecules contains 25 vertices from which 10 vertices are effective ($E_j = -1$), 5 vertices are noneffective ($E_j = +1$), and 10 vertices are irrelevant ($E_j = 0$). Now, if we consider a molecule M_i from this set that possesses 15 vertices (nonhydrogen atoms) from which 5 vertices are effective, 5 vertices are noneffective, and 5 vertices are irrelevant, then the MTD value for this molecule calculated according to Equation 2 would be:

$$MTD_{Mi} = 10 + \sum [s(-1) + 5(+1) + 5(0)] = 10$$

The factor $[s(-1) + 5(+1) + 5(0)]$ represents the number of cavity vertices not occupied by the considered molecule M_i , and $\sum [(+1)x_{ij}]$ represents the number of wall vertices occupied by the same molecule.

** The optimized map S^* depends strongly upon the starting map, S^o . When super ion position is done, the most probable conformer for every molecule is considered. However, when several starting maps are equally possible, all their corresponding optimized maps were calculated. From these, the map giving the best correlation indices for Equation 2 is selected.

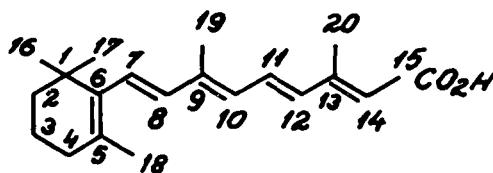


FIGURE 1. Numbering for retinoic acid. For structural comparison in the text, standard retinoid numbering has been used. Similar proton and carbon atoms in the aromatic analogs have been denoted by the subscript R.

For our purposes the biological parameter of ED₅₀ (*M*) in the TOC assay was used. The substituent constants for the lipophilicity were taken from the literature.^{33,34} Only π values were considered; a value of log P_{rel} of 0.0 was assigned to all-*trans*-RA. The use of π values instead of log P was justified by our aim to minimize the errors in the lipophilicity computation because for highly lipophilic molecules significant differences occur between log P_{calc} and log P_{exp}. We also checked whether or not this parametrization could affect the computational results, assuming for RA a log P_{calc} of 6.60 and for compound* **51** (Ro 13-7410) a log P_{calc} = 7.52. No significant differences for r (correlation index) and F (Fisher test) were obtained between the regression equations developed with the two sets of parameters [π , π^2 , and log P, (log P)²]. In some cases lipophilicities that were calculated only for the cyclic moiety and the middle polyenic side chain were used (π_{c+m}) rather than overall π .

Four additional parameters (indicator variables) were defined and used for the computation of correlation equations:

1. δ_1 (carboxylic group ionization), defined as 1 for an ionizable carboxylic group at pH 7.0 or as 0 otherwise, i.e. for esters, amides, etc.
2. δ_2 (degree of double bond saturation in the polyenic chain), defined as 1 if a double bond of the polyenic chain is saturated or as 0 for uninterrupted conjugation of the polyenic chain
3. δ_3 (possibility of the end group being transformed into a carboxylic group), defined as 1 for esters, aldehydes, primary and secondary amides, etc, or as 0 otherwise,
4. n_o (number of atoms acting as proton acceptors in hydrogen bonds at the polar terminal group level).

The correlational equations were developed according to the multilinear regression procedure using this methodology. The plus/minus value given for each coefficient represents the standard deviation.

To emphasize the contribution of the MTD parameter to the description of the biological activity, for each series of compounds pairs of regression analyses that were identical except for inclusion of the MTD parameter were calculated. The correlational indices of equations without this parameter were noted as r_o and F_o.

III. QSAR - MTD ANALYSIS OF RETINOIDS

The structures of the retinoids investigated in our first report⁵ were taken from the paper of Newton et al.²¹ on retinoid activity in the TOC assay. The series contains retinoids with different cyclic moieties (n = 19), various polyenic side chains (n = 11), and especially end group-modified derivatives of RA (n = 27). Their structures, as well as the main parameters used for analysis, are summarized in Table 2 (A,B,C,D,E).

* The numbering of compounds is that of Table 2.

TABLE 2A
Unified Set of Retinoid Congeners Used for QSAR Analysis (Series for Optimization)

No.	Structure, R ^{a,b}	$\text{R} \text{---} \text{CH}=\text{CH}_2 \text{---} \text{CH}=\text{CH}_2 \text{---} \text{CH}=\text{CH}_2 \text{---} \text{CO}_2\text{R}_1$					
		$\log \frac{1}{\text{ED}_{50}}$	δ_1	MTD ₁ ^c	MTD ₂ ^d	π	π_{c+m}
1		10.52	1	16	6	0.00	0.00
2	RA, R ₁ = CH ₃	9.52	0	16	6	0.38	0.00
3	RA, R ₁ = C ₂ H ₅	9.30	0	16	6	0.88	0.00
4		9.70	1	16	6	-0.87	-0.87
5		9.30	1	16	6	-1.71	-1.71
6		9.15	1	17	6	-1.34	-1.34
7		9.15	1	17	6	-1.73	-1.73
8		9.00	1	17	8	0.30	0.30
9		8.70	1	17	8	-1.38	-1.38
0		2	3	4	5	6	7
10	9, R ₁ = CH ₃	8.52	0	18	8	-1.00	-1.38
11		8.30	1	18	9	-0.29	-0.29
12	11, R ₁ = C ₂ H ₅	7.70	0	18	9	0.59	-0.29
13		7.70	0	18	9	-0.06	-0.94
14		8.00	1	18	9	0.00	0.00
15		8.00	1	19	9	-1.30	-1.30
16		6.52	1	19	9	-2.46	-2.46
17		6.00	1	19	9	-1.15	-1.15
18		6.00	1	19	9	-2.36	-2.36

TABLE 2A (continued)
Unified Set of Retinoid Congeners Used for QSAR Analysis (Series for Optimization)

No.	Structure, R ^{a,b}	$\log \frac{1}{ED_{50}}$	δ_1	MTD ₁ ^c	MTD ₂ ^d	π	π_{c+m}
19	 $R_1=CH_3$	6.70	0	19	8	-2.04	-2.42

36 ^a $R_1 = H$ when not otherwise stated.

37 ^b For all compounds $\delta_2 = 0$, $\delta_3 = 1$ and $n_o = 2$.

38 ^c Calculated for the Newton et al. series.²¹

39 ^d Calculated for the Dawson et al. series³⁶⁻³⁸ and for the unified sample of retinoids.

TABLE 2B

No.	Structure, R ^a	$\log \frac{1}{ED_{50}} (M)$	δ_1	δ_2	δ_3	n_o	MTD ₁	MTD ₂	π	π_{c+m}
20		10.52	0	0	1	2	16	6	0.00	0.00
21		9.70	0	0	1	2	16	6	0.00	0.00
22		9.30	0	0	1	2	16	7	-0.23	-0.30
23		9.00	0	0	1	2	17	7	-0.23	-0.23
24		8.52	0	0	1	2	17	9	0.82	0.82
25		8.00	1	0	1	2	16	6	0.30	0.30
26		8.00	0	0	1	2	17	9	-1.18	-1.18
27		7.00	1	0	1	2	16	6	0.30	0.30
28		6.00	0	0	1	2	19	12	-2.00	-2.00
29		9.70	1	0	1	2	—	7	1.40	1.40
30		7.50	1	0	1	2	—	9	0.26	0.26

TABLE 2B (continued)

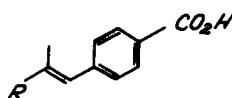
No.	Structure, R ^a	$\log \frac{1}{ED_{50}} (M)$	δ_1	δ_2	δ_3	n _o	MTD ₁	MTD ₂	π	π_{c+m}
31		7.50	1	0	1	2	—	9	0.03	0.03
32		9.7	0	0	1	2	—	7	0.65	0.23
33		9.52	0	0	1	2	—	7	1.45	0.57
34		9.52	1	1	1	2	—	7	-0.07	-0.07
35		9.7	1	0	1	2	—	7	-0.29	-0.29
36		9.40	1	0	1	2	—	8	0.39	0.39
37		9.00	0	0	1	2	—	7	0.13	0.13
38		9.70	1	0	1	2	—	7	-0.29	-0.29
39		8.00	1	0	1	2	—	7	-1.19	-1.19
40		10.00	1	0	1	2	—	7	-0.58	-0.58
41		10.00	1	0	1	2	—	7	-0.36	-0.36
42		9.00	0	0	0	0	—	7	0.04	0.39
43		8.00	0	0	0	0	—	8	0.04	0.39
44		8.22	0	0	0	0	—	8	0.04	0.39

TABLE 2B (continued)

No.	Structure, R*	$\log \frac{1}{ED_{50}} (M)$	δ_1	δ_2	δ_3	n_o	MTD ₁	MTD ₂	π	π_{c+m}
45		8.00	0	0	0	0	—	7	-2.33	0.39
46		8.00	0	0	0	0	—	7	-2.33	0.39

* The same explanatory notes as for Table 2A.

TABLE 2C



No.	Structure, R*	$\log \frac{1}{ED_{50}}$	δ_1	δ_3	n_o	MTD ₁	MTD ₂	π	π_{c+m}
47		10.52	1	1	2	—	6	0.23	0.23
48		9.70	1	1	2	—	6	-1.25	-1.25
49		10.30	1	1	2	—	6	-0.47	-0.47
50		9.00	1	1	2	—	7	-0.41	-0.41
51		11.0	1	1	2	15	6	0.95	0.95

* For all compounds $\delta_2 = 0$.

The retinoids were divided for computational purposes into two series of congeners: (a) 53 compounds for MTD computation and development of the QSAR (see Table 2 A,B,C,D,E), and (b) 15 compounds for the test series (see Table 3 A,B,C,D,E).

A. CONSTRUCTION OF THE HYPERMOLECULE

Models of all molecules were constructed using Dreiding stereomodels. The most active compound of the series, **51** (Ro 13-7410) with a rather rigid structure, was chosen as the basic compound for the superposition procedure. We considered most six-membered rings as superimposable, except for those of compounds **9**, **10**, and **14**. Larger variation appeared in the position of the two methyl groups at C₁.

The polyenic chain was assumed to be sufficiently flexible to allow rotations along the single bonds, which would have sufficiently low energy barriers to make such isomerizations

TABLE 2D

No.	Structure, R ^a	$\log \frac{1}{ED_{50}}$	δ_3	n _o	MTD ₁	MTD ₂	π	π_{c+m}
0	1	2	3	4	5	6	7	8
52	CHCH ₃	8.70	0	0	17	7	0.68	0.00
53	CHCH ₂ CH ₃	8.00	0	0	16	6	1.04	0.00
54	CHCOCH ₃	6.70	0	1	16	6	-0.23	0.00
55	O	8.00	0	1	17	8	-1.41	-1.41
56	CHCH ₂ OH	9.15	1	1	16	6	-0.71	0.00
57	CHCH ₂ OOCCH ₃	9.00	1	2	17	6	-1.46	0.00
58	CHCH ₂ O(n-C ₄ H ₉)	7.52	0	1	17	6	0.88	0.00
59	CHCH ₂ O ₂ C ₆ H ₅	7.52	0	1	18	6	0.73	0.00
60	CHCH ₂ NHCOCH ₃	8.18	0	1	17	6	-1.38	0.00
61	CHCH ₂ N(CH ₃)COCH ₃	9.00	0	1	16	6	-1.10	0.00
62	CHCHO	9.52	1	1	16	6	-0.33	0.00
63	CHCH=C(COCH ₃) ₂	8.70	0	2	17	6	-1.27	0.00
64		8.00	0	2	17	6	-1.50	0.00
65		10.00	0	2	16	6	-0.96	0.00
66	CHCONHC ₃ H ₇	9.70	1	1	15	6	-0.26	0.00
67	CHCONH(n-C ₄ H ₉)	9.15	1	1	16	6	0.76	0.00
68	CHCONH(t-C ₄ H ₉)	8.00	1	1	17	6	0.70	0.00
69	CHCONHC ₂ H ₄ OH	10.00	1	2	15	6	-1.60	0.00
70	CHCONH(CH ₂) ₂ CH ₂ OH	9.70	1	2	15	6	-1.10	0.00
71	CHCONH ₂ CH(OH)CH ₃	9.52	1	2	15	6	-1.10	0.00
72	CHCON(CH ₃)(CH ₂) ₂ CH ₂ OH	9.00	1	2	15	6	-0.80	0.00
73	CHCONHC ₆ H ₅	9.40	1	1	17	6	0.70	0.00
74	CHCONHC ₆ H ₄ -2-OH	9.00	1	1	16	6	0.03	0.00
75	CHCONHC ₆ H ₄ -2-CO ₂ H	10.00	1	2	16	6	0.18	0.00

^a For all compounds $\delta_1 = 0$, $\delta_2 = 0$.

TABLE 2E

No.	Structure	$\log \frac{1}{ED_{50}}$	δ_1	δ_2	δ_3	n _o	MTD ₁	MTD ₂	π	π_{c+m}
76		8.00	0	0	1	2	—	8	0.43	-0.45
77		9.00	0	0	1	2	—	6	0.28	-0.60

TABLE 3A
Unified Set of Retinoids Randomly Chosen for Test Series

	Structure, R ^a	$\log \frac{1}{ED_{50}}$	π	π_{c+m}	MTD ₁	MTD ₂	$\log \frac{1}{ED_{50}}$
78		10.00	0.88	0.00	—	6	10.42 ^c
79		9.52	-0.26	0.00	16	6	8.93
80		9.52	-1.60	0.00	16	6	9.52
81		10.00	-1.97	0.00	16	6	9.57
82		7.40	0.68	0.30	16	6	7.03
83		7.52	1.18	0.30	16	6	7.08
84		8.30	1.40	1.40	—	8	8.64 ^c
85		10.00	0.59	-0.29	—	7	9.21 ^c
86		9.10	-0.88	-1.76	—	7	8.36 ^b
87		9.00	-0.45	-0.45	—	7	9.41 ^b
88		9.22	-0.86	-1.74	—	7	8.37 ^b
89		9.00	-0.69	-0.36	—	7	8.47 ^b

^a The same explanatory notes as for Table 2A.

^b Calculated with Equation 11 when not otherwise stated.

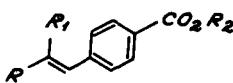
^c Calculated with Equation 13.

TABLE 3B

No.	Structure, R ^a	$\log \frac{1}{ED_{50}}$	π	π_{c+m}	MTD ₁	MTD ₂	$\log \frac{1}{ED_{50}}$
90	CHCH(COCH ₂ CH ₃) ₂	8.40	-0.56	0.00	17	6	8.34
91	CHCONHCH ₂ CH(OH)CH ₂ OH	9.70	-1.97	0.00	15	6	10.43
92	CHCONH(CH ₂) ₄ OH	9.70	-0.58	0.00	15	6	10.16
93	C(CH ₃) ₂	8.00	1.04	0.00	17	6	7.70
94	CHCH=NNCOCH ₃	9.40	-0.36	0.00	16	6	9.25
95	CHCH=NOH	8.00	-0.06	0.00	17	6	8.02
96	CHCONHC ₂ H ₅	9.00	-0.26	0.00	16	6	8.93
97	CHCH ₂ OCH ₃	8.52	-0.35	-0.71	17	6	8.02

^a The same explanatory notes as for Table 3A.

TABLE 3C

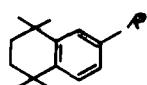
No.	Structure ^a	R, R ₁ , R ₂	$\log \frac{1}{ED_{50\exp}}$	π	π_{c+m}	TD ₁	MTD ₂	$\log \frac{1}{ED_{50}}$
98		R ₂ = C ₂ H ₅	11.00	1.83	0.95	15	6	10.16
99		R ₂ = C ₂ H ₅	9.40	-0.52	-1.31	—	7	8.87 ^c
100		R ₂ = C ₂ H ₅	9.30	1.11	0.23	—	7	9.51 ^c
101		R ₂ = C ₂ H ₅ 9-10-cis	8.00	1.11	0.23	—	9	7.43 ^c
102		R ₂ = C ₂ H ₅	9.40	0.47	-0.41	—	7	9.14 ^c
103		R ₁ = H R ₂ = C ₂ H ₅	8.52	0.11	-0.77	—	7	8.95 ^c

^a R₁ = CH₃, when not otherwise stated.

rapid with respect to the duration of the biological interaction. The polyenic chains and the terminal carboxylic groups in the different molecules were, therefore, superimposed as much as possible upon the structure of compound 51.

The orientation of the terminal functional groups of the retinoic acid derivatives was selected to avoid intramolecular steric repulsions.

TABLE 3D



No.	Structure, R ^a	$\log \frac{1}{ED_{50}}$	π	π_{c+m}	MTD ₁	MTD ₂	$\log \frac{1}{ED_{50}}$
104		11.52	0.82	0.82	—	6	11.19 ^c
105		8.70	1.70	0.82	—	8	8.82 ^c
106		9.70	0.83	0.82	—	7	9.35 ^c
107		9.70	1.19	0.82	—	7	9.56 ^c
108		7.50	1.14	0.82	—	9	7.78 ^c

^a The same explanatory notes as for Table 3A.

TABLE 3E

No.	Structure	$\log \frac{1}{ED_{50}}$	π	π_{c+m}	MTD ₁	MTD ₂	$\log \frac{1}{ED_{50}}$
109		8.40	-0.50	-1.38	18	8	7.41
110		8.00	-0.30	-0.30	—	8	8.46 ^c
111		8.40	-1.38	-1.38	—	6	9.91 ^c

B. THE MAPPING OF THE RECEPTOR SITE

Because of computational limitations, the MTD method was applied to the set of 53 compounds divided into three separate series A, B, and C, with M being the number of vertices employed in the building of the hypermolecule (for the number of the vertices, see Figure 2).

1. Series A

This series (Structures **1** to **19**, M = 15) is concerned only with structural variations to the retinoid ring terminus moiety affecting vertices 1 to 15. The compounds belonging to series A were chosen to study the influence of the stereochemistry of the ring on retinoid biological activities. Correlation equations were developed using MTD, δ_1 and π parameters. The best equation was

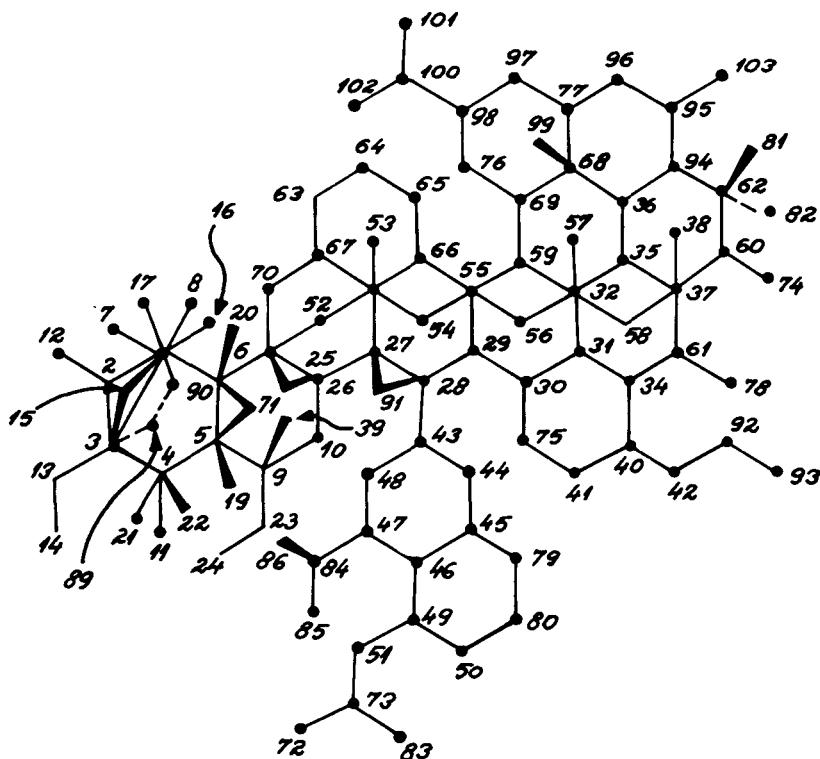


FIGURE 2. Numbering for the hypermolecules. For both hypermolecules (for the Newton et al.²¹ and Dawson et al.³⁸ series) the same numbering system as defined in this figure was used. There is no correlation between the hypermolecule and the standard chemical numbering.

$$\log 1/ED_{50} = 24.75 + 0.46(\pm 0.25)\delta_1 - 0.95(\pm 0.10)MTD_A + 0.19(\pm 0.11)\pi \quad (5)$$

$$\begin{array}{lll} n = 19 & r = 0.936 & F = 35.36 \\ r_o = 0.532 & & F_o = 3.15 \end{array} \quad (5)$$

In this equation and all subsequent regression analyses, r_o and F_o refer to a regression in which the MTD parameter is excluded. When instead of π we used π_{c+m} or $\log P$, correlation indices r equaling 0.936 and 0.934, respectively, were obtained.

The distribution of the effective vertices in the resultant hypermolecule (see Figure 3) suggested that one or two hydrophobic pockets could be located in this region of the receptor (i.e., one in the place occupied by the fused aromatic ring of compound 51 around vertex 3, and the other in the region of vertices 4 and 5).

2. Series B

This series (compounds 1, 21 to 29, and 51; $M = 19$) describes structural variations in the middle section of the molecules affecting vertices 16 to 27 and 31. This series contains the congeners exhibiting structural variations in the polyenic chain. The initial correlation of the activities of compounds 1, 21 to 29, and 51, with the MTD_B parameters was rather poor ($r = 0.733$, $s = 1.12$). However, the addition of the δ_2 term, which reflects the degree of saturation of the polyenic chain, greatly improved the correlation index as well as the variance ($r = 0.953$, $s = 0.60$). δ_2 could also be related to the probability of the polyenic chain adopting a more rigid conformation. The high activity of the 7,8-didehydro derivative 22 is consistent with this assumption. Hydrogenation of a double bond in this region of the

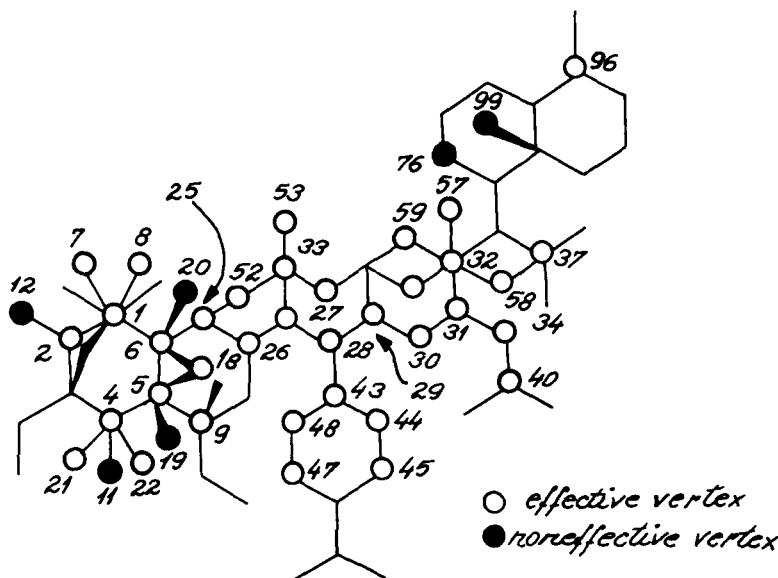


FIGURE 3. Distribution of effective (○) and noneffective (●) vertices in the hypermolecule computed for the Newton et al.²¹ series.

molecule destroyed conjugation and increased the flexibility of the side chain, leading to less active congeners (i.e., compounds **25** and **27**). Equations 6 and 7 are derived from this data:

$$\begin{aligned} \log 1/ED_{50} &= 30.79 - 2.40(\pm 0.42)\delta_2 - 1.31(\pm 0.17)MTD_B \\ n = 11 \quad r = 0.953 \quad F &= 39.90 \\ r_o = 0.435 \quad F_o &= 2.09 \end{aligned} \quad (6)$$

$$\begin{aligned} \log 1/ED_{50} &= 29.74 - 2.43(\pm 0.49)\delta_2 - 1.24(\pm 0.40)MTD_B \\ &\quad + 0.13(\pm 0.39)\pi - 0.01(\pm 0.45)\pi^2 \\ n = 11 \quad r = 0.954 \quad F &= 15.20 \\ r_o = 0.885 \quad F_o &= 8.46 \end{aligned} \quad (7)$$

The involvement of lipophilicity was poor in these correlations. Introducing this variable led to a significant decrease in the variance (Equation 7).

3. Series C

This series (compounds **1** to **3**, and **52** to **75**, M = 16) contains RA derivatives with modifications in the vertices of the polar terminal group. The MTD parameter alone did not describe the biological behavior of this group of RA derivatives ($r = 0.594$, $s = 0.77$). The introduction of a second term, n_o , reflecting the probability that a modified carboxylic group was involved in hydrogen bond formation with the receptor site, significantly improved the correlation ($r = 0.733$, $s = 0.68$). Inclusion of lipophilicity terms did not make a significant reduction in the variance ($F = 8.90$, compared to $F = 5.63$ for π and π^2 addition). The introduction of the parameter δ_3 , describing the probability that the end group is transformed into a carboxyl group, further raised the overall correlation index. However, the correlation remained unsatisfactory:

TABLE 4
Position of the Effective and Noneffective Vertices in the Retinoid
Hypermolecules Shown in Figures 3 and 5

Receptor map	Position of vertices ^{a,b}	
	Effective ($E_j = -1$)	Noneffective ($E_j = +1$)
S^* (Figure 3)	1,2,4—9,18,21,22,25—34,37,40, 43—45,47,48,52—54,56—59,96	11,12,19,20,76,99
S' (Figure 5)	1,4—10,18,89 ^c ,30—32,35—38,57,58	12,13,15—17 ^c , 40,55,61,63—91

^a For the numbering of vertices, see Figure 2.

^b The vertices not mentioned are irrelevant ones ($E_j = 0$).

^c Located in the cyclic moiety.

$$\begin{aligned} \log 1/ED_{50} &= 13.17 + 1.10(\pm 0.65)\delta_1 + 0.73(\pm 0.25)\delta_3 \\ &\quad + 0.28(\pm 0.20)n_o - 0.32(\pm 0.10)MTD_C - 0.03(\pm 0.12)\pi \\ n &= 27 \quad r = 0.777 \quad F = 6.38 \quad (8) \\ r_o &= 0.746 \quad F_o = 6.88 \end{aligned}$$

This result is consistent with the existence in hamster tracheal cells of various metabolizing systems^{21,35} that could convert primary or secondary amides or esters to carboxylic acids, retinyl acetate to retinol, as well as, retinal to RA. Because the conversion rates of various derivatives could significantly contribute to the overall rate of the process described by our equation, this lack of correlation is not unexpected. However, Equation 8 remains able to describe the general trend of the phenomenon. For instance, using Equation 8, the calculated activity of retinal is 9.07 (A_{exp} is 9.52, Table 2D) and only 8.53 if we neglect the δ_3 term.

4. Final Receptor Map

The final optimized map, S^* , of the receptor is given in Table 4. To obtain a reasonable map, without isolated effective vertices, the connectivity condition (as in the SIBIS method³¹) was introduced. In other words, it is reasonable to assume that there is a single cavity in the receptor and, therefore, that the $\epsilon_j = -1$ vertices should be grouped into a single connected graph.

The development of a "global" equation ($MTD = MTD_A + MTD_B + MTD_C + \text{constant}$), including all data, led to an unsatisfactory correlation (Equation 9), although the explained variance of ED_{50} remains around 70%.

$$\begin{aligned} \log 1/ED_{50} &= 21.86 + 0.44(\pm 0.20)\delta_1 - 2.31(\pm 0.49)\delta_2 + 0.32(\pm 0.20)n_o \\ &\quad - 0.93(\pm 0.09)MTD + 0.07(\pm 0.11)\pi - 0.13(\pm 0.08)\pi^2 \\ n &= 53 \quad r = 0.868 \quad F = 23.11 \quad (9) \\ r_o &= 0.568 \quad F_o = 4.49 \end{aligned}$$

The low contribution of lipophilicity terms to the overall biological effects was somewhat unexpected. However, mention should be made that we used only relative lipophilicities (i.e., $\log P$ as 0.0 for RA). Therefore, the absolute lipophilicity of the studied compounds varied around a $\log P$ value of 6.60 ± 2.00 , a variation of no more than 30% from the $\log P$ of RA that does not influence extensively the uptake of the considered congeners and the penetration across the cell membrane (passive diffusion).

The deletion of only one aberrant point (namely **54**, methyl-retinone) raised the correlation indices of Equation 9 to $r = 0.908$ and $F = 36.16$. The deletion of a second point [**72**, *N*-(1-methyl-3-hydroxypropyl)retinamide] gave even better results: $r = 0.918$ and $F = 39.41$, for $n = 51$. At this stage, it is difficult to state whether these compounds represented unusual congeners that could be studied for new leads or if they are poorly predicted.

In order to check the prediction probabilities of our computations, a test series was formed from 15 retinoids (**79** to **83**, **90** to **98**, and **109**) (see Table 3A, B, C) that were not included in series A, B, and C. The retinoids for the test series were randomly chosen from all 11 groups of compounds tested by Newton et al.²¹ For this purpose, we used the computed MTD values for this series on the basis of the final receptor map hypermolecule (Figure 3 and Equation 9). The experimental ED_{50} values were correlated to the computed ones according to the equation:

$$\log \frac{1}{ED_{50\text{exp}}} = 1.64 - 0.84 \log \frac{1}{ED_{50\text{calc}}} \quad (10)$$

$n = 15 \quad r = 0.916 \quad F = 60.25$

IV. ANALYSIS OF CONFORMATIONALLY RESTRICTED RETINOIDES

A similar methodology was followed for the analysis of a second series of 53 conformationally restricted retinoids synthetized by Dawson et al.³⁶⁻³⁸ This series contains compounds in which some double bonds corresponding to those of the tetraene chain of RA are held in a cisoid form, being included in aromatic rings. Mention should be made that our previous QSAR analysis of retinoids indicated that a cisoid conformation of the side chain of RA (as in Ro 13-7410) was necessary to obtain the best correlation indices for the computed equations.⁵ This suggestion is in agreement with experimental data that showed that molecules with very different side-chain stereochemistry (i.e., all-*trans*-RA, 13-*cis*-RA, or Ro 13-7410) were equally potent in the considered test. This result agrees also with Dawson's suggestion about the possibility that RA assumes an active conformation in which the 9,11,13*E*-double-bond systems are planar and cisoid.³⁸ For this reason the QSAR analysis of additional compounds with cisoid-fixed double bonds in the polyenic chain appears to be of very great theoretical interest to us.

Some differences were evident in this QSAR analysis compared to the previous series. Our first receptor map allowed a rather large variety of positions for the polyenic chain and terminal CO_2H group substituents without loss of biological activity. This map suggested the existence of two receptor moieties (with different cavities and a certain reciprocal mobility) as in the case of steroid receptor.⁴² In other words, we assumed a large hydrophobic pocket for the cyclic moiety of the molecule and a connected cavity for its terminal group. Therefore, we ascribed ϵ_j as 0 (irrelevant vertex) for as many as possible vertices in the middle range of the polyenic chain.

The construction of the hypermolecule was performed according to the rules described in Section III. The most active derivative Ro-13-7410 (**51**) was also selected as a basic structure for the superposition process. The polyenic chain was considered sufficiently flexible to allow rotations about C-C single bonds within short time intervals compared to those pertinent to the biological events. Orientation of the terminal groups was selected to avoid intramolecular steric repulsion as much as possible.

For the superposition procedure, Dreiding stereomodels and computer-simulated three-dimensional molecules were used. The X-ray structures of RA and Ro 13-7410⁴⁰ were also taken into account. In the aromatic ring of compound **51** and its adjacent vertices, the C_1 , C_4-C_6 , C_9 , C_{10} atoms are approximately coplanar (see Figure 4). Modification of the 5,6-

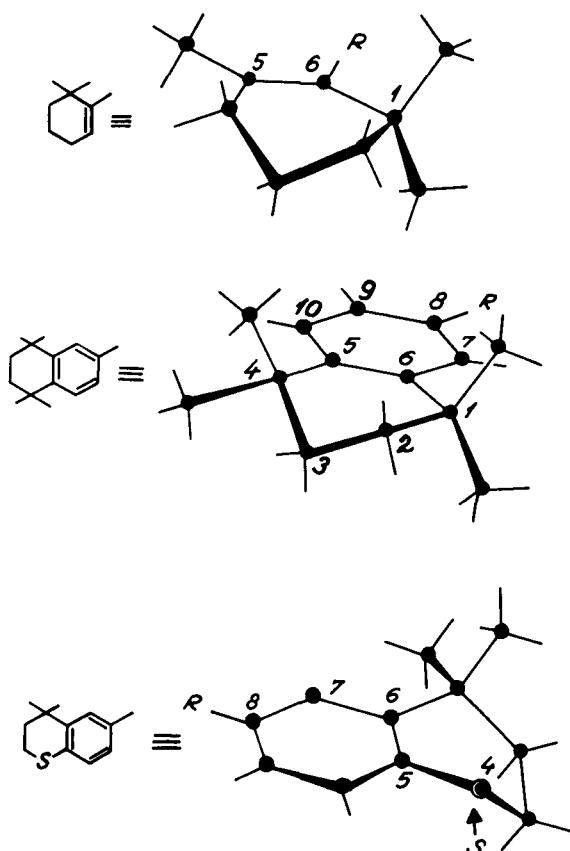


FIGURE 4. Structures of some cyclic moieties drawn by computer according to the three dimensional modeling of these molecules; (a) 1,1,5-trimethyl-6-cyclohexenyl ring; (b) 1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalenyl ring; (c) 4,4-dimethyl-3,4-dihydro-*2H*-1-benzothiopyran ring.

double bond (i.e. hydrogenation, epoxidation, etc.) produced a rather uniform displacement of the C₁–C₁₀ atoms compared to the positions occupied in Ro 13-7410. It was rather difficult to discern the equivalent positions that were within 0.5 Å distance of an average position and that could be contracted into a single vertex. A thorough comparison of Dreiding models and computer-generated structures resulted in the hypermolecule depicted in Figure 5. The superposition procedure in the regions of the middle of the chain and the polar terminus of the molecules did not pose such difficulties.

From the set of 53 compounds proposed for analysis, 29 compounds were retained for MTD-QSAR computations (compounds: **1**, **3**, **20**, **21**, **29** to **51**, and **76**). Initially, a randomly generated series of 21 compounds was used as a test series (compounds: **83** to **89**, **99** to **108**, and **110** to **113**). Three compounds were rejected as *ab initio* because of their biological responses. Two compounds (**112** and **113**) (see Figure 6) were deleted from the test series because their structures did not fit our hypermolecule. They will be discussed further.

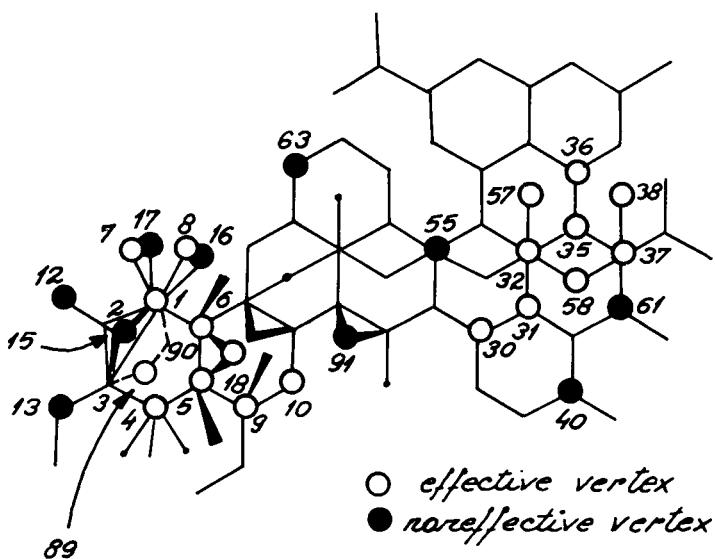


FIGURE 5. Effective (○) and noneffective (●) vertices in the hypermolecule computed for the Dawson et al.³⁸ series and for the unified set of retinoids.

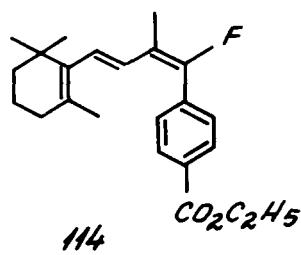
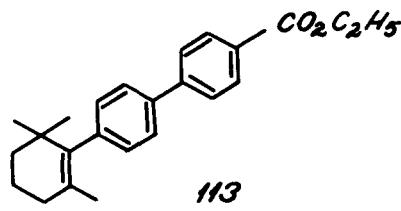
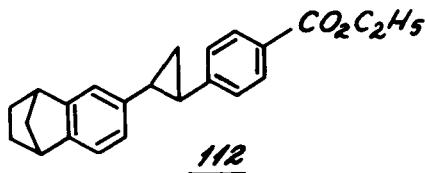


FIGURE 6. Compounds **112**, **113** and **114**.

Correlation equations were developed using MTD, δ_1 , δ_3 , and π terms. The best equation was:

$$\begin{aligned} \log 1/\text{ED}_{50} &= 16.48 + 0.80(\pm 0.18)\delta_1 - 0.33(\pm 0.23)\delta_3 \\ &\quad - 1.04(\pm 0.11)\text{MTD} + 0.58(\pm 0.10)\pi \\ n &= 29 \quad r = 0.918 \quad F = 32.04 \\ r_o &= 0.524 \quad F_o = 3.16 \end{aligned} \quad (11)$$

We want to emphasize that, as for the previous QSAR approach, a rapid *trans-cis* enzymatic isomerization was postulated for RA and 13-*cis* congeners. This assumption significantly improved the correlation indices in this series and might perhaps also explain the poor activity of compound **114** (Figure 6), in which the presence of a fluorine atom at position 10 of the side chain possibly blocked the isomerization.

The good correlation obtained for this series prompted us to examine more carefully the structures of the two rejected compounds (**112** and **113**). Computer generation of a simulated three-dimensional structure for compound **112** indicated that the CO₂H terminus lies in a region of the receptor situated at a distance of 7.09 Å from C₆* (under a C₅-C₆-C₁₅ angle of 35°45'), far away from the area of optimum activity (see Figure 8, Section VI). In this situation, even its borderline biological activity is difficult to explain without supplementary assumptions. In contrast, the CO₂H terminus of compound **113** is located 10.11 Å from C₆ and under an angle of 107°18' (C₅-C₆-C₁₅), and, therefore at the border of the region of the receptor that corresponds to the end group coordinates of the most effective compounds (as defined in Figure 8). This fact is consistent with its poor biological activity but does not fit with our computation (test series) that predicts a total lack of activity ($\log 1/\text{ED}_{50} < 7.00$) for such a structure. The only explanation at hand is that a torsion of the single bond (C₆-C₇ or/and C₁₀-C₁₁) occurs so that the phenyl rings are out of a plane with respect to each other and more effective vertices (E_j = -1) than calculated are fitted by this structure. This assumption was also suggested by X-ray data available for compound **51**, which showed such a phenomenon.⁴⁰

A larger test series, compared with our previous approach, was used to screen the prediction accuracy of Equation 11 (compounds **78**, **84** to **89**, **99** to **108**, **110**, and **111**). The following relationship was obtained between experimental and calculated data:

$$\begin{aligned} \log 1/\text{ED}_{50\text{exp}} &= 1.92 + 0.79(\pm 0.16) \log 1/\text{ED}_{50\text{calc}} \\ n &= 19 \quad r = 0.772 \quad F = 25.09 \end{aligned} \quad (12)$$

In this case the correlation was weaker than that obtained for the previous series of retinoids. However, if the single aberrant point (compound **111**) in the series were deleted, the correlation indices rose (r = 0.855, F = 43.59) (see Figure 7).

The absence of the δ_2 parameter in Equation 11, which was computed for the conformationally restricted retinoids, represents a major difference compared with Equation 9, which was obtained with the first sample of retinoids, and indicates that uninterrupted conjugation or a certain rigidity of the side chain is not a prerequisite condition for activity in this series. The fact that compound **34** is at least 15 times more effective than 7,8-; 9,10-; 11,12-; and 13,14-dihydroretinoic acids (**25**, **27**, **82**, and **83**, respectively), in the TOC assay is not easy to interpret. A tentative explanation is given in Section V.

V. ANALYSIS OF THE UNIFIED SET OF RETINOIDS

The computation of the unified set of both retinoid congeners and conformationally restricted retinoids was undertaken in order to clarify the following points:

* For numbering see Figure 1.

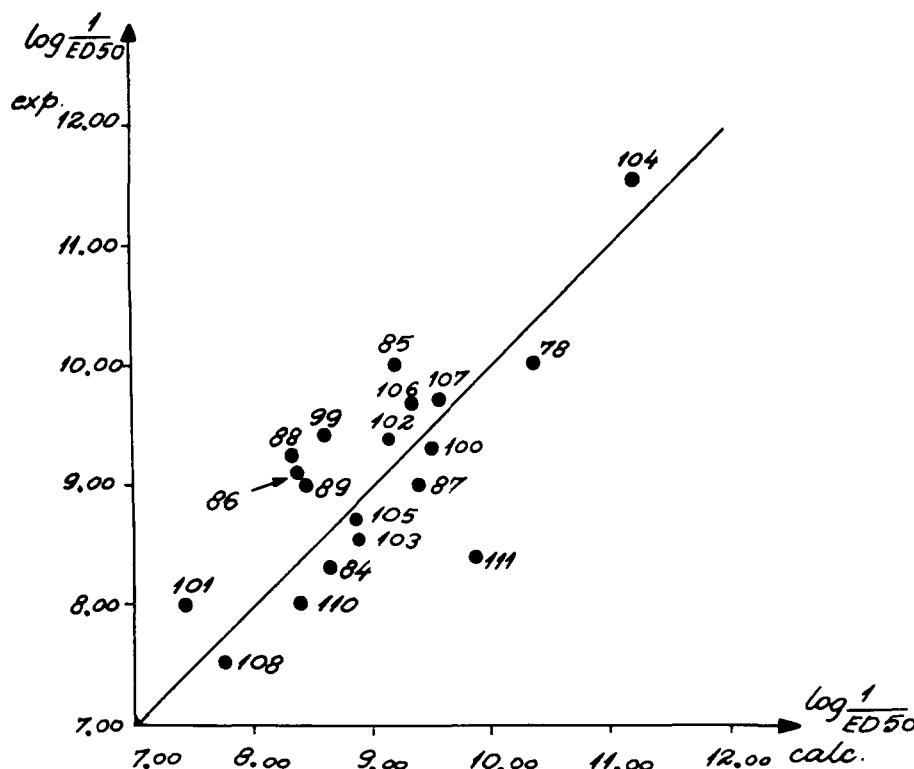


FIGURE 7. Correlation between the experimental and predicted activities for a test series ($n = 19$). The data were calculated using Equation 13 for the Dawson et al.³⁸ series. The compound numbering corresponds to that of Table 3 (A,B,C).

1. The effect that the origin of the TOC assay data had on the correlation indices.
2. Whether significant differences between the structural features responsible for biological activity in both series could be noted.

From the first series we retained 52 compounds. Methyl retinone, **54**, was deleted because it represented an aberrant point. The conformationally restricted retinoid series included in the unified set contains the same 29 compounds previously used. Five derivatives were common to both series. For these retinoids some differences existed between the measured values in the TOC assay but in almost all cases the ED_{50} values were within ± 0.5 log units of a mean value (Table 5). This mean value was employed in the computations. Finally, a unified sample of 75 compounds was obtained. The superposition procedure was the same as that employed for the series of conformationally restricted retinoids. The final hypermolecule possessed 102 vertices (Figure 4).

The compounds were grouped according to the same three structural areas defined in the first series:

1. The cyclic moiety ($n = 27$: **1** to **19**, **21**, **47** to **51**, **76**, and **77**)
2. The polyenic chain ($n = 34$: **1** to **3**, **20** to **33**, **35** to **45**, **47** to **50**, **76** and **77**)
3. The modified terminus ($n = 32$: **1** to **3**, **42**, **46**, and **52** to **75**)

The QSAR—MTD procedure was first applied separately to these three series of retinoids.

TABLE 5
Activity Differences Determined for the Same Compounds in Two
Different Laboratories^{21,38}

Compound ^a	Activity in the TOC assay ($\log 1/ED_{50}$) ^b	
	Series of Newton et al. ²¹	Series of Dawson et al. ³⁸
1 RA	10.5	11.0
3 RA ethyl ester	9.3	11.15
20 13-cis-RA	10.5	10.7
51 Ro 13-7410	11.0	11.7

^a The numbering of compounds is the same as in Tables 2 and 3.

^b For the unified set of retinoid congeners the average values of activity were used for the computations.

A. THE CYCLIC MOIETY SERIES

The correlational equations computed in this area are similar to those calculated for the retinoids in the Newton et al.²¹ series. The optimum is

$$\begin{aligned} \log 1/ED_{50} = & 13.98 + 0.77(\pm 0.22)\delta_1 - 0.73(\pm 0.08)MTD \\ & + 0.51(\pm 0.10)\pi - 0.10(\pm 0.06)\pi^2 \\ n = 27 \quad r = 0.938 \quad F = 40.62 \\ r_o = 0.660 \quad F_o = 5.93 \end{aligned} \quad (13)$$

The similarity of regression results based on π or π_{c+m} ($r = 0.941$, $F = 42.14$) warrants preferential use of the more specific index π_{c+m} . In this case, the lipophilicity contribution appears to be more important with respect to the equation containing only δ_1 and MTD parameters (unified set, $n = 27$, $r = 0.839$, $F = 26.19$). This result strengthened the suggestion about the highly lipophilic character of the receptor cavity containing the ring moiety. It also indicated that the ring moiety represents a very critical structural feature for the activity of the retinoids.

Accordingly, the best ring shape is represented by the 5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl* moiety, which possesses two supplemental effective vertices (vertices 9 and 10, see Figure 5) relative to the 1,1,5-trimethylcyclohexan-6-yl ring of RA. The introduction of a polar atom (i.e., sulfur and especially oxygen) in the 4_R-position has a negative influence upon activity.

B. THE POLYENIC CHAIN SERIES

If a unified set of conformationally restricted and nonrestricted retinoids is considered, a satisfactory correlation is obtained using δ_1 , δ_2 , MTD, and π parameters:

$$\begin{aligned} \log 1/ED_{50} = & 13.73 + 0.70(\pm 0.19)\delta_1 - 2.91(\pm 0.36)\delta_2 \\ & - 0.70(\pm 0.07)MTD + 0.33(\pm 0.10)\pi \\ n = 33 \quad r = 0.914 \quad s = 0.49 \quad F = 35.32 \\ r_o = 0.536 \quad s_o = 1.00 \quad F_o = 3.90 \end{aligned} \quad (14)$$

The use of π_{c+m} afforded no better correlations. The higher contribution of the δ_2 parameter emphasized the importance of the rigidity of the polyenic chain for the unified set of retinoid

* For numbering see Figure 1.

congeners. However, it should be noted that the cyclopropane ring fits this condition and the only aberrant point is represented by compound **34**, which is severely underpredicted. This last point by itself is not conclusive because we can argue that the Dawson series contains only one such compound (**34**) as compared to the four inactive 7,8-; 9,10-; 11,12-; and 13,14-dihydroretinoic acids previously reported. Nevertheless, if we assume that its biological effectiveness was properly determined, this fact does not agree with our previous interpretations.

The possibility of a dimeric protein receptor for retinoids was previously suggested (Section IV). Following this argument we may assume that the interaction of the terminal part of the molecule with the corresponding receptor moiety can be favored by the hydrophobic character of 11,12- and 13,14-double bonds of RA. Hence, the further increase of lipophilicity of this part of the molecule by the inclusion of the two cited double bonds in an aromatic nucleus (as in compound **32** or **34**) strengthens the binding between the terminal part of the retinoid molecule and the receptor moiety, thus reducing the rigidity requirements for the polyenic chain.

In other words, the enhancement of the hydrophobic interaction between a retinoid analog possessing an aromatic nucleus in the terminal part of the molecule and the receptor moiety allows a more flexible conformation of the side chain. From this explanation we may also infer that the 9,10-dihydro isomer of compound **34** must be equally effective.

C. TERMINAL GROUP SERIES

This series contains the same compounds as the C series of our first approach plus five additional compounds (**42** to **46**) belonging to the Dawson et al.³⁶ series. As expected, the best computed equations gave a poor correlation, but significantly better F values ($r = 0.772$, $F = 7.69$, $n = 32$) than that of Equation 8. This suggests that the enzymatic hydrolysis of the polar terminal group (probably to a carboxylic moiety) takes place before the rate-determining interaction described by our equations. This suggestion results from the increased contribution of the π_{c+m} term with respect to the overall π parameter in the obtained equations (the regression coefficient of π_{c+m} is 0.48 compared with only 0.02 for π). The enhanced role of δ_3 (the correlation index, r , of δ_3 alone is 0.691) in the final equations stresses the importance of the intracellular hydrolysis process for the biological activity of this series of congeners.

D. UNIFIED SET RECEPTOR MAP

The development of a global equation including all data ($n = 75$) led to an unsatisfactory correlation, not very different from that obtained for the retinoids in the Newton et al.²¹ series:

$$\begin{aligned} \log 1/ED_{50} = & 11.99 + 0.54(\pm 0.15)\delta_1 - 2.72(\pm 0.45)\delta_2 + 0.65(\pm 0.18)\delta_3 \\ & + 0.18(\pm 0.11)n_o - 0.57(\pm 0.06)MTD + 0.40(\pm 0.10)\pi_{c+m} \\ & - 0.12(\pm 0.05)\pi_{c+m}^2 \\ n = 75 \quad r = 0.847 \quad s = 0.63 \quad F = 24.27 \\ r_o = 0.810 \quad s_o = 0.81 \quad F_o = 20.41 \end{aligned} \quad (15)$$

The same comments as made for the terminal group series can be made here concerning the importance of the lipophilicity terms (π , π_{c+m}). Equation 15 was developed using the receptor map from Figure 5 and assuming a possible isomerization of the polyenic chain. However, it becomes obvious from these equations that the inclusion of the terminal group series in

the overall set significantly decreased their correlation indices because of the interference between the hydrolysis process (needed for the activation of the end group-modified retinoids) and the rate-determining step interaction. If this is true, then a set containing only ring and polyenic chain modified retinoid congeners will exhibit a significantly better correlation. In fact, considering such a set (**1** to **33**, and **35** to **51**) the following equation was obtained:

$$\begin{aligned} \log 1/\text{ED}_{50} = & 13.89 + 0.68(\pm 0.17)\delta_1 - 2.93(\pm 0.39)\delta_2 \\ & - 0.10(\pm 0.33)\delta_3 - 0.69(\pm 0.06)\text{MTD} + 0.42(\pm 0.08)\pi \\ & - 0.09(\pm 0.05)\pi^2 \\ n = 50 & \quad r = 0.915 \quad s = 0.55 \quad F = 37.03 \\ r_o = 0.611 & \quad s_o = 1.06 \quad F_o = 5.25 \end{aligned} \quad (16)$$

The very small coefficient and the large error limits for the δ_3 term in this equation (indicating that no enzymatic hydrolysis is needed for activation) is consistent with our assumption. The good correlation indices obtained for Equations 13, 14, and 16 confirmed the consistency between the biological activities in the TOC assay that were measured in two different laboratories.

The predictive power of Equation 16 was checked on a set of randomly chosen congeners belonging to both normal and conformationally restricted retinoids:

$$\begin{aligned} \log 1/\text{ED}_{50\text{exp}} = & 0.22 + 0.96(\pm 0.14) \log 1/\text{ED}_{50\text{calc}} \\ n = 23 & \quad r = 0.832 \quad s = 0.59 \quad F = 47.29 \end{aligned} \quad (17)$$

The obtained correlation was not a very encouraging one. The activity of two compounds (**87** and **111**) was poorly predicted by more than one order of magnitude. The deletion of compound **111**, which was also an aberrant point in Equation 12, afforded a better correlation ($r = 0.872$, $F = 63.90$).

In fact, the activity of compound **111** is severely overpredicted. The important hydrophobicity decrease produced by the introduction of an oxygen at the 4-position of the retinoid skeleton (Figure 1) cannot by itself account for the poor activity of this compound. Some other unidentified features might be involved.

VI. CORRELATION OF OUR DATA WITH THE POSSIBLE MECHANISMS OF ACTION OF RETINOIDS

The naturally occurring and synthetic retinoids modulate the differentiation of several cell types. However, despite the great amount of work spent in this area, their mode of action remains poorly understood. It is, therefore, of interest to compare the structure-activity relationships obtained here with some of the presumed mechanisms of action and also with theoretical models for cell differentiation.

From the experimental data yet available and based on contemporary concepts about cell regulation, it is most likely that two main sites are involved in the retinoid effect: the cell membrane and the nuclear chromatin.

Retinoids may modulate growth and differentiation via interaction with membrane-associated proteins, for instance by direct interaction with cyclic-AMP-dependent or calcium-dependent protein kinases associated with growth factors and their receptors.⁴¹⁻⁴³ Their direct interaction with the polypeptidic growth factors that control proliferation and differentiation was also suggested.^{40,41} There are also arguments against this possible mode of action.¹⁵

Another possible target of retinoids is the nucleus. The simplest explanation suggested

by the large amount of available experimental data is that retinoids are able to modulate the expression of the genes involved in both differentiation and proliferation.⁴⁴⁻⁴⁸ On this basis, it is logical to assume that an interaction with a receptor site at the nuclear level (by a steroid-like mechanism)^{10,44-48} is responsible for the carcinogenesis inhibitory properties of the retinoids. The existence of cellular specific retinoid-binding proteins (CRBP, CRABP, etc.) agrees with this mechanism of action. Whether the transport of retinoids through the cytoplasm and their translocation to the nucleus are dependent on these binding proteins or occur by passive diffusion remains an open question because it has recently been demonstrated that two highly retinoid-responsive cell systems—HL60 and 10T1/2 fibroblasts—are devoid of retinoid-binding proteins.^{15,49,50}

But, before discussing how our results substantiate one of the proposed mechanisms by which retinoids promote differentiation it is of interest to examine the data provided by the TOC assay. According to Newton et al.,²¹ this test is able to measure the intrinsic ability of retinoids to control epithelial cell differentiation and is a valuable procedure for the initial evaluation of the biological activity of new congeners. The TOC assay is believed to have significant predictive value for the prevention of epithelial cancer. Some features of this assay are important, namely: (1) its high specificity and sensitivity for retinoids,²¹ (2) the fairly good correlation of the activity of retinoids determined in this system with other *in vitro* test systems such as the inhibition of the growth of murine melanoma cells²⁰ and binding to cellular CRABP,⁴⁸ (3) the arguments that tracheal cells possess enzymes necessary to hydrolyze retinoid carbocyclic acid derivatives (e.g., esters and amides),^{21,35} and (4) according to Frolík et al.,⁵¹ the ability of tracheal cells to convert retinyl acetate to retinoic acid, via a retinal intermediate.

In the hamster tracheal organ culture test, the following steps could be important for the biological activity of retinoids: (1) retinoid uptake and penetration across the cell membrane; (2) transport through cytoplasm and nuclear translocation if these processes are mediated by a specific intracellular binding protein; (3) hydrolysis or metabolic conversion of the terminal group to a carboxylic group; and (4) interaction with a critical target site at the nuclear level.

Our equations describe the most important (rate-determining) process of the previously mentioned steps. They are also developed on the implicit assumption that all the considered retinoids act in the TOC assay by the same mechanism. This seems to be a reasonable hypothesis despite the suggestion that a retinoid may act by different mechanisms in different cell systems.¹⁵ Because of the important contribution of MTD terms in the equations developed in this study, an interaction with a given receptor appears to be the major event determining the biological activity of the retinoids; however, which of these two types of receptors—transport or regulatory proteins—are involved in this interaction is difficult to establish. It is therefore, interesting to speculate on some theoretical models for the regulation of gene activity and also to look upon some required characteristics of the transport and regulatory proteins and the way they could be modulated by effectors.

The mechanism of RA uptake by the cells is not yet elucidated.¹⁵ However, passive diffusion could be expected taking into account the high lipophilicity of the retinoids. The first conclusion of our QSAR study is that lipophilicity terms make a rather poor contribution to retinoid behavior. This conclusion agrees with the previously mentioned assumption and also suggests that the cellular uptake of retinoid derivatives is not a critical step in this system.

A second important conclusion of our approach is the necessity of an enzymatic *trans-cis* isomerization of the side chain of RA and several other congeners in order to assume an active conformation. This result explains why compounds with the terminal carboxyl group located in very different positions with respect to that of the most effective retinoid Ro 13-7410, i.e., all-*trans*-RA, 13-*cis*-RA, and also compounds with planar and *cisoid*-fixed conformations, are highly active.

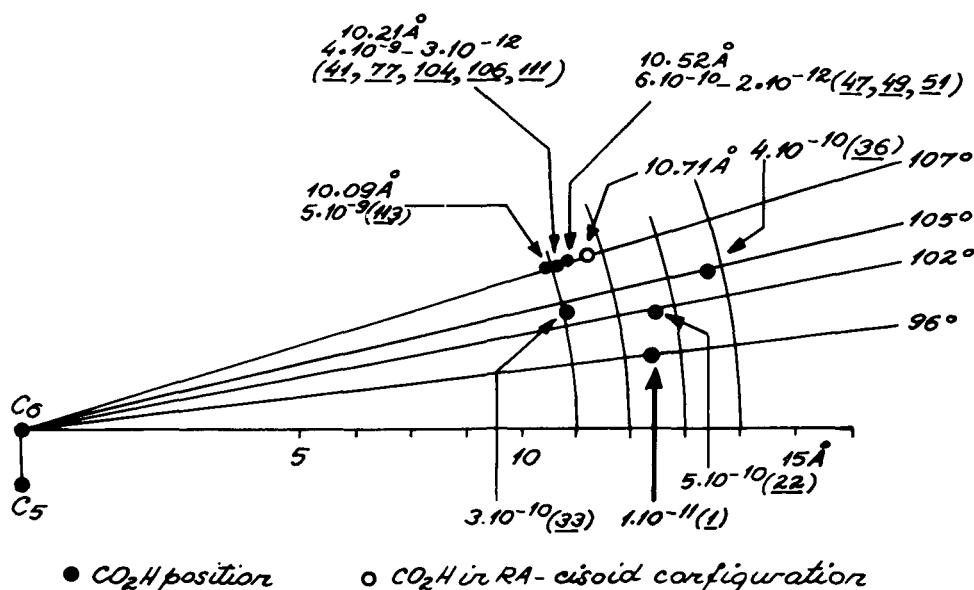


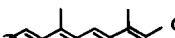
FIGURE 8. The location of the carboxylic acid termini of some effective retinoids in the receptor cavity, using polar coordinates (distance from C_6 atom and angle between $\text{C}_5-\text{C}_6-\text{C}_{\text{CO}_2\text{H}}$ atoms). Compound numbering corresponds to that of Tables 2 (A,B,C,D,E) and 3 (A,B,C). The activities, as ED_{50} (M), are shown.

Measurements of the chain length between the C_6 atom and the terminal carbon atom of the CO_2H group, as well as the $\text{C}_5-\text{C}_6-\text{C}_{\text{CO}_2\text{H}}$ angle using the computer three-dimensional simulated retinoid molecules indicate that biological effectiveness is strongly dependent upon these two parameters (Figure 8) involving a relatively restricted area for the location of the carboxylic acid end group of the retinoid in the receptor cavity that is correlated to high biological activity. This explanation is consistent with the activity of structurally different retinoids (Figure 8), for example the coordinates of the terminal moiety of RA for a planar cisoid conformation of the 11,12- and 13,14-double bonds are 10.7\AA and $107^\circ 18'$, which are very close to the position of the carboxylic group in Ro 13-7410 (Table 6). This argument remains valid regardless of whether or not the retinoid receptor is a transport protein (i.e., CRABP) or a regulatory one.

The interaction of retinoids with nuclear regulatory proteins appears as a very attractive hypothesis. Thus, it is known that gene activity in eukaryotes is regulated by specific activating nonhistonic proteins (NHP).^{52,53} These NHP should be coded by the so-called "master genes" of the hierarchical models for gene activity regulation. A model for a trigger of such a "master gene" with stable steady states corresponding to the inactive gene and to its transcriptional state was proposed by some of us.⁵⁴ The trigger was based upon the assumption that a certain NHP is able to activate its own gene (Figure 9). The behavior of such a trigger model (actually somewhat more complicated with three stable steady states, corresponding to the inactive gene, low and high transcriptional activity) was studied by computer simulation⁵⁴⁻⁵⁷ and the following conclusions were drawn:

1. Two NHP molecules must interact with a gene control sequence in order to allow for more than one stable steady state
2. Switches from the inactive to the active state are produced by temporary action of effectors, which increase the NHP-control sequence affinity at the DNA level
3. While the low activity state corresponds to about 10^3 to 10^4 specific NHP molecules per cell, activating switches can be produced by only a few NHP molecules per cell.^{56,57}

TABLE 6
Coordinates of the Carboxylic End Groups of
Several Retinoids^a

Side chain	Cyclic moiety		
 Å	11.42 ^b 96°11'	13.79 95°45'	—
 Å	10.52 107°24'	12.81 104°52'	—
 Å	10.37 107°32'	— —	— —
 Å	— —	10.09 105°11'	10.09 105°8'
 Å	— —	10.21 107°6'	— —
 Å	8.12 111°43'	10.36 107°27'	— —

^a Polar coordinates: distance between C₆ atom and the carbon atom of the carboxylic acid terminus; the angle between C₅-C₆-C_{CO2H} atoms;

^b For the cisoid structure of the 11,12- and 13,14-double bonds in RA the coordinates of the carboxylic group are: 10.71 Å and 107°14'.

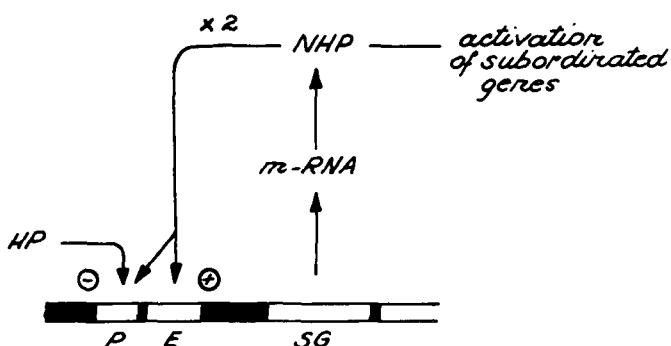
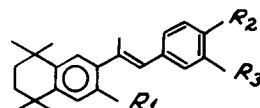


FIGURE 9. Trigger model. P, promoter; E, enhancer; SG, structural genes; NHP, nonhistonic (acidic) nuclear proteins, which control the activation of subordindated genes and also activate the control sequence (P + E); HP, histonic proteins blocking the transcription. Activation of the enhancer produces a higher transcription rate than that of the promoter but requires higher NHP concentration. This hypothetical trigger possesses three stable steady states corresponding to the three levels of gene activity.

TABLE 7
MTD Values Calculated for Retinoic Acid Derivatives Effective
Against F9 Teratocarcinoma Cells and Human HL-60 Promyelocytic
Leukemia Cells



No. ^a	Structure			Activity ^b against		MTD ^c parameter
	R ₁	R ₂	R ₃	F9 ^d	HL-60 ^e	
1	RA	—	—	6.7	7.0	6
51	H	CO ₂ H	H	8.5	6.5	5
115	H	H	CO ₂ H	5.0	6.0	11
116	H	H	H	5.0	6.0	9
117	CH ₃	CO ₂ H	H	6.7	7.7	7
118	C ₂ H ₅	CO ₂ H	H	5.4	6.7	8
119	n-C ₃ H ₇	CO ₂ H	H	5.0	6.0	9
120	i-C ₃ H ₇	CO ₂ H	H	5.4	6.0	9
121	CH ₃ O	CO ₂ H	H	6.7	7.5	8

^a The numbering of the compounds is the same as for Table 2 and 3.

^b Expressed as 1/log ED₅₀ (M).

^c MTD values were calculated according to the receptor map of Figure 5. The substituent R₁ contains noneffective vertices.

^d r = 0.882, n = 9, s = 1.80.

^e r = 0.534, n = 9, s = 1.80.

The receptor map obtained here for the retinoids, with several allowed active conformations for the cyclic and terminal moieties and mainly irrelevant vertices ($\epsilon_j = 0$) in the middle region of the retinoid molecule, suggests the coupling of the retinoid with two receptor sites. One of these should interact with the cyclic moiety, the other with the terminal CO₂H group and a few double bonds located in that region. This suggests that the retinoids act by favouring the dimerization of the two receptor units. A similar situation occurs in the case of estradiol interaction with its specific receptor, when a dimeric protein is also formed.³⁹ If we consider that the retinoid receptor acting in the rate-determining step is a NHP, then the enhancement of NHP-receptor dimerization by the retinoid in our trigger model⁵³⁻⁵⁷ increases the effective affinity of the receptor protein for specific chromatin binding, eliciting a biological response. Because of the few effector molecules needed for the activation of such a trigger, our model could also explain why retinoid binding proteins have not been detectable in cells in which retinoids suppress transformation.

Thus, a similar QSAR-MTD analysis of the results reported by Strickland et al.⁴⁰ on the ability of some aromatic retinoids to induce the differentiation of F9 teratocarcinoma and HL-60 human promyelocytic leukemia cells reveals a very interesting aspect. Using the MTD parameters computed for these compounds on the basic hypermolecule previously built for the analysis of the unified set of retinoids (Figure 5), we obtained a reasonable correlation (r = 0.88) between their activity against F9 teratocarcinoma cells and these MTD values (Table 7). In contrast, a similar approach failed to describe the behavior of the same retinoids in the HL-60 cell system. If we consider that F9 cells possess CRABP, in contrast with HL-60 cells, it is possible to infer that our equations describe retinoid-CRABP interactions. However, this conclusion has to be taken with caution, because of the limited number of compounds available for this analysis (n = 9) and of the inherent limits of such an approach. More work and additional experimental systems are needed in order to elucidate this point.

New data⁵⁸ indicate the presence of other receptors or binding proteins that are involved in retinoid expression in responsive cells. This possibility cannot be excluded.

VII. SUMMARY AND CONCLUSIONS

Quantitative structure-activity relationships were developed for a series of 111 retinoids having modified terminal groups, conformationally restricted and nonrestricted polyenic side chains, and various ring moieties. The predictive potential of these equations was checked on several test series, including 34 different randomly chosen retinoids.

The Minimum Topological Differences parameter makes a significant contribution to predicting the biological activity of the investigated compounds. The much better correlation indices (r, F) of the equations containing the MTD parameter compared with those (r_o, F_o) of the equations that do not contain this term support this position. The use of the more specific index π_{c+m} could also be of interest.

This approach allowed us to point out a series of structural features that are important for the cancerogenesis inhibitory properties of these compounds. The structure of the ring moiety, as well as that of the polar terminus and the neighbouring side chain (11,12- and 13,14-double bonds) are of particular importance for the biological activity of retinoids. The relative position of the carboxylic terminus (defined in polar coordinates) with respect to the ring moiety is another critical parameter for activity. The lipophilicity term has a reduced contribution in the computed equations. This fact agrees with a passive uptake of retinoids by tracheal hamster cells. Nevertheless, it appears as an important tool in describing the interaction of the cyclic moiety with the receptor cavity. These data may be of interest for the design of compounds with improved pharmacological effectiveness.

In order to obtain the best correlation indices for both series, a cisoid conformation for the terminal part (11,12- and 13,14-double bonds) of the side chain of RA and some of its congeners must be assumed. This assumption seems to be confirmed by experimental data.

Some hypotheses regarding the mechanisms of action of retinoids in promoting differentiation, as well as the nature and the possible structure of the receptor described by our equations, were made. Most likely the receptor involved in the rate-determining step interaction with the retinoids is a two-moiety protein molecule. One part of the receptor (probably including a strongly hydrophobic region) interacts with the ring moiety. This interaction might be enhanced by the introduction of a supplementary aromatic ring in this region (as for instance in Ro 13-7410, 51). The second part of the receptor interacts with the terminal region of the molecule. For highly effective retinoids, the carboxylic acid terminus lies in a restricted area located in this part of the receptor.

ADDENDUM

Some recent advances have been reported in the area of retinoid-binding proteins. Thus, the expression of CRBP, CRAP, CRAIBP (retinal-binding protein), and IRBP (interstitial binding protein) was investigated in freshly collected or cultured retinoblastoma cells.⁵⁹ The nuclear RA receptors have also been reported. However, these new findings do not alter our previous conclusions. It still remains difficult to designate whether the proteic receptor described by our computations would be a cytoplasmatic or a nuclear one on the basis of the transport kinetics of the processes. Binding affinities toward different types of receptors should be established for a series of RA derivatives to determine this. Unfortunately, no such data are available as yet.

A new series of 19 conformationally restricted retinoids from SRI International was studied recently using a similar approach. A new molecular descriptor δ_4 accounting for the repulsions appearing in sterically hindered neighboring groups (or atoms) was introduced

(this descriptor being 1 for steric repulsion and 0 otherwise), and the following equation (18) was obtained for a sample containing 42 retinoids (14 compounds of the new series and **1**, **21**, **29** to **31**, **33** to **41**, **47** to **51**, **76**, **77**, **84**, **89**, **99**, **100**, **103**, **104**, and **111**).

$$\log \frac{1}{ED_{50}} = 13.888 + 0.525\pi - 0.852\delta_4 - 0.628(MTD-1) \quad (18)$$

$$n = 42$$

$$r = 0.880$$

$$s = 0.476$$

$$F = 31.93$$

These computations are in progress.

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INDEX

A

Absorption maxima values of Schiff bases, 102
 Absorption spectrum
 of acyclic retinals, 141
 of 11-*cis*-retinals, 203—204
 of M intermediate, 142
 of rhodopsin, 30
 of 12-*s-cis*-locked retinals, 222
 Accutane, 477, see also 13-*Cis*-retinoic acid
 Accutane dysmorphic syndrome, 551, 564
 Acetylenic retinoids, 178—182, 186—190
 Acne, 477
 Acne treatment and teratogenicity, 551
 Actinic keratoses, 475—476
 Activated rhodopsin, see Rhodopsin R*
 Active-site methylated rhodopsin, 5—8
 Active-site modified rhodopsins, 9
 Acyclic polyene aldehydes, 138—140
 Acyclic retinals, 126—128, 141, 161
 Acyclic systems and 12-*s-cis* conformation, 206—207
 Adenosine triphosphate, see ATP
 Adjuvant-induced arthritis, 520—522, 527
 Alcohol dehydrogenase, 234
 Alcohol-dehydrogenase-negative deer mouse, 246
 Alcohol-dehydrogenase-positive deer mouse, 246
 Aldhydes, primary retinal-derived, 217
 Alkylcuprate approach to vinylallene functionality, 213—214
 3-Alkoxy derivatives of retinoid 30, 324—325
 Alkylated retinals, 57, 159
 Alkylation of aryl allyl sulfone ions, 416
 3-Alkyl derivatives of retinoid 30, 324—325
 Allene aldehyde, 214
 Allenic retinal analogs, 161
 Allfit program, 235
 All-*trans*/11-*cis* isomerization, 2, 10—23, 15, 158
 de novo, 18—23
 as energy-requiring process, 10
 in frog, 15
 All-*trans*-retinoic acid
 antiinflammatory activity of, 523
 ornithine decarboxylase inhibition and, 389—390
 in S91-C2 mouse melanoma cells, 253—254
 terata reduction and, 543
 Alpha-band UV absorption, 220, 222—223
 Alpha-difluoromethyltyrosine, 472
 Alpha-interferon, 472
 Alpha-retinals, 57, 61
 2-Aminopyridoimidazole, 276
 Ammonyx LO, 130
 AM80 retinoids, 303
 AM580 retinoids, 303
 Analog pigments, function of, 133—135
 Animal teratogenicity of synthetic retinoids, 551—556
 Anticarcinogenesis, see Antitumor activity; Cancer chemoprevention

Antiinflammatory/antiarthritic effects, 518—529
 Antipapilloma assay protocol, 428—429
 Antiproliferative effect of retinoids, 472
 Antirheumatic potential of retinoids, 520—533
 Antitumor activity, 89—93, 234, 468—470, see also
 Cancer chemoprevention and names of
 specific lesions
 Arachidonic acid metabolism, 527—529
 Aromatic amine containing drugs, 12—15
 Aromatic retinoids, 161
 design and synthesis of, 310—324
 Friedel-Crafts cycloalkylation of, 422
 structure/sensitivity relationships of, 240—245
 synthesis of, 423—425
 Arotinoids, 276
 Arthritis, 520—526, 533
 Artificial bacteriorhodopsin pigments, 103—105
 Aryl allyl sulfone ion alkylation, 416
 9-Aryl-2,4,6,8,-tetraenoic acids, 422
 Asp-83, 66
 Assay protocols, 428—429
 ATP synthesis, 8
 Attenburrow synthesis of retinoid 1A, 180
 Azobenzenecarboxylic acids, 280
 Azobenzenes and stilbenes, 280—281

B

Bacteriorhodopsin, 137
 Bacteriorhodopsin, 8
 artificial, 103—105
 binding restraints of vs. rhodopsin, 143
 binding site of, 136
 function of, 118
 molecular mechanism for, 100—119
 opsin shift in, 100—105
 photocycle of, 101, 108—115, 149
 pKa of, 109, 118
 protonated Schiff base and, 108—111
 spin-labeled pigment analogs of, 140—142
 Bacteriorhodopsin analogs
 retinal binding site and chromophore function in, 135—142
 ring-binding-modified, 169—170
 visual pigments and, 163—171
 Basal cell carcinomas, 476
 Basal cell layer of epidermis, 486
 Bathorhodopsin, 3, 111—113, 130—131
Belonesus sp. and visual pigments, 29
 Benzoic acid 65, 406
 Benzanilides, 277—280
 Benzoic acid analogs, 65, 297, 311—318, 324
 Benzoylaminobenzoic acids, 278—279
 BES2OB rat bladder cancer cell line, 284
 Beta-band UV absorption, 220—222
 Beta-carotene, 246, 541
 Beta ionylidene ring, 202
 Biaryl retinoids, 425—426
 Bicyclic retinal analogs, 85

- Bioassay of new retinoids, 276—277
 Biorganic chemistry of vision, 1—25
 Biosynthesis of interstitial retinol-binding protein, 39—40
 Bladder cancer, 502—504, 562
 Bleaching, 2, 6, 7, 78
 of bovine rhodopsin, 149
 intermediates in, 148
 Blindness, 12
 Blood-testes barrier, 232
 Bond isomerization and rotations in rhodopsins, 112—113
 Bone toxicity of retinoids, 477—478
 Bovine opsin
 isomers of, 59—62
 retinal analogs active toward, 63
 Bovine porphyropsin, 28
 Bovine rhodopsin, 2
 analogs of, 69
 bleaching of, 149
 photocycle of, 101, 111
 primary photochemical event in, 111—114
 visual pigment analogs of, 150—163
 Breast cancer, 472, 504—510
 Burns, 478—479
 10-Butyl-(9-pentyl) retinal, 61
- C**
- cAMP dibutryl, 235
 cAMP-dependent protein kinase and growth inhibition, 366
c-myc expression, 284
 Cancer chemoprevention, 239—240, 308, 353, 414, 504, 560—564, see also Antitumor effects and names of specific lesions
 12,13-cisoid side chain topography and, 357
 of mammary cancer, 505—510
 mode of administration and, 474
 toxic side effects and, 390
 Carboxylic acid-terminated retinoids, 414
 Carcinogenesis, 366, 468—470
 experimental, 502—516
 inhibitors of, 577
 in skin, 386—389
 Carcinoma, see specific types
 Carotenoids
 extraintestinal metabolism of, 234
 nontoxicity of, 540—541
 Cell differentiation, 308—309
 Cellular *c-myc* expression, 284
 Cellular retinoic acid-binding protein (CRABP), 261—262, 284, 367—369
 binding of, 243—244, 297, 369—370, 372
 as biological mediator, 369
 in cytosol, 374
 in epidermis, 490—491
 function of, 302
 $[^3\text{H}]$ retinoic acid specificity of, 367
 melanoma cell growth and, 260—262
 in mammary tissue, 513—514
 in mutant S91—C2 cells, 262
 in rat tissues, 232
 retinoic acid action and, 366—369
 retinoids and, 369—371
 transportation of, 472
 teratogenicity and, 559
 Cellular retinol-binding protein (CRBP), 41—44
 function of, 302
 in rat tissues, 232
 retinoid transport and, 472
 c-GMP hydrolysis, 2
 Chalcone-4'-carboxylic acid derivatives (Ch series), 281—282, 299
 Chalcones and flavones, 281—282
 CHAPS (detergent), 130, 150
 CHAPSO detergent, 150
 Chemical bleaching, see Bleaching
 Chemical carcinogenesis, see Carcinogenesis;
 Tumorigenesis
 Chemicals used in assays, 428
 Chemoprevention of cancer, see Cancer chemoprevention
 Chick epidermis assays, 308
 Chlorinated trimethylmethoxyphenyl (TMMP) and papilloma regression, 391—392, 402—404
 2'-Chloro-6'-fluoro-phenylretinal, 62
 10-Chlororetinal, 59
 12-Chlororetinal, 59
 14-Chlororetinal, 59
 Cholesterol sulfate, 289, 292
 Ch55 retinoids, 303
 Chromophore binding site of rhodopsin analogs, 126—130
 Chromophore of fly visual pigment, 86—89
 Chromophore/opsin interactions, 105—108
 C3H/10T 1/2 mouse fibroblast cell line, 471
 Ciliated cells, 288
 Circular dichroism spectrum of rhodopsin, 83—86
 11-cis-3-dehydroretinal, 28
 7-cis-isomers, 52—53
 9-cis-isomers, 53—54
 11-cis-isomers, 54, 202
 13-cis-isomers, 54—55
 11-cis-linked chromophores, 150—157
 13-cis-locked chromophores, 166
 11-cis-locked cyclopentatrienylidene retinals, 79—81, 83
 11-cis-locked cyclopentatrienylidene rhodopsin, 79—81
 13-cis-N-ethylretinamide, 239
 12,13-Cisoid side chain topography and cancer chemoprevention, 357
 9-cis-retinal, 134
 11-cis-retinal, 14, 126
 13-cis-retinal, 134 223
 11-cis-retinal palmitate, 19
 13-cis-retinoic acid
 antiinflammatory activity of, 523
 in arthritis, 521
 chemopreventative duration of, 474
 immunologic side effects of, 533

in melanoma cell inhibition, 255
 oral administration of, 476, 551—552
 squamous metaplasia and, 502
 teratogenicity of, 556
 topical vs. dietary administration of, 474
 toxicity of, 477
 tumor initiation and, 470, 474
11-cis-retinoids
 absorption spectra of, 203—204
 de novo synthesis of, 18—23
 isomerization and in eye, 10—23
9-cis-retinoyl fluoride, 3—5
9-cis-retro-gamma-rhodopsin, 78—79
11-cis-rhodopsin, 65
9-cis-rhodopsin absorption spectra, 163
13-cis-RSBH⁺, 117
 Clara cells, 288
 Clotrimazole, 239
 Cloudman S91 cell line, 472
 C14-methyl retinoids, 204
c-mos proto-oncogene, 284
 Collagen-induced arthritis, 522—526
 Combination chemotherapy, 509
 Conformational analysis of retinoids, 202—204
 Conformationally restricted retinoids, 591—594
 Congenital defects, 541—542; see also Teratogenicity
 CRABP, see Cellular retinoic acid-binding protein
 Craniofacial/CNS/thymic/cardiac malformation syndrome, 478
 CRBP, see Cellular retinal-binding protein
 Cross-linked envelope formation, 292
 Crystal structure of visual chromophores, 202
 Culture systems, disadvantages of, 240
 Cutaneous cancer, 468—479
 Cutaneous lupus erythematosus, 532—533
 Cyclohexal ring, 140
 Cyclohexene (beta-ionylidene) ring, 202
 Cyclosporin A, 524
 Cytosol CRABP, 374
 Cytosolic activity of retinoic acid, 233
 Cytotoxicity, 92, see also Antitumor activity

D

Dark adaptation, 2, 32, 135
 Dealkylated retinals, bovine-opsin-binding, 159
 3-Dehydroretinal, 61
 13-Demethylretinal, 61
 Design of new retinoids, 276—277
 13-Desmethylretinal, 134
 Detergent effects, 130
 Dexamethasone, 472
 Diaminophenoxyptane, 12—15, 17
 Dibutryl cAMP, 235
 9,13,-Di-cis-retinal, 133
 2-Difluoromethylomithine, 386
 Digitoxin, 130
 Dihydroretinals, 5—6, 135, 160
 18,18-Dimethyl-alpha-(5-isopropyl) retinal, 61
 7,12-Dimethylbenz/[α]-anthracene in mammary carcinogenesis, 504—507, 511

Dimethylcyclohexanes, 128
 Dimethylhydrazine, 386
 19,19,-Dimethyl-(9-isopropyl) retinal, 60
 3,7-Dimethyl-9-phenyl-2,4,6,8—nonatetraenal retinal, 62
 DNA, SQ10 and SQ37 recombinant, 291
 Dose-response in S91—C2 melanoma cells, 252—254, 260—262, 265
 Drug-resistant cell mutants, 262
 Dysplastic nevus syndrome, 471—477

E

Eglington, Glaser, Cadot-Chodkiewicz coupling, 180
 Eight-membered ring rhodopsin, see Rh8
 Electron spin resonance spectroscopy, 129
 Electrotoretinography, 133—134
 Embryogenesis, 540, see also Teratogenicity
 Embryonal carcinoma cells, 235—240
 Enzymatic labeling, 161
 Enzymes of visual cycle, 17
 Epidermal growth factor (EGF), 290
 Epidermal hyperplasia, 489—496
 Epidermal responses in vivo, 485—497
 Epidermis, 486—487
Epidermodysplasia verruciformis, 476
 Epithelial differentiation of cultured hamster trachea, 308—358
 4-[E-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl)-2-naphthalenyl]benzoic acid, 257—259, 369—371, 489—496
 Ethanol oxidation, 234
 Ethanoretinal, 57
 11,19-Ethano-(9,11-ring-locked) retinal, 61
 11,20,-Ethano-,11,13-ring-locked retinal, 61
 18-Ethyl,(5—n-propyl) retinal, 60
 10-Ethylretinal, 60
 Etretinate, 276, 288, 468
 antiinflammatory activity of, 523
 vs. ibuprofen, 529—530
 in psoriasis, 551
 Experimental carcinogenesis, 502—516
 Extraintestinal carotenoid metabolism, 234
 Eye, *trans/cis* retinoid isomerization in, 10—23

F

F9 cell differentiation, 241—243
 F9 cell line, 234, 284
 laminin secretion in, 235
 retinoic acid/retinol activity in, 238
 18,18,18-F3,(5-CF3) retinal, 60
 19,19,19-F3,(9-CF3) retinal, 60
 20,20,20-F3-(13-CF) retinal, 60
 10,12-F2-13-demethylretinal, 61
 13,14,F2-13-demethylretinal, 61
 F9 embryonal carcinoma cell, 235—237
 Fetal calf serum, retinoic acid in, 231
 Fetal malformations, 540—542, see also Teratogenicity
 Fetal retinoic acid, 231

- Fetal vs. postpartum epidermis, 486—487
 Fibrinolysis, 236
 Fish visual pigments, 28—29
F9/laminin/ELISA assays, 239
 Flavonecarboxylic acids, 282
 Fluorescence labeling, 161
 Fluorinated alcohols, 103
 Fluorinated retinals, 55—57
 10-Fluoro-alpha-retinal, 61
 12-Fluoro-3-dehydroretinal, 61
 10-Fluoromesityl-retinal, 62
 8-Fluororetinal, 59
9-cis-10-Fluororetinal, 66
 10-Fluororetinal, 59
 12-Fluororetinal, 59
 14-Fluororetinal, 59
 Fly visual pigment chromophore, 86—89
19F-NMR labeling, 69—71
 Freshwater vs. saltwater habitat and visual pigments, 28
 10,14-F₂-retinal, 60
 Friedel-Crafts cycloalkylation of aromatic systems, 422
 Frog porphyropsins, 28
 Frog rhodopsin, 30

G

- Gamma-band UV absorption, 222—223
 Gel-filtration chromatography, 37
 Gene expression and TPA, 473
 Geometrical shape and binding activity, 299
 Geometric isomers of retinal, 52—54
 Golden syrian hamster studies, 551—553
 Goldfish porphyropsins, 28
 G-protein activation, 9, 11
 Grignard derivative of retinoid 8, 180
 Growth control mechanisms, 472—473

H

- Hairless mouse, vitamin A and retinoid supplementation in, 488—491
[15-³H]all-*trans*-retinal, 16, 20—23
Halobacterium halobium, 100, 135, 148, 163, 204
4-Halo-retinals, 136
Hamster trachea
epithelial differentiation in, 308—358
organ culture of, 309—310
Harvey sarcoma viral infection, 474
2H-dihydroretinals, 160
18-Hexy-alpha-(5-heptyl) retinal, 62
High-performance liquid chromatography, 150, 238
HL 60 cell line, 277, 300
1H NMR data for retinals 79a-h, 219
Hormonal deprivation therapy, 510
Horner reactions, 54
Horner-Wadsworth-Emmons assays, 416—417
³H retinoic acid nuclear uptake of, 515
⁴H tetrahydroretinals, 160
Human blood/human plasma, retinoic acid in, 230

- Human interstitial retinol-binding protein, 36
Human malformation syndrome, 540, see also Teratogenicity
Human melanoma biopsy tissue studies, 472
Human papilloma virus 5, 476
Human promyelocytic leukemia HL 60 cell line, 278
Human rheumatic disease, 529—533
Hydrogenated retinals, 160—161, 166—169
Hydrogen bonding, 70
Hydrophobic pocket in opsin binding site, 64—65
Hydroxylamine decomposition of acyclic pigments, 141
2-Hydroxyretinal, 86—89
3-Hydroxyretinal, 86—89
4-Hydroxyretinal, 134, 239
Hyperkeratosis, 389
Hyperplasia of epidermis, 308, 488—496
Hyperpolarization of rod cells, 2
Hypervitaminosis A, 395, 477, 520

I

- Ibuprofen vs. etretinate, 529—530
[I,j]-sigmatropic hydrogen shift, 207—210
Illumination and visual pigments, 28—29
Imidazole antimycotics, 239
Immunocytochemical localization of interstitial retinol-binding protein, 35—36
Immunologic abnormalities and 13-*cis*-retinoids, 533
Immunosuppression, UV-induced, 478—479
Indomethacin, 522—523
Interphotoreceptor retinoid-binding protein, 39—40
genetic characteristics of, 36—39
immunocytochemical localization of, 35—36
in mammalian pineal gland, 40—41
purified, 36
in vertebrate species, 39
in visual cycle, 41
Intestinal retinol, 234
Intracellular retinoid binding proteins, 41—44
Invertebrate rhodopsin, 10
Invertebrate/vertebrate differences in visual cycle, 31—32, 34
In vitro activity of retinoids, 561
In vitro assay of hamster tracheal organ culture, 308—308
Isomers of retinal analogs and bovine opsin, 59—62
10-Isopropyl-3,7,11-trimethyl-2,4,6,8,10-dodecapentaenol, 61
Isoretinoin, see 13-*Cis*-retinoic acid
Isorhodopsin, 132

J

- JB6 C141 mouse cell line, 470—471
J625 intermediate, 112—113

K

- Keratin accumulation in vitamin A deficiency, 308

Keratinization, 308, 371—373
 Keratinizing dermatoses, 477—478
 Keratocanthoma, 476
 Ketoconazole, 239
 K610 intermediate, 112—113

L

Labeling, 161
 Laminin/ELISA assays, 235—236, 239
 Life cycle and rhodopsin/porphyrin ratio, 28
 Light bleaching, see Bleaching
 Linking chain conformations, 282—284
 L350 intermediate, 115, 118
 Lipophilicity, 325
 Liver adenomas, 562
 Locked retinoids, 204—205
 Lung cancer biopsy tissue studies, 472
 Lupus erythematosus, 532—533
 Lyngbyatoxin A, 276
 Lys 296, 2, 66

M

Mammalian cell lines, retinoic acid synthesis in, 233
 Mammalian rod and cone pigments, 3
 Mammary carcinogenesis, 504—510, 562
 Mammary gland action of retinoids, 513—516
 Mammary gland organ culture, 510—512
 Megadose vitamin A therapy, 542, see also
 Hypervitaminosis A
 Melanogenesis, 262—264, 274
 Melanoma cell growth inhibition, 252—272
 Melanoma S91 cells, 301
 Mercocyanines, 169
 Mesitylretinal, 62
 Metabolic activation/deactivation of retinoic acid, 325
 Metarhodopsin, 131—133
 Metarhodopsin II, 3—4
 3-Methycholanthene and squamous metaplasia, 308
 4-Methylpyrazole, 17
 10-Methylretinal, 60
 11-Methylretinal, 60
 12-Methylretinal, 60
 14-Methylretinal, 60
 Miconazole, 239
 M intermediate absorption, 142
 M412 intermediate, 115—116, 118
 ML1 leukemia cells, 284
 Mono Q chromatography, 368
 Mouse skin model of tumorigenesis, 473—475
 Mucociliary epithelium and vitamin A deficiency,
 288—291
 Mucous cells, 288, 309
 Murine melanoma inhibition, 252
 Murine melanoma S91—C2, 252—254, 260—262
 Mutant cells, 262

N

NADH as retinoic acid synthesis inhibitor, 234

Naphthalenecarboxylic acid retinoids, 318—323
 Natural retinoids and teratogenicity, 540—551
N-butyl-*N*-4-hydroxybutylnitrosamine, 502—503
 Neonatal tolerizing procedures, 478—479
 Neoplastic transformation, 470—471
 Neural cleft defects, 543
N-(4-hydroxyphenyl)retinamide, 495
 antiinflammatory activity of, 523
 in arthritis, 522—524, 533
 in mammary cancer, 505, 508—511, 513, 562
 Night blindness, 12
N-methyl-*N*'-nitro-*N*-nitroguanidine, 386
N-methyl-*N*-nitrosurea, 502—508, 514
 4-(*N,N*-dimethylamino)azobenzene, 276
 4-(*N,N*-dimethylamino)stilbene, 276
N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, 502—
 503
 Nonactive-site lysine modified rhodopsins, 8—11
 Nonaromatic retinoids and F9 cell differentiation, 240
 Nonolefinic retinoids, 427
 Nonsteroidal inflammatory drugs, 521—526
 19-Nor-9,10-didehydroretinal, 182—186
 19-Nor-7,8,9,10-tetrahydroretinal, 182—186
 19-Nor-9,10,11,12-tetrahydroretinal, 182—186
 Northern blot test of human interstitial retinol-binding
 protein, 38
Notemigous sp. and visual pigments, 29
 NRK 49F cell line, 284
 Nuclear magnetic resonance of 12-*s-cis*-locked
 retinals, 217—218
 Nuclear magnetic resonance studies, 69

O

Olefination reactions, 417
O-methyl ester of all-*trans*-retinol, 16
 Opitz trigonocephaly, 551
 Opsin binding sites
 characteristics of, 58—67
 hydrophobic pocket in, 64—65
 limitations within, 67—69
 in rat, 133
 visual pigment analogs and, 58—71
 Opsin/retinal interactions, 113—114
 Opsin shift, 3, 100—105
 Optic nerve and pigment regeneration, 18
 Organ culture methods, 308, see also Tracheal organ
 culture assays
 Ornithine decarboxylase, 366
 inhibition of, 388—395, 430—450
 in tumor promotion, 386—388
 Ornithine decarboxylase assay, 386, 428
 Ossification alterations as side effects, 478
 Osteoporosis as side effect of retinoid therapy, 477—
 478
 Ovarian cancer biopsy tissue studies, 472
 Ovariectomy in mammary cancer, 510

P

Palate/limb morphogenesis, 543

- Pancreatic carcinoma, 562
 Papilloma inhibition, 389
 Papilloma regression, 391, 396—400, 402—404, 406, 408—413, 563
 Passively transferred adjuvant-induced arthritis, 527
 Permethylated rhodopsin, 7
 Perturbation of the C=C bond, 106
 Pharmacoco-cosmetic applications, 486
 Pharmacology and toxicology of retinoids, 539—565
 Phenetidine-like compounds, 13
 Phosphatidylethanolamine, 31
 Phospholipid vesicles, 164
 Photoaffinity labeling, 161, 170, 245—246
 Photochemical reaction
 of 11-cis-locked cyclopentatrienylidene rhodopsin, 79—81
 of 9-cis-retro-gamma-rhodopsin, 78—79
 Photocycle
 rhodopsin pKa change in, 109
 of rhodopsins, 108—111, 114—115
 Pigment analogs, see specific compounds
 Pigment epithelium, 31
 Pigment epithelium, 10, 23, 31—33
 Pigment regeneration and optic nerve, 18
 Pineal gland, 40—41
 Placental permeability, 556—560
 Placental retinoic acid, 231
 Plasminogen activator assays, 236, 237
 Platelet-derived growth factor (PDGF), 473
 Polar-terminus-modified retinoids, 323—324, 405—406
 Polycyclic hydrocarbons, 308
 Polyene chain, 186, 202
 Polyene synthesis, 415, 419, 421
 Polyenoic acid analogs, 414—422
 Porphyrropsin, 28—31
 Postpartum vs. fetal epidermis, 486—487
 Postsurgical use of retinoids in mammary cancer, 508
 Prebathorhodopsin, 112
 Preneoplastic cells, 371
 Preneoplastic lesions, 475
 Prevention of neoplastic transformation, 470—471
 Prickle cell layer of epidermis, 486
 Primary retinol-derived aldehydes, 217
 Proliferative skin diseases, 414
 18-Propyl-(5-n-butyl) retinal, 60, 62
 Prostaglandin A, 472
 Prostatic epithelium, 308
 Protein dissociation in photocycle, 114
 Protein kinase C, 387
 Proto-oncogene *c-mos*, 284
 Psoriasis, 276, 391—392, 551
 Psoriatic arthropathy, 529—532
 Putrescine, 386
- Q**
- Q-cis-retinal, 23
 QSAR-MTD analysis
 of conformationally restricted retinoids, 591—594
 congeners used for, 580—581
- correlation of data in, 598—603
 methodology of, 576—579
 of retinoids, 579—591
 of unified set of retinoids, 594—598
 Quantitative structure-activity relationship/Minimal topical distances assay, see QSAR-MTD analysis
- R**
- Rabbit tracheal cells, 288—291
 Raman spectroscopy, 165—166, 168
 Rat liver, 231—233
 Rat opsin, 133
 Rats, vitamin A-deprived, see Vitamin-A deprived rats
 Rat tissues, retinoic acid in, 230—231
 Regeneration reaction, 126—127
 Reiter's disease, 532
 Reproductive function, 541
 Resonance Raman data, 165—166, 168
 Retina, threshold sensitivities of, 10
 Retinal
 analogs and visual pigments from, 59—62, see also specific compounds and structures
 dose-response curves for, 495
 micellaneous, 57—58
 synthesis of, 52—58
 isomers of, 52—55, 59—62, 115—117
 synthesis of, 52—58
 Retinal/opsin interactions, 113—114
 Retinal palmitate, 389, 479
 Retinal photoreceptors, 39
 Retinals
 alkylated, 57
 alpha-, 57
 fluorinated, 55—57
 hydrogenated, 160—161
 ring-binding-site modified, 161—163
 side-chain modified, 157—160
 Retinobenzoic acid, 276—285
 AM 80, 279—280
 AM 580, 279—280
 biological activity of, 284
 linking chains of, 282—284
 Retinoblastoma, 40, 41
 Retinoic acid
 affinity of for plastics, 231
 albumin binding of, 367
 biogenesis of, 230—246
 biological activity and CRABP binding of, 297
 biosynthesis of, 232—235
 cAMP-dependent protein kinase as target of, 366
 in cell differentiation, 325
 cytosolic activity of, 233
 elimination half-life of, 240—241
 endogenous origin of, 230—232, 366
 in fetal and placental tissue, 231
 function of, 366
 in human blood or plasma, 230—231

- mechanism of action, 371—379
 metabolic activation/deactivation of, 325
 murine melanoma and, 252
 pharmacologically dosed vs. endogenous, 230
 physiological occurrence of, 230—232
 physiological role of, 246
 polar terminus-modified analogs of, 323
 polyenoic acid analogs of, 414—422
 potency of vs. retinol, 237
 retinoic acid metabolites vs., 238—240
 in S91-C2 mouse melanoma cells, 255
 squamous cell differentiation and, 291
 structure of, 370, 429, 453—457
 synthesis of and NADH, 234
 tumor initiation and, 470
 Retinoic acid analog classification, 414
 Retinoic acid-resistant cells, 262
 Retinoic proteins, 366—367
 Retinoid activity and structural modification, 390—457
 Retinoidal butenolide synthesis, 89—93
 Retinoidal lactones, 89
 Retinoidal polyenes, 414—422
 Retinoidal 4-ylidenebutenolides, 90
 Retinoid inhibitory activities, 457
 Retinoid receptor, 353, 558
 Retinoids
 acytylenic, 178—197
 antiinflammatory mechanism of, 526—529
 as antirheumatic agents, 520—533
 aromatic, 240—245
 baryl, 425—426
 biological activity of, 299, 325
 cancer chemoprevention, see Cancer chemoprevention
 cell differentiation and in hamster, 308—309
 cellular mechanism of action of, 302—303
 cellular responses to, 470—473
 cellular retinoic acid-binding protein and, 369—371
 cholesterol sulfate accumulation by, 292
 C14-methyl, 204
 conformational analysis of, 202—204
 conformationally restricted, 405, 591—594
 CRABP binding affinity of, 369—370, 372
 cross-linked envelope formation by, 292
 definition of, 301
 design of, 276—277
 equilibrium positions of, 10
 extracellular transport of, 34—41
 in vitro activity of, 561
 in vivo epidermal responses to, 485—497
 locked, 204—205
 mammary gland action of, 513—516
 mammary gland development and, 511
 melanoma cell growth inhibition by, 252—272
 nomenclature, structure, and teratogenicity of, 544—550
 nonolefinic, 427
 ornithine decarboxylase inhibition by, 386—458,
 see also Cancer chemoprevention; Ornithine decarboxylase
 pharmacology and toxicology of, 539—565
 as photoaffinity probes, 245—246
 polar-terminus-modified, 405—406
 potency of, 240—245
 12-*S-cis* conformationally locked, 202—224
 side effects and toxicity of, 477—478
 SRI, 325, 353—356
 12-*s-trans*-locked, 205—206
 structure/activity relationships of, 291—302, 386
 sulfur analogs of, 406—407
 teratogenicity of, 276, 478, 554
 therapeutic and chemopreventive activities of, 560—564
 toxicity of, 276, 366
 transglutaminase inhibition by, 292
 UC Riverside, 356—357
 Retinoid synthesis
 acytylenic intermediates in, 177—197
 novel building blocks in, 190—197
 thermal rearrangement of vinylallene in, 207—210
 Retinol, see also Vitamin A
 dose-response curves for, 495
 metabolism of, 468
 potency of vs. retinoic acid, 237
 retinoic acid in EC cells vs., 237—238
 Retinol dehydrogenase, 234
 Retinol isomerase, 31—34
 Retinotoxic aromatic amines, 12—15
 Retinyl acetate, 506—514
 Retinyl ester formation in all-*trans*/11-*cis*
 isomerization, 24
 Retinyl esters
 hydrolysis of, 31
 metabolism of, 468
 mobilization of, 31
 synthesis of, 31
 Retinylidene diketones, 563—564
 Retinyl nitrones, 324
 Retinyl palmitate, 474, 505
 Retro-gamma-retinals synthesis, 78
 Rh7, 151—156
 absorption and circular dichroic spectrum of, 153
 photolysis of, 154
 picosecond absorption of, 154
 Rh8, 157
 Rh9, 157
 Rheumatic disease in humans, 529—533
 Rheumatoid arthritis, 533
 Rhino mouse, 389, 491—492
 Rhodopsin
 binding restraints of vs. bacteriorhodopsin, 143
 bleaching and regeneration of, 30—31
 bovine, see Bovine rhodopsin
 circular dichroism spectrum of, 83—86
 11-*cis*-locked cyclopentatrienylidene, 79—81
 conformational, see Rhodopsin R*
 developmental stage and, 28
 excited-state level of, 155—156
 fluorinated, 69—71
 8-membered ring, 157
 9-membered ring, 157

- 7-membered ring-containing, 157
 nonactive-site lysine modified, 8—10
 permethylated, 7
 single counterion model of, 157
 structure and function of, 2—10
 two-counterion model of, 157
 vertebrate vs. invertebrate, 10
- Rhodopsin analogs
 function of, 133—135
 retinal binding site and chromophore function in, 126—135
 uses of, 126
- Rhodopsin bleaching, see Bleaching
- Rhodopsin R*, 3—8
- Ring analogs and papilloma regression, 410—413
- Ring-binding site labeling, 161—163
- Ring methyls, 127—128
- Ring-modified retinoids, 420
- RNA, squamous-cell-specific, 289—291
- Ro13-7410, 297
- Ro17-0778 in mammary cancer, 506
- Rod and cone pigments in mammals, 3
- Rod cell hyperpolarization, 2
- Ro 13-6298 in psoriatic arthritis, 532
- RSBH⁺, 108—111, 116—118
- S**
- Sarcoma growth factors (SGF), 471
- S91 cell line, 284
- Schiff base formation, 2, 3—8, 13, 126
- Schiff base linkage chromophore/opsain interactions, 105—108
- Schiff base model systems, 223
- Schiff base protonation, 3
- Schiff bases, 102, 104
- 12-*s-cis* conformation in acyclic systems, 206—207
- 12-*s-cis* conformationally locked retinoids, 202—224, 407
- absorption spectrum of, 222
- spectroscopic properties of, 219—223
- thermal and photochemical conversions of, 217—219
- S91-C2 mouse melanoma cells, 252—254, 260—262—265
- Sephadex chromatography, 230, 368
- Serous cells, 288
- Sertoli cells, 232—233
- Serum retinol-binding protein, 366—367
- Seven-membered ring-containing rhodopsin, 157
- Side-chain alkylation, 157—160
- Side chain analogs and papilloma regression, 410—413
- Side-chain modified retinals, 157—160
- Side effects and toxicity in tumor therapy, 477—478
- Sigmatropic hydrogen shifts, 207—210
- Skate retina as model system, 133
- Skin carcinogenesis, 386—389
- Skin papillomas, 389
- Skin toxicity of *N*-(4—hydroxyphenyl)retinamide, 533
- S91 melanoma cells, 301
- Sodium dodecyl sulfate polyacrylamide gel electrophoresis of interstitial retinol-binding protein, 37
- Solid state magic angle spinning (MASS) experiments, 166
- Southern blot test of interstitial retinol-binding protein, 38
- Spectroscopy, 165
- Spermatids, 233
- Spermatocytes, 233
- Spermatogenesis, 232
- Spin-labeled bacteriorhodopsin pigment analogs, 140—142
- Spin-labeled rhodopsin pigments, 129—130
- Spontaneous abortion, 478
- SQ10 and SQ37 recombinant DNAs, 291
- Squamous cell carcinoma, 476
- Squamous cell differentiation in tracheobronchial epithelial cells, 288—304
- Squamous-cell specific RNAs, 289—291
- Squamous metaplasia, 288, 308, 389, 510
- SRI retinoids, 325, 353—356, 357
- Sterically hindered cycloalkanones, 416
- Steroid action vs. retinobenzoic acid action, 284
- Steroids, 524, 541
- Stilbenecarboxylic acids, 280—281
- Stilbene-4-carboxylic acids, 276
- STIR spectroscopy, 150
- Stratum corneum, 486
- Stratum granulosum, 486
- Stratum spinosum (prickle cell layer), 486
- Streptococcal cell wall-induced arthritis, 522—526
- Structural modification of retinoids, 414—428
- Structure/activity relationships in hamster tracheal culture, 324—325, 353—358
- 4-Substituted benzoate 66, 408—409
- Sulfone alkylation reactions, 416, 418
- Synthetic Schiff bases, 104
- Syrian hamster studies, 389, 551—552
- T**
- Teleocidin B-4, 276
- Teratocarcinoma cells, 235—237
- Teratocarcinoma stem cells, see Embryonal carcinoma cells
- Teratogenicity, 478
- biochemical basis of, 558
- generalizations regarding, 554
- of natural retinoids, 540—551
- of synthetic retinoids, 551—556
- Terephthalic anilides, 277—278, 299
- Terminal group series, QSAR-MTD analysis of, 597
- Testicular atrophy, 232
- 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 256—257, 386—389, 430—457, 473
- 5,6,7,8-Tetrahydroretinal, 60, 160
- T26 fibroblast cells, 284
- Therapeutic activities of retinoids, 560—564
- Thermal and photochemical conversions of 12-*s-cis*-locked retinals, 217—219
- Thermal isomerizations of retinal isomers, 115—117

Thin-layer chromatography, 230
 Threshold sensitivities of retina, 10
 Tigason, see Etretinate
 3T3 mouse cell line, 471
 O-Tolyretinal, 62
 Toxicity vs. control of cell differentiation, 366
 Toxicology and pharmacology of retinoids, 539—565
 Traceal organ culture system (TOC), 288, 309—310
 Tracheobronchial squamous cell differentiation, 288—304
 Transduction, 2
 Transglutaminase inhibition, 292
 13-*Trans*-locked chromophores, 166
Trans-retinoid isomerization, 12—23
Trans-retinol, see All-*trans*-retinal
 Transforming growth factor beta, 290
 Treacher Collins syndrome, 551
 Tretinoïn, see Retinoic acid
 13-Trifluoromethylretinal, 190
 3,7,11-Trimethylododeca-2,4,6,8,10-pentaenal, 61
 Tritium-release experiments, 16—18
 Triton X, 130
 Tumorigenesis, 386—390, 424, 468—470, 473—475
 Tumor prevention, see also Cancer chemoprevention, 424—475
 Tumor promotion, 424
 ornithine decarboxylase induction and, 386—388
 retinoid inhibitory effects on, 389—395
 Tumor promotion assay protocol, 428—429
 Tumor regression, 395—413
 Tyrosinase, 263

U

UC Riverside retinoids, 356—357
 U937 leukemia cells, 284
 Urinary bladder, see Bladder
 UV absorption of 12-*s-cis*-locked retinals, 220—223
 UV-induced immunosuppression, 478—479

V

Vertebrate/invertebrate differences in visual cycle, 31—32, 34
 Vertebrate retina, 28
 Vertebrate rhodopsin, 10
 Vertebrate interstitial retinol-binding protein, 34, 39
 Vinylallene
 functionality elaboration of, 210—214
 thermal rearrangement of, 207—210, 215
 Vinylcuprate approach to vinylallene functionality, 210—213
 Vinylic and allylic fluorine-labeled retinals, 69
 Vision, biorganic chemistry of, 1—25
 Visual chromophore crystal structure, 202

Visual cycle, 2, 10, 17
 drugs affecting, 12—15
 extracellular retinoid transport in, 34—41
 interstitial retinol-binding protein in, 41
 intracellular retinoid binding proteins in, 41—44
 molecular base of, 28—44
 vertebrate/invertebrate differences in, 31—32, 34
 Visual pigments, 28—31, see also specific pigments
 bacteriorhodopsin analogs and, 163—170
 developmental stage and, 28
 function of, 118
 illumination and, 28—29
 molecular mechanisms of, 100—119
 opsin binding and, 58—71
 regeneration of, 58
 species differences in, 28—29
 species differences in, 28—29
 Visual purple, see Rhodopsin
 Visual yellow, see All-*trans*-retinal
 Vitamin A, see also Retinol
 acetylenic analogs of, 178—182
 activity of, 366
 carbon oxygen bond of, 23
 epidermis and, 486—487
 functions of, 366
 natural and teratogenicity, 542—543
 spermatogenesis and, 232
 toxicity of, see Hypervitaminosis A
 transesterification of, 24
 water-miscible in pregnancy, 542
 Vitamin A acid, see Retinoic acid
 Vitamin A deficiency
 direct and indirect effects of, 309
 epithelial effects of, 408, 486—487
 mucociliary cells and, 288
 reproductive function and, 540—541
 supplementation in, 488—496
 teratogenic effects of, 540—542
 visual defects and, 540—541
 Vitamin A-deficient rats, 133—135, 389
 Vitamin D, 209

W

Wavelength regulation, 3, 169
 Wittig condensation of allene aldehyde, 214

X

Xanthopsin, see All-*trans*-retinol
 Xerophthalmia, 541

Z

Z-selectivity in Horner reactions, 54