DRUG STEREOCHEMISTRY

Analytical Methods and Pharmacology

Second Edition, Revised and Expanded

edited by
Irving W. Wainer

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Irving W. Wainer

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To my wife, Pamela Zulli—my best friend,
companion, and constant source of inspiration.

Series Introduction

In the beginning, medicinal therapy consisted of concoctions coadministered with incantations. Little thought was given to dissecting out the contributions of the pharmacology of the concoction from the spiritual consequences of the incantations. Eventually, potions and extracts were recognized to have predictable activity that could be observed and described objectively. Then came the capacity to estimate potency, leading to concentration and purification. And finally came chemical identification and synthesis and the currently fashionable demand that a modern medicament must be a pure single chemical entity. With synthesis replacing nature, the chemical aspect of pharmaceutical science had gone as far as it could go—or had it?

The longing to know exactly what is in a medicament is axiomatically rational beyond challenge. It is the first step in the intelligent preparation and use of a therapeutic agent. The task is much simplified if the medicinal is a single chemical entity. In some scientific medicinal circles, acceptability of a medicine is inversely proportional to the square of the number of its active components.

But what is meant by a simple active component? Most modern pharmaceuticals are weak acids or bases that exist in ionized and unionized form as a strict consequence of the nature of their solvent, and particularly the pH of aqueous solutions. These distinct forms are radically different in terms of several biologically critical characteristics, including physiological disposition and receptor binding. While such agents are generally formulated as a single substance, usually as a particular salt or as the free acid or base, on dissolution in the body there is rapid conversion into a specific equilibrium of charged and uncharged molecules. Neverthe-

less, such agents are quite properly considered a single component biologically, even though a mixture of chemical species exists.

But what about stereoisomers? Not too long ago the question was considered largely theoretical, since many synthetic drugs were racemates for which there were no practical means of resolution. Efforts to prepare and isolate pure enantiomorphs were generally perceived as interesting chemical exercises of little practical pharmaceutical importance because it was presumed that both enantiomorphs were equally active, or one of the pair was totally inert, or the enantiomorphs would spontaneously racemize in solution. Now we know better. The *d* versus *l* forms of amphetamine are perhaps one of our best and oldest examples of important pharmacological differences between enantiomorphs of the same agent.

Some natural products, such as heparin, have not yet succumbed to the onslaught of synthesis or genetic engineering and continue to be produced and used as a spectrum of closely related chemical entities, rather than a single species of precisely known chemical structure. Such exceptions to the single-component objective are becoming increasingly rare, and unresolved racemates as drugs are undergoing a similar fate.

In view of the current attitude toward mixtures or drug combinations, it is as difficult to justify the use of a racemate as it is to use a mixture of other analogs. With genetic engineering to help the synthetic chemist, even complex structures with a high degree of stereospecificity are being developed as the rule rather than the exception. It is therefore particularly timely to have available an in-depth review of the problems and opportunities resulting from our current knowledge of the stereochemical aspects of pharmaceuticals.

If one accepts the logic of seeking to deal with pure "single entity" drugs, should one also accept the proposal that drugs which undergo metabolic conversion should be avoided since, in effect, they behave as mixtures? Such a decision would indeed disrupt our present armamentarium of useful drugs. The prohibition of racemates would also be disruptive, since there are still instances where therapy with a cheaper, easy-to-make racemate is fully as effective as with a more expensive specific enantiomorph.

As long as we accept drugs that undergo metabolic conversion in vivo, we will continue to be obliged to accept some racemates and even mixtures of chemical analogs when it is hard to come by an equivalent single active component. Nevertheless, a single active moiety remains the preferred goal. The experts need to understand and evaluate how the recent advances in stereochemistry can both solve and create problems for the pharmaceutical manufacturer, research worker, and physician. In this revised and expanded second edition of *Drug Stereochemistry*, Dr. Wainer

has put together a volume that deserves careful review and will be a valuable resource to pharmaceutically oriented chemists, biologists, and clinicians who can no longer ignore the question of stereoisomerism in relation to drugs.

Murray Weiner

Preface to the Second Edition

The first edition of this book contained the observation that the stereoisomeric composition of drug substances was rapidly becoming a key issue in the development, approval, and clinical use of pharmaceuticals. During the past four years, this assertion has become a reality, and stereochemistry is now a major theme in all branches of pharmaceutical science. The interest in this topic is reflected in the content of articles appearing in natural and biological science journals and two new international journals, *Chirality* and *Tetrahedron Asymmetry*. Moreover, a variety of conferences and courses have been devoted to this subject. In addition, the European, North American, and Japanese drug regulatory agencies have expressed an interest in a closer examination of the stereoisomeric composition of chiral drugs and the therapeutic and toxicological consequences of this composition. The pharmaceutical industry has quickly responded to this situation by considering stereochemistry in its initial drug evaluation strategies. In fact, at the present time, a number of companies have made the decision to market only single-isomer drugs.

In light of these developments, we felt that it was necessary to review the content of the first edition and to update or supplement the information presented in this work. The new topics examined in this edition include: (1) enzymatic synthesis and resolution of enantiomerically pure compounds (Chapter 8); (2) toxicological consequences and implications of stereoselective biotransformations (Chapter 9); (3) stereoselective transport across epithelia (Chapter 10); and (4) assessment of bioavailability and bioequivalence of stereoisomeric drugs (Chapter 11). The chapter on stereoselective protein binding (Chapter 12) has been completely rewritten and new contributions are presented on the regulatory, industrial, and clinical aspects of stereoisomeric drugs (Chapters 13–16). In addition, the chapters

discussing stereoselective chromatographic separations (Chapters 4–6) have been revised and expanded.

As with all volumes of this type, this project could not have been completed without the contributors, and I would like to thank them for their superb efforts. I am indebted to the following people for their help: Ann Samson, David Lloyd, Camille Granvil, Karen Fried, Hiltrud Fieger, Tanja Alebic-Kolbah, Anne-Françoise Aubry, Gabriella Masolini, Xiao Ming Zhang, and Pierre Wong. The assistance of the publisher's staff is also appreciated, particularly the efforts of Sandra Beberman, Assistant Vice President, Editorial, and Ted Allen, the production editor.

Irving W. Wainer

Preface to the First Edition

The stereoisomeric composition of drug substances is rapidly becoming a key issue in the development, approval, and clinical use of pharmaceuticals. This volume is designed to cover the current debate on this topic from the academic, regulatory, and industrial points of view. We have endeavored to focus attention not only on what is already known about stereoisomeric drugs, but also on how this issue will be approached in the future. To accomplish this task, we have investigated three aspects of this question: (1) the preparation of stereochemically pure drugs, (2) the pharmacological differences between drug stereoisomers, and (3) perspectives on the use of stereochemically pure drugs.

The introductory section of this book covers the early history of stereochemistry and defines a number of stereochemical terms. These contributions are designed to provide the reader with a background for the concepts discussed in the rest of the work.

The second section addresses the issue that if a single isomer of a stereoisomeric drug is going to be used, it is necessary to prepare the substance and to be able to prove its purity. This section of the book includes chapters dealing with chromatographic and nonchromatographic methods for the resolution of drug enantiomers and for the determination of the purity of stereoisomeric drugs. The section also includes a discussion of the rapidly growing field of stereospecific synthesis.

The drive to produce stereoisomerically pure drugs is based on the recognition that there are pharmacological differences between drug stereoisomers. These differences are addressed in the third section of this book, which discusses the pharmacokinetic, plasma protein binding, efficacy, toxicity, and biotransformation of stereoisomeric drugs.

The debate on whether to produce and use only stereochemically pure

drugs is presented in the final section of this volume. Representatives from the Food and Drug Administration, pharmaceutical companies, and clinical practice have contributed to this discussion, giving the reader an overview of the situation as it stands today and where it is heading in the future.

This volume could not have been completed without the excellent efforts of the contributors. The asistance of the publisher's staff is also appreciated, particularly the efforts of the Executive Editorial Director, Sandra Beberman, and the production editor for this volume, Elaine Grohman.

Irving W. Wainer
Dennis E. Drayer

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DRUG STEREOCHEMISTRY

1

THE EARLY HISTORY OF STEREOCHEMISTRY

From the Discovery of Molecular Asymmetry and the First Resolution of a Racemate by Pasteur to the Asymmetrical Chiral Carbon of van't Hoff and Le Bel

Dennis E. Drayer* *Cornell University Medical College, New York, New York*

The first half of the nineteenth century was the great age of geometrical optics. Several French scientists studied diffraction, interference, and polarization of light. In particular, linear polarization of light and rotation of the plane of polarization very quickly attracted attention because of the possible relationship between these phenomena and the structure of matter. Optical activity, the ability of a substance to rotate the plane of polarization of light, was discovered in 1815 at the College de France by the physicist Jean-Baptiste Biot. In 1848 at the Ecole Normale in Paris, Louis Pasteur (Fig. 1) made a set of observations that led him a few years later to make this proposal, which is the foundation of stereochemistry: Optical activity of organic solutions is determined by molecular asymmetry, which produces nonsuperimposable mirror-image structures. A logical extension of this idea occurred in 1874 when a theory of organic structure in three dimensions was advanced independently and almost simultaneously by Jacobus Henricus van't Hoff (Fig. 2) in Holland, and Joseph Achille Le Bel (Fig. 3) in France. By this time it was known from the work of Kekule in 1858 that carbon is tetravalent (links up with four other groups or

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FIGURE 1 Pasteur. [From Vallery-Radot (17).]

FIGURE 2 van't Hoff. [From *Zeitschrift Für Physikalische Chemie, 31* (1899).]

atoms). van't Hoff and Le Bel proposed that the four valances of the carbon atom were not planar, but directed into three-dimensional space. van't Hoff specifically proposed that the spatial arrangement was tetrahedral. A compound containing a carbon substituted with four different groups, which van't Hoff defined as an asymmetric carbon (*asymmetrisch koolstof-atoom*), would therefore be capable of existing in two distinctly different nonsuperimposable forms. The asymmetric carbon atom, they proposed, was the cause of molecular asymmetry and therefore optical activity.

FIGURE 3 Le Bel. [From Snelders (18).]

The purpose of this chapter is to describe the observations and reasoning that led Pasteur, van't Hoff, and Le Bel to make these epochal discoveries. In several instances the protagonists will speak for themselves. More detailed accounts of their work are presented in Weyer (1), Partington (2), and Riddell and Robinson (3). Also, the three methods discovered by Pasteur to resolve for the first time an optically inactive

racemate into its optically active components (enantiomers) will be discussed. To truly appreciate the contributions of these three chemists, one should remember that during their time even the existence of atoms and molecules was questioned openly by many scientists, and to ascribe shape to what seemed like metaphysical concepts was too much for many of their contemporaries to accept.

Ordinary tartaric acid has been known since the eighteenth century and is a by-product of alcoholic fermentation obtained in great quantities from the tartar deposited in the barrels. This acid has been especially important in medicine and dyeing. Paratartaric acid (also called racemic acid), discovered in certain industrial processes in the Alsace region of France, came to the attention of chemists only in the 1820s, when Gay-Lussac established that it possessed the same chemical composition as ordinary tartaric acid. Because of their importance for the emerging concept of isomerism, the two acids thereafter attracted considerable notice. On January 20 and February 3, 1860, Pasteur gave lectures before the Council of the Société Chimique of Paris describing the principal results of his research (done from 1848–1850) on tartaric acid and paratartaric acid, from which evolved his proposals on the molecular asymmetry of organic products. The excerpts below are taken, with permission, from an English translation made by the Alembic Club (4). An English translation is also found in Pasteur (5). Additional insight is found in Mauskopf (6). The headings and interspersed comments below are mine. To better understand what follows, ordinary tartaric acid is now called dextro-tartaric acid and paratartaric acid is the racemate, *d,l*-tartaric acid.

I. HEMIHEDRAL CRYSTAL STRUCTURE

Pasteur begins his first lecture by discussing the precedents that led up to his research and then defines hemihedral crystals. These are cubical crystals with four little facets inclined at the same angle to the adjacent surfaces and arranged alternately so the same edge of the cube does not contain two facets (Fig. 4). Under these conditions, no point or plane of symmetry exists in the cube.

II. MOLECULAR ASYMMETRY AND OPTICAL ACTIVITY

Pasteur now describes the research that led to his conclusion about the causal relationship between molecular asymmetry and optical activity.

> When I began to devote myself to special work, I sought to strengthen myself in the knowledge of crystals, foreseeing the help that I should draw from this in my chemical researches. It seemed to me to be the simplest

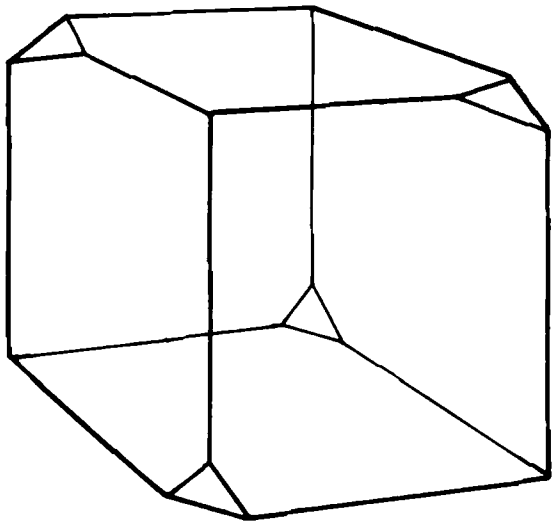

FIGURE 4 Hemihedral cube.

course, to take, as a guide, some rather extensive work on the crystalline forms; to repeat all the measurements, and to compare my determinations with those of the author. In 1841, M. de la Provostaye, whose accuracy is well known, had published a beautiful piece of work on the crystalline forms of tartaric and paratartaric acids and their salts. I made a study of this memoir. I crystallized tartaric acid and its salts, and investigated the forms of the crystals. But, as the work proceeded, I noticed that a very interesting fact had escaped the learned physicist. All the tartrates which I examined gave undoubted evidence of hemihedral faces.

This peculiarity in the forms of the tartrates was not very obvious. This will be readily conceived, seeing that it had not been observed before. But when, in a species, its presence was doubtful, I always succeeded in making it manifest by repeating the crystallisation and slightly modifying the conditions.

The German chemist Eilhard Mitscherlich published a note in 1844 in the *Reports of the Academy of Science* on the subject of the tartrate and paratartrate of sodium and ammonia. The importance of this note is now acknowledged by Pasteur.

I must first place before you a very remarkable note by Mitscherlich which was communicated to the *Academie des Sciences* by Biot. It was as follows:—

"The double paratartrate and the double tartrate of soda and ammonia have the same chemical composition, the same crystalline form with the

same angles, the same specific weight, the same double refraction, and consequently the same inclination in their optical axes. When dissolved in water their refraction is the same. But the dissolved tartrate deviates the plane of polarisation, while the paratartrate is indifferent, as has been found by M. Biot for the whole series of those two kinds of salts. Yet," adds Mitscherlich, "here the nature and the number of the atoms, their arrangement and distances, are the same in the two substances compared."

This note of Mitscherlich's attracted my attention forcibly at the time of publication. I was then a pupil in the Ecole Normale, reflecting in my leisure moments on these elegant investigations of the molecular constitution of substances, and having reached, as I thought at least, a thorough comprehension of the principles generally accepted by physicists and chemists. The above note disturbed all my ideas. What precision in every detail! Did two substances exist which had been more fully studied and more carefully compared as regards their properties? But how, in the existing condition of the science, could one conceive of two substances so closely alike without being identical? Mitscherlich himself tells us what was, to his mind, the consequence of this similarity:

The nature, the number, the arrangement, and the distance of the atoms are the same. If this is the case what becomes of the definition of chemical species, so rigorous, so remarkable for the time at which it appeared, given by Chevreul in 1823? In compound bodies a species is a collection of individuals identical in the nature, the proportion, and the arrangement of their elements.

In short, Mitscherlich's note remained in my mind as a difficulty of the first order in our mode of regarding material substances.

You will now understand why, being preoccupied, for the reasons already given, with a possible relation between the hemihedry of the tartrates and their rotative property, Mitscherlich's note of 1844 should recur to my memory. I thought at once that Mitscherlich was mistaken on one point. He had not observed that his double tartrate was hemihedral while his paratartrate was not. If this is so, the results in his note are no longer estraordinary; and further, I should have, in this, the best test of my preconceived idea as to the inter-relation of hemihedry and the rotatory phenomenon.

I hastened therefore to re-investigate the crystalline form of Mitscherlich's two salts. I found, as a matter of fact, that the tartrate was hemihedral, like all the other tartrates which I had previously studied, but, strange to say, the paratrate was hemihedral also. Only, the hemihedral faces which in the tartrate were all turned the same way were in the paratartrate inclined sometimes to the right and sometimes to the left. In spite of the unexpected character of this result, I continued to follow up my idea. I carefully separated the crystals which were hemihedral to the right from those hemihedral to the left, and examined their solutions separately in the polarising apparatus. I then saw with no less surprise than pleasure that the

crystals hemihedral to the right deviated the plane of polarisation to the right, and that those hemihedral to the left deviated it to the left (here Fig. 5); and when I took an equal weight of each of the two kinds of crystals, the mixed solution was indifferent towards the light in consequence of the neutralisation of the two equal and opposite individual deviations.

Thus, I start with paratartaric acid; I obtain in the usual way the double paratartrate of soda and ammonia; and the solution of this deposit, after some days, crystals all possessing exactly the same angles and the same aspect. To such a degree in this case that Mitscherlich, the celebrated crystallographer, in spite of the most minute and severe study possible, was not able to recognise the smallest difference. And yet the molecular arrangement in one set is entirely different from that in the other. The rotatory power proves this, as does also the mode of asymmetry of the crystals. The two kinds of crystals are isomorphous, and isomorphous with the corresponding tartrate. But the isomorphism presents itself with a hitherto unobserved peculiarity; it is the isomorphism of an asymmetric crystal with its mirror image. This comparison expresses the fact very exactly. Indeed, if, in a crystal of each kind, imagine the hemihedral facets produced till they meet, I obtain two symmetrical tetrahedra, inverse, and which cannot be superposed, in spite of the perfect identity of all their respective parts. From this I was justified in concluding that, by crystallisation of the double paratartrate of soda and ammonia, I had separated two symmetrically isomorphous atomic groups, which are intimately united in paratartaric acid. Nothing is easier to show than that these two species of crystals represent two distinct salts from which two different acids can be extracted.

The announcement of the above facts naturally placed me in communication with Biot, who was not without doubts regarding their accuracy. Being charged with giving an account of them to the Academy, he made me come to him and repeat before his eyes the decisive experiment. He handed over to me some paratartaric acid which he had himself previously studied with particular care, and which he had found to be perfectly indifferent to polarised light. I prepared the double salt in his presence, with soda and ammonia which he had likewise desired to provide. The liquid was set aside for slow evaporation in one of his rooms. When it had furnished about 30 to 40 grams of crystals, he asked me to call at the College de France in order to collect them and isolate them before him, by recognition of their crystallographic character, the right and the left crystals, requesting me to state once more whether I really affirmed that the crystals which I should place at his right would deviate to the right, and the others to the left. This done, he told me that he would undertake the rest. He prepared the solutions with carefully measured quantities, and when ready to examine them in the polarising apparatus, he once more invited me to come into his room. He first placed in the apparatus the more interesting solution, that which ought to deviate to the left. Without even making a measurement, he saw by the appearance of the tints of the two images, ordinary and extraordinary, in the

analyser, that there was a strong deviation to the left. Then, very visibly affected, the illustrious old man took me by the arm and said:

"My dear child, I have loved science so much throughout my life that this makes my heart throb."

Indeed there is more here than personal reminiscences. In Biot's case the emotion of the scientific man was mingled with the personal pleasure of seeing his conjectures realized. For more than thirty years Biot had striven in vain to induce chemists to share his conviction that the study of rotatory polarisation offered one of the surest means of gaining a knowledge of the molecular constitution of substances.

Let us return to the two acids furnished by the two sorts of crystals deposited in so unexpected a manner in the crystallisation of the double paratartrate of soda and ammonia. I have already remarked that nothing could be more interesting than the investigation of these acids.

One of them, that which comes from crystals of the double salt hemihedral to the right, deviates to the right, and is identical with ordinary tartaric acid. The other deviates to the left, like the salt which furnishes it. The deviation of the plane of polarisation produced by these two acids is rigorously the same in absolute value. The right acid follows special laws in its deviation, which no other active substance had exhibited. The left acid exhibits them, in the opposite sense, in the most faithful manner, leaving no suspicion of the slightest difference.

The paratartaric acid is really the combination, equivalent for equivalent, of these two acids, is proved by the fact that, if somewhat concentrated solutions of equal weights of each of them are mixed, as I shall do before you, their combination takes place with disengagement of heat, and the liquid solidifies immediately on account of the abundant crystallisation of paratar-

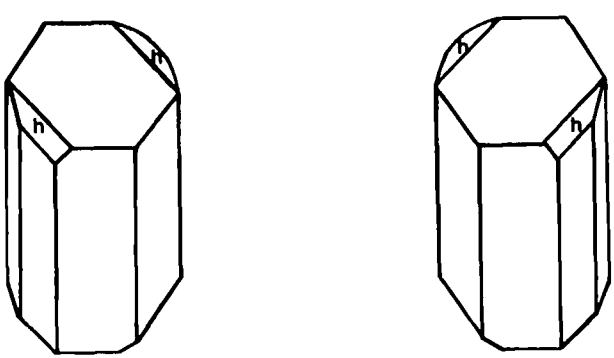

FIGURE 5 Paratarate of soda and ammonia formed by an equal mixture of hemihedral crystals of levo-tartrate (on left) and dextro-tartrate (on right). The anterior hemihedral facet "h" is on the left side of the observer in the levo-tartrate and on his or her right in the dextro-tartrate. [From Descour (17).]

taric acid, identical with the natural product. (This beautiful experiment called forth applause from the audience.)

Pasteur ends the first lecture with the following summary:

1). When the elementary atoms of organic products are grouped asymmetrically, the crystalline form of the substance manifests this molecular asymmetry in nonsuperposable hemihedry.

The cause of this hemihedry is thus recognised.

2). The existence of this same molecular asymmetry betrays itself, in addition, by the optical rotative property.

The cause of rotatory polarisation is likewise determined.

3). When the non-superposable molecular asymmetry is realised in opposite senses, as happens in the right and left tartaric acids and all their derivatives, the chemical properties of these identical and inverse substances are rigorously the same.

In the second lecture, Pasteur gives a further discussion of his fundamental idea that optical activity of organic solutions is related to molecular geometry. This insight was far ahead of the organic structural theory of the time.

We saw in the last lecture that quartz possesses the two characteristics of asymmetry—hemihedry in form, observed by Hauy, and the rotative phenomenon discovered by Arago! Nevertheless, molecular asymmetry is entirely absent in quartz. To understand this, let us take a further step in the knowledge of the phenomena with which we are dealing. We shall find in it, besides, the explanation of the analogies and differences already pointed out between quartz and natural organic products.

Permit me to illustrate roughly, although with essential accuracy, the structure of quartz and of the natural organic products. Imagine a spiral stair whose steps are cubes, or any other objects with superposable images. Destroy the stair and the asymmetry will have vanished. The asymmetry of the stair was simply the result of the mode of arrangement of the component steps. Such is quartz. The crystal of quartz is the stair complete. It is hemihedral. It acts on polarised light in virtue of this. But let the crystal be dissolved, fused, or have its physical structure destroyed in any way whatever; its asymmetry is suppressed and with it all action on polarised light, as it would be, for example, with a solution of alum, a liquid formed of molecules of cubic structure distributed without order.

Imagine, on the other hand, the same spiral stair to be constructed with irregular tetrahedra for steps. Destroy the stair and the asymmetry will still exist, since it is a question of a collection of tetrahedra. They may occupy any positions whatsoever, yet each of them will nonetheless have an asymmetry of its own. Such are the organic substances in which all the molecules have an asymmetry of their own, betraying itself in the form of the crystal. When the crystal is destroyed by solution, there results a liquid active towards

polarised light, because it is formed of molecules, without arrangement, it is true, but each having an asymmetry in the same sense, if not of the same intensity in all directions.

III. RESOLUTION OF RACEMATES

Pasteur devised three methods to resolve paratartaric acid: the first was manual, the second was chemical, and the third could be considered biological or physiological. Because paratartaric acid (also called racemic acid) was the first inactive compound to be resolved into optical isomers (enantiomers), an equimolar mixture of two enantiomers is now called a racemate.

A. Manual Separation

As indicated in the first lecture, Pasteur, using a hand lens and pair of tweezers, laboriously separated a quantity of the sodium ammonium salt of paratartaric acid into two piles, one of left-handed crystals and the other of right-handed crystals, and in this way accomplished the first resolution of a racemate. After purifying the free tartaric acids from the separate salt solutions, he found one acid to be identical to the previously characterized ordinary tartaric acid (which was dextrorotatory) and the other acid to be the previously unknown levorotatory isomer. Pasteur was extremely fortunate in this area of his research. The tartrate used by him is one of the very few substances that undergo a spontaneous separation into enantiomeric (hemihedral) crystals, thereby allowing resolution by hand. That is, most enantiomers do not form enantiomeric crystals. Moreover, this separation takes place only below 27°C (7). If Pasteur had been working in southern France during a torrid Mediterranean summer, rather than in Paris, we may have praised another chemist as being the first to resolve a racemate.

B. Chemical Formation of Diastereomers

The physical properties of enantiomers are identical in an achiral environment. However, chemical reactions that add another asymmetric center create a diastereomeric pair, each of which has physical properties that are not completely the same. Therefore, although an enantiomeric pair cannot be separated by ordinary chromatographic means or fractional recrystallization, the diastereomeric pair can often be separated easily by these means, as is indicated in the chapter by Joseph Gal. After separation, the pure enantiomers can then be regenerated by chemical means. This is today the most fundamental way of resolving a racemate.

Pasteur, in his second lecture, gives the following account, in which the optically active basic alkaloids quinicine or cinchonicine were used to convert the two enantiomeric tartaric acids into diastereomers:

We have seen that all artificial or natural chemical compounds, whether mineral or organic, must be divided into two great classes: non-asymmetric compounds with superposable image and asymmetric compounds with non-superposable image.

Taking this into account, the identity of properties above described in the case of the two tartaric acids and their similar derivatives, exists constantly, with the unchangeable characters which I have referred to, whenever these substances are placed in contact with any compound of the class with superposable image, such as potash, soda, ammonia, lime, baryta, aniline, alcohol, ethers—in a word, with any compounds whatever which are non-asymmetric, non-hemihedral in form, and without action on polarised light.

If, on the contrary, they are submitted to the action of products of the second class with non-superposable image—asparagine, quinine, strychnine, brucine, albumen, sugar, etc., bodies asymmetric like themselves—all is changed in an instant. The solubility is no longer the same. If combination takes place, the crystalline form, the specific weight, the quantity of water of crystallisation, the more or less easy destruction by heating, all differ as much as in the case of the most distantly related isomers.

Here, then, the molecular asymmetry of a substance obtrudes itself on chemistry as a powerful modifier of chemical affinities. Towards the two tartaric acids, quinine does not behave like potash, simply because it is asymmetric and potash is not. Molecular asymmetry exhibits itself henceforth as a property capable by itself, in virtue of its being asymmetry, of modifying chemical affinities. I do not believe that any discovery has yet made so great a step in the mechanical part of the problem of combination. . . .

Here is a very interesting application of the facts which have just been explained.

Seeing that the right and left tartaric acids formed such dissimilar compounds simply on account of the rotative power of the base, there was ground for hoping that, from this very dissimilarity, chemical forces might result, capable of balancing the mutual affinity of the two acids, and thereby supply a chemical means of separating the two constituents of paratartaric acid. I sought long in vain, but finally succeeded by the aid of two new bases, quinicine and cinchonicine, isomers of quinine and cinchonine, which I obtained very easily from the latter without the least loss.

I prepare the paratartrate of cinchonicine by neutralising the base and then adding as much of the acid as was necessary for the neutralisation, I allow the whole to crystallise, and the first crystallisations consist of perfectly pure left tartrate of cinchonicine. All the right tartrate remains in the mother liquor because it is more soluble. Finally this itself crystallises with an entirely different aspect, since it does not possess the same crystalline form as the left salt. We might also believe that we were dealing with the crystallisation of two distinct salts of unequal solubility.

C. Use of Living Organisms

Pasteur also discovered a method for resolving paratartaric acid while he was deeply involved in the study of fermentation. In essence, it depends on the capacity of certain microorganisms to discriminate between enantiomers and selectively to metabolize one instead of the other. This method is obviously less desirable than the chemical method since, at best, only one pure enantiomer can be obtained. The particular example described below by Pasteur in his second lecture grew out of his study of the fermentation of ammonium paratartrate.

> Knowing this, I set the ordinary right tartrate of ammonia to ferment in the following manner. I took the very pure crystallised salt, dissolved it, adding to the liquor a clear solution of albumenoid matter. One gram of albumenoid matter was sufficient for one hundred grams of tartrate. Very often it happens that the liquid ferments spontaneously when placed in an oven. I say very often; but it may be added that this will always take place if we take care to mix with the liquid a very small quantity of one of those liquids with which we have succeeded in obtaining spontaneous fermentation.
>
> So far there is nothing peculiar; it is a tartrate fermenting. The fact is well known.
>
> But let us apply this method of fermentation to paratartrate of ammonia, and under the above conditions it ferments. The same yeast is deposited. Everything shows that things are proceeding absolutely as in the case of the right tartrate. Yet if we follow the course of the operation with the help of the polarising apparatus, we soon discover profound differences between the two operations. The originally inactive liquid possesses a sensible rotative power to the left, which increases little by little and reaches a maximum. At this point the fermentation is suspended. There is no longer a trace of the right acid in the liquid. When it is evaporated and mixed with an equal volume of alcohol it gives immediately a beautiful crystallisation of left tartrate of ammonia.
>
> Let us note, in the first place, two distinct things in this phenomenon. As in all fermentation properly so called, there is a substance which is changed chemically, and correlatively there is a development of a body possessing the aspect of a mycodermic growth. On the other hand, and it is this which it is important to note, the yeast which causes the right salt to ferment leaves the left salt untouched, in spite of the absolute identity in physical and chemical properties of the right and left tartrates of ammonia as long as they are not subjected to asymmetric action.
>
> Here, then, the molecular asymmetry proper to organic substances intervenes in a phenomenon of a physiological kind, and it intervenes in the role of a modifier of chemical affinity. It is not at all doubtful that it is the kind of asymmetry proper to the molecular arrangement of left tartaric acid which is the sole and exclusive cause of the difference from the right acid, which it presents in relation to fermentation.

> Thus we find introduced into physiological principles and investigations the idea of the influence of the molecular asymmetry of natural organic products, of this great character which establishes perhaps the only well marked line of demarcation that can at present be drawn between the chemistry of dead matter and the chemistry of living matter.

Later qualified, modified, and generalized by others, Pasteur's new method became applicable to the separation of a number of other racemates (8).

Pasteur then ends his second lecture with the following:

> Such, gentlemen, are in co-ordinated form the investigations which I have been asked to present to you.
>
> You have understood, as we proceeded, why I entitled my exposition, "On the Molecular Asymmetry of Natural Organic Products." It is, in fact, the theory of molecular asymmetry that we have just established, one of the most exalted chapters of the science. It was completely unforeseen, and opens to physiology new horizons, distant, but sure.
>
> I hold this opinion of the results of my own work without allowing any of the vanity of the discoverer to mingle in the expression of my thought. May it please God that personal matters may never be possible at this desk. These are like pages in the history of chemistry which we write successively with that feeling of dignity which the true love of science always inspires.

Although popularly known chiefly for his great work in bacteriology and medicine, Pasteur was by training a chemist, and this work in chemistry alone would have earned him a position as an outstanding scientist.

The development of stereochemical ideas entered a new stage in 1858 when August Kekule (Fig 6) introduced the idea of the valence bond and the pictorial representation of molecules as atoms connected by valence bonds. His main thesis was that the carbon atom is tetravalent, and that a carbon atom can form valence bonds with other carbon atoms to form open chains and that sometimes the carbon chains can be closed to form rings (9). This led directly to his proposal for the structure of benzene. On the occasion of celebrations held in his honor, Kekule in 1890 delivered a speech before the German Chemical Society describing the origin of his idea of the linking of atoms (9).

> During my stay in London I resided for a considerable time in Clapham Road in the neighborhood of the Common. I frequently, however, spent my evenings with my friend Hugo Muller at Islington, at the opposite end of the giant town. . . . One fine summer evening I was returning by the last omnibus, "outside," as usual, through the deserted streets of the metropolis, which are at other times so fully of life. I fell into a reverie and lo, the atoms were gambolling before my eyes! Whenever, hitherto, these diminutive beings had appeared to me, they had always been in motion; but up to that

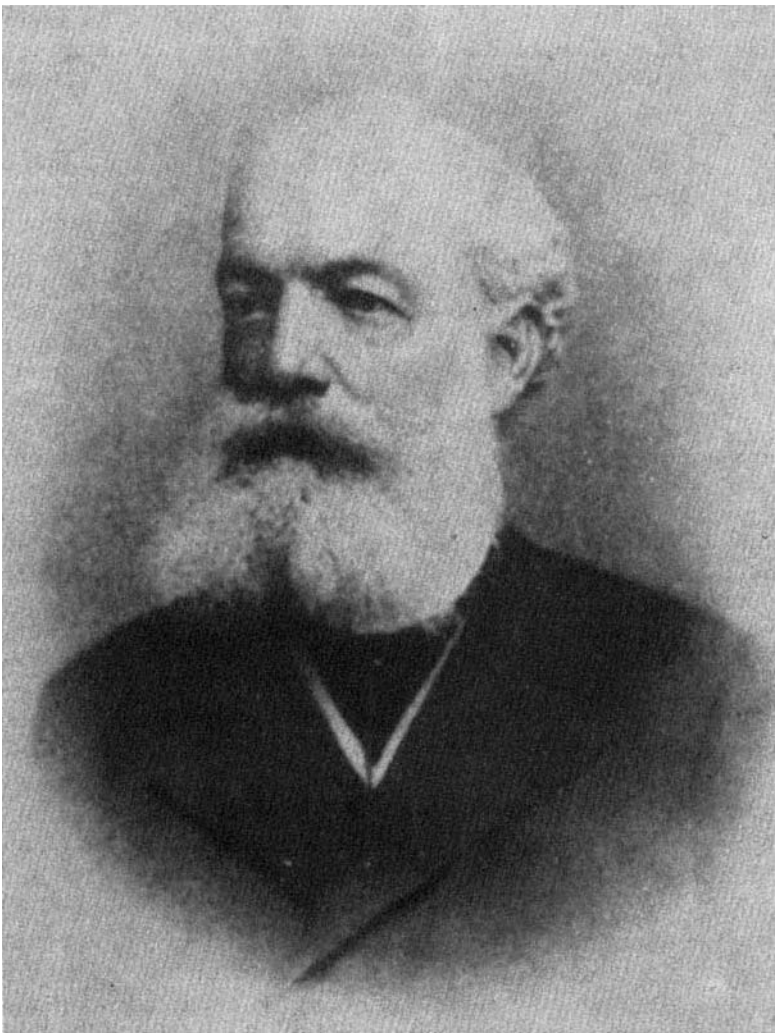

FIGURE 6 Kekule. [From Japp (9).]

time I had never been able to discern the nature of their motion. Now, however, I saw how, frequently, two smaller atoms united to form a pair; how a larger one embraced two smaller ones; how still larger ones kept hold of three or even four of the smaller; whilst the whole kept whirling in a giddy dance. I saw how the larger ones formed a chain, dragging the

> smaller ones after them, but only at the ends of the chain. . . . The cry of the conductor: "Clapham Road," awakened me from my dreaming; but I spent a part of the night in putting on paper at least sketches of these dream forms. This was the origin of the *Structurtheorie*.

Then he related a similar experience of how the idea for the structure of benzene occurred to him.

> I was sitting writing at my textbook, but the work did not progress; my thoughts were elsewhere. I turned my chair to the fire and dozed. Again the atoms were gambolling before my eyes. This time the smaller groups kept modestly in the background. My mental eye, rendered more acute by repeated visions of this kind, could now distinguish larger structures of manifold conformations; long rows, sometimes more closely fitted together; all twisting and turning in snake-like motion. But look! What was that? One of the snakes had seized hold of its own tail, and the form whirled mockingly before my eyes. As if by a flash of lightning I awoke; and this time also I spent the rest of the night working out the consequences of the hypothesis. Let us learn to dream, gentlemen, and then perhaps we shall find the truth . . . but let us beware of publishing our dreams before they have been put to the proof by the waking understanding.

In speculating on the kind of atomic arrangements that could produce molecular asymmetry, Pasteur, as already indicated, suggested tentatively in 1860 that the atoms of a right-handed compound, for example, might be "arranged in the form of a right-handed spiral, or situated at the corners of an irregular tetrahedron." But he never developed these suggestions. The solution to this problem of what is the cause of molecular asymmetry was presented in the publications of van't Hoff and Le Bel. On September 5, 1874, van't Hoff, while he was still a student at the University of Utrecht and only 22 years of age, published a pamphlet entitled "Proposal for the extension of the structural formulae now in use in chemistry into space, together with a related note on the relation between the optical active power and the chemical constitution of organic compounds" (10). An English translation is presented in van't Hoff (11). Starting with the ideas of August Kekule on the tetravalency of carbon, van't Hoff states, at the beginning of his pamphlet: "It appears more and more that the present constitutional formulas are incapable of explaining certain cases of isomerism; the reason for this is perhaps the fact that we need a more definite statement about the actual positions of the atoms." He then proposed that the four valences of a carbon atom are directed toward the corners of a tetrahedron with the carbon atom at the center, based on the concept of the isomer number, which is illustrated below.

For any atom Y, only one substance of formula CH_3Y has ever been

found. For example, chlorination of methane yields only one compound of formula CH_3Cl. Indeed, the same holds true if Y represents, not just an atom, but a group of atoms (unless the group is so complicated that in itself it brings about isomerism); there is only one CH_3OH, and only one CH_3CO_2H. This suggests that every hydrogen atom in methane is equivalent to every other hydrogen atom, so that replacement of any one of them gives rise to the same product. If the hydrogen atoms of methane were not equivalent, then replacement of one would yield a different compound than replacement of another, and isomeric substitution products would be obtained. In what ways can the atoms of methane be arranged so that the four hydrogen atoms are equivalent? There are three such arrangements (Fig. 7): a planar arrangement (I) in which carbon is at the center of a rectangle (or square) and a hydrogen atom is at each corner; a pyramidal arrangement (II) in which carbon is at the apex of a pyramid and a hydrogen atom is at each corner of a square base; a tetrahedral arrangement (III) in which carbon is at the center of a tetrahedron and a hydrogen atom is at each corner. By then comparing the number of isomers that have been prepared for di-, tri- and tetrasubstituted methanes with the number predicted by the above three spatial arrangements, it is possible to decide which one is correct.

For example, with a disubstituted compound CH_2R_2 (Fig. 8); (1) if the molecule is planar, then two isomers are possible. This planar configuration can be either square or rectangular; in each case, there are two isomers only. (2) If the molecule is pyramidal, then two isomers are also possible. There are only two isomers, whether the base is square or rectangular. (3) If the molecule is tetrahedral, then only one form is possible. The carbon atom is at the center of the tetrahedron. In actuality, only one disubstituted isomer is known. Therefore, only the tetrahedral model for a disubstituted methane agrees with the evidence of the isomer number.

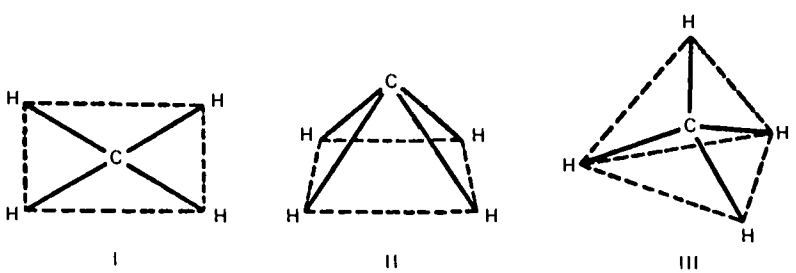

FIGURE 7 Spatial models for methane where the four hydrogen atoms are equivalent. I, planar; II, pyramidal; III, tetrahedral.

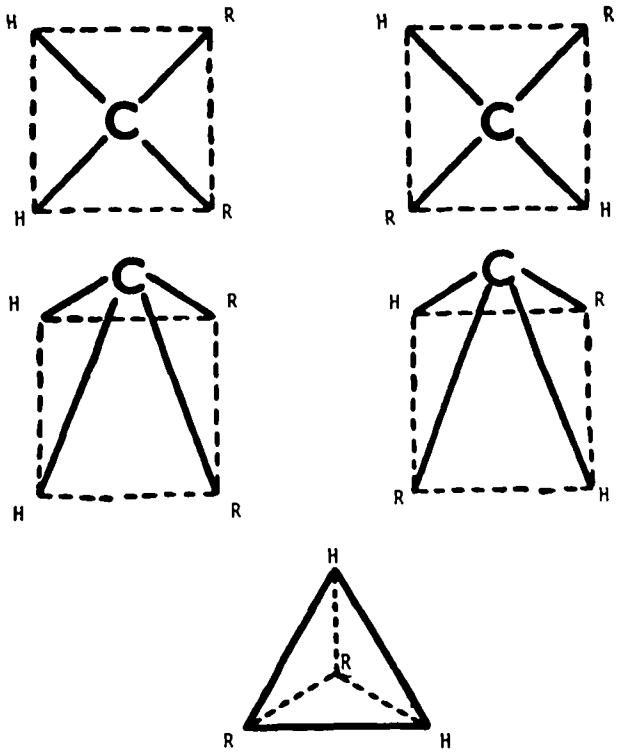

FIGURE 8 Spatial models for a disubstituted methane. Top, planar; middle, pyramidal; bottom, tetrahedral.

For tetrasubstituted compounds of the type $CR_1R_2R_3R_4$ (Fig. 9); (1) if the molecule is planar, then three isomers are possible. (2) If the molecule is pyramidal, then six isomers are possible. Each of the forms in Fig. 9, top, drawn as a pyramid, is not superimposable on its mirror image. Thus, three pairs of enantiomers are possible (one of which is shown in Fig. 9, middle). (3) If the molecule is tetrahedral, two isomers are possible, related to one another as object to mirror image. In actuality, only two tetrasubstituted isomers of methane are known (pair of enantiomers). This is strong evidence for the tetrahedral model for the carbon atom. Similar reasoning leads to the same conclusion for trisubstituted methanes.

The tetrahedral model for the carbon atom has withstood the test of time very well. Hundreds of thousands of organic compounds have been synthesized since it was first proposed. The number of isomers obtained has always been consistent with the concept of the tetrahedral carbon atom.

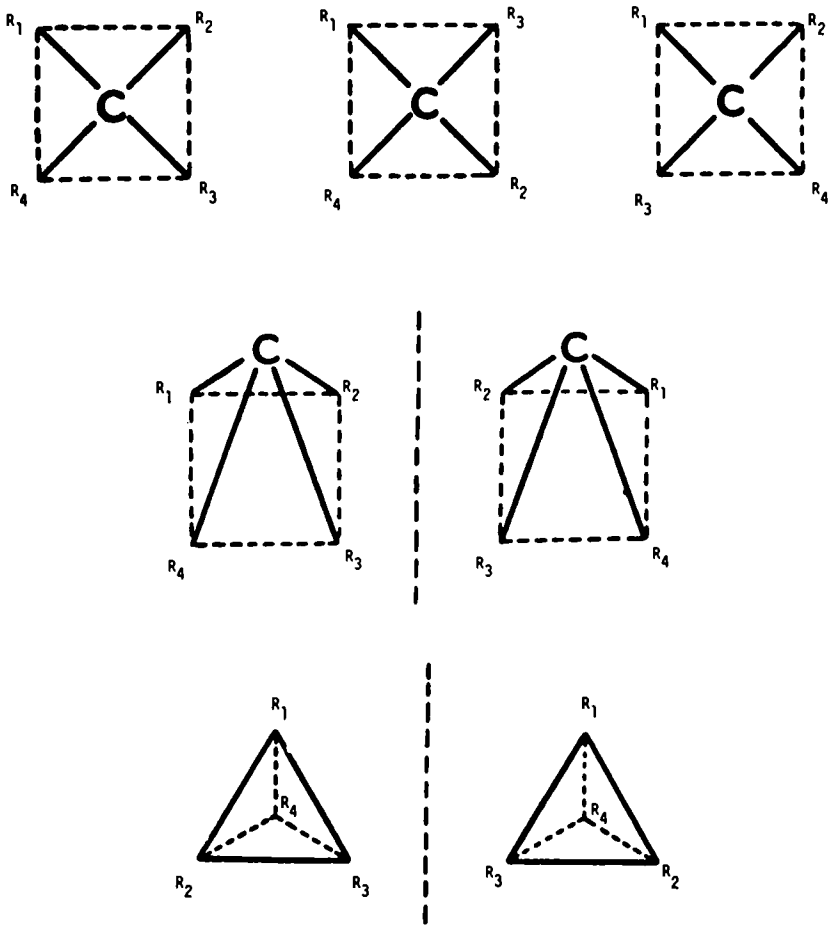

FIGURE 9 Spatial models for a tetrasubstituted methane. Top, planar; middle, pyramidal; bottom, tetrahedral.

van't Hoff then introduced the concept of the asymmetric carbon atom as follows: "When the four affinities of the carbon atom are satisfied by four univalent groups differing among themselves, two and not more than two different tetrahedrons are obtained, one of which is the reflected image of the other, they cannot be superposed; that is, we have to deal with two structural formulas isomeric in space." van't Hoff proposed that all carbon compounds that in solution rotate the plane of polarization possess an asymmetric carbon atom. He illustrated this for a great number of com-

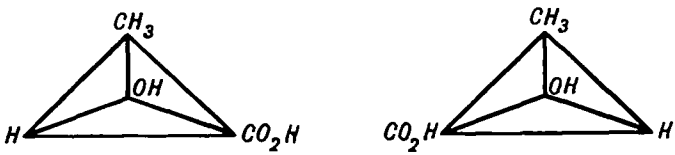

FIGURE 10 Tetrahedral model for lactic acid enantiomers (carbon atom is at the center of the tetrahedron) as envisioned by van't Hoff.

pounds: ethylidene lactic acid (now called α-hydroxypropionic acid), aspartic acid, asparagine, malic acid, glutaric acid, tartaric acid, sugars and glucosides, camphor, borneol, and camphoric acid.

Two compounds from this list are worthy of note: lactic acid (Fig. 10) and tartaric acid (Fig. 11). Wislicenus (Fig. 12) extensively investigated the isomers of lactic acid between 1863 and 1873, and was convinced that the number of isomers exceeded that allowed by the existing structural theory (12). However, due to experimental difficulties in obtaining pure samples of the isomers, in addition to the limits of the structural theory then known to him, he ended up going around in circles. van't Hoff studied the publications of Wislicenus on lactic acids and they led him to his own stereochemical ideas. In fact, lactic acid was the first concrete example of an optically active compound that van't Hoff discussed after his theoretical introduction. He pointed out that ethylidene lactic acid contains an asymmetric carbon. Therefore, it can exist as two pure enantiomers or a racemic mixture, which nicely cleared up the confusion surrounding the lactic acid

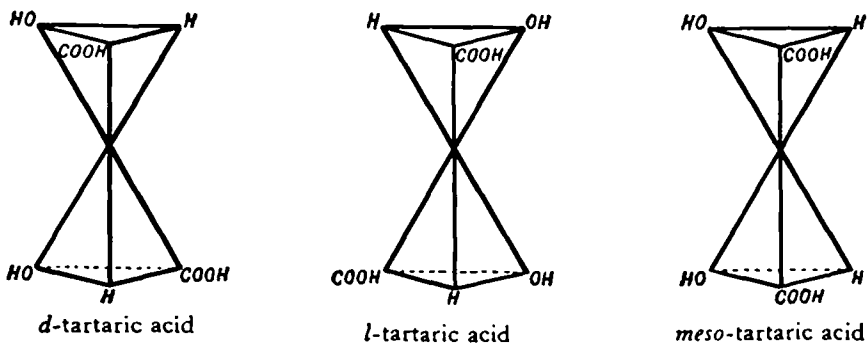

FIGURE 11 Structures for three tartaric acid isomers that are representative of the tetrahedral models used by van't Hoff.

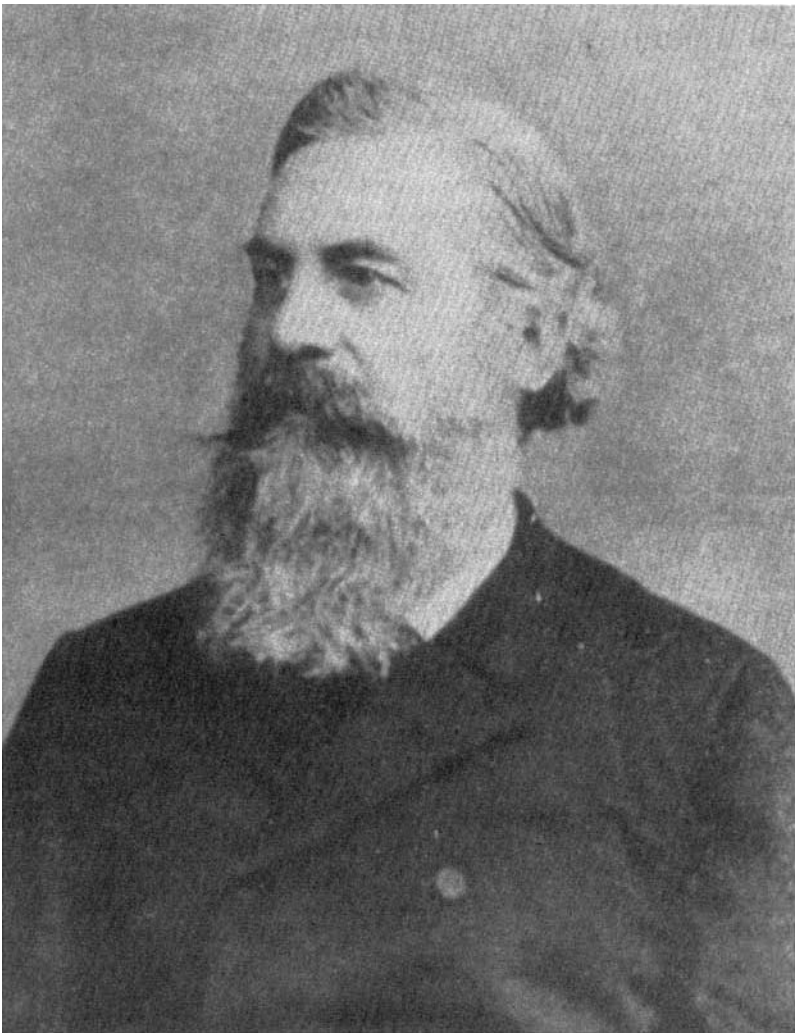

FIGURE 12 Wislicenus.

isomers. In a lecture, held much later in Utrecht on May 16, 1904, van't Hoff said the following:

> Students, let me give you a recipe for making discoveries. In connexion with what has just been said about libraries, I might remark that they have always had a mind-deadening effect on me. When Wislicenus' paper on lactic acid appeared and I was studying it in the Utrecht library, I therefore broke off my study half-way through, to go for a walk; and it was during this walk, under the influence of the fresh air, that the idea of asymmetric carbon first struck me.

These proposals of van't Hoff's came as a breath of fresh air to Wislicenus. No wonder that he was the first to welcome it enthusiastically, or that he sponsored the German translation that made it widely known, or that he was the first to make significant further use of the hypothesis, in his work on geometrical isomers of unsaturated compounds (13).

The other example of note is the optically active tartaric acids (Fig. 11). Tartaric acid contains two asymmetric carbon atoms. The dextro- and levo-tartaric acids are enantiomers. However, a third isomer is possible in which the two rotations due to the two asymmetric carbon atoms compensate and the molecule is optically inactive as a whole. That is, the molecule contains a plane of symmetry. This form, meso-tartaric acid, was also discovered by Pasteur, differs from the two optically active tartaric acids in being internally compensated, and is not resolvable. Thus, the tetrahedral model for carbon and the asymmetric carbon atom proposed by van't Hoff were able to completely explain the observations of Pasteur relating to the three isomers of tartaric acid.

Le Bel published his stereochemical ideas two months later, in November 1874, under the title, "The relations that exist between the atomic formulas of organic compounds and the rotatory power of their solutions" (14). An English translation is presented in Le Bel (15). Le Bel approached the problem from a different direction from van't Hoff. His hypothesis was based on neither the tetrahedral model of the carbon atom nor the concept of fixed valences between the atoms. He proceeded purely from symmetry arguments; he spoke of the asymmetry, not of individual atoms, but of the entire molecule, so that his views would nowadays be classed under the heading of molecular asymmetry. Only once does he mention the tetrahedral carbon atom, which he regarded as not a general principle but a special case. Today, substituted allenes, spiranes, and biphenyls are but a few examples of asymmetric molecules that do not contain any asymmetric carbons, thus confirming Le Bel's views on molecular asymmetry. The reason for the different approaches by van't Hoff and le Bel is easy to understand. van't Hoff came from the camp of structural chemists, and he

wished his hypothesis to be understood as an extension of the structural theory to spatial relationships. The tetravalent atomic models used by Kekule in his lectures presumably also prompted his pupil van't Hoff, possibly unconsciously, in the conception of the asymmetric carbon atom. Le Bel, on the other hand, was trained in the tradition of Pasteur (whose investigations he also mentioned expressly in his article), that is, he started out from Pasteur's considerations of the connections between optical rotation and molecular structure.

In 1877 Hermann Kolbe, one of the most distinguished of the older German chemists, published a diatribe in the *Journal für Praktische Chemie* after reading the work of van't Hoff (which had been translated into German by Felix Herrmann at the suggestion of Wislicenus). An English translation of this abusive attack is presented completely in Riddell and Robinson (3). Those individuals interested in seeing an example of the great personal attacks by editors that appeared in journals of the nineteenth century should read this translation. Although defamatory, this criticism served a useful purpose, since it made a decisive contribution to the dissemination of these ideas of van't Hoff. This was fortunate, since van't Hoff soon turned his genius away from stereochemistry to physical chemistry, for which he received the Nobel Prize.

We can now end this historical journey. We have walked through the early days of stereochemistry in the company of giants. In 1949, almost exactly 100 years after the first resolution of *d,l*-tartaric acid by Pasteur, the Dutchman Bijvoet (16), using x-ray diffraction, determined the actual arrangement in space of the atoms of the sodium rubidium salt of (+)-tartaric acid, and thus made the first determination of the absolute configuration about an asymmetric carbon. To further complete the link with the past, Bijvoet did this work while the Director of the van't Hoff Laboratory at the University of Utrecht.

In the intervening years since the first resolution of a racemate by Pasteur, many chromatographic and nonchromatographic methods have been developed for the resolution of racemic compounds. These methods are the subjects of many of the other chapters in this book.

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2

Stereochemical Terms and Concepts

An Overview

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I. INTRODUCTION

The terminology used to describe stereochemical relationships is often a maze of capital D's and L's, lowercase *d*'s and *l*'s, mixed in with (R)'s, (S)'s, (+)'s, and (−)'s. This chapter is designed to give the reader a quick overview of stereochemical terms and concepts and to lay a foundation for the more technical discussions to come. It is not meant to be an in-depth treatment of this topic and the interested reader is urged to consult one of the many fine texts on the subject.

II. SYMMETRY AND ASYMMETRY

Symmetry or the lack of it is one of the interesting features of geometric figures with two or more dimensions. We are often confronted with this phenomenon without realizing it. For example, the alphabet contains symmetrical and asymmetrical two-dimensional letters, some of which have different appearances when they are reflected in a mirror. Six of these letters and their mirror images are presented in Fig. 1. There is no difference between each of the symmetrical letters A, H, and M and the corresponding mirror image; however, the mirror images of the asymmetrical letters E, R, and S appear reversed.

The property of nonidentical mirror images also exists for figures with three dimensions. The human body, for example is an asymmetrical three-

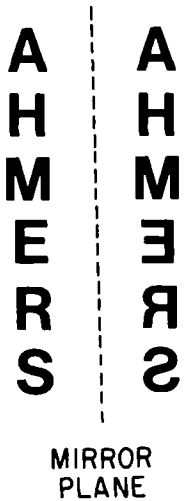

FIGURE 1 Symmetrical and asymmetrical letters of the English alphabet and their mirror images.

dimensional figure whose axis of dissymmetry lies between the front and back sides. The mirror image of the human body is also reversed, for example, the mirror image of the right hand appears to be on the left side of the body, etc. In addition, these mirror images cannot be made identical by simple spatial manipulations. The mirror image of an asymmetrical two-dimensional figure such as the letter R can be lifted out of the plane of the paper, turned over, and placed exactly on top of the original figure. A mirror image of a right hand cannot be placed exactly on top of a right hand (with both palms up or both palms down). Three-dimensional figures can exist as nonsuperimposable mirror images.

The spatial relationships that exist for the human body also exist at the molecular level because the molecules of nature exist as three-dimensional symmetrical and asymmetrical figures. One of the most common asymmetric molecules is a tetravalent carbon atom with four different ligands attached to it. The spatial arrangement of the atoms in this molecule is shown in Fig. 2. The carbon atom depicted in Fig. 2 is the asymmetric center of the molecule, and the molecule is a chiral stereoisomer. If the molecule and its mirror image are nonsuperimposable, the relationship between the two molecules is enantiomeric, and the two stereoisomers are enantiomers. Carbon is not the only atom that can act as an asymmetric center. Phosphorus, sulfur, and nitrogen are among some of the other atoms that can form chiral molecules.

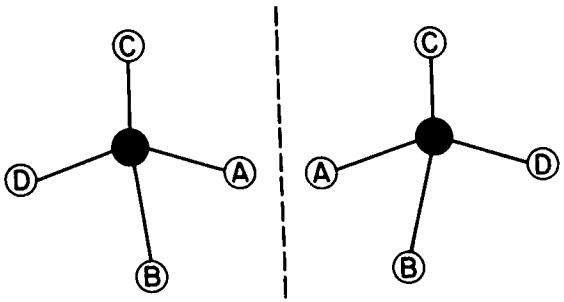

FIGURE 2 An asymmetric tetravalent carbon atom and its mirror image.

III. OPTICAL ROTATION

Because each member of a pair of enantiomeric molecules differs from the other only in the spatial arrangement of the ligands attached to the chiral center, their physical properties, that is, melting point, boiling point, refractive index, solubility, etc., are identical. The major difference between the isomers of an enantiomeric pair was first observed by Biot in 1815 when he noted that one form of tartaric acid rotated plane-polarized light, whereas another form did not (1).

Light is a form of electromagnetic radiation and is composed of electric and magnetic fields that oscillate in all directions perpendicular to each other and to the direction from which the beam is propagated. In plane-polarized light, the component electric and magnetic fields oscillate as in ordinary light, except that they are contained within two perpendicular planes. When the electrical part of the plane-polarized light interacts with an asymmetric molecule, the direction of the field is altered or rotated because of the dissymmetry of the molecule. The substance through which the light passes is said to be optically active.

Because enantiomers exist as mirror images, they interact with plane-polarized light to an equal but opposite extent. This situation is depicted in Fig. 3; in viewing this figure, the observer is looking directly at the beam of plane-polarized light, which is initially at position 0. One of the isomers rotates this beam in a counterclockwise direction. This isomer is defined as the levo-rotatory or *l* enantiomer, and the angle of the rotation α is defined as a negative (−) rotation. The other isomer rotates the beam of plane-polarized light in a clockwise direction and is defined as the dextrorotatory or *d* enantiomer, and α is defined as a positive (+) rotation.

The magnitude of α depends on the number and kind of molecules through which the beam of light passes. The observed rotation is defined

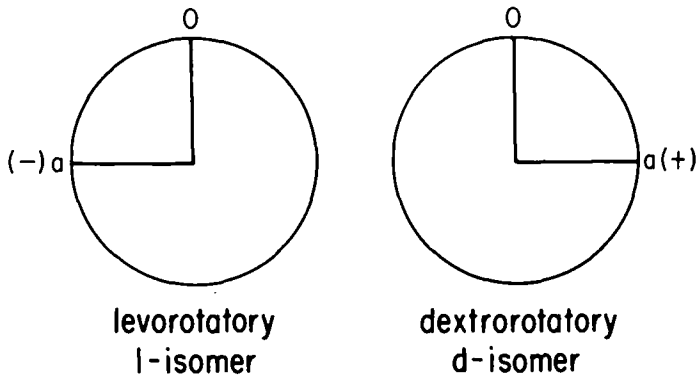

FIGURE 3 The rotation of plane-polarized light by the *l*- and *d*-enantiomers of an optically active substance. In the illustration the plane-polarized light is being propagated in a plane perpendicular to the plane of the page.

as the specific rotation [α] of a substance, and is expressed by the following formulas (2): For liquid substances, $[\alpha]^t_x = a/ld$; for solutions, $[\alpha]^t_x = 100\ a/lc$; where a is the corrected observed rotation in degrees at temperature t and wavelength x; l is the length of the polarimeter tube in decimeters; d is the specific gravity of the liquid at the temperature of observation; c is the concentration of the solution expressed as the number of grams of substance per 100 ml of solution. As indicated by these formulas, the specific rotation is dependent on the concentration of the solution or density of the pure liquid, the distance through which the light travels, the wavelength of the light, and the temperature at which the measurements are made.

IV. TYPES OF STEREOISOMERS

When molecules composed of the same constituents have the same structural formulas but differ only with respect to the spatial arrangement of certain atoms or groups of atoms, they are defined as stereoisomers. Stereoisomers can be optical isomers or geometrical isomers. Optical isomers are members of a set of stereoisomers, at least two of which are optically active or chiral; geometrical isomers are members of a set of stereoisomers that contains no optically active members. If the relationship between optical isomers is one of nonsuperimposable mirror images, the isomers are defined as enantiomers. Molecules having at least one pair of enantiomers are considered chiral. Optical isomers not related to each other as enantiomers are diastereomers.

A. Enantiomers

Enantiomeric compounds in which the asymmetric center is a tetravalent carbon, as in Fig. 2, represent the largest class of chiral molecules. The tetrahedral orientation of the bonds to a tetravalent carbon is such that when four nonidentical ligands are present, the mirror image of the molecule is nonsuperimposable, and the molecule is enantiomeric and chiral. When two of the ligands are identical, the mirror image is superimposable, and the molecule possesses a plane of symmetry and is achiral.

Molecules that do not possess an asymmetric center may still have nonsuperimposable mirror images and exist as enantiomers. These molecules contain a chiral plane or chiral axis and are dissymmetric with respect to either that plane or axis. The structures of the enantiomers of the sedative-hypnotic methaqualone are presented in Fig. 4. In this molecule there is a chiral axis between the nitrogen atom (N-1) and phenyl ring (C-1). The dissymmetry of the two forms of the molecule is a result of hindered rotation around this axis, which is due to steric interactions between methyl groups (M-1 and M-2). Other axially dissymmetric molecules include allene, biaryls, alkylidenecyclohexanes, and spiranes. Planar dissymmetric molecules are exemplified by molecules such as *trans*-cycloalkenes.

B. Diastereomers

Diastereomers are optical isomers that are not related as an object and its mirror image. Unlike enantiomers, the physical and chemical properties of diastereomers can differ and it is not unusual for them to have different melting and boiling points, refractive indices, solubilities, etc. Their optical rotations can differ in both sign and magnitude.

The most common diastereomeric molecule is one that contains two asymmetric carbons. This situation is illustrated by the compounds

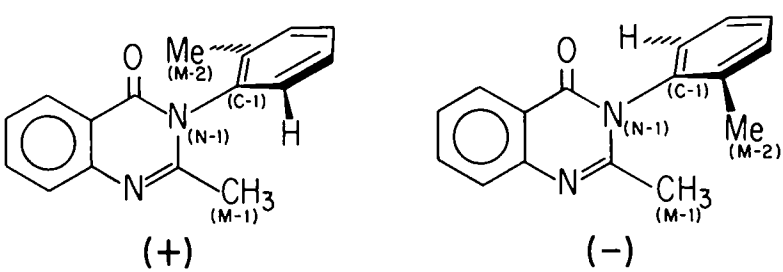

FIGURE 4 The enantiomers of methaqualone. The molecule is dissymmetric about the (N-1)—(C-1) axis.

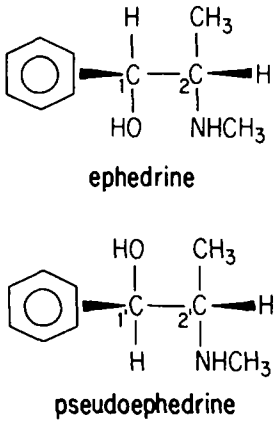

FIGURE 5 The structures of the diastereomeric molecules ephedrine and pseudoephedrine (only one enantiomer of each substance is presented).

ephedrine and pseudoephedrine (Fig. 5). In these molecules, the asymmetric carbons 2 and 2′ are identical, whereas carbons 1 and 1′ are mirror images. The different relationships between carbons 1 and 1′ and between carbons 2 and 2′ result in a non-mirror-image relationship between ephedrine and pseudoephedrine. It is noteworthy that each diastereomer (ephedrine and pseudoephedrine) exists as a member of an enantiomeric pair, that is, *d*- and *l*-ephedrine and *d*- and *l*-pseudoephedrine, respectively. Thus, diastereomeric molecules with two asymmetric centers are most often represented by four stereoisomers.

C. Geometrical Isomers

Molecules that contain a carbon-carbon double bond, alkenes, and similar double-bonded systems, C=N, for example, can exist as stereoisomers. Because each of these sets of stereoisomers contains no optically active members, these compounds are classified as geometrical isomers. This situation is illustrated by 2-butene (Fig. 6).

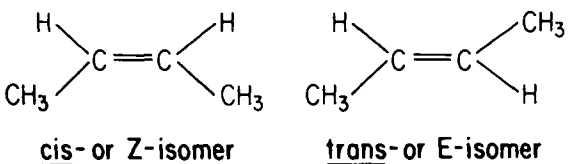

FIGURE 6 The isomers of 2-butene.

In this molecule, the two methyl groups can be found on the same or opposite sides of the double bond. When they are on the same side, the molecule is defined as the *cis* or *Z* isomer; when they are on the opposite sides, the *trans* or *E* designation is used. The descriptor *Z* comes from the German *zusammen*, which means together; *E* comes from the German *entgegen*, which means opposite.

V. NAMING OPTICALLY ACTIVE STEREOISOMERS

The earliest method of distinguishing one enantiomeric form from another was the sign of rotation of plane-polarized light, that is, the *d* and *l* or (+) and (−) forms. However, this does not describe the actual spatial arrangement about the chiral center, which is defined as the configuration.

The configuration of an asymmetric center was initially determined by the chemical transformation of the chiral molecule to an arbitrarily chosen standard, (+)-glyceraldehyde. This was the basis of the Fischer convention for the determination and designation of configuration. Recently, physical methods have replaced chemical transformations, and it is now possible to determine the absolute configuration of a molecule by using such techniques as nuclear magnetic resonance and x-ray crystallography. These advances have resulted in the development of a new system of nomenclature, the Cahn–Ingold–Prelog convention.

A. The Fischer Convention

The Fischer convention was designed by Emile Fischer in 1919 (3). The system operates by relating the configuration at the asymmetric center of the molecule under investigation to (+)-glyceraldehyde, which was arbitrarily assigned the D configuration that is presented in Fig. 7. Recent x-ray crystallographic studies have established that the assigned configuration is, in fact, correct.

To assign a configuration, the molecule under investigation must be chemically converted to glyceraldehyde or another molecule of known configuration. Once that is accomplished, the sign of rotation is determined and the D or L configuration is assigned accordingly.

The sign of rotation cannot be used a priori to assign a configuration, because they do not always correspond. For example, L-alanine has a (+) sign of rotation, whereas the sign of rotation for L-glyceraldehyde is (−).

The Fischer convention is widely used in sugar chemistry and for α-amino acids. For sugars, which contain a number of asymmetric centers, and for diastereomers with only two centers, the Fischer convention defines a series as D or L according to whether the configuration at the highest numbered asymmetric center is analogous to D- or L-glyceralde-

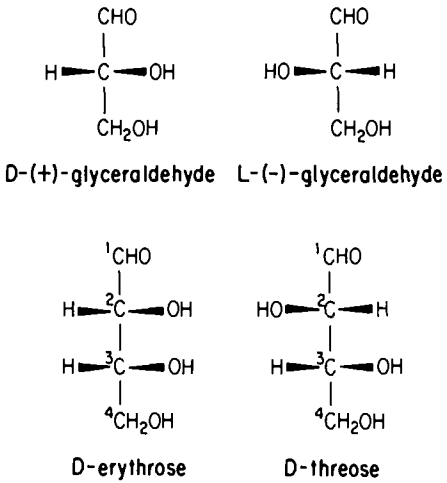

FIGURE 7 The configurations of D-(+) and L-(−)-glyceraldehyde, D-erythrose, and D-threose, according to the Fischer Convention.

hyde. In Fig. 7, the D configuration is assigned to the erythrose and threose series because of the D configuration at C-3.

The Fischer convention is often inexact and difficult to use, especially when complicated chemical transformations are required to convert the molecule under investigation into a molecule of known configuration. In addition, the assigned configuration, D or L, is often confused with the

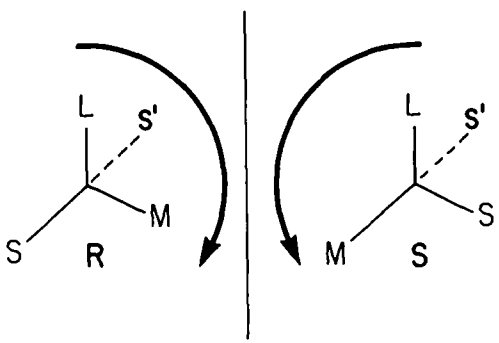

FIGURE 8 The Cahn–Ingold–Prelog sequence rule, where L is the largest group attached to the chiral center, followed in size by M, S, and s'.

TABLE 1 Commonly Used Sterochemical Terms

Stereoisomers	Molecules with the same consitution, but that differ in respect to the spatial arrangement of certain atoms or groups
Enantiomeric molecule	A molecule that is not superimposable on its mirror image
Chiral molecule	A molecule having at least one pair of enantiomers
Enantiomers	Stereoisomers that are related as nonsuperimposable mirror images
Diastereomers	Optical isomers that are not related as an object and its mirror image
Optical activity	A property of a chiral molecule—the ability to rotate a beam of plane polarized light
Optical rotation (α)	The angle that a beam of plane-polarized light is rotated by a chiral molecule
Dextrorotary, *d* or (+) rotation	A clockwise rotation of a beam of plane-polarized light by a stereoisomer, usually used to denote a specific enantiomer of a chiral molecule, that is *d*- or (+)-ephedrine
Levorotatory, *l* or (−) rotation	A counterclockwise rotation of a beam of plane-polarized light by a stereoisomer, usually used to denote a specific enantiomer of a chiral molecule, that is *l*- or (−)- ephedrine
Specific rotation [α]	A quantitative measure of the optical rotation, which is dependent on concentration or density of the chiral substance, length of the light path, temperature, and wavelength of light
Configuration	The description of the spatial arrangement about a chiral atom
Fischer convention (D, L)	The assignment of configuration about a chiral atom by comparison to a standard, (D) − (+) − glyceraldehyde, usually by actual chemical transformation of the molecule under investigation into the standard
Cahn–Ingold–Prelog convention (*R*, *S*)	The assignment of configuration about a chiral atom by designation of the sequence of substituents from the largest (L) to medium (M) to the smallest (S); a clockwise direction of the L-M-S sequence is assigned the *R* configuration and a counterclockwise direction is assigned the *S* configuration

observed sign of rotation, *d* or *l*. Because of this, the Fischer convention has been almost entirely replaced by the Cahn–Ingold–Prelog convention.

B. The Cahn–Ingold–Prelog Convention

The Cahn–Ingold–Prelog convention was designated by its originators as the "sequence rule," since it designates the sequence of substituents around the asymmetric center (4). In this method, the substituents at the chiral center are first ordered according to their atomic number from the largest to the smallest. In the example presented in Fig. 8, the order is L (large), M (medium), S (small), and s′ (smallest). The molecule is then oriented so that the smallest (s′) substituent is directed away from the viewer. The configuration is then determined by whether the sequence L—M—S goes in a clockwise or counterclockwise direction. A clockwise direction is specified as *R* (*right*), whereas the counterclockwise direction is designated as *S* (*sinister*).

This convention can be used to rapidly and unambiguously specify the configuration of a chiral center. As would be expected, for an enantiomeric molecule the direction of the sequence for one enantiomer is reversed for the other enantiomer. If one enantiomer has an *R* designation, its mirror image has the *S* configuration.

The Cahn–Ingold–Prelog convention is also extremely useful for describing diastereomers. In this case, each chiral center is designated independently and the configuration of the whole molecule can be easily assigned. For example, instead of *d*- and *l*-pseudoephedrine, the assigned configurations are (*R*,*S*)- and (*S*,*R*)-ephedrine and (*R*,*R*)- and (*S*,*S*)-pseudoephedrine. The enantiomeric relationships within the ephedrine and pseudoephedrine molecules and the diastereomeric relationship between ephedrine and pseudoephedrine are readily discernible.

A summary of the nomenclature used in defining stereochemical configurations and related terms is presented in Table 1. For further rules and definitions, the reader is referred to a publication by the International Union of Pure and Applied Chemistry (IUPAC) (5).

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3. E. Fischer, *Chem. Ber.*, *52*: 129–138 (1919).
4. R. S. Cahn, C. K. Ingold, and V. Prelog, *Angew. Chem. Int. Ed. Engl.*, *5*:385–415 (1966).
5. IUPAC, *J. Org. Chem.*, *35*:2849–2867 (1970).

3

Enantiomer Analysis by Competitive Binding Methods

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I. INTRODUCTION

It is only when presented with an asymmetric environment, for example, another chiral molecule, that differences in the structure of enantiomers permit their separation for analysis. Because antibodies and receptors are themselves chiral molecules, nature can provide the analyst with a variety of substances that would be expected to bind drug molecules in a selective manner based on their stereochemistry. The complexes formed by binding of individual enantiomers to a chiral immunoglobulin may be considered diastereoisomers and will have different physical properties, including affinity constants. Because a criterion for demonstrating a specific receptor for a given drug is its stereoselectivity, it is also obvious that receptors can be used to distinguish between enantiomers. Both receptors and antibodies can therefore be used in competitive binding assays to selectively analyze one enantiomer in the presence of the other.

This review will not attempt to be an exhaustive compilation of all references to competitive binding analysis of enantiomers. However, it is my intention to give sufficient examples of this area of research to give the reader some feel for the potential advantages and problems associated with enantiomers in competitive binding assays.

II. DEFINITION OF TERMS

Various immunoassays and receptor assays have been referred to as being stereoselective, enantioselective, stereospecific, or enantiospecific. I generally prefer the suffix selective rather than specific, based on the fact that

most binding proteins exhibit at least some affinity for isomers of a compound, even though that affinity may be minimal. Although from a practical point of view, binding assays can be specific for one isomer, the term selective is a more accurate one and I will use it throughout this chapter. The choice between the use of stereo- and enantio- may depend on the context. Enantiomers are a special case of stereoisomers. Stereoisomers differ in their relative stereochemistry at least at one point in the molecule, but only if two isomers are mirror images of each other are they enantiomers. Stereoselective may be used to describe the ability to distinguish between, for example, *cis* and *trans* isomers of olefins or cyclic compounds or between diastereoisomers. Thus, an antibody for quinidine (1) is properly termed stereoselective, since it was able to distinguish quinidine and its diastereomer quinine. Its enantioselectivity (ability to distinguish *R*- and *S*-quinidine) was not determined. Assays for *R*- and *S*-isomers, described below, are both stereoselective and enantioselective.

III. IMMUNOASSAYS FOR ENANTIOMERS

A. Immunoassay Methodology

1. *Literature*

Immunoassays are a form of competitive binding assay in which a ligand such as a drug molecule interacts in a reversible manner with the binding site of a specific antibody. If the ligand bears a distinguishing label, then displacement by unlabeled drug may be measured and related to the concentration of drug present.

Since the introduction of radioimmunoassay (RIA) by Yalow and Berson in 1959 (2), numerous books and review articles have been written on immunoassays. The reader is referred to the literature for a selection of reviews on general immunoassay methodology (3), RIA (4–7), theory [8] and statistical analysis (9,10), synthesis of immunogens (5,6,11,12), enzyme immunoassays (13,14), fluorescence immunoassay techniques (15), miscellaneous labels (16) and separation techniques (17). A brief discussion of general techniques is presented below.

2. *Basic Requirements*

Basic requirements for a competitive binding assay are a selective, high-affinity binding site, a ligand with a detectable label, and a means of distinguishing bound and unbound labeled ligands. Given the availability of these factors, competitive binding assays have many advantages. These include sensitivity, speed of analysis, simplicity of procedures, and often high selectivity for a given analyte.

3. *Antibodies and Their Production*

Antibody molecules can provide the required high-affinity binding site. Antibody binding sites may be obtained that can recognize any kind of organic compound ranging in molecular mass from perhaps 200 to 1000 Da and in any shape or conformation that such amount of matter can assume. For larger molecules, antibodies are capable of recognizing a portion of the molecule and binding to that. Antigen-antibody binding is noncovalent and involves hydrophobic, ionic, and van der Waals interactions, as well as hydrogen bonding and steric repulsive forces (18).

Small molecules such as drugs are not immunogenic per se, but conjugation of the small molecule (hapten) to a large molecule such as a protein (carrier) results in the formation of immunogenic material (19). When injected into an animal, this immunogen stimulates the formation of antibodies capable of binding the drug. Introduction of a linking group inevitably results in at least some change in the overall structure and electronic configuration of the small molecule, and its presence will influence the affinity and selectivity of the resulting antibodies. Although there seems to be no really definitive studies on the effects of link structure on resulting antibody affinity and selectivity, it may be reasoned that the link should be relatively small in volume, rather uniform in structure, and should facilitate extension of the small molecule away from the surface of the protein.

No definitive study of the effects of protein carrier on response is available, and many carrier proteins have been used, including globulins, albumin, hemocyanin, thyroglobulin, and fibrinogen. The optimal number of haptens, or the epitope density, is also controversial, but a density of 8 to 25 haptens per bovine serum albumin molecule is probably optimal (12).

The classical studies of Landsteiner (19) and many reports since that time show that antibodies resulting from conjugates are generally most selective for those portions of the small molecule that are not involved in the link to protein. However, different animals injected with the same immunogen can still give rise to a wide range of antibody selectivity. Even with the same animal, a range of antibodies of varying selectivity and affinity will be produced on immunization. The antiserum composition will thus vary from one animal to the next and indeed from one bleeding of an animal to the next.

In principal, such problems may be overcome by the hybridoma technique of Kohler and Milstein (20). Immunization of a mouse or other animal will stimulate the production of a series of B cells producing antibodies directed at various portions of the immunogen. Such cells do

not grow well in culture, but they may be fused with myeloma cells, which do grow readily. The resulting hybridoma cells have both the immortality of the myeloma cell line and the antibody-producing capability of the B cell. These cells can be isolated by growing them in a medium that kills the unfused myeloma cells and allows the unfused B cells to die. Cloning by limiting-dilution procedures and selection of the appropriate hybridoma by screening techniques then permit one to develop a cell line that can produce a desired uniform antibody. The cell line can be grown by tissue culture techniques or injection into mice, where it forms ascites. The resulting ascites fluid is rich in the desired antibody.

The hybridoma technique has had an enormous impact on immunology in general, and many antibody reagents are now based on monoclonal antibodies. However, much work is still done with polyclonal antibodies from whole animals. When one recognizes that a few milliters of good antiserum from a rabbit permit thousands or even hundreds of thousands of analyses, it is apparent that generation of polyclonal antibodies, which is much less labor-intensive than generation of monoclonal antibodies, will still be useful for development of immunoassays for several years to come. Nevertheless, the hybridoma technique has much to recommend it and will certainly increase in importance for development of selective immunoassays.

4. Detection of Bound or Unbound Ligand

It is necessary to be able to distinguish the labeled ligand bound to antibody from that which is not bound. This may be done by either some physical separation of bound and unbound labeled ligand, or some change in property of the ligand upon binding to the antibody. Separation techniques have relied heavily on the use of adsorbents, second antibodies, etc. Centrifugation then separates the liquid and solid phases and the appropriate phase can be measured. Other techniques such as the use of magnetizable particles have come into being to facilitate automation of the separation procedure (17).

Although the most common label is still the radioactive hydrogen or iodine atom, the problems associated with radioactivity have led to a continual search for other techniques that may be in some ways preferable to it. Fluorescence, in principal, can be detected with the same sensitivity as radioactivity; however, unlike radioactivity, there is a very significant natural background fluorescence in biological material. A variety of procedures have been called on to get around this problem (15), and some of these have made their way into commercial instrumentation.

One commercial system is based on fluorescence polarization. Molecules absorbing polarized light emit polarized fluorescent light. The de-

gree of polarization is related to molecular size, which in turn controls molecular rotation. Thus, the degree of polarization from a fluorophor-labeled ligand will change when it binds to a macromolecule such as an antibody. This procedure permits a homogeneous assay. Another system is based on the fact that the fluorescence of most serum components is very short-lived. Therefore, if a ligand is labeled with a fluorophor in which the fluorescence has a long lifetime, it is possible to pulse the sample with radiation, measure fluorescence emission after an appropriate interval, and thereby overcome the problem of background fluorescence (21).

The amplification ability of enzymes has led to many schemes for enzyme immunoassay (13,14). The enzyme may be labeled with a drug in such a manner that when the drug portion of the conjugate binds to an antibody, enzymatic activity is inhibited. By measuring the product of the enzyme reaction in the presence of added substrate, one may obtain a measure of the amount of free enzyme-drug conjugate present in solution, which will be related to the competition of this conjugate with the drug in solution.

Alternatively, one may attach the drug to an enzyme in such a way that enzymatic activity is not inhibited. If the conjugate is allowed to bind to the antibody on a solid phase, excess enzyme may be washed away, and then by measurement of enzymatic activity the concentration of enzyme conjugate (and by deduction, therefore, the concentration of competitive drug in the original solution) may be measured. The drug may also be labeled with the enzyme substrate, an enzyme cofactor, an enzyme inhibitor, an enzyme activator, or even with a prosthetic group for an enzyme. Alternatively, a second antibody may be labeled with an enzyme.

Radioactivity, however, is still a very sensitive means of measuring the presence or absence of a given material. Assay methodology has now come full circle, to the development of an ultrasensitive enzyme RIA. In this technique, an antigen is bound to a solid phase. Antibody will bind to the antigen, which could be a drug-protein conjugate, and the presence of bound antibody is detected by means of a second antibody coupled to alkaline phosphatase. So far this is the standard enzyme-linked immunosorbent assay (ELISA). However, if the substrate is tritium-labeled adenosine monophosphate, it is converted by the enzyme to tritium-labeled adenosine, which may be readily separated and measured. The detection limit for this assay for cholera toxin is approximately 600 molecules of the toxin (22).

5. *Enantioselective Antisera: Historical*

As is true for many of the procedures and concepts on which immunoassays are based, Landsteiner's classical studies first pointed the way

toward the use of antibodies for analysis of enantiomers. He produced antisera to D-tartaric acid and showed that the antibodies could discriminate between D- and L-tartaric acid (19). Although this gives a clear indication of the possibilities of enantioselective immunoassay, for many years after the introduction of the immunoassay technique there was little, if any, systematic effort to explore this particular aspect of antibody properties.

B. Development and Use of Enantioselective Antisera from Racemic Immunogens

1. *Introduction*

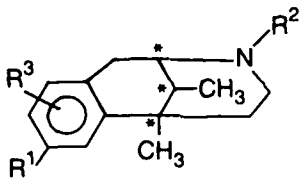
When an animal is immunized with a drug-protein conjugate, a mixture of heterogeneous antibodies may be formed. The immune system may recognize haptenic determinants from the drug itself, or those involving portions of the drug molecule and the protein to which it is conjugated. However, in a typical immunoassay, further selectivity may be achieved by the choice of the labeled ligand. This component of the assay permits the analyst to observe only those antibodies for which it has high affinity.

Conjugation of a racemic drug to a protein increases the possibility for heterogeneous antisera because at least two haptens (*R* and *S*) are introduced. The immune system could respond by producing antibodies to only one enantiomer, or varying amounts of antibodies to each enantiomer. Each antibody may also have affinity for the opposite enantiomer. The use of racemic radioligands further complicates the situation. These problems have been discussed by Maeda and Tsuji (23), Cook et al. (24), and Rominger and Albert (25).

2. *Cyclazocine: A Compound with Multiple Asymmetric Centers*

Rabbits were immunized with a conjugate of racemic cyclazocine (Fig. 1 [1a]) and competitive binding studies carried out with tritium-labeled *d*, *l*-cyclazocine (23). Various types of antibody mixtures were obtained from different animals. One animal immunized with the compound in the form [1c] shown in Fig. 1 exhibited almost no affinity for the *d* isomer of cyclazocine. In such an instance one would expect that the displacement curve for the *d*,*l* mixture would be displaced to higher concentrations than the displacement curve for the *l* isomer, and this indeed was observed. If the antibodies produced were completely nonenantioselective, then displacement curves for *d*, *l*, and the *d*,*l* mixture should be identical. This case was not observed.

Antiserum from an animal immunized with the conjugate [1e] shown



[1]

*Denotes asymmetric center

a. R^1 = –OH, R^2 = $-CH_2$—▽, R^3 = H
b. R^1 = $-OCH_2CONH(BSA)_{1/n}$, R^2 = $-CH_2$—▽, R^3 = H
c. R^1 = $-OCH_2CH_2-NH(BSA)_{1/n}$, R^2 = $-CH_2$—▽, R^3 = H
d. R^1 = –OH, R^2 = $-CH_2CH_2CO-NH-(BSA)_{1/n}$, R^3 = H
e. R^1 = –OH, R^2 = $-CH_2$—▽, R_3 = $-CH_2NH-(BSA)_{1/n}$

FIGURE 1 Cyclazocine and immunogens based on cyclazocine.

in Fig. 1 gave displacement curves in which both the *d* and *l* isomers displaced radioligand at higher concentrations than was observed for the racemate. Such an antiserum is consistent with a model in which there are equal amounts of *d*- and *l*-selective antibodies, each of which cross-reacts to some extent with the opposite enantiomer. In a third animal (immunized with the form of cyclazocine [1a] shown in Fig. 1), the displacement curve for the racemate showed the normal S-shaped characteristics of a standard RIA curve; however, the curves for the individual *d* and *l* isomers, although displaced to higher concentrations, became saturated at lower concentrations than occurred with the *d,l* mixture. This suggests the presence of a mixture of highly enantioselective antibodies. Thus, each antibody binds only its own specific enantiomeric radioligand, and saturation occurs at binding percentages that correspond to the ratio of *d*- and *l*-selective antibodies in the antiserum (23).

Although the three different types of antisera described above each was derived by challenge with a different immunogen, there are insufficient data to determine whether this is done to the immunogen structure or the individual animal.

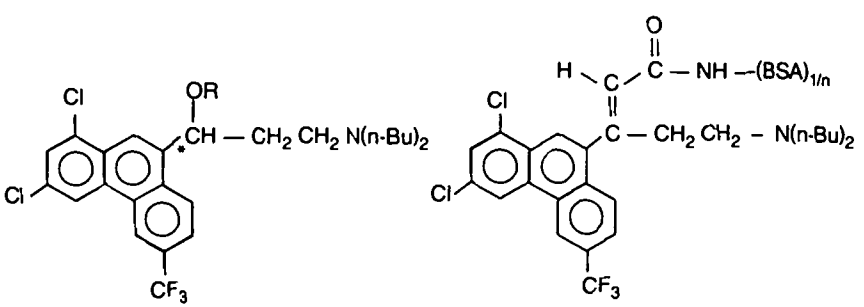
When dogs were administered cyclazocine and their serum was analyzed by RIA, markedly different values were obtained depending on which antiserum was used. Such a result is, of course, to be expected and serves as a warning to those who develop immunoassays from racemic

immunogens. For example, in animals administered *d,l*-cyclazocine, a peak plasma concentration of somewhat over 600 pg/mL was observed 0.5 hr after administration when the *l*-selective antiserum was used. Analysis with an antiserum that contained an apparent mixture of crossreacting *d* and *l* antibodies indicated a peak concentration of about 400 pg/mL at 1 hr after drug administration (23).

3. *WR 171,669: A Compound with a Single Chiral Center*

Cyclazocine has three asymmetric centers and thus the *d* and *l* isomers differ in stereochemistry at three separate points on the molecule. However, a similar situation has been shown to exist in a compound (Fig. 2 [2a]) with a single asymmetric carbon atom: 1-(1,3-dichloro-6-trifluoromethyl-9-phenanthryl)-3-*N*,*N*-dibutyl-aminopropan-1-ol (WR 171,669), an antimalarial compound (24). Conjugation of the hemisuccinate ester of the racemic drug with bovine thyroglobulin gave an immunogen, shown to contain equal amounts of the *d*- and *l*-WR 171,669 (Fig. 2 [2b]), which was injected into rabbits. When tested with tritium-labeled racemic WR 171,669 as the radioligand, antisera from two rabbits challenged with the immunogen exhibited equivalent 50% displacement values for the racemate and *l* isomer, with markedly higher values for the *d* isomer (Fig. 3A). A third rabbit showed a similar trend but differences were smaller.

Comparison of displacement curves from one antiserum was made by



a. R = H, WR 171,669
b. R = CO–CH_2 CH_2 CO–NH–$(BSA)_{1/n}$

*Chiral center

FIGURE 2 WR 171,669 and related immunogens.

use of the program ALLFIT (26). This program permits simultaneous analysis of families of sigmoidal curves that may be fitted by the general logistic equation $y = D + (A - D)/[1 + (x/C)^B]$. Initially, individual parameters are fit to each curve without constraint. The curves can then be forced to share one or more parameters in common. If the constraints chosen do not significantly degrade the quality of fit (F test), the curves are assumed to have those parameters in common. This test showed that (as expected from inspection) the *d* and *d,l* displacement curves could not be constrained to share all the parameters ($p < 0.001$). Even the *l* and *d,l*

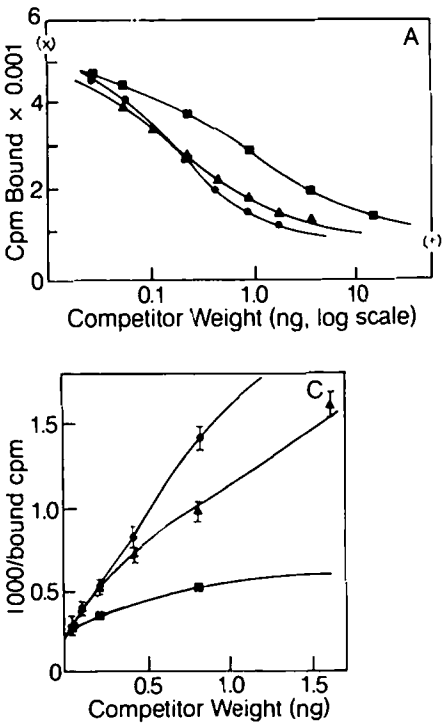


FIGURE 3 Competition with binding of racemic tritium-labeled WR 171,669 expressed as counts per minute of radioligand bound as function of log of competitor weight (A) or reciprocal of counts per minute of radioligand bound as a function of competitor weight (C). × = binding in absence of competitor; + = nonspecific binding; ■ = *d*-, ▲ = *l*, ● = *d,l*-WR 171,669. (From Ref. 23, used with permission.)

displacement curves could not share all parameters ($p < 0.001$). Although they have a superficial resemblance and almost identical values for parameter C (the 50% displacement intercept), B (the slope parameter) differed significantly (0.76 vs. 0.99).

Cook et al. (24) also applied the method of Pratt et al. (27) for evaluating cross-reaction. In this procedure the reciprocal of bound radioligand is plotted vs. competitor concentration. This provides a very sensitive test for divergence of standard curves, which may not be observed in the normal standard displacement plot. The Pratt plot showed rapid divergence of all three lines (*d*, *d,l*, and *l*), although divergence between *d,l* and *l* lines was less than between *d,l* and *d* lines (Fig. 3C).

For individual rabbits the relative enantioselectivity of the antisera appeared to remain fairly constant over time. However, an insufficient number of animals were studied for definitive conclusions.

4. *Mathematical Description of Multiple Antibodies and Ligands*

A mathematical description of the situation involving two enantioselective antibodies and a racemic radioligand in the RIA procedure is complex. Rominger and Albert (25) have examined this situation from a theoretical basis and have reported equations that can be used to describe the situation, although they cannot be solved explicitly. By assuming two different antibody concentrations, different affinities of the antibodies for the corresponding enantiomers of the tracer and of the ligand [one antibody with a very low cross-reactivity and one with a relatively high cross-reactivity (10%)], Rominger and Albert arrived at a plot for the *d*, *l*, and *d,l* mixture displacement curves that resembles closely the experimental plot (Fig. 3A) presented by Cook et al. (24).

5. *Conclusions*

The above work indicates that a wide variety of responses may be obtained using antisera from immunization of animals with a racemic immunogen and competitive binding studies with racemic radioligand. This point seems not to have been considered by all investigators. In a sampling of 20 papers in which immunization was carried out with the *d,l*-immunogen, 7 made no mention of cross-reactions of the individual isomers in the binding studies; 3 simply made the statement that there was no discrimination of isomers; 3 reported that, based on 50% inhibition of binding, the *d*, *l*, and *d,l* forms had equivalent displacement of racemic radioligand; 2 indicated that the *d* and *l* forms showed 50% cross-reaction when compared with the racemate; and 3 papers studied these interactions in more detail.

This is an area that should receive considerably more attention. The data of Maeda and Tsuji (23) show the very significant errors in plasma concentrations that can result from use of inappropriately enantioselective sera in immunoassay of plasma from animals given racemic drug. The data of Cook et al. (24) demonstrate that even in cases in which the cross-reaction calculated at 50% inhibition is identical between an isomer and the racemate, the slopes of the two curves may differ significantly. Thus, it would behoove those who generate "nonselective" antisera from racemic immunogen to show that the resulting antisera will indeed give correct values for total concentration of the drug under the conditions of the experiment. Simple 50% cross-reaction data may not be sufficient to establish coincidence of curves, and an approach such as the use of ALLFIT (26) or Pratt-type plots (27) should be carried out to establish lack of enantioselectivity of an assay.

In connection with the above recommendation, it should be recognized that the purpose of an assay is to give valid data. An enantiomerically mixed assay can still give valid results if the ratio of enantiomers in the biological system measured does not vary appreciably from unity. Rominger and Albert (25) suggest determining whether the isomer ratio differs from unity by analysis of serial dilutions of the sample vs. the racemate standard curve. This is a standard approach to establishing identity of analyte and standard and may be helpful in cases in which it is not known whether the enantiomer ratio varies due to differences in enantiomer pharmacokinetics.

For compounds with a single asymmetric center, it might in principle be possible to generate antisera that are not enantioselective by using an immunogen that is a nonchiral analog of the drug in question (24). Thus, when the tetrahedral secondary alcohol carbon of WR 171,669 was converted to a trigonal carbon analog (Fig. 2 [3]) and this substance used for immunization, the resulting antisera showed relatively little enantioselectivity in a competitive binding assay with the *d,l* radioligand. Because antibodies are chiral molecules, they could nevertheless exhibit enantioselectivity even when generated to a nonchiral conjugate. In the case of WR 171,669, the enantioselectivity was small. This indicates that the binding site was sufficiently flexible to accommodate the change from the essentially planar trigonal template of the immunogen to the tetrahedral structure of the analyte, and that the binding site was insensitive to changes in the drug molecule close to the site of the hapten-protein link in the immunogen. This flexibility was observed even though the apparent affinity constant remained high (approximately 10^{10} L/mol). This approach may be useful in other instances where a single asymmetric center is

present (24). Obviously, when two or more asymmetric centers are present, the likelihood of being able to devise a nonchiral analog for immunogen formation becomes much smaller (25).

C. Development and Use of Antisera for a Single Enantiomer

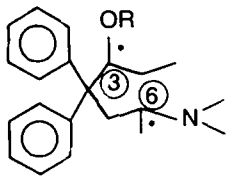
1. *Introduction*

In a number of cases, investigators have prepared immunogens from one of two enantiomers and used these for analysis of that particular enantiomer. In some cases, the radioligand was enantiomerically pure; in others, racemic radioligand was used. Usually, this was done because of some specific information regarding the differential pharmacological effect of the enantiomer. In other cases, the drug itself is already administered as a single enantiomer and thus is often readily available for conjugation to protein. Most RIAs for steroids are developed by means of immunogens incorporating the naturally occurring or hormonally effective steroid enantiomer. The voluminous literature on this type of assay will not be further discussed. Likewise, a number of natural products that are generally obtained in the form of a single enantiomer have been subjected to RIA development. Unless there is special information regarding the cross-reaction of enantiomers, this also will not be covered.

2. *Psychoactive Drugs*

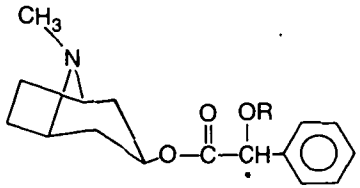
A 1974 report described the development of an antiserum for *d*-amphetamine. Inhibition studies were carried out by means of displacement of tritium-labeled *d*-amphetamine, and the cross-reaction for *l*-amphetamine was 4.5% (28). Later an antiserum to *d*-methamphetamine was reported to have no significant cross-reaction with the *l* isomer (29).

RIA for *L*-α-acetyl methadol (LAAM) has been reported (30). The *O*-acetyl group of this compound (Fig. 4 [4a]) was replaced by an *O*-succinyl moiety, which was coupled to bovine thyroglobulin by mediation with a soluble carbodiimide reagent to give an immunogen (Fig. 4 [4b]). Tritium-labeled LAAM was used as a radioligand. A sensitivity of 50 pg was recorded with no serum interference. LAMM has two asymmetric centers, both of the *S* configuration. The diastereoisomer formed by change of the 3-*S* configuration (which bears the acetate residue and was involved in linkage to the protein) to the 3-*R* configuration exhibited a cross-reaction 25% that of LAMM. When the 6 carbon atom in the chain (Fig. 4) was changed from *S* to *R*, the cross-reaction dropped to 0.033% and the 3-*R*, 6-*R* compound (enantiomer of LAAM) had a cross-reaction of 0.17%. Thus, in this case, we see the general trend toward greater differen-



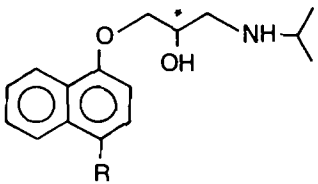
[4]

a. R = $-COCH_3$ (LAAM)
b. R = $-COCH_2CH_2CONH$-thyroglobulin



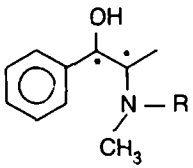
[5]

a. R = H (Atropine)
b. R = $-COCH_2CH_2CO-NH-(BSA)_{1/n}$



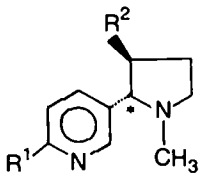
[6]

a. R = –H (Propranolol)
b. R = –OH (4-hydroxy propranolol)



[7]

a. R = H (pseudoephedrine)
b. R = $CH_2CH_2CONH(BSA)_{1/n}$



[8]

a. $R^1 = R_2 = H$ (nicotine)
b. $R^1 = -NHCO-C_6H_4-N=N-(BSA)_{1/n}$, $R^2 = H$
c. $R^1 = H$, $R^2 = -CH_2O-COCH_2CH_2CONH(BSA)_{1/n}$

*Chiral center

FIGURE 4 Structures of drugs and immunogens.

tiation as one goes further from the link to protein. This antiserum had good selectivity vs. the isomeric methadols, but exhibited 12% cross-reaction with the *N*-desmethyl metabolite norLAMM. When norLAMM levels were twice those of LAMM, a 25% positive error was introduced. Such ratios can occur as early as 11 hr after LAMM administration (unpublished results) and may be more likely on repeated dosage, due to the longer half-life of the metabolite. This highlights the fact that enantioselectivity alone is not enough. Selectivity vs. metabolites and other closely related compounds must also be taken into consideration.

3. *Atropine*

S-atropine (Fig. 4 [5a]) is markedly more potent than the *R* isomer in a series of tests (31) and binds much more strongly to the muscarinic receptor (32). Indeed, given the propensity of this particular compound for racemization (33) and therefore its lack of complete optical purity, it would appear that essentially all the biological activity resides in the *S* enantiomer.

Various preparations of antisera to atropine have been reported. A racemic hemisuccinate ester was prepared and conjugated to bovine serum albumin by the carbodiimide technique. Antisera formed to the original immunogen selectively bound the *R* isomer (34), but a later antiserum prepared by this approach was reported to bind both *R* and *S* forms with "equal efficiency" (35). *R,S*-atropine was treated with diazotized *p*-aminobenzoic acid, and the resulting compound (which was not further characterized) was used for conjugation to bovine serum albumin by means of a carbodiimide-mediated reaction. Antisera resulting from use of this material were quite selective for the *R* isomer, with a cross-reaction of only about 2% for the *S* isomer (36). Virtanen et al. followed this procedure with *S*-atropine. Their antiserum bound equally to *S*- and *R,S*-atropine, as measured by displacement of tritium-labeled *R,S*-atropine (37). In another study (31), both racemic atropine and the *S* isomer were coupled to human serum albumin by the technique of Wurtzburger et al. (36). Antisera were obtained that were selective for both the *R* and *S* isomers (33).

The work with atropine presents a confusing picture. Use of racemic radioligand in these studies may contribute to the observed cross-reactivity picture. The rather facile enolization and hence racemization of atropine-type structures is another problem. Finally, the structure of the product of atropine and diazotized *p*-aminobenzoic acid has never been established conclusively. For these reasons, the radioreceptor assay for this compound (see below) is generally preferable.

4. *Propranolol*

The importance of propranolol (Fig. 4 [6a]) has led to the development of a number of immunoassays for it. Most of the workers dealt with the

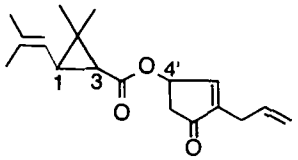
racemic compound. However, Kawashima et al., in an early demonstration of the utility of enantioselective assays, developed a procedure for analysis of the *l* isomer (38). These workers made a hemisuccinyl derivative of *l*-propranolol and coupled it to bovine serum albumin by a mixed anhydride technique. Antisera were generated in rabbits, and the radioligand was racemic tritiated propranolol. With this antiserum, this group measured plasma concentrations of the *l* isomer in rat blood (38), as well as in mice (39). Because they also had generated an antiserum against the racemic material that had little capability for distinguishing *d*-, *l*-, or *d,l*-propranolol, they also determined total propranolol and by difference could obtain an estimate of the *d* isomer as well. In the rat they found a higher initial concentration of *d* than *l* isomer, but much higher amounts of the *l* isomer in the heart. This was attributed to selective uptake of the *l* isomer by receptors. Because of the relatively high (7%) cross-reaction of the *d* isomer with the *l* antiserum, concentrations of the *l* isomer were corrected. The actual concentration of *l* was equal to [(measured concentration of *l* isomer) − 0.07(measured concentration of *d,l*-propranolol)]/0.93.

5. *Pseudoephedrine*

Findlay and co-workers developed an RIA for *d*-pseudoephedrine (Fig. 4 [7a]) (40). The compound was allowed to add to methyl acrylate. After hydrolysis, the resulting carboxylic acid was conjugated to bovine serum albumin by means of a carbodiimide procedure to form an immunogen (Fig. 4 [7b]). The radioligand for the binding studies was either tritium-labeled *d,l*-pseudoephedrine (5 Ci/mmol) or a conjugate of the *d*-pseudoephedrine-methyl acrylate adduct (analogous to [7b]) with tyrosine methyl ester labeled with ^{125}I. Charcoal or polyethylene glycol was used to separate free and bound ligand. With the tritium-labeled radioligand, serum could be analyzed directly, but plasma had to be extracted if the ^{125}I radioligand was used. The plasma extract was treated with methyl acrylate to convert the *d*-pseudoephedrine to a compound more closely resembling the original immunogen. By this procedure it was possible to achieve sensitivities down to 0.2 ng/mL. When the iodinated radioligand was used, the cross-reaction with *l*-pseudoephedrine was less than 0.003%. With the tritium-labeled *d,l* radioligand, the cross-reaction with the *l* isomer was 0.01–0.05%. *l*-Ephedrine, a diastereoisomer, cross-reacted to the extent of 0.13–0.32%.

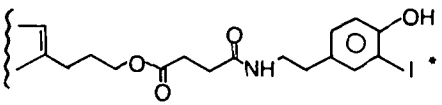
6. S-*Bioallethrin*

S-Bioallethrin (Fig. 5 [9a]) is a synthetic pyrethroid insecticide with three asymmetric centers. The *S* designation refers to the 1-*R*, 3-*R*, 4′-*S* isomer and is not a true designation of stereochemistry. Conversion of the terminal allylic group to $—CH_2CH_2CH_2OH$, formation of hemisuccinate,

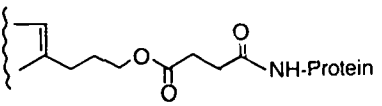


[9]

a. 1–R, 3–R, 4′–S
b – h. See Table 1.



[10]



[11]

FIGURE 5 Bioallethrin structures.

and conjugation to protein led to an immunogen (Fig. 5 [11]) by means of which stereoselective antibodies were obtained. A ^{125}I-labeled tyramine conjugate (Fig. 5 [10]) was used as the radioligand. All eight isomers were studied for their ability to inhibit radioligand binding. As Table 1 shows, the enantiomer [9b] of compound [9a] (Fig. 5) had less than 1% cross-reaction. Changing only the asymmetric center at the 4′ position is less critical for binding than changing the orientation of the groups on the

TABLE 1 Stereochemistry and Antibody Binding in the Bioallethrin Series[a]

Compound	Cyclopropane substituent orientation	Absolute configuration 1	3	4′	Percent cross-reaction at 50% inhibition
[9a]	*Trans*	*R*	*R*	*S*	100
[9b]	*Trans*	*S*	*S*	*R*	0.8
[9c]	*Trans*	*R*	*R*	*R*	57
[9d]	*Trans*	*S*	*S*	*S*	5
[9e]	*Cis*	*R*	*S*	*S*	<0.1
[9f]	*Cis*	*S*	*R*	*S*	2
[9g]	*Cis*	*R*	*S*	*R*	3
[9h]	*Cis*	*S*	*R*	*R*	≪0.1

[a]The antiserum was generated from a conjugate of an analog of compound Fig. 5[9a].
Source: Wing and Hammock (41).

cyclopropane ring (compare Fig. 5 [9c] vs. [9d] or [9c] vs. [9e] or [9f]). Although the authors attribute this to the closer proximity of the 4′ position to the conjugation link (41), the 4′ position is well removed from the link. An alternative explanation would be the flexibility of rotation around the 4′ *O*—*C* bond compared to the rigidity of the cyclopropane ring.

7. *Nicotine*

Although *l*-nicotine is the predominant natural isomer, the occurrence of some *d*-nicotine makes it of interest to develop enantioselective assays for this compound. A review of nicotine and cotinine assays has been given (42). An early report of an RIA for *l*-nicotine described an immunogen prepared from 6-aminonicotine. This compound was converted to 6-(*p*-aminobenzamido)nicotine and conjugated to albumin (Fig. 4 [8b]) by diazotization (43). The enantiomeric purity of the 6-aminonicotine was not discussed, and the synthesis of 6-aminonicotine is reported to cause racemization (44). However, the antiserum eventually obtained showed only 6% cross-reaction with *d*-nicotine (43).

A racemic nicotine analog, *trans*-3-hydroxymethyl-nicotine, was converted to the hemisuccinate, which was conjugated to protein to form an immunogen (Fig. 4 [8c]). The resulting antiserum was used with tritiated *l*-nicotine as radioligand. With this radioligand the assay was highly selective for *l*-nicotine, with less than 0.01% cross-reaction with *d*-nicotine. Similar enantioselectivity is claimed for *l*-cotinine (42).

Hybridomas producing monoclonal antibodies to *S*-nicotine were obtained from mice immunized with conjugated racemic 3′hydroxymethyl-nicotine. Affinity constants were around 10^8 M^{-1} with 4% cross-reaction to *R*-nicotine (67).

D. Development and Use of Antisera for Both Enantiomers

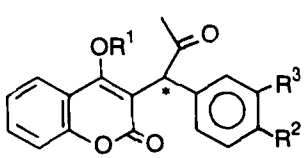
1. *Introduction*

In not all instances in which racemic drug is administered is only one enantiomer of interest. Even when the major portion of the activity is associated with a single enantiomer, knowledge of concentrations of the other enantiomer may be of interest because of potential for toxicity or other side effects. Furthermore, it would be anticipated that the best selectivity would be achieved if both immunogen and labeled ligand were enantiomerically equivalent and optically pure. The enantiomeric immunogen would favor formation of enantioselective antibodies, and the enantiomerically pure labeled ligand would further enhance enantioselectivity by allowing the analyst to observe and use those antibodies with highest

affinity for the enantiomer. A few examples of this approach have been published.

2. *Warfarin*

A method for the determination of both warfarin enantiomers based on enantioselective immunoassay has been reported (45). Warfarin analogs (Fig. 6) containing a 4′-carboxyethyl group (as the methyl ester) were converted to their diastereoisomeric camphor-sulfonates (Fig. 6 [12b]). These were separated chromatographically. Base-catalyzed hydrolysis then removed the camphor-sulfonyl group to yield the pure enantiomeric warfarin analogs. These were individually conjugated to bovine serum albumin by a mixed anhydride procedure to yield an immunogen (Fig. 6 [12c]). For radioligands, halogenated warfarin derivatives were resolved in a similar manner and then reduced with tritium gas to yield radioligands (Fig. 6 [12d]) of high optical purity (25–31 Ci/mmol). To demonstrate that racemization had not occurred under the catalytic conditions of the reduction, the tritiated *S* enantiomer was mixed with unlabeled *R,S*-warfarin. The *d*-10-camphor-sulfonates were synthesized and separated chromatographically, and it was shown that the tritium in the diastereoisomer containing the *R*-warfarin enantiomer was less than 1% that in the diastereoisomer containing the *S*-warfarin enantiomer.



[12]

a. $R^1 = R^2 = R^3 = H$ (warfarin)
b. R^1 = Camphorsulfonyl
$R^2 = CH_2CH_2COOMe$
$R^3 = H$
c. $R^1 = R^3 = H$
$R^2 = CH_2CH_2CONH(BSA)_{1/n}$
d. $R^1 = R^2 = H$
$R^3 = T$
*Chiral center

FIGURE 6 Warfarin, analogs, and immunogen.

R-warfarin exhibited a cross-reaction of only about 0.3% with the *S*-antiserum and *S* radioligand. *S*-warfarin cross-reacted to the extent of 3% with the *R*-warfarin antiserum and *R* radioligand. Various warfarin metabolites were shown to have low cross-reactions, with the exception of 4′-hydroxy warfarin. This latter compound is not a human metabolite, although it is found in rats in low concentration after a single dose of warfarin. The method could be used to determine the half-life of the individual enantiomers in rats given racemic drug; the resulting ratios of half-lives were in accord with those previously reported after administration of individual enantiomers. This enantioselective immunoassay was used in humans to demonstrate that vaccination against influenza did not change the relative pharmacokinetics of warfarin enantiomers (46).

The development of the warfarin immunoassays illustrates several points that are of value in development and use of enantioselective assays. In assays of this type, not only must enantioselectivity be considered, but also the cross-reaction with metabolites is still of importance. As in any RIA, high-specific-activity radioligand is required for the best sensitivity. The use of optically pure radioligand is a further advantage in enantioselectivity. The standard samples used for competitive binding assays must also be essentially optically pure. Otherwise, misleadingly high cross-reactions may be observed.

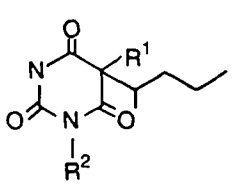
Two rabbits were challenged with each immunogen, and all four rabbits gave highly enantioselective antisera, thus indicating that the use of enantiomerically pure immunogens can be relied on to generally produce antisera of high enantioselectivity.

Unpublished results of immunoadsorption studies indicate that the low cross-reactions between enantiomers are probably not due to the presence of small amounts of antiserum selective for the opposite enantiomer. Such antibodies might be generated if an animal responds strongly to a very small amount of the opposite enantiomer present as an impurity. It was observed that even a low cross-reaction may still result in some inaccuracies, particularly in instances in which one enantiomer is present in much higher concentrations than the others. Also, cross-reaction curves are not generally parallel. Thus, in the assay of *R*- and *S*-warfarin in rat plasma, it was necessary to set up procedures for correction of the observed cross-reaction. In the rat, the *R* isomer, which yields the highest cross-reaction with the opposite enantiomer, is also the one with higher plasma concentration and longer half-life. The observation that corrections are necessary, and the earlier problems discussed with antisera to racemic immunogens constitute a strong argument for preparing and using antisera to both enantiomers of a drug.

3. *Pentobarbital and Its Analogs*

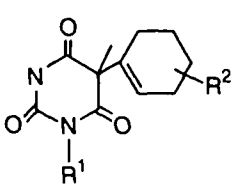
A class of barbiturates possessing the 2′-pentyl side chain all exhibit similar optical isomerism. These include pentobarbital (Fig. 7 [13a]), thiopental, thiamylal, and secobarbital (Fig. 7 [13b]). Differences in pharmacological activity between enantiomers of these compounds have been shown in humans (47) and animals (48).

R and *S* forms of pentobarbital have been synthesized (49,50) and alkylated to yield eventually the corresponding *N-trans*-crotonic acids. The latter were conjugated to bovine serum albumin, and the resulting conjugates (Fig. 7 [13c]) used to immunize rabbits. Enantiomerically pure tritium-labeled *R*- and *S*-5-propyl-5-(2′-pentyl)-barbituric acids (Fig. 7 [13d]) were made by catalytic reduction of the enantiomers of secobarbital with tritium gas, for use as radioligands. The resulting antisera were quite selective, with only about 1% cross-reaction of each antiserum with the opposite enantiomer and less than 1% cross-reaction with the hydroxy metabolite of pentobarbital (51). The resulting system could also be used to analyze the other closely related 2′-pentyl barbiturates. For example, the *S* isomer of secobarbital showed 83% cross-reaction with the antiserum to *S*-pentobarbital and only 2% cross-reaction with the *R* antiserum and could be used to analyze these isomers in human subjects given racemic secobarbital (52). The assay methodology was checked by adding the values for the two enantiomers of pentobarbital and comparing them with those from gas/liquid chromatography (GLC), which did not resolve the enantiomers (68).



[13]

a. R^1 = Et, R^2 = H (Pentobarbital)
b. R^1 = $CH_2CH{=}CH_2$, R^2 = H (Secobarbital)
c. R^1 = Et
R^2 = $CH_2CH^{tr}{=}CHCONH(BSA)_{1/n}$
d. R^1 = CH_2CHTCH_2T
R^2 = H



[14]

a. R^1 = CH_3, R^2 = H (Hexobarbital)
b. R^1 = CH_3, R_2 = T
c. R^1 = $CH_2CH_2CH_2CH_2NH(BSA)_{1/n}$
R^2 = H

FIGURE 7 Barbiturate structures.

4. *Hexobarbital*

In the case of both warfarin and pentobarbital, the molecule has a single asymmetric center in a nonrigid position of the molecule. When one considers that, in the case of pentobarbital, an exchange of positions between a hydrogen atom and methyl group reverses the stereochemistry, the selectivity of the antibodies for such substances is impressive. If the single asymmetric center were part of a more rigid system, such as a ring, one might anticipate even greater possibilities for enantioselectivity. This was shown to be the case with hexobarbital (Fig. 7 [14a]) in which the asymmetric center is the 5 carbon atom of the ring.

Synthesis of enantiomeric analogs of this drug made use of stereoselective reactions beginning with the enantiomers of 2,2,2-trifluoro-1(9-anthryl)-ethanol. Esterification of each enantiomer with 2-cyano-2-cyclohexylidene acetic acid yielded an optically active compound, which was methylated to create an additional asymmetric center. The methylation was stereoselective, and one diastereoisomer could be crystallized out in high optical purity. Reaction of the optically pure esters with methylurea led to optically pure enantiomers of hexobarbital, which by bromination/reduction procedures could be converted to tritium-labeled hexobarbital (Fig. 7 [14b]). If 4,4-dimethoxybutylurea was used in place of methylurea, eventually an optically active analog having the nitrogen of the hexobarbital linked to a butyraldehyde moiety resulted. This could then be conjugated to the amino groups of bovine serum albumin to give an immunogen (Fig. 7 [14c]). Possibly because of the greater rigidity of the asymmetric center, the *d*-hexobarbital cross-reacted with the antiserum to the *l* immunogen to the extent of only 0.005%; the cross-reaction of *l*-hexobarbital with the *d* antiserum was only 0.0005% (53).

5. *Methadone*

To develop assays for *d*- and *l*-methadone, the hemisuccinates of α-*l*-methadol (Fig. 4 [4a]) were conjugated to bovine thyroglobulin (54). The resulting immunogens (Fig. 4 [4b]) caused formation of antisera that were quite selective when used with enantiomerically pure tritium-labeled *d*- or *l*-methadone. The cross-reaction of the *l* isomer with the *d* antiserum was less than 1%, and cross-reaction of *l*-methadone with the *d* antiserum was about 3%. The racemate exhibited cross-reactions of 56–57% (55).

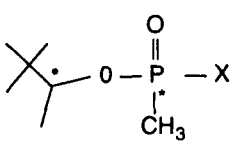
6. *Ephedrine*

Ephedrine is a diastereoisomer of pseudoephedrine (Fig. 4 [7a]) having the erythro configuration. The enantiomeric immunogens of this compound were prepared (57) in a manner similar to that described for *d*-pseudoephedrine (40). Tritium-labeled *d,l*-ephedrine was used as the

radioligand. Cross-reactions of antisera with the opposite enantiomer were less than 2%. When plasma from a subject given *d,l*-ephedrine was analyzed by RIA, the sum of the two enantiomer concentrations agreed closely with the total ephedrine concentration determined by GLC-electron capture detection (57).

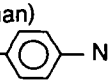
7. *Monoclonal Antibodies to Soman*

With the advent of hybridoma technology, the question arises as to whether, given a racemic immunogen, one would be able to generate hybridomas producing monoclonal antibodies to both isomers with high enantioselectivity. This is supported by the observed enantioselectivity of polyclonal antisera to racemic immunogens discussed earlier and the results obtained with nicotine (67). The selectivity of such antibodies was less clear cut with a diastereoisomeric compound. Monoclonal antibodies were developed to the nerve agent soman (Fig. 8 [15a]) by immunizing mice with a phenyldiazonium analog of soman conjugated to keyhold limpet hemocyanin or bovine serum albumin (Fig. 8 [15b]). Soman has two asymmetric centers: one on carbon [C(±)] and one on phosphorus [P(±)]. One antibody-producing clone was obtained from each protein. A competitive inhibition enzyme assay was used to test binding affinity. The first monoclonal antibody had the following order of affinity: C(+)P(+) ≤ C(−)P(+) < C(−)P(−) < C(+)P(−). The second antibody also exhibited a preference for the more toxic P(−) diastereoisomers. Unfortunately, the observed affinity constants were very low (covering a range from 5×10^3 L/mol to $< 10^3$ L/mol for the first antibody and from 5.9×10^5 to 5.2×10^4 for the second antibody). Other selectivity observations indicated that only two loci on the soman molecule (the P=O oxygen and *t*-butyl group)



[15]

a. X = F (Soman)

b. X = — O —  — N = N — (Protein)$_{1/n}$

• Chiral center

FIGURE 8 Soman and immunogen based on soman.

provided the major source of interaction with the antibodies. Because three loci are needed for enantioselectivity, the rather poor range of enantioselectivities may be explained. The immunogen used for this coupling contains a highly immunodominant azophenyl residue, and it is not surprising that the resulting antibodies have low affinity for the actual soman molecule itself (56).

IV. RECEPTOR BINDING ASSAYS

A. Introduction

Receptors for drug molecules generally exhibit stereoselectivity, including enantioselectivity. Thus, receptors are in many ways ideally suited for the development of enantioselective binding assays. A property of receptors that often makes them different from antibodies is the fact that receptors will tend to bind to a series of drugs in a manner correlated with their pharmacological activity. Pharmacologically active metabolites may be therefore analyzed in a receptor binding assay. Results from such assays are therefore best quoted in terms of drug equivalents unless it has been established for certain that only one analyte is involved. This property has the disadvantage that the actual analyte may be a poorly defined mixture of substances. On the other hand, it can be argued that the analysis result may have a better correlation with drug effects than a more precise measurement of a single substance, which may not represent all the pharmacologically active material present. Brief reviews of radioreceptor assays have been given (58,59).

B. Antimuscarinic Drugs

A good example of the potential advantages of radioreceptor assay (RRA) is illustrated by the case of atropine (Fig. 4 [5a]). As has been previously discussed, the generation of enantioselective antisera for this particular compound has presented problems; the drug has often been analyzed using a nonenantioselective immunoassay, even though most, if not all, of the biological activity resides in the *S* isomer. Metcalfe (60) isolated a fraction of porcine brain that contained muscarinic binding sites and had high affinity for tritium-labeled quinuclidinyl benzilate, which was used as the radioligand (40 Ci/mmol). The receptor fraction could be kept up to 1 yr at −20°C without significant loss of activity. Assay sensitivity was increased by using sequential addition (allowing the receptor to react first with the atropine and then with the radioligand). Radioligand bound to receptor was separated from unbound material by filtration through glass fiber disks, and the radioactivity in the disk was measured. Nonspecific binding (a difficulty sometimes associated with RRA) was less than 5%.

Plasma proteins were precipitated with methanol, and the methanol extract was evaporated before analysis. The sensitivity of the assay was 300 fmol in the assay tube (equivalent to 87 pg of atropine), and plasma samples could be measured down to 1.4 ng/mL (60). The assay in general responded to muscarinic antagonists and was reportedly considerably more sensitive to (−)-hyoscyamine (*S*-atropine) (60) than an RIA procedure (36).

Aaltonen et al. (61) compared RRA and RIA for atropine. These workers obtained preparations of receptor from rat brain and lyophilized them to a stable, dry form. They used the tritium-labeled quinuclidinyl benzilate at 35 Ci/mmol. The affinity constant was 0.48 nM, and by analysis of 25-μL serum samples they could obtain a sensitivity down to 1.25 ng/mL in serum. Nonspecific binding was again quite reasonable (4%) and a filtration-type separation was used. The *d* isomer of an atropine did not bind, and therefore, the cross-reaction of the *d,l* compound was 50% that of the *l* isomer. For comparison they used RIA developed by the method of Virtanen et al. (37). The immunogen was an *l*-hyoscyamine-bovine serum albumin conjugate, but the antiserum was sensitive to both *d,l* and *l* isomers. Racemic tritium-labeled atropine was used as the radioligand.

When plasma samples were analyzed by both techniques, the RIA showed much greater concentrations in plasma than the RRA. The AUC calculated from RIA was 104 μg·h/L but only 28.9 μg·h/L when calculated from RRA. The RRA results fit a three-compartment model and the volume of distribution by RRA was two times that found by RIA. Half-lives calculated from the two methods were about the same, but the clearance by the RRA was about three times that calculated from the RIA results (61). Clearly, in this case, more valuable results were obtained by an enantioselective RRA than a poorly selective or nonselective RIA.

C. β-Adrenergic Antagonists

The beta blocker drugs often contain an asymmetric center, since many of them are based on an aminodihydroxypropane structure. Nahorski et al. (62) obtained membranes from bovine lung tissue that contained beta receptors. In combination with tritium-labeled (−)-dihydroalprenolol (48–58 Ci/mmol), these membrane fragments were used to develop an assay applicable to several beta blocking drugs. *l*-Propranolol (Fig. 4 [6a]) competed very well with the radioligand for binding sites (K_i 0.8 nM vs. k_d of 0.95 nM for dihydroalprenolol). *d*-Propranolol competed only about 1.3% as well, with a K_i of 60 nM. A series of other beta blocking drugs were examined. Although in several instances, the *l* isomer was tested (timolol, isoprenoline, adrenaline, and noradrenaline), the *d* isomer was not sim-

ilarly examined. In general, however, the compounds tested displaced the radioligand with affinities that matched their pharmacological potency. The assay was used to measure *l*-propranolol in volunteers who took 40 mg of *d,l*-propranolol. The lack of interference of the *d* isomer was established by analyzing plasma from volunteers given 40 mg of *d*-propranolol. In this case, the apparent concentration of propranolol was essentially indistinguishable from zero. Proteins did not have significant effects on the analysis of *l*-propranolol. This suggests that dissociation from plasma proteins occurs during the assay, since propranolol is highly protein bound.

4-Hydroxypropanolol (Fig. 4 [6b]) is an active metabolite that contributes to the beta blocking effect of propranolol. Its affinity (as the racemate) for the beta receptor in this instance was only 5% that of propranolol. If we assume that its enantioselectivity was similar to that of the parent drug, this would be equivalent to about 10% cross-reactivity for the *l* metabolite. It would appear that 4-hydroxypropanolol would contribute little to the apparent propranolol equivalents in plasma unless its concentration was markedly greater than that of the parent drug.

Another radioreceptor assay for propranolol used an ^{125}I radioligand (iodohydroxybenzylpindolol) and β-receptor sites were obtained from turkey erythrocyte membranes (63). The membranes were stable indefinitely when stored at −80°C in buffer and could be repeatedly frozen and thawed. Glass fiber filters were used to separate free and bound radioligand. Serum samples inhibited binding to the membrane very readily, and it was necessary to extract propranolol from serum before assay. Indications were that some constituent of serum destroyed the ability of the radioligand to bind to the β-receptors. Most of the interference could be avoided by heating serum to 95°C. The sensitivity limit of the assay was about 0.25–0.5 ng/mL. The assay was selective for the *l*-isomer, although high concentrations of *d*-propranolol could slightly inhibit binding. It was more sensitive to propranolol than to its metabolites 4-hydroxypropanolol or desisopropylpropanolol. The conditions used for treatment of plasma and extraction appeared to get rid of these compounds, so the authors concluded that mainly *l*-propranolol was being analyzed (63).

The method correlated well with both a high-performance liquid chromatography and an RIA procedure, with a slope approximating 1 in each case. The HPLC procedure at least measures both enantiomers. The most probable explanation of this result is that the enantiomers differ little in concentration in human plasma under the conditions of the experiment. Since racemic propranolol was used for generating the standard displacement curve, inability to detect the *d* isomer would be compensated for.

This procedure was modified to measure total β-adrenergic blocking

activity in plasma as well as the separate contributions of propranolol and 4-hydroxypropanolol (64). The cross-reaction of 4-hydroxypropanolol with this receptor protein is about 25% that of propranolol itself (probably 50% with the *l* enantiomer, since racemic 4-hydroxypropanolol was used as a standard). Pharmacologically inactive metabolites of propranolol do not react with the receptor. 4-Hydroxypropanolol was stabilized in plasma by addition of sodium bisulfite and could then be recovered quantitatively by extraction. Alternatively, oxidation of plasma with dilute hydrogen peroxide destroyed 4-hydroxypropanolol without affecting propranolol concentration. Thus, by use of both of these techniques, it was possible to obtain 4-hydroxypropanolol concentrations by difference.

In spite of the demonstration that at least the receptor assays using receptor from bovine lung measure very specifically *l*-propranolol (62), most other workers appear to have used the racemic material as a standard and to have recorded assay results in terms of racemic substance. That this procedure can give usable results is supported by the generally good correlation between the receptor assay methodology and other nonenantioselective techniques such as GLC or fluorometry. However, it would seem that given the enantioselective properties of the receptor system, it would behoove researchers to analyze and report *l*-propranolol or *l*-propranolol equivalents whenever possible.

D. Miscellaneous

Calcium channel antagonist drugs are a relatively new class of therapeutic agents, all of which influence the binding of tritium-labeled nitrendipine to receptor sites. Many of these drugs are not asymmetric but others, such as verapamil, are. RRA are available for these compounds (65,66).

Other drug classes for which RRA have been reported include benzodiazepines, neuroleptics, and tricyclic antidepressants. Many of the drugs in these classes are not asymmetric and information on enantioselectivity is sparse. Barnett and Nahovski (59) give references to these assays.

E. Conclusions

As more receptors are identified and isolated, the importance of RRA will likely increase, and they will be applied to enantiomer determination. As with enantioselective immunoassays, careful attention must be paid to the optical purity of standards and radioligands. The potential for interference by the inactive enantiomer should be considered. When possible, results should be obtained by use of a standard curve of the enantiomer and reported as concentration of the enantiomer. A disadvantage of RRA is its lack of ability to measure the pharmacologically inactive enantiomer, which may be important for toxicological reasons. The ability of RRA to

measure drug equivalents, including active metabolites, may help or hurt, depending on the purpose of the assay.

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4

Indirect Methods for the Chromatographic Resolution of Drug Enantiomers

Synthesis and Separation of Diastereomeric Derivatives

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I. INTRODUCTION

When two chiral compounds, racemic A and racemic B, react to form covalent adducts in a reaction not affecting the asymmetric centers, the stereochemical course of the reaction may be depicted as follows:

$$(\pm)\text{-A} + (\pm)\text{-B} \rightarrow \underset{[1]}{[+A+B]} + \underset{[2]}{[+A-B]} + \underset{[3]}{[-A+B]} + \underset{[4]}{[-A-B]}$$

Four different products, [1–4], are possible. Derivatives [1] and [4] are enantiomers of each other, as are [2] and [3]. Any two of the derivatives that are not enantiomerically related, for example, [1] and [3], are diastereomeric pairs. Nonchiral chromatographic systems can separate diastereomers, but not enantiomers. Thus, chromatographic analysis of a mixture of [1–4] in a nonchiral chromatographic system can yield, in principle, two peaks: one consisting of [1] and [4], and the other of [2] and [3].

The above forms the basis for the chromatographic separation of enantiomers via diastereomeric derivatives. A mixture of the enantiomers of A is reacted, before chromatographic analysis, with a pure enantiomer of B, for example, (−)-B, to produce, in this case, diastereomers [2] and [4]. In this theoretical example, A may represent a sample of a drug whose enantiomeric composition is to be determined, and B is the chiral derivatizing agent (CDA) used to convert the enantiomers of A to diastereomeric

derivatives. The diastereomeric products are then separated using a nonchiral chromatographic system. The ratio of diastereomeric peak areas (if we assume equal response to the diastereomers by the detector used) is the enantiomeric ratio of A in the sample analyzed. This approach to enantiomeric analysis is often termed the indirect method, inasmuch as the enantiomers are converted to diastereomers, and it is the diastereomeric species, rather than the enantiomers per se, that are separated chromatographically.

The indirect chromatographic separation of enantiomers has been used for many years (1–7). However, the spectacular developments during the past 10 years in the synthesis and application of chiral chromatographic stationary phases have revolutionized the field of enantiospecific analysis and provided a wide array of alternatives to the indirect method. Nevertheless, as predicted several years ago (7), the indirect approach continues to enjoy wide popularity in enantiospecific drug analysis. There are several reasons for this popularity of indirect chromatographic separation methods. Thus, despite the increased availability of direct methods—based primarily on chiral stationary phases—many separations have been achieved only via an indirect method; in many cases, the increased sensitivity in detection that may be provided by the CDA is essential; some of the new chiral high-performance liquid chromatography (LC) columns, although providing the desired enantioselectivity for a given separation problem, may not offer adequate chemical selectivity, thereby requiring additional purification steps (e.g., column-switching, etc.); some of the chiral columns have a relatively short lifetime and/or are expensive; in many cases, the use of a chiral column still requires derivatization (with a nonchiral reagent) in order to obtain chiral recognition, good chromatographic behavior, or sufficient sensitivity; a large variety of CDAs are available; in many cases, both enantiomers of the CDA are available, an advantage not shared by most chiral LC and gas-liquid-chromatography (GLC) columns. In summary, then, while it is unquestionable that the direct separation of enantiomers is conceptually superior to the indirect approach, the latter has a well-deserved niche in the armamentarium of the biomedical analyst.

In this chapter, the use of derivatization with CDAs will be reviewed, with emphasis on pharmaceutical and pharmacological applications. The intent here is not the exhaustive listing of publications in this field but rather the illustration of principles and applications, including discussion of recent and interesting examples.

II. GENERAL CONSIDERATIONS

There are several important requirements that must be met for the successful use of the indirect chromatographic separation of enantiomers.

A. The Derivatization Reaction

Derivatization with a CDA results in the chemical modification of the analytes. It is important, therefore, to ascertain that the expected products are obtained. Thus, when a new derivatization reaction is carried out or a new CDA is first used, it is essential to confirm rigorously the structure of the derivatives using appropriate analytical techniques (mass spectrometry, nuclear magnetic resonance, elemental analysis, etc.). This is particularly important in complex cases, for example, when more than one functional group in the analyte may react with the CDA.

The derivatization reaction should proceed under sufficiently mild conditions to avoid significant degradation of the reactants. Most important, under the reaction conditions used, racemization or epimerization of the chiral components, that is, the drug to be derivatized, the chiral reagent, or the derivatives, must not occur. Ideally, the reaction of the analyte enantiomers with the CDA should proceed at the same rate. The derivatization reaction should proceed until completion, that is, until all the compound to be derivatized has reacted with the CDA. This will obviate any concerns about kinetic resolution, that is, unequal rates of reaction of the enantiomers with the CDA. In practice, this requirement usually means use of excess CDA. This, in turn, may have significant implications for the chromatographic separation of the excess CDA from the derivatives formed. It is important, when developing a derivatization reaction, to study the time course of the reaction, that is, to demonstrate that under the conditions used the reaction proceeds to completion.

In rare instances, the derivatization with a CDA was carried out enzymatically (see below). Clearly, the requirements for optimization of such reactions are different from those of nonenzymatic chemical transformations and should be carefully examined before an enzymatic derivatization reaction is routinely applied for quantification.

B. The Chiral Derivatizing Agent

The CDA must also meet some important requirements. It should be of high chemical purity and stable under the derivatization conditions used, as discussed above. Furthermore, the CDA should also be chemically and stereochemically stable under the storage conditions used. In addition, the CDA should react with the target functional group readily and selectively. It is highly desirable that the derivatives formed should produce an equal response by the detection system used.

A critically important question in using a CDA is its enantiomeric purity. If we refer to the original theoretical example of the equation above, it is clear that if CDA (−)-B were contaminated with a small amount of (+)-B, the products of the reaction with a mixture of the enantiomers of A

would include, in addition to [2] and [4] derived from (−)-B, compounds [1] and [3] derived from (+)-B. As pointed out earlier, in nonchiral chromatographic systems [1] is not separable from [4], and [2] is not separable from [3]. Thus, the presence of contaminant (+)-B in (−)-B will cause a contamination of the peaks of [2] and [4] with [3] and [1], respectively. Obviously, this will result in a false value for the enantiomeric composition of A.

It may be argued that if the actual extent of enantiomeric contamination of a CDA is known accurately, the reagent may be safely used, because the appropriate correction in diastereomeric peak ratios can be made. An objection (5) to this argument is that if the enantiomerically impure CDA is present in excess, differences, if any, between the CDA enantiomers in their reaction rates with the analyte enantiomers (i.e., diastereoselective kinetics) will still result in an error in the determination of the enantiomeric ratio. In practice, however, such kinetic differences are usually negligible. A more precise but cumbersome solution to this problem is to separate the four stereoisomeric derivatives using chiral chromatographic conditions, for example, a chiral stationary phase. Under such conditions, four distinct peaks are obtainable as a matter of principle (whether the four stereoisomers are actually resolved depends, of course, on the chromatographic conditions chosen). A review of the literature indicates that small (1–2%) enantiomeric contamination of a CDA may not necessarily render the CDA useless in many applications. It is clear, nevertheless, that the CDA used should be enantiomerically pure whenever possible. This simplifies the analysis and eliminates any uncertainty associated with enantiomeric contamination. There is, in fact, an application in which enantiomerically impure CDAs cannot be used safely: the determination of trace enantiomeric impurity in an analyte. If the CDA used is itself enantiomerically contaminated, the accurate determination of the extent of trace enantiomeric contamination of the analyte may be difficult if not impossible.

Although the danger posed by enantiomeric contamination of the CDA has been pointed out frequently, it must be emphasized that many CDAs are available in optically pure form. For example, CDAs derived from natural products, such as those based on D-glucose, (−)-methanol, etc. (see below), are often available in enantiomerically pure form.

The enantiomeric purity of a CDA is best determined by chromatographic means. This may be accomplished (1) by chromatographic separation of the enantiomers of the CDA using direct methods, that is, a chiral stationary phase or chiral mobile phase; for this method, both enantiomers of the CDA should be available to demonstrate reliably their separability; or (2) indirectly, by derivatizing the CDA with a compound known to be enantiomerically pure. Here also, both enantiomers of the CDA, or alter-

natively, those of the compound used in the derivatization, should be available to demonstrate chromatographic separation and thereby detectability of enantiomeric contamination.

There are other requirements the CDA must meet. The derivatives produced by the CDA must have suitable chromatographic properties. In GLC applications, for example, the derivatives must have adequate volatility. Because derivatizations with CDAs frequently involve acylation, esterification, etc., of such polar centers as hydroxyl, carboxyl, amino, etc., groups of the analytes, the resulting derivatives, often display improved chromatographic behavior relative to the parent compounds. In some cases, however, the diastereomeric derivatives formed still contain functional groups that interfere with the chromatographic separation, for example, via insufficient volatility, peak tailing, inadequate resolution of diastereomers, etc. Such additional functional groups must also be derivatized or masked if the diastereomers are to be successfully separated. This usually involves masking the troublesome polar groups via derivatization with nonchiral agents. Thus, carboxyl groups may be esterified with diazomethane, hydroxyl groups silylated, etc. Clearly, however, such additional derivatization requirements increase the complexity of the analytical method.

It may also be necessary, especially in biological applications, for the CDA to increase the detectability of the analyte. Some CDAs have, in fact, been designed with sensitive detection in mind, for example, to produce electron-capturing derivatives for GLC or fluorescent derivatives for LC. Sensitive and selective detection is of utmost importance in applications in drug metabolism and pharmacokinetics, where drugs present at low concentrations in complex biological fluids are to be identified and quantified.

Finally, a significant consideration in the use of a particular CDA is its availability. A CDA that must be synthesized via an elaborate route and resolved into its enantiomers before it can be used clearly is not a practical candidate in many laboratories. Fortunately, however, many of the CDAs described in the literature are available from commercial suppliers. Others, while not commercially available, are easily prepared from commercially available resolved starting materials.

C. Types of Applications

Chromatographic resolution of enantiomers, including the indirect approach, can be used in several different types of applications. The recent surge of interest (8–12) in the stereochemical aspects of drug action and disposition has been accompanied by a rapidly increasing need for synthetic and analytical methods for drug stereoisomers. Regarding analyti-

cal methods, chromatographic separation of enantiomers, directly or indirectly, has clearly become the technique of choice. As for the preparation of optically active compounds, although asymmetric synthesis is now a practical possibility in many cases (13), preparative LC separation of enantiomers is rapidly becoming an important alternative to chemical resolution and asymmetric synthesis, at least on a laboratory scale (3).

1. *Determination of Enantiomeric Composition*

The enantiomeric purity or composition of drugs, synthetic intermediates, analogs, etc., in bulk samples can be determined via chiral derivatization in laboratory research, manufacturing processes, dosage forms, quality control, etc. In these applications, limitations on the amount of material available are usually nonexistent, allowing for convenient derivatization and ready detection. In such derivatizations, 0.1–1.0 mg is typically the amount of analyte derivatized.

Another major role for the chromatographic analysis of enantiomeric composition is in the areas of drug metabolism and pharmacokinetics. Many drugs, in research or in clinical use, are applied in their racemic form; chiral or nonchiral drugs may be metabolized to chiral metabolites, etc. Thus, there is a need for the stereospecific detection, identification, and quantification of the individual enantiomers of drugs and their metabolites in various biological media. The concentration of these analytes can often be as low as a few nanograms per milliliter in complex biological fluids containing many other substances. Sensitivity and specificity are therefore critical factors in such analyses. Derivatization with a CDA, in addition to permitting stereochemical analysis, can also improve the chromatographic behavior and detectability of the analytes, an important consideration in the analysis of xenobiotics in biological media. It is important to recognize, however, that derivatization with a CDA in such trace analysis procedures can be very different from derivatizing, for example, a milligram of a pure standard. In fact, the limitations of chiral derivatization in this respect are in many ways very similar to those encountered in nonchiral chemical derivatization for chromatographic trace analysis, which have been discussed (14,15).

2. *Determination of Absolute Configuration*

Determination of absolute configuration is another important application of the chromatographic separation of diastereomeric derivatives of enantiomers. This can be accomplished in several different ways. Obviously, if a standard of known configuration is available for comparison, the configuration of a compound of unknown stereochemical identity in a given sample can be readily assigned. If an authentic standard of known

configuration is not available, the absolute configuration of the analyte may be determined by separating and isolating the derivatives using LC, followed by determination of the configuration using physical measurements such as circular dichroism and nuclear magnetic resonance (NMR).

Still another approach to the determination of absolute configuration is based on the elution order of the diastereomeric derivatives. When a series of closely related chiral compounds is derivatized with the same CDA, it is often observed that the order of elution of the two diastereomers is the same for all members of the series, that is, the elution order is correlated with configuration. Indeed, this phenomenon was recognized early (16), and empirical correlations of elution order with configuration are frequently used to assign absolute configurations (17,18). Caution must be exercised, however, in using this approach to assign configurations, since occasionally an "unexpected" reversal in the elution order of the derivatives is observed (19). Indeed, Pirkle and Finn have pointed out that in order to assign with confidence the absolute configurations on the basis of elution order of the diastereomers, one must know the absolute configuration of the CDA and the mechanism of chromatographic separation for the relevant stereoisomers (3).

3. *Preparative Chromatography*

The recovery of useful amounts of the chromatographically separated components is a capability provided by a variety of chromatographic techniques, including GLC, thin-layer chromatography (TLC), and LC. It is the latter technique, however, that can most readily serve in the preparative mode for the isolation of relatively large amounts of material. There is no doubt, however, that the requirement in the indirect approach to cleave chemically the derivatizing moiety from the derivatives after chromatographic separation is a significant disadvantage and deterrent to the preparative use of this technique. Nevertheless, such preparative separations have been described in the literature and in many instances can provide useful alternatives to preparative direct separations, especially when one considers the lack of availability of preparative-size versions of many chiral LC columns or the often prohibitively high price of those that are available.

There are several important considerations in the design of preparative separations via derivatization with CDAs. First, the CDAs should produce diastereomeric derivatives that, after the chromatographic separation, can be cleaved chemically to yield the original drug enantiomers. The components resulting from the reaction used to cleave the derivatives should be separable without undue difficulty. An important factor is the enantiomeric purity of the CDA, since enantiomeric contamination of the CDA

will result in enantiomeric contamination of the drug stereoisomer(s) retrieved. Depending on the scale used, the cost of the CDA may become a consideration.

III. SEPARATIONS

A. General Considerations

Two different measures of the extent of chromatographic separation of two peaks are used in characterizing stereoisomer separations. The separation factor α is the ratio of the retention times corrected for the retention time of an unretained substance, whereas the peak resolution R is a measure of the extent of overlap of the two peak areas (20). The two parameters α and R contain different information (6,21). If two equal-sized peaks have $R > 1.5$, the two peaks are essentially completely separated (22). When developing a chromatographic separation for the enantiomers of a given drug, one should determine R under the chromatographic conditions used to be able to judge the extent of peak separation. Ideally, R should be > 1.5 for the racemate if the chromatographic peaks are to be used in quantification.

The chromatographic separation of enantiomers as diastereomers was first developed using GLC (1). Subsequently, many separations using GLC were reported, but modern LC has dominated the field of enantiospecific drug analysis in recent years. Nevertheless, the arrival of high-resolution capillary GLC has revived interest in the use of indirect enantiomer separation via this type of chromatography, and today GLC remains important in the analytical separation of enantiomers after derivatization with CDAs. The availability of sensitive detection methods, for example, mass spectrometry, electron capture, etc., enhances the applicability of GLC in indirect enantiospecific drug analysis.

The advent of modern column LC in the 1970s rapidly led to the use of this chromatographic technique in the separation of enantiomers as diastereomeric derivatives. Today, most of the reported new developments in indirect enantiomer resolutions use LC, and LC is particularly important in the resolution of chiral pharmaceuticals.

TLC has also been used for the separation of diastereomeric derivatives of enantiomers, but this form of chromatography has not attained widespread use in indirect resolutions. Other chromatographic techniques, for example, supercritical fluid chromatography, capillary electrophoresis, countercurrent chromatography, etc., have not received much attention in indirect enantioseparation.

A variety of compounds have been resolved via the indirect method, and a large variety of chromatographic systems and conditions have been

used in the separations. Relatively few of these chromatographic resolutions have been examined in detail to elucidate the physiochemical mechanism responsible for the separation of the diastereomers. Indeed, many of the pharmaceutical applications of indirect resolution were developed empirically, without available rational information on suitable CDAs and chromatographic conditions that would likely produce optimal separation. Much of the pioneering work aimed at elucidating the mechanisms responsible for the separation of diastereomers in GLC was carried out by Karger and his associates (23,24). In LC resolutions, Pirkle et al. expended considerable effort in developing models that explain the separation of diastereomeric amides, carbamates, and ureas on normal-phase columns (3,19,21,25). Other investigators have also studied the factors underlying the separability of diastereomers, and the reader is referred to recent detailed discussions of the subject (3–5,26). Additional studies on separation mechanisms, particularly in reversed-phase (RP) LC, could provide further useful information for the rational selection of conditions for the indirect resolution of pharmacologically active agents.

B. Examples of Separations

In this section, applications of the indirect-resolution approach to compounds of primarily pharmaceutical, pharmacological, or toxicological interest will be reviewed. Because a fundamental aspect of the method is the chemical reaction between the analyte and the CDA, it is convenient to divide the applications according to the drug functional groups involved in the derivatization.

1. Resolutions via Derivatization of Amino Groups

The amino group is present in a wide variety of asymmetric drugs. Furthermore, this functional group undergoes a variety of chemical reactions, many of which have been exploited in derivatizing with CDAs.

Formation of Amides. Carboxamide bond formation (Eq. 1, CDA is first reactant) appears to be the most common reaction used in chiral derivatization of primary and secondary amino groups.

$$\text{R-CO-X} + \text{HNR'R''} \rightarrow \text{R-CO-NR'R''} \qquad (1)$$

This is not surprising, since amide-forming reactions between amines and (activated) carboxylic acids proceed readily, and many optically active carboxylic acids are readily available. Furthermore, the diastereomeric amides produced are often well-resolved by LC or GLC. A variety of leaving groups (X in Eq. 1) have been used in the acylation reaction, for example, chloride, imidazole, carboxylate, etc.

N-perfluoroacyl derivatives of L-proline, such as (*S*)-*N*-trifluoroacetyl-

prolyl chloride (corresponding to L-proline), [5] (see structures at end of chapter), have been used as CDAs (27–35). The use of some of these reagents illustrates several of the potential problems associated with the indirect method. CDA [5] is well known (29,34–36) to be contaminated with the *R* enantiomer formed via racemization during synthesis and storage of the reagent. Commercial samples of [5] have been reported to contain varying amounts, sometimes as much as 15% (35), of the enantiomeric contaminant. Some investigators found that CDAs such as [5] racemized during the derivatization reaction if triethylamine was present as the catalyst (32,33). Lim et al., however, were able to develop aqueous reaction conditions under which racemization of the *N*-heptafluorobutyryl analog of [5] was negligible (33).

Other attempts to circumvent the enantiomeric contamination of CDAs such [5] include the work of Silber and Riegelman, who carefully synthesized [5] in > 98% enantiomeric purity and found that derivatization at −78°C resulted in <2% racemization (35). Others, on the other hand, found that the *N*-heptafluorobutyryl analog of [5] racemized during the derivatization reaction even at −78°C (33). Liu et al. used [5] to derivatize racemic methamphetamine and separated the four derivatives on a chiral GLC column (30). It was concluded that an achiral column is adequate for the determination of the methamphetamine enantiomers, provided that the extent of enantiomeric contamination of [5] is known (30). Attempts to use leaving groups other than chloride in [5] have been disappointing, leading to reagents that reacted sluggishly and stereoselectively with the analyte enantiomers and were still not free from racemization problems (29). More recently, however, the *N*-heptafluorobutyryl analog of [5] has been successfully used in the resolution of several primary and secondary amines. Thus, several amphetamine derivatives and ephedrines were resolved (37,38), and the determination of the concentration of methoxyphenamine and several of its metabolites by GLC and ECD were reported (39). It would seem, nevertheless, that the possibility of racemization of these reagents must be carefully checked during their use. That the problems are specific to structures such as [5] is illustrated by observations that other L-proline-based CDAs, such as [6] (40) and [7] (41), are free from racemization problems.

Reagent [6] was developed for the determination of the enantiomeric composition of amphetamines in pharmaceutical dosage forms using LC separation. The derivatives show strong UV-light absorption, but baseline separation of the diastereomers was not achieved (40). Compound [7], containing the pentafluorophenyl group, produces derivatives that elicit a high response from the electron capture detector (41) and has proven valuable in drug metabolism studies (42,43). For example, the absolute

configuration of α-methyldopamine formed from α-methyl DOPA in a hypertensive patient could be determined using [7]. The metabolite, isolated from urine, was derivatized with [7], and its GLC retention time was shown to correspond exactly to that of the derivative of an authentic sample of known absolute configuration (42).

Krull and his co-workers developed a new and promising approach in derivatizations of amino groups with CDAs (44). The CDA, an amino acid derivative, was covalently linked via esterification of the carboxyl group to an insoluble, structurally rigid organic polymer backbone. The amino group of the CDA moiety was linked to a fluorescent group, for example, fluorenylmethoxycarbonyl, in order to provide sensitive detectability to the derivatives. In the derivatization reaction (aminolysis of an ester), the amino group of the analyte reacted at the labile-activated ester link between the CDA and the polymer support, severing the link with release of the derivatives, in which the analyte was now covalently attached to the CDA via the newly formed amide bond. The derivatization can be carried out on-line or off-line with respect to a LC system used for the separation of the diastereomeric derivatives. The authors carefully evaluated the nature of the CDA, its immobilization on the polymer backbone, the conditions of derivatization, etc. Such an approach has several potential advantages: The absence of excess reagent in the analyte mixture is highly advantageous, especially when trace amounts of the analyte are measured; the analysis is amenable to automation; the CDA includes a built-in moiety for sensitive detectability. Clearly, this technique has considerable promise.

α-Methoxy-α-trifluoromethylphenylacetyl chloride, [8], is a widely used and highly useful CDA. Both enantiomers of the parent, [9], are commercially available and can be converted in a simple one-step procedure to [8] (36,45). Lacking a hydrogen α to the carboxyl group, [8] is stereochemically highly stable. Applications of this CDA for the resolution of amines using packed-column GLC include the resolution of amphetamine and several related compounds (36) and DOPA and α-methylDOPA (46), studies on the stereochemical course of the metabolism of amphetamines in vivo (36,47) and in vitro (48,49), and investigations of the stereoselective disposition of the antiarrhythmic agent tocainide in animals (50) and humans (51–53). More recently, the reagent has been used in conjunction with capillary GLC. Changchit et al. derivatized 3-amino-1-phenylbutane, a metabolite of labetalol, with [8] and separated the diastereomeric derivatives on capillary GLC columns with detection by mass spectrometry (54).

The reagent has also been evaluated for the resolution of amphetamines by RP LC, but for several compounds incomplete separation of the diastereomers was obtained (55). On the other hand, *N*-acylation of prazi-

quantel, a chiral anthelmintic agent, with [8] produced diastereomers that were well resolved (α = 1.18) using normal-phase LC in a study of the absolute configuration of the drug (56).

Coleman (57) synthesized the anhydride of acid (*R*)-[9] and used it to determine the enantiomeric purity of oxfenicine [(*S*)-(+)-*p*-hydroxyphenylglycine], a therapeutic agent that promotes carbohydrate oxidation. The anhydride had to be used in the derivatization since the use of acid chloride [8] led to racemization of the drug due to the presence of the required base catalyst. The diastereomers were separated by silica gel LC and detected at 254 nm (57).

The above-mentioned antiarrhythmic agent tocainide has also been resolved with (*R*)-(−)-*O*-methylmandelic acid chloride, [10], using normal-phase LC in a study of the stereoselective disposition of the drug (58). In the assay, CDA [10] was dissolved in the organic solvent used for the extraction of tocainide from urine (58). The *S* form of this CDA was used by Helmchen and Strubert in early studies on indirect resolutions of chiral amines using LC (59). Both enantiomers of the parent acid of [10] are commercially available.

(−)-Camphanic acid chloride, [11], was used in the RP LC determination of the enantiomeric purity of ^{11}C-labeled D- and L-methionine synthesized for positron-emission tomography studies of the brain (60). CDA [11] has received limited attention despite its commercial availability, but interest in its use appears to be increasing. Nichols et al. used [11] and capillary GLC to determine the enantiomeric purity of resolved samples of the hallucinogenic compound 1-(3,4-methylenedioxyphenyl)-2-aminopropane (MDA) and several of its homologs (61). CDA [11] has also been applied and to the determination of the enantiomeric purity of nicotine, the stereochemical aspects of which are of interest. Jacob et al. (62) developed an elegant procedure in which nicotine is *N*-demethylated to nornicotine, and the latter in turn is derivatized with [11]. The resulting diastereomers are conveniently separated on a capillary GLC column, thus providing a method for the determination of the enantiomeric composition of both nicotine and nornicotine (62). CDA [11] is a readily available agent of high enantiomeric purity, stereochemical stability, and high reactivity toward amines (and alcohols, see below), which should prove useful in the chromatographic resolution of a variety of chiral compounds.

Another readily available and potentially useful chiral reagent that has received very little attention is τ-butyloxycarbonyl-L-leucine-*N*-hyroxysuccinimide ester, [12]. This reagent was used to acylate the amino group of the chiral drug γ-vinyl-γ-aminobutyric acid, followed by trifluoroacetic acid-catalyzed removal of the t-butyloxycarbonyl group (63). The diastereomeric derivatives were resolved on a C8 column, with an α value as

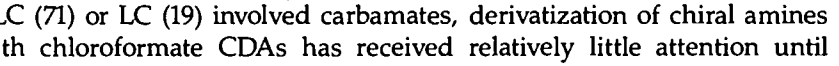
high as 5.75 (63). A related CDA, γ-butyloxycarbonyl-L-leucine anhydride was described by Hermansson (64,65). Acylation with this reagent produces the same derivatives as those produced by [12], the difference between the two CDAs being only in the identity of the leaving group. Hermansson's reagent was used in the determination of the enantiomers of propranolol and several related β-adrenergic antagonists in blood plasma (64,65). Interest in this area is intense as a result of the stereoselective pharmacology and disposition of these drugs. The assay procedure described by Hermansson permitted the simultaneous quantification of the propranolol enantiomers in plasma at a concentration as low as 1.0 ng/mL using LC with fluorometric detection, the fluorescence being due to the drug moiety in the derivatives rather than the CDA (64).

An interesting fluorescent CDA was developed by Weber et al. (66). These workers converted racemic benoxaprofen, [13], to the corresponding *N*-(*R*)-α-methylbenzylamide diastereomers, which were preparatively separated on silica gel LC columns. The separated diastereomers were acid-hydrolyzed to retrieve the individual enantiomers of benoxaprofen. Either enantiomer could then serve as CDA for the resolution and determination of chiral amines. The diastereomeric derivatives of several amines were highly fluorescent and well resolved on silica gel columns or by TLC. One disadvantage of the procedure was that the CDAs had slightly less than 100% enantiomeric purity.

Goto et al. (67) synthesized the succinimidyl ester [14] of (−)-α-methoxy-α-methyl-1-naphthaleneacetic acid for the normal-phase LC resolution of chiral amines. The reagent permitted the determination of the enantiomers of an amphetamine derivative in blood plasma after administration of racemic drug to rabbits. With detection at 280 nm, the lower limit of sensitivity was 5 ng/mL for each enantiomer (67). Several chiral acids from the "profen" group of nonsteroidal antiinflammatory drugs have been adapted as CDAs. One of these, naproxen, [15], is the *S* enantiomer and is commercially available as the resolved acid; several of these acids have the advantage of providing fluorescent derivatives (68,69).

Demian and Gripshover evaluated several CDAs for the LC resolution of 3-aminoquinuclidine, an intermediate in the synthesis of several pharmaceuticals (70). It was found that the best resolution was provided by *O,O*-dibenzoyltartaric anhydride, [16], a CDA originally developed for the derivatization of alcohols by Lindner and his co-workers (see below). Interestingly, this reagent, although commercially available as the (*R,R*) and also the (*S,S*) enantiomer, has not received much attention for amines.

Formation of Carbamates. The reaction of amines with chloroformates bears some similarity to amide formation and produces carbamates (Eq. 2)



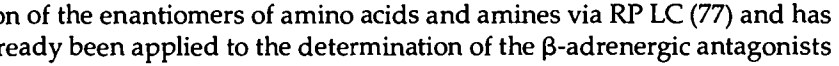
$$RO\text{-}CO\text{-}Cl + HNR'R'' \rightarrow RO\text{-}CO\text{-}NR'R'' \quad (2)$$

Although some of the early work on the separation of diastereomers by GLC (71) or LC (19) involved carbamates, derivatization of chiral amines with chloroformate CDAs has received relatively little attention until recently in pharmaceutical/pharmacological applications. This is surprising, inasmuch as chiral chloroformates can be readily synthesized from chiral alcohols, many of which are commercially available. There are indications, however, that interest in the use of chloroformates is increasing, and several reports on such reagents have appeared in recent years.

(−)-Menthyl chloroformate, [17], is commercially available and has been used in several applications. For example, Seeman et al. (72) carried out the preparative chromatographic resolution of nornicotine using [17] as a CDA. The diastereomeric carbamates formed via the reaction of racemic nornicotine with [17] were separated by preparative silica gel LC, and the pure enantiomers of nornicotine were liberated by acid-catalyzed hydrolysis of the carbamates (72). Several reports have described the use of [17] in the enantiospecific determination of drug concentrations in biological fluids; thus, the reagent has been used in the analysis of encainide and some of its metabolites (73), flecainide (74), and propranolol (75), and other drugs (76).

An interesting chloroformate CDA that appeared recently is (+)-1-(9-fluorenyl)ethyl chloroformate, [18]. This reagent is suitable for the separation of the enantiomers of amino acids and amines via RP LC (77) and has already been applied to the determination of the β-adrenergic antagonists propranolol (78) and atenolol (79) in biological fluids. In these drugs, there is a hydroxyl group in addition to the secondary amino functionality; it was shown that only the amino group reacted when propranolol was derivatized with [18] (78). A significant advantage of [18] is that it produces fluorescent derivatives. A disadvantage, on the other hand, is that the reagent is rather expensive.

A unique property of chloroformates is their ability to *N*-dealkylate tertiary amines to produce the carbamate of a corresponding secondary amine (Eq. 3)



$$RO\text{-}CO\text{-}Cl + NR'_3 \rightarrow RO\text{-}CO\text{-}NR'_2 \quad (3)$$

Depending on the nature of the substituents on nitrogen, this reaction may give a complex product mixture, but in some cases such derivatization could be the basis of a useful indirect enantiomer separation. When one or two of the three *N*-substituents are methyl, for example, demethylation may be favored, and the reaction may be usable as a precolumn derivatization for chromatographic resolution. This approach was used in the analysis of encainide, a tertiary-amine antiarrhythmic drug (73).

Formation of Ureas. Primary and secondary amino groups undergo facile reaction with isocyanates to give the corresponding ureas (Eq. 4), and this transformation has been exploited for the derivatization of amines

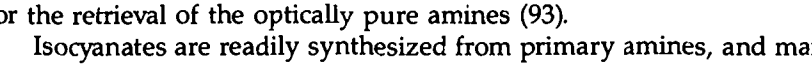
$$\text{R-N=C=O} + \text{HNR'R''} \rightarrow \text{RNH-CO-NR'R''} \quad (4)$$

Two commercially available optically active isocyanates have been extensively used as CDAs in LC: 1-phenylethyl isocyanate ([19], also known as α-methylbenzyl isocyanate) and 1-(1-naphthyl)ethyl isocyanate, [20]. Both enantiomers of each of these CDAs are commercially available. Thompson et al. examined the products of the reaction of propranolol, an amino-alcohol, with [19], and found that the amino group of the drug was derivatized, whereas the hydroxyl group did not react under the reaction conditions used (80). There are many reports on the use of CDA [19] and its naphthalene analog [20] for the resolution of amine drugs by LC; many of these describe applications to β-adrenergic antagonists or related drugs that are β-amino alcohols (81–89), and many of the applications involve determination of the enantiomers in biological fluids. In addition to the above β-adrenergic antagonists, several other amine drugs have been derivatized with one or the other enantiomer of [19] or [20] and the derivatives resolved on analytical and/or preparative LC columns, for example, flecainide (90), cathinone (91), noreseroline (92), mecamylamine (93), tocainide (94), fluoxetine and its metabolite norfluoxetine (95), and others (96). It is noteworthy that the naphthalene moiety in [20] provides fluorescent derivatives, an advantage over [19]. Other chiral isocyanates have also been evaluated as CDAs for amines (97).

It was shown by Shonenberger et al. that thermal decomposition of the separated diastereomeric (α-methylbenzyl) urea derivatives produced by the reaction of [19] and chiral secondary amines is a convenient technique for the retrieval of the optically pure amines (93).

Isocyanates are readily synthesized from primary amines, and many such amines are available in resolved form. It is somewhat surprising therefore that only relatively few chiral isocyanates have been used as CDAs. On the other hand, such lack of interest in other isocyanates may be the reflection of the considerable success afforded by [19] and [20].

Formation of Thioureas. If the isocyanate in Eq. 4 is replaced by an isothiocyanate, the products formed are thioureas (Eq. 5).



$$\text{R-N=C=S} + \text{HNR'R''} \rightarrow \text{RNH-CS-NR'R''} \quad (5)$$

This reaction is selective for primary and secondary amino groups, and hydroxyl groups do not react under conditions in which the amine is readily derivatized. A significant difference between isothiocyanates and isocyanates is that the former react with water or alcohols much more

slowly than the latter. This difference is an advantage when the derivatization of amino acids, catecholamines, and other highly polar compounds is considered: Isothiocyanate CDAs are compatible with aqueous reaction media, in which such polar analytes have greater solubility.

Nambara et al. described in 1978 the synthesis of two optically active terpene isothiocyanates and their use in the LC resolution of amino acids (98). These CDAs did not receive further attention, but in 1980 Nimura et al. reported on the use of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate, [21], for the LC resolution of amino acid esters (99). The use of this CDA was then extended to amino acids (100) and catecholamines (101). Concerning the latter, [21] was also shown to be suitable for the determination of the enantiomeric purity of (−)-epinephrine in cardiac and dental dosage forms (102,103). CDA [21] is commercially available, and in recent years its use in indirect resolutions by LC has greatly expanded (104). Many β-adrenergic antagonists (105), other amino alcohols (106–109), several antiarrhythmic primary and secondary amines (110), and amphetamines (55) have been resolved via derivatization with CDA [21]. Many of the separations were achieved on RP LC columns, and the reagent has been shown to be applicable to problems of stereoselective drug metabolism and pharmacokinetics (104). Of particular interest in this regard is the report by Walle et al. (111) on the enantiomeric composition of the sulfate conjugates of 4-hydroxypropranolol. The intact sulfate conjugates could be derivatized with [21], and the derivatives were well resolved by RP LC. With detection at 313 nm, the minimum detectable amount of each enantiomer of the conjugate was 20 ng. By using this method, the plasma concentration of the enantiomeric sulfate conjugates of 4-hydroxypropranolol in a patient on chronic propranolol therapy could be determined (111). The technique could also be applied to in vitro studies of the conjugation of 4-hydroxypropranolol (112). Clearly, this is a promising method for the investigation of the stereoselectivity of the formation and disposition of those drug conjugates that are amenable to derivatization with [21].

Particularly good separation of the diastereomers derived from amino alcohol structures was achieved with [21] (104–110). In the derivatization of such compounds, only the amino group reacts with the isothiocyanate moiety. It is interesting to compare the resolution of the amino alcohol ephedrine (*R,S/S,R* racemate), [22], with that of methamphetamine, [23], which lacks the hydroxy group. Derivatization of racemic ephedrine with the isothiocyanate [21] produces derivatives that are well resolved (α = 1.19) by RP LC (106), whereas the derivatives of methamphetamine are minimally resolved under similar conditions (no α given) (113). An analogous comparison can be made for norephedrine and amphetamine (55,106), which are the *N*-demethyl analogs of [22] and [23], respectively.

Although a detailed separation mechanism that would account for these differences has not been proposed, it is clear that the hydroxyl group in amino alcohols plays a crucial role in producing differences between the diastereomers in their partition characteristics. A likely mechanism could be the formation of an intramolecular hydrogen bond involving the hydroxyl group, which would result in the molecule favoring a more rigid conformation. Such reduction in conformational mobility by intramolecular binding via various forces (hydrogen bonding, dipole-dipole, etc.) is thought to be important in maximizing physiochemical differences between diastereomers and appears to explain a variety of diastereomer separations (3,5). It should be noted that the considerable success of the isocyanate reagents in the resolution of aminoalcohols discussed in the previous section above may also be due to similar intramolecular hydrogen-bonding mechanisms.

The successful use of [21] in a variety of resolutions, as outlined above, has stimulated the search for other optically active isothiocyanate CDAs, partly because [21] failed to resolve some racemates. Several promising alternatives to [21] have emerged, for example, (*R*)-α-methylbenzyl isothiocyanate, [24] (114), (*S*)-1-(1-naphthyl)ethyl isothiocyanate [25] (110,115), (*R*)-1-(2-naphthyl)ethyl isothiocyanate [26] (110), and (*S*,*S*)-2,2-dimethyl-4-phenyl-5-isothiocyanato-1,3-dioxane [27] (54); these CDAs are suitable for the LC resolution of a variety of amines using RP conditions. Interestingly, significant differences were observed between the regioisomeric α-naphthyl and β-naphthyl CDAs [25] and [26] in their abilities to resolve some of the amines (110,116). One of the new isothiocyanates, reagent [24], is commercially available (114).

It appears, then, that isocyanate and isothiocyanate CDAs are useful in LC resolutions of amines. Several of these agents are commercially available, and many others can be readily prepared from a variety of commercially available optically active primary amines. The stereochemical stability of these CDAs is excellent, their reaction with amines is rapid and convenient, and the derivatives are readily and sensitively detected. These reagents are likely, therefore, to enjoy continued popularity.

Formation of Isoindoles. In 1971 Roth described a sensitive analytical method for amino acids based on their reaction with *o*-phthaldialdehyde (OPA) and a thiol, 2-mercaptoethanol (117). The reaction produces an intensely fluorescent isoindole derivative of the amino acid and is specific for the primary amino group (118,119). Subsequently, the OPA method was extended to the enantiospecific LC analysis of amino acids by substituting an optically active (single enantiomer) thiol for 2-mercaptoethanol in the derivatization reaction (120–122). This results in the formation of two

diastereomeric isoindoles derived from the enantiomeric amino acids, respectively, and the derivatives are then separated on conventional (non-chiral) LC columns. This procedure is a sensitive and convenient method for the enantiospecific analysis of amino acids, and it is surprising, therefore, that until recently it attracted little attention in the resolution of other primary amines. More recently, however, a study of the applicability of the OPA method to non-amino-acid primary amines was reported (123). Several thiol CDAs were evaluated for the resolution of a series of primary amines: *N*-acetyl-L-cysteine (NAC), *N*-acetyl-D-penicillamine, and 2,3,4,6-tetraacetyl-1-thio-D-glucose, and it was found that the RP LC separation of the enantiomers of a series primary amines, for example, several amphetamines, 1-phenylethylamine, 3-amino-1-phenylbutane, and mexiletine, could be obtained via the OPA/chiral thiol derivatization method (123). This approach to enantiospecific chromatographic analysis of primary amines has several advantages: The derivatives are fluorescent and detectable with high sensitivity; the CDAs are commercially available; the reaction is selective, simple, rapid, and convenient. Since many drugs are primary amines and many non-primary-amine drugs are metabolized to primary amines, it is likely that the OPA method will gain popularity in their enantiospecific analysis, especially in drug metabolism and pharmacokinetic studies.

Formation of Other Derivatives. Other CDAs have also been used for resolution of amines. Souter described (124) the LC resolution of amphetamine and several of its analogs after derivatization with (+)-10-camphorsulfonyl chloride, but this CDA, although commercially available, has received little further attention. In a novel approach, Knorr et al. used a chiral alkylating agent to derivatize the adrenergic agent etilefrin, producing diastereomeric *N*-alkyl derivatives, which were separated by RP LC (125). The utility and scope of this derivatization remain to be defined. An unusual CDA, optically active antihead-to-head coumarin dimer, was synthesized by Saigo et al. (126). Each molecule of the reagent reacts with two molecules of an amine, and racemic amines produce three diastereomeric derivatives, which could be used to determine enantiomeric composition via LC separation (126). The scope and usefulness of this more complex approach remain to be studied. Pirkle and Simmons developed optically active *cis*-4,5-diphenyl-2-oxazolidone-3-carbamyl chloride as a CDA for the resolution of amines (21).

2. Resolutions via Derivatization of Hydroxyl Groups

The hydroxyl group is present in many asymmetric drugs, natural products, environmental pollutants, etc. In addition, oxidative, reductive, and hydrolytic biotransformations can introduce hydroxyl groups into

molecules. It is not surprising, therefore, that stereochemical considerations, including stereospecific analysis, of hydroxyl-containing xenobiotics have received considerable attention. The hydroxyl group undergoes several of the reactions discussed above for the amino groups, and many of the above-cited CDAs have also been applied to the chiral derivatizations of hydroxyl compounds.

Formation of Esters. In view of the chemistry of the hydroxyl group, it is not surprising that the most frequently used chiral derivatizations of alcohols involve the formation of ester derivatives. Many chiral acids (or derivatives thereof) have been used for this purpose. Ester bound formation in the derivatization may use the CDA in the free acid form, or more frequently, in an activated form, for example, as the acid chloride, anhydride, etc. (Eq. 6)

$$\text{R-CO-X} + \text{HOR}' \rightarrow \text{R-CO-OR}' \qquad (6)$$

$$\text{X} = \text{OH, Cl, O-CO-R, etc.}$$

When the carboxyl moiety of the CDA is thus activated, often to the acid chloride, (partial) racemization of the reagent may be a possibility, depending on the nature of the acid used and reaction conditions of activation. In some of the reports using such activation, it was ascertained that no significant racemization occurred, but in many other studies no such check was made.

Initial studies on the GLC separation of enantiomeric alcohols as esters of various chiral acids used packed columns (1,4). A major advance in GLC separations was the advent of capillary columns, and most of the recently published indirect GLC separations used capillary columns.

N-trifluoroacetyl amino acids or their derivatives have been used as CDAs. Kruse et al. (127), for example, resolved several chiral alcohols and several insect pheromone terpenols after derivatization with *N*-trifluoroacetyl-L-alanine, [28]. It was found that when the acid chloride of [28] was used in the acylation of the terpenols, decomposition and side-product formation occurred; the problem was avoided by using acid [28] with dicyclohexylcarbodiimide as a condensing agent. Separation of the diastereomeric esters was achieved on glass capillary GLC columns (127). In another study of insect pheromones, the absolute configuration of 3-octanol isolated from the mandibular gland of ants was determined via capillary GLC separation of diastereomeric esters (128). Of the several derivatives evaluated, the *trans*-chrysanthemate esters {derived from (+)-[29]} were found to be most useful. A disadvantage of the procedure was the less than 100% optical purity of the CDA (128).

Doolittle and Heath published the results of an extensive study com-

paring three optically active acid chlorides, [8], [30], and [31] in the chromatographic resolution of a variety of chiral alcohols (129). Five capillary GLC columns and one LC column were screened. The data obtained provided a good starting point for the selection of the appropriate chromatographic system and conditions for a particular resolution problem (129).

The earlier-cited CDA [8] has been used successfully and extensively in the chromatographic resolution of chiral alcohols. In addition to its value in the GLC resolution of alcohols (130) and hydroxy-fatty acids (131), the reagent is also useful in the LC resolution of hydroxyl compounds, as shown, for example, by the work of Doolittle and Heath (129) and others (132). In this connection, the applications of [8] in studies of the chemistry and biology of carcinogenic polycyclic aromatic hydrocarbons (PAHs) are worthy of consideration. These hydrocarbons, for example, benzo[a]-pyrene, undergo biotransformation to toxic metabolites; furthermore, the biotransformations and toxicity of the metabolites can be highly stereoselective. Among the metabolites involved are arene epoxides that undergo rapid conversion to vicinal dihydroxy dihydro derivatives of the aromatic hydrocarbons. Derivatization of the diols with [8] and chromatographic separation of the resulting diastereomeric bis-esters have been used in the determination of the enantioselectivity of biotransformations (133,134) and the preparation of the pure enantiomers (135–137). The latter applications involved preparative LC separation of the diastereomers, followed by hydrolysis of the bis esters (135–137). The separations were usually carried out on silica gel LC columns, although in some cases TLC separation of the diastereomeric esters was also feasible (137). Bromohydrin analogs of the diols, useful in the synthesis of some of the chiral metabolites, have been similarly resolved (138).

Another CDA that has been similarly applied (139–143) in the separation of dihydrodiol derivatives of PAHs is the acid chloride [32] of (−)-menthoxyacetic acid. This acid is commercially available from several sources. CDA [32] has also been used in the separation of the isomers of the new antihypertensive agent nipradilol. The chemical structure of this drug includes two asymmetric centers, and therefore the drug is a mixture of four stereoisomers, that is, two diastereomeric racemates. Derivatization of nipradilol, an amino alcohol, with [32] produced the four *N,O*-bis-menthoxyacetyl derivatives, which could be cleanly separated using silica gel LC, with detection at 275 nm (144).

The important anticoagulant drug warfarin is chiral and is administered clinically as the racemic mixture. Significant pharmacodynamic and pharmacokinetic differences between the enantiomers have generated considerable interest in the stereochemical aspects of this drug (145).

Banfield and Rowland developed an elegant procedure for the chromatographic resolution of the warfarin enantiomers (146). The enol hydroxyl group in the molecule was esterified with carbobenzoxy-L-proline, [33], a commercially available agent, and the derivatives were separated on silica LC columns. The procedure could be adapted to pharmacokinetic studies of warfarin using a UV detector at 313 nm (146). The original procedure was subsequently modified to use fluorescence detection to achieve greater sensitivity and permit monitoring of metabolites of the drug (147). The modification involved postcolumn on-line reaction of the eluted derivatives to generate fluorescent species. The procedure was recently adapted to the stereospecific analysis of a related chiral drug, nicoumalone (148,149) and large pharmacokinetic differences between its enantiomers were found (149).

Camphanic acid chloride, [11], described above in the resolution of amines, has also been used in the derivatization and separation of hydroxyl compounds. The bronchodilator proxyphylline was resolved after derivatization with [11] using RP LC in a study of the optical purity of synthetic samples of the enantiomers (150).

Other optically active acid chlorides have also been used in chromatographic separations of hydroxyl-containing drug enantiomers. Shimizu et al. (151) synthesized and resolved 2-(2-naphthyl)-propionic acid and used the acid chloride [34] of the dextrorotatory acid to acylate the hydrolyzed derivative of the calcium antagonist drug diltiazem. The derivatives were separated using RP LC. The purpose of the procedure was the determination of the enantiomeric purity of diltiazem, since this drug is used clinically as a pure enantiomer. The disadvantage of the procedure is the requirement to synthesize and resolve the parent acid of the CDA. Indeed, the same workers recently published a new procedure for the same purpose: The new CDA used, [35], was the (*S*)-*N*-1-(2-naphthylsulfonyl) derivative of L-proline. This CDA is readily prepared in a simple procedure from the resolved and inexpensive precursor, L-proline. No racemization occurred during the synthesis of the CDA and its optical purity was high. The CDA afforded derivatives of diltiazem that were well separated by silica (152). Reagent [35] merits further attention.

Several derivatives of mandelic acid have been used as CDAs for the resolution of chiral alcohols as the corresponding esters. Thus, in a study of the steric course of the cytochrome P-450-mediated hydroxylation of phenylethane (ethylbenzene), White et al. derivatized the enzymatically formed 1-phenylethanols with (*S*)-*O*-propionylmandelyl chloride, [36], and separated the diastereomeric derivatives by GLC (153). Comber and Brouillette synthesized the enantiomers of carnitine via derivatization of a racemic precursor alcohol with (*R*)- or (*S*)-α-methoxyphenylacetic [(*O*)-

methylmandelic] acid (see acid chloride [10]). After adding a required methyl group to the derivatives, the diastereomeric esters were separated via flash chromatography, followed by hydrolysis of the individual diastereomeric esters to remove the esterifying CDA moiety and thereby obtain the desired individual enantiomers (154).

Several lactic acid derivatives were used by Gessner et al. for the determination of the enantiomeric purity of flavor substances such as chiral alcohols from natural sources. Diastereomeric *O*-acetyl-, propionyl-, and hexanoyllactic acid esters of the chiral alcohols were separated by GLC (155). A report from the same laboratories described characterization of several chiral aroma substances that are δ-lactones. The lactones were hydrolyzed to the corresponding hydroxy acids, and the acid moiety was esterified to the isopropyl ester. The remaining hydroxyl group was esterified with (*R*)-2-phenylpropionic acid chloride or [30], and the diastereomeric derivatives were separated using preparative silica gel LC. The derivatives were also separated on an analytical scale by GLC (156).

A new type of CDA for the normal-phase LC resolution of hydroxyl compounds was developed by Goto et al. (157). The reagent ([37], both enantiomers were prepared) is chiral by virtue of restricted rotation around the binaphthyl bond. Hydroxyl groups react with the carbonyl nitrile moiety of the CDA to give the esters. The resolution of hydroxyl acids (157) and β-adrenergic antagonists (158) was carried out with [37] using normal-phase LC. The ester derivatives formed are highly fluorescent, whereas the reagent itself is nonfluorescent, a great advantage indeed.

A series of CDAs was reported by Lindner et al. (159). The reagents, optically pure symmetrically *O,O*-disubstituted (*R,R*)- or (*S,S*)-tartaric acid anhydrides, were developed for the LC separation of amino alcohols. Under the derivatization conditions used, only the hydroxyl group reacts with the anhydride to produce a monoester. The derivatives were well resolved by RP LC, and the zwitterionic nature (amino and carboxyl groups) of the derivatives was thought to be important to their separability. The procedure was applied to a series of β-adrenergic antagonists, and the presence of 0.2% of enantiomeric contamination of either enantiomer of propranolol could be determined, illustrating the power of the indirect method, provided an essentially 100% optically pure CDA is used (159). In an application of the method, Demian and Gripshover used (*R,R*)-dibenzoyltartaric anhydride to derivatize 1-methyl-3-pyrrolidinol at the hydroxyl group and separated the diastereomeric esters on a C_8 RP LC column. The procedure was used in the determination of the enantiomeric purity of the substrate, an intermediate in the synthesis of several drugs (160).

The earlier-mentioned chiral acid (*S*)-naproxen [15] has also been used, as the acid chloride derived from the *S* acid, to form diastereomeric esters

of 2-pentanol, 2-hexanol, 2-heptanol, 2-octanol, and 2-nonanol, and the diastereomers were separated on packed OV-17 GLC columns (69). Walther et al. recently described a new CDA for the resolution of enantiomeric alcohols. The reagent, now available commercially as (*S*)-Trolox™ methyl ether [38], appears superior to several other CDAs for the same purpose in that unlike the latter, [38] provided enantiomer separations by capillary GLC of several primary alcohols, that is, alcohols in which the stereogenic (asymmetric) center was not the hydroxyl-bearing carbon (161).

Formation of Carbonates. The reaction of an alcohol with a chloroformate produces the corresponding carbonate (Eq. 7)

$$\text{RO-CO-Cl} + \text{HOR}' \rightarrow \text{RO-CO-OR}' \tag{7}$$

This reaction was among the earliest used for the GLC resolution of enantiomeric alcohols: Studies 20 years ago showed that the reaction of (−)-menthyl chloroformate, [17], with chiral alcohols produced diastereomeric carbonates that could be resolved on packed columns (71,162). Despite its commercial availability, this CDA has been little used for the resolution of alcohols. Prelusky et al. (163) used [17] in a study of enantioselective metabolic ketone reductions. The metabolite alcohols were derivatized with [17] and the derivatives were separated by capillary GLC. Good resolution was obtained under the chromatographic conditions used (163). The derivatization of warfarin with [17] and LC of the derivatives was described, but the peaks were not fully separated, even with retention times of 80–100 min (164). Brash et al. used CDA [17] to determine the enantiomeric composition of several enantiomeric pairs of hydroperoxyeicosatetraenoic acids using silica LC, but some of the derivatives could not be resolved (165). It appears that [17] may be useful in the resolution of hydroxyl compounds and the evaluation of capillary GLC in the separations deserves attention.

Formation of Carbamates. Chiral isocyanates were discussed above as CDAs for the derivatization of amines, but these reagents have also proven useful for hydroxyl compounds. The reaction (Eq. 8) of an isocyanate with an alcohol yields a carbamate (sometimes also referred to as a urethane)

$$\text{RN=C=O} + \text{HOR}' \rightarrow \text{RHN-CO-OR}' \tag{8}$$

In pioneering work by Pereira et al., the derivatization of racemic alcohols with (+)-[19] was carried out to produce diastereomeric carbamates that could be resolved on stainless steel capillary GLC columns coated with Carbowax 20M or OV-225 stationary phase (166). The retention times, however, ranged between 28 and 90 min, rendering the technique somewhat inconvenient. Gal et al. used capillary GLC columns to separate

carbamate derivatives of (−)-[19] with retention times of 15 min or less and applied the procedure to the investigation of the stereoselectivity of ketone reduction by rat- and rabbit-liver cytosol (50,167). The CDA was also used in a study of the pharmacokinetics of misonidazole, using RP LC to separate the carbamate derivatives (168). The naphthalene analog, [20], has also been used in the separation of enantiomeric alcohols (169–173). Pirkle and his associates carried out extensive studies on the LC separation of diastereomeric carbamate derivatives using adsorption chromatography (3). Sakaki and Hirata (174) studied the separation by supercritical fluid chromatography of diastereomeric derivatives of several chiral secondary alcohols after derivatization with [20]. The role played by the stationary phase, mobile phase, and structure of the analyte was studied, and it was shown that useful resolutions could be obtained for several alcohols (174).

Dehydroabietyl isocyanate, [39], was used by Falck et al. to determine the absolute configuration of hydroxyeicosatetraenoic acid methyl esters in a study of the enzymatic epoxidation of arachidonic acid (175). In another study of similar metabolites, the stereochemical identity of 12-hydroxy-5,8,10,14-eicosatetraenoic acid derived from the lesional scale of patients with psoriasis was studied via separation of the enantiomers after derivatization with [39] (176). In these studies (175,176), LC separation of the derivatives was used. CDA [39] can be prepared from commercially available resolved dehydroabietylamine. It would be worthwhile to examine the applicability of this CDA to the resolution of other compounds.

Formation of Other Derivatives. The reactivity of isothiocyanates toward alcohols is less than that of isocyanates (177), and it appears that chiral isothiocyanates have not received attention as CDAs for the resolution of hydroxyl compounds.

A common metabolic biotransformation of alcohols is conjugation with D-glucuronic acid at the hydroxyl group, and it has been often reported that when the substrate alcohol is chiral, the diastereomeric glucuronide conjugates of the two enantiomeric alcohols are separable by LC. It could be expected, therefore, that this "natural CDA method" would be exploited for indirect resolutions; however, little work has appeared on enzymatic derivatizations for indirect resolutions. One reason for this paucity of interest may be that enzymatic reactions are more complex than nonenzymatic derivatizations. Gerding et al. described (178) the derivatization of compound [40], a D-2 dopamine agonist, with uridine-5′-diphosphoglucuronic acid (UDPGA), the natural co-factor involved in the conjugation of alcohols with glucuronic acid. The enzyme required, glucuronyl transferase, was obtained from fresh bovine livers.

The diastereomeric conjugates were well separated by RP LC, and the procedure could be used to determine the enantiomeric purity of the drug enantiomers in the range of 99.84–99.98%, illustrating the utility of this approach. Furthermore, this work is another exception to the generalization that trace-enantiomeric-impurity determinations cannot generally be performed reliably with the indirect method.

Meyers et al. (179) described a procedure for the determination of the enantiomeric composition of 2-substituted-1,2-glycols. The glycols were reacted with optically pure *S*-(+)-2-propylcyclohexanone, [41], to form diastereomeric ketals. A new asymmetric center is formed in the reaction, and therefore, a racemic glycol gives, upon reaction with [41], four diastereomers. For several glycols the four derivatives could be separated by LC, and therefore, the enantiomeric composition could be determined (179). Because some drugs are metabolized to glycols (180), this procedure may be of interest in drug disposition studies.

3. *Resolutions via Derivatization of Carboxyl Groups*

Many chiral drug molecules contain the carboxyl group. This functional moiety can also be generated metabolically from other structures via such biotransformations as hydrolysis of esters, oxidation of alkyl groups, alcohols, aldehydes, amines, etc. Of particular interest in recent years has been the stereoselectivity in the action and disposition of a group of nonsteroidal antiinflammatory drugs (NSAIDs), for example, ibuprofen, fenoprofen, flurbiprofen, etc. (181). These drugs have the 2-arylpropionic acid moiety in their structure and are chiral by virtue of the asymmetric center α to the carboxyl group. Most such drugs are used clinically in the racemic form, even though it is known that the desired activity resides primarily in the *S* isomers, the *R* enantiomers possessing low or no activity (181). Furthermore, it has been shown that many of these NSAIDs undergo in vivo metabolic inversion of the chiral center of the inactive *R* enantiomer to the active *S* antipode (181), and this unusual and interesting biotransformation has been the subject of many investigations (181). As a result of such interest, much of the recently published work on chiral derivatization of carboxylic acids deals with arylpropionic acid NSAIDs.

Formation of Esters. One frequently used approach to the formation of diastereomeric derivatives of carboxylic acids is esterification with an optically active alcohol (Eq. 9)

$$\mathrm{ROH} + \mathrm{HOOCR'} \rightarrow \mathrm{RO\text{-}OC\text{-}R'} \qquad (9)$$

A variety of alcohols, for example, 2-octanol (182,183), (−)-2-butanol (184), (+)-3-methyl-2-butanol (185), (−)-menthol (186), (*S*)-ethyl lactate (187), (*R*)-1-phenylethanol (188), etc., have been used. Several different sets

of reaction conditions have been used in the derivatization. Esterification of the acid with the alcohol via catalysis by anhydrous HCl (184–186) or concentrated sulfuric acid (182) is commonly used; formation of the ester from the acid and alcohol in the presence of a base and condensing agent is effective (187); the alcohol may be activated by conversion to an isourea derivative and the latter reacted directly with the acid (189); the acid may be converted to the acid chloride, which is then used to acylate the alcohol (190). The resolution of amino acids (186), hydroxy acids (185,187,189), hydroxydicarboxylic acids (184), monoesters of dicarboxylic acids (187), 2-alkyl-substituted carboxylic acids (185), etc., have been carried out as diastereomeric esters. The technique has been applied to NSAIDs, including studies of the stereoselective disposition of ibuprofen (191,192) and the determination of the enantiomeric purity of naproxen, a NSAID marketed as the (+) enantiomer (182). Both GLC (184–187) and LC (182–191) have been used for the separation of the diastereomeric esters.

Formation of Amides. The formation of diastereomeric amides from the enantiomers of carboxylic acids and amine CDAs has been used more frequently than the above-described ester syntheses, perhaps because of the greater stability of amides over esters and because the diastereomeric amides are often more readily separated than the corresponding esters. Both GLC (193–196) and LC (17,18,197–202) have been used for the separation of the amide derivatives. A variety of optically active amines have been employed in the derivatization, but the enantiomers of 1-phenylethylamine, [42] (18,193,194,200–203), and of 1-(1-naphthyl)ethylamine, [43] (17,202,204), appear to have emerged as the most commonly used. These CDAs are commercially available. Blessington et al. have compared packed and capillary GLC columns in the resolutions of NSAIDs and several other carboxylic acids as their derivatives formed with [42] (205). Singh et al. found the enantiomers of amphetamine to be useful alternatives to [42] and used (*S*)-(+)-amphetamine to study the pharmacokinetics of tiaprofenic acid in humans (195,196). It was found that the *R* enantiomer of the NSAID does not undergo inversion to the *S* isomer commonly seen with most other arylpropionic acid NSAIDs (196). (*S*)-(+)-Amphetamine was also found useful as a CDA in the capillary-column-GLC resolution of etodolac, a NSAID that differs from the "profens" in chemical structure (206). (*S*)-1-(4-Dimethylaminonaphthalen-1-yl)ethylamine, [44], has also been used, its advantage being the fluorescence of its derivatives, but it is not commercially available (197,198). Goto et al. recently described (207) the five-step synthesis and chemical resolution of 1-(1-anthryl)ethylamine and the 2-anthryl analog. The resolved enantiomers were used as CDAs

for carboxyl compounds with LC resolution, and the derivatives produced were highly fluorescent. The use of these potentially important CDAs is limited, however, by the lack of their ready availability at this time. In an interesting innovation, Shimada et al. (208) developed two new CDAs, 1-ferrocenylethylamine and 1-ferrocenylpropylamine, for the RP LC resolution of carboxylic acids. The derivatization reaction was carried out in the presence of a water-soluble carbodiimide and 1-hydroxybenzotriazole. An important advantage of the method is that the derivatives are electrochemically detectable (208). Other chiral amines have also been evaluated as CDA for the resolution of carboxylic acids (7).

To form the amide derivatives, the acid and amine are condensed in the presence of such agents as *N,N'*-carbonyldiimidazole (18) or a carbodiimide and 1-hydroxybenzotriazole (17). The amides can also be formed via the acid chlorides (200,201). Bjorkman (199) described a novel approach to the formation of diastereomeric amides: The NSAID indoprofen was coupled by means of ethyl chloroformate to L-leucinamide in a reaction that is complete in 3 min. The derivatives were separated by RP LC, and the procedure was used to study the disposition of the drug in surgical patients (199). Others have adopted this derivatization scheme (209). The chloroformate activation method has also been used with (*R*)-[42] for resolution of several acids (210). It was found that when hydroxyacids were derivatized, not only did the reaction produce the desired amide moiety at the carboxyl group, but the hydroxyl group was converted to the carbonate derivative of the chloroformate (210).

In addition to the resolution of amide derivatives of NSAIDS, diastereomeric amides of terpenoid (202), pyrethroid (201), and other (200) acids have also been resolved chromatographically.

It is clear that amine CDAs are highly useful in the chromatographic analysis of the enantiomeric composition of chiral carboxylic acids. Since such acids must also be derivatized (with a nonchiral reagent) for analysis on many of the available chiral LC columns, it would seem that the indirect method is advantageous and is likely to remain popular.

4. *Resolutions via Derivatization of Epoxides*

Metabolic epoxidation of carbon-carbon double bonds in alkenes and arenes is a fundamentally important biotransformation of foreign compounds. The primary epoxide (oxirane) metabolites formed generally undergo further biotransformation to more polar and readily excreted metabolites via conjugation with glutathione or epoxide-hydrolase-mediated hydrolysis to diols. Thus, epoxidation can be considered the first step in a metabolic detoxification scheme. On the other hand, epoxidation is also a

toxification pathway, inasmuch as epoxides are often highly reactive compounds that can react with various cellular nucleophiles to produce, ultimately, serious toxic effects (211).

In recent years, there has been considerable interest in the stereochemical aspects of metabolic epoxidation, prompted by the recognition that the toxicity, metabolic formation, and further metabolism of epoxides can be highly stereoselective (211). As discussed earlier, when the epoxide formed is an arene oxide (i.e., epoxidation of an aromatic double bond), it is often rapidly converted to dihydrodiols, and such hydroxyl compounds have been analyzed by the indirect resolution approach. However, when the metabolite epoxide is more stable, it is often possible to examine its stereochemistry. For this purpose, several methods for the chromatographic separation of enantiomeric epoxides have been developed, including some indirect methods.

The thiol nucleophile glutathione is known to react with many epoxides in a biotransformation catalyzed by glutathione-*S*-transferases (GST). In the reaction, the thiol nucleophile reacts at one and/or the other of the epoxide-ring carbons to give ring-opened derivatives, and such conjugations can be markedly regio- and enantioselective (212,213). Under appropriate conditions, the ring-opening reaction can also take place nonenzymatically. Such reactions form the basis of derivatizations of epoxides, with glutathione serving as the CDA. Glutathione is a chiral compound of high enantiomeric purity that has been found to be suitable for use in enzymatic and nonenzymatic reactions as a CDA for epoxides. Armstrong et al. (214) and van Bladeren et al. (215) used this approach to study the stereoselectivity of the cytochrome P-450-catalyzed epoxidation of PAHs. The enzymatically formed epoxides were reacted nonenzymatically with glutathione, and the derivatives were separated by RP LC. The CDA was found to add to the epoxides in *trans* fashion, but often in a nonregiospecific manner, each enantiomer yielding two positionally isomeric adducts, crating a challenging chromatographic separation problem (214,215). In subsequent investigations, glutathione was replaced by *N*-acetylcysteine as the thiol nucleophile, but regioisomerism in the reaction of the CDA with the epoxides was still a complication, and complete chromatographic separation of all the adducts was not possible in some cases (216,217). Nevertheless, the thiol-CDA approach to the derivatization of arene oxides was used successfully in studies of the stereochemical course and nature of enzymatic epoxidations (214–217).

Foureman et al. took a somewhat different approach in using glutathione as the thiol CDA in an in vitro study of the stereoselectivity of the oxidation of styrene catalyzed by purified cytochrome P-450 and by microsomes from rat liver (218). After incubation of styrene with the P-450

preparation, glutathione and a preparation containing GST were added, and the epoxide metabolite was converted enzymatically to the glutathione adducts. Here again, attack by the thiol occurred nonregiospecifically, and therefore, each enantiomer of the epoxide gave two adducts. Nevertheless, the analytical method was suitable for the determination of the stereoselectivity of the epoxidation of styrene (219).

A different approach was taken by Panthananickal et al., who studied the steric course of enzymatic epoxidations of PAHs (220,221). These investigators chose a chiral amino compound, polyguanylic acid, to serve as the CDA. In the reaction, the exocyclic amino group of the guanine moiety in the CDA reacted regio-specifically (i.e,. at only one of the two ring carbons) with the epoxides, but both *cis* and *trans* addition occurred, so that each epoxide enantiomer yielded two adducts. The initial adducts were hydrolyzed with potassium hydroxide and then digested with alkaline phosphatase to guanosine derivatives, which could be separated by RP LC (220,221).

Gal developed a procedure for the determination of the enantiomeric composition of several types of epoxides (222). Nonchiral simple aklylamines, for example, isopropylamine, were used in the ring-opening reaction to produce enantiomeric amino alcohols, which were then derivatized with the optically active isothiocyanate [21], and the derivatives were resolved by RP LC (222). Despite the two-step derivatization, the procedure is simple and practical to carry out and could be adapted to the determination of the enantioselectivity of the rat-liver-microsomal epoxidation of an alkene (223).

5. *Resolutions via Derivatization of Other Functional Groups*

Other functional groups have been derivatized with CDAs for the purpose of analytical or preparative chromatographic resolution. Isocyanates and isothiocyanates, for example, can be derivatized with chiral amines serving as CDAs (114). Similarly, the enantiomeric purity of isocyanates may be determined via derivatization with an optically pure alcohol (167). Isocyanates may also be derivatized with chiral 2-oxazolidones [21]. Pirkle et al. described derivatization and resolution of lactams using chiral isocyanates such as [19] and [20] (224).

The natural product gossypol, [45], a polyphenolic binaphthyl, is a male antifertility agent of considerable current interest. The available evidence suggests that the antifertility action of [45] may reside only in the (−) enantiomer (225). Procedures have been devised for the chromatographic separation of the enantiomers of [45] as diastereomeric derivatives of optically active β-amino alcohols (226,227). L-Phenylalaninol was used in one procedure (227), whereas (−)-norepinephrine was employed in

another (226). The latter CDA produced the bis-Schiff base upon reaction with the aldehyde groups of gossypol (226), whereas the former CDA was thought to yield the bis-oxazolidine derivative (227). This difference in the reaction course of [45] with the two CDAs may be the result of a difference between the two CDAs in steric hindrance around the hydroxyl groups.

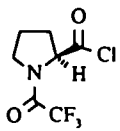
Reid et al. developed an interesting albeit complex derivatization scheme for the enantiospecific analysis of cyclophosphamide (228). Cyclophosphamide [46] is an antineoplastic drug that is chiral by virtue of the stereogenic phosphorus center. In the derivatization scheme, the drug is reacted with chloral to produce the secondary alcohol derivative [47]. In this reaction a new asymmetric center is created, and thus, two diastereomeric products are possible from each enantiomer of cyclophosphamide. However, the authors found that either only one of the two diastereomers was formed or the two diastereomers were not separable under the chromatographic conditions used, as only a single product peak was obtained regardless of the stereochemical identity of the drug enantiomer. The secondary alcohols were then reacted with the acid chloride of (+)-naproxen, and the resulting diastereomeric esters were separated by normal-phase or RP LC. The technique could be applied to the determination of the enantiomeric composition of cyclophosphamide in human serum (228). It should be recognized that although this derivatization scheme is indeed complex, the chemistry of cyclophosphamide precludes simple one-step derivatizations.

Chiral olefins were reacted with optically platinum complexes, and the diastereomeric derivatives formed were separated by LC (229); thiols may be derivatized with optically active acetals for chromatographic resolution (230). The chromatographic separation of enantiomeric sugars, a rather specialized field, has been reviewed (231).

IV. SUMMARY AND CONCLUSIONS

The enantiomers of a large number of compounds of pharmaceutical, pharmacological, or toxicological interest have been resolved as diastereomeric derivatives. A variety of functional groups can be derivatized, and a large variety of chiral derivatizing agents are available. The indirect method can be used to determine enantiomeric composition or purity, assign absolute configuration, and isolate the enantiomers on a preparative scale. GLC, TLC, and LC have been used in the separations; the majority of new applications use LC, although capillary GLC is also gaining popularity.

Some CDAs suffer from racemization problems and should therefore be abandoned, but most CDAs described have demonstrated excellent



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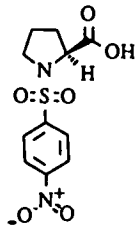

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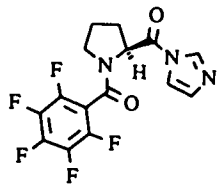

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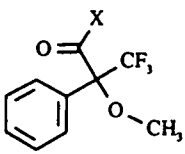
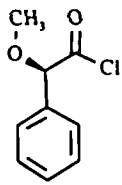


[8] X = Cl
[9] X = OH



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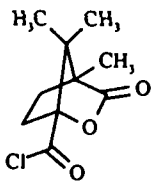

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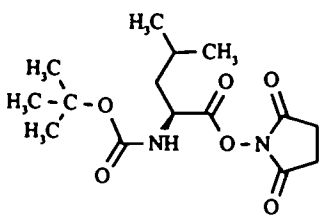

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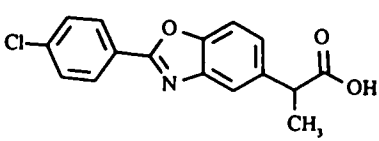

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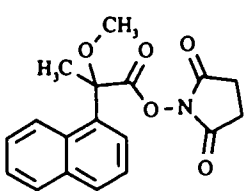

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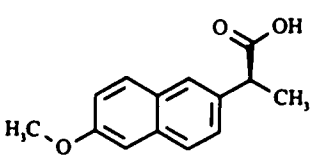

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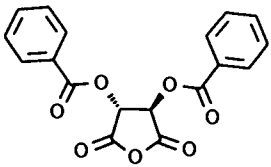

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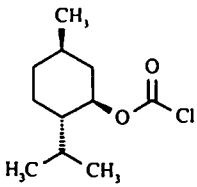

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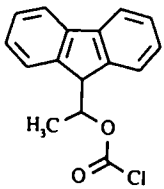

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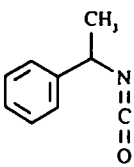

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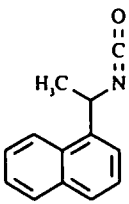

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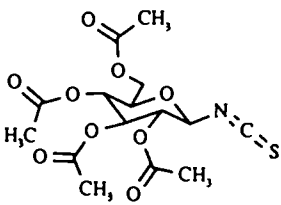

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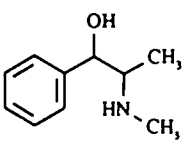

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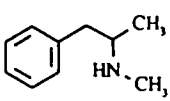

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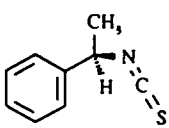

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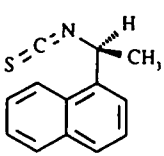

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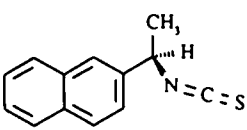

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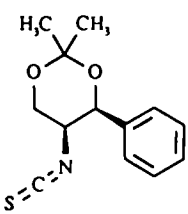

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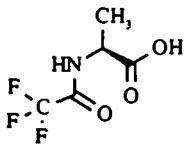

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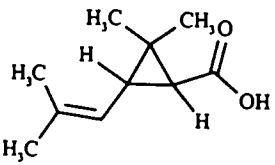

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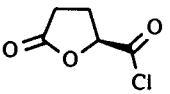

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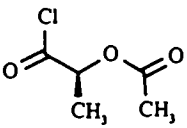

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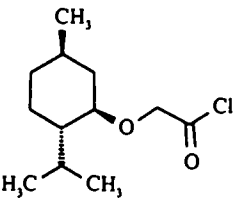

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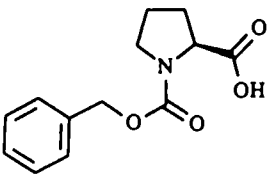

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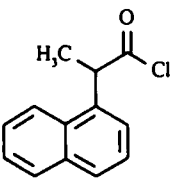

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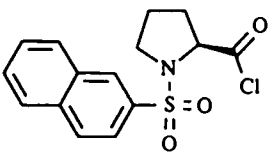

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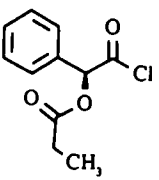

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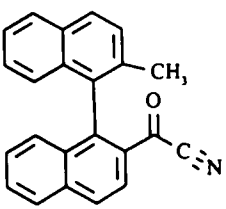

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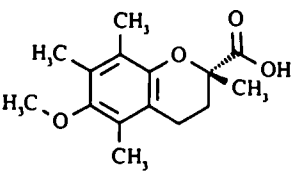

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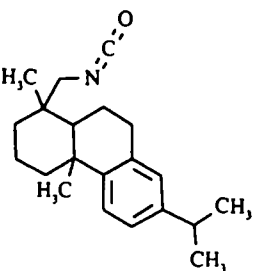

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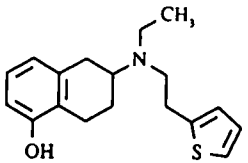

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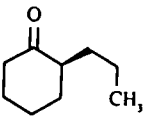

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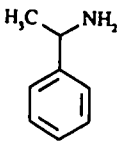

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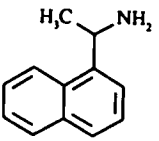

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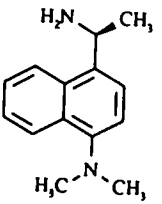

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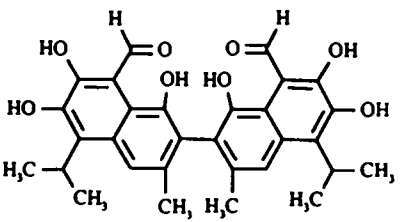

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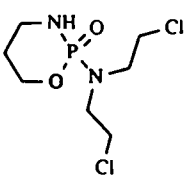

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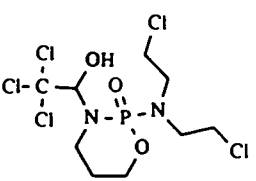

[47]

stereochemical stability. The enantiomeric purity of some CDAs described has been slightly less than 100%, but in many applications this is not a serious drawback. Ideally, however, enantiomeric purity approaching 100%, within experimental error, is desirable; many available CDAs do meet this goal.

The use of excess CDA in the derivatization eliminates the potential danger of unequal reaction rates of the enantiomers with the CDA, and kinetic resolution is generally not a problem. The commonly used chromatographic detectors usually produce an equal response to the diastereomeric derivatives.

The large number and variety of applications of the chiral derivatization approach attest to the success, viability, and importance of the technique. It is expected that despite the predictable advances to be realized in the near future in the development of direct chromatographic—mainly chiral-stationary-phase-based—separations of enantiomers, the indirect approach will continue to be widely used to solve stereochemical problems in the pharmaceutical, pharmacological, and toxicological arenas.

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5

THE DIRECT RESOLUTION OF ENANTIOMERIC DRUGS BY CHIRAL-PHASE GAS CHROMATOGRAPHY

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I. INTRODUCTION

Similar to many other cases of biologically active compounds, stereochemistry influences the pharmacological effect of a chiral drug. This can be explained by the fact that there is only one energetically favorable (specific) interaction of an active molecule with its receptor, both being chiral structures. Qualitative and quantitative differences are caused by different receptor affinities as demonstrated in Fig. 1 (1). The metabolism (biotransformation) of drugs is mainly caused by enzymes, which are chiral macromolecules and discriminate between substrate molecules of different stereochemistry. This may result in metabolites of different activity and in different pharmacokinetics, resorption, and excretion. Therefore, racemic drugs should be looked on as a 1:1 mixture of two different compounds.

About 40% of synthetic pharmaceuticals are chiral, but only approximately 10% of them are used as pure stereoisomers, whereas the rest are applied as racemic mixtures. A disastrous accident with racemic thalidomide combining the sedative action of the *R* enantiomer with the highly teratogenic properties of the *S* enantiomer has brought the importance of stereochemistry to public attention.

There are many less dramatic cases of different activities of stereoisomers. Even in cases where only one of the enantiomers is active, its inactive optical antipode should be avoided and considered as "enantio-

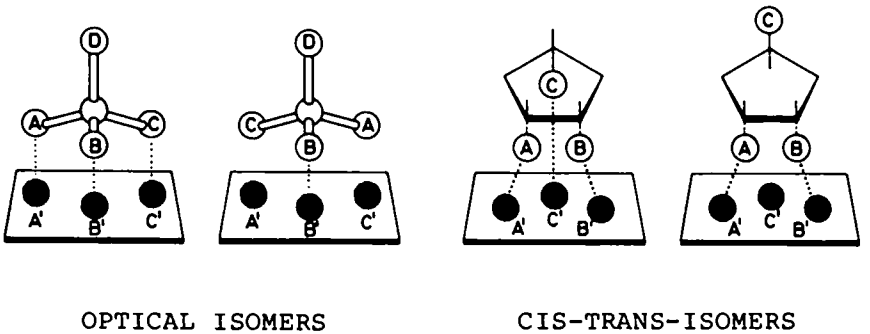


FIGURE 1 Schematic representation of the molecular interaction of a receptor with different stereoisomers of drugs. (From Schunack, Mayer, and Haake [1]).

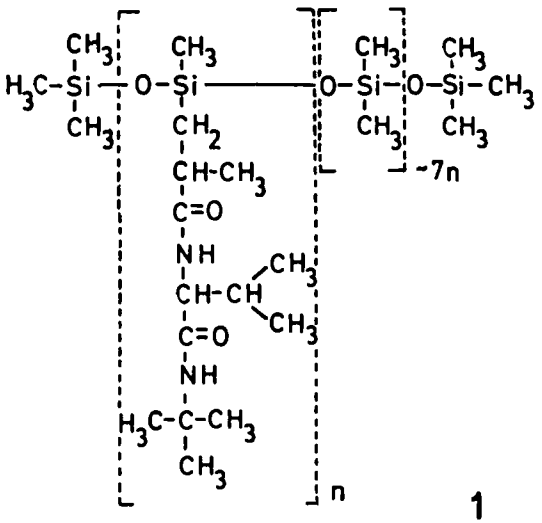
meric waste," which needs to be metabolized by the organism of a patient and ultimately contaminates the environment. There is also a possibility that negative side effects may not be obvious and sometimes hard to recognize. In addition, findings exist of synergistic effects of two enantiomers or of different although favorable effects. Recently, toxicity studies on both enantiomers of a racemic drug have begun to be demanded by the regulatory authorities of most countries before release of a racemic drug.

Growing awareness of the relevance of drug stereochemistry has not only greatly stimulated the development of methods for asymmetric synthesis of enantiomerically pure drugs as well as the preparative separation of racemic pharmaceuticals, but also initiated the development of methods for precise and sensitive determination of enantiomeric proportions. On the other hand, access to pure stereoisomers has enabled scientists to study physiological activity and stereoselective metabolism of enantiomerically pure drugs.

II. ENANTIOSELECTIVE GAS CHROMATOGRAPHY

A. Chiral Diamide Phases

Capillary gas chromatography with optically active stationary phases became a well-established technique for stereochemical analysis after the pioneering work of Gil-Av and his associates in the mid–1960s (2). Thermal stability of the initially low-molecular-weight amino acid and peptide derivatives was greatly improved after the introduction of chiral polysiloxanes by Frank, Nicholson, and Bayer (Chirasil-val [1], 1977) (3) and of



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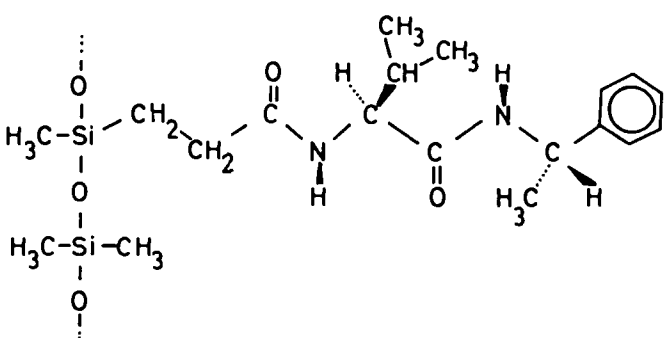
similar optically active polymer diamide phases (e.g., XE-60-L-valine-(*S*)- and (*R*)-α-phenylethylamide, [2], 1981) by König et al. (4). As in all chiral diamide phases, the stereoselective interaction of the enantiomers with the chiral selector molecules (diasteromeric interaction) is mainly based on hydrogen-bonding forces. The ability to separate enantiomers using this type of phase is, therefore, with only few exceptions restricted to sub-



XE-60-L-VAL-(S)-α-PEA

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strates with hydrogen donor or acceptor sites. Gil-Av et al. have proposed the formation of transient five- and seven-membered cyclic association complexes for explaining enantioselective selector-selectand interaction (5). By special derivatization procedures (formation of carbamates, urethanes, cyclic carbonates, oxazolidin-2-ones, or oximes), the range of compound classes susceptible to enantiomeric resolution could be enlarged (6–8). The results obtained with diamide-type chiral stationary phases were summarized in a monograph (9). A large number of chiral sympathomimetic and β-receptor-blocking drugs could be resolved after conversion of the amino alcohols to cyclic oxazolidin-2-ones with phosgene or phosgene-substituting reagents (10,11). A comprehensive summary of these studies was published in the previous edition of this work (12).

B. Cyclodextrin Derivatives

In 1983 Koscielski et al. reported on gas chromatographic enantiomer separations with cyclodextrins (cyclic glucose oligomers with 6, 7, or 8 α-1→4-linked glucose units, Fig. 2) dissolved in formamide as chiral stationary phases in packed-column gas chromatography (13). In the fundamental studies of Smolkova-Keulemansova (14,15), the unique "topological" selectivity of cyclodextrins toward guest molecules that could be included in the macrocyclic cavity (Fig. 2, formation of inclusion complexes) had already been indicated, although separation efficiency of these columns was rather poor. Enantioselectivity of the complex formation of guest molecules with cyclodextrins was demonstrated by Cramer who separated enantiomers by fractionated crystallization as early as 1952 (16). Szejtli et al. were able to improve gas chromatographic efficiency by using per-*O*-methylated cyclodextrins and capillary columns (17,18). Due to the high melting points of these cyclodextrin derivatives, the operation temperature had only a very limited range. The great potential of cyclodextrins was again demonstrated by Armstrong et al. who applied cyclodextrins bonded to silica gel as chiral support in liquid chromatography and succeeded in the separation of chiral pharmaceuticals with mainly aromatic substituents (19).

A breakthrough in the application of cyclodextrins in capillary gas chromatography was achieved in 1988 by substitution of the sugar hydroxylic groups with alkyl, for example, pentyl, residues and by preparing selectively alkylated/acylated derivatives (20,21). The resulting hydrophobic compounds are liquid at room temperature, soluble in unpolar solvents, temperature-stable up to 400°C, and highly enantioselective. At the same time, it was shown that the rather hydrophilic per-*O*-methylated cyclodextrins could be used over a temperature range of about 70–220°C after dilution with a polysiloxane, particularly OV 1701 (22). With both

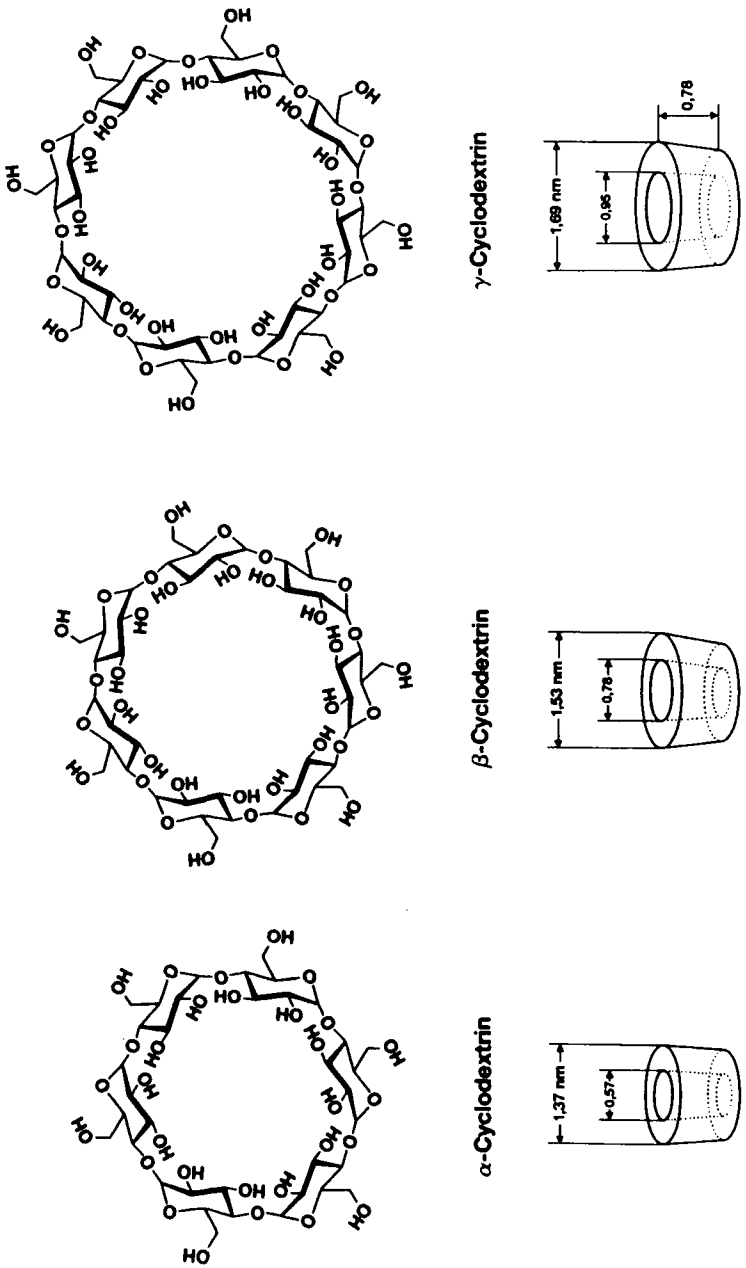


FIGURE 2 Chemical structure and molecular dimensions of α-, β-, and γ-cyclodextrin.

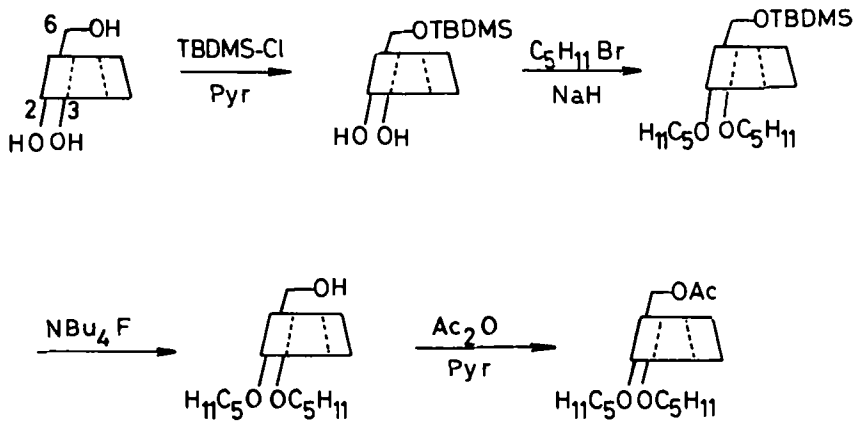
approaches, enantiomeric resolutions of a wide variety of racemic compounds could be achieved, including enantiomers of many compound classes, which had never been resolved by any chromatographic method before (23,24). Somewhat later several other research groups, including those of Armstrong, Li, and Pitha (25), Mosandl et al. (26), Fischer et al. (27), and others, joined in research activities on cyclodextrin phases and an increasing number of papers in this field are published.

Today mainly fused silica tubing is used to prepare highly efficient and well-deactivated capillary columns, although Pyrex glass proved to be equally suited, except that handling of glass capillaries is not common knowledge any more. The most recent advancement was reported by Schurig, who chemically immobilized per-*O*-methylated cyclodextrins and obtained capillary columns that can be used in supercritical fluid chromatography (28), which may be particularly useful for the investigation of drugs with volatilities insufficient for gas chromatography.

III. PREPARATION OF HYDROPHOBIC CYCLODEXTRIN DERIVATIVES

Hydroxy groups of cyclodextrins can be regioselectively alkylated in positions 2 and 6 of the glucose residues due to the high reactivity of these hydroxy groups. The 3-positions are much less reactive and can be alkylated or acylated under more drastic conditions. The preparation of pure per-*O*-alkylated cyclodextrin derivatives, which are mainly used for the separation of unpolar compounds (alkanes, alkenes, spiroacetals, alkyl halides, etc.) as well as a method for characterization of these phases have been described in detail (29).

Particularly useful for enantiomer separation of more polar compounds are 2,6-di-*O*-pentylated/3-*O*-acylated (acetyl, butyryl) cyclodextrin derivatives. They are prepared by acylation of the 2,6-di-*O*-pentylated intermediates (30–32). Substitution of hydroxy groups of cyclodextrins not only causes hydrophobicity and solubility in unpolar solvents and a decrease in the melting point, but also changes the conformation of the macrocyclic system, which again has a great influence on the selectivity toward the structure of a guest molecule. We found that all the selectively different-position di-pentylated/methylated cyclodextrin derivatives have quite different properties and are generally very useful chiral stationary phases. Cyclodextrin derivatives with selective alkylation in the 6- or 2-position can be prepared by using removable protecting groups (33,34). An example is shown in Scheme 1. Some of the hydrophobic cyclodextrin derivatives that were prepared and evaluated in our group are shown in



SCHEME 1 Reaction pathway for the preparation of hydrophobic cyclodextrin derivatives with "inverse" substituion pattern. (TBDMS-Cl = tert-butyldimethylsilyl chloride, NBu_4F = tetra-*n*-butylammonium fluoride.) [From König et al. (33).]

Fig. 3. It should be emphasized that a careful examination of the cyclodextrin derivatives must be performed after the final purification. Several authors have indicated that incomplete derivatization may be the reason for irreproducible results. Except for thorough NMR investigations (^{1}H- and ^{13}C-NMR), the "reductive cleavage" of cyclodextrin derivatives and subsequent quantitative gas chromatographic analysis of the 1,5-anhydrosorbitol derivatives as developed by Mischnick-Lübbecke and Krebber (35) have proved a sensitive and reliable method to detect even traces of under- or over-alkylated derivatives.

IV. APPLICATION OF ENANTIOSELECTIVE GAS CHROMATOGRAPHY TO PHARMACEUTICALS

The introduction of "inclusion" as a new principle of selectivity has considerably extended the range of applications of enantioselective gas chromatography in enantiomer separation of drugs, although systematic investigations have not yet been published. In many cases, chiral pharmaceuticals suffer from insufficient volatility, however, in some cases, derivative formation may be a way to enhance volatility to the degree that gas chromatography becomes applicable. This is particularly true for polyfunctional compounds as amino acids (e.g., L-DOPA), amino alcohols (e.g., adrenergic drugs), amines (e.g., amphetamine), and diols (*trans*-

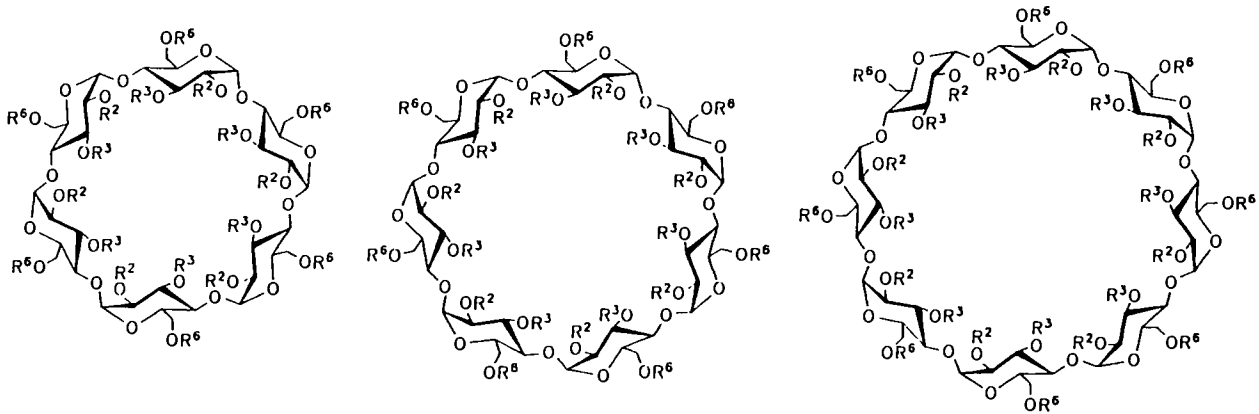


FIGURE 3 Structures of hydrophobic α-, β-, and γ-cyclodextrin derivatives:
$R^2 = R^3 = R^6 =$ *n*-pentyl
$R^2 = R^6 =$ *n*-pentyl, $R^3 =$ methyl
$R^2 = R^3 =$ *n*-pentyl, $R^6 =$ methyl
$R^2 = R^6 =$ *n*-pentyl, $R^3 =$ acetyl
$R^2 = R^3 =$ *n*-pentyl, $R^6 =$ acetyl
$R^2 = R^6 =$ *n*-pentyl, $R^3 =$ butyryl
$R^2 = R^3 =$ *n*-pentyl, $R^6 =$ butyryl

sobrerol). For other compounds missing sites for derivatization, for example, compounds with heterocyclic structures (hydantoins, succinimides, barbiturates), enantioselective gas chromatography using cyclodextrin derivatives has proved to be superior to the formerly used chiral polysiloxane stationary phases.

Although *high-pressure liquid chromatography* (HPLC) with chiral stationary phases is certainly more popular in stereochemical drug analysis, enantioselective gas chromatography may be beneficial in certain cases where sensitivity and precision are major issues. Also, once the separation conditions are elaborated, gas chromatography will provide highly reproducible results, whereas HLPC conditions for optimal resolutions may be more sensitive to operational parameters. As will be shown, in certain cases enantioselective gas chromatography is the only way to separate a racemic pharmaceutical.

A. Amino Alcohol-Type Structures and Sympathomimetics

A great number of compounds of this type, including β-receptor blocking agents and sympathomimetic drugs, have been resolved on capillary columns with XE-60-L-valine-(*R*)-α-phenylethylamide (10,11). For the separation of most of the important β-blockers meanwhile, efficient HPLC-methods have been established and gas chromatography has lost some of its attractiveness because of the necessity to derivatize samples. It was shown, however, that ephedrine, pseudoephedrine, and norephedrine can be easily resolved using 3-*O*-acylated-2,6-di-*O*-pentylated β-cyclodextrin (Lipodex D®; Fig. 4, (36)).* Probably this chiral stationary phase would be suitable to separate many more compounds of this type. Results of systematic investigations have not yet been published.

B. Amine-Type Drugs

Lipodex D is the chiral phase of choice to investigate amine-type pharmaceuticals (30). Figure 5 demonstrates the separation of some trifluoroacetylated compounds. Amphetamine is a nervous system stimulant. Its *S*-enantiomer is three to four times more potent than the *R*-enantiomer (38). Mexiletin is used as an antiarrhythmic drug, pholedrine is a sympathomimetic agent, which could not be separated on chiral diamide phases due to the lack of a hydrogen-bonding donor site. An extraordinarily large separation effect is observed for the enantiomers of tranylcypromine, a drug used as an antidepressant. In this case, the different

*Capillary columns with this phase are available from Macherey-Nagel, D-5160 Düren, Germany.

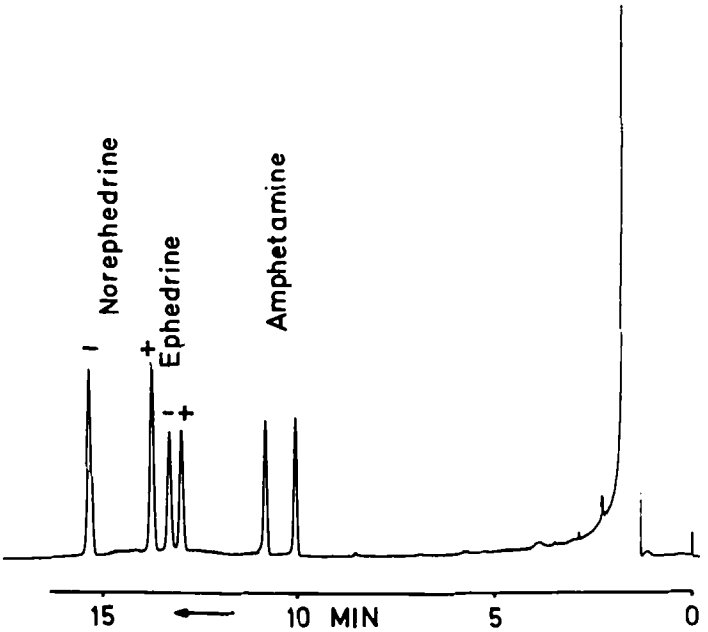
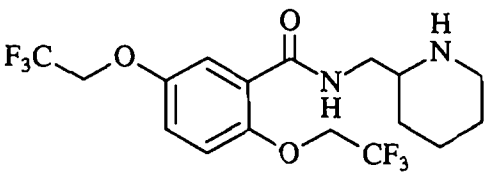


FIGURE 4 Enantiomer separation of (trifluoroacetylated) amphetamine, ephedrine, and norephedrine on a 40-m Pyrex glass capillary with heptakis(3-*O*-acetyl-2,6-di-*O*-pentyl)-β-cyclodextrin at 150°C. Carrier gas (as in all other figures) is hydrogen. [Trifluoroacetylation with trifluoroacetic acid anhydride in dichloromethane (1:4, v/v) for 30 min at 100°C.]

physiological activities of the individual enantiomers have been well documented (39).

The potency of enantioselective gas chromatography became apparent in the pharmacokinetic studies of flecainide [3] and two of its chiral metabolites as carried out by Fischer et al. (40). Trace amounts of these compounds in plasma samples were screened for enantiomeric discrimination.



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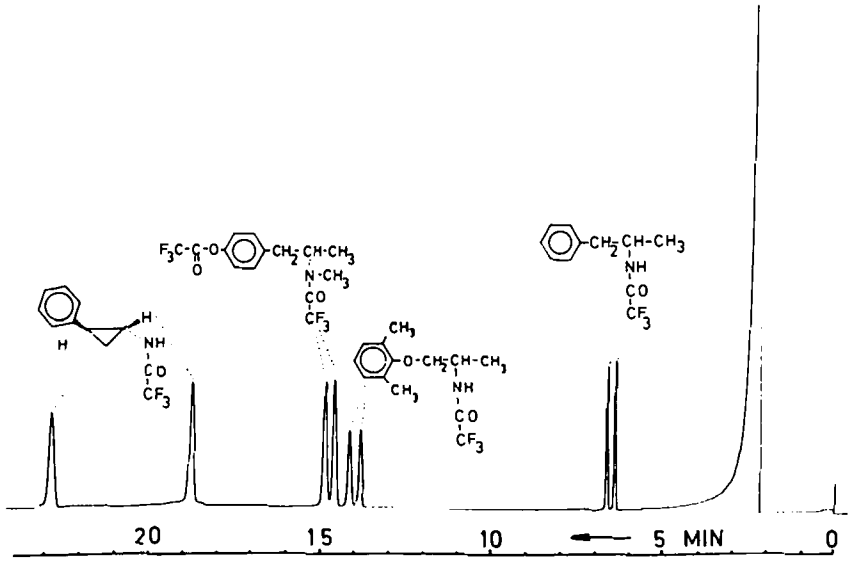


FIGURE 5 Enantiomer separation of trifluoroacetylated amphetamine, mexiletin, pholedrine and tranylcypromine. 45-m Pyrex glass capillary with 3-ac-2,6-pe-β-CD at 175°C.

C. Amino Acid-Type Drugs

Although enantiomer separation of amino acids has always been a domain of chiral diamide phases such as Chirasil-val [1] or XE-60-L-val-(*S*)-α-phenylethylamide [2], the development of hydrophobic cyclodextrin derivatives has affected even this field of applications of enantioselective gas chromatography. Octakis(3-*O*-butyryl-2,6-di-*O*-pentyl)-γ-cyclodextrin [Lipodex E®, (32) (see footnote on p. 115)] is highly selective for amino acid enantiomers. Since hydrogen bonding is not necessary for a determination of the enantiomers, some unusual amino acids can be separated that could not be resolved before, including β-amino acids, α-alkylated, and *N*-alkylated amino acids.

Proof of enantiomeric purity is necessary in the case of L-3,4-dihydroxyphenylalanine (L-DOPA), a drug used to treat Parkinson's disease, because of the high toxicity of the D enantiomer. Previously, D,L-DOPA was resolved by Frank, Nicholson, and Bayer using Chirasil-val (41). Figure 6 shows the separation of D,L-DOPA on a column with perpentylated α-cyclodextrin. In the case of α-methyl-DOPA (Aldomet) [4], a drug that is used in its L form for its antihypertensive activity, the enan-

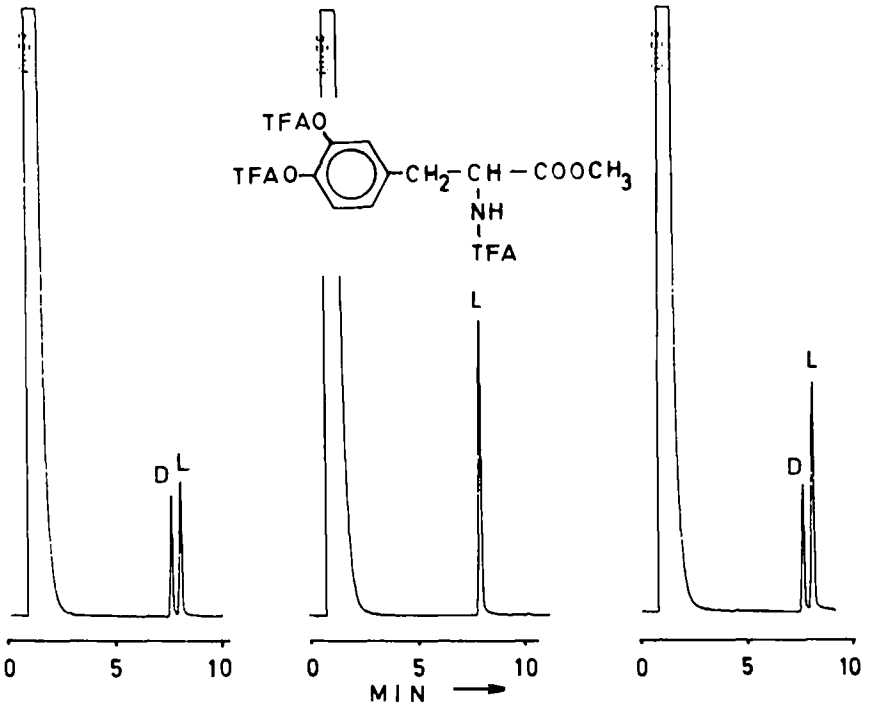
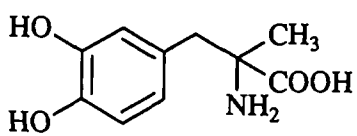


FIGURE 6 Enantiomer separation and proof of enantiomeric purity of DOPA (trifluoroacetyl/methyl ester derivative). 25-m Fused silica capillary with per-pe-α-CD at 150°C.

tiomers could be resolved on columns with Lipodex E. In Fig. 7 the enantiomer separation of (trifluoroacetylated) tocainide, a drug with antiarrhythmic properties, is shown using a Lipodex E capillary. In this case, the *R* enantiomer is the more potent drug and the enantiomers are metabolized enantioselectively (39).

The enantiomer separation of another unusual amino acid is given in Fig. 8. 4-Amino-3-hydroxy-5-phenylpentanoic acid is an intermediate in the synthesis of a statin analogue. Statin-type amino acids are essential



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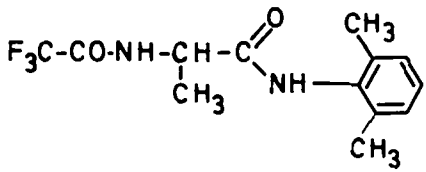


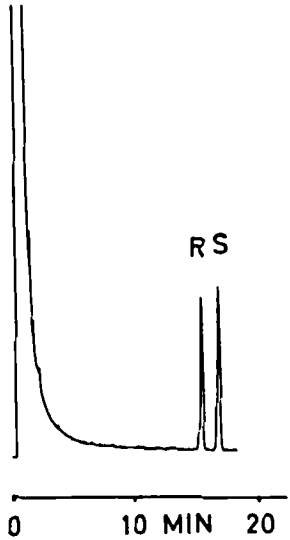
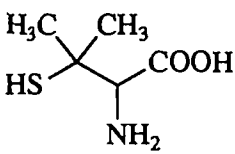


FIGURE 7 Enantiomer separation of trifluoroacetylated tocainide on a 10-m fused silica capillary with 3-bu-2,6-pe-γ-CD [= octakis(3-*O*-butyryl-2,6-di-*O*-pentyl)-γ-cyclodextrin] (60:40, w/w, mixture with polysiloxane OV 1701).

constituents of pepstatins, synthetic renin inhibitors with antihypertensic properties.

The control of enantiomeric purity is also important in the case of penicillamine [5], a drug administered during therapy for arthritis and other rheumatic diseases. The enantiomer separation of penicillamine was achieved after esterification and formation of a thiazolidin-2-one derivative with phosgene on a capillary with XE-60-L-val-(*R*)-α-phenylethylamide (42).



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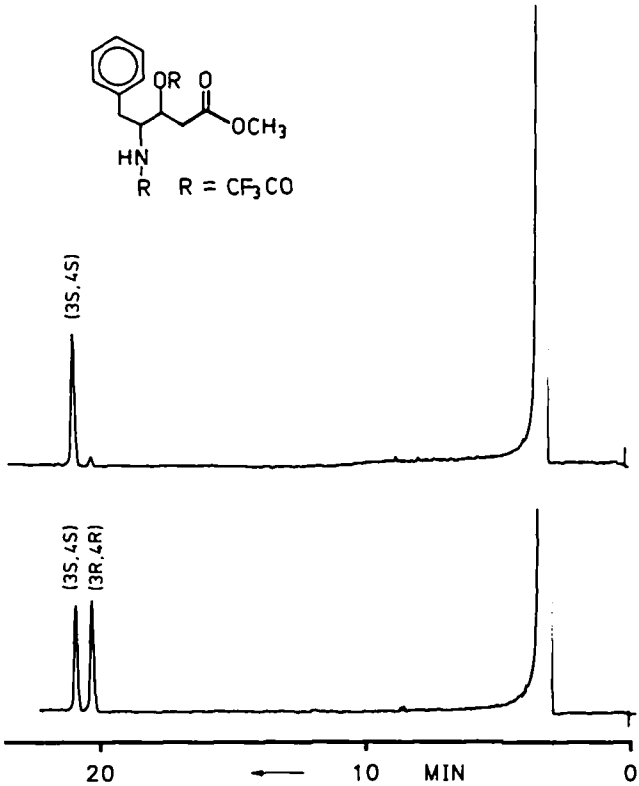
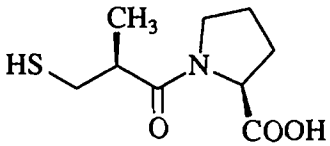


FIGURE 8 Enantiomer separation of 4-amino-3-hydroxy-5-phenyl-pentanoic acid and determination of enantiomeric purity of the (3*S*,4*S*) isomer (sample courtesy of Dr. H.-E. Radunz, Merck AG). 50-m Pyrex glass capillary with 3-bu-2,6-pe-γ-CD at 170°C.

One of the *most successful* drugs of recent years is captopril [1-(3-mercapto-2-(*S*)-methyl-1-oxopropyl)-(*S*)-proline] [6]. It is an inhibitor of the angiotensin-converting enzyme and is widely used for the treatment of hypertension and congestive heart failure. The stereochemical purity of captopril was investigated after esterification and acylation by enantioselective gas chromatography using a XE-60-L-val-(*S*)-α-phenylethylamide column (43).

D. Pharmaceuticals with Other Functional Groups

Enantiomer separation of trifluoroacetylated panthenol can be carried out perfectly using XE-60-L-val-(*S*)-α-phenylethylamide (44), but was recently



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also performed with Lipodex E columns (23; Fig. 9). The D enantiomer (Dexpanthenol) is used in treating skin irritations, inflammations of the respiratory system, and gastritis. The high sensitivity and precision of the determination of an enantiomeric impurity by gas chromatography are typically demonstrated in this example. D-Panthenol is prepared synthetically from D-pantolactone, which can also be separated on different cyclodextrin derivatives, for example, Lipodex D (Fig. 10).

trans-Sobrerol, a terpenoid diol, is formed by autoxidation of α-pinene in natural turpentine oil. It has mucoregulatory properties and is used in

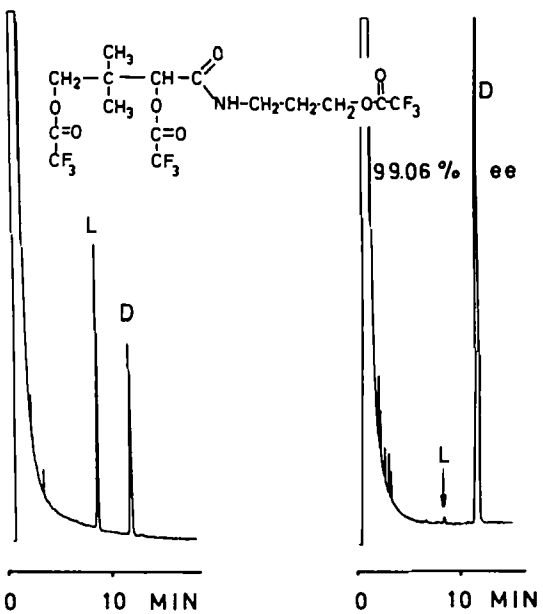


FIGURE 9 Enantiomer separation of panthenol (trifluoroacetyl derivative) and determination of enantiomeric purity of D-panthenol. 10-m Fused silica capillary with 3-bu-2,6-pe-γ-CD at 140°C. [Trifluoroacetylation with trifluoroacetic acid anhydride in dichloromethane (1:4, v/v) for 10 min at room temperature.]

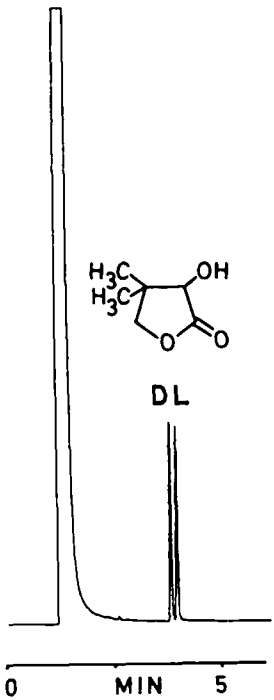


FIGURE 10 Enantiomer separation of pantolactone on a 25-m fused silica capillary with 3-ac-2,6-pe-β-CD [= heptakis(3-*O*-acetyl-2,6-di-*O*-pentyl)-β-cyclodextrin] at 185°C.

racemic form in the treatment of respiratory diseases. The enantiomers can be resolved as *bis*-trifluoroacetates using octakis(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin [(33, Fig. 11) (see footnote on p. 115)]. An HPLC method for the separation of the different stereoisomers of this drug was advanced by Italia, Schiavi, and Ventura (45).

Another pharmaceutical administered as a racemic mixture to treat liver diseases is α-lipoic acid (Thioctacid). Practically nothing is known about the activity of the individual enantiomers, although naturally occurring (+)-α-lipoic acid has an *R*-configuration. The first enantiomer separation of α-lipoic acid methyl ester was demonstrated on columns with Lipodex D (Fig. 12, 46). α-Lipoic acid is involved in the oxidative decarboxylation of pyruvic acid to activated acetaldehyde, which is transferred to coenzyme A to form acetyl coenzyme A.

In the case of ibuprofen, an analgesic drug used as a racemate, solely

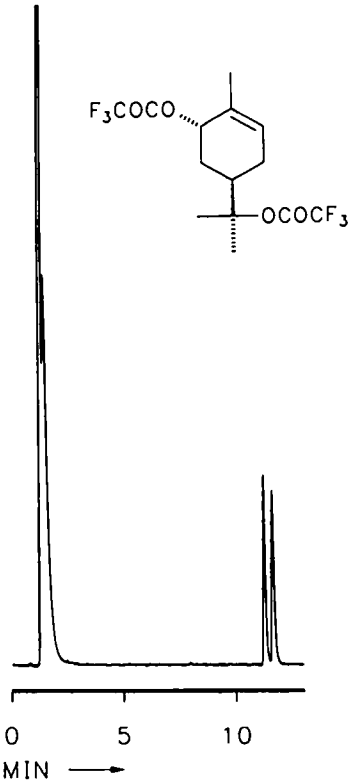


FIGURE 11 Enantiomer separation of *trans*-sobrerol (TFA derivative). 25-m Fused silica capillary on Pyrex glass capillary with 6-me-2,3-pe-γ-CD [= octakis(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin] at 75°C. (Trifluoroacetylation as in Fig. 9.)

the *S* enantiomer was found to have *in vitro* activity, whereas both enantiomers are equally active *in vivo*, due to enzymatic inversion of the configuration in living organisms (47). The enantiomer separation of the methyl ester of ibuprofen using a mixture of per-*O*-pentylated and per-*O*-methylated β-cyclodextrin is shown in Fig. 13.

Enantioselective gas chromatography was also successfully applied for diagnostic purposes. While L-arabinitol is a metabolite common to higher (including human) organisms, its enantiomer is a metabolite of many microorganisms. A microdetermination method for the discrimination of D- and L-arabinitol from human serum was proposed by Roboz, Nieves, and Holland (48). A significant increase in the proportion of the D enantiomer was found in patients infected with *Candida* species (e.g.,

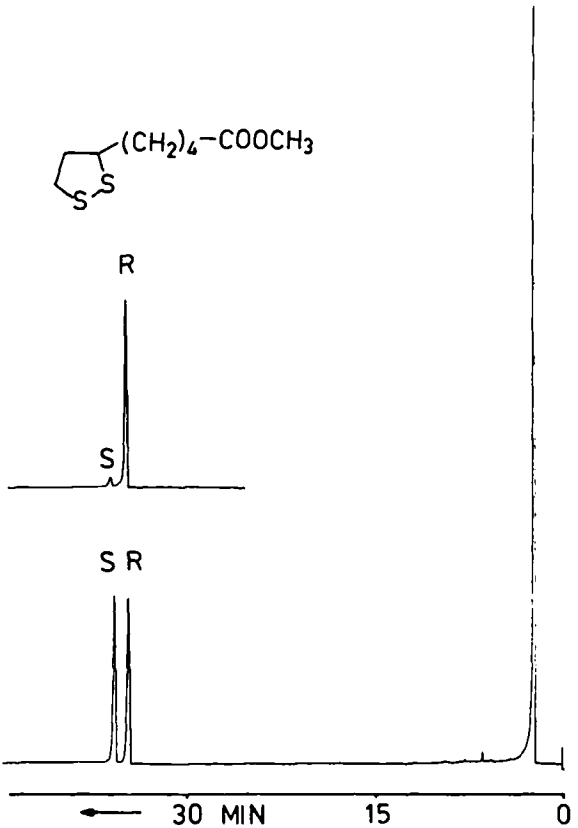


FIGURE 12 Enantiomer separation of α-lipoic methyl ester (esterification with diazomethane) and determination of enantiomeric purity of *R* enantiomer. 40-m Pyrex glass capillary with 3-ac-2,6-pe-β-CD at 180°C.

Candida albicans) after they received high doses of cytotoxic, immunosuppressive, or corticosteroid drugs. By monitoring the D/L arabinitol ratio, fungal infections can be diagnosed early enough to permit effective—in some cases, life-saving—treatment. The separation of trifluoroacetylated arabinitol is depicted in Fig. 14.

Blaschke et al. reported on metabolic studies of the anticancer drug ifosfamide [7] using Chirasil-val as a chiral stationary phase (49). In urine extracts of cancer patients, the *R*-enantiomer was found to be enriched.

Halothane, enflurane, and isoflurane are widely used inhalation anesthetics currently administered in racemic form. Nothing is known about

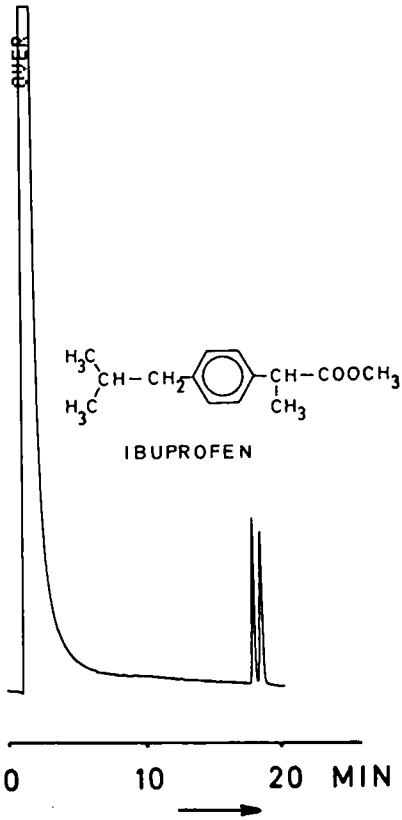


FIGURE 13 Enantiomer separation of analgesic drug ibuprofen as methyl ester (esterification with diazomethane). 25-m Pyrex glass capillary with per-pentyl-β-CD and per-methyl-β-Cd (1:1, w/w) at 140°C.

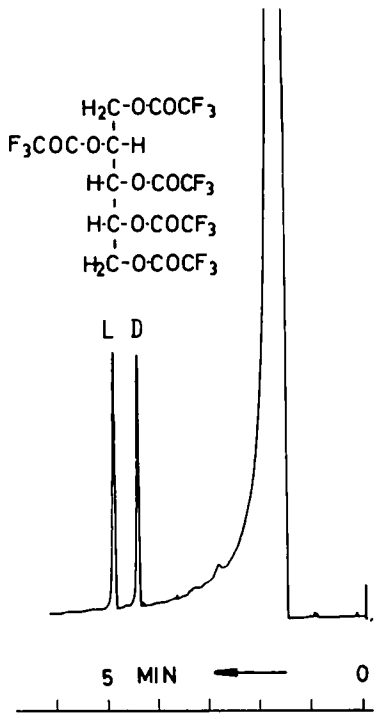
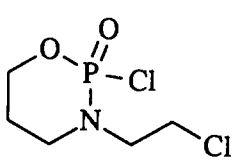


FIGURE 14 Enantiomer separation of arabinitol (trifluoroacetyl derivatives) on a 40-m Pyrex glass capillary with per-pe-a-CD [= hexakis(2,3,6-tri-O-pentyl)-α-cyclodextrin] at 110°C.



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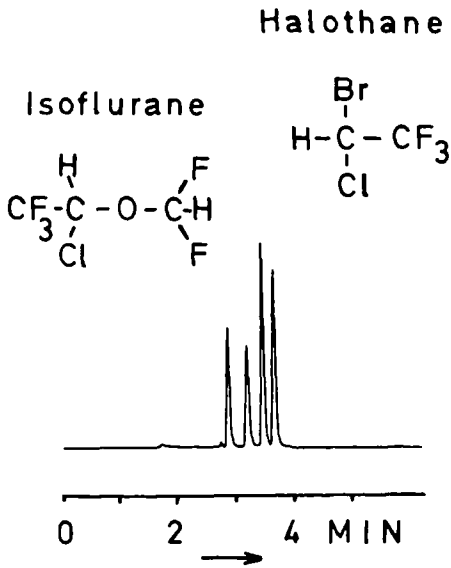


FIGURE 15 Enantiomer separation of inhalation anesthetics halothane and isoflurane on a 25-m fused silica capillary with per-pe-α-CD at 35°C.

the specific activity or enantioselective metabolism of these compounds. After being able to resolve the enantiomers (Fig. 15), it should be possible to answer these questions, as soon as sufficient amounts of the pure stereoisomers are available.

E. Pharmaceuticals with Heterocyclic Structures

Quite a large number of pharmaceuticals has heterocyclic structures, including barbiturates, hydantoins, succinimides, and other nitrogen-containing compounds. We have found that the enantiomers of *N*-alkylated barbiturates can best be resolved on per-*O*-pentylated (37) (or -hexylated) α-cyclodextrin (46) at column temperatures between 180 and 220°C, as demonstrated in Fig. 16. In all separations, the *R* enantiomers were eluted prior to the *S* enantiomers. Some chiral barbiturates have also been separated on XE-60-L-val-(*R*)-α-phenylethylamide (50). In liquid chromatography, hexobarbital was resolved on triacetylcellulose by Koller et al. (51) and the same compound together with other barbiturates by Yang et al. (52) in a Pirkle-type phase.

It was proved by Knabe et al. (53) that the narcotic effects of *N*-methylated barbiturates differ markedly. In some examples, one of the enan-

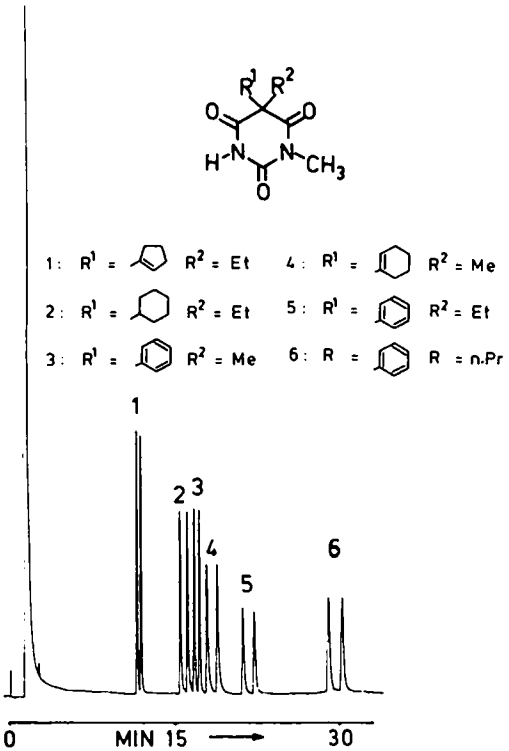


FIGURE 16 Enantiomer separation of some *N*-alkylated barbiturates (*R* enantiomers are eluted before *S* enantiomers). 36-m Pyrex glass capillary with per-*O*-hexyl-α-CD at 180°C.

tiomers even showed convulsive properties. The enantiomers also have different pharmacokinetics.

Some hydantoin-type antiepileptic drugs like ethotoin and mephenytoin could be separated on different cyclodextrin phases, as shown in Figs. 17 and 18. Several succinimide-type structures like mesuximide, phensuximide, and ethosuximide are also used as antiepileptic agents. Their separation is depicted in Figs. 19 and 20. Gluthetimide, a drug with hypnotic activity, can be resolved on per-*O*-pentyl-α-cyclodextrin, per-*O*-methyl-β-cyclodextrin, 3-*O*-butyryl-2,6-di-*O*-pentyl-γ-cyclodextrin, and 2-*O*-methyl-3,6-di-*O*-pentyl-β-cyclodextrin, as demonstrated in Fig. 21. Both enantiomers are metabolized selectively and the sedative activity of the (+) enantiomer is two to three times greater than that of the (−) enantiomer (54). The absolute configuration *R*-(+) of gluthetimide was

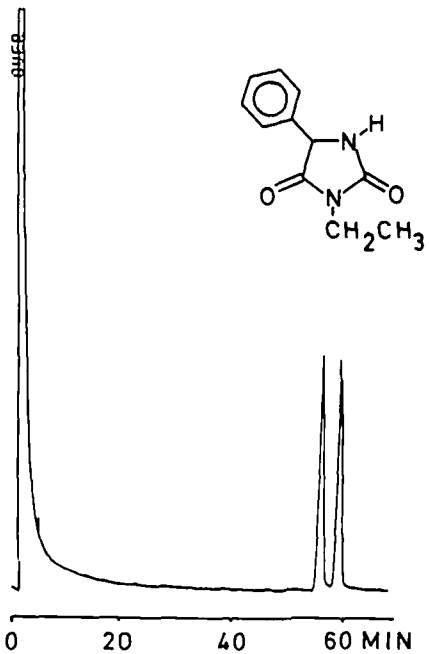


FIGURE 17 Enantiomer separation of ethotoin on a 25-m fused silica capillary with per-pe-α-CD at 170°C.

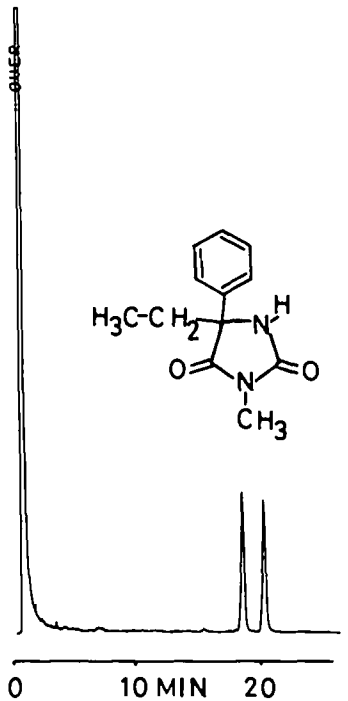


FIGURE 18 Enantiomer separation of mephenytoin on a 10-m fused silica capillary with 3-bu-2,6-pe-γ-CD (60:40, w/w in OV1701) at 160°C.

determined by Knabe and Reischig (55). The enantiomers of methyprylon, also a hypnotic drug, are separated on capillaries with per-O-methyl-β-cyclodextrin or on 2-O-methyl-3,6-di-O-pentyl-β-cyclodextrin, as shown in Fig. 22.

F. Essential Oils

With the advent of hydrophobic cyclodextrin derivatives, many chiral constituents of essential oils became the subject of a detailed investigation of their enantiomeric composition. Several essential oils are used as additives in pharmaceutical formulations, for example, pine needle oil or peppermint oil. They are not only used for their pleasant flavor or fragrance, monoterpene hydrocarbons like α-pinene [8], β-pinene [9], and

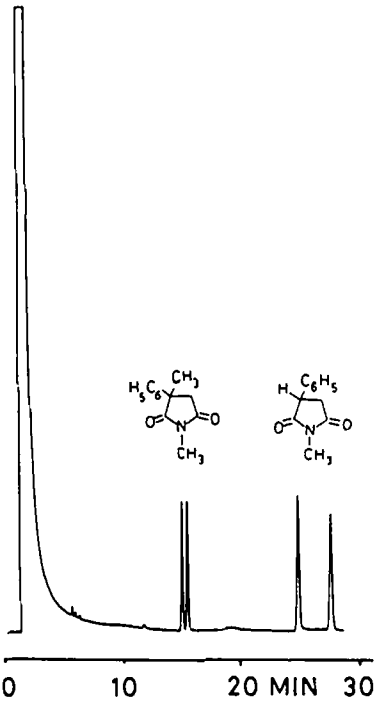


FIGURE 19 Enantiomer separation of mesuximide (left) and phensuximide (right) on a 25-m fused silica capillary with 3-ac-2,6-pe-β-CD at 180°C.

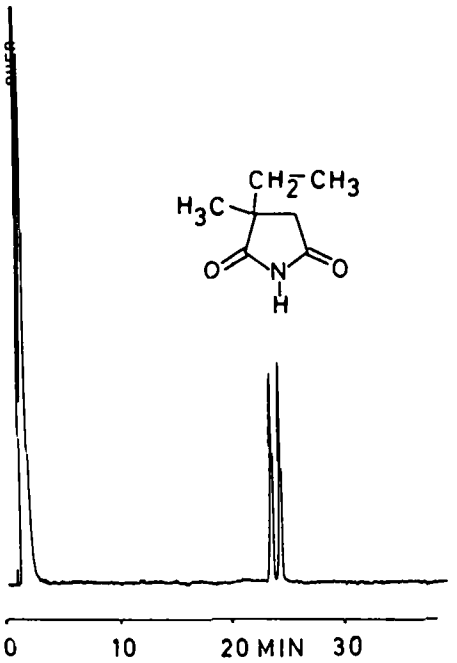
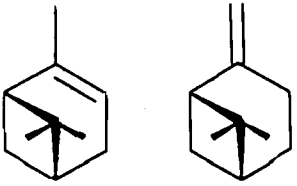


FIGURE 20 Enantiomer separation of ethosuximide on a 25-m fused silica capillary with 6-me-2,3-pe-γ-CD at 115°C.



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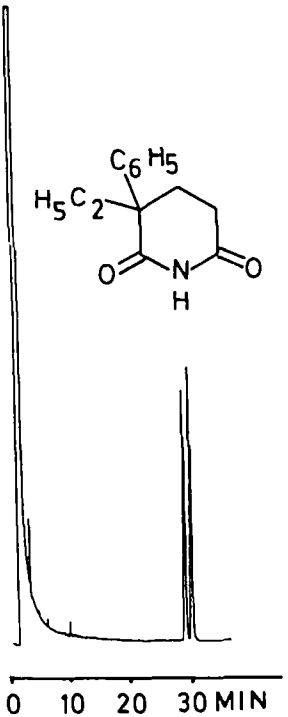


FIGURE 21 Enantiomer separation of glutethimide on a 25-m Pyrex glass capillary with 2-me-3,6-pe-β-CD at 168°C.

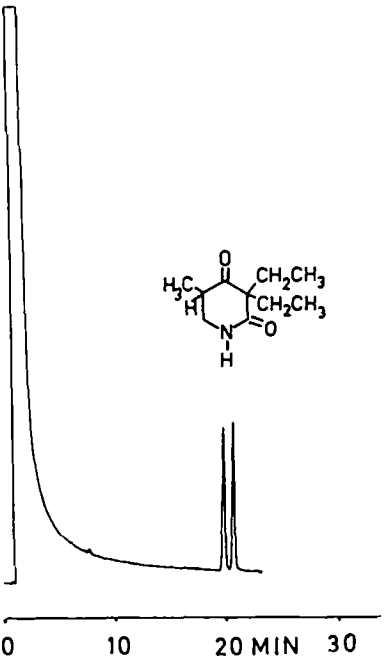
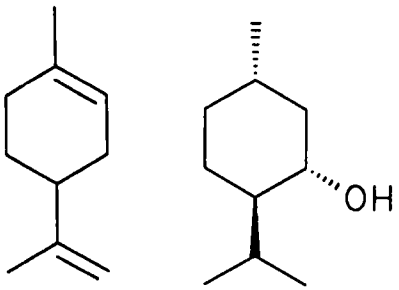


FIGURE 22 Enantiomer separation of methyprylon on a 25-m Pyrex glass capillary with 2-me-3,6-pe-β-CD at 140°C.

limonene [10], major constituents of all conifer needle oils, are also known for their antibacterial or antifungal activity. Figure 23 shows the separation of the enantiomers of these monoterpenes. Pharmacological activities are also attributed to (−)-menthol [11], the main constituent of peppermint oil, including antiseptic and anesthetic properties. It is an important ingredient of pharmaceutical formulations for the treatment of respiratory infections. Enantioselective gas chromatography allows a differentiation of these constituents with respect to their enantiomeric composition (33, 56, 57) (Fig. 24), thus providing a good means to detect adulterations. Because of the complexity of essential oils, multidimensional gas chromatography may be necessary to obtain unambiguous results (58). (−)-



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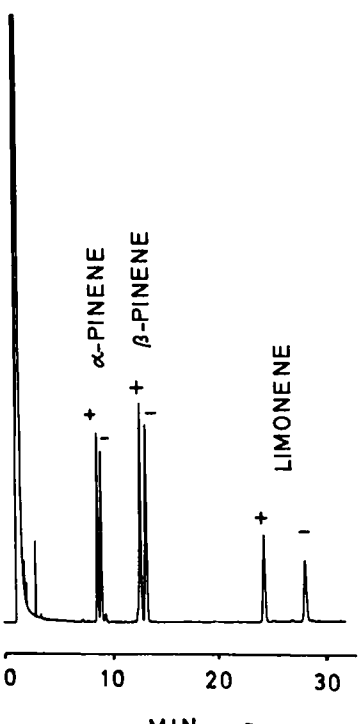

FIGURE 23 Enantiomer separation of α-pinene, β-pinene, and limonene on a 25-m Pyrex glass capillary with 6-me-2,3-pe-γ-CD at 55°C.

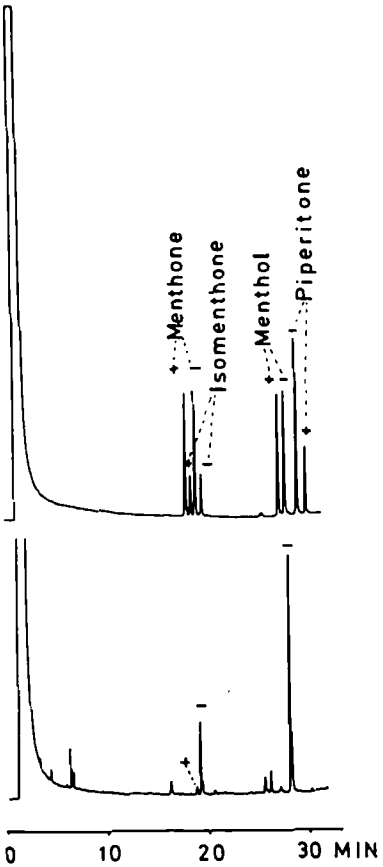

FIGURE 24 Enantiomer separation of menthone, isomenthone, menthol, and piperitone (top) and determination of enantiomeric composition of these compounds in peppermint oil (bottom). 25-m Fused silica capillary with 6-me-2,3-pe-γ-CD at 75°C (15 min), temperature program 2°C/min to 150°C.

Menthyl acetate, also a major component of peppermint oil, should be enantiomerically pure. The presence of the (+) enantiomer is considered an indication of adulteration (59, Fig. 25).

G. Enantioselective Gas Chromatography in Purity Control of Synthetic Building Blocks

A major hurdle on the road to enantiomerically pure drugs is the development of efficient methods of asymmetric synthesis. In many cases, small chiral compounds of synthetic or natural origin are used as starting

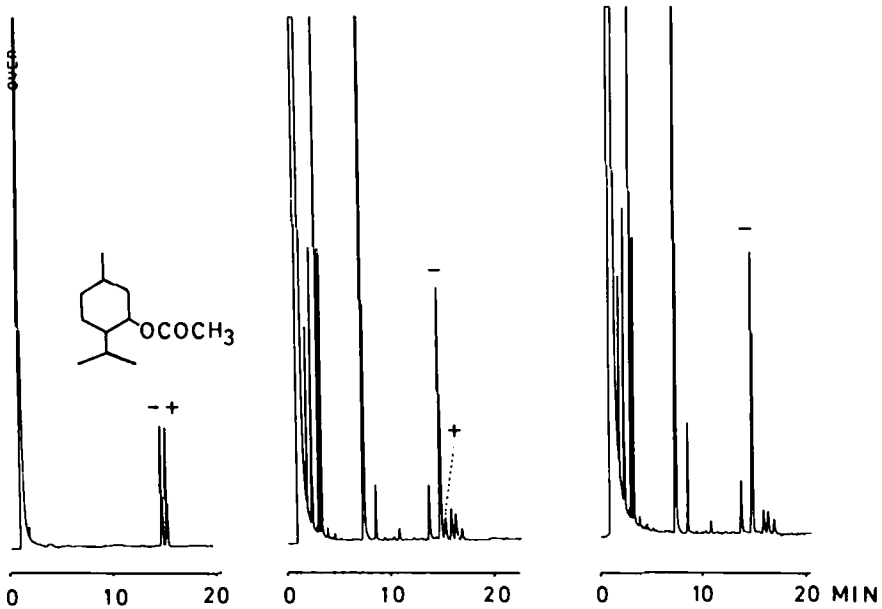

FIGURE 25 Enantiomer separation of menthyl acetate (left) and determination of enantiomeric purity of menthyl acetate in two commercial peppermint oil samples. The presence of (+)-menthyl acetate in one of the samples (middle) may be an indication of adulteration. 22-m Pyrex glass capillary with 6-me-2,3-pe-β-CD at 85°C.

materials or chiral auxiliaries for multistep syntheses. To obtain products of high enantiomeric purity, it is important to have highly pure synthons. A typical example may demonstrate the importance of enantioselective gas chromatography to control the enantiomeric purity of chiral building blocks. Enantioselective epoxidation of allylalcohols according to Katsuki and Sharpless (60) may serve as an important primary step in an asymmetric synthesis. Besides many other applications, this procedure may be used to prepare in only few steps (*S*)-propranolol, an important β-blocker, as shown in Fig. 26 (61). Starting from allylalcohol, Sharpless epoxidation results in glycidol (epoxypropanol) of high enantiomeric purity, which can easily be proved by enantioselective gas chromatography (37). The separation of the enantiomers is demonstrated in Fig. 27.

V. CONCLUSION

It should be emphasized that only a small fraction of separation problems of chiral pharmaceuticals has so far been addressed by enantioselective

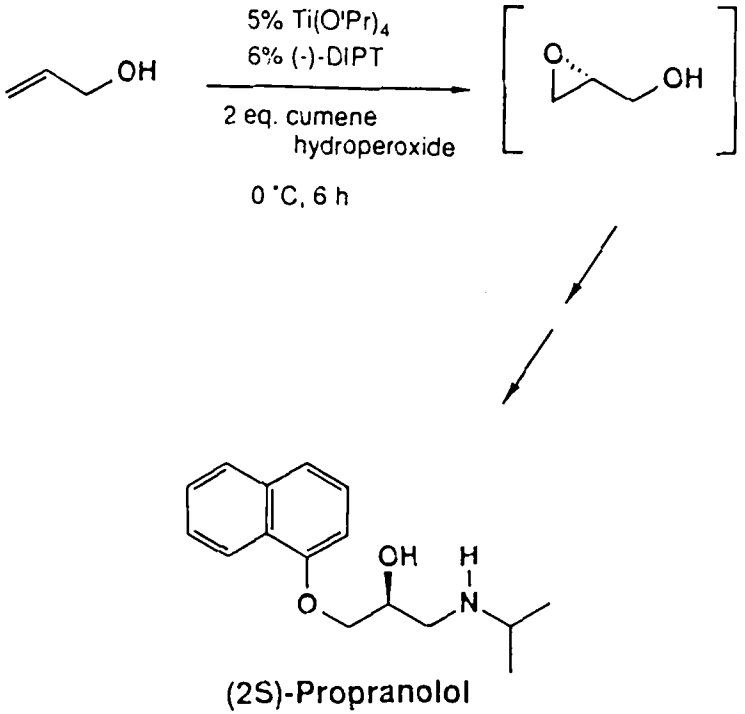

FIGURE 26 Schematic pathway for the synthesis of (*S*)-propranolol with glycidol as an intermediate according to Klunder, Ko, and Sharpless (61).

gas chromatography. From the high thermal stability of cyclodextrin derivatives, it can be expected that even pharmaceuticals with low volatility, now a domain for liquid chromatography, will be susceptible to enantiomer separation by gas chromatography in the future. The problems related to low volatility may also be overcome by the preparation of capillary columns with immobilized cyclodextrin derivatives, which can be applied for chromatography with supercritical mobile phases.

ACKNOWLEDGMENTS

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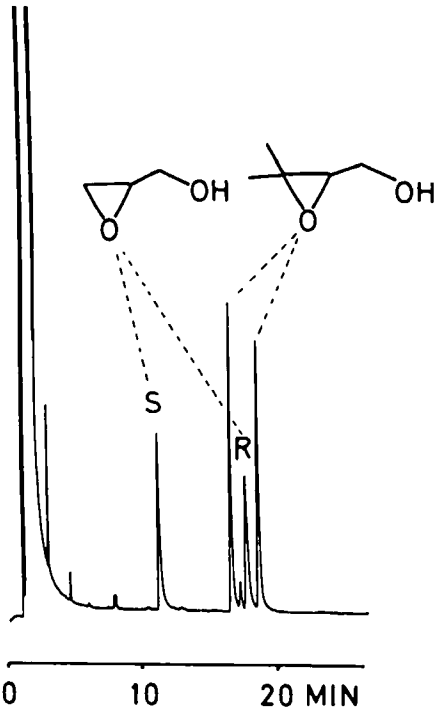

FIGURE 27 Enantiomer separation of glycidol and 3,3-di-methyl-glycidol on a 25-m fused silica capillary with per-pe-α-CD at 40°C.

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6

HPLC Chiral Stationary Phases for the Stereochemical Resolution of Enantiomeric Compounds

The Current State of the Art

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I. INTRODUCTION

In the first edition of this work, the conclusion of this chapter stated

> The HPLC chiral stationary phases (CSPs) that are already available, and those that will shortly be on the market will play a large role in future regulatory and pharmacological applications, and the last half of the 1980s should see the continued rapid expansion of the use of HPLC-CSPs. By the end of the decade, these columns should be a routine analytical tool in most analytical, regulatory, and pharmacological laboratories (1).

To a great extent this has occurred.

In the four years since the publication of the first edition, the European, North American, and Japanese drug regulatory agencies have expressed an interest in a closer examination of the stereoisomeric composition of chiral drugs and the therapeutic and toxicological consequences of this composition (2–4). The pharmaceutical industry has quickly responded to this situation, incorporating the consideration of stereochemistry into its initial drug evaluation strategies (5). In fact, at the present time, a number of companies have made the decision to market only single-isomer drugs (5).

These events have been driven by technological developments in the

chromatographic separation of stereoisomeric compounds and, in particular, the spectacular growth in HPLC-CSPs for the separation of enantiomeric molecules. In the past four years, the number of commercially available HPLC-CSPs has grown from 35 to over 50 and will surely increase again by the time this manuscript is published.

The large number of available HPLC-CSPs presents the user with a broad range of possibilities and the problem of deciding which phase to use. These problems can be minimized by grouping the HPLC-CSPs according to the chiral recognition process taking place on the column. The approach described and illustrated below was initially proposed in 1986 (6), revised in subsequent publications up to 1988 (1,7,8), and is now presented in its current form. What is missing from this presentation are the extensive compilations of applications included in the first edition. The use of HPLC-CSPs has grown to such an extent that whole volumes have been dedicated to this topic and the interested reader is directed to some of the recent works in this area (9–11).

II. CLASSIFYING HPLC-CSPS USING THE CHIRAL RECOGNITION MECHANISM

A. The Solute/CSP Complex

HPLC-CSPs are based on molecules of known stereochemical composition immobilized on liquid chromatographic supports. Single enantiomorphs, diastereomers, diastereomeric mixtures, and chiral polymers (such as proteins) have been used as the chiral selector. The chiral recognition mechanisms operating on these phases are the result of the formation of temporary diastereomeric complexes between the enantiomeric solute molecules and immobilized chiral selector. The difference in energy between the resulting diastereomeric solute/CSP complexes determines the magnitude of the observed stereoselectivity, whereas the sum total of the interactions between the solute and CSP, chiral and achiral, determines the observed retention and efficiency.

A simplified version of this process is presented in Fig. 1. In this example, the two solute/CSP complexes, *d*-solute-CSP and *l*-solute-CSP, have different free energies, with the *d*-solute-CSP complex being more stable, that is, the one with the lower free energy. As a result, the *d* isomer will remain on the column longer than the *l* isomer, and the two enantiomers will be resolved.

B. Formation of the Solute CSP Complex

The chiral recognition mechanism can be broken down into parts if one remembers that the parts are interdependent and cannot exist apart from one another. These parts are (1) how the solute/CSP complexes are formed

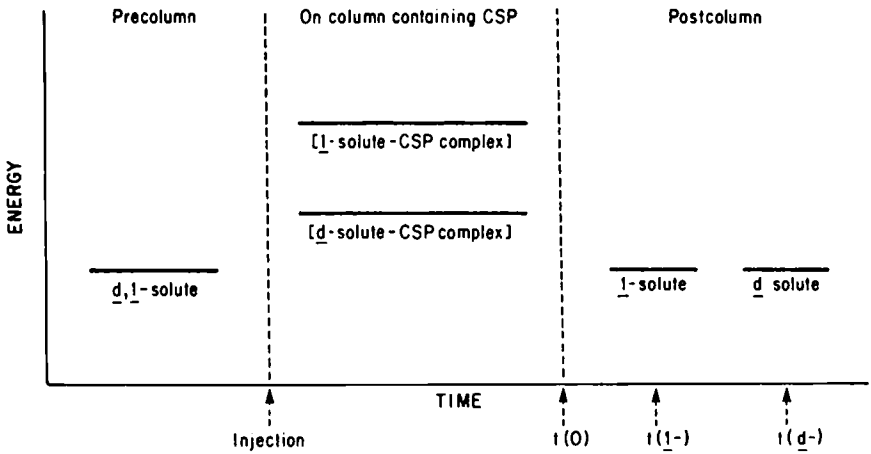


FIGURE 1 A general scheme for the mechanism of chiral resolution on an HPLC chiral stationary phase. Enantioselectivity (α) = $\frac{t(d\text{-}) - t(0)/t(0)}{t(l\text{-}) - t(0)/t(0)}$.

and (2) how the stereochemical differences are expressed during and after the formation of these complexes. The *key step* is the formation of the diastereomeric complex, and the current HPLC-CSPs can be broken down into five basic types on the basis of the solute/CSP bonding interactions (8). These are the following:

Type I. When the solute/CSP complexes are formed by attractive interactions, hydrogen bonding, π-π, dipole stacking, etc., between the solute and CSP

Type II. When the primary mechanism for the formation of the solute/CSP complex is through attractive interactions, but inclusion complexes also play an important role

Type III. When the solute enters into chiral cavities within the CSP to form inclusion complexes

Type IV. When the solute is part of a diastereomeric metal complex (chiral ligand-exchange chromatography)

Type V. When the CSP is a protein and the solute/CSP complexes are based on combinations of hydrophobic and polar interactions

C. Chiral Recognition Within Solute/CSP Complexes

Within the solute/CSP complex, chiral recognition is based on the "three-point interaction" model proposed by Dalgliesh (12). According to this mechanism, three interactions occur between the solute and chiral selec-

tor, and at least one of these interactions must be dependent on the stereochemical structure of the solute.

Although this appears to be a rather simple process, it is actually extremely complicated. During the formation of the solute/CSP complex, the solute and CSP are positioned in space relative to one another, depending on the bonding interactions. If these interactions change, producing a different spacial orientation, then the "stereochemically dependent" interaction can also change, leading to a different outcome of the chiral recognition process.

This situation has been described by Pirkle et al. (13,14) for a type I CSP, (*R*)-*N*-(10-undecanoyl)-α-(6,7-dimethyl-1-naphthyl)-isobutyl amine, and for a homologous series of solutes based on dinitrobenzoyl derivatives of 1-phenylalkyl amines. The experimental results indicated that two different interaction mechanisms were responsible for the formation of the solute/CSP complexes. One mechanism was based on dipole–dipole interactions that resulted in diastereomeric complexes where the (*R*)-solute/CSP complex was more stable, whereas the other was due to hydrogen-bonding interactions and produced a situation where the (*S*)-solute/CSP complex was more stable.

Two bonding mechanisms have also been proposed for the stereochemical resolution of enantiomeric amides and anilides on a type I CSP based on (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine (15–18). Both the mechanisms are based on dipole-dipole interactions between the solute and CSP. The difference arises from the positioning of the solutes relative to the CSP within the solute/CSP complexes. Pirkle and McCune (15) labeled the two possibilities "head to head" and "head to tail" dipole stacking.

Although multiple chiral recognition processes can occur on most CSPs, they take place within the context of a single class of the bonding interactions described above, that is, type I–V CSPs. Thus, the general guidelines presented in this chapter can be used to select the appropriate CSP as a starting point for enantioselective separations with the caveat that the enantiomeric elution order cannot be assumed even within a homologous series. The only exceptions are the "mixed-mode" cyclodextrin CSPs created by Armstrong et al. (19). Depending on the mobile phase, these CSPs operate either as a type I (hexane:isopropanol) or type III (aqueous) CSP.

III. TYPE I CSPs

Type I CSPs comprise the largest section of commercially available HPLC-CSPs, and at the present time, 28 are marketed (see Table 1). The majority of these phases are based on amino acid derivatives and contain functional

TABLE 1 Commercially Available Type I CSPs

Chiral stationary phase	Chiral selector
(D)-Phenylglycine	(*R*)-*N*-(3,5-Dinitrobenzoyl)phenylglycine
(L)-Phenylglycine	(*S*)-*N*-(3,5-Dinitrobenzoyl)phenylglycine
(D)-Phenylglycine (ionic)	(*R*)-*N*-(3,5-Dinitrobenzoyl)phenylglycine
(D,L)-Phenylglycine	(*R*,*S*)-*N*-(3,5-Dinitrobenzoyl)phenylglycine
(D)-Leucine	(*R*)-*N*-(3,5-Dinitrobenzoyl)leucine
(L)-Leucine	(*S*)-*N*-(3,5-Dinitrobenzoyl)leucine
(D)-N2N-Naphthylalanine	(*R*)-*N*-(2-Naphthyl)alanine
(L)-N2N-Naphthylalanine	(*S*)-*N*-(2-Naphthyl)alanine
(D,L)-N2N-Naphthylalanine	(*R*,*S*)-*N*-(2-Naphthyl)alanine
(S)-N1N-Naphthylleucine	(*S*)-*N*-(1-Naphthyl)leucine
α-Burke 1	(*R*)-Dimethyl-*N*-3,5-dinitrobenzoyl-α-amino-2,2-dimethyl-4-pentyl phosphonate
β-Gem 1	(*S*,*S*)-*N*-3,5-Dinitrobenzoyl-3-amino-3-phenyl-2-(1,1 dimethylethyl)propanoate
(*tert*)-Buc-(S)-leucine	*N*-(*tert*-Butylaminocarbonyl)-(*S*)-leucine
Chyrosin-A	(*S*)-*N*-(3,5-Dinitrobenzoyl)tyrosine-O-(2-propen-1-yl) butylamide
OA-1000	(*S*)-(1-Naphthyl)ethylaminoterephthalic acid
OA-2200	(1*R*,3*R*)-*trans*-Chrysanthemoyl-(*R*)-phenylglycine
OA-2500	(*R*)-*N*-(3,4-Dinitrobenzoyl)-1-naphthylglycine
OA-3000	*N*-(*tert*-Butylaminocarbonyl)-(*S*)-valine
OA-3100	*N*-3,5-Dinitrophenylaminocarbonyl-(*S*)-valine
OA-3200	*N*-3,5-Dinitrophenylaminocarbonyl-(*S*)-(*tert*)-leucine
OA-4000	(*R*)-*N*-(1-Naphthyl)ethylaminocarbonyl-(*S*)-valine
OA-4100	(*S*)-*N*-(1-Naphthyl)ethylaminocarbonyl-(*S*)-valine
OA-4400	(*S*)-(1-Naphthyl)ethylaminocarbonyl-(*S*)-proline
OA-4500	(*R*)-(1-Naphthyl)ethylaminocarbonyl-(*S*)-proline
OA-4600	(*R*)-(1-Naphthyl)ethylaminocarbonyl-(*S*)-(*tert*)-leucine
OA-4700	(*S*)-(1-Naphthyl)ethylaminocarbonyl-(*S*)-(*tert*)-leucine
(R)-Naphthylethylurea	(*R*)-1-(Naphthyl)ethylurea
(S)-Naphthylethylurea	(*S*)-1-(Naphthyl)ethylurea

groups that provide the interaction sites between the solute and CSP. These sites can include π-π interaction sites provided by an aromatic π-base (a naphthalene moiety) or aromatic π-acid (3,5-dinitrobenzene), and a hydrogen-bonding and/or dipole interaction site provided by an amide, urea, or ester moiety.

A. Solute Structure

A limiting factor in the use of these CSPs is the fact that the formation of the solute/CSP complex is dependent on the existence of complimentary interaction sites on the solute. However, this is not a problem with a wide variety of enantiomeric compounds. Type I CSPs have been used to stereochemically resolve alkyl carbinols, aryl-substituted hydantoins, lactams, succinimides, phthalides, sulfoxides, and sulfides (20).

If the interaction sites are not present in the solute, they must be added through derivatization. When derivatization is required, the standard approach is to convert an alcohol, amine, or carboxylic acid to an aromatic amide, urea, or carbamate. For example, amphetamine was resolved as its naphthoyl amide (21), a series of α-methylaryl acetic acid antiinflammatory agents were resolved after conversion to their 1-naphthalenemethylamides (22), and aliphatic alcohols can be resolved as 3,5-dinitro-carbamates (23). Other derivatives such as oxazolidones (24) and oxazolidines (25) have also been used.

The situation often occurs where more than one derivative can be used to resolve a particular compound. For example, racemic ephedrine has been successfully resolved on type I CSPs as an oxazolidine (25), a naphthylurea (26), and a naphthylcarbamate (27). With such a wide range of possibilities, the choice of derivative can be based on either the availability of a particular type I CSP or the availability of various derivatizing agents.

B. Mobile Phases

The type I CSPs are most often used with nonpolar mobile phases composed of hexane and an alcoholic polar modifier such as 2-propanol or dichloromethane:ethanol. These mobile phases are used to optimize the polar attractive interactions between the solute and CSP.

The effect of the structure, type, and concentration of the polar mobile phase modifier (mpm) on retention (k′) and stereoselectivity (α) has been studied by a number of investigators (28–30). In general, an increase in the steric bulk of the mpm results in an increase in the observed enantioselectivity. An example of these studies is the work reported by Zief et al. (28) in which the solute was 2-trifluoro-1-(9-anthryl)ethanol and the mobile phases were composed of hexane and either ethanol, 2-propanol, or *t*-butanol as the mpm. Of the three alcohols tested, the use of *t*-butanol as

the mpm gave the best stereoselectivity, α = 1.62 vs. 1.56 for 2-propanol and 1.33 for ethanol.

The authors concluded that an increase in the bulk of the alcohol enhances the ability of the solute enantiomers to displace the modifier from the CSP that stabilizes the diastereomeric solute/CSP complexes. The enhanced stability of the two solute/CSP complexes magnifies the energy differences between them, resulting in an increase in the observed enantioselectivity. A similar effect can be produced by lowering the temperature of the chromatographic system (31).

Type I CSPs can also be used under supercritical fluid chromatographic (SFC) conditions. Macaudiere et al. (30) reported on the resolution of a series of enantiomeric amides using subcritical CO_2 modified with an alcohol. The results of the study also indicated that at constant k′, the stereo-selectives are greater for alcohols with large steric hindrance close to the hydroxyl moiety.

Type I CSPs have also been used with aqueous mobile phases. Pirkle et al. (32) have reported on the resolution of *N*-(3,5-dinitrobenzoyl) derivatives of α-amino acids and 2-aminophosphonic acids on an (*R*)-*N*-(2-naphthyl)-alanine-derived CSP using a mobile phase composed of methanol-aqueous phosphate buffer. The utility of achiral alkyltrimethylammonium ion-pairing reagents was also investigated. Other examples include the following: (1) The recently commercialized α-Burke 1 CSP resolves the enantiomers of a number of underivatized β-blockers using an ethanol-dichloromethane-ammonium acetate mobile phase (33); (2) an (*R*)-1-naphthylethylurea CSP was used to resolve *N*-(3,5-dinitrobenzoyl)-substituted amino acids and 3,5-dinitrobenzoyl amide derivatives of ibuprofen, naproxen, and fenoprofen with acetonitrile-sodium acetate mobile phases (34).

C. Pharmaceutical Applications

The type I CSPs have been used to stereochemically resolve a vast number of compounds (9–11). However, most of the pharmaceutical applications involve bulk substances and only a relatively small number in vivo or in vitro studies. Some examples of the use of type I CSPs metabolic and pharmacokinetic studies are the following:

1. "The determination of (*R*)- and (*S*)-glutethimide and the corresponding 4-hydroxyglutethimide metabolites in human serum and urine using a Pirkle-type chiral stationary phase" (35). In this study, a validated assay was developed using an (*S*)-*N*-(3,5-dintrobenzoyl)leucine CSP and gradient elution with hexane:isopropanol:acetonitrile mobile phases.

2. "High performance liquid chromatographic method for the analysis of debrisoquine and its *S*-(+)- and *R*-(−)-hydroxy metabolites in

urine" (36). An assay was developed and used in the study of metabolic phenotyping using an (*R*)-*N*-(3,5-dinitrobenzoyl)-phenylglycine CSP and a mobile phase composed of hexane:ethanol:*t*-butyl methyl ether.

3. "Species differences in the generation of the chiral sulfoxide metabolite of albendazole in sheep and rats" (37). The chiral metabolites of the prochiral anthelmintic drug albendazole were determined on an (*S*)-*N*-(3,5-dinitrobenzoyl)-tyrosine-*O*-(2-propen-1-yl) *n*-butylamide chiral stationary phase using a mobile phase composed of hexane:ethanol.

4. "The determination of the enantiomers of mefloquine in plasma and whole blood using a coupled achiral-chiral HPLC system" (38). A

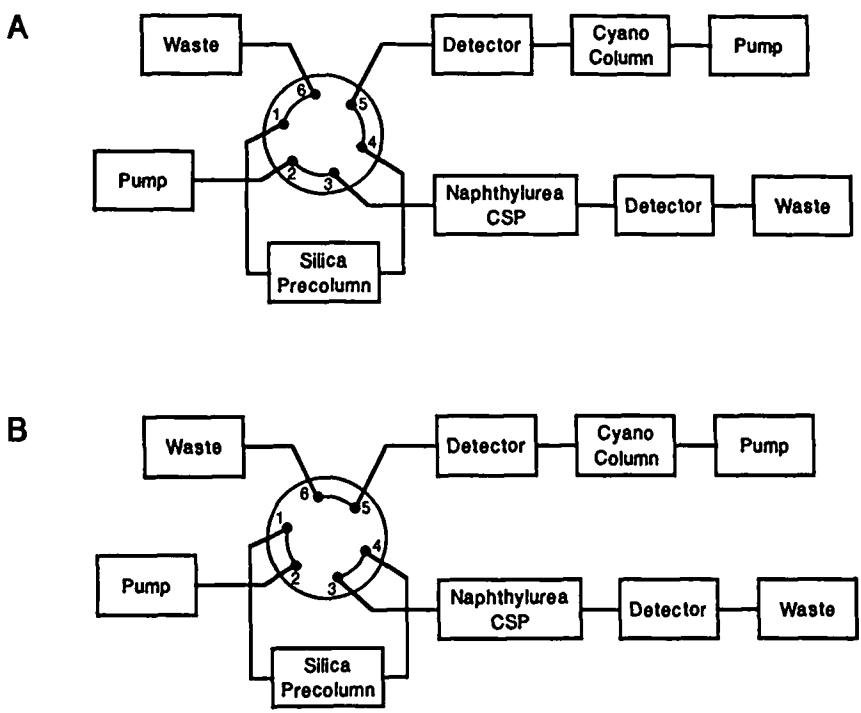


FIGURE 2 The chromatographic system used for the pharmacokinetic study of the enantiomers of mefloquine (MFQ), where A = system configuration used to direct the column eluent containing MFQ on the silica precolumn after separation from metabolites and other interfering compounds, and B = system configuration used to determine total MFQ concentrations on the achiral cyano column before switching and the enantiomeric composition of MFQ on the naphthylurea CSP after switching. [From Gimenez (38).]

coupled achiral-chiral HPLC system was developed and validated for the determination of mefloquine (MFQ) in plasma and whole blood. The MFQ was separated from an interfering component in the biological matrix and quantified on a cyano-bonded phase and the enantiomeric composition was determined on an (*S*)-naphthylethylurea CSP. The two columns were connected by a switching valve equipped with a silica precolumn. The precolumn was used to concentrate the MFQ in the eluent from the achiral column before backflushing onto the CSP. The analytical system is presented in Fig. 2.

IV. TYPE II CSPs

Cellulose is a highly crystalline polymer composed of D-β-glucose units that are arrayed in chains with a polar axis and directionality (39). The two major crystalline forms of this polymer are native cellulose (form I) and regenerated or mercerized cellulose (form II). The present view is that, for cellulose I, all the chains in a crystal point in the same direction, that is, they are parallel, whereas for cellulose II, the chains in adjacent layers of a crystal are antiparallel (39).

Cellulose is also a chiral polymer consisting of chiral subunits, D-β-glucose, a number of chiral environments created within the polymer including the areas (or cavities) formed between adjacent glucose units, and the spaces (or channels) between the polysaccharide chains. Chiral recognition can take place through attractive interactions between the solute and the D-β-glucose units or by inclusion into the cavities or channels. Both these interactions take place on the type II CSPs. The commercially available type II CSPs are listed in Table 2.

A. Microcrystalline Cellulose I: Cellulose Triacetate Derivative

Microcrystalline cellulose I is an excellent CSP for some forms of chromatography, such as paper chromatography. However, it lacks sufficient mechanical strength to withstand the pressures associated with HPLC and, thus, cannot be used with this technique. This problem was overcome by Hesse and Hagel (40) through the synthesis of a triacetyl derivative of cellulose I, CTA-I, which can be used in HPLC.

CTA-I can be used as an analytical and a preparative support and has been employed for the preparation of various racemic drugs or drug intermediates (9–11,41–43). The advantages of this phase have been delineated by Francotte and Wolf (41). They are (1) easy preparation of the phase; (2) practically unlimited source; (3) high loading capacity; (4) high chiral recognition; (5) broad applicability; (6) low production costs.

TABLE 2 Commercially Available Type II CSPs

Chiral stationary phase	Chiral selector
Type I—Cellulose	
CTA	Microcrystalline cellulose triacetate
CTB	Microcrystalline cellulose tribenzoate
Type II—Cellulose	
Chiralcel OA	Cellulose triacetate
Chiralcel OB	Cellulose tribenzoate
Chiralcel OC	Cellulose trisphenylcarbamate
Chiralcel OD	Cellulose tris(3,5-dimethylphenyl carbamate)
Chiralcel OF	Cellulose tris(4-chlorophyl carbamate)
Chiralcel OG	Cellulose tris(4-methylphenyl carbamate)
Chiralcel OJ	Cellulose tris(4-methyl-benzoate)
Chiralcel OK	Cellulose tricinnamate
Type II—Amylose	
Chiralpak AD	Amylose tris(3,5-dimethylphenylcarbamate)
Chiralpak AS	Amylose (*S*)-α-methylbenzylcarbamate

1. *Solute Structure*

Although CTA-I has been used to resolve a large number of compounds, the solutes appear to be limited to molecules that contain a phenyl group. This limitation may be due to the fact that it is the phenyl moiety that enters the chiral cavity to form the solute/CSP complex, as postulated by Hesse and Hagel (40). Francotte and Wolf (41) have recently reported the results of a study on the effect on enantioselectivity of *para*-substituents in the aromatic moieties of a series of compounds. They concluded that there are (41)

> . . . Multiple interaction sites in CTA-I, each exhibiting different chiral discriminations depending upon the local sterical and electronic configuration. In fact, this conclusion is quite conceivable considering the supramolecular structure of CTA-I, which consists of a lamellar arrangement of polysaccharide chains offering a multitude of structurally different cavities available to small intruding molecules.

Enantiomeric cyclic amides and imides, esters, ketones, δ- and γ-lactones, and alkyl-substituted indenes can be resolved on CTA-I without derivatization. Chiral alcohols can also be resolved without derivatization, but the best results are obtained when they are converted to esters. In general, *para*-nitrobenzoic esters are recommended if the hydroxyl group is directly attached to the stereogenic center; benzoate or *para*-bromobenzoate

derivatives if the hydroxyl moiety is not at the stereogenic center; and acetate derivatives for benzyl alcohols (8,43). Other recommended derivatives include benzyl amides for amines, *N*-benzyl amide-methyl esters for α-amino acids, and benzyl esters for carboxylic acids (8,43).

Chiral molecules with an axis of dissymmetry (atrop isomers) can also be stereochemically resolved on the CTA-I CSP. The resolution and pharmacological testing of the enantiomers of methaqualone illustrate this type of application (44). Methaqualone (Fig. 3) is a quinazolinone derivative that possesses hypnotic and anticonvulsive activities. The molecule exists in two enantiomeric forms, M(+) and M(−), due to the hindered rotation around the *N*-phenyl bond. Mannschreck et al. (44) were able to partially resolve this compound using the CTA-I CSP to optical purities of 0.75 for the (+) isomer and 0.68 for the (−) isomer. The anticonvulsive activity of the two enantiomers was evaluated by the mouse electroshock test, and the (−) isomer was found to be significantly more potent than the (+) isomer.

2. *Mobile Phases*

An aqueous or predominately aqueous mobile phase cannot be used with the CTA-I CSP. Anhydrous ethanol or ethanol modified with up to 5% water are the most commonly used mobile phases. Other alcohols such as methanol and 2-propanol can be used and dramatic changes can be seen with these solvents (8,43,45,46).

Koller et al. (45) investigated the effect of the structure of the alcohol on the retentions (k′) and enantioselectivities (α) for three enantiomeric solutes on the CTA-I CSP. In the series methanol, ethanol:water (96:4), 2-propanol, the k′ of each enantiomer increased through the series, whereas the effect on the observed α's varied. The authors concluded that the polarity of the eluents may not be the key to their elutropic strength. Instead, the elutropic strength of the solvent may be a function of the steric bulk of the alcohol. By binding to sites near or at the site of inclusion, the alcohol can affect the steric environment of the channels and cavities, thereby changing the fit on the solutes into these cavities. This, in turn, would alter both k′ and α.

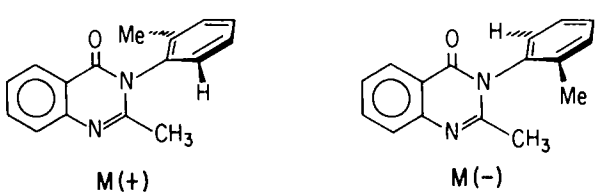


FIGURE 3 The enantiomers of methaqualone.

Nonpolar mobile phases composed of hexane modified with 2-propanol have also been used with the CTA-I CSP (43,45).

B. Microcrystalline Cellulose I: Cellulose Tribenzoate Derivative

Francotte and Wolf (46) have recently reported on the synthesis of a CSP based on tribenzoyl cellulose (TBC). The TBC CSP is composed of beads containing pure TBC and no inorganic carrier such as silica.

1. *Solute Structure*

This phase can be used in the preparative and analytical stereochemical resolution of a wide variety of compounds, including acetate derivatives of alcohols and diols; benzylic alcohol derivatives; *para*-substituted *d*-phenyl-*d*-valerolactones; 3,4-dihydro-2H-pyran-2-carboxylic acid derivatives; phenylvinylsulfoxide; *trans*-stilbene oxide (46).

2. *Mobile Phase*

The TBC CSP has been used with a mobile phase composed of hexane:2-propanol (90:10).

C. Microcrystalline Cellulose II: The Cellulosic Phases

Microcrystalline cellulose II, like the cellulose I form, does not possess enough mechanical strength to be used as an HPLC-CSP. Although its triacetate derivative, CTA-II, can be used as an HPLC support, it is not as useful a CSP as CTA-I (47). Ichida et al. (48) were able to improve on the properties of derivatized cellulose II by depolymerization, followed by coating on macroporous silica gel. In this manner, they created a number of new CSPs, the cellulosic CSPs, based on ester and carbamate derivatives of cellulose (see Table 2).

Amylose is another polysaccharide that has been used to create CSPs. Amylose is a D-α-glucose polymer with high helical content. Two amylose-based CSPs are commercially available (Table 2): amylose tris(3,5-dimethylphenylcarbamate), the AD CSP, and amylose (*S*)-α-methylbenzylcarbamate, the AS CSP.

The chiral recognition mechanisms that operate on the cellulosic CSPs have been studied by Wainer and co-workers (49,50). In one study, the chiral recognition of amides on the OB CSP was examined (49). The results indicated that the solute/CSP complexes formed between the OB CSP and the amide solutes were based on attractive hydrogen bonding, π-π, and dipole–dipole interactions. Chiral recognition within the solute/CSP complex was due to the differential inclusion (or fit) of the solute into a chiral cavity or ravine on the CSP. However, studies with aromatic alcohols

suggest that the inclusion of the aromatic moiety of the solute in a chiral cavity or ravine was the key step in the formation of the solute/CSP complex (50). Thus, it appears that the cellulosic CSPs use two different but related mechanisms.

1. *Solute Structure*

The type II CSPs have been used to resolve a wide variety of pharmacologically active compounds, both with and without derivatization (9–11). Some of the compounds that have been resolved without derivatization are hexobarbital (OA-CSP) (51), glutethimide (OB, OC, OK) (51), warfarin (OB, OC) (51), compounds containing a chiral sulfur atom (OB, OC) (52), verapamil (AD) (53), propranolol (OD) (54), and flurbiprofen (AD) (55). The resolution of the latter two compounds, propranolol, an α,β-amino-alcohol, and flurbiprofen, a carboxylic acid, is representative of new applications for the cellulosic CSPs that are primarily used with hexane: alcohol mobile phases. In the case of propranolol, the mobile phase was modified with *N,N*-dimethyloctyl amine, and when flurbiprofen was chromatographed, trifluoroacetic acid was used as the modifier.

Most solutes that can be resolved on the type II CSPs contain one or more aromatic rings or polar π-bonded groups such as carbonyl, sulfinyl, or nitro moieties (9–11). This reflects the two chiral recognition mechanisms discussed above in which hydrophobic moieties are required for inclusion in hydrophobic cavities or ravines and polar groups are required for attractive hydrogen-bonding, dipole, and π-π interactions. Aliphatic mono- or poly-functional alcohols that do not contain these interaction sites must usually be derivatized. In addition, amine and carboxylic acid moieties are often derivatized to improve the chromatographic efficiency of the CSPs. The resolution of the enantiomeric benzylamide derivatives of amphetamine is presented in Fig. 4.

2. *Mobile Phases*

The cellulosic CSPs are most often used with nonpolar mobile phases composed of hexane and a polar modifier such as 2-propanol. These mobile phases are used to maximize the attractive interactions between solute and CSP. Since these interactions contain a polar component, an increase in the polarity of the mobile phase, for example, a lager alcohol content, will reduce the retention and vice versa. These phases have also been used with aqueous mobile phases and a new reversed-phase version of the OD CSP is currrently being marketed (56).

The effect of the structure, type, and concentration of the polar mobile phase modifier (mpm) on retention (k′) and stereoselectivity (α) has been studied (57,58). Wainer et al. (57) chromatographed a series of enantio-

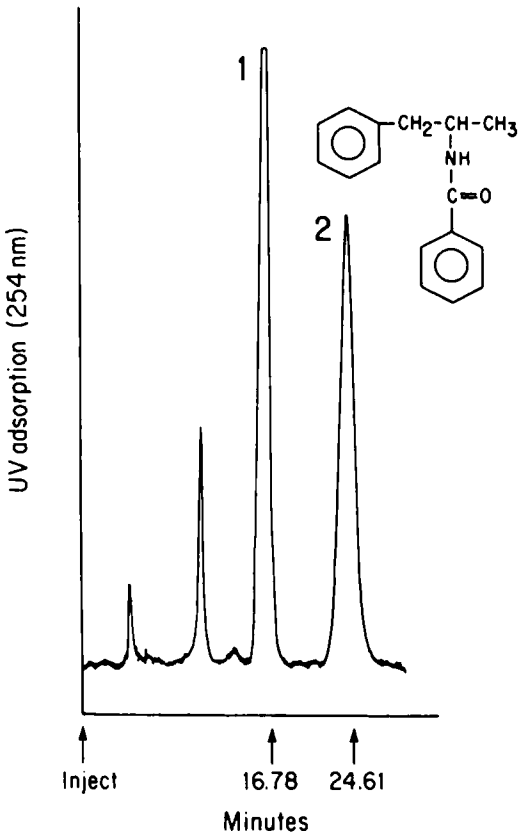


FIGURE 4 The chiral resolution of the benzylamide derivatives of (*R*)- and (*S*)-amphetamine on the OB CSP, where 1 = (*R*)-amphetamine, and 2 = (*S*)-amphetamine.

meric amides on the OB CSP using mobile phases composed of hexane and two homologous series of primary and secondary alcohols. The results of the study indicate that the alcoholic mpms compete with the solutes for achiral and chiral binding sites and that the steric bulk around the hydroxyl moiety of the mpm plays a role in this competition. Increased steric bulk tends to result in increased k′ and α. However, the results also suggest that the effect of the alcoholic mpms on stereoselectivity may also be due to binding to achiral sites near or at the chiral cavities of the CSP that alters the steric environment of these cavities. The latter conclusions are consistent with the observations reported by Koller et al (45) from experiments with the CTA-I CSP.

In a related experiment, Gaffney et al. (58) used the same CSP and mobile phases with three different enantiomeric solutes: phenyl vinyl sulfoxide, glutethimide, and 2-phenylpropanoic acid methyl ester. Reversals in the enantiomeric elution order were observed for the methyl ester solute associated with changes in the chain length of the mpm. The results of the study suggested that at least two chiral recognition mechanisms (attractive interactions and hydrophobic insertion) operate on the OB CSP and that the mpm can change the relative importance of these mechanisms. The results also point out the importance of determining the enantiomeric elution order after each alteration in the composition of the mobile phase.

3. *Pharmaceutical Applications*

The cellulosic CSPs have been used to stereochemically resolve a vast number of compounds (9–11). However, most of the pharmaceutical applications involve bulk substances and only a relatively small number in vivo or in vitro studies. Some examples of the use of cellulosic CSPs in metabolic and pharmacokinetic studies are the following:

1. "Direct enantiomeric separation of betaxolol with applications to the analysis of bulk drug and biological samples" (59). The enantiomers of betaxolol were resolved on the OD CSP using a mobile phase composed of hexane:2-propanol:diethylamine (87:13:0.05, v/v/v). This method was used to determine the concentration of the enantiomers of betaxolol after incubation of a racemic mixture of the compound with rat hepatocytes.

2. "Measurement of underivatized propranolol enantiomers in serum using a cellulose-tris(3,5-dimethylphenylcarbamate) high performance liquid chromatographic (HPLC) chiral stationary phase" (54). A method for the direct measurement of the enantiomers of propranolol in human serum was developed using the OD CSP and a mobile phase composed of hexane:2-propranol:*N*,*N*-dimethyloctylamine (92:8:0.01, v/v/v). The assay was validated for use in pharmacokinetic and metabolic studies and was subsequently used in the investigation of the effect of cimetidine on the metabolism and clearance of propranolol enantiomers (60).

3. "Analytical and preparative high-performance liquid chromatographic separation of the enantiomers of ifosfamide, cyclophosphamide and trofosfamide and their determination in plasma" (61). Three racemic anticancer drugs—ifosfamide (IFF), cyclophosphamide (CTX), and trofosfamide (TFF)—were resolved on the OD CSP and gram quantities of (*R*)-IFF and (*S*)-IFF were prepared for pharmacological testing. An analytical assay was also developed for the determination of the enantiomeric composition of IFF and CTX in plasma using achiral–chiral coupled column

chromatography. A racemic form of another CSP, the D,L- naphthylalanine CSP, was used as the achiral precolumn.

4. "Simultaneous direct determination of the enantiomers of verapamil and norverapamil in plasma using a derivatized amylose HPLC chiral stationary phase" (53). An AD CSP was used to determine the plasma concentrations of the enantiomers of verapamil and its major metabolite norverapamil. After a liquid–liquid extraction, the analytes were resolved on the amylose column using a mobile phase composed of heptane:2-propanol:ethanol, (85:7.5:7.5, v/v/v) modified with 1.0% triethylamine, a flow rate of 1.0 mL/min, and a column temperature of 30°C. The assay was validated for use in human pharmacokinetic studies. A representative chromatogram is presented in Fig. 5.

V. TYPE III CSPs

In type III CSPs, the solute enters into chiral cavities within the CSP to form inclusion complexes and the relative stabilities of the resulting diastereomeric complexes are based on secondary attractive (e.g., hydrogen-bonding) or steric interactions. The driving force for the insertion can be hydrophobic (cyclodextrin and polymethacrylate CSPs) or electrostatic (crown ether CSP). The commercially available CSPs based on these mechanisms are presented in Table 3.

A. Cyclodextrin-Based CSPs

Cyclodextrins (CD) are cyclic oligosaccharides composed of α-D-glucose units linked through the 1,4 position. The molecules are formed by the action of *Bacillus macerous* amylase on starch (62). The three most common forms of this molecule are α-, β-, and γ-cyclodextrin, which contain six, seven, and eight glucose units, respectively. Because of the α-D-glucose units, the cyclodextrin molecule has a stereospecific, toroidal (doughnut-shaped) structure. The interior cavity of the cyclodextrin molecule is relatively hydrophobic, and a variety of water-soluble and insoluble compounds can fit into it to form inclusion complexes (63). All three forms of the molecule have been attached to silica and successfully used as CSPs for the resolution of a variety of enantiomeric compounds as well as geometrical isomers such as *cis*- and *trans*-stilbene (64), structural isomers such as *m*- and *p*-nitroaniline (64), and sugar anomers (65).

The chromatographic utility of the CD CSPs has been expanded through the development of derivatized CD supports. The currently available derivatized cyclodextrin-based CSPs are listed in Table 3 and include acetylated (in α-, β-, and γ-CD forms); (*S*)- and (*R*,*S*)-hydroxypropyl ether (β-CD); (*S*)- and (*R*)-naphthylethyl carbamate (β-CD); 3,5-

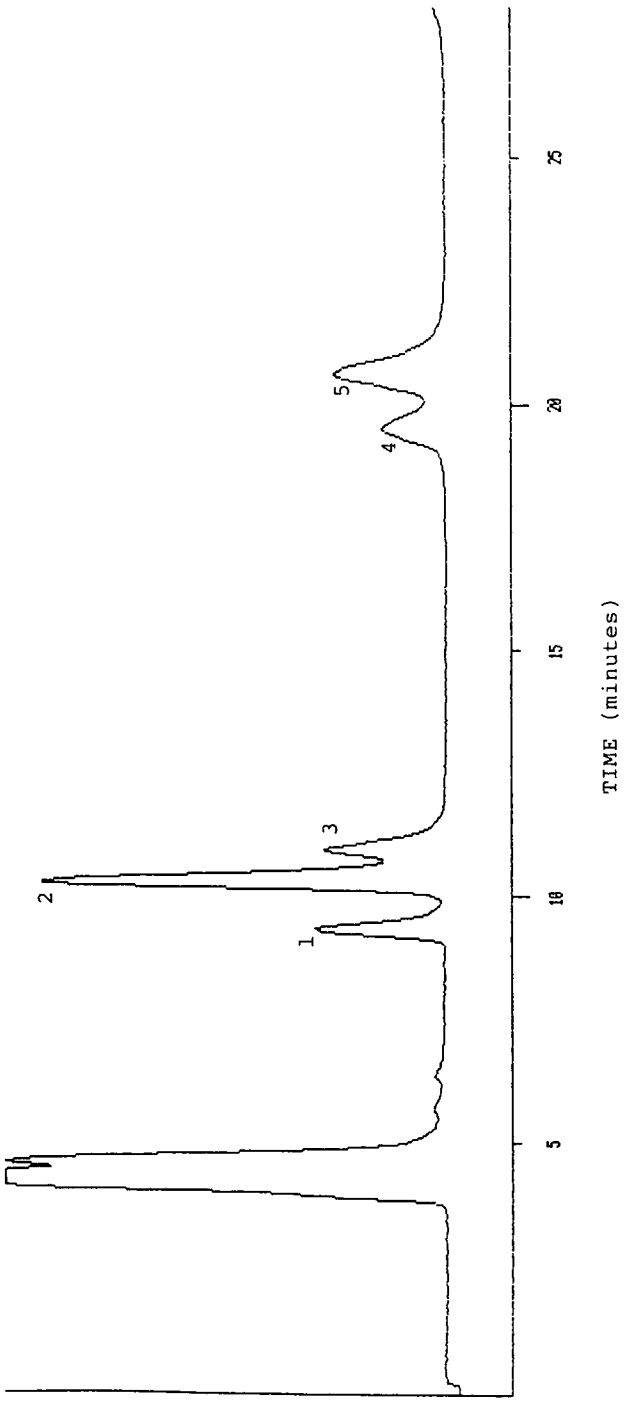


FIGURE 5 Chromatogram of plasma from a healthy volunteer 4 hr after the administration of 240 mg of a sustained-release formulation of racemic verapamil, where 1 = (*S*)-verapamil, 2 = (*R*)-verapamil, 3 = internal standard, 4 = (*S*)-norverapamil, 5 = (*R*)-norverapamil. [From Shibukawa and Wainer (53).]

TABLE 3 Commercially Available Type III CSPs

Chiral stationary phase	Chiral selector
Cyclodextrin	
Cyclobond I	β-Cyclodextrin
Cyclobond II	γ-Cyclodextrin
Cyclobond III	α-Cyclodextrin
Cyclobond I Ac	β-Cyclodextrin acetylate
Cyclobond I SP	β-Cyclodextrin (*S*)-hydroxypropyl ether
Cyclobond I RSP	β-Cyclodextrin (*R*,*S*)-hydroxypropyl ether
Cyclobond I SN	β-Cyclodextrin (*S*)-naphthylethyl carbamate
Cyclobond I RN	β-Cyclodextrin (*R*)-naphthylethyl carbamate
Cyclobond I DMP	β-Cyclodextrin 3,5-dimethylphenyl carbamate
Cyclobond I PT	β-Cyclodextrin *para*-toluoyl ester
Cyclobond II Ac	γ-Cyclodextrin acetylate
Cyclobond III Ac	α-Cyclodextrin acetylate
Synthetic polymers	
Chiralpak OT (+)	Poly(triphenylmethyl methacrylate)
Chiralpak OP (+)	Poly(2-pyridyldiphenylmethyl methacrylate)
ChiraSpher	Poly-*N*-acryloyl-(*S*)-phenylalanine ethylester
Crown ether	
CR (+)	Chiral 18-crown-6 ether, structure not reported
CR (−)	Chiral 18-crown-6 ether, structure not reported

dimethylphenyl carbamate (β-CD); and *para*-toluoyl ester (β-CD). Unlike the standard CD CSPs, the derivatized CD CSPs can be used with both reverse and normal phases and can operate with a variety of chiral recognition mechanisms. In fact, the (*S*)- and (*R*)-naphthylethyl carbamate-CD CSPs have been described as mixed-mode CSPs and will be discussed in Sec. VI.

1. Solute Structure

To achieve chiral separations on CD CSPs, a part or all of the solute molecules must enter the cyclodextrin cavity. In most cases, the solutes that are successfully resolved contain an aromatic moiety at or adjacent to the stereogenic center, and it is the aromatic portion of the molecule that inserts itself into the chiral cavity of the cyclodextrin molecule to form the inclusion complex (66,67). The size of the aromatic moiety and cyclodextrin cavity determine which CD CSP will form the best inclusion complex, and single aromatic rings fit best in the α-CD, naphthylrings in the β-CD and aromatic system larger than naphthyl in the γ-CD (68). In addition to the

aromatic system, the solutes successfully resolved on the CD CSPs also contain either a hydrogen-bonding group or an additional π system (carbonyl moiety or aromatic ring) at the stereogenic center (68). The structural requirements for the derivatized CD CSPs often differ and are discussed below in Sec. IV.

2. *Mobile Phases*

The most common mobile phases used with the CD CSPs are composed of water modified with methanol, ethanol, or acetonitrile. Buffers such as 0.5–1% triethylammonium acetate and phosphate can also be used and tend to improve efficiency and reduce retention of anionic and cationic solutes. As a general rule, the greater the aqueous content of the mobile phase, the greater the retention and stereoselectivity.

Normal phases composed of hexane modified with 2-propanol have been successfully used with the 3,5-dimethylphenyl carbamate and *para*-toluoyl ester forms of the CD CSP. The β-CD CSP has been used in subcritical fluid chromatography with a mobile phase composed of carbon dioxide modified with methanol, ethanol, or 2-propanol (69). Under these conditions, racemic amides and phosphine oxides were enantiomerically resolved.

3. *Pharmaceutical Applications*

The CD CSPs have been used to stereochemically resolve a vast number of compounds (9–11). However, most of the pharmaceutical applications involve bulk substances and only a relatively small number in vivo or in vitro studies. Some examples of the use of CD CSPs in metabolic and pharmacokinetic studies are the following:

1. "The stereoselective metabolism of phenytoin in children" (70). McClanahan and Maguire have reported on a study of the metabolism of phenytoin that is widely prescribed for the treatment of epilepsy, 4-hydroxyphenyl metabolites of phenytoin (Fig. 6). Although phenytoin itself

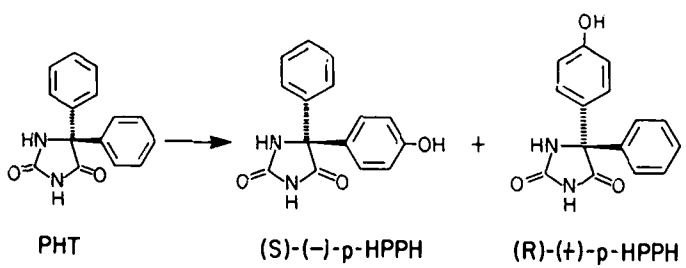


FIGURE 6 The metabolism of phenytoin to 5*S*-(−)- and 5*R*-(+)-5-(4-hydroxyphenyl)-5-phenylhydantoin, e.e., (*S*)-(−)-*p*-HPPH and (*R*)-(+)-*p*-HPPH, respectively.

is not a chiral compound, the 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH) metabolites, *S*-(−)-*p*-HPPH and *R*-(+)-*p*-HPPH, are enantiomeric because of the difference between the hydroxylated and nonhydroxylated phenyl rings.

The stereoselective metabolism of the drug was monitored using a β-CD CSP and mobile phase of water/acetonitrile (80:20) (see Fig. 7). The chronic treatment of 22 pediatric patients with phenytoin alone or in combination with phenobarbital was monitored using this method, and it was found that the *S*-(−) metabolite was favored over the *R*-(+) metabolite by a ratio of approximately 9:1.

2. "Enantiomeric analysis of a new antiinflammatory agent in rat plasma using a chiral β-cyclodextrin stationary phase" (71). The *R,R*; *S,S* enantiomers of the investigational antiinflammatory agent *trans*-6,6a,7,10,10a,11-hexahydro-8,9-dimethyl-11-oxodibenz[b,e]-oxepin-3-acetic acid (HOA) were resolved on the β-CD CSP after extraction from rat plasma. The mobile phase was a mixture of 0.05 *M* potassium dihydrogenphosphate (pH 5.3) and methanol (35:65, v/v); the flow rate was 1.0 mL/min; and the temperature was 22 ± 1°C. The results of the study indicated that the inactive (−) enantiomer was cleared from the plasma more rapidly than the active (+) isomer.

3. "Determination of drug enantiomers in biological samples by coupled column liquid chromatography and liquid chromatography-mass spectrometry" (72). The direct separation and determination of the enantiomeric composition of terbutaline in human plasma at the nmol/L level

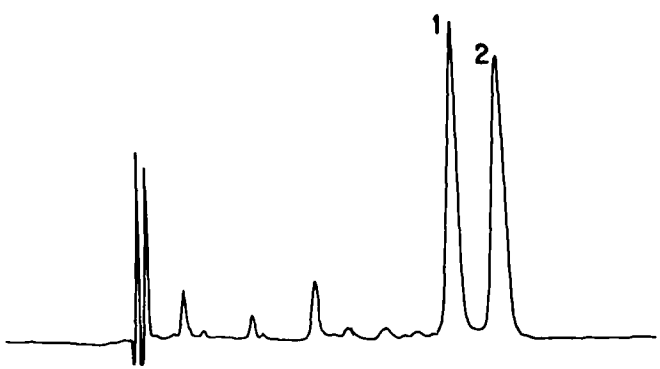


FIGURE 7 The enantioselective resolution of (*S*)-(−)-*p*-HPPH and (*R*)-(+)-*p*-HPPH on a β-CD CSP, where 1 = (*R*)-(+)-*p*-HPPH and 2 = (*S*)-(−)-*p*-HPPH. See Fig. 6 and McClanahan and Maguire (70).

were accomplished by both coupled column liquid chromatography and coupling a β-CD CSP to a mass spectrometer.

In the first approach, an extracted plasma sample was initially chromatographed on a phenyl column and the terbutaline separated from interfering matrix components. The eluent containing the terbutaline was directed onto the β-CD CSP through a switching valve. The mobile phase used on the phenyl column [0.01 *M* ammonium acetate (pH 4.6)] was not strong enough to elute the terbutaline from the β-CD CSP and, therefore, significant peak compression was accomplished on the chiral phase. The enantiomers were then resolved and eluted from the β-CD CSP using a mobile phase containing methanol:0.05 *M* ammonium acetate (pH 6.0) (10:90, v/v). With this method, nmol/L concentrations of both enantiomers were detected and quantified.

In the second approach, the β-CD CSP was connected directly to the mass spectrometer using a thermospray interface. A mobile phase of 0.1 *M* ammonium acetate (pH 5) and flow rate of 1.2 mL/min were employed during the analysis. By using deuterium-labeled racemic terbutaline as the internal standard, linear calibration curves were obtained down to 4 pmol.

B. Polymethacrylate CSPs

A number of CSPs have been developed that are based on optically active synthetic helices formed by the asymmetric polymerization of methacrylate monomers. These polymers have been formed using either chiral monomers such as (*S*)-acryloylphenyl-alanine (73) and *N*-methylacryloyl-(*S*)-cyclohexylethylamine (73), or achiral monomers such as triphenyl methacrylate (74) and diphenyl-2-pyridyl-methyl methacrylate (74). In the latter case, the polymers were prepared using chiral cation catalysts including (−)-spartene-butyllithium and (+)-6-benzylsparteine-butyllithium complexes (74). The commercially available forms of these CSPs are listed in Table 3.

1. *Solute Structure*

The formation of the solute-CSP diastereomeric complexes in these CSPs usually requires the insertion of an aromatic moiety on the solute into the chirality of the optically active polymer. Thus, the solutes should contain an aromatic moiety near or at the stereogenic center. Enantiomeric molecules containing the necessary aromatic moiety and one of the following functionalities have been resolved on these CSPs: alcohol, amide, ester, ether, and ketone (9–11).

If the solute does not contain the appropriate aromatic moiety, this functionality can often be added through derivatization. Chiral aliphatic

amines have been resolved after conversion to benzamide derivatives; enantiomeric 1,2- and 1,3-dicarboxylic acids have been resolved as benzyl esters or benzamides; and racemic alkyl alcohols, 1,2- and 1,3-diols, and some sugars have been resolved as benzoate esters (9–11).

Solutes containing asymmetric sulfur atomes or asymmetric phosphorous atoms can be resolved with or without an aromatic moiety in the molecule. For example, the anticancer drug ifosfamide, an oxazaphosphorane not containing an aromatic moiety, has been resolved on this type of CSP (73). In addition, these are excellent CSPs for the resolution of enantiomeric molecules with an axis of dissymmetry (atrop isomers) (9–11).

2. *Mobile Phases and Temperature*

The most common mobile phases used with these CSPs are methanol modified with 2-propanol or mixtures of toluene and dioxane. The CSPs derived from triphenyl methacrylate and diphenyl-2-pyridyl-methyl methacrylate operate best at a temperature of 15°C or less.

C. Chiral Crown Ethers

Crown ethers are synthetic macrocyclic polyethers that can form selective complexes with various cations. An 18-crown-6 ether is illustrated in Fig. 8, where 18 indicates the total number of atoms in the polyether ring and 6 the number of oxygen atoms. Chiral crown ethers have been synthesized by

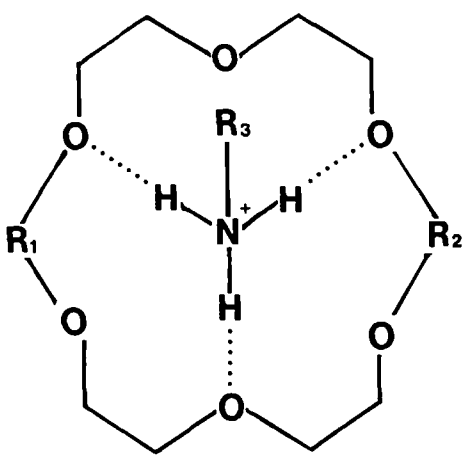


FIGURE 8 The structure of an 18-crown-6 ether ammonium complex, where R_1 and R_2 = the chiral elements of the crown ether and R_3 = rest of the included molecule.

adding substituents to the ether ring that hinder its rotation about an axis of dissymmetry, that is, producing atrop isomers, and have been immobilized on chromatographic supports, yielding CR CSPs (75).

1. *Solute Structure*

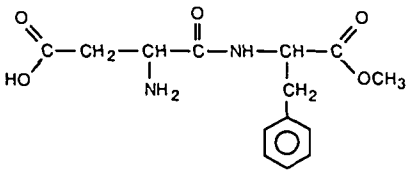
Compounds based on the 18-crown-6 ring have the ability to form stable complexes with potassium and ammonium ions (Fig. 8). This is a function of the cavity size and the electrostatic interactions between the charged ion and free electrons on the oxygen atoms. Thus, the solutes that can be successfully resolved on the CR CSP must contain a primary amine moiety at or near the stereogenic center. Steric bulk, especially aromatic moieties, that are close to the stereogenic center also enhance chiral recognition. Given these requirements, the CR CSP is ideally suited for the stereochemical resolution of free and *O*-derivatized amino acids, small peptides, and many pharmaceutically interesting compounds containing primary amine moieties.

2. *Mobile Phase*

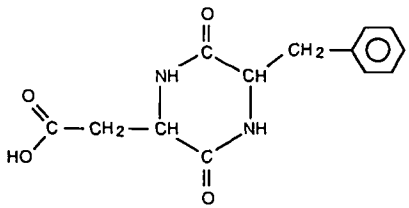
The mobile phases used on the CR CSP are limited to perchloric acid (usually around 10 *mM*) modified with small amounts of organic modifiers such as alcohols or acetonitrile. Although the mobile phases are limited, retention (k′) and stereoselectivity (α) can be altered by varying the pH between 1 and 3 and adjusting the temperature. Udvarhelyi and Watkins (75) have investigated these parameters on the chromatography of a series of racemic phenylglycines. The results of this study indicated that when the pH was raised from 1–3, the k′s decreased dramatically, as did most of the observed α′s. However, the magnitude of the change in α, as well as the direction, was a function of solute structure. The effect of temperature was consistent throughout the series and an increase from 5–25°C resulted in a decrease in k′ and α.

3. *Applications*

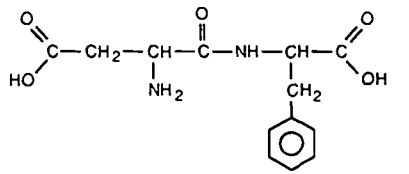
At the present time, the reported applications of the CR CSP have been limited to the separation of amino acids and some dipeptides as bulk substances. One example of the use of the CR CSP in a complex matrix was the direct stereochemical resolution of aspartame stereoisomers and their degradation products in coffee and diet soft drinks (76). Aspartame (*N*-DL-α-aspartyl-DL-phenylalanine methyl ester) is a dipeptide whose L,L-isomer is a low-calorie sweetener sold under the name NutraSweet. The structure of aspartame and its major degradation products are presented in Fig. 9 and the stereochemical separation of these compounds on the CR CSP in Fig. 10A. The resolutions were accomplished using a mobile phase



Aspartame (APM)



Diketopiperazine (DKP)



Aspartyl-phenylalanine (Asp-Phe)

FIGURE 9 The structure of aspartame and its major degradation products.

composed of aqueous perchloric acid (pH 2.8) modified with 1.5% 2-propanol, a flow rate of 0.6 mL/min, and a temperature gradient (10–40°C). The application of this method to the determination of L,L-aspartame and its major decomposition product, L,L-diketopiperazine, in a diet cola is presented in Fig. 10B.

VI. MIXED-MODE CSPs

A mixed-mode, or multimodal, CSP is one that has been designed to operate with two or more different types of chiral recognition mechanisms. Armstrong et al. (19,78) have developed such a CSP by reacting β-cyclodextrin with (*R*)- or (*S*)-naphthyl-ethylisocyanate. The resulting naphthylethylcarbamate derivative of β-cyclodextrin, the NEC-β-CD CSP, can function as either a type I CSP or type III CSP.

When the NEC-β-CD CSP is used with a nonpolar mobile phase such as hexane modified with 2-propanol, the solute/CSP complexes are formed

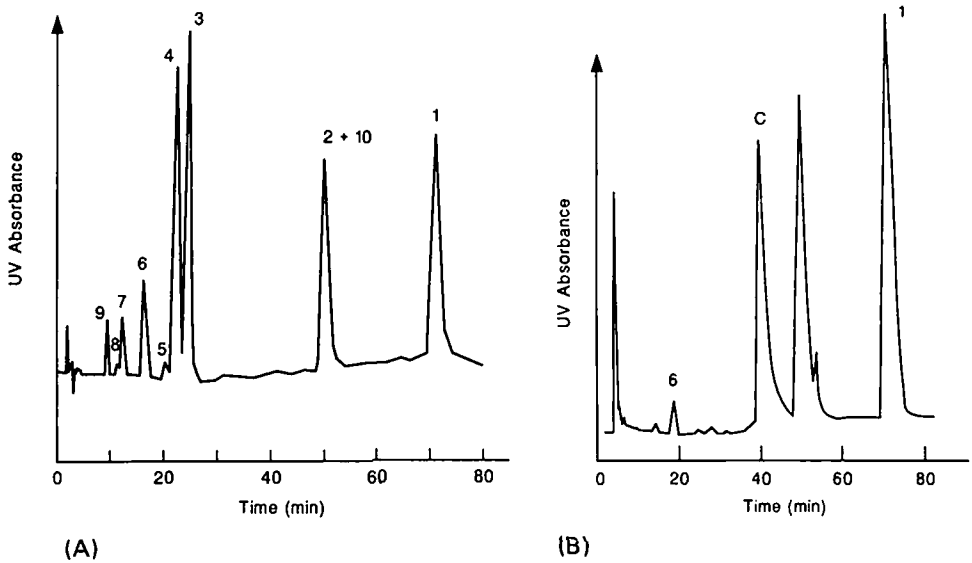


FIGURE 10 (A) Chromatogram of a 1-day-old synthetic mixture of the four stereoisomers of APM (0.1 mM of each isomer), where 1 = L,L-APM; 2 = L,D-APM; 3 = D,D,-APM; 4 = D,L-APM; 5 = L-Asp-D-Phe; 6 = D,D-DKP + L,L,-DKP; 7 = D,L-DKP + L,D-DKP; 8 = D-Asp-D-Phe; 9 = D-Asp-L-Phe; 10 = L-Asp-L-Phe. Column: Crownpack CR(+), Daicel, 150 × 4 mm i.d.; injection loop: 20 μl; mobile phase: aqueous $HClO_4$ pH = 2.8/2-PrOH, (98.5:1.5), (v:v); flow rate: 0.6 ml/min.; temperature gradient: 0 to 26 min, T° = 10°C; 26 to 70 min, linear gradient from T° = 10°C to T° = 40°C; detector: = 210 nm, RS = 0.02 AUFS, ATT = 2^5. (B) The determination of L,L-aspartame and its decomposition products in a diet cola, where 1 = L,L-aspartame; 6 = L,L-diketopiperazine; C = caffeine. [From Motellier and Wainer (76).]

by attractive interactions, hydrogen bonding, π-π, dipole stacking, etc., between the solute and CSP, type I interactions. These interactions take place between the naphthylethylcarbamate moiety and complimentary functionalities on the solute. As with the other type I CSPs, if the solutes do not contain the appropriate interaction sites, they must be added through derivatization. Thus, nonaromatic amines and alcohols, amino acids, and carboxylic acids should be converted to their dinitrophenyl derivatives before chromatography on the NEC-β-CD CSP.

For solutes meeting the requirements for use with a standard or derivatized β-CD CSP, the NEC-β-CD CSP can be used with aqueous mobile phases modified with acetonitrile or another organic modifier. Since chiral recognition is a function of the interaction of the solute with the chirality of the CD as a whole, the configuration about the stereogenic

center of the naphthylethyl moiety can affect the observed stereochemical resolutions. Thus, the (*R*)-NEC-β-CD and (*S*)-NEC-β-CD CSP can display different enantioselectivities.

A number of solutes have been stereochemically resolved on this multimodal CSP, using both chiral recognition mechanisms (19), and a flowchart has been developed to help in the application of the NEC-β-CD CSP (19). This chart is presented in Fig. 11.

VII. TYPE IV CSPs

Chiral ligand-exchange chromatography is based on the formation of diastereomeric ternary complexes that involve a transition metal ion (M), usually copper II; a single enantiomer of a chiral molecule (L), usually an amino acid; and the enantiomers of the racemic solute (*R* and *S*). The diastereomeric mixed chelate complexes formed in this system are represented by the formulas L-M-*R* and L-M-*S*. When these complexes have different stabilities, the less stable complex is eluted first, and the enantiomeric solutes are separated.

Chiral ligand-exchange chromatography can be accomplished using an achiral chromatographic support and a mobile phase containing M and L. The efficiency and reproducibility of the chromatography can be improved through the immobilization of the chiral ligand (L) on a chromatographic support that produces the type IV CSPs. The commercially available type IV CSPs are presented in Table 4.

A. Solute Structure

The solutes resolved by the Type IV CSPs must be able to form coordination complexes with transition metal ions. This limits the classes of compounds to α-amino acids and mono- and dicarboxylic acids containing an α-hydroxyl moiety. The most common solutes resolved on these CSPs are underivatized amino acids, although a number of derivatized amino acids can be resolved, including *N*-acetyl, amide, *N*-carbamoyl, *N*-carbobenzoxy, and hydantoin derivatives, as well as complex amino acids such as the biochemical modulator BSO (79).

B. Mobile Phases

The Type IV CSPs are used with aqueous mobile phases that contain copper(II) sulfate (CS). The usual starting concentration of CS is 0.25 *mM* and retention times can be manipulated by increasing or decreasing the CS concentration; an increase in CS concentration usually shortens retention, whereas a decrease has the opposite effect. Concentrations as low as 0.05 *mM* and as high as 20.0 *mM* have been used.

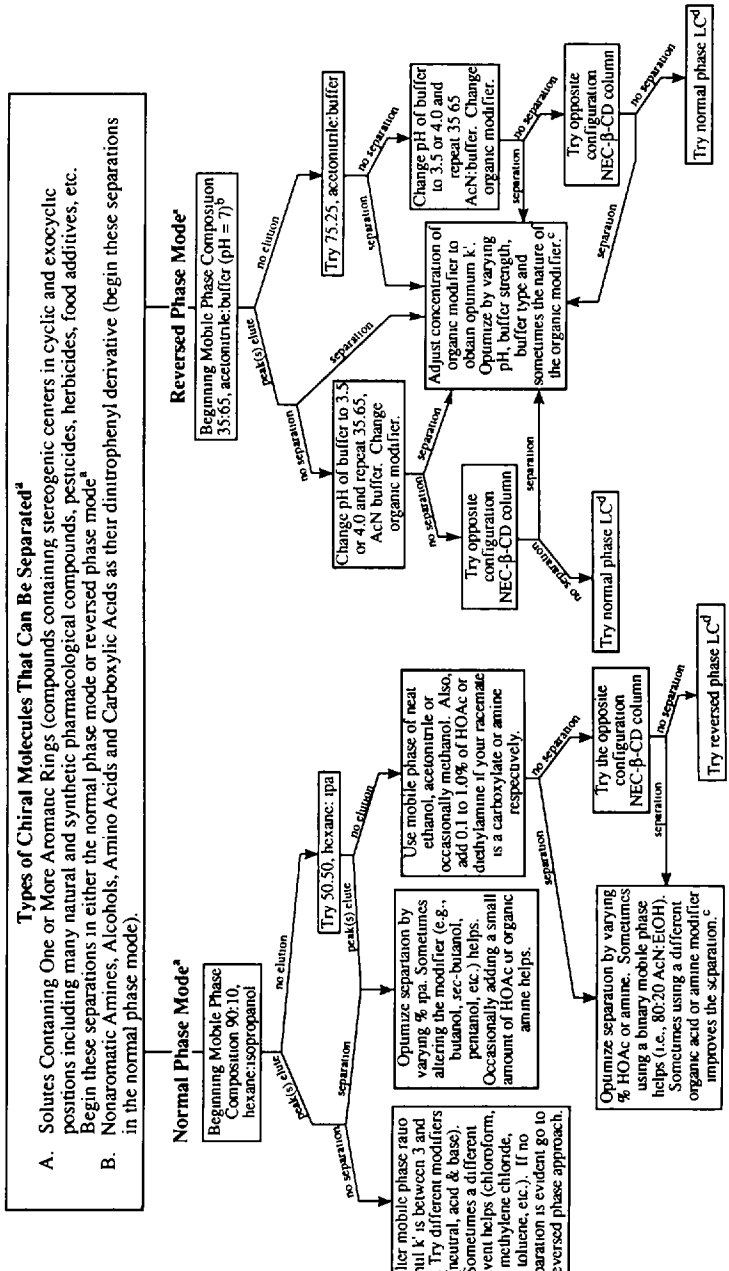


FIGURE 11 Basic flowchart for the use and optimization of the NEC-β-CD multimodal CSP. [a]Before choosing whether to begin the separation in the normal phase or reversed phase mode, one must know the solubility and stability of the racemate in these mobile phases. For example, there is no sense in trying a normal phase separation if the compound is insoluble in that mobile phase. [b]Recommended buffers include ammonium nitrate and triethylammonium acetate. [c]Two additional things can be done to optimize the separation. One is to decrease the flow rate. The other is to lower the temperature. Selectivity factors (α's) usually increase at lower temperatures. [d]If the separation does not occur in any mode, another CSP must be used. If a racemate is slightly volatile (or a volatile derivative can be made), gas chromatography is an excellent option. [From Armstrong et al. (19).]

TABLE 4 Commercially Available Type IV CSPs

Chiral stationary phase	Chiral selector
WE	*N*-Carboxymethyl-(1*R*,2*S*)-diphenylamino ethanol
WH	L-Proline
WM	L-*tert*-Leucine
MCI gel	Amino acid, structure not reported

Chromatographic retention on a type IV CSP can be manipulated by altering the pH of the mobile phase, adding mobile-phase modifiers, or changing the temperature. Examples of these effects are the following: (1) The pH of the mobile phase can be varied between 3 and 7, and the lower the pH, the shorter the chromatographic retention. (2) The addition of methanol and acetonitrile usually increases retention, whereas sodium chloride has the opposite effect. (3) The chromatography is usually carried out at 50°C and a reduction in temperature increases the retention time.

VIII. TYPE V CSPs

Proteins are complex polymers of high molecular weight that are composed of chiral subunits (L-amino acids). These biopolymers play a variety of different roles in a biological system, including the uptake and transport of drug substances. One of the key aspects of the uptake and transport mechanisms is the reversible binding of small molecules to the protein and this process is often stereospecific.

An example of this stereospecificity is the in vitro binding of propranolol to α_1-acid glycoprotein (AGP) and human serum albumin (HSA) (80,81). When human AGP is the binding protein, (*S*)-propranolol is bound to a greater extent than the *R*-isomer (80), whereas the reverse is found with HSA (81). Other serum albumins, including bovine serum albumin (BSA), have also displayed stereo-specific binding (82,83).

The ability of proteins to stereoselectively bind small molecules has been used to develop a series of commercially available protein-based CSPs (the type V HPLC CSPs), including phases that contain immobilized AGP (84), HSA (85), BSA (86), and ovomucoid (OVM) (87) (see Table 5). All these CSPs are useful in the HPLC resolution of enantiomeric compounds and appear to have an extremely wide range of applications, and the AGP CSP seems to have the broadest utility of any of the current CSPs (9–11). However, although the type V CSPs display high enantioselectivities, they also have low capacities due to the relatively small amounts of the chiral selector that can be immobilized per g silica. Thus, these CSPs are useful

TABLE 5 Commercially Available Type V CSPs

Chiral stationary phase	Chiral selector
AGP	α_1-Acid glycoprotein
OVM	Ovomucoid
HSA	Human serum albumin
BSA	Bovine serum albumin

analytical tools, but not applicable to the large-scale preparation of pure enantiomers.

A. General Mechanism

Type V CSPs are normally used with an aqueous mobile phase containing a phosphate buffer at pH 7.0. Under these conditions, hydrogen bonding and hydrophobic interactions take place between the protein part of the CSP and the solute. In addition, electrostatic interactions can occur. For example, the isoelectric points of AGP and BSA are 2.7 and 4.7, respectively, and both proteins have a net negative charge at neutral pH. Cationic and anionic solutes are also changed at this pH; this situation suggests the possibility of ion-exchange and/or ion-pairing types of interactions between the solute and CSP. Studies by Schill et al. (88) have shown that, under certain conditions, charged compounds are retained on the AGP CSP as ion pairs.

The binding interactions between the solute and protein usually involves stereospecific and nonstereospecific mechanisms. These mechanisms make the type V CSPs sensitive to the composition of the mobile phase, temperature, flow rate, and pH. These parameters can be adjusted to improve the chromatography and stereoselectivity of specific solutes on the AGP CSP (88,89), OVM CSP (90,91), BSA CSP (92,93), and HSA CSP (94).

B. The AGP CSP

1. Solute Structure

The AGP CSP can be used with a wide range of cationic and anionic compounds. The cationic compounds include relatively simple molecules such as methylphenidate, tetrahydroxoline, and terbutaline; α,β-amino alcohols such as ephedrine, labetalol, and nadolol; and complex molecules such as atropine, methorphan, and verapamil (9–11). Anionic molecules such as the α-methylarylacetic acid antiinflammatory agents ibuprofen, fenoprofen, and naproxen can also be resolved on the AGP CSP (9–11). All these compounds can be resolved without precolumn derivatization.

2. Mobile Phases

The basic mobile phase used with the AGP CSP is composed of phosphate buffer and one or more modifiers. The type and concentration of the mobile phase modifier are extremely important and both k′ and α can be altered by changing the mobile phase (9–11).

1. *Mobile-phase modifiers*. The modifiers that have been used with the AGP CSP encompass uncharged and charged compounds and include 2-propanol, *N,N*-dimethyloctylamine, tetrapropyl- and tetrabutyl-ammonium bromide, and octanoic acid (see Fig. 12). The addition of these modifiers to the mobile phase usually results in a decrease in the retention of solutes, with an accompanying decrease in enantioselectivity (α). However, in some instances, the addition of a modifier results in an increase in α.

The effect of various mobile-phase modifiers on α is the result of a highly complex relationship involving the structure of the solute, as well as the structure and type of modifier, and may also involve changes in the conformation of the AGP (95). The complexity of this relationship is illustrated by the effect of various modifiers on the chiral resolution of two closely related compounds, atropine and *N*-methylhomatropine (Fig. 13).

For *N*-methylhomatropine, the use of the cationic modifier dimethyloctylamine reduces the value of α relative to the value obtained when the uncharged modifier 2-propanol is used. The same result occurs when the anionic modifier octanoic acid is used. However, the addition of the tetrabutylammonium bromide almost doubles the enantioselectivity relative to the value of α obtained with 2-propanol. When atropine is the solute, poor enantioselectivity is observed with neutral and cationic modifiers, whereas the addition of an anionic modifier, octanoic acid, results in an α of 1.6.

2. *The effect of pH*. In addition to the use of a modifier, adjusting the pH of the mobile phase can change the retention and enantioselectivity. The magnitude of the effect depends on the nature of the solute and composition of the mobile phase. The resolution of cyclopentolate illustrates the effect of pH on α (89). When the mobile phase contains sodium chloride (0.1 *M*) and 2-propanol (0.33 *M*), a decrease in the pH from 7.5–6.5 produces a decrease in α from 1.96–1.79. However, when tetrabutylammonium bromide (0.003 *M*) is the modifier, a corresponding decrease in the pH produces an increase in α from 1.70–2.09.

3. Pharmaceutical Applications

Because of its broad enantioselectivity, the AGP CSP has been used in a large number of pharmacokinetic studies. A representative selection is presented below:

UNCHARGED MODIFIERS		
	monovalent alcohols	1-propanol
		2-propanol
		ethanol
	diols	ethylene glycol
		propylene glycol
		1,2-butanediol
	amino acids	6-aminohexanoic acid
		β-alanine
		leucine
CATIONIC MODIFIERS		
	tertiary amines	N,N-dimethyloctylamine
		N,N-dimethylethylamine
	quaternary amines	tetrapropylammonium bromide
		tetrabutylammonium bromide
	amino acid	1,2-diaminobutyric acid
ANIONIC MODIFIERS		
	carboxylic acids	octanoic acid
		butyric acid
		decanoic acid
	amino acid	aspartic acid
	sulfamic acids	cyclohexylsulfamic acid

FIGURE 12 Mobile-phase modifiers used with the AGP CSP.

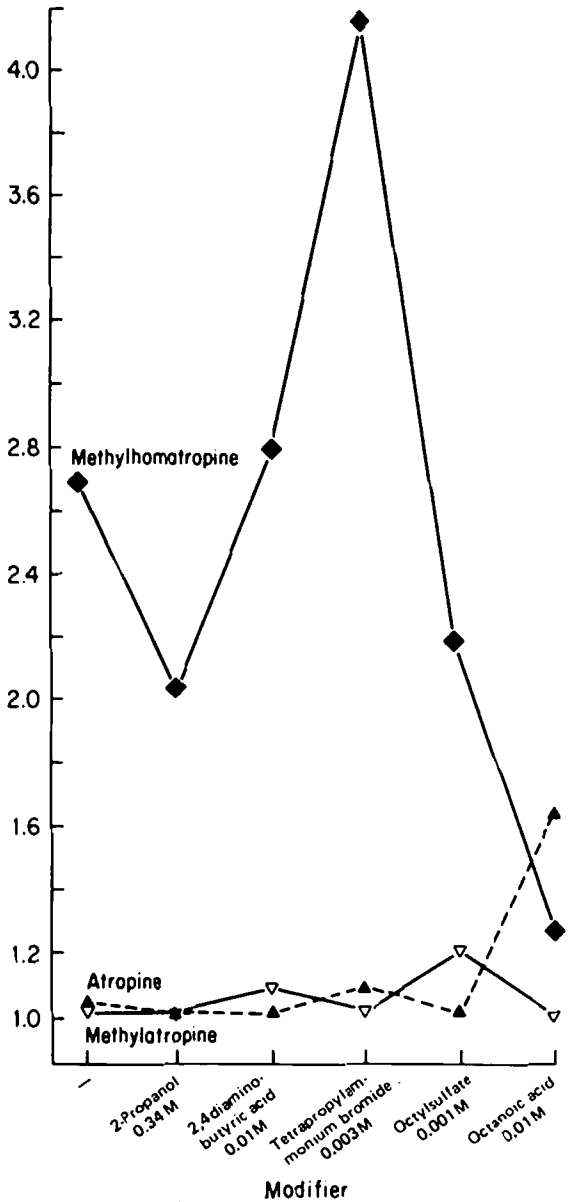


FIGURE 13 The effect of mobile-phase modifiers on the enantioselective resolution of atropine, methylatropine, and *N*-methylhomatropine.

1. "Separation and quantitation of (*R*)- and (*S*)-atenolol in human plasma and urine using an α_1-AGP column" (96). (*R*)- and (*S*)-atenolol were extracted from human serum and urine using dichloromethane containing 3% heptafluorobutanol; acetylated using acetic anhydride; separated on an AGP CSP using a mobile phase of phosphate buffer (0.01 *M*, pH 7.1) modified with 0.25% (v/v) acetonitrile. Quantitation was performed using fluorescence detection and the limit of detection was less than 6 ng/mL. The acetylation was required to eliminate a chromatographic overlap with endogenous materials and metabolites. The plasma concentration-time curves for (*R*)- and (*S*)-atenolol did not differ significantly in two subjects who received a single oral dose of 50 mg rac-atentolol and the *R/S* ratio of atenolol in the urine was approximately 1.

2. "Improved performance of the second generation α_1-AGP columns: Applications to the routine assay of plasma levels of alfuzosin hydrochloride" (97). The concentrations of (*R*)- and (*S*)-alfuzosin in rat plasma were determined by (1) addition of 1 mL 0.1 *N* sodium hydroxide to 1 mL rat plasma; (2) extraction with 7 mL of dichloromethane:ethyl ether (3:4, v/v); (3) evaporation of the organic phase and redissolution in 80 μL of the mobile phase; (4) chromatography on the AGP CSP using a mobile phase composed of phosphate buffer (0.05 *M*, pH 7.4; containing 0.025 *M* tetrabutyl ammonium bromide):acetonitrile (96:4, v/v); (5) fluorimetric detection. The chromatography took less than 10 min and plasma concentrations of 5 ng/mL could be quantitated. Initial pharmacokinetic studies demonstrated no significant difference in the plasma concentration-time curves of the two enantiomers.

C. The OVM CSP

Miwa and co-workers (87) have recently reported on the synthesis of a CSP based on ovomucoid, OVM CSP, an acid glycoprotein found in chicken egg white. The primary, secondary, and most of the tertiary structure of the protein have been elucidated (98). The molecule consists of a single 186 amino acid chain divided into three tandem homologous domains by 9 disulfide bonds, carbohydrate moieties [4–5 glycosylated asparagine residues (99)], and sialic acid moieties composing 0.5–1.0% of the total weight of the protein (100).

As with the other protein-based CSPs, initial studies indicate a relationship between the structure of the protein and chromatographic properties of the OVM CSP (101). When the sialic acid residues were enzymatically removed from the protein, the capacity factors (k') of the enantiomers of an acidic solute (ketoprofen) were reduced, whereas the k's of the enantiomers of a basic solute (chlorpheniramine) were un-

affected (101). The observed enantioselectivities for both solutes remained unchanged. However, when the carbohydrate moieties were chemically removed, the k's of the enantiomers of both solutes were reduced and the enantioselectivities lost.

1. Solute Structure

Since ovomucoid is a glycoprotein the solutes that can be stereochemically resolved on the OVM CSP are similar to the basic, neutral, and acidic compounds separated on the AGP CSP. The major differences between the two CSPs appears to be quantitative (i.e., the magnitude of the chiral separation), rather than qualitative. The two CSPs have been recently compared and a representative series of successfully resolved solutes is contained in this article (102).

2. Mobile Phases

The basic mobile phase used with the OVM CSP is composed of phosphate buffer and one or more modifiers. The type and concentration of the mobile-phase modifier as well as the pH are extremely important and both k' and α can be altered by changing the mobile phase (102).

1. *The effect of pH.* Okamoto and Nakazawa (90) have observed an unexpected decrease in the retention of abscisic acid enantiomers when the pH of the mobile phase was lowered from 4.0–3.5. This was attributed to a change in the properties of the protein. Ovomucoid has been shown to undergo a low-pH transition, during which the protein adopts a more folded and ordered configuration (99,101).

2. *The effect of organic modifiers.* The effect of organic mobile-phase modifiers on retention and stereoselectivity of abscisic acid enantiomers has also been examined using methanol, ethanol, and 2-propanol (91). The results of this study indicate that hydrophobic interactions are involved in the retention, but not the stereochemical resolution of the solutes. Miwa and co-workers (91) have also postulated that hydrophobic and hydrogen-bonding interactions are important in the retention of basic compounds.

The effects of alcoholic modifiers and pH on the chromatographic properties of the OVM CSP have been investigated using acidic, basic, and neutral solutes (103). A series of primary, secondary, and tertiary alcohols and pH's ranging from 3.5–6.0 were used in this study. The results indicate that both the shape and hydrophobicity of the alcoholic modifier affect retention (k') and enantioselectivity (α). In general, an increase in the hydrophobicity of the modifier results in a decrease in k's and α's. However, this is not the case when *t*-butanol is the modifier, suggesting that the size of the alkyl moiety attached to the carbinol carbon also contributes to the chromatographic results. The pH studies indicated that

Coulombic interactions play a role in the retention of the acidic and basic solutes. The results also suggest that in addition to ethanol and 1-propanol, *t*-butanol should be considered during optimization and that maximum efficiencies may be obtained at pH 5.0

3. *An approach to optimization.* A systematic approach to the determination of the optimum chromatographic conditions has recently been published (104) and is presented in Fig. 14.

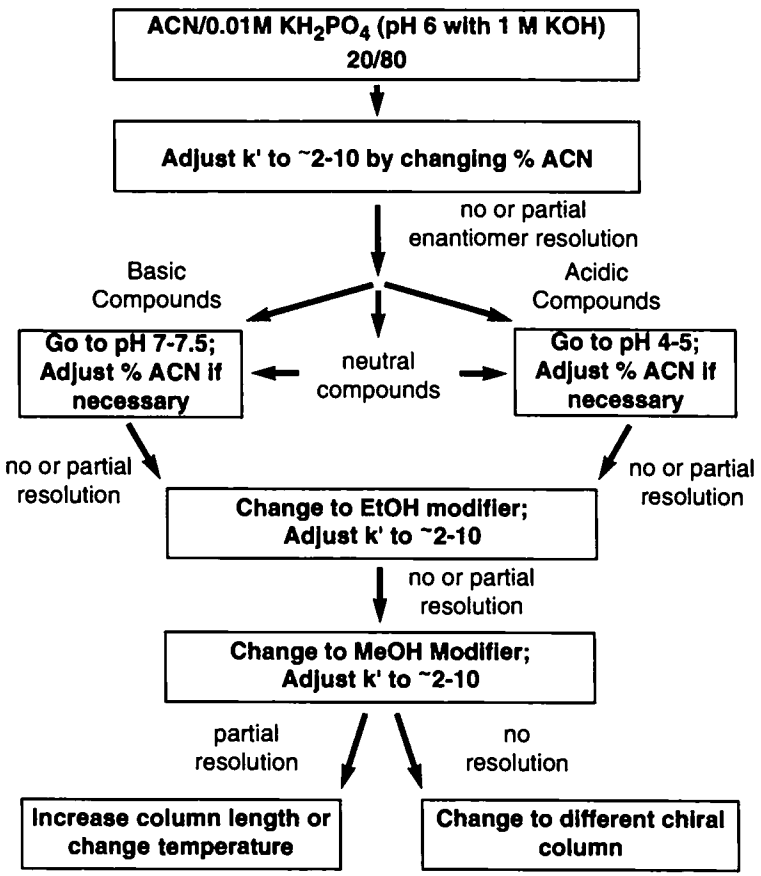


FIGURE 14 Basic flowchart for the use and optimization of the OVM CSP. [From Kirkland et al. (104).]

3. *Pharmaceutical Applications*

Because the OVM CSP is a relatively new CSP, only one pharmacokinetic application has been reported. Because of the utility of this phase, it is evident that such a situation will quickly change.

1. "The determination of the enantiomers of halofantrine and monodesbutylhalofantrine in plasma and whole blood using sequential achiral/chiral high performance liquid chromatography" (105). An HPLC method employing sequential achiral–chiral chromatography was developed for the determination of enantiomers of halofantrine (HF) and its monodesbutylated metabolite (HFM) in plasma and whole blood. After extraction from the biological matrix, HF and HFM were separated and quantitated on an achiral RP2 HPLC column. The eluent fractions containing HF and HFM were collected and reinjected onto the chiral HPLC system that was composed of an OVM CSP. The enantiomers of HF and HFM were stereochemically resolved on the OVM CSP and the enantiomeric ratios determined. The assay has been validated and applied to a pilot study of the pharmacokinetics of halofantrine in humans.

D. The HSA CSP and BSA CSP

Human serum albumin and bovine serum albumin are closely related proteins and, consequently, the chromatographic properties of the CSPs based on these proteins are similar. The only difference between the two phases appears to be due to inherent differences in stereoselectivity between HSA and BSA. For example, on the HSA-CSP, (*S*)-warfarin elutes before (*R*)-warfarin, whereas on the BSA CSP, the opposite elution order is observed (85). This is consistent with the enantioselectivities of the native proteins (106). However, even though there are differences between the CSPs, the selectivity, mobile-phase effects, and chromatographic properties of the HSA CSP and BSA CSP are so similar that the two phases will be discussed together.

1. *Solute Structure*

A wide variety of anionic and neutral compounds can be resolved on serum albumin (SA) CSPs, but not cationic compounds and, in general, the solute should contain aromatic and polar moieties (92). Some representative classes of compounds are the following:

1. *Amino acids*. Some aromatic amino acids such as kynurenin can be resolved on the BSA CSP without derivatization (92). However, most amino acids require precolumn derivatization of the amine moiety. The *N*-derivatives that have been used include acetyl, benzenesulphonyl, phthalimido, DANSYL, 2,4-dinitrophenyl, and 2,4,6-trinitrophenyl (85,92,107,108).

2. *Sulfur-containing compounds.* Molecules containing asymmetric sulphoxide or sulphoximine moieties and an aromatic group near or at the stereogenic center have been resolved (92,107).

3. *Coumarin derivatives.* Phenprocoumon and warfarin have been resolved (85,92).

4. *Benzodiazepine derivatives.* A wide variety of chiral benzodiazepines including oxazepam, lorazepam, and temazepam as well as their hemisuccinate and acetate derivatives have been resolved (85,92).

5. *2-Aryl propionic acid nonsteroidal antiinflammatory drugs.* The compounds from this class of agents that have been stereochemically resolved include ibuprofen, flurbiprofen, naproxen, benoxaprofen, pirprofen, suprofen, fenoprofen, indoprofen, and ketoprofen (94).

6. *Reduced folates.* Leucovorin and 5-methyltetrahydrofolate have been stereochemically resolved on SA CSPs (85,109).

7. *Neutral compounds.* Some neutral compounds including benzoin have been separated on SA CSPs (92).

2. Mobile Phases

The basic mobile phase used with the SA CSP is composed of phosphate buffer and one or more modifiers. The buffer concentration can range from 0.01–0.20 *M* and the pH from 4.5–8.0. These parameters can affect both retention (k′) and enantioselectivity (α). In addition, both k′ and α can be manipulated using organic and inorganic mobile-phase modifiers.

1. *The effect of pH.* In general, for *N*-derivatized amino acids, an increase in pH will result in a decrease in retention and stereochemical resolution (107,108). This effect is believed to be due to the fact that a decrease in the pH will result in a corresponding decrease in the net negative charge of the BSA. This means that the Coulomb interaction between the BSA and *N*-derivatized amino acids or other uncharged carboxylic acids will increase, resulting in an increase in k′ (108). The same effect was found for the enantiomers of oxazepam hemisuccinate (85).

A similar effect of pH of k′ has been found for the enantiomers of warfarin, but the effect on α was opposite that found with the *N*-derivatized amino acids (110). An increase in the pH of the mobile phase from 5.0–8.0 resulted in a decrease in k′ for (*S*)-warfarin from 33.6–3.4 (a 90% drop) and a decrease in k′ for (*S*)-warfarin from 38.6–4.6 (an 88% drop), the net result being an increase in α from 1.15 (pH 5.0) to 1.35 (pH 8.0).

2. *The effect of buffer concentration.* For solutes containing a carboxylic acid moiety, the general rule appears to be that the lower the buffer

concentration, the higher the retention (85,92,107,108). This holds for buffer concentrations ranging from 1–100 *mM*. However, when *N*-benzoyl-amino acids were chromatographed with buffer concentrations of 200 *mM* and above, an increase in buffer concentration resulted in an increase in k′ (108).

3. *Alcohols as mobile-phase modifiers.* As with the other type V CSPs, the addition of an alcohol to the mobile phase appears to reduce the hydrophobic interactions between the solute and SA CSP, which results in lower k′ values and reduced α's (9–11). An example of this phenomenon are the effects of ethanol, 1-propanol, and 1-butanol on the retention and enantioselective resolution of *N*-benzoyl-D,L,alanine on the BSA CSP (111). The addition to a mobile phase composed of phosphate buffer (50 *mM*, pH 7.0) of 2% (v/v) of ethanol reduced the k′ of the last eluting enantiomer by 33% and the observed α by 6%. When ethanol was replaced by 1-propanol, the observed reductions were 67 and 40%, respectively, and when 1-butanol was the modifier, the observed reductions were 80 and 77%, respectively. In practice, 1-propanol appears to be the most commonly used alcoholic modifier.

4. *Other mobile-phase modifiers.* In general, any species that binds to SA can be used to modify retention and stereoselectivity on an SA CSP. The observed effects are due to either direct competition with the solutes for binding sites on the protein, or through a change in the conformation of the protein that alters the affinity of the protein for the solute (an allosteric interaction). These interactions present the chromatographer with a broad array of mobile-phase modifiers that can be used to tailor the analytical methods to the solutes.

The compounds which can be used as modifiers include trichloroacetic acid and cyclamic acid (112). The utility of these additives is demonstrated by their effect on the chromatography of the enantiomers of warfarin (WAR) on the BSA CSP. When a mobile phase composed of phosphate buffer (0.2 *M*, pH 7.5):1-propanol (97:3, v/v) was used, the k's for (*S*-WAR and (*R*)-WAR were 18.08 and 21.83, respectively, and α = 1.21. The addition of trichloroacetic acid (5 *mM*) reduced the k's by almost 50% [9.42 (*S*)-WAR, 10.96 (*R*)-WAR], whereas the enantioselective fell by only 4% to α = 1.16. The addition of cyclamic acid (7 *mM*) resulted in the same 4% reduction in α, but a smaller reduction in retention, a 25% decrease in both k's.

Fatty acids such as octanoic and lauric acids have also been used to reduce k′ (94,112). An example of the effect of octanoic acid on the retention and enantioselectivity of ibuprofen enantiomers on the HSA CSP is presented in Fig. 15 (94). In this instance, without the addition of the

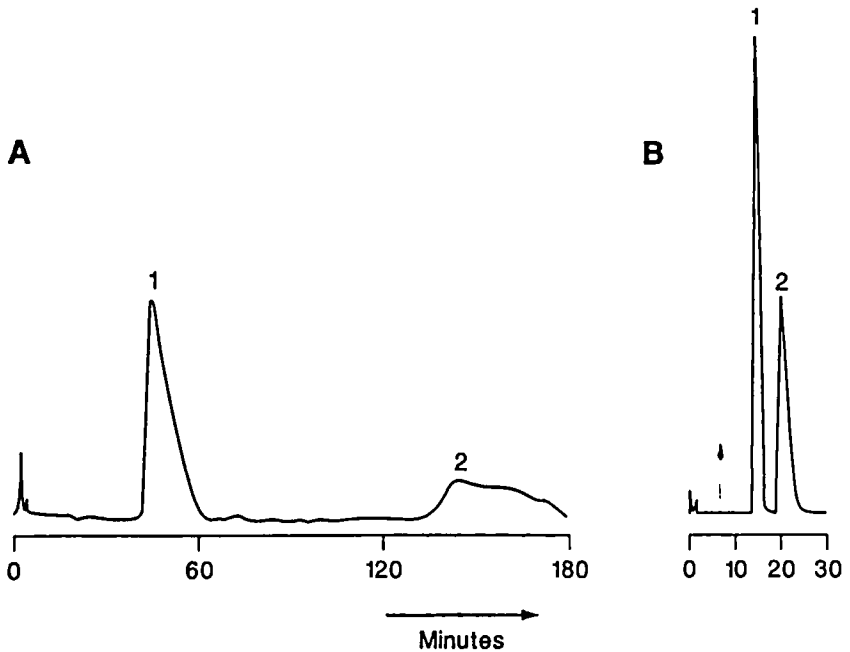


FIGURE 15 Effect of octanoic acid on the retention and enantioselective resolution of (*R*)- and (*S*)-ibuprofen on the HSA CSP, where: 1 = (*S*)-ibuprofen and 2 = (*R*)-ibuprofen. A, Without octanoic acid in the mobile phase. B, With 4 *mM* octanoic acid added to the mobile phase. [From Noctor et al. (94).]

modifier, the enantioselectivity is high, but the column efficiency is very low, and the resulting separation is useless to the analyst.

When substances that themselves bind to specific sites on SA are added to the mobile phase, competitive displacements, that is, a lowering of k′ and α, are not the only possibilities. There is also the potential for an allosteric interaction to occur in which the affinity of the protein for the solute is increased by the addition of the modifier. For example, the addition of 10 μM (*S*)-WAR to the mobile phase increased the k′ of the *S*-enantiomers of lorazepam and lorazepam hemisuccinate by 4 and 72%, respectively (113). The k′s of the *R*-enantiomers were not affected and, therefore, the observed α's increased by 5 and 76%, respectively. These results not only increased the chromatographic separation of the respective enantiomers, but also indicated that there was an allosteric interaction between WAR and (*S*)-lorazepam and (*S*)-lorazepam hemisuccinate.

3. Pharmaceutical Applications

The SA CSPs have been used in a variety of pharmacokinetic and pharmacodynamic studies. Because of the nature of these columns, they are most often used in coupled achiral–chiral systems. Two examples of this approach are the following:

1. "The measurement of warfarin enantiomers in serum using coupled achiral/chiral high-performance liquid chromatography" (110). An assay for the serum concentrations of (*R*)-warfarin and (*S*)-warfarin was developed using the BSA CSP coupled to a Pinkerton internal-surface reverse-phase (ISRP) achiral column. The ISRP column was used to separate (*R*,*S*)-warfarin from the serum components and warfarin metabolites and to quantitate the total warfarin concentration. The eluent containing the (*R*,*S*)-warfarin was then selectively transferred to the BSA CSP, where the enantiomers were enantioselectively resolved ($\alpha = 1.19$) and the enantiomeric composition determined.

2. "Determination of low levels of the stereoisomers of leucovorin and 5-methyltetrahydrofolate in plasma using a coupled chiral-achiral high performance liquid chromatographic system with post-chiral column peak compression" (114). A method for the determination of low levels of the stereoisomers of leucovorin (LV) and 5-methyltetrahydrofolate (5-MTH) was developed and validated for plasma levels of both compounds ranging from 15–500 ng/mL. The assay involved initial chromatography on a BSA CSP, followed by postcolumn peak compression and elution on two C_{18} columns. The BSA CSP separated LV and 5-MTH from interfering plasma components and from each other and achieved the stereochemical resolution of the diastereomeric (6*S*)- and (6*R*)-LV. The eluent containing (6*S*)-LV was directed onto one C_{18} column and the eluent containing (6*R*)-LV and 5-MTH was directed onto the other. This was followed by sequential rapid gradient elution of the target compounds from the respective C_{18} columns.

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7

Synthesis of Enantiomerically Pure Drugs

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The need to prepare a chiral organic molecule that is to be used as a drug, in enantiomerically homogeneous form, has been amply justified in other sections of this monograph. Here, the synthetic chemical and biochemical methods available for preparing these compounds will be reviewed and illustrated.

I. METHODOLOGY

A. Synthetic Analysis and Design

The synthesis of an organic molecule generally proceeds in a series of logically connected individual stages. First, obviously, is definition of the target. For the medicinal chemist this includes, in the case of a chiral molecule, a decision on whether to prepare the compound in racemic or enantiomerically homogeneous form.

The design of a synthesis is based on a careful analysis of the structure sought. This process, termed retrosynthetic analysis by Corey, who is responsible for its formalization, can be performed manually or in a computer-aided fashion (1). It involves consideration of all potential bond breakings—thus, retrosynthesis—of the target. Each is evaluated in terms of the probability of success, based on known reactions, of the reverse, synthetic transformation. In its more sophisticated forms, the computer program will provide an estimate of the probability of success of the proposed transformation, as well as relevant literature citations.

The first generation retrosynthetic analysis provides the initial branches of a tree. Similar analysis of each branch, representing a target precursor, is then carried out. A judicious choice of which branches to terminate

leads, ultimately, to a compound (starting material) that is commercially available or whose synthesis is known.

Usually, several routes to the target will be generated in this fashion. The choice of which one is to be attempted is often subjective, based on the prejudices of the chemist involved. On a more logical basis, the factors leading to the synthesis choice can involve the cost and availability of the starting material, the length of the synthesis, the overall probability of success, and the options available should one reaction not occur as predicted.

The preparation of an enantiomerically homogeneous chiral molecule adds another element of difficulty to the retrosynthetic analysis. Either the analysis must include a specific step for obtaining one enantiomer, or it must lead ultimately to an enantiomeric starting material. These possibilities are examined more fully below.

B. Introduction of Chirality

The practicing organic chemist now has available a variety of synthetic tools for preparing enantiomerically pure compounds (2). These methods all derive, ultimately, from a naturally occurring chiral molecule. The means by which this natural chirality is applied to preparing other chiral molecules varies widely in concept and execution. These concepts fall, however, into three general areas: resolution, asymmetric synthesis, and the use of the chiral carbon pool. Comprehensive reviews of these methods exist (3–5), and thus only a brief outline of each will be presented here.

1. Resolution

Classical Resolution and Variants. Resolution is the process by which a chiral recemic molecule is combined with a second chiral, but enantiomerically homogeneous, molecule. The resultant mixture of diastereomers is separated and the appropriate diastereomer is then cleaved to recover the resolving agent and the desired enantiomer. As opposed to enantiomers, diastereomers have different physical properties, for example, melting points and solubilities, thus allowing for separation.

The most classical of resolutions is exemplified by the separation, by crystallization, of the diastereomeric salts formed by treatment of a racemic acid with one enantiomer of a chiral base, typically an alkaloid such as quinine. Unfortunately, despite significant recent advances (3,6), the relative solubilities of two diastereomers, and thus the probability for success of a classical resolution, are difficult to predict. It thus remains, for most chemists, a largely empirical method. On the other hand, a successful resolution often provides both enantiomers, even when both enantiomers of the resolving agent are not at hand, by recovery from the enriched

mother liquors. A careful study of the pharmacological and toxicological properties of the individual enantiomers can determine whether, in fact, the cost of separation is necessary or justified.

The development of newer separation techniques, in particular preparative gas and liquid chromatography, has broadened the scope of resolution in recent years. An alternative to the acid–base salt separation by crystallization, for example, would be formation of the covalent amide linkage, chromatographic separation of the diastereomers, and then chemical hydrolysis.

Resolution, by its very nature, is an inefficient process. The maximum obtainable yield is 50%; in practice, inefficient separation requiring more than one crystallization or chromatography and/or mechanical losses during processing often make the actual yield significantly lower. As a practical matter of synthetic strategy then, it is important to carry out the resolution as early in the synthesis as possible, when the material to be lost carries the minimum value. For economic viability, a drug synthesis involving a resolution usually must contain an efficient recycle of the wrong enantiomer. In most cases, this recycle is effected by racemization and reresolution. There are examples, however, where clever synthetic design allows the carrying forward of both enantiomers of a chiral intermediate; such syntheses have been termed chirally economic (7).

Second-Order Asymmetric Transformations. A modification of the classical resolution occurs in the specific case where equilibration of the chiral center can be achieved during the resolution. By judicious choice of reaction conditions, one diastereomeric salt can be induced to crystallize under the equilibration conditions. As this material precipitates, solution equilibrium is reestablished by racemization of the now-major isomer remaining. In the best cases, over 90% of a single diastereomeric salt can be obtained.

Examples of second-order asymmetric transformations are relatively rare. By far the best known case (Fig. 1) is the preparation of methyl

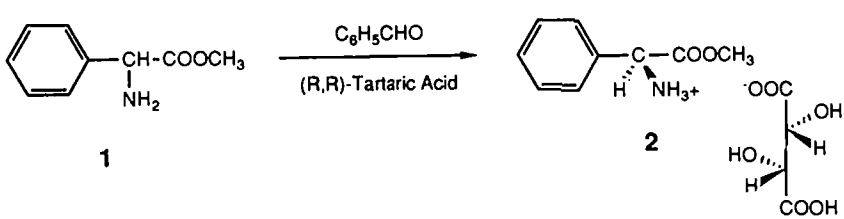

FIGURE 1 Second-order asymmetric transformation.

R-phenylglycinate-*R*,*R*-hydrogen tartrate [2], a key building block for the β-lactam antibiotic, ampicillin. The addition of one mole each of benzaldehyde and *R*,*R*-tartaric acid to a 10% solution of racemic methyl phenylglycinate [1] in ethanol results in precipitation, after 24 hr, of the desired salt [2] in 85% yield. Reuse of the salt mother liquors as feed in subsequent runs results in, ultimately, an overall 95% conversion to the desired material (8). The presence of benzaldehyde greatly facilitates the racemization process by forming, reversibly, a Schiff base.

The finding of a second-order asymmetric transformation involves not only the empiricism of the classical resolution but also the finding of resolution conditions that simultaneously allow the diastereomeric interconversion. It is not, then, surprising that these rigid criteria have kept the number of demonstrated examples small.

Kinetic Resolution. The selective reaction of one member of a racemic pair with a chiral reagent is the basis for a kinetic resolution. This reaction provides recovered starting material in one enantiomeric series with a product in the opposite series.

The reagents giving a kinetic resolution can be either chemical or enzymatic. The most generally useful of such reagents, to date, have been enzymes (9). Perhaps the best known example is the acylase-mediated hydrolysis of, for example, racemic *N*-acetylphenylalanine [3] (Fig. 2). The process gives *S*-amino acid [5] of, usually, very high enantiomeric purity, as well as recovered *R*-*N*-acetyl amino acid [4]. As in classical resolution, the obtainable yield is 50%, and recycle of the unwanted enantiomer is required for maximum efficiency. Fortunately, there are several simple methods available for racemization of *N*-acyl amino acids and, thus, by recycling, an excellent yield of *S*-amino acid is often achieved. This methodology is now practiced industrially, principally in Japan, yielding many tons annually of synthetic amino acids (10). The industrial applications are particularly elegant in that often an immobilized enzyme is used. The kinetic resolution is effected by simply

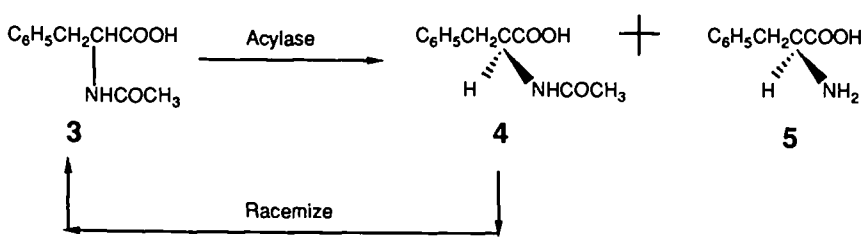

FIGURE 2 Enzymatic kinetic resolution of amino acids.

passing a solution of the racemate through a column containing the immobilized enzyme.

Other enzymic kinetic resolutions are known. Of particular value to the synthetic chemist are the lipase and/or esterase-mediated hydrolyses of esters of chiral racemic alcohols (11) or acids (12). The resultant product alcohols or acids and recovered esters are often of high enantiomeric purity.

Methods for chemical kinetic resolution to give products of high enantiomeric purity are less well known. Perhaps the most successful, and one complementary in terms of the products obtained with the enzymic methods, is the epoxidation of a racemic secondary allylic alcohol (13). When this epoxidation is carried out using *t*-butylhydroperoxide as oxidant in the presence of a titanium catalyst that is chirally modified by an ester of tartaric acid, the selectivity for one enantiomer of the starting alcohol is often virtually complete.

Thus, a chiral secondary alcohol, extremely useful as an intermediate for many synthetic targets, can be prepared by either a chemical or enzymatic kinetic resolution. The choice depends on the particular molecule sought and the prejudices of the chemist involved. Recycling of the unwanted enantiomer in these cases is simple, involving oxidation, then reduction to the racemate.

2. *Asymmetric Synthesis*

Asymmetric synthesis is the chemical or biochemical conversion of a prochiral substrate to a chiral product. In general, this involves reaction at an unsaturated site having prochiral faces (C=C, C=N, C=O, etc.) to give one product enantiomer in excess over another. The reagents effecting the asymmetric synthesis are used either catalytically or stoichiometrically. Clearly, the former is to be preferred, for economic reasons, when applicable. The reagents can be either chemical or enzymatic.

Asymmetric synthesis is, in itself, a very active and exciting field for scientific exploration, with major discoveries being reported continually. The reader is referred to the five-volume treatise by Morrison (4) for a comprehensive review and an assessment of recent developments.

The methodologies for asymmetric synthesis have now matured to the extent that they form the basis for commercial syntheses of several chiral compounds (14). Two such examples involve the preparation of pharmaceuticals. Shown in Fig. 3 are the key chirality-introducing steps in the synthesis of L-dopa [8] and cilastatin [11].

L-dopa, used in the treatment of Parkinson's disease, is best prepared by asymmetric catalytic hydrogenation (15) of the enamide [6]. The hydrogenation, performed with a soluble rhodium catalyst modified with the

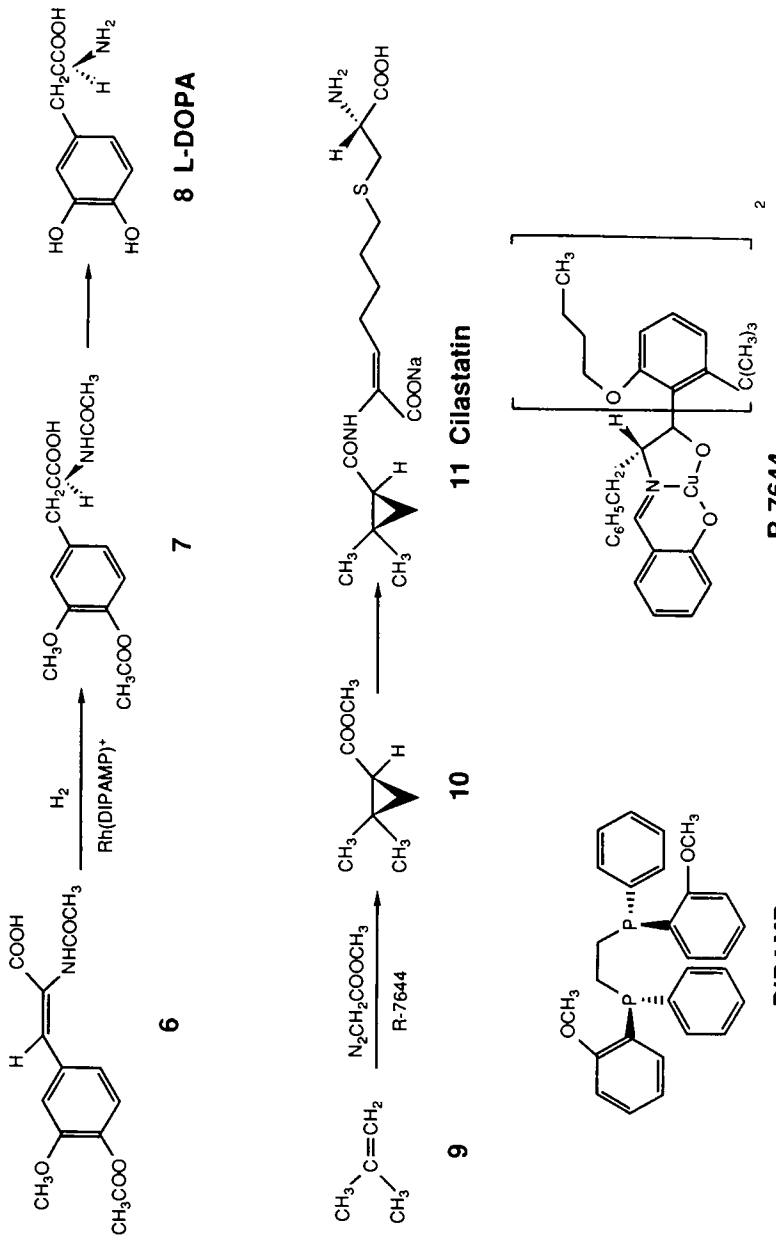

FIGURE 3 Industrial asymmetric syntheses of pharmaceuticals.

chiral bisphosphine DIPAMP, gives the protected amino acid [7] in 94% enantiomeric excess (e.e.). Enantiomeric enrichment and removal of the protecting groups then provide the desired amino acid. It was this industrial preparation of L-dopa that firmly established asymmetric synthesis as a viable synthetic tool, rather than an exotic curiosity, in the minds of most organic chemists.

Thienamycin and its derivatives are exciting new antibiotics. Their clinical use is limited, however, by their susceptibility to the kidney enzyme dehydropeptidase I. Reversible inhibition of this enzyme is provided by cilastatin [11]. The preparation of the *S*-cyclopropane portion [10] of cilastatin is achieved (16) by decomposition of ethyl diazoacetate in isobutylene [9] in the presence of the chiral copper catalyst R-7644. The product [10] is obtained in 92% e.e. and then further processed to cilastatin. Cilastatin is now marketed in combination with the thienamycin derivative imipenem as a very-broad-spectrum antibiotic.

Asymmetric synthesis, when applicable, is a very valuable tool for chiral drug synthesis. Although the number of examples giving high e.e.'s is growing, it is still limited, and the method will not be applicable in all cases. Of particular concern in any asymmetric synthesis is the fact that no such reported reaction yet gives absolute (i.e., 100%) introduction of chirality, and thus asymmetric synthesis must be paired with an enantiomeric enrichment step. A reaction giving a 95% e.e. may be of little use in drug synthesis if a method for reaching enantiomeric homogeneity cannot be found.

3. *Chiral Carbon Pool*

The third major source of chiral pharmaceuticals involves synthesis using naturally occurring chiral molecules as starting materials (5,17). Those compounds most generally used are carbohydrates, amino acids, terpenes, and smaller, microbiologically derived compounds such as lactic acid or tartaric acid. In addition, the synthetic chemist now has in his or her repertoire a variety of rather standard building blocks derived by manipulation of the natural substances; a list of such compounds has been compiled (5).

A retrosynthetic analysis may well lead to a molecule recognizably derived from the chiral carbon pool. Presumably, the resulting synthesis will then be subject only to the vagaries encountered in the preparation of any target molecule, chiral or not. Unfortunately, the actual situation is not always that simple. If the target molecule contains more than one chiral center, the introduction of the later centers must be highly stereoselective to avoid diastereomer formation. As noted above, though, diastereomers usually are separated fairly readily and the loss of a small amount of

material as a diastereomer usually can be tolerated. Synthetic operations offering the possibility of racemization are, of course, to be avoided if at all possible.

Of most concern in using the chiral carbon pool, however, is the enantiomeric homogeneity of the natural products themselves. Although it is generally accepted that most carbohydrates and amino acids are enantiomerically pure, it is known that many terpenes are not. The small molecules may or not be enantiomerically pure. The only sure method of avoiding a nasty surprise during the projected synthesis is to use a starting material, the enantiomeric composition of which is known with certainty.

A further limitation of the chiral pool approach may be the availability of only one member of an enantiomeric pair. Strategies that circumvent this problem are available in certain cases, however (5).

II. EXEMPLIFICATION

The prostaglandins are extremely bioactive substances. Their availability in only very small amounts from natural sources, as well as their potential use in pharmacology in their native or altered form, has made them the subject of intense synthetic interest in recent years. These syntheses amply illustrate, as a coherent whole, the methods outlined above for obtaining chiral molecules.

It is by no means possible here to describe all synthetic work on prostaglandins. The reader is referred to a leading review (18) for that purpose. The examples chosen were those best illustrating the ingenuity of the synthetic chemist who needed to prepare a complex and relatively unstable chiral molecule. Emphasis in the discussion and figures is placed on the means used for introduction of chirality.

A. Corey Lactone

A by now classic retrosynthesis of prostaglandins $PGF_{2\alpha}$ and PGE_2 (Fig. 4) leads to the bicyclic lactone [12], five-carbon phosphonium salt [13], and phosphonate [14] (19). These compounds contain all the carbon atoms of the prostaglandins and, in [12], all but one of the chiral centers. Lactone [12] has come to be known generically as the Corey lactone, and its synthesis in one enantiomeric form has been the subject of numerous complementary investigations.

Several of the seminal routes to the lactone, as devised by Corey, are summarized in Fig. 5. Diels–Alder reaction of (methoxymethyl)cyclopentadiene [15] with chloroacrylonitrile and then basic hydrolysis gave the bicyclic ketone [16] (20). Ring expansion in a selective Baeyer–Villiger reaction led to lactone [17] that was then hydrolyzed to hydroxy acid [18].

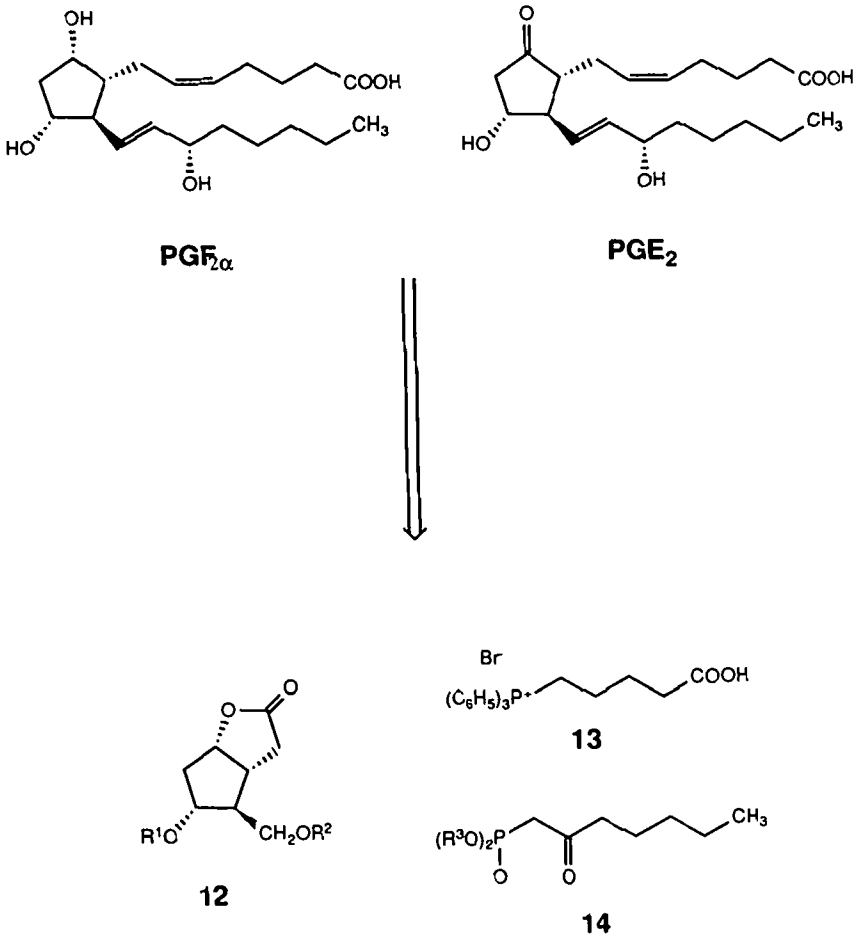

FIGURE 4 Prostaglandin retrosynthetic analysis, part I.

Resolution (21) with 2*S*,3*R*-ephedrine provided acid [19] of the correct absolute configuration. Iodolactonization gave the lactone [20] which was readily transformed to the Corey lactone.

An approach to lactone [12] similar in concept to that just described, but not requiring a resolution, involved asymmetric Diels–Alder reaction of (benzyloxymethyl)cyclopentadiene [21] with the chiral ester of acrylic acid and 8-phenylmenthol (22). The adduct [22] was obtained in undetermined but apparently quite high e.e. Oxidation of the ester enolate of [22], followed by lithium aluminum hydride reduction, gave diol [23] as an

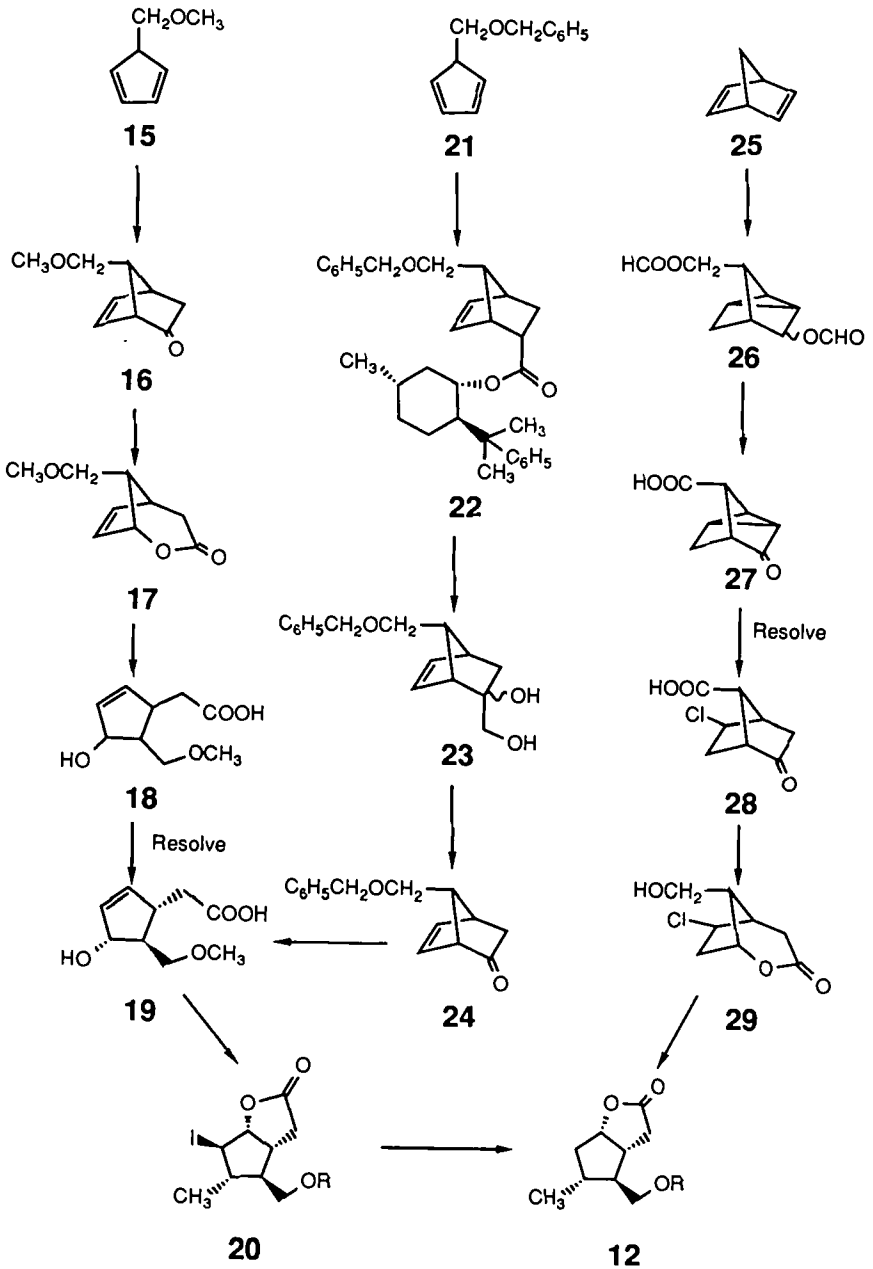

FIGURE 5 Corey approaches to lactone [12].

endo/exo mixture. As a by-product of this reaction, the 8-phenylmenthol could be efficiently recovered for reuse. Oxidative removal of the excess carbon atom gave ketone [24]. This synthetic equivalent to the resolved form of [16] was oxidized with basic hydrogen peroxide to hydroxy acid [19], from which the desired Corey lactone is readily obtained. Crystallization of this compound gave enantiomerically pure material; the enantiomer and any diastereomers, if present at all, were lost in this operation.

A third Corey approach involving bicyclic compounds started with the reaction of norbornadiene [25] with paraformaldehyde and formic acid, catalyzed by sulfuric acid (23). The mixture of formates [26] so produced could be directly oxidized with Jones reagent to keto acid [27]. Classical resolution, requiring two to three crystallizations, was effected with *S*-α-methylbenzylamine. One conversion of the resolved acid [27] to the Corey lactone involved cleavage with hydrochloric acid to chloro ketone [28]. Baeyer-Villiger oxidation followed by selective reduction of the acid functionality gave lactone [29]. After protection of the alcohol as its tetrahydropyranyl ether, base-catalyzed ring opening and relactonization with expulsion of chloride gave the desired Corey lactone.

A route to the Corey lactone that was devised by a Hoffmann-La Roche group (24) also involved bicyclic intermediates and a resolution (Fig. 6). However, use of the "meso trick" made introduction of the necessary chirality an efficient process. Thus, treatment of the symmetrical and hence achiral diol [30] with phosgene and then isobornylamine gave the mixture of diastereomers [31] and [32]. These urethanes were separable by fractional crystallization. Although the isolated yield of the desired diastereomer [32] was only 25%, the mother liquors, enriched in [31], were recycled by hydrolysis to the starting material, diol [30]. A continued resolution/recycle led to a quite efficient overall conversion of [30] to [32]. Elaboration of alcohol [32] to the one-carbon-homologated nitrile, followed by hydrolysis, gave lactone [33] and recovered isobornylamine. A several-step series of reactions involving ring opening and amide formation with pyrrolidine, oxidation to the aldehyde, epimerization, reduction, and ether formation led to amide [34]. Ozonolysis and oxidation then generated the diacetate [35] possessing the desired stereochemistry and oxidation level. Conversion of [35] to [12] was straightforward.

An application of the meso trick that does not, in principle, require recycling, has been provided (Fig. 7) by a Japanese group (25). Reaction of *cis*-2-cyclopenten-1,4-diol [36] with *N*-mesyl-*S*-phenylalanyl chloride gave, in addition to diester and recovered starting material, the diastereomeric esters [37] and [38]. Separation was effected by either chromatography or crystallization. Conversion of the free alcohol of [37] to its tetrahydropyranyl ether and saponification gave alcohol [39]. Transfer of the

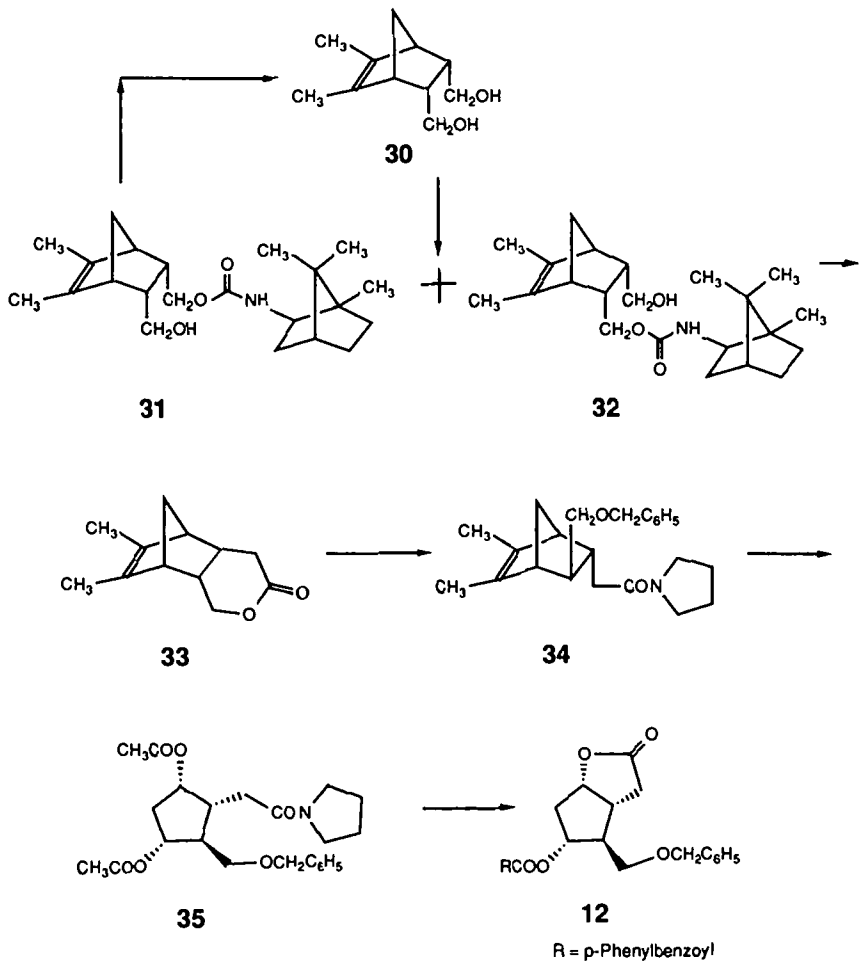

FIGURE 6 Fischli synthesis of Corey lactone.

C—4 chirality to C—2 with concomitant introduction of a two-carbon chain was carried out by Claisen rearrangement, using triethyl orthoacetate. Deprotection and ring closure gave bicyclic lactone [41], which has been converted (26,27) to the Corey lactone and a synthetic equivalent.

To use diastereomer [38] of [37] for synthesis of [41], a different sequence was required. As for [37], ester [38] was first converted to the tetrahydropyranyl ether [41]. The stereochemistry was corrected by esterification and THP cleavage to give benzoate [42], in the same stereo-

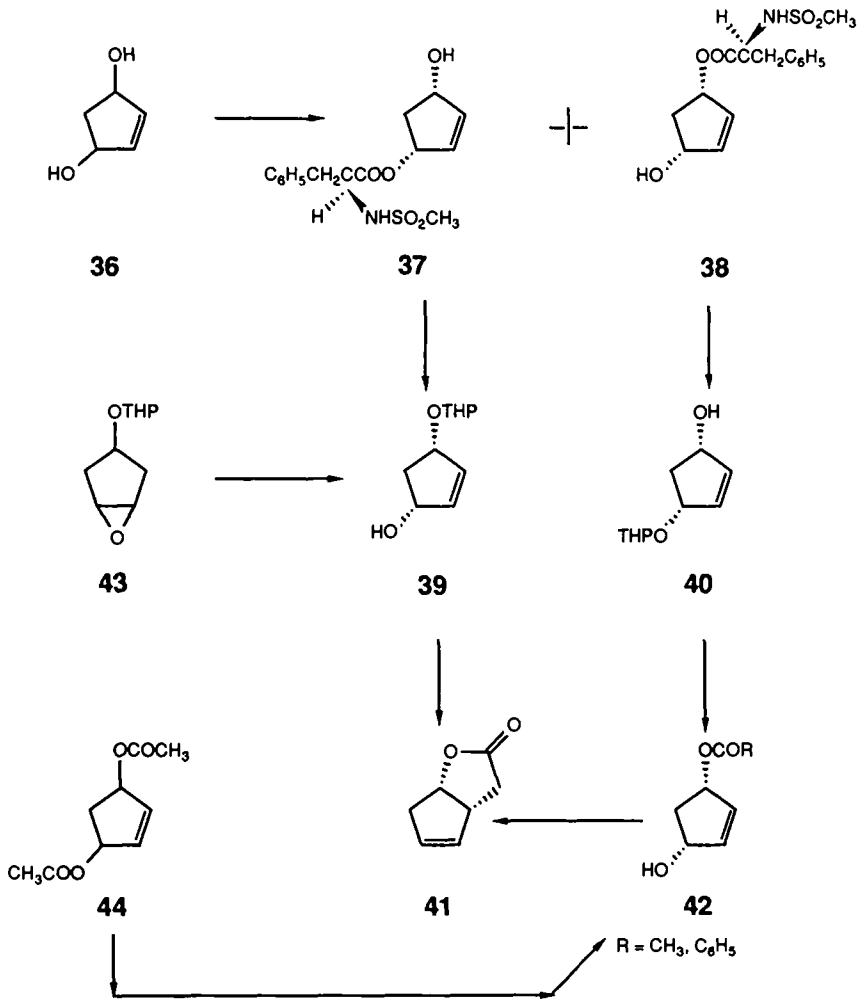

FIGURE 7 Yamada synthesis of Corey lactone.

chemical series as [39]. In the same manner as [39], Claisen rearrangement, deprotection, and lactonization gave the desired [41]. Thus, by maintaining by means of protecting groups the nonequivalence of the hydroxyl groups of [36], it was possible to convert an achiral starting material entirely to an enantiomerically homogeneous product.

Two entirely different approaches to intermediates in the Yamada synthesis have been reported. Opening of the symmetrical epoxide [43]

with the lithium amide of *S*-2-(pyrrolidinomethyl)pyrrolidide gave alcohol [39] in up to 90% e.e. (28). On the other hand, selective hydrolysis of diacetate [44] by immobilized pig liver esterase (29) gave monoacetate [42] in about 80% e.e., as calculated from optical rotations. Upgrading to enantiomeric homogeneity was possible by crystallization.

Syntheses of the Corey lactone using materials from the chiral carbon pool have been described. Johnson (30) chose *S*-malic acid ([45], Fig. 8) in his approach. Conversion to the acetoxysuccinyl chloride [46] was followed by malonate chain extension to the *bis*(keto ester) [47]. Cyclization was highly regioselective, giving a 4:1 mixture of [48] and its regioisomer. The remaining stereocenters were then introduced. Hydrogenation (*cis*, but accompanied by isomerization of the β-keto ester center to the more stable *trans* configuration) gave [49]. Sodium borohydride reduction under carefully controlled conditions led to alcohol [50], which was induced to lactonize with anhydrous potassium carbonate. Manipulation of the protecting groups and oxidation level of the resultant lactone [51] led without incident to the Corey lactone.

D-Glucose ([52], Fig. 9) has served as an intriguing educt for preparation (31) of the Corey lactone equivalent [59] (32). The iodo compound [53] was readily available from glucose in four steps. Reductive fragmentation, induced by zinc in ethanol, gave the unsaturated aldehyde [54]. Reaction with *N*-methylhydroxylamine was followed by a spontaneous nitrone cycloaddition to provide the oxazolidine [55]. Catalytic reduction of the *N-O* bond was accompanied by the unexpected loss of tosylate and aziridine formation. Olefin formation from [56] via the *N*-oxide and chain extension gave acid [57]. Iodolactonization and tri-*n*-butyltin hydride reduction in the standard fashion led to lactone [58]. After saponification of the benzoates, stereoselective epoxide formation gave epoxy lactone [59].

An extremely efficient synthesis of lactone [41] is provided (33,34) by asymmetric synthesis (Fig. 10). Alkylation of the anion of cyclopentadiene with methyl bromoacetate gave the unstable diene [59]. Immediate asymmetric hydroboration with (+)-di-3-pinanylborane gave, after oxidative workup, the hydroxy ester [60] in about 95% e.e. Lactonization involved conversion to mesylate [61] and saponification. The crystalline lactone [41] was readily brought to an enantiomerically pure state. This route is apparently the basis for commercial quantities of compound [41], the Corey lactone, and other prostaglandin intermediates offered by the Hungarian firm Chinoin.

The final approach to the Corey lactone to be discussed (Fig. 11) is not of particular interest of itself. It is, however, unique and of some value in other approaches to prostaglandins. Reduction of racemic bicyclic ketone

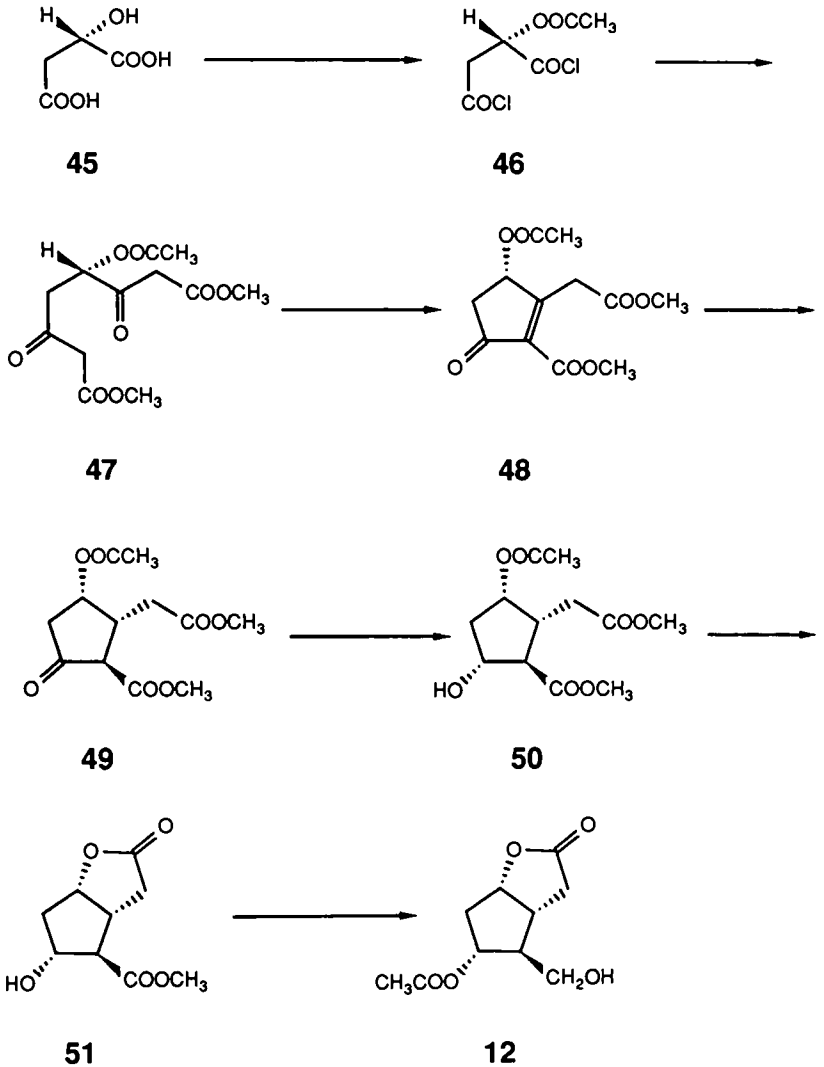

FIGURE 8 Johnson synthesis of Corey lactone.

[62] with actively fermenting baker's yeast (35) gave a roughly 2:1 mixture of alcohols [63] and [64]. The latter compound, of unknown e.e., was isolated by chromatography. Two-stage oxidation (Jones' reagent, then Baeyer–Villiger) gave lactone [41], which was brought to enantiomeric purity by crystallization.

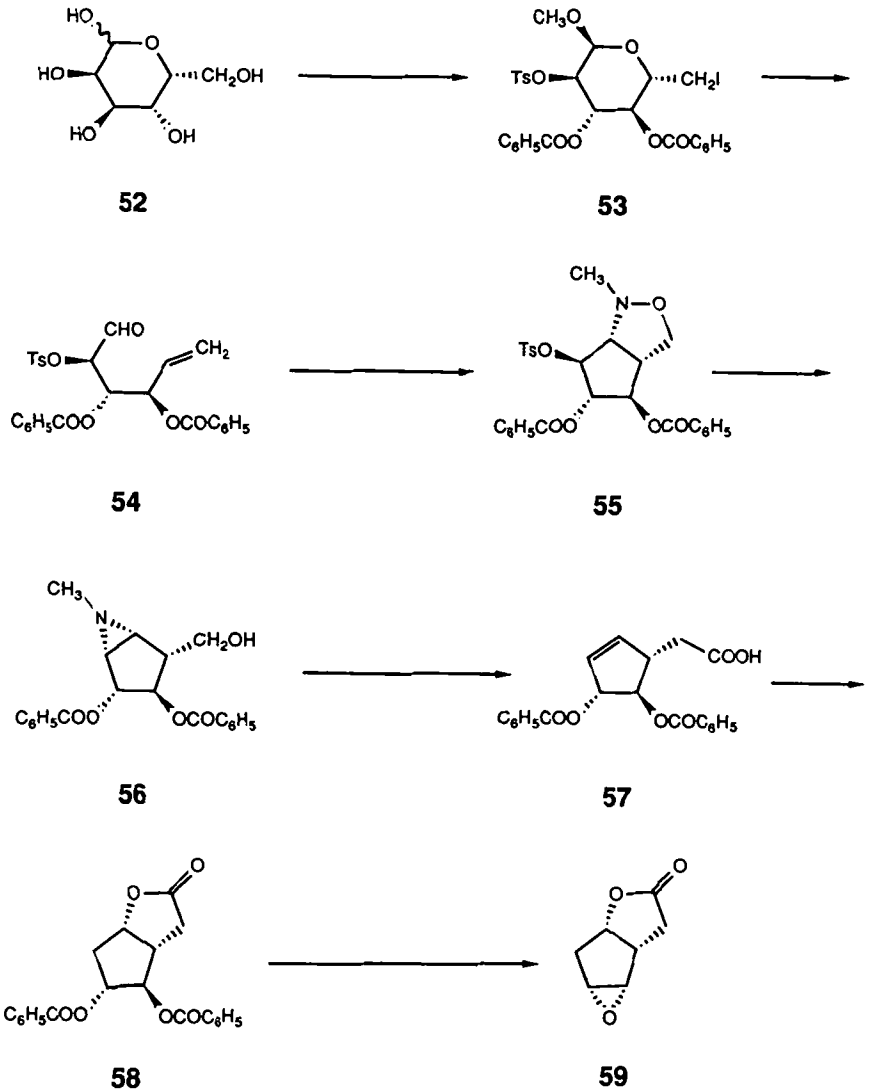

FIGURE 9 Ferrier synthesis of a Corey lactone equivalent.

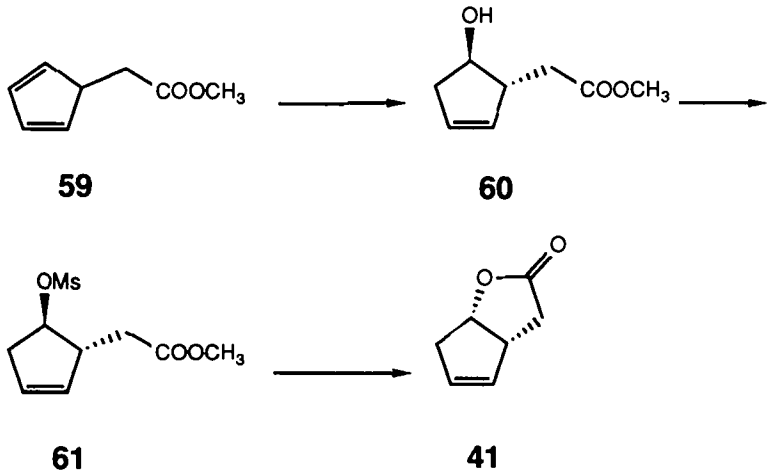

FIGURE 10 Partridge synthesis of bicyclic lactone [41].

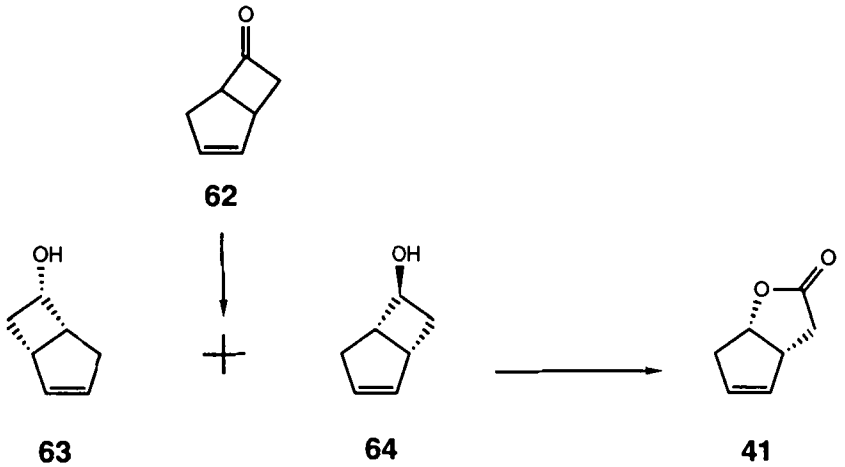

FIGURE 11 Newton and Roberts synthesis of bicyclic lactone [41].

B. Cyclopentenone Conjugate Addition Approach

A second major retrosynthetic disconnection of prostaglandins $F_{2\alpha}$ and E_2 (Fig. 12) leads to the cyclopentenone [65]. The 1,4 addition to [65] of a nucleophile representing the lower side chain, followed by capture of the resulting ketone enolate with an electrophile representing the upper side

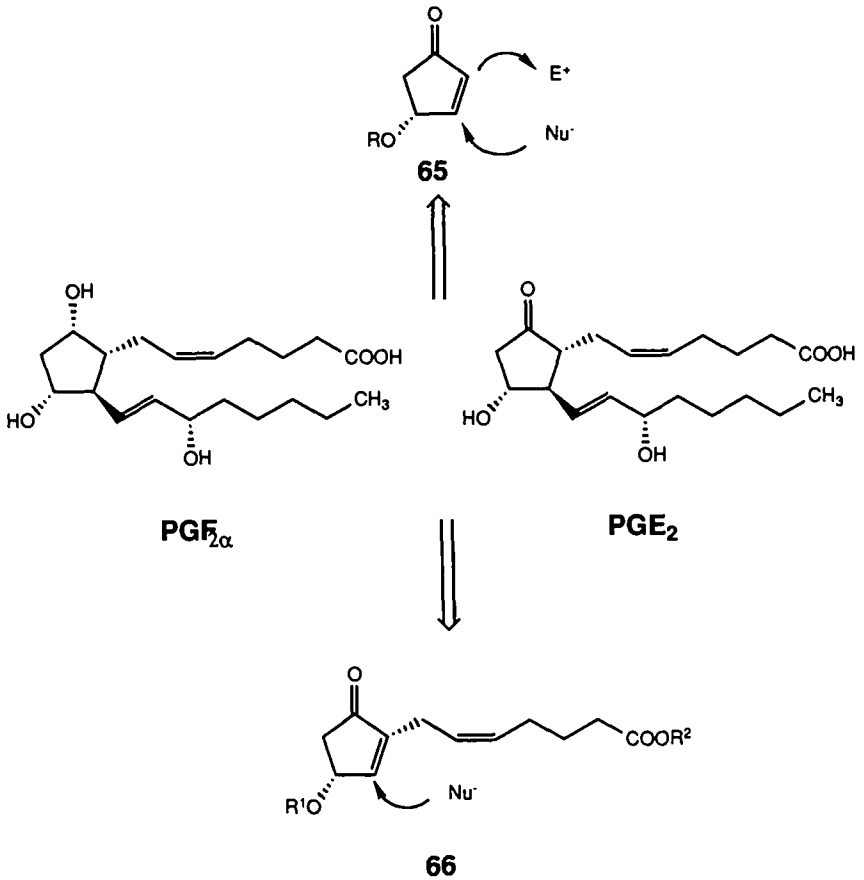

FIGURE 12 Prostaglandin retrosynthetic analysis, part II.

chain, has been recognized (36) for a substantial time as a prostaglandin synthesis that would be of considerable value due to its convergent nature. However, it is only recently that the fully convergent synthesis has been achieved.

In earlier work, a less convergent variant was developed in which a nucleophile was added in the 1,4 fashion to enone [66] containing the preformed upper chain. As practiced by the Sih group (37,38), the enone [66] (Fig. 13) was prepared starting from ethyl acetoacetate [67]. A nine-step chain-lengthening provided keto ester [68]. Reaction of this material with diethyl oxalate, followed by acidic hydrolysis and reesterification, gave the trione [69]. The key, chirality-inducing step involved reduction

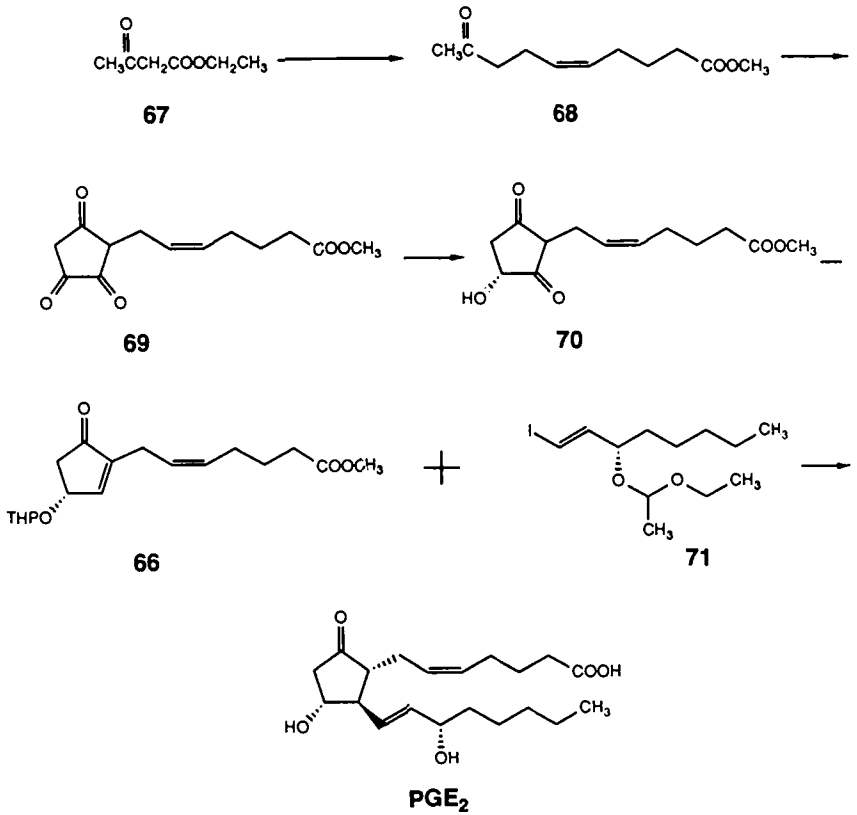

FIGURE 13 Sih synthesis of PGE_2.

of [69] with *Dipodascus uninucleatus* to the 4*R*-alcohol [70]. Apparently, the reaction was enantiospecific, although no evidence was presented to support this claim. Conversion of [70] to enone [66] involved reduction of the derived enol mesitylenesulfonate with sodium *bis*(2-methoxyethoxy)-aluminum hydride, acidic rearrangement and elimination, and THP ether formation. Addition of the cuprate prepared from the enantiomeric iodide [71] gave, after removal of the ether protecting groups with acid and microbiological ester hydrolysis, PGE_2.

An alternative preparation of the unprotected alcohol corresponding to structure [66] involved elaboration of the enantiomer of the bicyclic lactone [41] (Figs. 7 and 10) (39).

For the synthesis shown in Fig. 13 to be of value, a source of the enantiomeric vinyl iodide [71] or its equivalent must be available. A

number of solutions to the problem have been devised. The initial approaches involved resolution. The hydrogen phthalate of racemic *E*-3-hydroxy-1-iodo-1-octene was resolved with *S*-α-methylbenzylamine (40). Alternatively, resolution in a similar manner of 1-octyn-3-ol (41) and then conversion of the acetylenic unit to the *E*-1-iodoalkene gave the same result (40). In either case, the overall efficiency of the resolution, due to the necessary chemical manipulations, was quite low.

Sih (38) has described the reduction of *E*-1-iodo-1-octen-3-one with *Penicillium decumbens* to give the desired *S*-alcohol. Based on optical rotation, the e.e. was about 80%. An asymmetric chemical reduction of this same ketone, using lithium aluminum hydride that had been partially decomposed by one mole each of *S*-2,2′-dihydroxy-1,1′-binaphthol and ethanol (42), gave the desired alcohol in 97% e.e. This reagent also reduced 1-octyn-3-one in 84% e.e. to the corresponding alcohol (43). A 92% e.e. could be obtained with B-3-pinanyl-9-borabicyclo[3.3.1]nonane as the reducing agent (44).

The more convergent prostaglandin synthesis in which the two side chains are added to enone [65] in a one-pot operation has been difficult to achieve (36) because the second step (reaction of the ketone enolate with an electrophile) initially failed. A variety of solutions (45) to the problem with varying degrees of sophistication have been developed. One memorable step along the way was Stork's synthesis (46,47) of $PGF_{2\alpha}$ (Fig. 14). The racemic 4-cumyloxy-2-cyclopentenone [72], upon reaction with the organocuprate derived from iodide [73] and subsequent trapping with formaldehyde, a very powerful electrophile, gave a 1:3 mixture of diastereomeric keto alcohols [74] and [75]. Mesylation and base-induced elimination gave [76]. This enone successfully underwent a second conjugate addition, this time with the cuprate from the *Z*-iodide [77]. Manipulation of the protecting groups and oxidation level of the resulting adduct [78] gave $PGF_{2\alpha}$ methyl ester [79]. At this point, the offending diastereomer was removed by chromatography.

The ultimate in the three-component coupling approach to prostaglandins has now been achieved by Noyori (48). As illustrated in Fig. 15, the cuprate derived from iodide [82] was added to enone [80] in the usual fashion. Then, after addition of hexamethylphosphoramide, triphenyltin chloride was used to effect enolate interchange. As opposed to lithium (or copper) enolates, the tin enolate is cleanly alkylated with allylic iodide [81]. The protected PGE_2 [83] was obtained in 78% yield. Two-step deprotection to PGE_2 was straightforward.

For the cyclopentenone conjugate addition approach to prostaglandins to be useful, good syntheses of the chiral lower chain and cyclopentenones must be available. Some preparations of the former have already

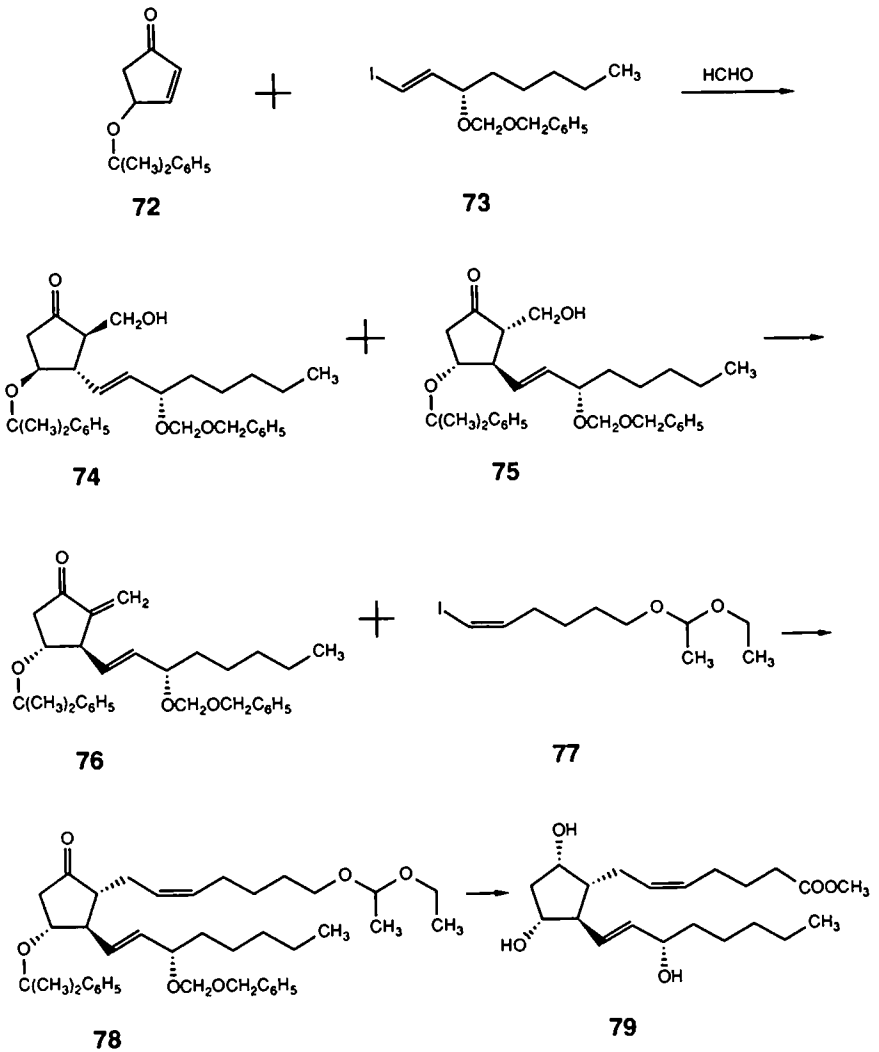

FIGURE 14 Stork synthesis of $PGF_{2\alpha}$ part I. Although diastereomer separation was postponed to the $PGF_{2\alpha}$ stage, only the desired isomers of compounds [76] and [78] are shown, for convenience.

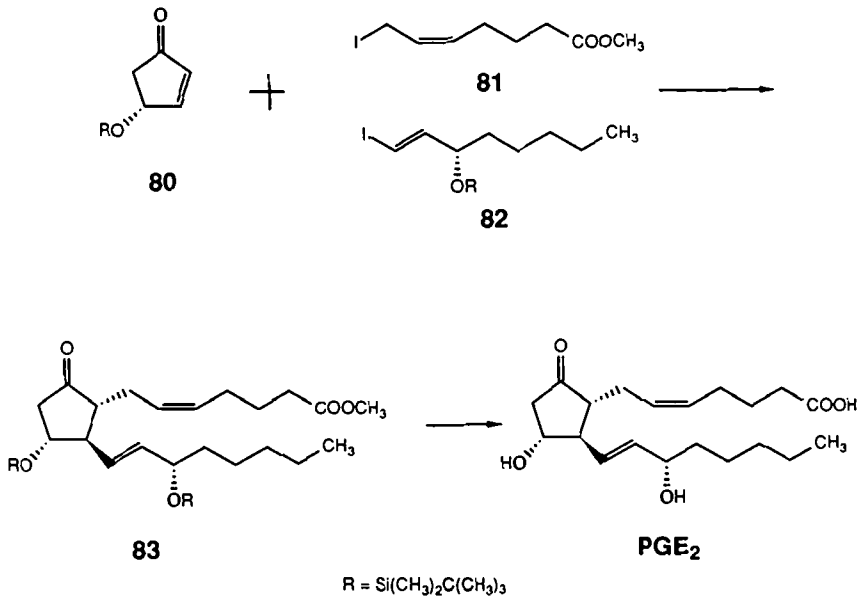

FIGURE 15 Noyori synthesis of PGE_2.

been discussed. A few of the more interesting approaches to the latter are shown in Figs. 16–18.

One synthesis of cyclopentenone [80], requiring a resolution, involved initial ring contraction of phenol when treated with alkaline hypochlorite (49). Resolution of the resulting *cis* acid [85] was effected with brucine. The desired enantiomer [86] formed the more soluble brucine salt and was thus obtained from the mother liquors of the initial resolution. Oxidative decarboxylation with lead tetracetate, partial dechlorination with chromous chloride, and alcohol protection gave chloro enone [87]. Zinc-silver couple (50) dechlorinated [87] to the desired cyclopentenone [80].

Use of the chiral carbon pool for cyclopentenone preparation is also known. The fungal metabolite terrein [88] was selectively monoacetylated and then reduced with chromous chloride to enone [89]. Acetylation and olefin cleavage with ruthenium tetroxide and sodium periodate led to aldehyde [90], which was readily decarbonylated to [65] (51). An alternative route (52) began with the less common *S,S*-tartaric acid [91], converted in four steps to diiodide [92]. Dialkylation of methyl methylthiomethyl sulfoxide with [92] gave the cyclopentane derivative [93]. Treatment of [93]

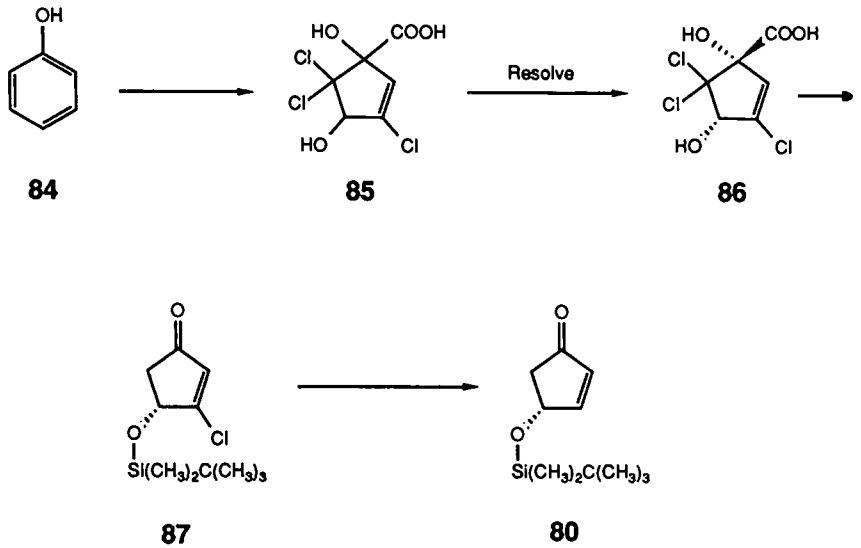

FIGURE 16 Cyclopentenone synthesis involving resolution.

with sulfuric acid in ether liberated the masked carbonyl and caused elimination and deprotection of the C—3 alcohol to give directly [65]. The material thus obtained had an e.e. of about 85%, as estimated by nuclear magnetic resonance.

An intriguing synthesis of chiral cyclopentenone [100] from D-glucose has recently been described (53). The readily available diacetone glucose [94] was benzylated, selectively deprotected, and oxidatively cleaved to the aldehyde, which was condensed with nitromethane to adduct [95]. Acidic hydrolysis of the product gave hemiacetal [96], cleaved with periodate in methanol to aldehyde [97]. Aldol-type cyclization was effected with triethylamine; subsequent dehydration to [98] was induced by mesylation. The nitro olefin [98], upon treatment with activated lead in an acidic media, was converted to ketone [99]. Mesylation in the presence of triethylamine then led directly to cyclopentenone [100].

Two asymmetric synthesis approaches to chiral cyclopentenone derivatives can be envisaged. The first, reduced to practice by Noyori (43), involved reduction of cyclopentene-1,4-dione with lithium aluminum hydride chirally modified with binaphthol to give *R*-4-hydroxycyclopent-2-en-1-one in 94% e.e. Alternatively, manganese dioxide oxidation of allylic alcohol [40] (Fig. 7), in analogy to the *cis* isomer (54), would be expected to give the same enone.

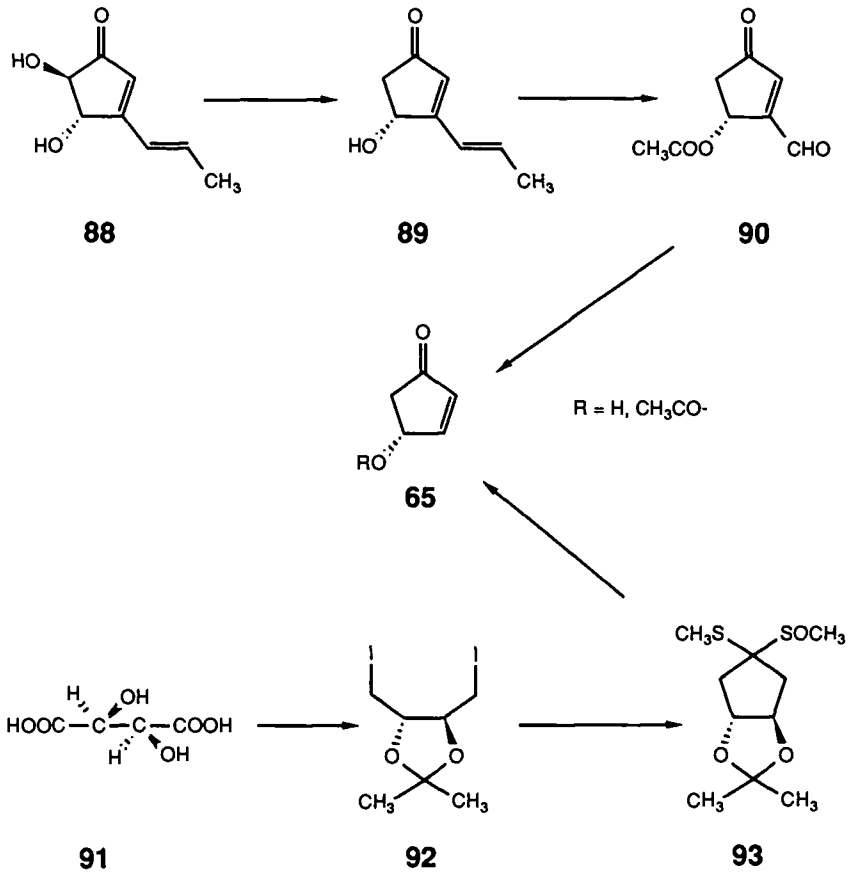

FIGURE 17 Chiral carbon pool approaches to cyclopentenones, part I.

C. Stork Synthesis

There is one synthesis of $PGF_{2\alpha}$ that cannot be classified with any others. It is, however, such an elegant route to $PGF_{2\alpha}$ both in concept and execution, that its inclusion in this discussion of prostaglandin syntheses is mandatory. Stork (55) initiated the synthesis with lactone [101] (Fig. 19), a commercially available material obtained from D-glucose [52] by homologation with cyanide, followed by hydrolysis. Lactone partial reduction with sodium borohydride and *bis*(isopropylidenation) gave [102]. Further reduction with borohydride, selective acetylation of the primary alcohol, and elimination of the vicinal hydroxyl groups by heating with dimethyl-

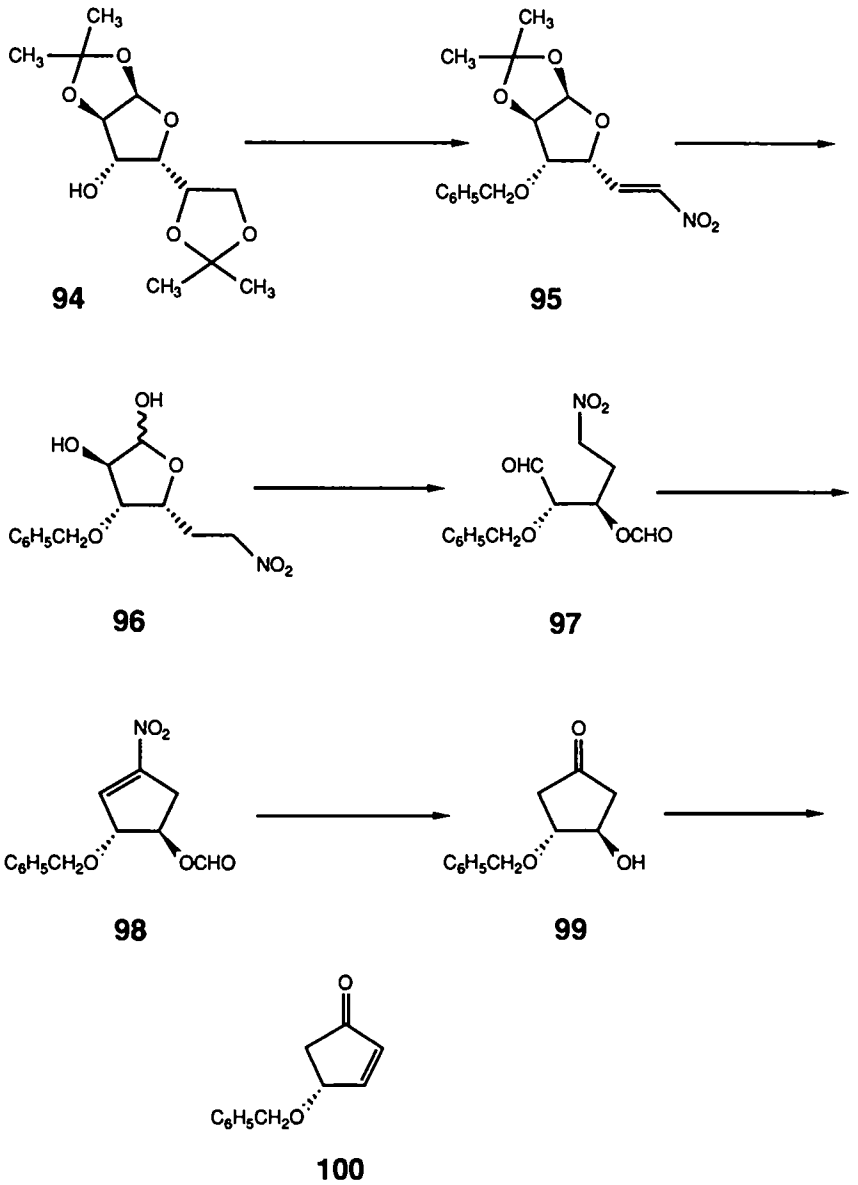

FIGURE 18 Chiral carbon pool approaches to cyclopentenones, part II.

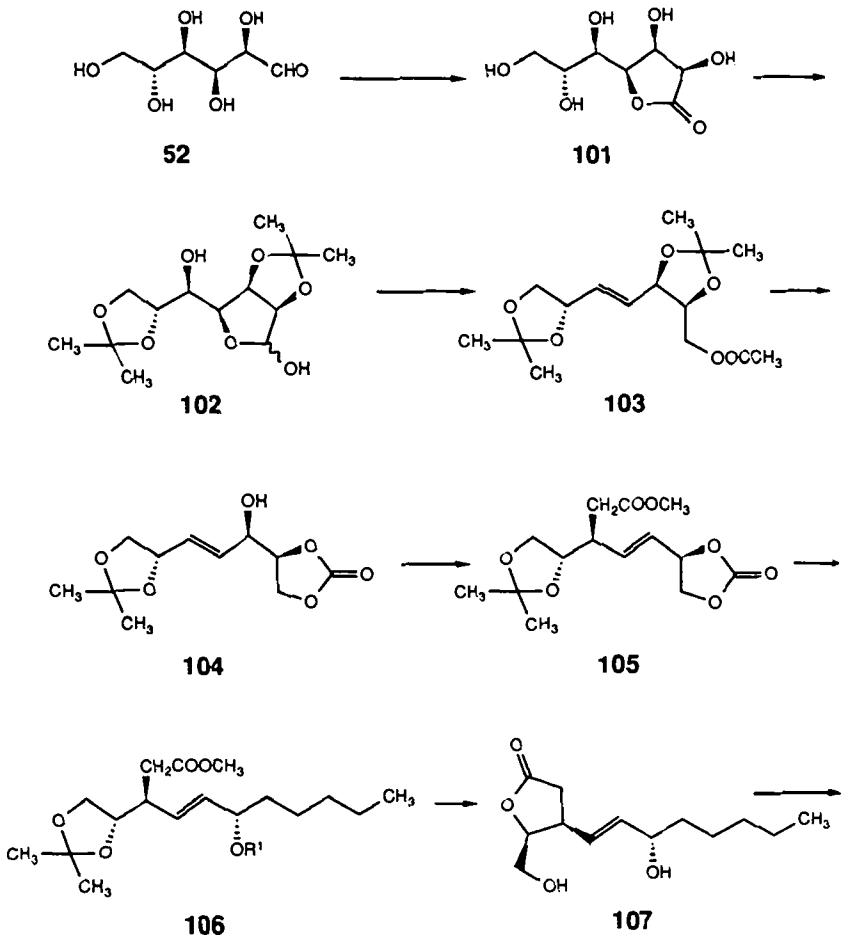

FIGURE 19 Stork synthesis of $PGF_{2\alpha}$, part II.

formamide dimethyl acetal led to olefin [103]. The allylic alcohol [104], needed for a projected orthoester Claisen rearrangement, was obtained from [103] by ester hydrolysis, treatment with methyl chloroformate to give the mixed carbonate, isopropylidene group hydrolysis (with concomitant cyclic carbonate formation), and reprotection of the terminal glycol. The Claisen rearrangement proceeded as anticipated, with complete transfer of asymmetry, to give ester [105].

The lower side chain of $PGF_{2\alpha}$ was elaborated by selective cleavage of

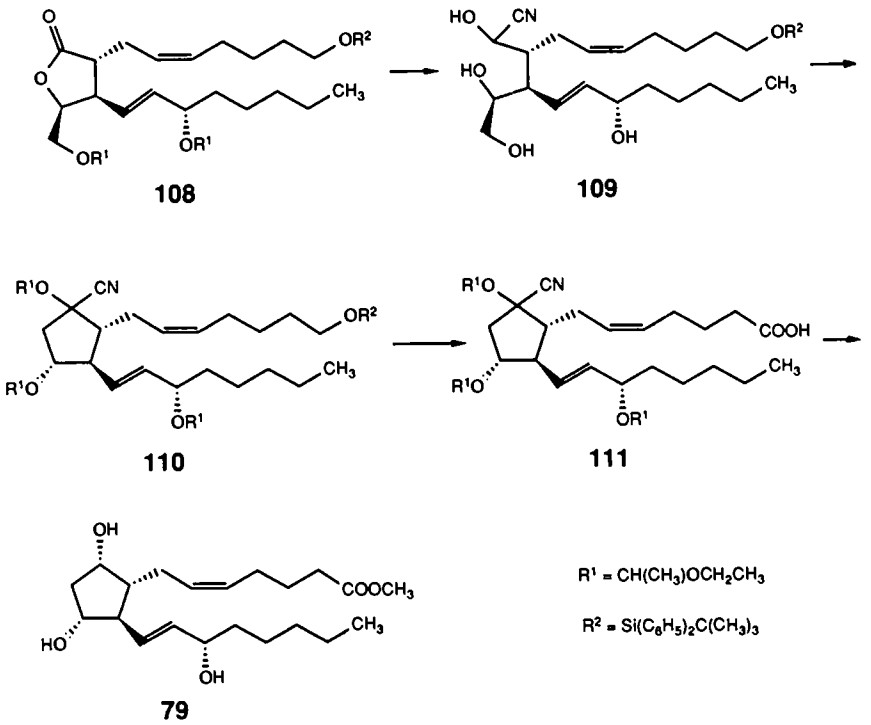

FIGURE 19 Continued

the carbonate, protection of the primary and secondary hydroxyl groups as a tosylate and ethoxyethyl ether, respectively, and reaction with lithium dibutylcuprate. Treatment of [106] with acid led to deprotection and lactonization to [107]. Protection of the alcohol groups and alkylation of the lactone enolate with the diphenyl-*t*-butylsilyl either of Z-7-bromo-5-hepten-1-ol introduced the top side chain.

At this point, formation of the cyclopentanone ring was undertaken. Lactone to lactol reduction of [108] was followed by cyanohydrin formation, giving monoprotected pentaol [109]. The primary alcohol was selectively tosylated and the remaining three alcohols converted to their ethoxyethyl ethers. Base-induced cyclization gave protected cyclopentanone [110]. Selective cleavage of the silyl protecting group and oxidation gave acid [111]. Ether hydrolysis (with cyanohydrin reversal) and selective ketone reduction completed the synthesis of $PGF_{2\alpha}$, which was characterized as its methyl ester [79].

It is worthy or note that, of the five chiral centers in lactone [101], two (C—2 and C—6) appear in the final product as C—11 and C—15.

III. CONCLUSION

The preparation of a chiral pharmaceutical in enantiomerically homogeneous form is clearly a viable proposition. The tools—resolution, asymmetric synthesis, and the chiral carbon pool—are available. As exemplified by the prostaglandins, the manner in which these tools are used is limited only by the imagination and inventiveness of the chemist.

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8

Enzymatic Synthesis and Resolution of Enantiomerically Pure Compounds

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I. INTRODUCTION

Biocatalysis is one of a number of forms of chemical catalysis (Fig. 1) that can be utilized to synthesize a variety of organic chemicals. Over 60% of the 135 MM tons of organic chemicals produced in the United States involve a catalytic step somewhere in their manufacture (1,2). In recent years many reports and reviews extolling the virtues of biocatalysis for the production of chemicals have been released (e.g., 3–9). However, there have still been very few examples of commercial chemical processes introduced in the last few years that utilize a biocatalyst, for example, the acrylamide process (10–12). There has been small but growing concern as to the validity of the expectations placed on bioconversion-based chemical process (13).

The two major advantages that a biocatalyst may offer over a chemical counterpart can be summarized as mild reaction conditions and catalytic specificity. Enzyme/substrate interactions can significantly lower the activation energy of a chemical reaction. For this reason, enzymes exhibit high catalytic activities under mild reaction conditions, low temperatures and pressures, primarily around neutral pH. These egregious reaction conditions also result in the production of less toxic/undesirable by-products, that is, waste minimization, a growing advantage to biology-based processes.

The reaction specificity associated with enzymes reinforces the benefit of waste minimization due to their inherent ability to produce a homoge-

1. HOMOGENEOUS

2. HETEROGENEOUS
   —nonuniform, e.g. Co, Rh, Pd on supports
   —uniform, e.g. zeolites
   —biocatalysis

FIGURE 1 Types of chemical catalysis.

neous product. Enzymes can also exhibit remarkable regiospecificity toward their substrate. They can differentiate between equivalent position or groups of similar reactivity. Finally, there is one form of enzymatic specificity that most observers, as well as practitioners, agree is perhaps the single attribute of greatest potential: stereoselectivity (13–15).

II. METHODS OF PRODUCTION OF CHIRAL COMPOUNDS

Process options for the production of homochiral compounds are summarized in Fig. 2. The three basic routes are separation of racemic mixture, synthesis using a naturally occurring chiral synthon, and asymmetric synthesis using a prochiral intermediate. Historically, the efficiency of asymmetric synthesis has been capricious in terms of chemical and optical yield. Hence, from a practical, commercial process perspective, resolution via diastereomer crystallization has remained important for many commercial scale processes, for example, diltiazem.

Asymmetric synthesis has advanced significantly in recent years with the advent of optically active reagents, auxiliaries, and catalysts. Both chemical and biological systems have been developed during this time. For example, Fig. 3 illustrates a recently published synthetic route to diltiazem, an important calcium channel blocker (16). The process starts with the chiral auxiliary (1*R*,2*S*)-2-phenylcyclohexanol, and proceeds through a chiral (aryl)oxirane, with two chiral centers formed by induction from the original auxiliary. The chiral epoxide is then opened with 2-aminobenzenethiol and the original chiral auxiliary recovered (Step 4). Finally, the molecule is cyclized, aminoalkylated, and acylated, resulting in the desired product.

Similarly, an example of a biological asymmetric synthesis is shown in Fig. 4. In this case, α-methylbenzylamine is synthesized in high chiral

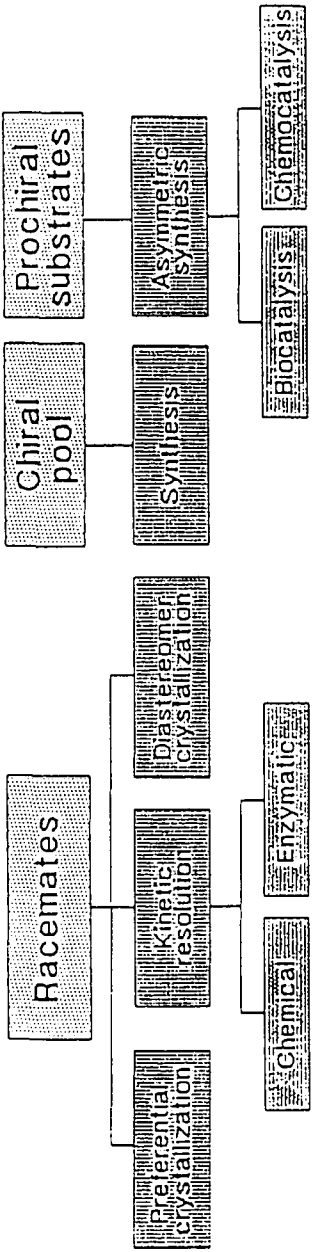

FIGURE 2 Routes to optically active compounds.

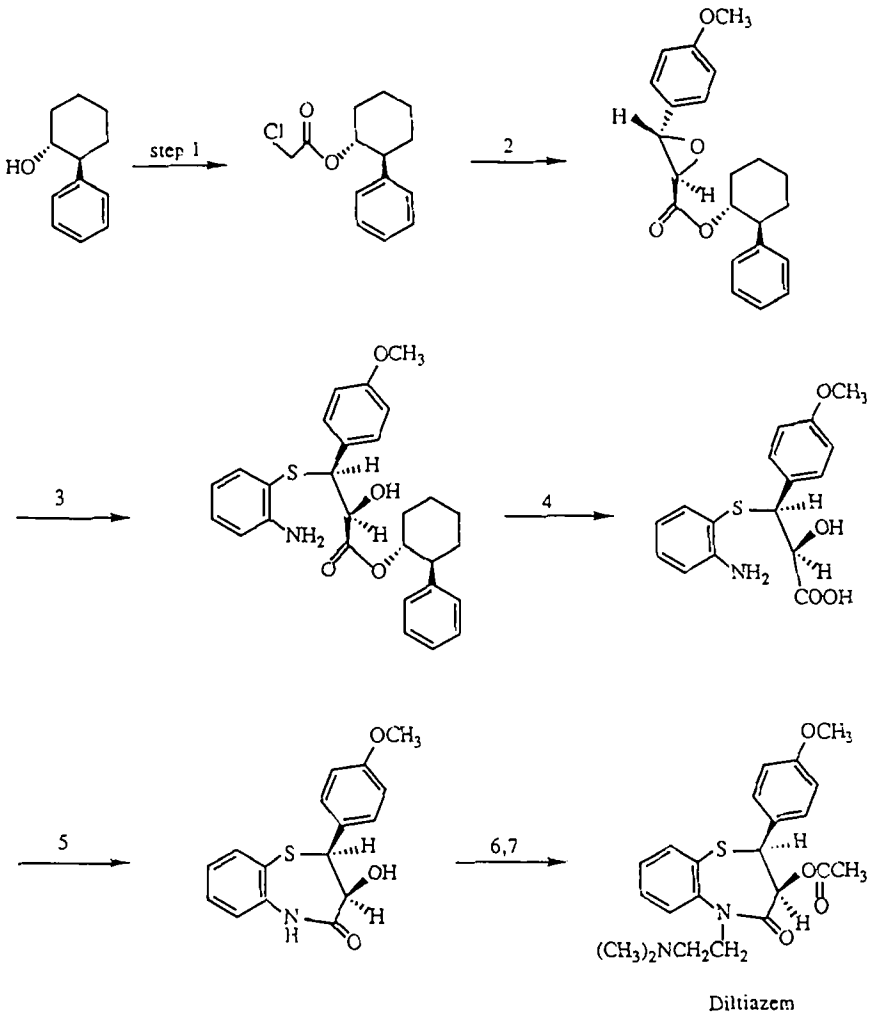

FIGURE 3 Synthesis of diltiazem using a chiral auxiliary.

purity (>99% e.e.) using the prochiral ketone acetophenone and a suitable amino donor (17,18). The chiral catalyst in this case is an aminotransferase.

The resolution of racemic mixtures through fractional crystallization of diastereomeric salts is traditionally used for organic acids and bases (19). Often the separation requires multiple recrystallizations and, in addition to the desired product, results in the production of the unwanted isomer

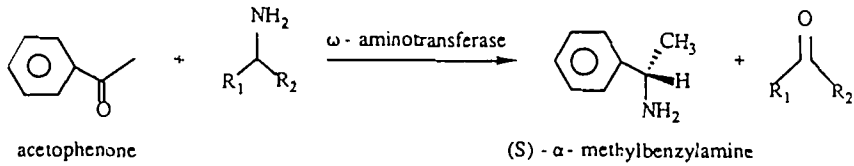

FIGURE 4 Asymmetric synthesis of (*S*)-α-methylbenzylamine using an aminotransferase.

that has to be racemized and recycled. This type of chemical resolution can be improved if the selective crystallization of the desired diastereomeric salt is combined with an in situ racemization. Such a scheme was used to good effect in the synthesis of a potent, periferal Cholecystokinin antagonist by Merck scientists (20). A critical chiral amine center was racemized using 3,5-dichlorosalicylaldehyde in the presence of the resolution agent, camphor sulfonic acid, resulting in a resolution yield of >90%.

Resolution by entrainment can sometimes be used to separate racemic mixtures when there are distinct differences in the rates of crystallization of the two optical isomers. This preferential crystallization is initiated by seeding with the crystals of one enantiomer. This technique has been shown to be effective in the production of thiamphenicol (21).

Kinetic resolution incorporates many of the attributes of asymmetric synthesis. These processes involve the selective destruction of one of the two enantiomers through discriminate consumption by an optically active reagent or catalyst. Since 1980, a number of reports have appeared describing chemical kinetic resolutions (22–24). An obvious advantage exists when the kinetic resolution agent is catalytic in nature. Consequently, the prototypical kinetic resolution catalyst would be an enzyme. A major advantage of kinetic resolution is the ability to control the optical purity of the product. Figure 5 depicts an aminotransferase-based kinetic resolution where the relative rates on the *S* and *R* enantiomers are 20:1, respectively. Therefore, if an enantiomeric excess (e.e.) greater than 99% is desired, then about 60% conversion is required. If the enzyme reaction is reversed (Fig. 5) and the *S* amine synthesized from the prochiral ketone, then the e.e. of the product is independent of conversion and is determined by the inherent enantioselectivity of the reaction, i.e., 95% e.e.

A potential disadvantage of a kinetic resolution-based process vs. an asymmetric synthesis is the requisite recycle of the by-product or unwanted enantiomer. Examples discussed later demonstrate the production of an unwanted enantiomer (Fig. 6) or undesirable by-product (Fig. 7). These potential yield losses can be avoided if either the unwanted enantio-

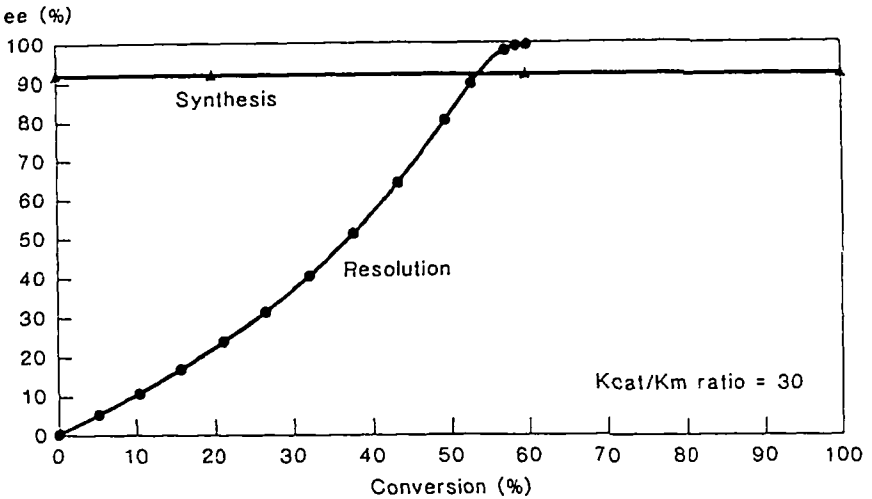

FIGURE 5 Kinetic resolution and chiral synthesis using an aminotransferase.

mer can be racemized in place or the coproduct easily recycled. Figure 8 shows an example where in situ racemization was possible and provided an efficient process for the production of amino acid (25). Figure 9 shows the kinetic resolution of racemic α-methylbenzylamine using an ω-amino acid aminotransferase (17). The coproduct in this process is the prochiral

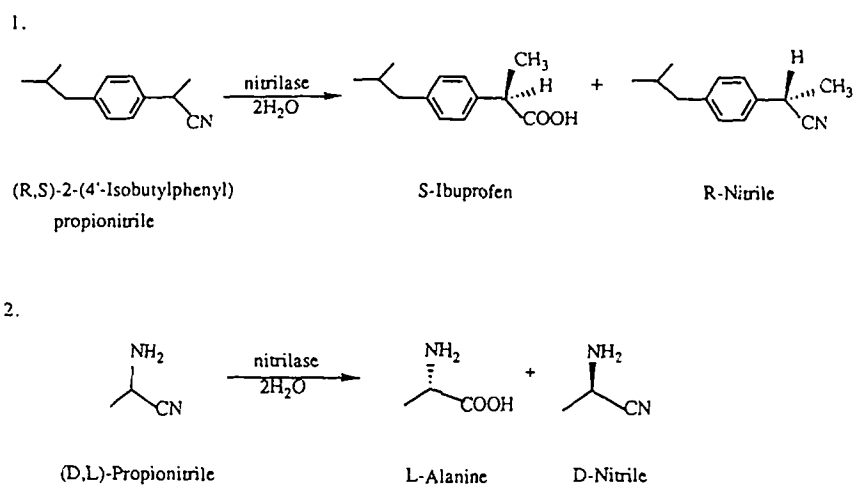

FIGURE 6 Use of nitrilase to produce chiral carboxylic acids.

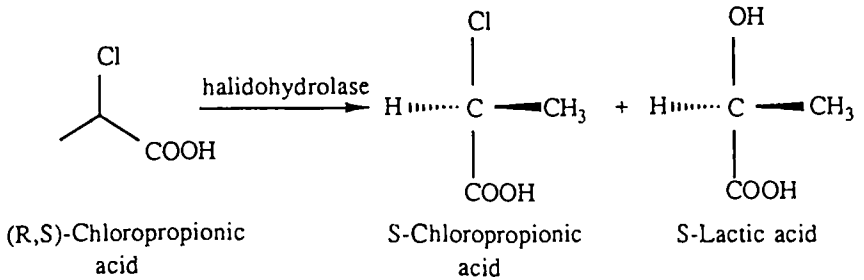

FIGURE 7 Production of L-chloropropionic acid.

ketone acetophenone that can be directly recycled into the reductive animation step used to prepare the racemic raw material. Such a facile recycle results in both a simple process and a high yield based on prochiral ketone consumption, hence a more economic process.

III. ENZYME-BASED PRODUCTION OF HOMOCHIRAL COMPOUNDS

It has been estimated that biochemists have isolated and characterized over two-thirds of the projected 2000 naturally occurring enzymes (26). Enzymes are known to catalyze a myriad of different chemical reaction types. Table 1 summarizes the classification of enzymes based on function and provides examples of the kind of stereoselective reactions exhibited by

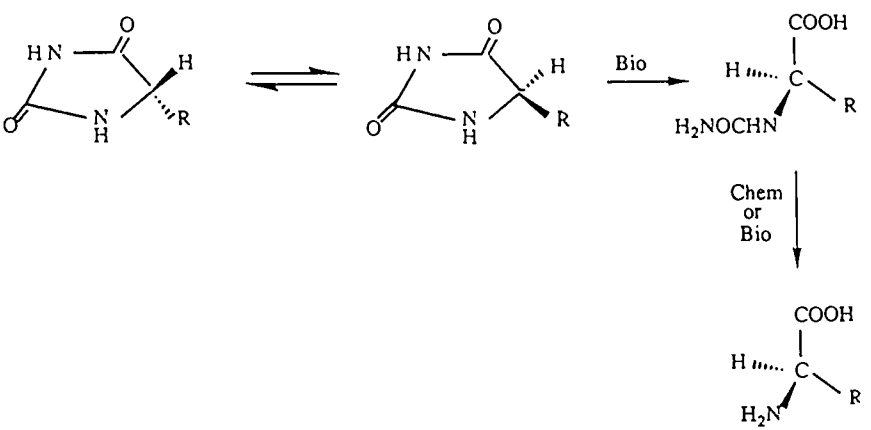

FIGURE 8 Production of optically active α-amino acids via enzymatic hydrolysis of the corresponding hydantoins.

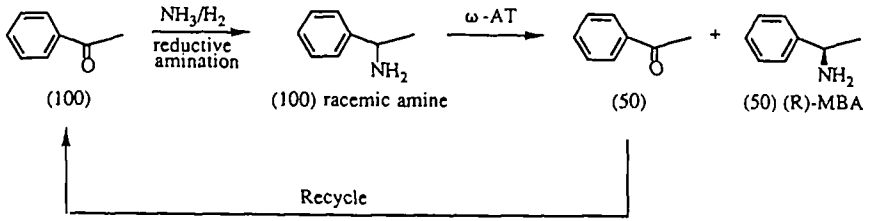

FIGURE 9 Kinetic resolution of α-methylbenzylamine using an ω-aminotransferase.

TABLE 1 Enzyme Types, Function, and Potential Products

Enzyme group	Reaction types	Potential chiral products
Oxidoreductase	Oxidation	Alcohol
		Epoxide
		Sulfoxide
		Amino acid
		Lactone
	Reduction	Alcohol
		Lactone
Transferase	Hydroxymethyl transfer	Hydroxyamino acid
	Amino group transfer	Amino acid
		Amine
Hydrolase	Ester hydrolysis	Alcohol/carboxylic acid/carboxylic ester
	(*Trans*) esterification	Alcohol/carboxylic acid/carboxylic ester
	Nitrile/amide hydrolysis	Carboxylic acid
	Hydantoin hydrolysis	Amino acid
	Alkylhalide hydrolysis	Haloalkanoic acid
		Alcohol/epoxide
Lyase	C-C formation	Amino acid
		Acyloin
		Cyanohydrin
	C-O formation	Alcohol
		Amino acid
	C-N formation	Amino acid
Isomerase	Lactone formation	Lactone

the various groups. The following potpourri of enzyme-catalyzed chiral resolutions and syntheses is not intended to be comprehensive in nature, but rather to exemplify the diverse yet prodigious selectivity exhibited by these biocatalysts.

A. Oxidoreductase: Oxidation

The insertion of an oxygen atom(s) into a molecule in a regioselective/stereoselective manner can be catalyzed by a broad group of enzymes. The target molecules are diverse in nature as illustrated by the following examples. The source of oxygen can be dioxygen or water.

1. *Alcohol*

Oxygenases can insert one or both atoms of dioxygen into many types of compounds and in some cases creating chiral centers (27). Toluene dioxygenase has been shown to oxidize toluene (Fig. 10) to produce *cis*-dihydrodiol (28,29). The overall oxidation involves the insertion of two atoms of oxygen, producing two chiral alcohol centers. Interestingly, the same enzyme can catalyze the stereoselective mono-hydroxylation of certain substrates. Figure 11 shows the products obtained from the oxidation of deuterated indene using toluene dioxygenase (30).

2. *Epoxide*

Many mono-oxygenases have been shown to epoxidize a variety of olefinic substrates (27). In a few cases, this fortuitous enzyme reaction has been demonstrated to be stereoselective in nature. The ω hydroxylase from *Pseudomonas oleovorans* was reported to oxidize 1,7-octadiene, producing the *R* enantiomer of the epoxide in high enantiomeric excess (31). Workers at Shell Research Limited showed that a similar *Pseudomonas oleovorans* mono-oxygenase system could produce a key epoxide intermediate used for the production of the β-adrenergic receptor-blocking drug (*S*)-atenolol (Fig. 12) (32).

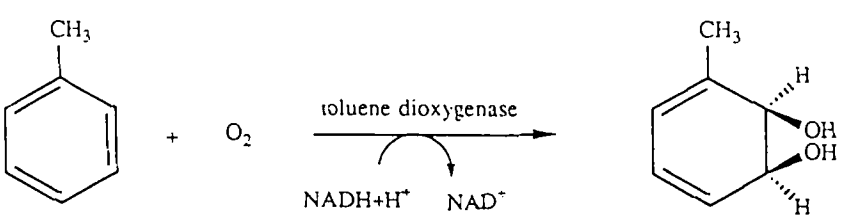

FIGURE 10 Oxidation of toluene.

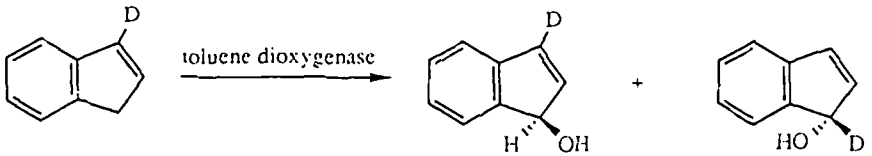

FIGURE 11 Benzylic mono-oxygenation of indene.

3. *Sulfoxide*

Reports on the ability of microorganisms to selectively oxidize various types of sulfides to the corresponding chiral sulfoxides have grown significantly in the last few years (33). Chiral sulfoxides are evident in a variety of pharmaceutical and agricultural chemical compounds (33). The ω hydroxylase from *Pseudomonas oleovorans* has been shown to produce a variety of chiral aliphatic sulfoxides from corresponding sulfides (34). Vinyl sulfox-

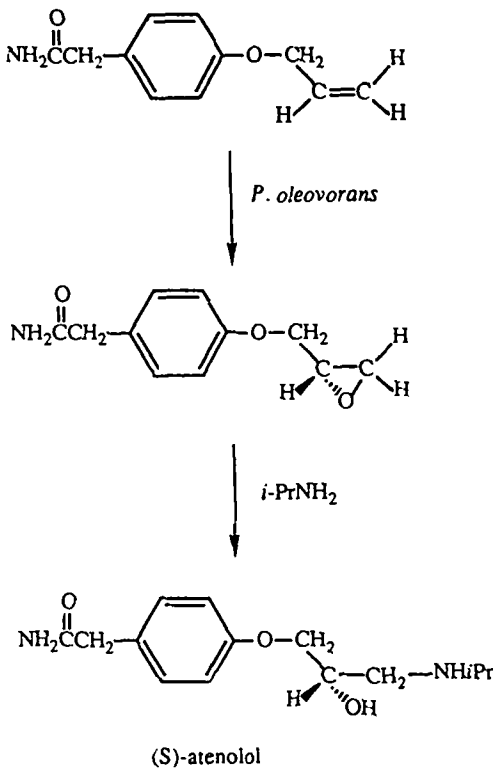

FIGURE 12 Microbial epoxidation route to (*S*)-atenolol.

ides can be synthesized using the fungus *Mortierella isabellina* (33). Figure 13 shows the enantioselective sulfoxidation of the substrate vinyl sulfide, which gave a 45% yield and an enantiomeric excess of 95% (33).

4. Amino Acid

Microbial enzymes catalyzing the oxidative deamination of primary amines are classified into two groups: flavoprotein amine oxidases and copper-containing amine oxidases. Some of the copper-dependent enzymes have recently been shown to contain the prosthetic group pyrroloquinoline quinone (PQQ) (35,36). Amino acid oxidases show a stereoselective preference for either the D or L isomers of amino acids. The enzymes catalyze the dioxygen-dependent oxidation of the amino acid to the corresponding α-keto acid, ammonia, and the concomitant reduction of molecular oxygen to hydrogen peroxide.

5. Lactone

Chiral lactones can be formed from ketones via the Baeyer–Villiger reaction. Such lactones are potentially useful synthons for a number of natural products (37). Many of the examples of enantioselective lactone formation have been demonstrated using cyclohexanone oxygenase isolated from various *Acinetobacter* species (37,38). Figure 14 shows the enzymatic lactonization of methylcyclohexanone, which gave an 80% yield with an enantiomeric excess greater than 98%.

B. Oxidoreductase: Reduction

1. Alcohol

Many microorganisms possessing alcohol dehydrogenases that are capable of reducing ketones and diketones have been demonstrated to produce chiral alcohols. Examples of such enantioselective reductions have been reviewed on many occasions (3–8). The main advantage of a secondary alcohol dehydrogenase for the production of chiral alcohols

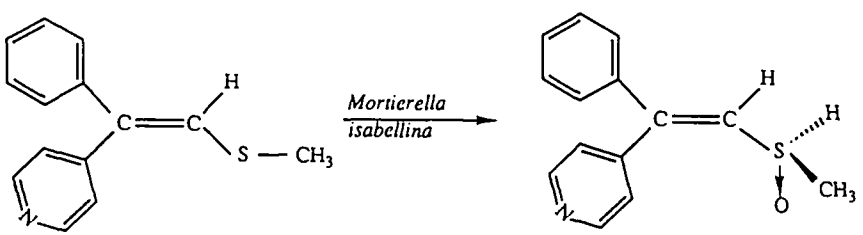

FIGURE 13 Enantioselective microbiological sulfoxidation.

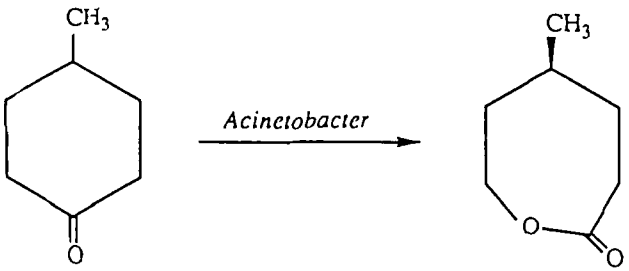

FIGURE 14 Enzymatic Baeyer–Villiger reaction.

over indirect routes such as ester resolutions is the overall yield. Being an asymmetric reduction, the yield should approach 100% as opposed to 50% for the resolution methods. The inherent disadvantage of dehydrogenase systems is the obligatory cofactor requirement, that is, nicotinamide adenine dinucleotide. This has restricted the use of such enzymes to mainly whole-cell-based processes. These systems generally have poor productivities plus unwanted side reactions and by-products.

For these reasons, recent efforts have been aimed at cheap, efficient cofactor recycle systems for use with cell-free alcohol dehydrogenase preparations. Regeneration of the nicotinamide adenine dinucleotide cofactor required for alcohol synthesis can be catalyzed by a second enzyme or reduced by the same enzyme, provided the overall equilibrium is favorable.

Formate dehydrogenase in conjunction with polyethyleneglycol-immobilized nicotinamide adenine dinucleotide has been used to good effect as a cofactor recycle system (39). The alcohol dehydrogenase from *Thermoanaerobium brockii* catalyzed the reduction of ketones independently when driven by the cooxidation of isopropanol (40,41).

Lactone. Various fungi, including Bakers' yeast and *Geotrichum candidum*, have been shown to produce optically active lactones, useful chiral synthon, via the stereoselective reduction of suitable unsaturated precursors (42). Figure 15 shows the production scheme for a chiral, substituted diketone, a synthon for optically active carotenoids.

C. Transferase: Hydroxymethyl Transfer

1. α-Hydroxyamino Acid

Considerable efforts have been made over many years toward developing a microbial process (whole-cell and enzyme-based) for the production of L-serine (43–46). The enzyme of choice for these processes has been

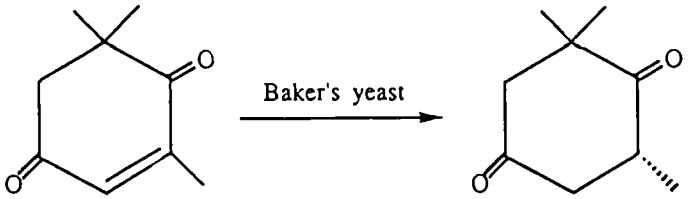

FIGURE 15 Stereospecific reduction of oxo-isophorone.

serine hydroxymethyltransferase that is found in many different types of microorganisms (43,45). The enzyme catalyzes the reversible aldol condensation between glycine and formaldehyde, yielding L-serine. The activity of the enzyme is dependent on pyridoxal-5′-phosphate, a covalently bound coenzyme. In addition, tehrahydrofolate is required as a free coenzyme serving as the C_1 acceptor. Some of these enzymes have been shown to be active with other α-hydroxylamino acids, for example, L-threonine, L-phenylserine (47).

D. Transferase: Amino Group Transfer

Aminotransferases are a ubiquitous group of enzymes that are not only important in intermediate metabolism, but also are critical for the maintenance of nitrogen homeostasis. In addition to their critical role in nature, they are finding prominence as industrial biocatalysts (48). Aminotransferases catalyze the transfer of an amino group, a proton, and a pair of electrons from a primary amine substrate to the carbonyl group of an acceptor molecule. Figure 16 shows the overall reaction sequence catalyzed by these enzymes, indicating the reversible nature of the reaction. Most aminotransferases investigated have been shown to be pyridoxal-5′-phosphate-dependent enzymes and the reaction mechanism has been well characterized (48). As the primary metabolic role of these proteins is the transfer of an amino group from an α-amino acid to a 2-keto acid acceptor,

FIGURE 16 Aminotransferase reaction.

their industrial exploitation has until recently been focused on the production of α-amino acids.

1. Amino Acid

The potential and actual use of aminotransferases for the commercial synthesis of amino acids has been well documented (49–58). The vast majority of this work was focused on L-α-amino acids, using both whole-cell and free enzyme systems. The inherent equilibrium problem associated with the reversible nature of the reaction has been cleverly overcome by the use of coupled enzyme systems. When L-aspartic acid is used as the amino donor for the transamination reaction catalyzed by aspartate aminotransferase, the products are the desired α-amino acid plus oxaloacetic acid (Fig. 17). This 2-ketoacid by-product can be efficiently removed using oxaloacetate decarboxylase, producing pyruvic acid and carbon dioxide. Coupling the two enzymes in this manner results in driving the conversion of the amino acceptor to the desired amino acid product with a high overall yield (>90%), for example, L-phenylalanine with whole-cell systems (52,53) or enzyme-based systems (51,55).

2. Amine

It has recently been shown that aminotransferases can be used to produce homochiral amine products that do not contain an α-carboxylic acid (17,18,48). Enzymes capable of catalyzing such reactions belong to the group of ω-aminotransferases. The reversible nature of the transamination reaction makes it possible for both enantiomers of a given amine to be produced using a single enzyme (Fig. 18). The racemic amine can be

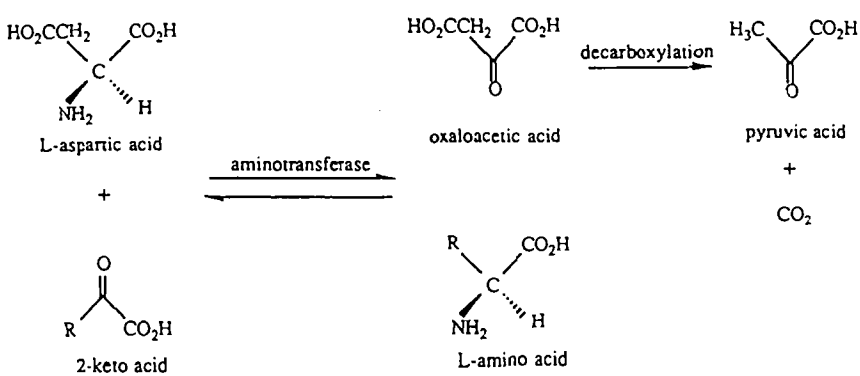

FIGURE 17 Scheme for production of L-amino acids.

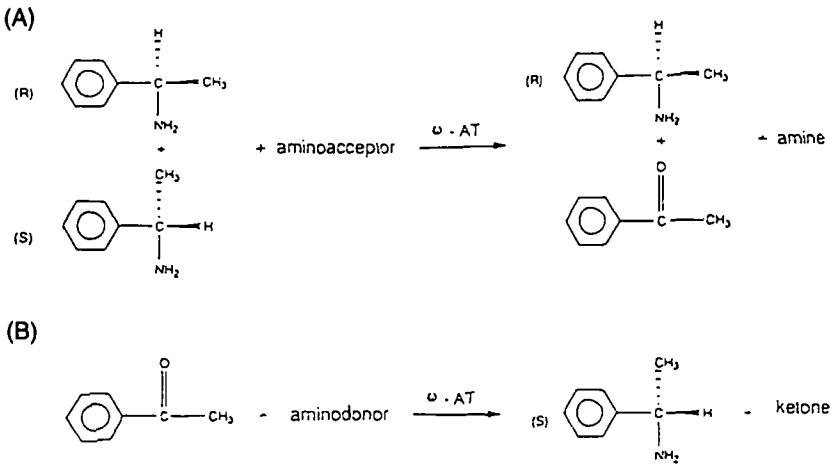

FIGURE 18 Production of both enantiomers of α-methylbenzylamine in high e.e. using a single enzyme.

resolved to yield one enantiomer (Fig. 18A), or conversely, the other enantiomer can be synthesized using the corresponding prochiral ketone as the amino acceptor (Fig. 18B) (17). This technology has been developed and applied to the commercial production of a wide variety of homochiral amine products, for example, pharmaceutical products/intermediates, chiral auxiliaries, and resolution agents (18,48).

E. Hydrolase: Ester Hydrolysis

Hydrolytic enzymes, for example, esterases and lipases, are ostensibly well suited for synthetic applications due to the lack of cofactor/coenzyme involvement and their typically broad substrate specificity. In addition, some of these enzymes have the ability to effect the enantiotopically selective hydrolysis of prochiral substrates with enantiotopic ester groups (59–61). The major potential drawback for the application of most esterases is their often modest enantioselectivity. Therefore, to develop a process for a particular product may necessitate the screening and evaluation of many enzymes. Another significant factor driving the application of lipases/esterases is their commercial availability. Over 20 such catalysts are currently on the market, mainly derived from fungal sources (62). Most can be purchased in kilogram quantities ranging from $100–10,000 per kg depending on source and activity (62).

1. *Alcohol/Ester*

A process utilizing a stereospecific ester cleavage yielding the product *R*-glycidylbutyrate in high optical purity has recently been scaled to commercial levels (63). The process catalyzed by porcine pancreatic lipase is shown in Fig. 19. *R*-glycidylbutyrate is a useful chiral synthon for a variety of commercial products, for example, β-blockers.

2. *Carboxylic Acid*

Two groups of chiral aromatic carboxylic acids are important commercial intermediates for the agricultural and pharmaceutical industries. The *R* enantiomer of α-phenoxypropionic acids confers biological activity for a number of herbicides. The *S* enantiomer of a variety of arylpropionic acids is the biologically active form of the nonsteroidal antiinflammatory products labeled profens. Racemic mixtures of the alkyl esters of these propionic acid derivatives have been effectively resolved to yield the desired optically active carboxylic acids (64–66). Figure 20 shows examples of the resolution of aromatic propionic acid esters.

F. Hydrolase: (*Trans*)Esterification

It is generally held that an aqueous environment is desirable, although not a prerequisite, for the optimal activity of enzymes. It is assumed that in most cases, placing an enzyme in an organic medium will cause deleterious effects to the tertiary structure of the protein. However, if this was not the case, there would be potential advantages for enzyme catalysis in an organic medium. It would clearly facilitate conversion of water-insoluble substrates and aid in product recovery. The thermodynamic equilibria of certain reactions are unfavorable in aqueous systems. Enzyme stereoselectivity has been shown to improve in some nonaqueous enzyme transformations (67,68).

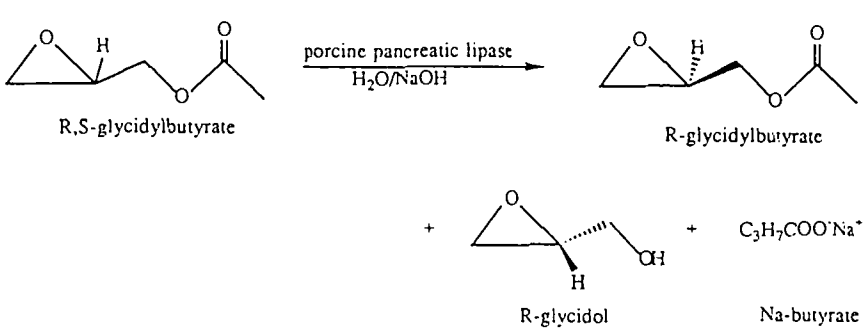

FIGURE 19 Resolution process for the production of *R*-glycidylbutyrate.

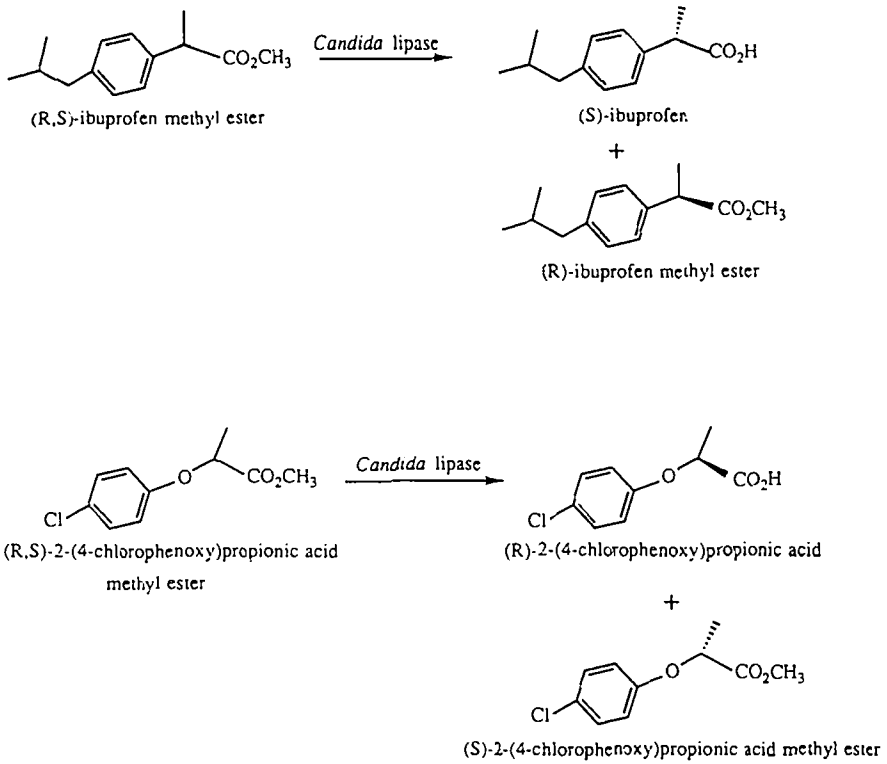

FIGURE 20 Lipase solution of (*R,S*)-arylpropionic acid and (*R,S*)-α-phenoxypropionic acid esters.

The types of solvent systems employed range from water-miscible cosolvents, for example, acetone, dimethylformamide, through biphasic water/water-immiscible solvent systems to anhydrous organic media with less than 1% water present. Guidelines are beginning to emerge as to the choice of organic solvent plus desirable water content of the medium for a particular enzyme and reaction (9,67). The vast majority of enzymes used for preparative purposes in organic media have been hydrolases.

1. *Alcohol/Carboxylic Acid/Ester*

Racemic mixtures of carboxylic acids can be resolved by esterification using lipases in organic media. Figure 21 illustrates such a process when racemic α-bromopropionic acid is stereoselectively esterified with *n*-butanol using a lipase from the yeast *Candida cylindraceae* (68). This reaction was carried out in virtually anhydrous hexane.

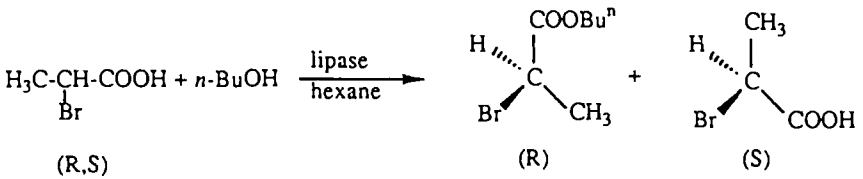

FIGURE 21 Stereoselective esterification of α-bromopropionic acid.

Similarly, enantiomeric mixtures of carboxylic acid esters can be separated using transesterification (Fig. 22). An *n*-butanol/water biphasic medium aryloxypropionic acid methyl ester was transesterified stereoselectively, yielding the butylester of the *R* enantiomer (69).

G. Hydrolase: Nitrile Hydrolysis

The enzymatic hydrolysis of nitriles to yield either the corresponding amides or carboxylic acids has been studied in some detail over the last 10 yr (70,71). The enzymatic hydrolytic cleavage of nitriles can be achieved by two types of hydrolase: nitrile hydratase or nitrilase (Fig. 23). Nitrile hydratase has been commercially exploited for the production of various amides, the most notable being acrylamide (10–12,71,72).

1. *Acid*

Various optically active carboxylic acids can be produced via the activities of nitrile-metabolizing enzymes, for example, from the corresponding cyanohydrins, α-hydroxy acids, α-amino acids, and arylalkanoic acids. Figure 6 shows two types of products derived from the enantioselective action of nitrilase. (*S*)-ibuprofen, the nonsteroidal antiinflammatory agent, can be prepared in high enantiomeric excess by resolving the corresponding racemic nitrile using a nitrilase system from an *Acinetobacter* sp. (73). (*S*)-nitrile is hydrolyzed in one step to (*S*)-ibuprofen. Similarly, (*S*)-aminopropionitrile can be selectively hydrosized by a nitrilase in a single step to L-alanine (74).

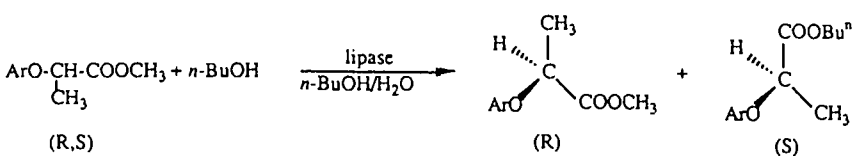

FIGURE 22 Stereoselective *trans*-esterification of aryloxypropionic acid methyl ester.

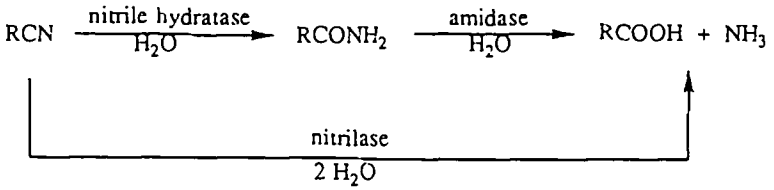

FIGURE 23 Microbial metabolism of nitriles.

Chiral carboxylic acids can also be obtained via a two-enzyme step process involving nitrile hydratase and amidase. Figure 24 shows the scheme for producing L-amino acids using a nonenantiospecific nitrile hydratase and L-enantiospecific amidase in combination (75). An amino peptidase from a *Pseudomonas putida* strain was shown to stereoselectively hydrolyze amides of L-amino acids, yielding the corresponding amino acids (76). In this particular process, the racemic amides were prepared chemically from the nitriles under alkaline conditions in the presence of a catalytic amount of acetone.

H. Hydrolase: Hydantoin Hydrolysis

1. *Amino Acid*

Dihydropyrimidinase- (hydantoinase-) based processes have been successfully employed for the production of D-amino acids, particularly D-*p*-hydroxyphenylglycine (7,25,77,78). D,L-hydantoins are chemically synthesized from the corresponding aldehydes using the Bucherer–Berg

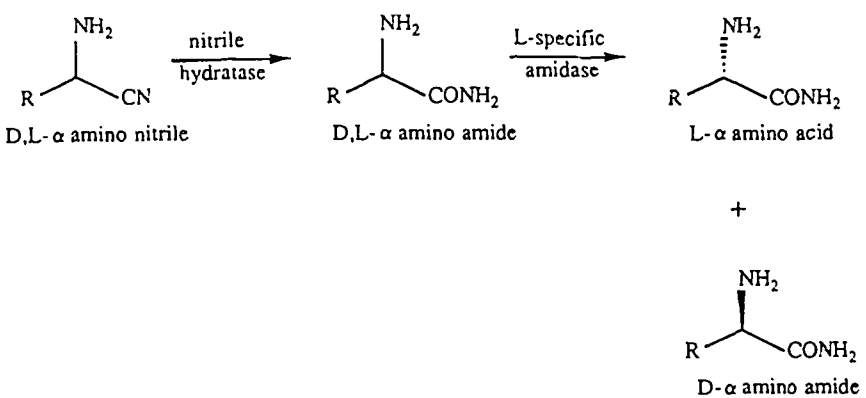

FIGURE 24 Production of L-amino acids using a nonselective nitrile hydratase and a L-stereospecific amidase.

reaction. A D-specific hydantoinase hydrolyzes the D-hydantoin to the D-*N*-carbamyl amino acid. The free D-amino acid is recovered either by treatment with nitrous acid (7,25) or enzymatically using an amidohydrolase (77,78).

The advantage of a hydantoin-based approach for the production of D-amino acids is that the remaining unhydrolyzed *R*-hydantoin is easily racemized under reaction conditions. Therefore in principle, an overall yield approaching 100% is possible. Kanegafuchi has a commercial process based on hydantoinase technology for the production of D-*p*-hydroxyphenylglycine. It produces the racemic hydantoin using an amidoalkylation reaction between phenol, glyoxylic acid, and urea. This circumvents the need to use the relatively expensive *p*-hydroxybenzaldehyde as a raw material for Bucherer–Berg synthesis.

I. Hydrolase: Alkylhalide Hydrolase

Halidohydrolases that catalyze halide hydrolysis are rapidly becoming important commercial catalysts for the synthesis of chiral products and also the bioremediation of industrial waste (79).

1. *Haloalkanoic Acid*

Stereoselective dehalogenation of 2-haloalkanoic acids has been demonstrated for a number of halidohydrolases (80–82). Figure 77 details the production of an L-haloacid intermediate used in the production of phenoxypropionic acid herbicides. The *R* enantiomer of chloropropionic acid is selectively hydrolyzed to (*S*)-lactic acid due to an inversion of configuration that occurs during the hydrolysis (83). (*S*)-2-chloroproprionic acid is used as a chiral synthon to produce a number of (*R*)-phenoxypropionic acid herbicides, for example, Fusilade 2000 (ICI).

2. *Alcohol/Epoxide*

Racemic 2,3-dichloropropan-1-ol has been used as a source of (*S*)-2,3-dichloropropan-1-ol, which is subsequently converted to (*R*)-epichlorohydrin, a very useful chiral synthon (84). Figure 25 shows the overall process whereby a *Pseudomonas* sp. containing an (*R*)-2,3-dichloropropan-1-ol-specific halidohydrolase metabolizes the *R* enantiomer of the raw

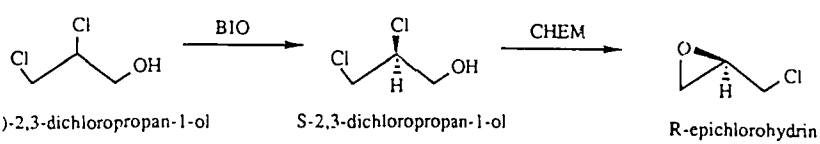

FIGURE 25 Production of (*S*)-2,3-dichloropropan-1-ol.

material, leaving the desired product. The *S*-dichloropropanol can be easily converted chemically to the chiral epichlorohydrin.

J. Lyase: C—C Formation

Lyases are enzymes that cleave C—C, C—O, and C—N bonds by reactions other than hydrolysis or oxidation. They utilize two substrates in one direction but only one in the other. In the latter case, a molecule is eliminated, leaving an unsaturated moiety. When the reverse reaction is the most important, that is, condensation, the enzymes are often designated as synthases.

1. Amino Acid

L-threonine aldolase (L-threonine acetaldehyde-lyase) catalyzes the reversible condensation of acetaldehyde and glycine to form L-threonine. The enzyme has been shown to be an activity distinct from serine hydroxymethyltransferase that also catalyzes the above reaction (85,86). The substrate specifically of the adolase has been demonstrated to be flexible with respect to the aldehyde involved. The enzyme has been shown to form phenylserine derivatives from substituted benzaldehydes and glycine (86).

Tyrosine phenol lyase (β-tyrosinase) has been shown to catalyze the efficient synthesis of the L-amino acids L-tyrosine and L-dopa from pyruvate, ammonia and phenol, or catechol, respectively (87–89).

2. Acyloin

Pyruvate decarboxylase catalyzes the nonoxidative decarboxylation of pyruvate to acetaldehyde and carbon dioxide. When an aldehyde is present with pyruvate, the enzyme promotes an acyloin condensation reaction. The mechanistic reason for this fortuitous reaction is well understood and involves the aldehyde outcompeting a proton for bond formation with a reactive thiamine pyrophosphate-bound intermediate (90,91). When acetaldehyde is present, the product formed is acetoin. Benzaldehyde results in the production of phenylacetylcarbinol (Fig. 26). Both of these condensations are enantioselective, forming the *R* enantiomer preferentially in both cases.

The ability of pyruvate decarboxylase from many microbial sources to produce phenylacetylcarbinol has been exploited for many years in the synthesis of ephedrine, a natural adrenergic compound (92). The acyloin is reductively aminated to produce the ethanolamine product, (1*R*,2*S*)-ephedrine, with two chiral centers.

3. Cyanohydrin

The enzyme mandelonitrile lyase (oxynitrilase) isolated from almond flour has been shown to catalyze the stereospecific cyanohydrination of

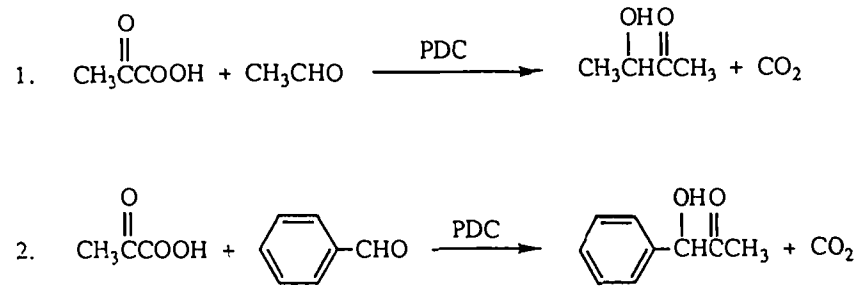

FIGURE 26 Fortuitous reactions of pyruvate decarboxylase (PDC).

aldehydes, yielding the (*R*)-cyanohydrins (Fig. 27) (93). Due to spontaneous nonselective chemical additions of hydrogen cyanide to benzaldehyde, high optical purity of the cyanohydrin is difficult to obtain. The purity can be improved by the judicious use of certain organic solvents (94).

Recently, it has been demonstrated that the enzymatic synthesis of (*S*)-cyanohydrins was possible using an oxynitrilase isolated from *Sorghum biocolor* (95,96). These optically active cyanohydrins can be subsequently converted chemically to chiral α-hydroxyacids, aminoalcohols, and acyloins.

K. Lyase: C—O Formation

1. Alcohol

The stereospecific addition of water to fumaric acid catalyzed by the enzyme fumarase yields optically pure L-malic acid (Fig. 28). A *Brevibacterium flavum* strain with high fumarase activity has been used industrially for the commercial production of L-malic acid (97). The substrate specificity of fumarase is narrow and hence its broader application in organic synthesis has been somewhat limited. However, it has been shown to synthesize L-*threo*-chloromalic acid in very high optical purity (98).

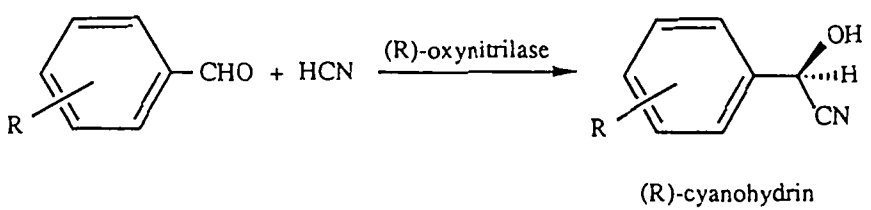

FIGURE 27 Formation of chiral cyanohydrins.

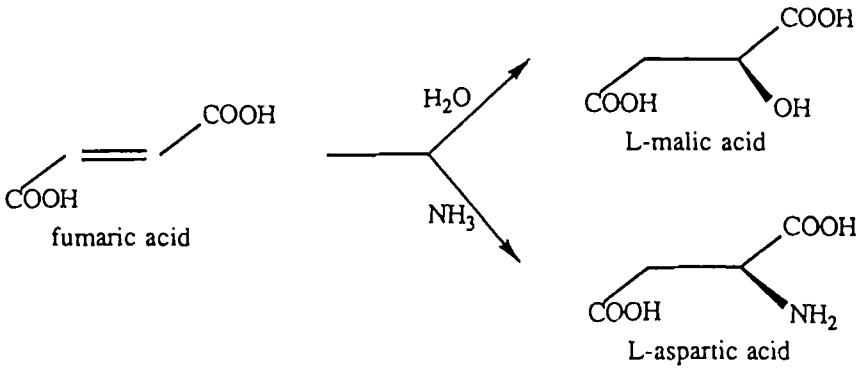

FIGURE 28 Asymmetric biotransformations of fumaric acid.

2. *Amino Acid*

L-Serine and L-threonine dehydratases dehydrate and subsequently deaminate the amino acid to the corresponding α-keto acid. These enzymes are known to require pyridoxal-5′-phosphate as a coenzyme. They can function in a biosynthetic or catabolic manner (99). Both enzymes can cause problems for the whole-cell-based production of L-serine (100).

L. Lyase: C—N Formation

1. *Amino Acid*

A commercial process for the production of L-asparatic acid has been developed based on *Escherichia coli* strains with high aspartase activity (101–103). Aspartase catalyzes the stereospecific addition of ammonia to fumaric acid (Fig. 29). L-aspartic acid can be enzymatically decarboxylated to yield the product L-alanine (104).

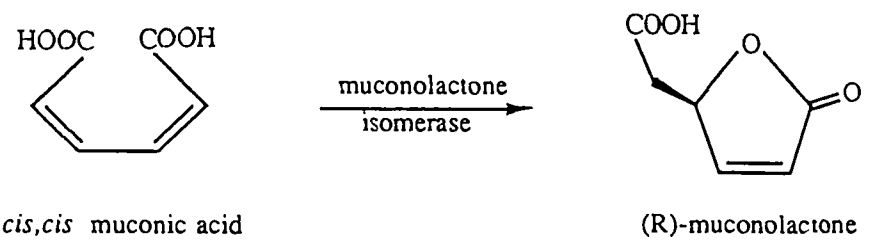

FIGURE 29 Conversion of *cis,cis*-muconic acid to muconolactone.

Various commercial routes for the production of L-phenyalanine have been developed because of the utilization of this amino acid in the dipeptide sweetener Aspartame. One route that has been actively pursued is the synthesis of L-phenylalanine from *trans*-cinnamic acid using the enzyme phenylalanine ammonia lyase (105,106). This enzyme catalyzes the reversible, nonoxidative deamination of L-phenylalanine and can be isolated from various plant and microbial sources (107,108).

M. Isomerase: Rearrangement

1. *Lactone*

Isomerases catalyze conversions within one molecule. For example, the *cis,cis*-muconate lactonizing enzyme (cycloisomerase) catalyzes the chiral conversion of *cis,cis*-muconic acid to (*R*)-muconolactone (Fig. 29). It is a key enzyme in the degradation of benzoate via the β-ketoadipate pathway (109). Chiral lactones could be useful as chiral synthons.

IV. CONCLUSION

It is clear that enzyme systems can and will play an ever-increasing role in the industrial production of chiral compounds. Not all classes of enzymes were represented in this review, for example, ligases; only enzymes with demonstrated industrial potential were discussed. Hydrolases are currently the most exploited group of biocatalysts, largely due to their commercial availability and relative simplicity of use. However, other groups of enzymes with greater catalytic versatility, in particular the lyases, are growing in commercial significance.

The exploitation of biotransformations to date has been hampered by a number of factors, including the nonjudicious choice of target products. Often, the large cost in time and money for the development of such processes far exceeds the final financial reward. A manufacturing issue that is often overlooked or underestimated is the eventual integration of the biotransformation step with conventional chemical process technology. Many problems can be minimized with the careful amalgamation of the appropriate disciplines required to turn an idea into a product. It is hoped that industry will learn to capitalize more on the inherent asymmetric nature of enzymes for everyone's benefit, including the environment.

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9

STEREOSELECTIVE BIOTRANSFORMATION

Toxicological Consequences and Implications

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I. INTRODUCTION

Over the last decades toxicologists have felt that the appropriate prevention of or intervention with toxic reactions requires knowledge about the molecular mechanisms by which the toxic responses of drugs and other xenobiotic chemicals occur. In the development of toxicity, different stages can be distinguished: (1) toxicokinetics (absorption, distribution, and elimination), (2) biotransformation, resulting in activation or inactivation of xenobiotics, (3) reversible or irreversible interactions with cellular or tissue components, (4) protection or repair mechanisms, and (5) nature and extent of the toxic effect for the organism.

When a toxic effect occurs, stereochemical factors may play a significant role at all stages mentioned, and therefore they will have to be considered when one is interested in understanding the role of stereochemistry in the occurrence of toxic effects (for recent reviews, see Vermeulen and Breimer, 1983; Ariens, 1984; Vermeulen, 1986; Testa, 1986; Eichelbaum, 1988; Caldwell et al., 1988; van Bladeren, 1988; Jamali et al., 1989; Williams, 1990).

Stereoisomerism manifests itself in various forms, such as those related to enantiomers, epimers, diastereoisomers, meso compounds, geometrical isomers, etc. Generally speaking, the consequences of stereoisomerism for the disposition and biological action of xenobiotics in living

organisms are difficult to predict, because they are complex and of a multifactorial origin. Recent developments in analytical techniques, enabling the separation of stereoisomers with HPLC, GC, and the use of stable isotopes with GC-MS and LC-MS (Blaschke, 1986; Schill et al., 1986; Vermeulen and Testa, 1988), as well as advancements in stereocontrolled synthesis (Beld and Zwanenburg, 1986; van der Goot and Timmerman, 1988), have certainly contributed to the increased interest in stereoselective aspects of biotransformation and biological activities of drugs and other xenobiotics.

A. Stereoselectivity and Toxicity: The Role of Biotransformation

The prominent role of stereochemical factors in toxicity is clearly seen with chemotherapeutic drugs. The desired toxicity of these compounds (cytostatic effect), as well as undesired toxicity toward nontumor tissue (cytotoxic effect), depends directly on the configuration of the parent drug. For instance, platinum drugs require a *cis* and not *trans* configuration to coordinate bifunctionally to DNA, thus inhibiting DNA replication and transcription (Singh and Koropatnick, 1988). The folate antimetabolite, methotrexate, a potent inhibitor of dihydrofolate reductase, also demonstrates high stereochemical requirements related to interaction with a biological template. In a number of other cytostatic drugs, stereochemistry indirectly plays a crucial role, since substrate- or product-stereoselective biotransformation is involved. For instance, gossypol, a polyhydroxylated 2,2′-binaphthalene originating from cotton seeds (Fig. 1), was found to have in vitro and in vivo cytotoxic properties. The effect of (−)-gossypol was at least twofold greater than that of the (+)-enantiomer (Joseph et al., 1986). Multidrug-resistant MCF/Adr cells appeared more resistant to (−)-gossypol than the parent cell line, which was tentatively attributed to the inactivation of (−)-gossypol by glutathione transferases present in the MCF/Adr cells. Interestingly, (−)-gossypol was a threefold more potent inhibitor of glutathione transferase α- and π-isoenzymes than the (+) enantiomer (Benz et al., 1990).

Metabolism of the cytostatic drug cyclophosphamide (CP; Fig. 1) involves hydroxylation at the C-4 position by cytochrome P450. Subsequently, a number of detoxification reactions can occur (oxidation to the 4-keto derivative, dechloroethylation, formation of a carboxylic acid). The phosphoramide mustard resulting from spontaneous decomposition of 4-hydroxy-CP is thought to be the cytotoxic chemotherapeutic species. The chiral nature of the phosphorus atom resulted in a twofold greater therapeutic index (LD_{50}/ED_{90}) for the *S*-(−)-enantiomer (against the ADJ/PC6 plasma cell tumor in mice) without detectable differences in metabolism

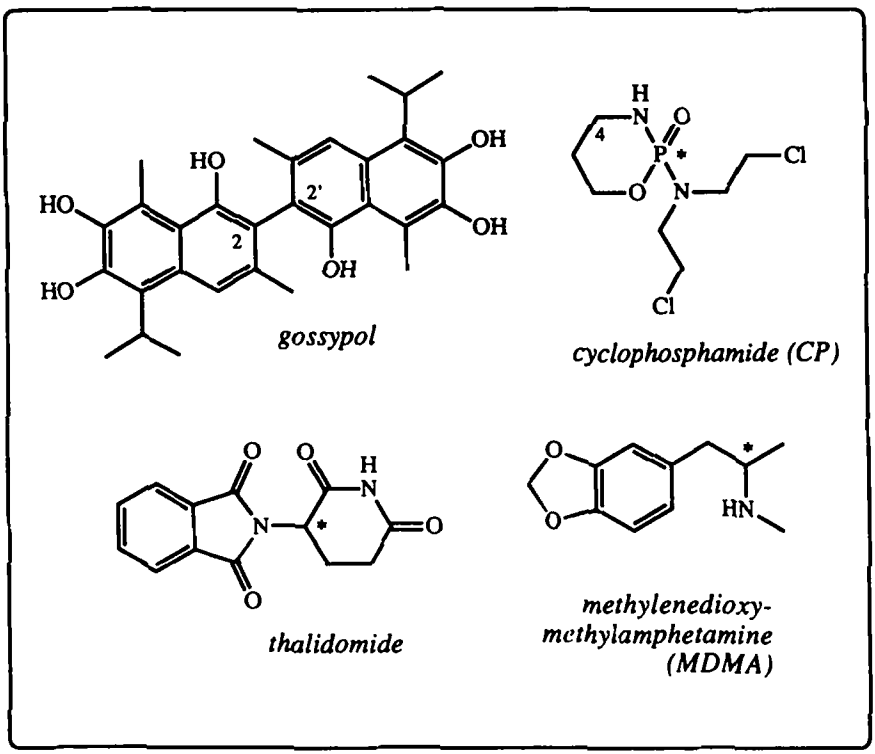

FIGURE 1 Examples of compounds that exert stereoselective toxicity. * = chiral center.

by rat liver microsomes (Cox et al., 1976). Several studies revealed considerable species differences in the metabolism of CP enantiomers, both in the initial 4-hydroxylation and in the later oxidative detoxification pathways (Farmer, 1988). Preliminary studies in man showed that the geometrical isomer iphosphamide undergoes more stereoselective metabolism than CP (Farmer, 1988). Analogues of CP were synthesized to improve the therapeutic properties. Modification of the ring structure of CP introduces a second chiral center. For instance, a methyl or phenyl substitution at the 4-position results in four stereoisomers (two *trans* isomers, *RR* and *SS*, and two *cis* isomers, *RS* and *SR*). 4-Methyl-CP isomers lacked a difference in metabolism by rat liver microsomes and chemotherapeutic efficiency against ADJ/PC6 plasma cell tumors in mice. In contrast, it was suggested that the *trans* isomers of 4-phenyl-CP are metabolized to phosphoramide

mustards, whereas the *cis* diastereromers are not, in agreement with the therapeutic activity of *trans*-phenyl-CP against L1210 lymphoid leukemia in mice, which is lacking for the *cis* diastereomers (Boyd et al., 1980). Similarly, *cis*-5-fluoro-CP was hardly metabolized by mouse liver microsomes (in contrast to *trans*), corresponding to low tumor inhibitory activity (in contrast to *trans*; see Foster et al., 1981).

In addition, toxicity-related stereoselectivity can be illustrated with the classical example of thalidomide (Fig. 1), of which the underlying mechanism has as yet not been completely elucidated. At the end of the 1950s, the drug was marketed as a hypnotic agent and in the early 1960s it was withdrawn because of embryotoxic and teratogenic effects. Only in more recent years has it become clear that in various species, both thalidomide enantiomers are transformed in vivo into 1-*N*-phthaloylglutamine and 1-*N*-phthaloylglutamic acid. Reports have been published on the sensitivity of different mouse and rat strains toward thalidomide toxicity. For instance, in SWS mice only the glutamic acid metabolite derived from the *S*(−)-enantiomer was embryotoxic and teratogenic and not the one derived from the *R*(+)-isomer (Ockzenfelz et al., 1976). More recently, however, a rapid racemization of thalidomide enantiomers of α-C-substituted analogues (Blaschke and Graute, 1987) and of a phthalimide analogue of thalidomide (Schmahl et al., 1989) was suggested to occur under physiological conditions. Moreover, in rabbits both thalidomide enantiomers were found to be equally teratogenic. The suggestion that the use of *R*-thalidomide would have avoided the toxicity problems associated with the racemic drug is therefore premature at this time. Another example of stereoselective teratogenicity is presented by 2-ethylhexanoic acid, an environmental pollutant resulting from the plasticizer di-(2-ethylhexyl)-phthalate. While the *R*(−)-enantiomer was highly teratogenic (60% of the fetuses exhibited exencephaly), no teratogenic or embryotoxic effects of *S*(+)-ethylhexanoate were observed at the same dose in mice (Hauck et al., 1990).

Stereoselective neurotoxicity was reported for methylenedioxymethylamphetamine (MDMA; Fig. 1). It causes degeneration of serotonergic and, to a lesser extent, catecholaminergic neurons. MDMA is a widely abused amphetamine derivative (Ecstasy), of which the *S*(+)-enantiomer has the highest pharmacological as well as toxicological potency. Following cytochrome P450-mediated demethylation, 3,4-methylenedioxyamphetamine (MDA) is formed; it also has been shown to be a serotonergic neurotoxin. *S*(+)-MDMA undergoes this demethylation to a far greater extent than the *R*(−)-enantiomer (Fitzgerald et al., 1989). The profile of neurotoxicological reactions is identical to that of *para*-chloroamphetamine (pCA; Schmidt, 1987). Altogether, stereoselective formation of an as yet unknown metabo-

lite of MDMA (and of MDA and pCA) seems to be involved in the stereoselective toxicity of MDMA.

Pharmacokinetic consequences of stereoselective metabolism can be illustrated by the hypnotic drug hexobarbital (HB), at present still used as a model substrate to assess cytochrome P450 enzyme activities. Large differences between the intrinsic hypnotic activities of the enantiomers of this highly lipophilic chiral drug exist; the *S*(+)-enantiomer is the most active enantiomer in most species. In addition, substrate- and product-stereoselective metabolism occurs. With a pseudoracemic mixture of HB, consisting of a mixture of *S*(+)- and N_1-trideutero-*R*(−)-HB, and GC-MS analysis (van der Graaff et al., 1985) the stereoselective disposition of HB enantiomers was studied upon simultaneous administration to the rat (Table 1). The intrinsic clearance of *R*(−)-HB (CL_{int}) was about sevenfold greater than CL_{int} of the *S*(+)-enantiomer (van der Graaff et al., 1988). A similar ninefold difference in the CL_{int} of the HB enantiomers was reported in humans. Interestingly, the observed age-related decline in stereoselectivity was attributed to a decline in various cytochrome P450 isoenzyme activities, resulting in a decrease in CL_{int}, in particular of *R*(−)-HB (Chandler et al., 1988).

Many other drugs and xenobiotics demonstrate stereoselectivity in toxicological or pharmacological effects (Tables 2 and 3) (Vermeulen, 1986; Caldwell et al., 1988; Jamali et al., 1989; Vermeulen, 1989; Eichelbaum and Gross, 1990; Williams, 1990). In addition to direct stereoselective effects of

TABLE 1 Pharmacokinetic Parameters and Recoveries of Unconjugated Metabolites in Rat Urine of *S*(+)-HB and *R*(−)-HB after p.o. and i.a. administration of 25 $mg \cdot kg^{-1}$. Values (n = 6) represent the mean ± s.e.m.

	S(+)-HB p.o.	*S*(+)-HB i.a.	*R*(−)-HB p.o.	*R*(−)-HB i.a.
$t_{1/2}$ (min)	13.4 ± 0.8	15.7 ± 1.5	16.7 ± 0.6	13.3 ± 0.4
AUC ($mg \cdot min^{-1} \cdot ml^{-1}$)	16.4 ± 2.2	366 ± 37	129 ± 22	444 ± 30
CL ($mL \cdot min^{-1} \cdot kg^{-1}$)	1621 ± 197	73.9 ± 7.5	226 ± 36	54.4 ± 2.4
V ($mL \cdot kg^{-1}$)		1670 ± 233		1042 ± 56
E	0.94 ± 0.01		0.68 ± 0.03	
OH-HB (% dose)	15.6 ± 0.9	20.6 ± 2.2	10.9 ± 1.3	16.9 ± 2.3
K-HB (% dose)	19.3 ± 0.8	17.3 ± 1.7	56.2 ± 1.8	54.9 ± 9.0
DMBA (% dose)	9.1 ± 1.4	7.8 ± 1.2	11.8 ± 1.9	11.6 ± 4.8
Total (% dose)	44.0 ± 1.8	45.7 ± 3.3	78.9 ± 2.9	83.4 ± 6.6

Source: Data derived from Van der Graaff et al., 1988.

TABLE 2 Pharmacokinetic Parameters for Propranolol Enantiomers in Dog and Man after i.v. Administration of a Deuterium-Labeled Pseudoracemate and Their Bioavailability and Urinary Excretion upon Oral Administration

	Dog[a]		Man[a]	
	(+)-P	(−)-P	(+)-P	(−)-P
Cl_s (mL/min)	364	451[b]	1210	1030[b]
$t_{1/2}$ (hr)	1.67	1.55	3.57	3.53
V_d (L/kg)	4.65	5.43[b]	4.82	4.08[b]
Protein bound f_p (%)	16.7	13.0[b]	20.3	17.6[b]
Oral bioavailability (%)[c]	19.9	10.5[b]	19.4	28.0[b]
Unchanged drug in urine (%)[d]	0.26	0.14[b]	0.16	0.24[b]

[a]N = 4–6 in the dog and 5 in man.
[b]Significantly different from (+)-P; $p < 0.05$ (Student's test).
[c]$AUC_{oral}/AUC_{i.v.} \times dose_{i.v.}/dose_{oral}$.
[d]Percentage of total recovery of propranol dose in urine.
Source: Adapted from Walle, 1985.

the parent compounds, stereoselective bioactivation or bioinactivation through enzymes seems to be of major importance. Therefore, knowledge of the stereoselectivity of biotransformation of substrates will give insight into the toxicological role and catalytic mechanism of these enzymes, and vice versa. Ultimately, such knowledge will allow one to design, more efficiently and on a more rational basis, safer and more selective drugs and tools for the prevention of or intervention with toxicity.

II. STEREOSELECTIVE BIOTRANSFORMATION

A. Mixed-Function Oxidases

One of the most important drug metabolizing enzyme systems in mammals and nonmammals is the MFO system, consisting of a large family of cytochrome P450 isoenzymes (Testa, 1988; Ortiz de Montellano, 1989; Guengerich, 1991) with a broad range of substrates (substrate selectivity), as well as products that can be formed from one substrate (product selectivity). For instance, the anticoagulant warfarin is metabolized to dehydrowarfarin, 4′-, 6-, 7-, 8-, and 10-hydroxy-warfarin by a number of cytochrome P450 isoenzymes. Regio- and stereoselective hydroxylation reactions of $R(+)$- and $S(-)$-warfarin have been suggested to be useful as a probe for the functional characterization of the multiplicity of hepatic cytochrome P450 isoenzymes (Kaminsky et al., 1984; Thijssen and Baars,

TABLE 3 Mean Pharmacokinetic Parameters for Warfarin Enantiomers in the Rat and Human upon i.v. Administration of *R*(+)− or *S*(−)− Warfarin

	Rat		Human	
	R(+)-W	*S*(−)-W	*R*(+)-W	*S*(−)-W
Total plasma clearance (mL/hr/kg)	7.8	4.0	2.6	4.0
Plasma clearance of free drugs (mL/hr/kg)	6.94	491	480	940
Elimination half-life (hr^{-1})	13.3	23.8	37	25
Volume of distribution (mL/kg)	139	132	140	140
Plasma free fractions (× 100%)	1.07	0.84	0.56	0.43

Source: Adapted from Yacobi et al., 1984.

1987). In addition, regio- and stereoselective oxidation, for instance of steroids, can be used as a tool to obtain information on the catalytic mechanism of steroids such as testosterone and progesterone cytochrome P450 isoenzymes (Swinney et al., 1987; Waxman, 1988; Wood et al., 1983). Studies on the regio- and stereoselectivity of steroid hydroxylation resulted in the suggestion that residues Leu^{58} and Ile^{114} of cytochrome P450-IIB2 (located at the *N*-terminal part of the protein) are involved in the orientation of the substrate at the active site (Aoyama et al., 1989). Studies on suicidal heme-alkylation of cytochrome P450-IIB1/IIB2 with alkynes and *trans*-1-[1^2H]-octene have led to a simplified model of the active site of these isoenzymes, explaining that oxidation is selectively occurring at the *re* face of the olefin double bounds (Ortiz de Montellano et al., 1983).

Mechanistical studies, for example, on the stereochemistry of the 1′-hydroxylation of (*R*)- and (*S*)-1′-deuterated-phenylethane by purified cytochrome $P450_{LM2}$, have demonstrated that the product stereoselectivity most probably results from constraints of the substrate binding site provided by the protein environment, rather than the intrinsic hydroxylation mechanism of cytochrome P450 (White et al., 1986).

Among the best documented examples of the stereoselective action of cytochrome P450 isoenzymes are provided by studies on the stereoselective bioactivation of polycyclic aromatic hydrocarbons (PAHs) to carcinogens by cytochrome P450-IA1 (Jerina et al., 1985). This isoenzyme among others was found to catalyze with high regio- and stereoselectivity the epoxidation of benzo[a]pyrene to its (+)-7*R*,8*S*-epoxide, which upon

hydrolysis by epoxide hydrolase (−)-7*R*,8*R*-dihydrodiol-benzo[a]pyrene is converted by the same cytochrome P450 isoenzyme to *anti*-(+)-benzo[a]pyrene-7*R*,8*S*-dihydrodiol-9*S*,10*R*-epoxide in over 85% diastereomeric excess (*anti* refers to the relative orientation of the benzylic hydroxyl group at position-7 and the 9,10-epoxide group; Fig. 2).

The stereoselectivity of a large number of PAHs was studied in detail by Jerina and co-workers and allowed them to propose a model for steric requirements of the catalytic site of the cytochrome P450-IA1 (Fig. 3A, Jerina et al., 1985). This model was demonstrated to be also valid in predicting the stereoselective metabolism of other PAHs like phenanthrene, chrysene, and benz[a]anthracene. More recently, this model of the active site of cytochrome P450-IA1 was reevaluated by superimposing the structures of enantiomeric epoxides of other PAHs, including specific isomers of non-bay-region or K-region epoxides (Kadlubar and Hammons, 1987; Yang, 1988). This led to model B (Fig. 3B) and, when a certain degree of flexibility was allowed in the angle of oxidation toward the substrate, model C (Fig. 3C). The above models for the PAHs are as yet not applicable to other, non–PAH substrates of cytochrome P450-IA1, however. More research on this aspect is necessary.

Studies on the consequences of stereoselective PAH metabolism in respect to mutagenicity and carcinogenicity were also shown to be strongly dependent on the relative and absolute configuration of the PAH metabolite (Conney, 1982; Thakker et al., 1982). For example, (−)-7*R*,8*R*-dihydrodiol benzo[a]pyrene has a 10-fold higher tumor-initiating activity in female CD-1 mouse skin than the (+)-7*S*,8*S*-enantiomer, and it produces

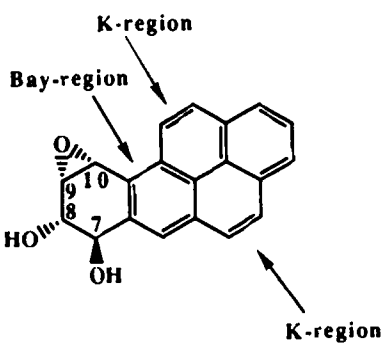

FIGURE 2 Benzo[a]pyrene is metabolized highly stereoselectively to the ultimate carcinogen (+)-benzo[a]pyrene-*anti*-7*R*,8*S*-dihydriol-9*S*,10*R*-epoxide by cytochrome P450-IA1 and epoxide hydrolase. (From Jerina et al., 1985.)

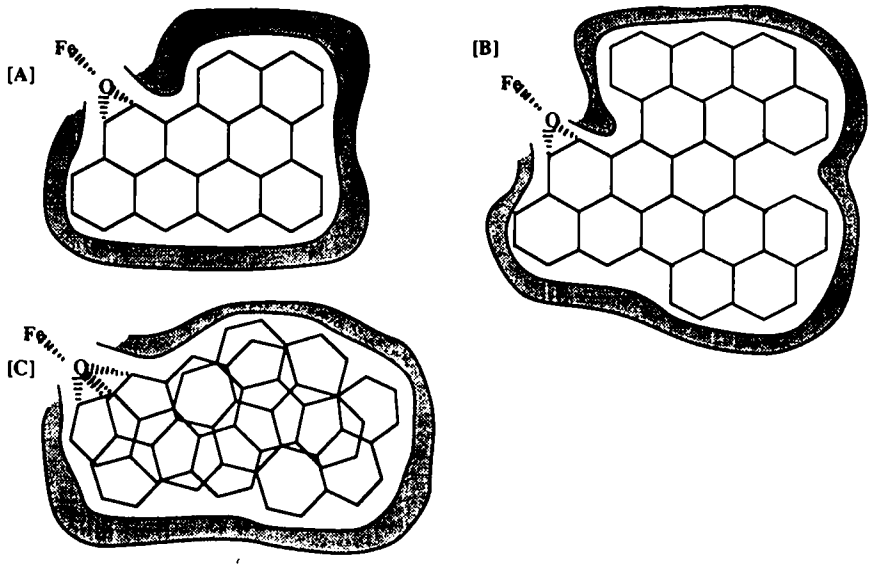

FIGURE 3 A, steric model of the active site of cytochrome P450-IA1 based on the metabolism of benzo[a]pyrene; the binding site is asymmetrically positioned toward the activated iron-bound oxygen species. (From Jerina et al., 1985.) B, expanded model of A in order to accommodate also non-bay-region or K-region epoxides. (From Kadlubar and Hammons, 1987; Yang, 1988.) C, proposed model in which some flexibility in the angle of oxygen addition to the substrate is allowed. (From Kadlubar and Hammons, 1987.)

over 14 times more lung tumors in newborn mice. Among the four stereoisomeric diol-epoxides, the (+)-7*R*,8*S*-dihydrodiol-9*S*,10*R*-epoxide was severalfold more mutagenic to Chinese hamster V79 cells than the corresponding (−) enantiomer and, in addition, it was the only isomer with high carcinogenic activity on mouse skin and in newborn mice. Thus, the 7*R*,8*S*-diol-9*S*,10*R*-epoxide was established as the ultimate carcinogen of benzo[a]pyrene. At the DNA level, stereoselectivity in adduct-formation between the diol-epoxides and the exocyclic 2-amino group of guanine has been suggested to be responsible for the observed stereoselectivity in this type of toxicity. From comparative investigations on the tumorigenicity of various PAHs (benzo[a]pyrene, benz[a]anthracene, chrysene), it has been derived that a bay-region diol-epoxide functionality is of crucial importance in the expression of tumorigenicity and, furthermore, that common stereochemical factors such as relative configuration (pseudodiequatorial hydroxyl groups) and absolute configuration (*R* configuration at benzylic

carbon atom) appear to be far more important in this regard than chemical reactivity (Chang et al., 1983; Jerina et al., 1985; Yang and Bao, 1989). The carcinogenicity and mutagenicity of dibenz[c,h]acridine further support the idea that the highest biological activity is attributable to a bay-region diolepoxide that is superimposable on the highly active *R,S,S,R* bay-region diolepoxides of the PAHs mentioned above (Wood et al., 1986). More recent studies on PAHs larger than benzo[a]pyrene seem to be in agreement with these findings. For instance, cytochrome P450-mediated metabolism of the potent carcinogen dibenzo[a,l]pyrene results, among other metabolites, in the 11,12-dihydrodiol (which corresponds to benzo[a]pyrene 7,8-dihydrodiol). Interestingly, the isomeric dibenzo[a,e]pyrene, a very weak tumor initiator, was not metabolized to the corresponding proximate dihydrodiol (Devanesan et al., 1990).

In recent years, immunochemical detection with monoclonal or polyclonal antibodies has been applied to DNA adducts of benzo[a]pyrene diolepoxide metabolites. Investigations in relation to PAH exposure of animals and humans have shown that the (+)-*anti*-diolepoxide-2 deoxyguanosine DNA adduct formed in vivo was more persistent in mouse epidermis than rat epidermis (Rojas and Alexandrov, 1986). This observation is in agreement with the relative resistance of the rat to the carcinogenic action of benzo[a]pyrene. In contrast, the relative tumor-initiating activities of four stereoisomeric 3,4-diol-1,2-epoxides of benzo[c]phenanthrene seem to be related to a DNA adduct at deoxyadenosine, instead of deoxyguanosine (Dipple et al., 1987). Clearly, apart from stereoselectivity at the level of biotransformation, stereoselectivity is also occurring at the level of toxicologically relevant molecular interactions between reactive metabolites and biological macromolecules.

B. Oxidation Polymorphism

Cytochrome P450s involved in biotransformation of numerous drugs and other xenobiotics are a major determinant of interindividual differences in their rate of elimination and, consequently, their pharmacological or toxicological response. For a number of enzymes (cytochromes P450, glutathione transferases, *N*-acetyltransferases; see Eichelbaum and Gross, 1990; Meyer et al., 1990; Guengerich, 1991), the genetically determined absence of enzyme activity in a fraction of the population was reported [individuals called poor metabolizers (PMs) in contrast to normal extensive metabolizers (EMs)]. For example, an epidemiological study in 245 bronchogenic carcinoma patients suggested a causal genetic relationship between a polymorphic cytochrome P450 isoenzyme, catalyzing the 4-hydroxylation of debrisoquine, and the risk of lung cancer in heavy smokers (Ayesh et al., 1984). However, there are also reports of failure to observe such a

relationship (Speirs et al., 1990). Among compounds whose oxidation has been reported to be impaired in PM phenotypes is the β-adrenoceptor antagonist bufuralol (Fig. 4A). It is a chiral drug for which 1′-hydroxylation is under genetic control and selective for the (+)isomer. Hydroxylation of bufuralol at the 4- and 6-positions is under the same genetic control and favors the (−)isomer. The stereoselectivity for the aliphatic 1′-and aromatic

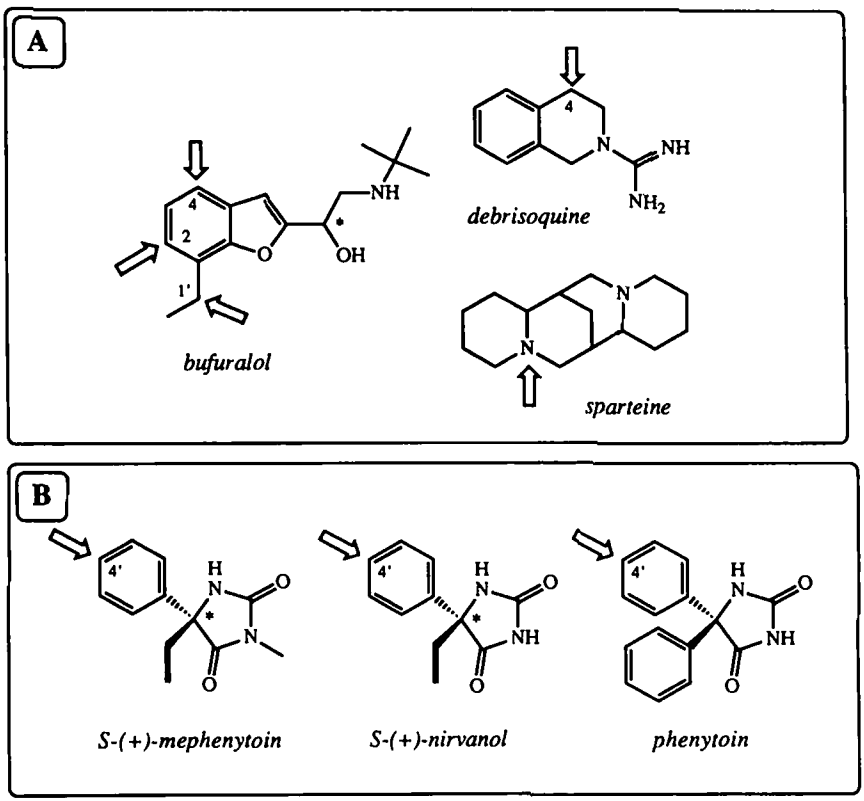

FIGURE 4 Polymorphic drug oxidations by cytochrome P450. A, substrates subject to debrisoquine/sparteine polymorphism. *R*(+)-bufuralol is 1′-hydroxylated by P450-IID6; the *S*(−)-enantiomer undergoes hydroxylation at the 2- and 4-positions; debrisoquine is hydroxylated at the prochiral C4-atom to *S*(+)-hydroxy-debrisoquine; sparteine metabolism by P450-IID6 consists of *N*-oxidation. B, substrates subject to hydantoin polymorphism (4′-hydroxylation). Extensive metabolizers convert *S*(+)-mephenytoin and -nirvanol to the 4′-hydroxy derivative (indicated by the arrow). Similarly, EMs metabolize the prochiral drug phenytoin to *R*(+)-4′-hydroxyphenytoin. * = chiral center.

2,4-oxidations is virtually abolished in PM phenotypes. Apart from these oxidation reactions, glucuronidation of the hydroxyl group in the side chain and renal elimination appeared to be stereoselective for (+)-bufuralol and particularly important in poor metabolizers (Dayer et al., 1985; Meyer et al., 1986).

Various investigations (e.g., Meyer et al., 1986) have provided evidence for the hypothesis that 1′-hydroxylation of bufuralol is mediated by a polymorphic cytochrome P450 isoenzyme, cytochrome P450-IID6, which is also stereoselectively catalyzing the hydroxylation at the prochiral benzylic C_4-position of debrisoquine (Fig. 4A; Meese et al., 1988), *N*-oxidation in sparteine (Fig. 4A), *O*-demethylation of dextrometorphan, E-10-hydroxylation of nortriptyline, *trans*-4′-hydroxylation of perhexiline, α-hydroxylation of (+)-metoprolol, and several other reactions in various substrates (e.g., Boobis et al., 1985; Distlerath et al., 1985). Based on the common structural features of these substrates, Meyer et al. (1986) derived a preliminary substrate model for this polymorphic cytochrome P450 isoenzyme. Surprisingly, however, the polymorphic pathways of the classical substrates, debrisoquine and sparteine, could not be predicted with it.

More recently, Koymans et al. (1992) used 16 substrates, accounting for 23 regio- and stereochemically controlled metabolic reactions, and molecular modeling techniques to derive a more advanced predictive substrate model for cytochrome P450-IID6 (Fig. 5). The predictive value of the model was evaluated by investigating the P450-IID6-mediated metabolism of 4 new compounds (alfentanil, astemizole, resperidone, and nebivolol), comprising 14 oxidative metabolic routes, 13 of which proved to be correctly predicted as far as the involvement of P450-IID6 was concerned (Koymans et al., 1992).

Similar findings on stereoselectivity have been made with other cytochrome P450 isoenzymes and other classes of drugs, for example, 5-substituted-5-phenyl-hydantoins (Fig. 4B; see Küpfer et al., 1981; Meyer et al., 1986). For instance, the 4′-hydroxylation of *S*(+)-mephenytoin (*N*-methyl-5-ethyl-5-phenyl-hydantoin) is subject to genetic polymorphism. In PMs, *S*(+)-mephenytoin is not significantly *para*-hydroxylated, has a $t_{1/2}$ of about 70 hr, and, like the *R* enantiomer is available for *N*-demethylation. In EMs, extensive 4′-hydroxylation of *S*(+)-mephenytoin occurs (at greater rates than *N*-demethylation). In contrast, *R*(−)-mephenytoin is metabolized by *N*-demethylation (affording pharmacologically active *R*(−)-5-ethyl-5-phenyl-hydantoin, also known as nirvanol, Fig. 4B). Nirvanol hydroxylation also favors the *S* enantiomer and cosegregates with *S*(+)-mephenytoin hydroxylation in humans (Küpfer et al., 1984). Phenytoin (5,5,-diphenyl-hydantoin), a prochiral drug, also undergoes polymorphic 4′-hydroxylation (Fig. 4B). In humans, cytochrome

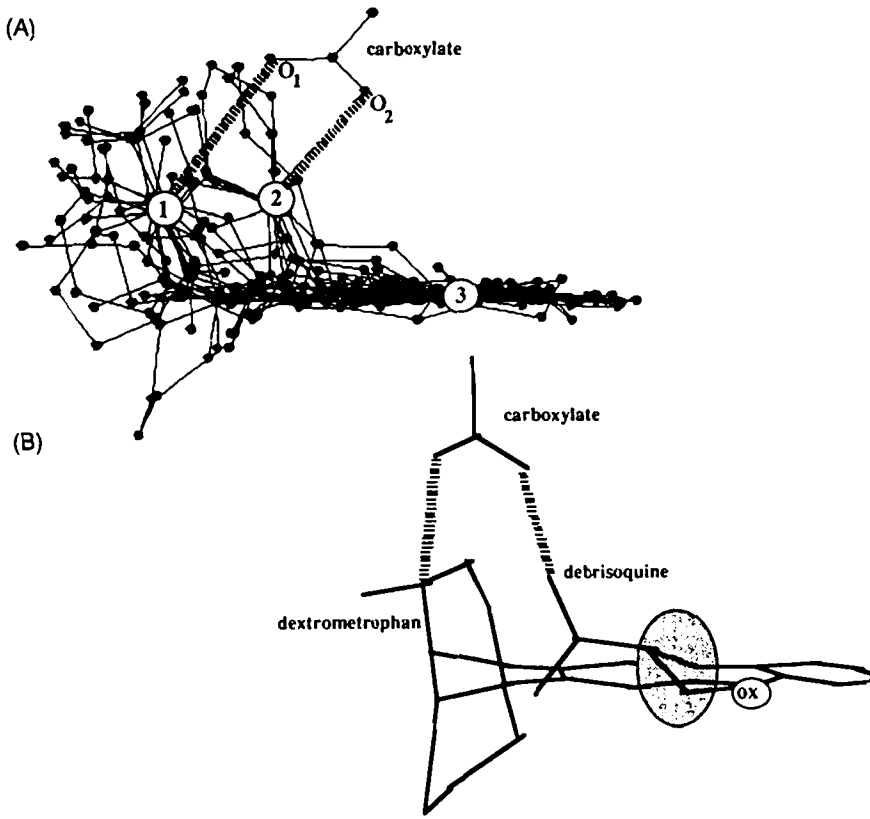

FIGURE 5 Active-site model of cytochrome P450-IID6 with 16 substrates (23 metabolic reactions (A) fitted onto the template molecules debrisoquine and dextrometorphan (B). Basic nitrogen atoms ① and ②, oxidation sites ③, negative molecular electrostatic potentials ⊗ as well as carboxylate interaction with the protein (O_1 and O_2) are indicated. (From Koymans et al., 1992.)

P450-mediated metabolism of phenytoin to 5-(4′-hydroxyphenyl)-5-phenylhydantoin results almost exclusively in the *S*(−)-enantiomer (95%). Interestingly, the formation of the small amount (5%) of *R*(+)-metabolite cosegregates with the (*S*)(+)-mephenytoin hydroxylation polymorphism: Both hydroxylation reactions are lacking in PMs (Fritz et al., 1987). The product enantioselectivity in this reaction appears to be controlled by the relative affinities of the prochiral diphenylhydantoin to two different cytochrome P450 isoenzymes, most likely belonging to the P450-IIC isoenzyme subfamily (Relling et al., 1990).

Another most important polymorphically regulated cytochrome P450 isoenzyme family showing stereoselective metabolism is the P450-IIIA isoenzyme subfamily (for a review, see Guengerich, 1991). P450-IIIA4 or P450-nifedipine appears to play a major role in the oxidation of many drugs, including nifedipine oxidation, *R*(+)-warfarin 9,10-dehydrogenation, *S*(−)-warfarin 10-hydroxylation, quinidine *N*-hydroxylation, cortisol 6β-hydroxylation, testosteron 6β-hydroxylation, but also in the bioactivation of aflatoxin B1 and G1, 6-aminochrysene, benzo[a]pyrene, and pyrrolizidine alkaloids (see Guengerich, 1991).

In these studies, a stereoselective metabolism has been used as a tool to phenotype individuals for (iso)enzymes or enzyme systems that, in principle, can activate xenobiotics into chemical carcinogens. Obviously, information on stereoselectivity and structure biotransformation relationships may be worthwhile to predict this type of toxicity-related biotransformation.

C. Flavin-Containing Monooxygenases

About a decade ago, studies on the chirality of sulfoxidation by microsomal FAD-containing monooxygenase (FMO) and cytochrome P450 oxygenase isoenzymes (MFO) were published (Waxman et al., 1982). The latter enzyme system can oxygenate carbon, nitrogen, and sulfur atoms, the former only nitrogen and sulfur. Using 4-tolyl ethyl sulfide as a prochiral model compound, rat liver microsomal FMO yielded predominantly the *R*(+)-enantiomer of 4-tolylethyl sulfoxide, whereas rat liver microsomes (containing FMO as well as MFO) yielded both the *R*(+)- and *S*(−)-enantiomers. *S*(−)-sulfoxide was formed by cytochromes P450, as was demonstrated with two purified cytochrome P450 isozymes (PB-1 and PB-4) isolated from phenobarbital-induced rat livers. Oxidation of the substrate at benzylic carbon atoms was performed only by cytochrome P450 and, as to be expected, not the FAD-monooxygenase. The stereoselectivity of sulfoxidation was strongly dependent on substituents at the sulfur atom (Waxman et al., 1982) and on the relative participation of cytochrome P450 and FMO. The stereoselective nature of FMO and cytochrome P450 to produce sulfoxides of opposite absolute configuration from *prochiral* sulfides was recently extended with studies on *chiral* sulfides. Thus, it was found that the enantioselectivity of *S*-oxidation of P450-IIB1 is not always opposite that of purified hog liver FMO (Cashman et al., 1990). From the fact that hog liver FMO only oxidized *pro-S*-configured sulfur and P450-IIB1 the *pro-S* and *pro-R* positions (depending on the substituent), it was concluded that FMO possesses significant active-site restraints and requires rigid orientation of the substrate, whereas P450-IIB1 has substantially more flexibility at the active site (Fig. 6).

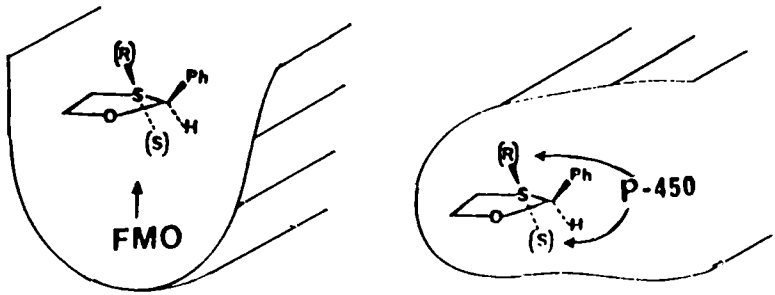

FIGURE 6 Stereoselective sulfoxidation of 2-aryl-1,3-oxathiolanes by hog liver flavine-containing monooxygenase (FMO) and cytochrome P450-IIB1. (Taken from Cashman et al., 1990.)

Meanwhile, there are many substrates of FMO already known, including tertiary and secondary amines, hydroxyamines, thiols, sulfides, thiocarbamides, and thioamides (for a review, see Ziegler, 1991). Compounds accepted by this mono-oxygenase have one chemical property in common: All are soft nucleophiles readily oxidized by per-acids. Apparently, the nucleophilic centers are oxidized by an ionic rather than a radical mechanism in an active site requiring more than one point of contact (Ziegler, 1991).

D. Glutathione Transferases

GSH transferases (GSTs) catalyze the conjugation of the optically active tripeptide glutathione (GSH, γ-Glu-Cys-Gly) to many hydrophobic and electrophilic substrates possessing leaving groups, such as aliphatic and aromatic epoxides, phosphates, sulphates and halogenides, organic peroxides and ozonides, quinones, etc. The mechanism of the conjugation reaction, which is generally catalyzed by a cytosolic system of GSH transferases, is consistent with a S_N2-type single-displacement substitution mechanism or Michael addition mechanism (with olefins and quinones) involving a direct attack of the sulfhydryl group of GSH at the electrophilic atom (Armstrong, 1987; Mannervik and Danielson, 1988; Pickett and Lu, 1989; van Bladeren, 1988).

The stereoselectivity of GSTs toward a considerable number of substrates has been described (Fig. 7) (Vermeulen and Breimer, 1983; Armstrong, 1987; van Bladeren, 1988; te Koppele and Mulder, 1991). With regard to rigid epoxides, it was found by identifying and quantifying mercapturic acids excreted in the urine of rats treated with the model compound cyclohexene oxide, that only two *trans*-2-hydroxy-cyclohexyl-

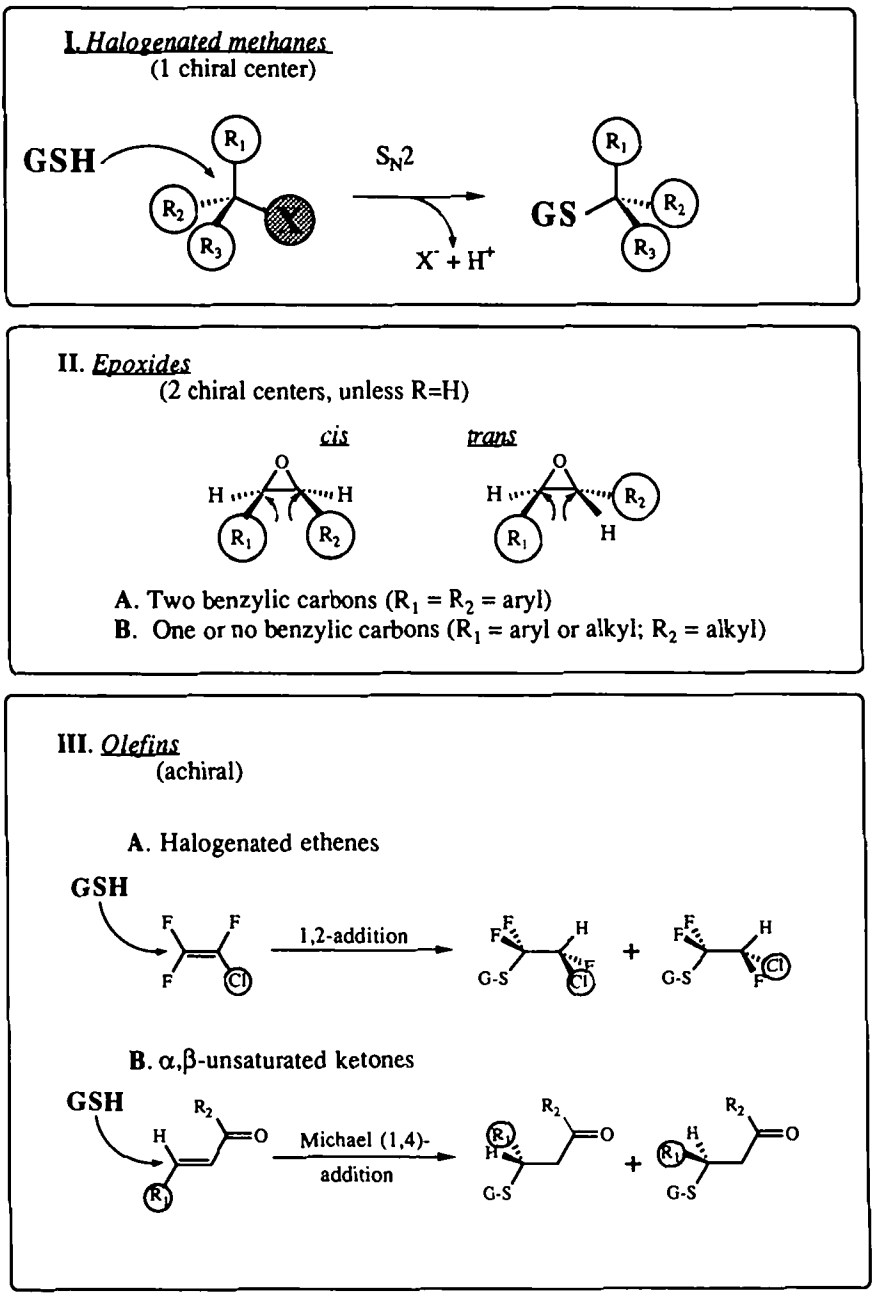

FIGURE 7 Types of substrates for GST enzymes that undergo stereoselective GSH conjugation. (Taken from te Koppele and Mulder, 1991.)

mercapturic acids and no *cis* isomers were excreted (Vermeulen et al., 1980; van Bladeren et al., 1981). In alkylhalogenides rat liver cytosolic GSH transferases conjugated chiral α-phenylethyl-chloride and -bromide with a high degree of substrate stereoselectivity. *S* enantiomers were far better substrates than *R* enantiomers, and the chloride analogue showed a higher stereoselectivity than the bromide analogue, a finding that was suggested to be related to the enantioselectivity of GSH transferase isoenzymes from the α and μ family (Mangold and Abdel-Monem, 1983).

A *trans*-addition mechanism involving S_N2-substitution was also found for the enzymatic conjugation of (±)-benz[a]pyrene-4,5-oxide using rat liver cytosol as a source of transferase activity. Both positional isomers (at C_4 and C_5) were produced, however, with a preference for two diastereoisomers. Subsequent investigations with optically active (+)-4*S*,5*R*- and (−)-4*R*,5*S*-benzo[a]pyrene-4,5-oxide have shown that the rat liver cytosolic GSH transferases preferentially catalyze the formation of the 5-glutathionyl-isomer (4*S*,5*S*) from the (+)benzo[a]pyrene-4,5-oxide and the 4-glutathionyl-isomer (4*S*,5*S*) from the (−) isomer. Apparently, these GSH transferases prefer to attack the oxirane at the carbon atom with the *R* configuration (Cobb et al., 1983).

Unless different isoenzymes possess the same kind of stereoselectivity toward a substrate, it may be anticipated that a mixture of isoenzymes will exhibit a diminished stereoselective metabolism. In the case of GSH transferases, stereoselection is even more complicated since one isoenzyme may exist as a homo- or heterodimeric protein of two subunits, each of the subunits potentially having structurally different active sites apart from their GSH-binding site. In a detailed study with purified rat isoenzymes 3–3, 3–4, and 4–4, Armstrong and co-workers (Cobb et al., 1983) reported that subunit 4 was more stereoselective in the conjugation with arene and aza-arene epoxides (with more than a 99% attack at the oxirane carbon with the *R* configuration) than subunit 3. It was suggested that hydrophobic interactions between the substrate and enzyme surface distal to the oxirane ring, rather than electronic differences between the two oxirane carbons, are important in determining stereoselectivity. Recently, it was shown that cytosolic GSH transferases from rat hepatic and extra-hepatic tissues varied markedly in their stereoselectivity toward a number of polycyclic arene and alkene oxide substrates. This stereoselectivity was found to correlate roughly with known tissue differences in isoenzyme composition (π transferases preferentially attacking *S*-configured carbon atoms of K-region epoxides), and it was suggested to use this stereo- and enantioselectivity for the functional characterization of GSH-transferase isoenzymes in different tissues (Dostal et al., 1986). In Fig. 8, a schematic representation of binding interactions of some arene and alkene oxides is presented. Studies with GST 4-4 catalyzed conjugation of *trans*-

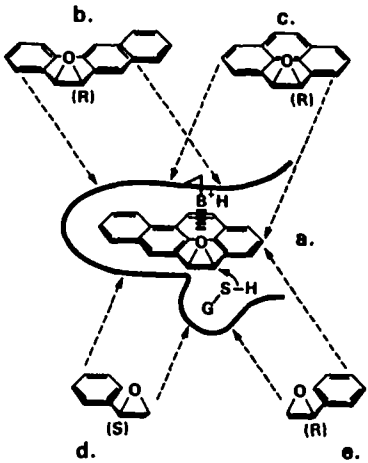

FIGURE 8 Schematic representation of binding interactions of arene oxides at the H site of GST: A, (4*R*,5*S*)-benzo[a]pyrene oxide; B, (5*S*,6*R*)-benzo[a]anthracene oxide; C, (4*R*,5*S*)-pyrene oxide; D, (7*S*)-styrene oxide, and E, (7*R*)-styrene oxide. (Taken from Dostal et al., 1986.)

and *cis*-stilbene oxide demonstrated a highly stereoselective conjugation of the carbon atom with the *R* configuration of both substrates, suggesting that the H site of this isoenzyme can accommodate phenyl rings out of the plane, perpendicular to the epoxide (Armstrong, 1987).

That stereoselective metabolism by cytosolic GSH transferases may be important from a toxicological point of view was illustrated several years ago by experiments in which the GSH transferases were bioactivating nontoxic substrates. In this context, the mutagenic behavior of *cis*- and *trans*-1,2-dichlorocyclohexane was investigated with and without metabolic activation of rat liver fractions (van Bladeren et al., 1979). By using the Ames test with *Salmonella typhimurium* cells, it was shown that, in contrast to the *trans* isomer, the *cis* isomer demonstrated mutagenic activity that was significantly increased upon the addition of 9000 g and 100,000 g supernatant of a rat liver homogenate. The explanation of this stereoselective effect was given in terms of the stereospecific action of glutathione transferases present in the liver homogenate fractions and catalyzing the substitution of the vicinal chlorine atoms according to a S_N2-substitution mechanism. Only in *cis*-1,2-dichlorocyclohexane can this conjugation re-

action lead to the formation of a reactive thiiranium ion, which is suspected to be responsible for the mutagenic activity.

Apart from stereoselective bioactivation, stereoselective bioinactivation may be important too from a toxicological point of view. For instance, in contrast to the basic (α-ε), the human near neutral (μ) and acidic (π) classes of GSH transferases were efficient in the conjugation of benz[a]-pyrene-7,8-diol-9,10-epoxides and, in particular, the carcinogenic (+)-anti-enantiomer (and other (+)-*anti*-enantiomers of the bay-region diol-epoxides of benz[a]anthracene and chrysene) (Jernström et al., 1985; Robertson et al., 1986).

In recent studies of the rat, GSH conjugation of the chiral hypnotic drug α-bromoisovaleryl-urea was also shown to proceed with a high degree of stereoselectivity in vivo (Fig. 9, te Koppele et al., 1987). Most interestingly, the subunits 3 and 4 (μ-type isoenzymes) were particularly selective for the *R* enantiomer, whereas the subunits 1 and 2 (α-type isoenzymes) were found to be particularly active toward the opposite *S* enantiomer. Since this drug possesses the profile of a relatively ideal model substrate for in vivo applications, it has been proposed as an in vivo probe to assess the activity of different GSH transferases, including the polymorphic and toxicologically potentially important μ class of isoenzymes (te Koppele, 1988).

In recent years, the existence of a microsomal GSH transferase has also been established firmly (Morgenstern et al., 1983). Apart from the usual substrates for the cytosolic GSH transferases, compounds like hexachloro-1,3-butadiene and tetrafluoroethylene seem to be the better substrates for the microsomal enzyme. Dohn et al. (1985) were the first to demonstrate with ^{19}F-NMR the stereochemical control of an enzymatic addition reaction between the pro-chiral chlorotrifluoroethene (CTFE) and GSH. By a microsomal rat liver fraction, predominantly one diastereomeric GSH conjugate (the *S*-configured chlorotrifluoroethyl GSH conjugate; see Hargus et al., 1991) was formed, whereas a cytosolic fraction produced an equimolar fraction of two diastereomeric 2-chloro-1,1,2-trifluoroethyl GSH conjugates. Similarly, the formation of diastereomeric GSH conjugates was shown to be highly product-stereoselective in the case of a GST 4-4 catalyzed Michael addition of GSH to phenylbutenones (Zhang and Armstrong, 1990). 4-Hydroxyalkenals, toxic products of lipid peroxidation, might be anticipated to behave similarly. Further studies of the (regio- and) stereospecificities of GSH transferases might reveal information on the respective mechanisms of these reactions.

In summary, GSH transferase isoenzymes operate highly stereoselectively and this may have important consequences for the detoxification or toxification of chiral or pro-chiral substrates.

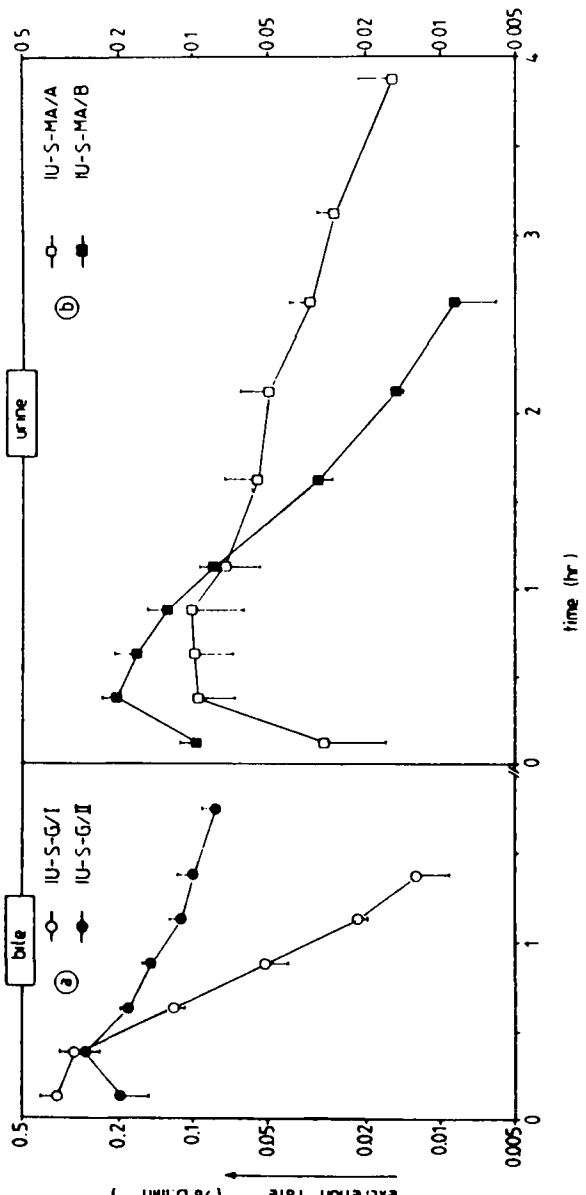

FIGURE 9 Excretion rates of BIU metabolites in bile and urine in anesthetized rats. a, diastereomeric glutathione conjugates in bile (n = 4). b, diastereomeric mercapturates in urine, (n = 3). Glutathione conjugate I and mercapturate B are derived from R(+)-BIU. The conjugates IU-S-G/II and IU-S-MA/A result from S(−)-BIU. (Taken from te Koppele et al., 1987.)

E. Epoxide Hydrolases

Epoxide-containing compounds are often rather reactive and, in many cases, they therefore exhibit strong and direct mutagenic or carcinogenic properties. Thus, the *R* and *S* enantiomers of epoxystyrene exhibit strongly different mutagenic activity in *Salmonella typhimurium* TA 100, although their chemical reactivity toward nucleophiles, such as 4-(p-nitrobenzyl)pyridine, does not differ considerably (Seiler, 1990). This difference in intrinsic mutagenic activity has been attributed to qualitative differences in binding to DNA, rather than to differences in indirect factors, such as metabolic detoxication by GSH-*S* transferases or epoxide hydrolase. Epoxide hydrolases catalyze the hydrolysis of alkene and arene oxides into dihydro-diols. For instance, the widely used anticonvulsant phenytoin, following the cytochrome P450-mediated formation of the 3′,4′-epoxide is stereoselectively hydrated by epoxide hydrolase. The (+) epoxide is metabolized to a greater extent than the (−) enantiomer (Maguire et al., 1980). Microsomal, membrane-bound (Oesch, 1979), as well as cytosolic, soluble epoxide hydrolases have been isolated and identified (Hammock et al., 1980). Substrate selectivities of both hydrolases are broad and largely overlapping. The cytosolic hydrolase with lower activity seems to possess a greater activity toward aliphatic epoxides. In contrast, the microsomal epoxide hydrolase has a higher degree of substrate stereoselectivity toward such epoxides (Belluci et al., 1989b). Mechanistically, the enzymatic hydration of epoxides most likely proceeds according to the general base-catalyzed nucleophilic addition of "activated water." A unionized histidine residue has been implicated as essential for the deprotonation of the attacking water molecule (Fig. 10, Armstrong, 1987). An electrophilic (acid-catalyzed) mechanism was ruled out by experiments with $H_2{}^{18}O$, which demonstrated regiospecific hydration at the 2-position of unlabeled styrene and nafthaleneoxides and by experiments that demonstrated the inability of the epoxides to accept nucleophiles other than water (Hanzlik et al., 1976).

Stereochemical studies have shown that the hydration of epoxides (of cyclic olefins as well as arenes) is performed by epoxide hydrolase in such a way that *trans*-diols are formed. Aliphatic epoxides have also been shown to undergo *trans*-hydration, which means that *trans*-epoxides yield *erythro*-glycols and *cis*-epoxides result in *threo*-glycols (Hammock et al., 1980).

Varying degrees of substrate enantioselectivity have been described for microsomal epoxide hydrolases. For example, for benzo[a]pyrene-4,5-oxide a 40-fold difference between the rates of hydration for the (+) and (−) enantiomers was observed in vitro (Armstrong et al., 1980). Monosubstituted epoxides (such as 1,2-epoxyhexane and its geometrical isomer,

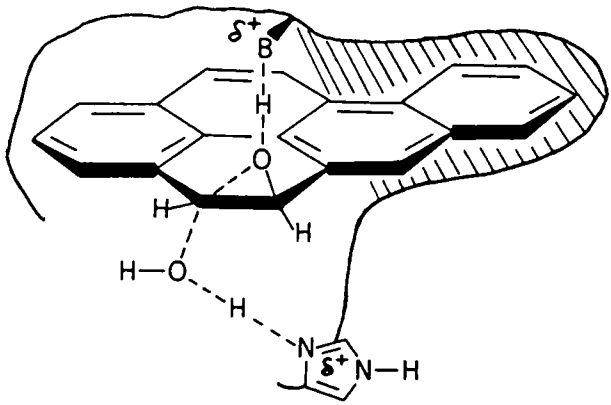

FIGURE 10 Possible transition state for the reaction of an epoxide hydrolase-oxide-H_2O complex, illustrating a general acid–base catalysis mechanism for the hydrolysis of benzo[a]pyrene-4,5-oxide. (Taken from Armstrong, 1987.)

3,3-dimethyl-1,2-epoxybutane) are good substrates for microsomal epoxide hydrolase. Racemic mixtures of C_6 and C_{10} linear-chain epoxides resulted in diols with low enantiomeric excess. In contrast, epoxides with *tert*-butyl substituents were hydrated with a high degree of product-stereoselectivity (excess of *R*-configured diols). Experiments with $H_2{}^{18}O$ showed that the epoxide was attacked preferentially at the unsubstituted, a-chiral C_1-position of the epoxide (Belluci et al., 1989a).

The enantioselectivity and regioselectivity of the microsomal epoxide hydrolase have been studied in detail with stereoisomers of (*cis*- and *trans*-) 3-*tert*-butyl-1,2-epoxycyclohexane (Belluci et al., 1982) and tetrahydro-epoxides and tetrahydro-diolepoxides derived from benz[a]anthracene (Sayer et al., 1985). From these and other studies, a model of the active site was deduced: Stereoisomeric epoxides are oriented at the active site by a polar group of the enzyme and a hydrophobic pocket (interacting at the epoxide and planar aromatic moieties of the substrate, respectively) so that it is bound in a proper orientation for the nucleophilic attack of water (Fig. 10, Armstrong, 1987). This relatively simple model is very similar to that proposed for GSH transferases (Fig. 8) and is essentially based on the same type of interactions between substrates and enzyme (one hydrophobic and one polar interaction). Similar to GSH transferase isoenzymes, the initial attack of the epoxide can occur regio- as well as stereospecifically. In contrast to the GSH transferases, no clear preference was observed in regio- and or stereoselectivity, however. Whereas GSH transferase 4-4 preferentially attacks epoxides with two benzylic carbon atoms at the

R-configured carbon atom (*vide supra*), microsomal epoxide hydrolase attacks at *R*- as well as *S*-configured carbon atoms. With some arene oxides, both enantiomers are attacked at the same carbon, irrespective of configuration (e.g., benzo[c]phenanthrene). Other arene oxides (e.g., benzo[a]-pyrene 4,5-oxide) undergo the primary reaction, predominantly at *S*-configured carbon (Yang, 1988).

Recently, sterically differently substituted epoxides, such as reactive metabolites of the pesticides vinclozolin, rotenone, and phenothrin, were investigated in terms of their ability to inhibit epoxide hydrolase. Mono- and di-substituted oxiranes were good substrates and strong noncompetitive inhibitors of hepatic epoxide hydrolase, whereas tri-substituted epoxides were virtually inactive in this regard (Cova et al., 1986). Obviously, selective inhibition of epoxide hydrolase by epoxide metabolites could interfere with the natural protection of the organism against other toxic epoxides.

Intrinsically, epoxides have been shown as able to exert various kinds of toxicity. Bay-region diol-epoxides are the most well-known ultimate carcinogenic metabolites of polycyclic aromatic hydrocarbons. With respect to aliphatic epoxides, it has been shown that the following decreasing order of mutagenicity exists for mono- > *cis*-1,2-di ≫ 1,1-di- > *trans*-1,2-tetra-substituted oxiranes (Wade et al., 1978). Generally speaking, both steric and electronic factors will determine the reactivity and toxicity of oxiranes. The toxicity of oxiranes, however, is influenced by multiple reactions. On the one hand, stereoselective metabolism of arenes by cytochrome P450 is involved. Subsequently, detoxication by epoxide hydrolase and GSH transferase determine the overall outcome with respect to toxicity. Although different isoenzymes of cytochrome P450 have different degrees of stereoselectivity in the formation of epoxides (e.g., Yang et al., 1988), the catalytic activity and stereoselectivity of epoxide hydrolases and GSH transferases also play a major role (van Bladeren et al., 1985; Armstrong, 1978; te Koppele and Mulder, 1991).

F. Glucuronyl Transferases

UDP-β-D-glucuronic acid is an important optically active cofactor for microsomal UDP-glucuronyl-transferases (GTs), which catalyze the conjugation (and inactivation) of substrates containing *O*-, *N*-, or C-functional groups to this glucuronic acid. In a number of cases, however, glucuronidation is thought to play a significant role in the generation of toxic effects (Mulder et al., 1990). Apart from increasing the instability and/or reactivity of pro-carcinogens (e.g., *O*-glucuronide of *N*-hydroxy-2-acetaminofluorene), the glucuronides may serve as transport forms of pro-carcinogens to various tissues (e.g., the *N*-glucuronide of *N*-hydroxy-2-naphtylamine).

Apart from substrate-stereoselective glucuronidation of hydroxylated hexobarbital, considerable enantioselective differences and species differences have been shown in vitro for propranolol (see the references in Vermeulen, 1986), fenoterol (Koster et al., 1986), carvedilol (Fujimaki and Hakusui, 1990), dihydrodiols of polycyclic aromatic hydrocarbons (Armstrong et al., 1988), and oxazepam (Table 4) (Sisenwine et al., 1982). (−)-Morphine has been shown to be glucuronidated exclusively at the 3-hydroxy position, whereas (+)-morphine is preferentially glucuronidated at the 6-hydroxy position. In vivo, enantioselectivity is often less pronounced than in vitro. 3-Methylcholanthrene, β-naphtoflavone, inducers of the so-called GT_1 isoenzyme, and ethanol have been shown to increase enantioselectivity in the formation of diastereomeric glucuronides from racemic oxazepam in hepatic microsomes of rabbits and to produce a large increase in enantioselectivity, whereas inducers of the GT_2 isoenzyme, such as phenobarbital, only increased oxazepam glucuronidation without affecting enantioselectivity. Inducers of forms other than GT_1 and GT_2, such as trans-stilbene-oxide and clofibric acid, generally cause increases in activities, coupled with increases in *R/S* ratios. Measurement of enantiomeric selectivity in the glucuronidation of racemic oxazepam was suggested to be a useful tool to characterize the nature and selectivity of inducers of the glucuronyltransferase system (Yost and Finley, 1985).

As of yet, very little is known about the active sites of these enzymes. The enzyme mechanism is probably consistent with a single displacement, S_N2-type nucleophilic attack of the aglycone on UDP-glucuronic acid in a ternary complex. Further studies seem necessary for a better understanding of the stereochemical behavior and active-site geometry of UDP-glucuronyl transferases (Armstrong, 1987; Armstrong et al., 1988; Mulder et al., 1990).

G. Sulfotransferases

Sulfotransferases catalyze the transfer of a sulphate group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to nucleophiles such as alcohols, phenols, and amines. Several isoenzymes, grouped into two classes based on substrate selectivity toward phenols and alcohols, have been purified (Mulder et al., 1990). How the active sites of sulfotransferases participate in the catalysis is not known (Armstrong, 1987).

Generally speaking, conclusions regarding stereoselective sulfation in vivo are difficult to draw, because of competition between (stereoselective) glucuronidation and sulfation of the same substrates. Stereoselective sulfation of 4′-hydroxypropranol enantiomers was demonstrated in vitro in hepatic tissue of various species (Christ and Walle, 1985). Enantiomeric (−/+)-4′-hydroxypropranolol sulfate ratios varied from 1.07 in the rat and

TABLE 4 In vitro Glucuronidations of Oxazepam Enantiomers (nmol/hr/g liver)

Species	*S*(+)	*R*(−)	Ratio *S*/*R*
Rhesus monkey	238	596	0.4
Dog	826	43	19.1
Miniature swine	41	29	1.4
Rabbit	178	92	1.9
Rat	424	22	19.3

Source: Sisenwine et al., 1982.

0.73 in the dog to 0.62 in the hamster. Terbutaline and prenalterol, a structural analogue of terbutaline, both showed preferential sulfation for the respective (+)-enantiomers in human liver cytosol (Walle and Walle, 1990). Both the species-dependent preference as well as the degree of apparent stereoselectivity are probably due to the activity of multiple sulfotransferase isozymes present in the supernatant preparations. At least three isozymes have been isolated from rat liver. No examples are yet known, demonstrating the causal relationship between sulfation and stereoselective toxicity. Metabolic activation, however, has been shown to occur, for example, in the case of 7-hydroxymethyl-12-methylbenz[a]anthracene, the sulfation of which is followed by alkylation of DNA via the sulfate ester (Watabe et al., 1985).

H. Cysteine Conjugate β-Lyase

Several cysteine conjugates are metabolized by β-lyase to produce thiols, ammonia, and pyruvate. Cytosolic β-lyases have been isolated from the kidney, liver, and intestinal microflora. Since it has become clear that β-lyases can play a decisive role in the bioactivation of cysteine conjugates (such as those from hexachloro-1,3-butadiene or other halogenated alkenes) to nephrotoxic agents, the scientific interest in this enzyme has increased considerably (Elfarra and Anders, 1984; Commandeur et al., 1987).

As for stereoselectivity, relatively little is known as yet, except that the natural *R* configuration in the cysteine residue is a prerequisite for a cysteine conjugate to be a substrate of β-lyase. This fact has been used as a tool to ascertain the role of β-lyase in the development of nephrotoxicity by cysteine conjugates. No data are presently available with regard to the substrate-stereoselective effects of the noncysteine part of the thioether substrates of β-lyase (Commandeur and Vermeulen, 1990). Such stereoselective effects may be anticipated, however, since, for example, the regioisomeric 1,2- and 2,2-dichlorovinyl-L-cysteine conjugates have also

been shown to cause strongly different β-lyase-mediated mutagenic and nephrotoxic effects (Commandeur et al., 1991).

III. STEREOSELECTIVE PROTECTION AGAINST TOXICITY

The antagonism or prevention of toxic effects may also benefit from stereochemical principles. Thus, optically active flavanones have been shown to inhibit the metabolic activation of the carcinogen benzo[a]pyrene to metabolites that bind covalently to DNA (Chae et al., 1992). Moreover, the (+) enantiomers of 3-*O*-methylcatechin and catechin have been demonstrated to protect stereoselectively against lipid peroxidation due to paracetamol (Devalia et al., 1982).

Alternatively, *R*-cysteine is utilized for the biosynthesis of cosubstrates for several conjugation reactions, such as glutathione, sulfate (PAPS), and taurine. The unphysiological isomer *S*-cysteine has been used to investigate the mechanistic aspects of physiological processes involving cysteine. In rats, the sulfoxidation rates of *R*- and *S*-cysteine, as well as the sulfation rates of the test substrate harmol sulfate, were found to be very similar, so that stereoselectivity for the amino acid does not seem to play a role in these reactions (Glazenburg et al., 1984). Since *S*-cysteine, in contrast to the *R*-isomer, did not increase the taurine concentration in serum, this type of stereoselectivity can be used to selectively enhance sulfate availability in vivo.

Glutathione, as the main intracellular nonprotein sulfhydryl, plays an important role as a cosubstrate for conjugation or a reductant in the detoxication of electrophilic compounds or radicals in organisms, as well as in the repair of various kinds of cellular injury. Consequently, increased levels of glutathione may exert beneficial effects in some cases. *N*-acetyl-*R*-cysteine, which is rapidly hydrolyzed intracellularly to *R*-cysteine, more successfully promotes glutathione synthesis in vivo than *R*-cysteine itself and is known to protect more efficiently against paracetamol-induced hepatotoxicity than *N*-acetyl-*S*-cysteine. Alternatively, 2-alkyl- or 2-aryl-substituted thiazolidine-4-*R*-carboxylic acids (Fig. 11), especially those derived from the condensation of *R*-cysteine with even alkyl carbon aldehydes, that is, aldehydes that are readily metabolizable to nontoxic products, can serve as precursors of *R*-cysteine and protect against paracetamol toxicity (Nagasawa et al., 1984). The incorporation of *R*-cysteine into GSH and not the liberation of sulfhydryl groups per se appears to be important, since analogues with a 4-*S* configuration, which are converted into unphysiological *S*-cysteine, were found to be totally ineffective as protecting agents. *R*-2-oxothiazolidine-4-carboxylate has been shown to increase brain cysteine levels and has been suggested as useful to protect, for

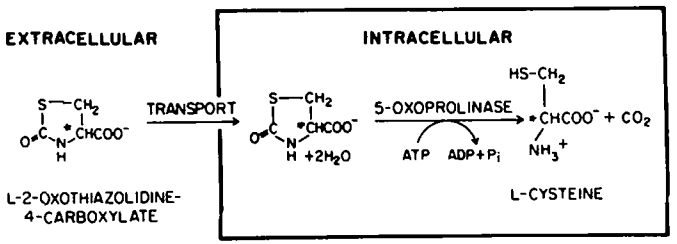

FIGURE 11 Chemical structure, transport, and metabolic degradation of 2-oxothiazolidine-4-carboxylate. * = chiral center. (Taken from Anderson et al., 1989.)

instance against the neurotoxicity of 6-hydroxydopamine (Anderson and Meister, 1989).

IV. GENERAL CONCLUSIONS

This chapter has demonstrated that it is generally recognized at present that stereochemical factors play a significant role in the disposition and pharmacological or toxicological action of drugs and other xenobiotics. As for the toxicity of xenobiotics, stereoselectivity in biotransformation (i.e., bioactivation or bioinactivation) is probably a factor of major importance. As a consequence, the biological actions of xenobiotics, in general, are difficult to predict because of the complex regulation of the processes involved.

In recent years, considerable progress has been made in the elucidation of stereochemical mechanisms of several important biotransformation enzymes, despite the fact that the multiplicity, a widely varying substrate selectivity as well as secondary metabolism (which in itself can be stereoselective), considerably complicates such studies. In principle, the same factors do complicate the correlation of data on stereoselective effects obtained in vitro with those obtained in vivo, even when it concerns the same species. The prediction of stereoselective effects from one species to another or from one chemical to another is even more difficult.

As this review also has demonstrated, new insights into stereochemical mechanisms at a molecular level [i.e., at the level of binding of substrate molecules to active (or binding) sites of isolated and purified (iso)-enzymes (or other proteins)] in some cases provided more or less simplified models or working hypotheses that might be helpful in predicting stereoselective biotransformations. Progress in research along these lines will be most promising, from not only an academic but also a more practical point of

view, namely, that concerned with predictability. From a toxicological point of view, it is important to stress that knowledge on stereoselective effects at the different levels of the development of biologically active drugs or other chemicals should be obtained as early as possible. Without this knowledge, it is almost impossible to interpret the toxicodynamics and toxicokinetics of chiral compounds.

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10

Stereoselective Transport of Drugs Across Epithelia

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I. INTRODUCTION

Drug absorption and disposition are regulated, in part, by transport across epithelial barriers. For example, both the skin and the gastrointestinal tract consist of epithelial barriers important to drug absorption. The major organs responsible for the elimination of drugs, the liver and kidney, contain epithelial cells that play essential roles in drug elimination. The renal tubules are composed of monolayers of epithelial cells that function in the active secretion and reabsorption of endogenous substances as well as xenobiotics. In recent years, it has become increasingly clear that the liver, widely recognized as the major organ responsible for drug metabolism, is an important epithelia in drug transport. Transport systems in the canalicular and sinusoidal membrane mediate drug transport into the bile. Drug distribution may also involve transport across epithelial cells. For example, distribution into the cerebrospinal fluid may involve transport systems in the choroid plexus epithelium. Transport across the specialized capillaries of the blood brain barrier is important in the distribution of drugs to the brain. The placenta is an epithelia and transport of drugs into the fetus necessarily involves placental transport. Drug excretion into the milk occurs via the mammary gland, a specialized secretory epithelium.

In the past decade, our knowledge of transport mechanisms in epithelial cells has increased dramatically. In the 1980s considerable information about the kinetics of many transport processes was obtained. Recently, molecular biology techniques have been used to clone several important transport proteins for endogenous substances and it is likely that in the next decade other transport proteins will be cloned and sequenced. With

the identification of genes encoding transport proteins, information about structure-function relations is beginning to emerge.

Whereas considerable knowledge has been obtained about stereoselective drug metabolism, comparably little is known about stereoselective drug transport. To date, our knowledge of stereoselective transport is based on kinetic studies in vivo, as well as studies in isolated tissue preparations, cells, and membrane fragments. Most of the studies have focused on the stereoselective transport of endogenous compounds; considerably less is known about stereoselective drug transport. This chapter focuses on stereoselective transport processes. Initially, we review the fundamentals of membrane transport and properties of epithelial cells. Afterward, stereoselective transport of drugs and endogenous substances is discussed. Particular attention is paid to transport processes in the kidney.

II. TRANSPORT MECHANISMS

The transport of molecules across the plasma membrane of a cell occurs by three major mechanisms: simple diffusion across the membrane, facilitated diffusion through channels or carriers, and active transport of molecules by carriers and pumps. The latter form of transport requires energy from the cell and is divided into two major types: primary active and secondary active (1,2).

For the simple diffusion of a substance through a membrane, no energy from the cell is required. The net direction of flow is determined by the concentration gradient of the substance across the membrane, from high to low concentrations. For nonelectrolytes, the rate of transport of the compound undergoing simple diffusion is linearly related to the magnitude of the gradient (Fig. 1) and can be fit to the following equation:

$$J = P(\Delta S) \tag{1}$$

where J is the net flux or transport of the substance, P the permeability coefficient for the substance in the membrane, and ΔS the concentration gradient across the membrane (1,2). The permeability coefficient should be determined for each substance and includes many factors, such as lipophilicity and molecular weight of the substance, viscosity and width of the membrane, and temperature. The net flux of the substance across the permeable membrane is composed of two opposing forces: the influx and efflux. At equilibrium, the concentration of the substance is equal on both sides of the membrane. The influx and efflux rates are equal and no net flux occurs. Because simple diffusion is not mediated by any cellular event and the thermal motion of one molecule is not dependent on any other

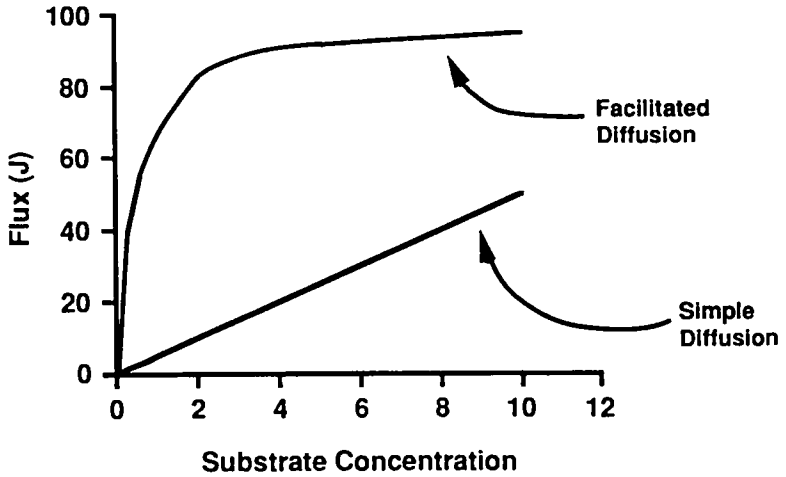


FIGURE 1 Computer-generated curves of the flux vs. increasing concentrations of substrate transported by either a simple diffusion process fit to the equation J = 5(S) or by a facilitated diffusion process fit to the equation J = 100(S)/(0.4 + S).

molecule, the diffusion of one species is not influenced by the presence of another. Since enantiomers have identical physicochemical properties, their P values are identical. Therefore for a substance transported only by simple diffusion, the rate of transport of the *l* and *d* enantiomer must be equal.

The diffusion of charged ions is more complicated because of the law of electroneutrality, which states that the sum of the positive charges on each side of the membrane must equal the sum of the negative charges. In addition to the concentration gradient, the electrical potential difference determines the final equilibrium of a substance across the membrane. Therefore at equilibrium, the concentration of an ionic species may be unequal across the membrane and this gradient will balance the electrical difference across the membrane. The driving force for transport in this situation is defined as the electrochemical potential. The Nernst equation describes the equilibrium situation for ions

$$\Delta Y = \left(\frac{2.303\ RT}{ZF}\right)\log\left(\frac{S2}{S1}\right) \tag{2}$$

where ΔY is the electrical potential difference, R is the universal gas constant, T is the temperature, Z is the valence number of the substance, F is Faraday's constant, and S2 and S1 are the concentrations of the ionic

species on each side of the membrane (1,2). For a monovalent ion at 25°C, a tenfold concentration difference balances a 59-mV electrical potential difference across the membrane.

The transport of most drugs occurs by simple diffusion. Drug transport across cells is a combination of lipid and aqueous diffusional pathways. More lipophilic substances diffuse through the membrane bilayer (transcellular route), whereas more hydrophilic substances diffuse through aqueous pathways between cells (paracellular route), resulting in a higher dependency on the size of the molecule. For example, the rate of absorption of a substance through the intestinal mucosa is related to its lipophilicity; the more lipophilic the substance, the greater the rate of absorption. If the transport rate of a substance does not follow these general laws of simple diffusion, a more complicated model, such as a facilitated mechanism, must be proposed for its transport.

Experimentally, it has been observed that many substances are transported across plasma membranes by more complicated mechanisms. Although no energy is expended by the cell and the net flux is still determined by the electrochemical potential, some substances are transported at a rate faster than predicted by their permeability coefficients. The transport of these substances is characterized by a saturable kinetic mechanism; the rate of transport is not linearly proportional to the concentration gradient. A facilitated mechanism has been proposed for these systems. Substances interact and bind with cellular proteins, which facilitate transport across the membrane by forming a channel or carrier. The two basic models of facilitated diffusion, a channel or a carrier, can be experimentally distinguished (1,2).

For a substrate that is transported by a facilitated diffusion mechanism, the kinetics follow a rectangular hyperbola (Fig. 1) and can be fit to the following Michaelis–Menten equation:

$$J = \frac{J_{max} \quad S}{K_t + S} \tag{3}$$

where J_{max} is the maximal transport rate observed for the substance at a given temperature and K_t the concentration of the substrate that produces half the maximal rate. The J_{max} for a transport protein (transporter) is a measure of the actual number of transporters in a membrane and its turnover number (or how efficient the transporter is in making a complete cycle—binding to a substrate, transport to the other side of the membrane, release of the substrate, and a return to be available to bind to another substrate). The affinity (inverse of the K_t) of a substance for a transport protein is commonly used to determine how well it binds to and interacts with the transporter (1,2). Therefore, a substance with a high affinity for a

transporter will exhibit a low K_t and vice versa. Unlike simple diffusion, the facilitated diffusion of a substance may be affected by other substances. Because the substrate must bind to the transporter, structurally similar substances can inhibit its binding and therefore its transport (1,2).

Two basic models for facilitated diffusion have been developed: the channel and the carrier. The protein forming the channel (or pore) must traverse the entire length of the membrane. When opened by a specific signal, a channel allows for the flood of substances through the aqueous hole (Fig. 2) (3). Most channels mediate the flux of inorganic ions such as Na^+, K^+, or Cl^- and are specific for either positively or negatively charged ions. Although selective in the sense that any molecule larger than the opening of the channel will not be allowed to traverse the pore, most channels are less selective than carriers because any substance with the proper charge and size should flow through the channel. What a channel gives up in selectivity, it gains in speed. The turnover number of most channels is in the range of 10^7 molecules/sec (1,2).

A major feature of the carrier model is that the binding site for the substrate must not be exposed to both sides of the membrane at the same time. The binding of a substrate to a carrier is thought to cause a conformational change in the protein that will allow the substrate to traverse the membrane (Fig. 3) (4). The binding of the substrate to the carrier may also be inhibited by structurally similar substances (Fig. 4) (5). Because the binding pocket or active site of the transporter is a three-dimensional site, as opposed to an open aqueous pore, the stereochemistry of the substrate is important. The transport rates of many stereoisomers have been shown to be greatly different (Fig. 5) (1). Although the carrier is much more selective for individual substances, the turnover number (range 10–10,000 molecules/sec) is much lower than that of a channel (1,2).

The transport of D-glucose across the membranes of erythrocytes fulfills all the characteristics of a facilitated carrier mechanism (6). Glucose is transported down its concentration gradient at a rate greater than predicted by the permeability coefficient. Its transport is saturable and inhibited by structurally similar sugars such as o-methyl-D-glucoside. The facilitated glucose transporter of red blood cells is also selective for D- over L-glucose.

Active transport has been observed in all living cells and exhibits characteristics similar to those of facilitated diffusion, except that the substance is transported into or out of the cell against its concentration gradient at the expense of energy lost by the cell. Two major types of active transport systems, primary and secondary active, have been identified. The transport of a substance against its concentration gradient that is directly coupled to the breakdown of ATP or the absorption of light energy

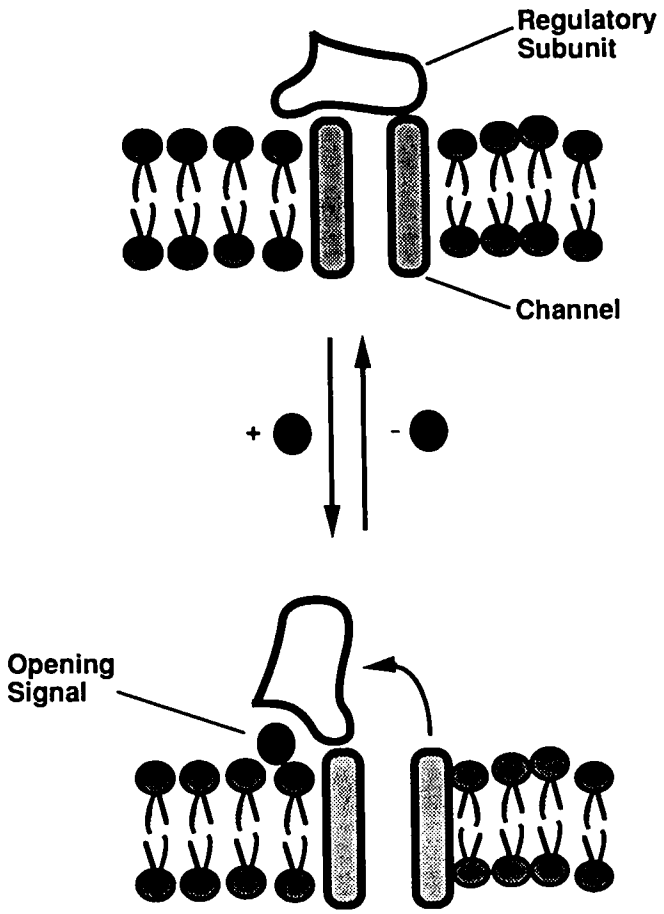


FIGURE 2 Model of an ionic channel and a regulatory subunit. Addition of an opening signal induces a conformational shift in the regulatory subunit, exposing the channel. [Adapted from Dusinsky (3).]

is an example of primary active systems (1,2). These systems are normally termed pumps, such as the Na^+K^+ATPase pump and the H^+ATPase pump (Fig. 6).

Secondary active transport mechanisms involve the flow of one substance against its concentration gradient coupled to the flow of another substrate down its concentration gradient. The substance moving down its concentration gradient supplies the energy, allowing the other substance to be transported against its concentration gradient. Direct evidence of such a

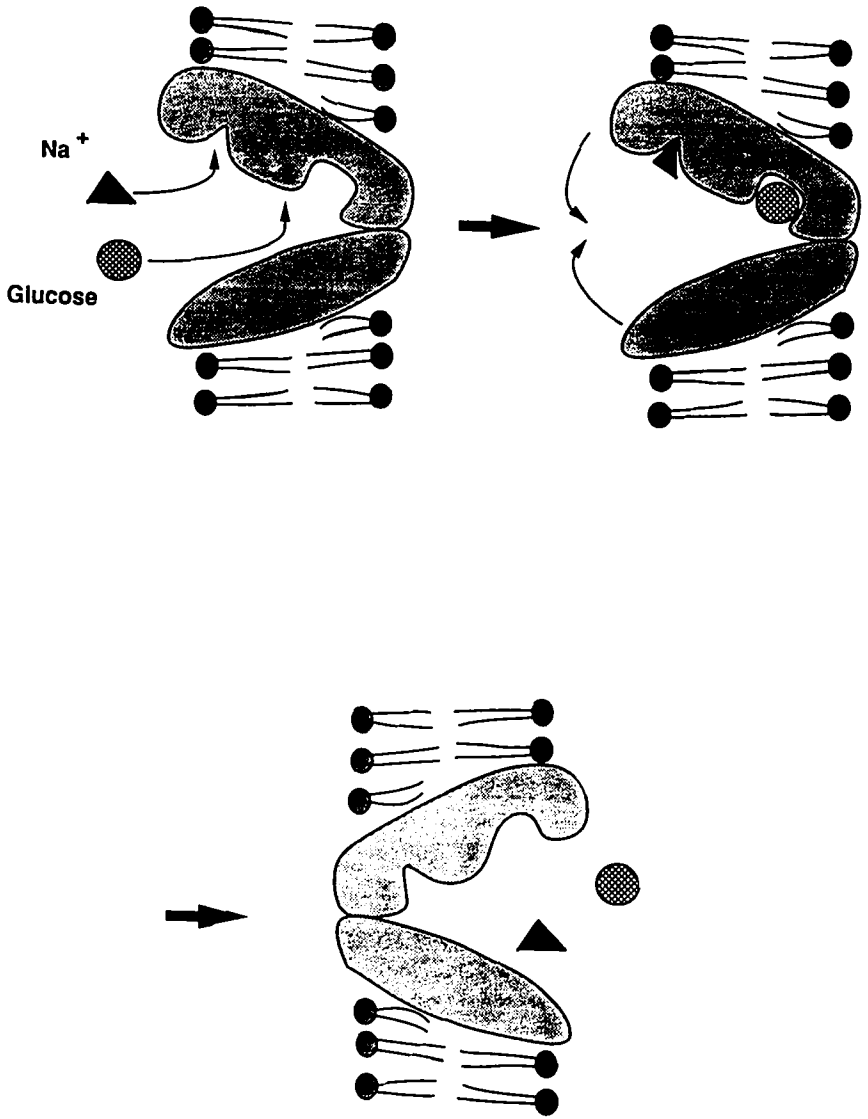


FIGURE 3 Model of the sodium-coupled glucose cotransporter. Binding site for both sodium and glucose is alternatively exposed at opposite membrane surfaces. [Adapted from Silverman (4).]

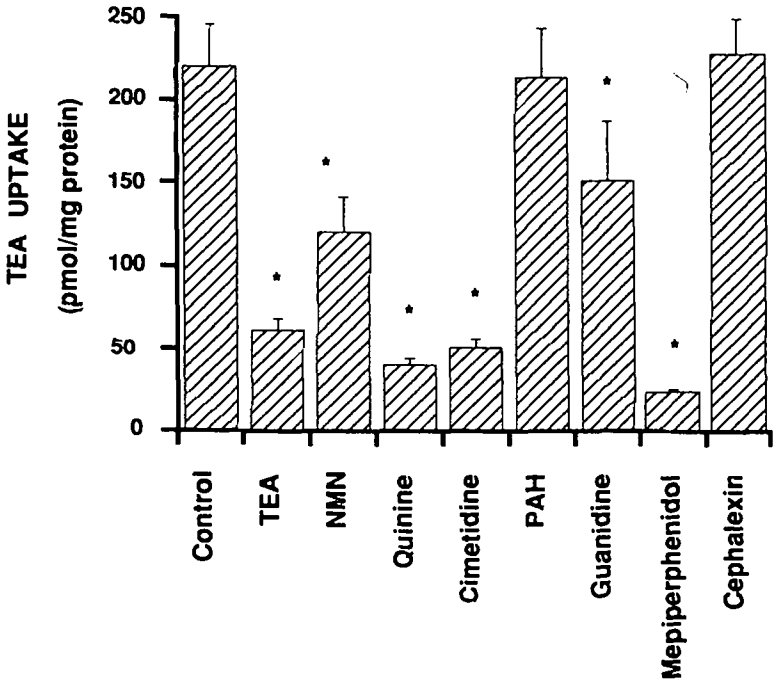


FIGURE 4 Effect of various compounds on the uptake of TEA in OK cell monolayers in the absence (control) and presence of TEA, NMN, quinine, cimetidine, *p*-aminohippuric acid (PAH), guanidine, mepiperphenidol, or cephalexin (all at 100 μM). The asterisk indicates results significantly different from the control. [Reprinted with permission from Yuan et al. (5).]

mechanism can be determined in studies in isolated plasma membrane vesicles. The organic cation tetraethylammonium (TEA) in human renal brush border membrane vesicles accumulates over time, reaching an apparent equilibrium in 60 min (Fig. 7) (7). When an outwardly directed proton gradient is present (pH in = 6.0, pH out = 7.4), the accumulation of TEA is enhanced transiently, producing an "overshoot" above its equilibrium value. This "overshoot phenomenon," reflecting the transient accumulation of TEA in response to the proton gradient, occurs because of the direct coupling of the downhill transport of protons to the uphill transport of TEA.

Many sodium-coupled systems located in the proximal tubule of the kidney exhibit an analogous mechanism (see Fig. 3). When the transport of both substances, the substrate going uphill and the coupled substrate going downhill, is flowing in the same direction, the transporter is termed

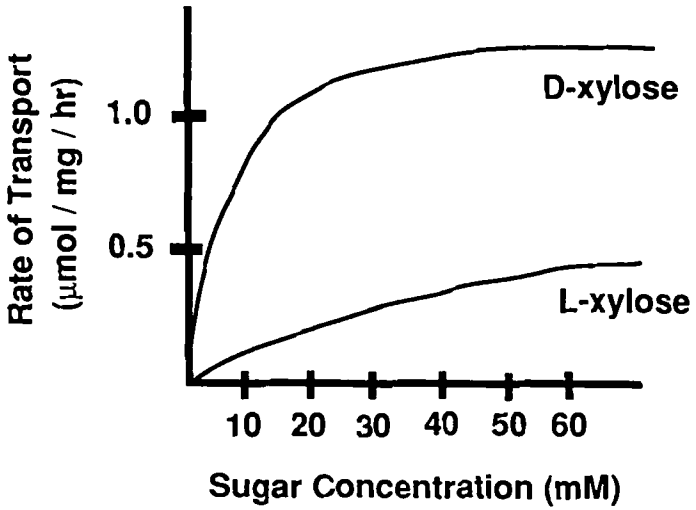


FIGURE 5 Kinetics of transport of D- and L-xylose in the yeast *Rhodotorula gracilis*. [Adapted from Dusinsky (1).]

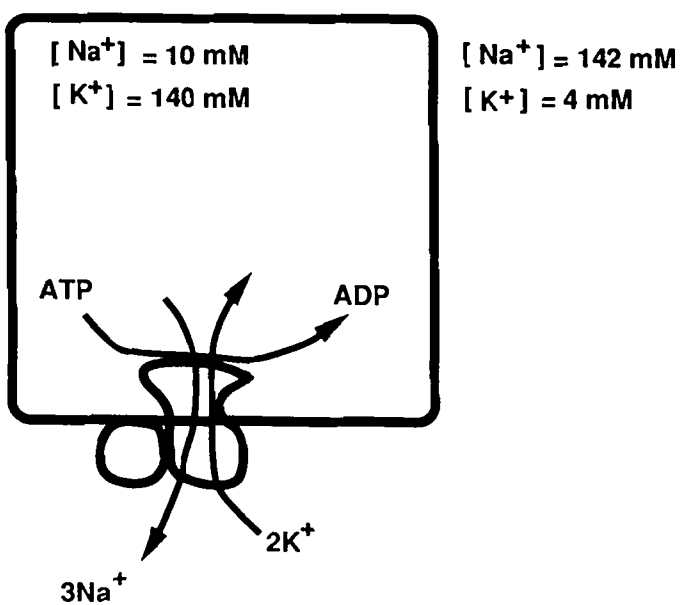


FIGURE 6 Model of the Na^+K^+ATPase pump showing the coupling ratio of Na^+ and K^+. Intracellular and extracellular concentrations of Na^+ and K^+ normally maintained by the pump are included.

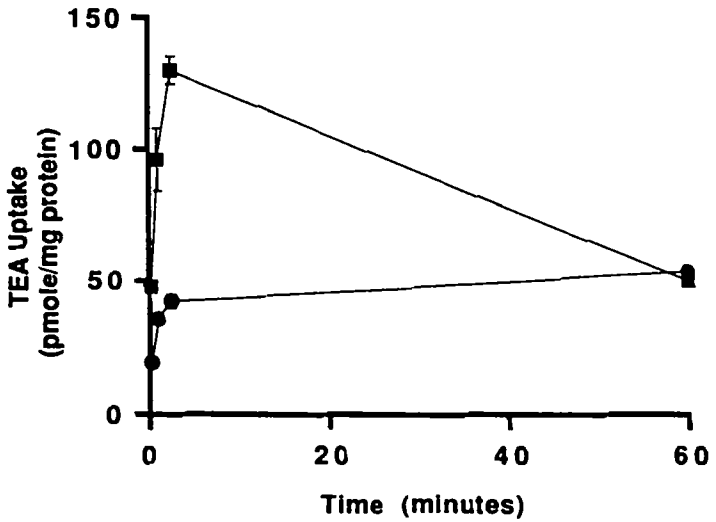


FIGURE 7 TEA uptake into human brush border membrane vesicles in the presence of an outwardly directed proton gradient (square) and in the absence of a proton gradient (circle). [Reprinted with permission from Ott et al. (7).]

a cotransporter. When the substances are transported in opposite directions across the membrane, the transporter is termed an antiporter. The passive facilitated glucose transporter in the red blood cells mentioned previously, involving a single substrate, is called a uniporter (1,2). Except for the requirement of energy, active transport systems have the same properties of facilitated diffusion systems.

For enantiomeric recognition by a protein to occur, the substrate must bind to the protein at three or more sites (8). Fewer than three interactions will not promote a stereoselective binding event (Fig. 8). When the protein of interest is a transporter, there are several stages of the transport process that could exhibit stereoselectivity (Eq. 3). Although stereoisomers would not affect the number of transporters, the J_{max} could be affected in a stereoselective manner if enantiomers produce differences in the turnover number or translocation rate of the transporter. The complex rate constant K_t, which is a function of the rate of formation and rate of breakdown of the substrate-transporter complex along with the rate of translocation of the complex, can be severely affected by stereoisomeric structural changes. First, the binding affinity may differ between enantiomers when a three-point binding interaction is involved. Second, the rate of release of the substrate from the complex may differ between enantiomers. Finally, the

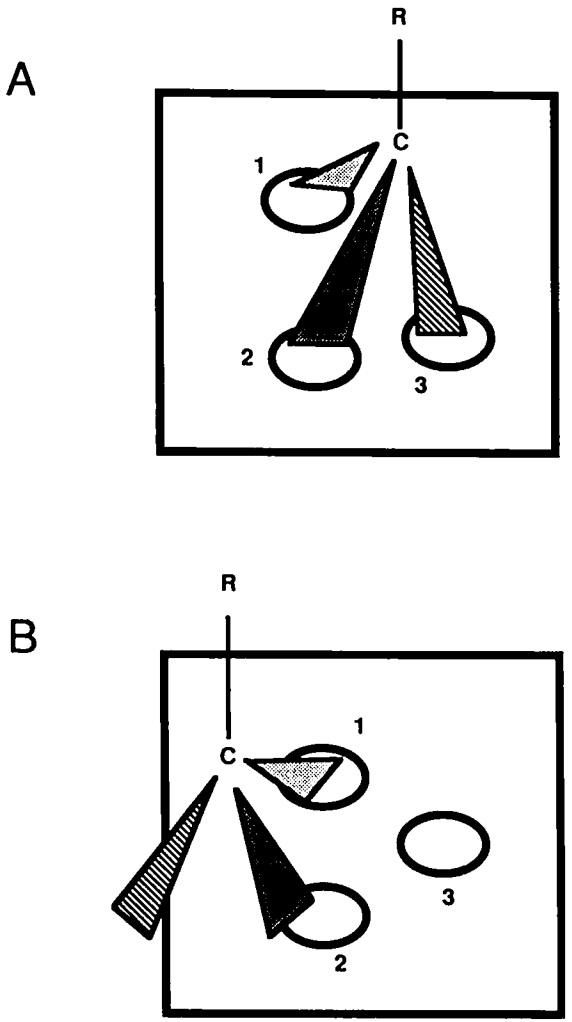


FIGURE 8 Model of substrate binding to protein showing a proper three-point binding interaction (A) and of the enantiomer showing an improper three-point binding interaction (B). [Adapted from Christensen (8).]

ability of the protein to change its conformation may be different for two enantiomers after substrate binding, thereby affecting the translocation rate of the transporter.

III. PROPERTIES OF EPITHELIAL CELLS

A major function of epithelia is to transport substances from one body cavity to another, causing the net movement of many essential ions, nutrients, and potential toxins. For this net vectorial transport to occur, the single layer of epithelial cells lining the body's cavities must have polarized membranes exhibiting different transport mechanisms. The mucosal membrane (also termed brush border, luminal, or apical) faces the outside of the body or cavity and usually has many fingerlike projections or microvilli (Fig. 9). These membrane processes increase the surface area of the cell membrane dramatically and thus facilitate the transfer of large quantities of substances. The serosal membrane (also termed basolateral, peritubular, or antiluminal) faces the blood and lies on a structural support of glycoproteins and collagen (basement membrane) that is not a significant barrier for the movement of substances. The two membrane faces are separated physically by tight junction proteins (zona occludens) that appear to prevent the movement of transmembrane proteins between the two polarized faces (9). For vectorial transport across epithelia, the substance must enter the cell, diffuse across it, and exit at the other membrane. Net movement from the basolateral through the apical membrane is called secretion and from the apical to the basolateral is referred to as absorption or reabsorption. For net flux of a nonmetabolized substance, the transport mechanisms at the two membranes must be different and active at one or both membranes. The commonly used scheme is called a pump-leak arrangement (10). For example, a substance is actively transported across one membrane into the cell, which leads to a concentration gradient across the other membrane. The exit membrane contains a transporter that allows passive facilitated diffusion out of the cell. Alternatively, simple diffusion of the substance out of the cell down its concentration gradient occurs. Cellular metabolism of the substance can also occur, complicating the above scheme. For many drugs, cellular metabolism may be important and stereoselective drug "transport" may actually represent stereoselective drug metabolism.

The major important organic electrolytes and nonelectrolytes transported by epithelial cells include sugars, amino acids, nucleosides, organic cations, and organic anions. Transport systems have significant implications for the absorption, distribution, elimination, and pharmacokinetic properties of many clinically important drugs. The major epithelial tissues

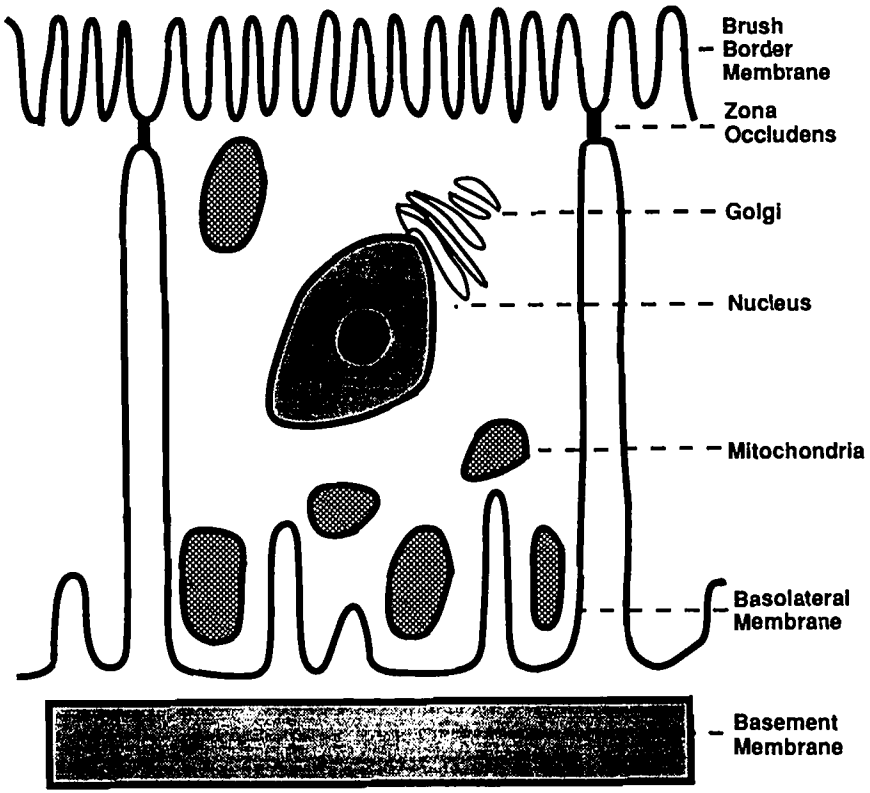


FIGURE 9 Epithelial cell showing the polarized membranes and cellular components.

important in drug absorption and disposition include the kidney, the entire gastrointestinal tract, the choroid plexus that lines the ventricles of the brain, placenta, liver hepatocytes, and the linings of the lungs (10). The remainder of this chapter will focus on the transporters located in the kidney and how the use of stereoselective mechanisms promote the specific transport of organic substances. Some discussion of transport in other epithelia is also included.

IV. KIDNEY

The major functions of the kidney include the regulation of water, electrolyte and nutrient balance of the body, and the excretion of metabolic waste products and foreign substances. These functions are carried out by a

series of complex interactions involving the filtration, reabsorption, and secretion of plasma water and its constituents. The major structural and functional unit of the kidney is the nephron (Fig. 10).

Each kidney is composed of approximately 1 million nephrons (9). A complete nephron consists of a glomerular capillary network, Bowman's capsule, proximal tubule, loop of Henle, distal tubule, and collecting duct. The glomerular capillary network and Bowman's capsule are responsible for the formation of the initial ultrafiltrate of the blood. The plasma water and all non-protein-bound constituents are forced through the capillary network and into the Bowman's space formed by the capsule. Most plasma proteins and red blood cells are usually retained in the capillary. The rate of plasma filtered by the glomeruli is referred to as the glomerular filtration rate (GFR) and in a normal healthy adult is usually around 125 mL/min (9). The final urine flow averages 1 mL/min (9). Therefore, over 99% of the filtered plasma is reabsorbed in its passage through the kidney tubules. The ultrafiltrate composition changes dramatically as it flows through the

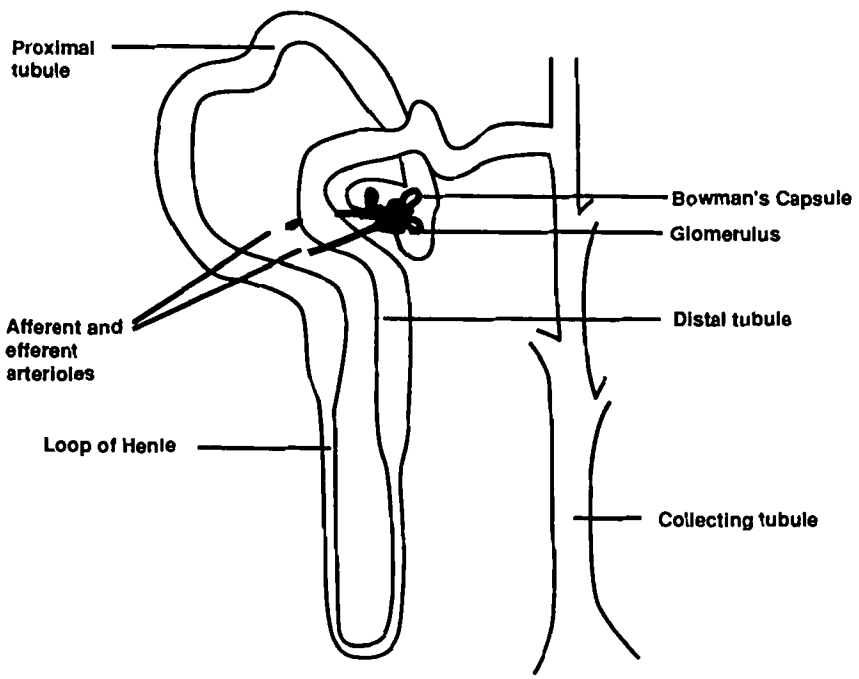


FIGURE 10 Diagram of a nephron.

extensive tubule system of the nephron. Based on the requirements of the body at any specific time, the exiting urine can be diluted to 50 mOsm/kg H_2O or concentrated to 1200 mOsm/kg H_2O, suggesting that the secretion and reabsorption of inorganic ions is an active and highly regulated process (9).

The renal excretion rate of a compound is determined by a combination of three factors: filtration at the glomeruli, tubular reabsorption, and tubular secretion (11) and is described by the following equation:

$$\text{renal excretion rate} = \text{rate of filtration} - \text{rate of reabsorption} + \text{rate of secretion} \tag{4}$$

and the renal clearance of a compound is defined by the following equation:

$$\text{renal clearance} = \frac{\text{renal excretion rate}}{\text{plasma concentration of compound}} \tag{5}$$

Therefore, the renal clearance of a compound is

$$\frac{\text{rate of filtration} - \text{rate of reabsorption} + \text{rate of secretion}}{\text{plasma concentration}} \tag{6}$$

The rate of filtration for a substance is equal to GFR*fu*C, where GFR is the glomerular filtration rate, fu the fraction of the drug unbound in the plasma, and C the plasma concentration (11). The GFR can be experimentally determined by measuring the renal clearance of inulin, an exogenous polysaccharide that is not bound to plasma proteins (fu = 1) and is not reabsorbed, secreted, or metabolized by the tubules of the kidney. Therefore, the renal clearance of inulin is equal to the GFR (11). By comparing the renal clearance of a drug to its clearance by filtration (GFR*fu), one can determine if the compound undergoes net reabsorption or secretion by the kidney. Therefore, if the clearance of a drug is lower than the GFR*fu, net reabsorption must have occurred. This does not exclude the possibility that simultaneous secretion of the drug also occurred. Conversely, if the clearance of a drug is higher than GFR*fu, the compound must be actively secreted by the kidney (simultaneous reabsorption is also possible, but secretion must predominate). The fact that many organic nutrients such as glucose (which is present in plasma and not bound to plasma proteins) are almost completely absent from the urine suggests active reabsorption by the kidney. Therefore, reabsorption and secretion of compounds may be active or passive, and the processes may occur simultaneously and in different sections of the nephron. For example, potassium is usually reabsorbed in the proximal tubule and the loop of Henle, whereas it may

be secreted in the distal tubule and reabsorbed again in the collecting duct (9).

The renal clearance of two enantiomers will be different if any of the abovementioned processes exhibit stereoselectivity (12–14). The filtration clearance (GFR*fu) will reflect differences between enantiomers if a compound is bound stereoselectively to plasma proteins (12–14). The rate of reabsorption or secretion of enantiomers can be different if transport proteins at either the basolateral or brush border membrane exhibit stereoselective kinetics. Stereoselective cellular metabolism is a well described process and is dealt with in depth elsewhere in this book. However, the possibility of stereoselective metabolism occurring within the renal cells cannot be ignored as a potential mechanism responsible for differing renal clearance between two enantiomers.

Many methodologies have been developed to study transport processes in the renal tubules. The total renal clearances of various substances can be determined in human or whole animal studies and in isolated perfused kidneys. Transport systems along the nephron are usually located by stop-flow or microperfusion studies. Isolated perfused or nonperfused tubules can be used to determine specific transport mechanisms at individual membranes. For example, in the nonperfused tubule that has a collapsed lumen, transport reflects the process occurring at the basolateral membrane only. In the last decade, isolated plasma membrane vesicles, either of the brush border or basolateral membrane, have been widely used to study transport mechanisms at each of the polarized membranes. Vesicles are excellent for studies of drug transport since potential problems related to the metabolism of substances by the whole cells are eliminated. The use of primary or continuous renal cell lines is particularly suited to the study of the effects of long-term exposure to regulatory or toxic agents on transport proteins.

The proximal tubule is responsible for the reabsorption of over 65% of the sodium and water of the glomerular ultrafiltrate (9). Most if not all the organic nutrients such as glucose and amino acids are also reabsorbed in the proximal tubule (9). It has been experimentally determined that the renal handling of exogenous substances such as organic cations and organic anions that are generally secreted also occurs within the proximal tubule (15). Therefore, the rest of this section will be concerned with the stereoselective handling of organic substance within the proximal tubule.

A. Monosaccharides

Glucose is almost completely and actively reabsorbed within the first third of the proximal tubule of the kidney (9). Because of this and the importance of glucose and its urinary excretion in the monitoring of diabetes, much

work has been conducted on the specific mechanisms responsible for glucose reabsorption. The transport of glucose across the brush border membrane is active and coupled to the downhill movement of Na^+ across the membrane by the Na^+-glucose cotransporter (Figs. 11 and 12), with the Na^+ gradient supplied by the Na^+K^+ATPase pump (16). The transport of glucose down its concentration gradient across the basolateral membrane into the blood is thought to occur by a passive uniporter similar to that seen in red blood cells (Fig. 12) (17).

This pump-leak arrangement for glucose is so efficient that only in cases such as diabetes, does any glucose escape reabsorption. The Na^+-glucose cotransporter is selectively inhibited by phloridzin (18–25), whereas the glucose transporter at the basolateral membrane is selectively inhibited by phloretin (16). Many studies have been conducted on the specificity of the two transporters, and for both transporters a D-glucopyranose ring in the chair conformation with the C1 hydroxyl in the equatorial position is required (18–25). In addition, the brush border membrane cotransporter appears to have essential requirements for free C2 and C3 hydroxyl in the equatorial positions (Fig. 13) (26).

The stereoselective requirements for the transport of sugars by these transporters are quite restrictive. The D isomers of mannose, fructose, and galactose appear to interact with both systems; however, only D-galactose, a methyl-D-glucoside, and 6-deoxy-D-glucose appear to be transported

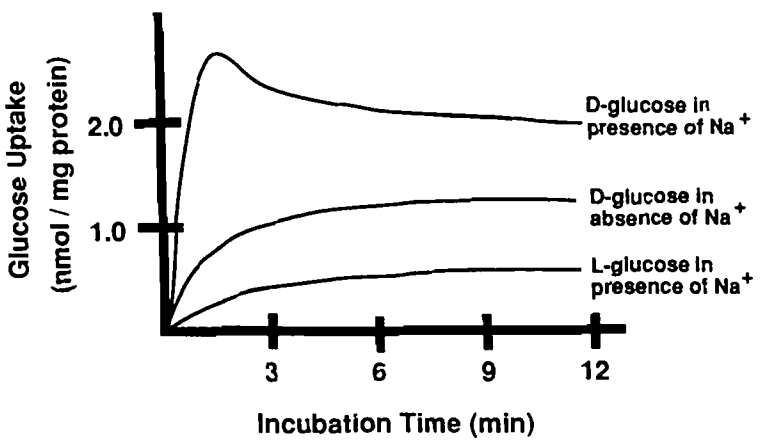


FIGURE 11 Uptake of D- and L-glucose in rat renal brush border membrane vesicles in the presence or absence of a sodium gradient [Adapted from Kinne et al. (16).]

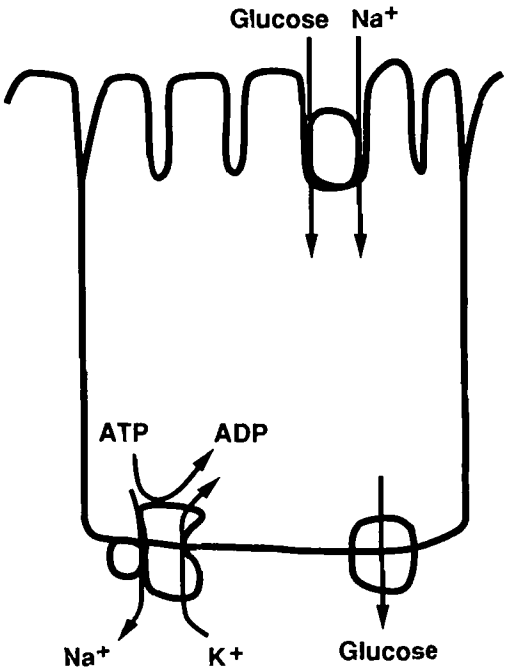


FIGURE 12 Model of transepithelial flux of glucose in the proximal tubule.

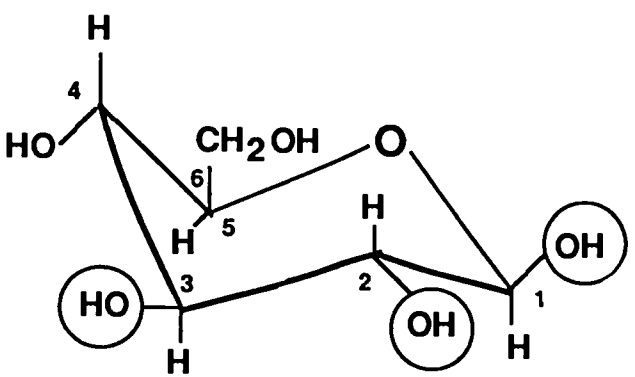


FIGURE 13 Structural requirements of monosaccharide transport across the brush border membrane of the proximal tubule. Hydroxyl groups in circles are essential for transport. [Adapted from Deetjen and von Baeyer (26).]

by the Na^+ system (18–25). The L isomer of glucose is not transported across either membrane by mediated mechanisms and appears to cross the membrane by simple diffusion (Fig. 11). There has been one report of L-arabinose interacting with the basolateral uniporter (27). Therefore, the stereoselective requirements for sugar transport in the kidney are very restrictive and only a few structural changes to the ribose ring are allowed.

B. Amino Acids

Amino acids, like glucose, are very efficiently reabsorbed by the early proximal tubule via a pump-leak arrangement, with a Na^+-cotransporter located in the brush border membrane (Fig. 14) (28). However, there are some additional complicating factors affecting amino acid transport. Brush border membrane aminopeptidases can split peptides within the lumen, increasing the concentration of amino acids after filtration (29). Under certain conditions, such as hereditary hyperamino aciduria, amino acids can undergo net secretion in the tubules by mechanisms not completely understood, involving both active and leak pathways (29). In addition, there are three different types of amino acids: acidic, basic, and neutral, and there are amino acids with aliphatic, aromatic, amide, and sulfur-containing side chains. There is one transporter for acidic amino acids, one for basic amino acids, and at least two transporters for neutral amino acids

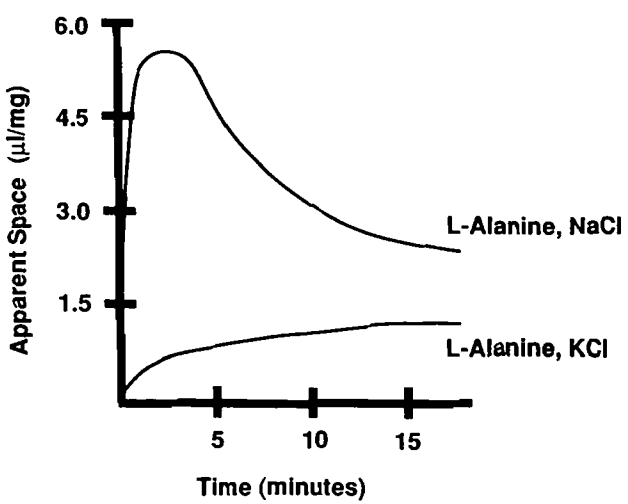


FIGURE 14 Uptake of L-alanine in rabbit renal brush border membrane vesicles in the presence and absence of an inwardly directed sodium gradient. [Adapted from Mircheff et al. (28).]

TABLE 1 Stereoselectivity of Alanine Uptake[a]

Incubation	Uptake (pmol/30 sec/mg protein)
L-alanine	2.48 ± 0.41
D-alanine	2.45 ± 0.43
L-alanine + NaCl	8.39 ± 1.15
D-alanine + NaCl	3.41 ± 0.36

[a]Rabbit renal brush border membrane vesicles were incubated for 30 sec at room temperature.
Source: Fass et al. (38).

in the brush border membrane (29–37). The naturally occurring L isomers are transported through the brush border membrane at a much greater rate than the D forms (Table 1) (38). However, at high concentrations, the D isomers can inhibit the transport of L isomers and vice versa. Therefore, the D isomers do appear to be actively transported by these systems, albeit with a much lower affinity (38). The acidic amino acid carrier appears to exhibit pronounced stereoselectivity. D-glutamate has almost no affinity for this transporter; however, D-aspartate inhibits the transport of both L-glutamate and L-aspartate (29).

C. Organic Cations

The renal handling of organic cations and bases has received considerable attention during the last decade due to the identification of many clinically relevant drug–drug interactions occurring in the kidney. The active tubular secretion of organic cations was discovered in the 1940s (39); however, because of their detrimental effects on the cardiovascular system (dopamine, norepinephrine, neostigmine, histamine, and epinephrine), mechanisms of organic cation transport were not elucidated until the advent of in vitro methodologies, including the isolation of plasma membrane vesicles and of perfused and nonperfused tubules.

In 1981, a model for the transepithelial secretion of organic cations was proposed by Holohan and Ross (40). Organic cations such as TEA and N^1-methylnicotinamide (NMN) are transported into the proximal tubule cell across the peritubular membrane by a facilitated, passive electrogenic carrier-mediated system and accumulate within the cell because of the favorable electrochemical gradient across that membrane (40). The transport of organic cations into the lumen of the tubule involves a secondarily active system driven by a counterflow of protons. This system is referred to as the organic cation/proton antiporter (7,40–46). The Na^+/H^+ antiporter, also located in the brush border membrane, is thought to be largely

responsible for the proton gradient that exists from lumen to cell. The Na^+ gradient across the apical membrane is produced by the Na^+K^+ATPase system in the basolateral membrane (40). The complete model for organic cation secretion is detailed below (Fig. 15). Microperfusion studies, isolated tubules, and membrane vesicle studies have localized this transport system to the proximal tubule, with more transport occurring in the first segment (47).

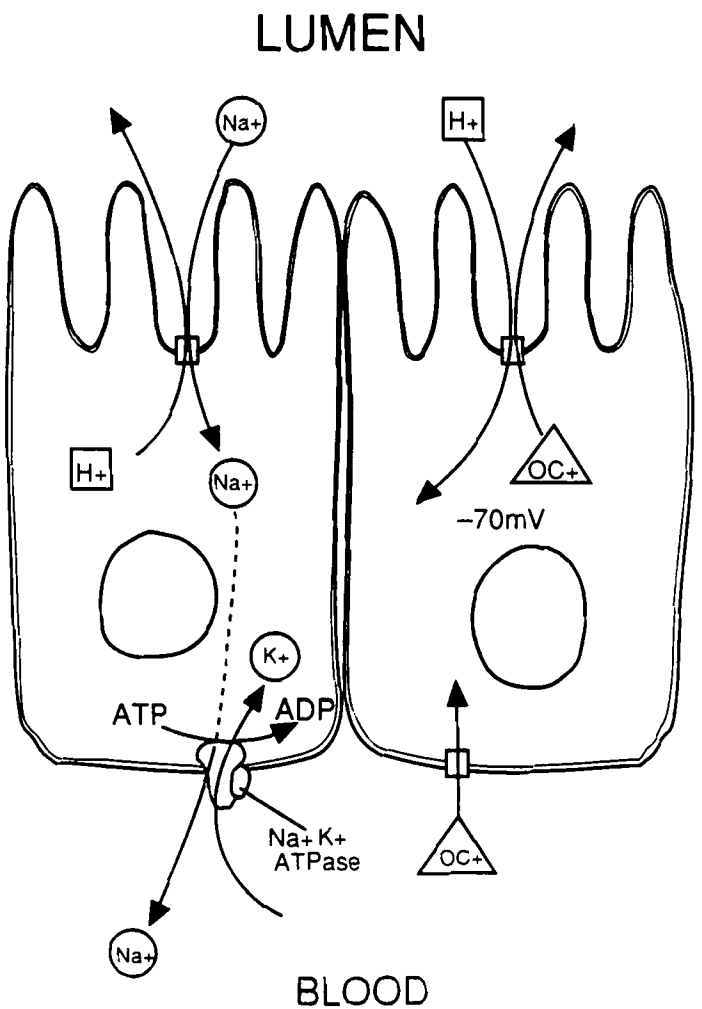


FIGURE 15 Model of the transepithelial flux of organic cations in the proximal tubule.

Many exogenous, pharmacologically active, clinically important organic cations including cimetidine, procainamide, quinine, quinidine, triamterene, and pindolol are actively secreted (renal clearance > GRF*fu) (48–52). Drug interactions between many of these substances have been documented in vivo in humans. For example, cimetidine has been shown to decrease the renal clearance of procainamide, pindolol, ranitidine, cephalexin, and amiloride (53–55). Competitive inhibition of the organic cation/proton antiporter at the brush border membrane has been suggested as the mechanism for these drug–drug interactions. Consistent with this mechanism, in vitro studies of the organic cation/proton antiporter demonstrate the competitive inhibition between many of these organic cations (Fig. 16) (5). This, however, does not preclude interactions at the basolateral membrane.

Stereoselective biotransformation of drugs has long been observed. However, the possibility of stereoselective renal excretion of exogenous compounds has only been recently proposed (56,57). Pindolol, a β-adrenoreceptor blocking agent, is actively secreted (renal clearance > GRF*fu) by the organic cation transport system, and recent clinical studies have suggested that it is stereoselectively eliminated. The *S*(−) enantiomer of pindolol had a 30% higher renal clearance than the *R*(+) form (Fig. 17) (56).

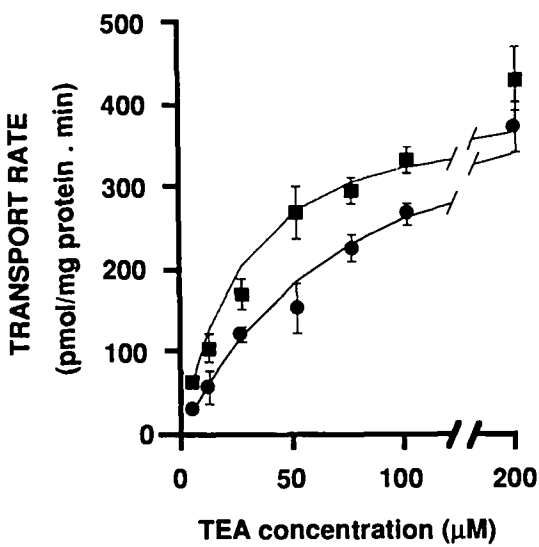


FIGURE 16 Effect of increasing TEA concentration in the absence (square) or presence (circle) of 100 μM NMN on the rate of TEA uptake in OK cell monolayers. [From Yuan et al. (5).]

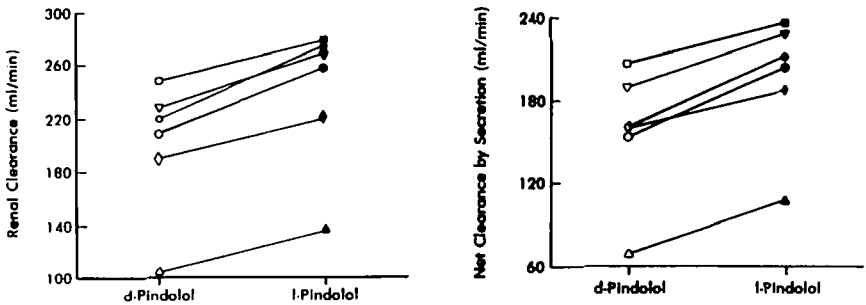


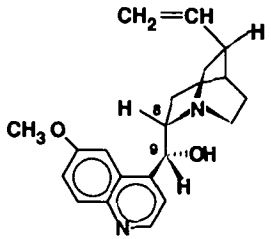
FIGURE 17 Renal clearance (left) and net clearance by secretion (right) of *l*- and *d*-pindolol in six subjects [From Hsyu and Giacomini (56).]

Possible mechanisms responsible for this stereoselective renal clearance of pindolol appear to be stereoselective renal metabolism or stereoselective renal secretion (stereoselective binding to plasma proteins was not observed). Recently, Somogyi et al. investigated the effect of coadministration of cimetidine on the renal clearance of the two enantiomers of pindolol (58). Cimetidine significantly reduced the renal clearance of both enantiomers, but reduced the renal clearance of the *R* enantiomer by a greater extent than the *S* enantiomer. These data are consistent with the stereoselective renal elimination mechanism for pindolol, with the *S* enantiomer being preferentially cleared.

Although diastereoisomers, both quinine and quinidine, have similar physical properties (Fig. 18). In clinical studies, the renal clearance of quinidine was fourfold greater than that of quinine (57). No stereoselective differences in plasma protein binding were observed. The renal filtration and passive reabsorption of these two diastereoisomers should be similar since the compounds have similar octanol-water partition coefficients and pKa values (57). Therefore, stereoselective active renal secretion may be the mechanism responsible for the observed differences in the renal clearances of quinine and quinidine.

Further clinical examples of potential stereoselective renal secretion of organic cations have been recently reported. A major metabolite of verapamil (D-617) is actively secreted by the kidney (59). Upon coadministration of cimetidine, the renal clearance of the *S*-D-617 isomer was significantly decreased, whereas the clearance of the *R*-D-617 metabolite was unaffected by cimetidine administration (59). Stereoselective renal secretion was suggested as the mechanism of this effect. However, it is not known if this metabolite is actually secreted by the organic cation transport system. The renal clearance of unbound *S*(+) disopyramide was

QUININE (8S,9R)



QUINIDINE (8R,9S)

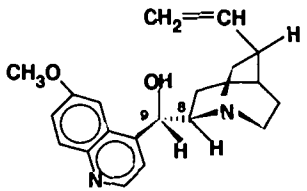
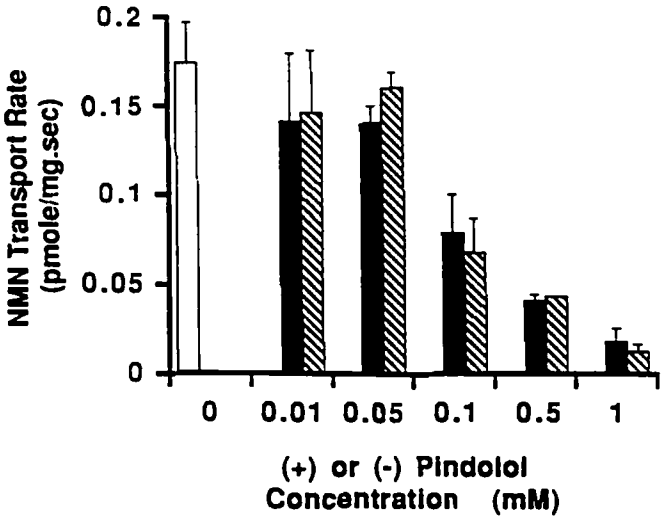


FIGURE 18 Structures of quinine and quinidine.

much greater than that of the $R(-)$ enantiomer (60). In addition, stereoselective plasma protein binding may produce stereoselective renal clearance of the enantiomers of disopyramide (61).

The results of these studies suggest that there may be stereoselective secretion by the renal organic cation transport system. However, the potential concomitant stereoselective renal metabolism of these drugs was not addressed in these studies. The use of isolated plasma membranes eliminates the metabolic machinery of the renal cell (62), thereby allowing the direct examination of the interactions of enantiomers with organic cation transport proteins located at individual membranes. We examined the effect of $R(+)$ and $S(-)$ pindolol and the diastereoisomers quinine and quinidine on the uptake of NMN in renal brush border membrane vesicles from the rabbit (63). Our rationale was that if these substances interacted stereoselectively with the organic cation/proton antiporter, the K_I or IC_{50} values should reflect this interaction. All four compounds significantly inhibited the transport of NMN in brush border membranes vesicles (Fig. 19). The IC_{50} values of the inhibitors were $R(+)$ pindolol = 0.14 μM, $S(-)$ pindolol = 0.12 μM, quinine = 2.5 μM, and quinidine = 2.4 μM. However, there was no significant difference in the IC_{50} values for the enantiomers of



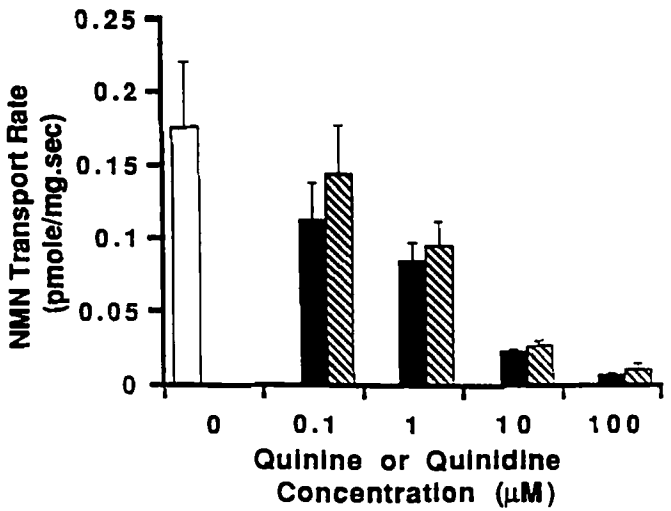


FIGURE 19 Uptake of NMN in rabbit brush border membrane vesicles in the presence of various concentrations of quinine and quinidine (lower panel) and *d*- and *l*-pindolol (upper panel). The clear bar in each panel is the control, the black bars represent either quinine (lower panel) or *d*-pindolol (upper panel) and the shaded bars represent either quinidine (lower panel) or *l*-pindolol (upper panel). [From Ott et al. (63).]

pindolol or the IC_{50} values of quinine and quinidine, suggesting no stereoselective interaction with the organic cation/proton antiporter. This same approach was undertaken by Bendayan et al. (64) using dog renal brush border membrane vesicles. They determined that the uptake of NMN was inhibited by quinine with a K_I of 7 μM and quinidine with a K_I of 0.7 μM. Although not stated if these values were significantly different, the authors suggest that the organic cation/proton antiporter demonstrates a degree of stereospecificity.

Wong et al. examined the effect of quinine and quinidine on the uptake of the organic cation amantidine (65), using rat renal cortical slices and proximal tubule suspensions. They observed a stereoselective inhibition of amantidine, with quinine being the significantly more potent inhibitory species. This is in direct conflict with the previous studies, which demonstrated either no stereoselective inhibition between the diastereoisomers or that quinidine is the more potent species in terms of renal transport.

Clearly, the results to date using in vitro methods are conflicting. Of the three studies detailed, each produced significantly different findings and conclusions regarding the potential stereoselectivity of the organic cation transport system. These conflicting data may be a result of species differences in transport mechanisms or in differences in experimental methods. Although the method employed by Wong et al. includes the basolateral membrane (proximal tubule suspensions and cortex slices), no study has specifically addressed the potential interactions occurring at the basolateral membrane. Further studies are required to determine potential stereoselective interactions for this system. The studies until now have relied on the inhibition potencies (IC_{50}) of enantiomers. Studies directly examining the transport of enantiomers are needed; they may reveal differences in binding and translocation rates.

D. Organic Anions

The active secretion of organic anions by the proximal tubule of the kidney has been studied extensively. *p*-Aminohippuric acid has been the model compound of organic anions for many decades. Transporters in the two membranes of proximal cells appear to be arranged opposite to that observed for organic cations (66,67). The active step is located in the basolateral membrane, but the exact mechanism is still very controversial. Some studies have suggested that the active transport of organic anions involves a Na^+ cotransport mechanism, whereas others have suggested organic anion exchange with endogenous organic anions (66,67). The brush border membrane is thought to contain a downhill facilitated carrier-mediated step in exchange for urate (66,67). Probenecid has become the

model inhibitor of the organic anion transporter (68). Organic anions transported by this system include salicylates, penicillins, and cephalosporins (68,69). Although much work has been conducted on the structural specificity of this system for organic anions, there have been few reports detailing the stereoselectivity of this system, possibly due to the fact that both model compounds for this system are achiral. Ullrich et al. demonstrated that the *N*-a-acetyl-D isomers of many amino acids have a much higher affinity for the basolateral membrane than the L forms (70). Cox et al. observed no stereoselective renal handling of the nonsteroidal anti-inflammatory agent, ibuprofen, in rat isolated perfused kidney (71). However, Ahn et al. (72) demonstrated that *R*(−) ibuprofen is cleared more rapidly in the isolated perfused rat kidney than its *S*(+) enantiomer, suggesting differences in tubular transport or renal drug metabolism. These conflicting results suggest that further studies are required to determine potential stereoselective interactions of the organic anion transport system.

E. Reabsorption of Drugs in the Kidney

As mentioned previously, both the secretion and reabsorption of compounds can occur simultaneously and in different areas of the kidney, complicating the net handling of the drugs by the kidney. The kidney appears to actively reabsorb most endogenous nutrients, including inorganic ions, sugars, amino acids, and vitamins. However, in general for many exogenous compounds, reabsorption appears to occur by simple diffusion and is dependent on the degree of ionization of the drug, lipophilicity of the drug, and urine pH and flow. The driving force for the passive reabsorption of drugs is reabsorption of water. As water is reabsorbed along the nephron, the concentration of drug within the lumen increases, causing the required concentration gradient to be imposed across the membrane. Drug reabsorption will then occur passively by the laws of simple diffusion.

V. OTHER EPITHELIA

A. Small Intestine

The absorption of sugars and amino acids appears to follow the same basic mechanisms as previously described for the proximal tubule of the kidney (73–77). An additional D-fructose-facilitated carrier mechanism has been located in the brush border membrane that is stereoselective and specific (78). It is Na^+-independent and does not accept D- or L-glucose. After transport into the cell, the intracellular fructose concentration is decreased

by metabolism, which promotes further passive reabsorption through the brush border membrane (78).

The absorption of most exogenous compounds occurs by a first-order simple diffusion process. Therefore, the physicochemical properties of the drug, molecular weight, degree of lipophilicity, and concentration gradient will determine the extent of absorption (11). Also important is the pH of the lumen solution, which determines the percent of the nonionizable drug. Additional factors determining the extent of absorption include metabolism of the drug in the gut lumen due to enzymatic or chemical degradation, gastric emptying, and local blood flow (11). A few exogenous drugs are transported in the small intestine by carrier-mediated systems. 5-Fluorouracil and methotrexate are structural analogs of endogenous compounds and are thought to be transported by those systems (11). A carrier-mediated mechanism has also been observed for aminocephalosporins such as cefadroxil and cephradine (79). These drugs show a saturable and inhibitable transport mechanism. These compounds are also inhibited by dipeptides, suggesting that certain drugs may have some affinity for the dipeptide transport system in the small intestine. This system transports dipeptides coupled to an inwardly directed proton gradient, independent of a sodium gradient. It has recently been documented that the *cis* isomer of ceftibuten is markedly concentrated by this system, whereas the *trans* isomer is not readily absorbed in the small intestine and is not stimulated by a proton gradient (80). Therefore, it appears that some exogenous compounds may be absorbed through the small intestine by carrier mechanism and a report suggests some limited stereoselectivity.

B. Liver

Studies have demonstrated multiple transport carriers in hepatocytes for organic cations, organic anions, and neutral charged compounds (81–83). For biliary excretion to occur, compounds must transverse two different membranes of the hepatocyte, the sinusoidal and canaliculi membranes. Many large positively charged (monovalent and bivalent) lipophilic compounds with a minimum molecular-weight threshold in the range of 200–600 daltons are actively transported into the bile from the blood through hepatocytes (81–83). These include procainamide ethobromide, vecuronium, *N*-methylimipramine, and tubocurarine. Therefore, the pharmacokinetics of anticholinergics, neuromuscular blocking agents, psychotropic amines, and local anesthetics can potentially be affected by biliary excretion. Also, bile acids and salts, steroid hormones, bilirubin, fatty acids, and cardiac glycosides have been demonstrated to be transported by system in the liver cells (81–83). Proton cotransport along with sodium dependent

and independent mechanisms have been suggested for transport. Because the major structural feature affecting the selectivity of transport appears to be lipophilicity, only one study showing stereoselectivity has been documented (84). Clearly, more work is needed in this area in not only the determination of ionic driving forces, but also potential stereoselective structural requirements.

VI. SUMMARY

There are many examples of stereospecific epithelial transport. Especially significant are the transport of endogenous compounds and nutrients, such as glucose and amino acids. Less well understood and identified are those transport processes involving exogenous compounds, including racemic drugs. To clearly understand the pharmacokinetics and pharmacodynamics of drugs, transport processes involving absorption, distribution, and excretion must be clearly delineated. Further work on the various aspects of stereoselective transport needs to be done for those compounds in which transport processes greatly affect the handling of these drugs.

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11

VERAPAMIL

A Chiral Challenge to the Pharmacokinetic and Pharmacodynamic Assessment of Bioavailability and Bioequivalence

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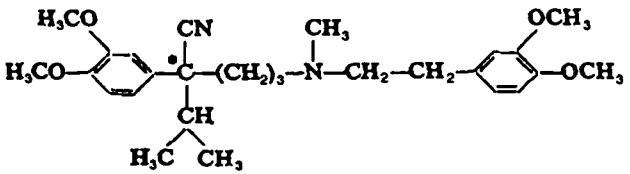
I. INTRODUCTION

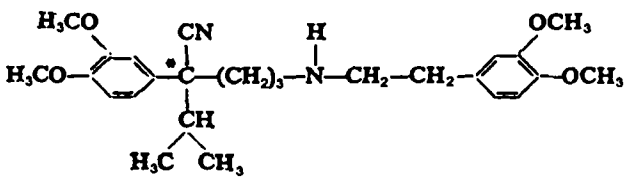
Like all calcium channel blockers, verapamil modulates ionic calcium influx across cell membranes of conductile and contractile myocardial cells, as well as arterial smooth muscle. The modulation of calcium influx slows atrioventricular conduction, reduces myocardial contractility and systemic vascular resistance, and results in coronary and peripheral vasodilation. Verapamil is currently indicated for controlling angina, hypertension, paroxysmal supraventricular tachycardia, and rapid ventricular atrial flutter or fibrillation (1–5).

The single stereogenic or chiral center in the chemical structure of verapamil results in two stereoisomers of verapamil: *S*(−)-verapamil and *R*(+)-verapamil (Fig. 1). These enantiomers have different pharmacokinetic and pharmacodynamic properties (3,6–9). Although both enantiomers have similar types of pharmacologic activity, the *S* enantiomer has been shown to be the more potent with respect to several of the effects (3,6–8).

Currently, all marketed verapamil products are racemic mixtures of *R* and *S* enantiomers (7,8). The total pharmacological activity of a verapamil dose is determined by the combined bioavailability of both individual enantiomers. The *S* enantiomer is preferentially metabolized after oral dosing, resulting in the *R* enantiomer being the more prevalent in the systemic circulation (8). The actual value of the enantiomeric ratio (*R*:*S*) in the plasma, however, is determined by a combination of factors. Pharmacokinetic distinctions between the two enantiomers (e.g., volumes of



VERAPAMIL



NORVERAPAMIL

FIGURE 1 Molecular structures of verapamil and its *N*-demethylated metabolite, norverapamil. The carbon chiral center is designated by the asterisk.

distribution, clearances, protein binding) (10,11) are the ultimate source of differences, but external factors such as the route of administration (e.g., intravenous or oral) and the rate of absorption are also critical (12,13). The type of dosage form (e.g., capsule or tablet) is also a factor influencing the enantiomer ratio ultimately observed in the systemic circulation because it can influence the absorption rate of the drug from the gastrointestinal tract by controlling the dissolution rate of the drug (e.g., immediate or sustained release).

This chapter reviews the pharmacokinetics and pharmacodynamics of the enantiomers of verapamil and examines their impact on verapamil bioavailability. In addition, new information on the influence of product formulation on the differential bioavailability of the enantiomers of verapamil is presented and discussed.

II. PHARMACOKINETICS OF VERAPAMIL

Mean plasma concentration-time curves for total verapamil (i.e., the mixture of both enantiomers) as measured in 20 healthy volunteers receiving

either 10-mg intravenous verapamil or 160-mg oral verapamil racemic doses appear in Fig. 2 (1). The study compared the pharmacokinetics of three oral doses (80, 120, and 160 mg) and a single intravenous dose (10 mg) and found no difference between treatments in values for the terminal half-life and volume of distribution. Clearance was somewhat decreased ($p < .05$) after intravenous administration (0.71 L/hr/kg), compared to oral (0.82–0.86 L/hr/kg). The maximum plasma concentration (C_{max}) was dose-dependent in subjects given oral verapamil, with the 120-mg oral dose producing a C_{max} similar to that of 10-mg intravenous verapamil. The

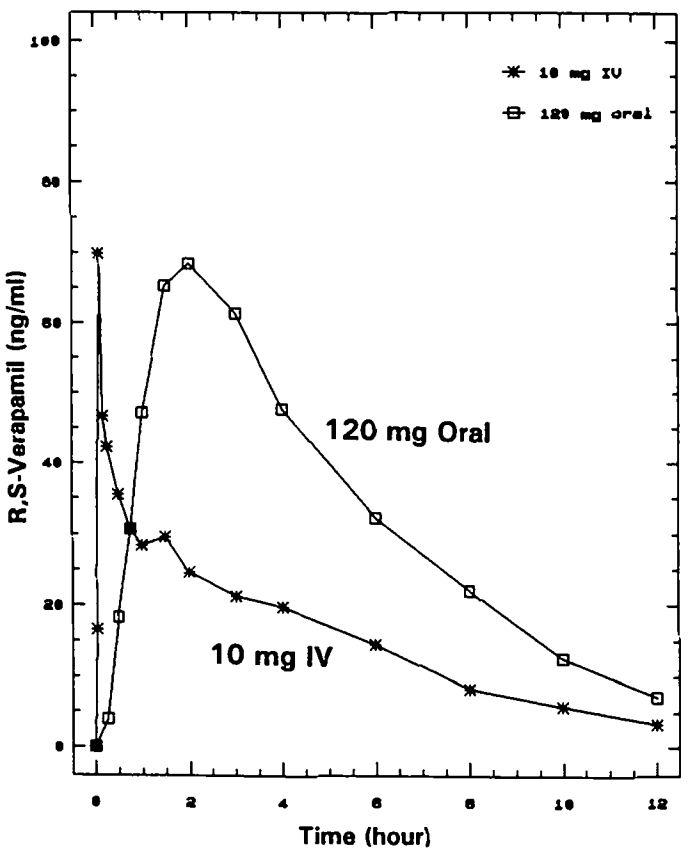


FIGURE 2 Mean concentrations of verapamil following a 10-mg intravenous dose (*) or 120-mg oral dose (□) of racemic verapamil in 20 normal volunteers. Verapamil concentrations were determined using a nonstereospecific assay. [Adapted from McAllister and Kirsten (1).]

bioavailability of oral verapamil was similar after all three oral doses and ranged from 18–20.4%.

Investigators in early studies addressing the conversion of patients on intravenous verapamil therapy to oral therapy found that verapamil, when administered intravenously, appeared to be two to three times more potent than the oral formulations in causing changes in conductivity in the heart (as measured by PR interval prolongation) (3,12). This route dependency on the potency of verapamil puzzled investigators for several years, but since the early 1980s has been recognized as the direct result of unequal oral bioavailabilities of *R*- and *S*-verapamil (12). The biologic receptors mediating the effect did not then, and do not now, regard the stereoisomers as equals. The apparent discordance between the assayed concentrations and observed pharmacodynamic effects arose from the inability of the nonstereospecific assay that was used in the early studies to discriminate between the relative concentrations of the two enantiomers, yielding only a misleading summed concentration measurement.

In the studies comparing intravenous and oral therapy, the intravenous dose resulted in equal amounts of the two enantiomers (50% *R*-verapamil and 50% *S*-verapamil) in the body, whereas oral tablets and solutions resulted in approximately 75% of the drug in the body being the less active *R*-verapamil and only about 25% being the more active *S*-verapamil (11). Without an awareness that the concentrations of the more active molecular entity differed substantially for the two treatments, or that those differences could not be detected by the presumably sufficiently sensitive assays, it is no wonder that in early studies verapamil appeared to violate some of the basic premises of pharmacological action. In retrospect, this and similar clinical paradoxes with other drugs have been partially responsible for forcing pharmacologists and drug researchers into a recognition of stereoisomers, and for a growing appreciation of the range of interactions that can be traced to stereoselectivity.

In addition to the difference in bioavailabilities for the two enantiomers of verapamil, other pharmacokinetic parameters show an enantiomer effect. Although the actual extent of binding of verapamil to plasma proteins appears to be dependent on the route of administration, the free fraction of *S*-verapamil (approximately 12% after intravenous dosing and 23% after oral dosing) is always about twice that of *R*-verapamil (approximately 6% after intravenous dosing and 13% after oral) (7,10,11). This difference in the free fractions contributes to the volume of distribution of *S*-verapamil being about twice that of *R*-verapamil (6.4 L/kg vs. 2.7 L/kg), and the plasma clearance of *S*-verapamil being about twice that of *R*-verapamil (18 mL/min/kg vs. 10 mL/min/kg) (7,11). The terminal half-lives at approximately 4–5 hr are similar, but not identical for the two enantiomers

(11). There are also substantial differences in the pharmacokinetic parameters associated with hepatic metabolism for the two enantiomers. The differences are the source of the differential bioavailability described above and will be discussed in greater detail in subsequent sections of this chapter.

III. PHARMACODYNAMICS OF VERAPAMIL

The two stereoisomers of verapamil differ extensively in their pharmacodynamics. The *S* enantiomer is the more pharmacologically active of the two relative to the cardiovascular system (3,6–9). The cardiovascular pharmacodynamic effects and relative potency of *S*- and *R*-verapamil are summarized in Table 1.

Prediction of the pharmacodynamic effects associated with verapamil dosing in the clinical setting is further complicated by the formation of norverapamil, an active metabolite and optically active molecule (Fig. 1). Norverapamil is reported to have approximately 20% of the vasodilatory activity of the parent drug (1,4), and it achieves plasma concentrations of both its *R* and *S* enantiomers that are similar to concentrations of the parent drugs (*R*- and *S*-verapamil, respectively). Not all pharmacological activities differ for *R*- and *S*-verapamil. For instance, the two enantiomers have been shown to have nearly identical activities in overcoming multiple-drug resistance (MDR) to several oncologic agents in drug-resistant cell lines (14).

IV. RELATIONSHIP BETWEEN CLINICAL PROPERTIES OF VERAPAMIL AND PHARMACOKINETICS

As noted before, one of the more striking clinical features of verapamil is that following intravenous administration, verapamil appears to be more potent than following oral administration. This is illustrated in Fig. 3,

TABLE 1 Summary of the Pharmacodynamic Effects of Verapamil and Relative Potencies of the *S* and *R* Enantiomers

Pharmacodynamic effect	Greater potency of *S* vs. *R*
Negative dromotropic	10–20 times greater (3, 6–9)
Negative inotropic	15 times greater (6)
Negative chronotropic	5 times greater (6)
Vasodilation	2.5 times greater (6)

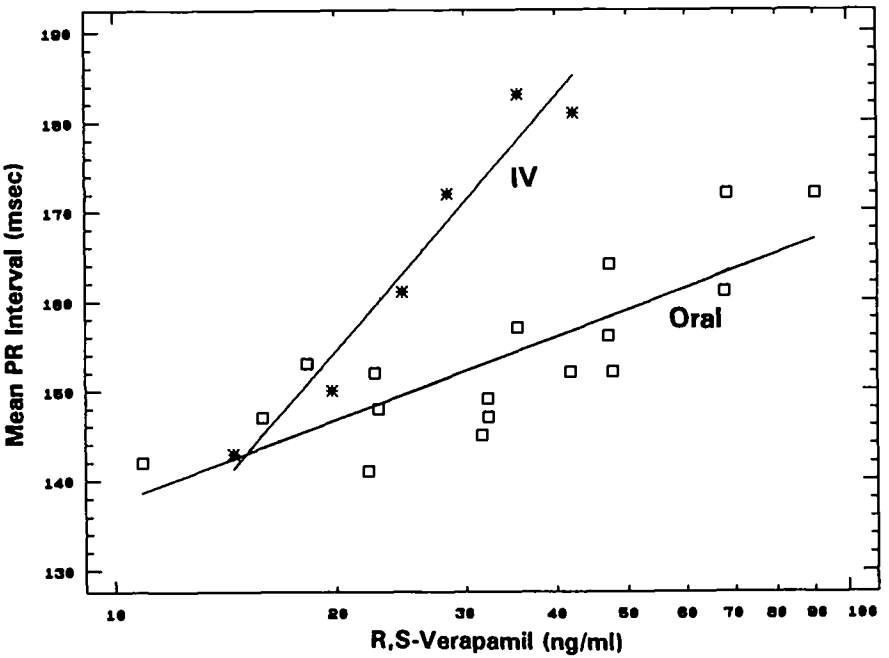


FIGURE 3 Relationships between prolongation of the PR interval and verapamil concentration following a single 10-mg intravenous dose of racemic verapamil (*), or either an 80-, 120-, or 160-mg oral dose of immediate release racemic verapamil (□). Verapamil concentrations were determined using a nonstereospecific assay. [Adapted from McAllister and Kirsten (1).]

where for instance, an approximately threefold higher plasma concentration of total verapamil (68 ng/mL vs. 22 ng/mL) is needed to obtain the same effect on the PR interval as the intravenous formulation (an increase from 143–163 msec). When administered orally, a greater fraction of the absorbed *S*-verapamil is metabolized by the liver than the *R*-verapamil. This results in there being three to ten times greater concentrations of *R*-verapamil than *S*-verapamil in the systemic circulation (3,6–9,11,15).

Although the larger volume of distribution for *S*-verapamil means that even without differential metabolism of the enantiomers, the observed *S*-verapamil plasma concentrations would only be about 50–65% that of *R*-verapamil (15), the faster hepatic metabolism of *S*-verapamil dramatically increases the concentration difference between enantiomers. Results from a study utilizing human liver microsomes suggest that the enzymes responsible for the primary metabolites of verapamil saturate at lower

concentrations of *S*-verapamil than *R*-verapamil, even though the maximum metabolism rates (V_{max}) of the two enantiomers appear to be similar (16).

The saturable nature of the metabolic pathways for the enantiomers of verapamil provides the theoretical basis for qualitatively explaining and perhaps someday quantitatively describing (1) the large differences between the enantiomer concentrations following oral dosing and (2) the kinds of variability in the enantiomer ratios that have been observed with verapamil.

The amount of verapamil presented to the liver, and its effective concentration in the region of the hepatic enzymes soon after oral dosing, are related to the rate at which verapamil is absorbed from the gastrointestinal tract into the portal vein and to the flow rate of blood in the portal vein to the liver. For instance, by hypothesizing a Michaelis-Menten metabolic process, when the absorption rate is slow and concentrations in the portal vein and liver are low, the hepatic metabolism of both enantiomers will be approximately first-order. Under these conditions, the *R*:*S* ratio of the unmetabolized enantiomers leaving the liver will be closely related to the ratio of the Michaelis-Menten saturation constants (K_m) for the enantiomers. The observed more rapid metabolism of *S*-verapamil than *R*-verapamil (i.e., *S*-verapamil has the lower systemic concentrations) is consistent with the lower K_m reported for *S*-verapamil (16).

At the other extreme of the absorption rate spectrum, when the absorption rate is high and portal vein concentrations are high, the presentation rates of the enantiomers to the liver exceed the maximum velocities (V_{max}) of their metabolic pathways. This scenario results in saturation of the metabolic enzymes and large, and approximately equal, fractions of both enantiomers will pass through the liver unchanged, and the *R*:*S* bioavailability ratio will approach 1:1. Given the difference in the enantiomer volumes of distribution, this will produce an *R*:*S* plasma concentration ratio of approximately 2:1, just as is observed following intravenous administration.

Between the very low and very high extremes in the absorption rate is a wide intermediate range. In this intermediate range, increasing the absorption rate increases verapamil concentrations in the portal vein. This, in turn, increases the presentation rate of verapamil to the liver, where the degree of saturation of the enzymes will be increased, thus leading to a smaller fraction of the incident verapamil being metabolized and a larger fraction escaping unchanged. However, the bioavailable fractions will not increase exactly in parallel because their effective K_m parameters for the metabolizing enzymes are not identical. Over the intermediate range, the ratio of the bioavailabilities of the two enantiomers should decrease from the high *R*:*S* ratios seen with slow absorption rates, to a low ratio of about

2:1 expected at high absorption rates. Consistent with this analysis is our observation that a sustained-release dosage form of racemic verapamil yields an *R*:*S* ratio of about 4:1, whereas an immediate release dosage form yields an *R*:*S* ratio of about 3:1 (plots B and C in Fig. 4). The sustained-release dosage form with its intentionally lower drug release rate (average release rate approximately 30 mg/hr) has a lower absorption rate and, as predicted, a greater *R*:*S* ratio than the immediate release dosage form (average release rate approximately 40 mg/hr).

Modulation of the *R*:*S* ratio about some characteristic average value that is dependent on the particular design of a verapamil formulation is to

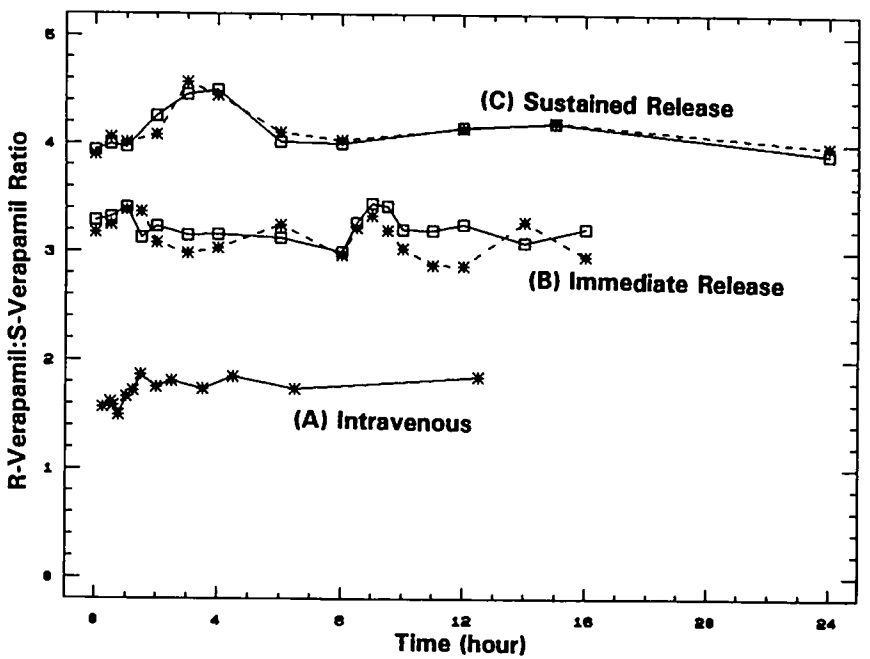


FIGURE 4 Mean *R*-verapamil:*S*-verapamil ratio observed following intravenous and oral dosing of racemic verapamil. A, After intravenous administration of a single 15-mg dose in 8 volunteers (personal communication from A. Rasymas, Univ. of Toronto, Canada). B, After oral administration of two different 120-mg immediate release formulations dosed every 8 hr to 22 normal volunteers in a cross-over design study and measured at steady state over two dosing intervals (-*-, test formulation; □, reference formulation). C, After oral administration of two different lots of a 180-mg once daily sustained-release formulation to 48 normal volunteers in a cross-over design study and measured at steady state (-*-, new manufacturing site; □, reference manufacturing site).

be expected. At this time, little information has been published that allows careful examination of how sensitive the relative plasma concentrations of *R*- and *S*-verapamil actually might be to small changes in the release characteristics of an oral formulation. One would expect that sustained-release formulations, which have slower *average* release rates but where the instantaneous release rate often fluctuates substantially over the release period, would very likely show formulation-dependent variation in the *R*:*S* ratio with time. However, the possibility that immediate release products show analogous variability in their release rate cannot be ruled out, and such formulation-specific variability may be the source of the slightly different time profiles for the *R*:*S* ratio observed with the two different immediate release formulations shown in Fig. 4 (plots B). As more studies of this type are conducted on a wider range of formulations, we will be able to confirm whether particular formulations can be associated with a signature *R*:*S* ratio.

The possibility that the modulation in the *R*:*S* ratio is simply an artifact of the formulation and not mediated through the absorption rate and presentation rate of drug to the liver has been addressed in our laboratory. Figure 5 shows results from a study in which 24 subjects were given the same sustained-release formulation of racemic verapamil at two different doses. In one treatment period, the dose was 120 mg/day to steady state, and in another it was 480 mg/day, also to steady state. The fourfold difference in absorption rates associated with the two different doses produced dramatically different profiles for the *R*:*S* ratios during the dosing interval. As predicted in the preceding discussions, the greatest *R*:*S* ratio was observed with the slower average absorption rate (low dose). However, the lowest *R*:*S* ratios were also observed with that low dose (at the beginning and end of the dosing interval). The mechanism(s) responsible for this wider fluctuation in the *R*:*S* ratio over the dosing interval at a lower dose have not yet been identified.

We are just beginning to understand and address, in a systematic fashion, the interactions in the pharmacokinetic processes that control the relative concentrations of two enantiomers in vivo. The above discussion, which is based on verapamil as a model substrate, is too simplistic for predicting enantiomer ratios in general. Until now the discussion has concentrated on the relative bioavailabilities of the two enantiomers, that is, the fraction of the dose that escaped first-pass metabolism. We have ignored other pharmacokinetic processes. If absorption is not the dominant process, drug distribution and systemic clearance will have a greater role in determining the relative systemic concentrations of the two enantiomers and the resulting *R*:*S* ratio. For verapamil, the distribution phase is rapid and the primary systemic clearance mechanisms are the same ones

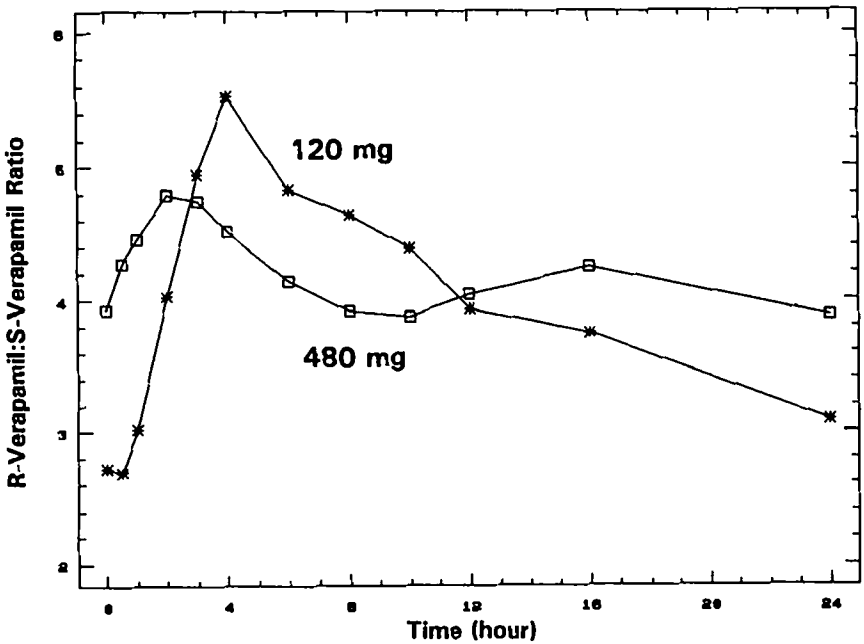


FIGURE 5 Mean *R*-verapamil:*S*-verapamil ratios observed at steady state during once daily dosing of either 120-mg (*) or 480-mg (□) racemic verapamil in 24 normal volunteers.

responsible for the first-pass effect. These limitations probably permitted our predictions above to be relatively close qualitatively. For drugs that have substantial multicompartmental kinetics, or where the primary systemic elimination pathway is different from that responsible for the first-pass effect (e.g., when renal clearance dominates elimination), the likely patterns have not yet been explored, or the important factors identified.

V. CLINICAL STUDIES: BIOEQUIVALENCE OF VERAPAMIL FORMULATIONS

Due to (1) the differential pharmacokinetics of the enantiomers of verapamil within each formulation, (2) differences in the rate of metabolism between various oral formulations, (3) large interindividual variability in drug effect (as measured by the PR interval), and (4) large interindividual variability in plasma verapamil concentration, the assessment of the bioequivalency of different verapamil formulations is not a trivial task (1,4,11).

Traditionally, the presence of enantiomers in the administered dosage form and the possibility that differential metabolic kinetics may influence systemic concentrations of the enantiomers have been ignored in trying to establish the bioequivalence of two racemic drug formulations, even for verapamil (17). Recently, however, verapamil has attracted attention as a model compound for a group of chiral drugs in which it may be necessary to use stereoselective assays to unambiguously evaluate bioequivalence. Verapamil is a prototype of a chiral drug with a high first-pass clearance (i.e., there is rapid hepatic metabolism), where the high clearance metabolic pathways display saturable kinetics and the saturable pathways also show stereoselectivity. In addition, because verapamil is an example in which the two enantiomers have different pharmacologic potencies, knowledge of the concentrations of the individual enantiomers is required to predict the total effect.

Other drugs warranting serious consideration for stereospecific bioequivalence evaluations are (1) chiral drugs shown to have a potency that depends on the route of administration; (2) chiral drugs undergoing hepatic metabolism and available in formulations with obviously different release rates; and (3) chiral drugs used in formulations that contain other chiral molecules as excipients, binders, or a retarding matrix that may promote stereoselective release rates from the dosage form.

Our first stereospecific bioequivalence evaluation using verapamil formulations was a pilot study conducted in 24 healthy male volunteers. The study compared the bioavailability of two sustained-release dosage forms and an immediate release formulation of racemic verapamil in a three-way cross-over study. Table 2 summarizes the results observed for verapamil, Table 3 the results for norverapamil. First, pharmacokinetic

TABLE 2 Achiral and Chiral Verapamil Bioequivalence Comparisons of Pellatized Capsule and Gel Tablet Formulations of Verapamil

	R, *S* Ratio (90% CI) power	*R* Ratio (90% CI) power	*R* Ratio (90% CI) power
AUC	94.3 (100.2, 88.5) 0.99	80.2 (92.0, 68.4) 0.72	72.3 (82.4, 52.3) 0.75
Cmax	91.4 (99.0, 83.7) 0.99	84.7 (99.7, 69.7) 0.50	79.0 (91.5, 56.5) 0.61
Tmax	73.2 (84.0, 62.3) 0.84	75.0 (81.7, 62.9) 0.88	76.9 (87.6, 56.2) 0.75
Cmin	108.7 (121.3, 96.2) 0.71	71.4 (86.7, 56.3) <0.50	76.4 (98.1, 54.8) <0.50

TABLE 3 Achiral and Chiral Norverapamil Comparisons Following Pellatized Capsule and Gel Tablet Formulations of Verapamil

	R, *S* Ratio (90% CI) power	*R* Ratio (90% CI) power	*R* Ratio (90% CI) power
AUC	96.8 (102.8, 90.8) 0.99	87.8 (97.3, 78.2) 0.88	62.6 (112.6, 87.4) 0.74
Cmax	99.2 (106.7, 91.7) 0.99	87.3 (98.8, 75.8) 0.74	92.3 (113.9, 86.1) 0.64
Tmax	81.5 (92.3, 70.8) 0.84	78.7 (88.1, 69.2) 0.89	100.0 (113.0, 87.0) 0.71
Cmin	100.0 (111.8, 88.3) 0.77	74.8 (88.3, 61.4) 0.62	58.9 (119.7, 80.4) <0.50

parameters generated from concentration data obtained with a nonstereospecific assay were used to compare the verapamil and norverapamil bioavailabilities. The two sustained-release formulations were concluded to have verapamil and norverapamil pharmacokinetic parameters that were generally not statistically different. However, when the pharmacokinetic parameters generated from concentration data obtained with a chiral assay were compared for a 12-subject subset of that study population, statistically significant differences were noted in some of the pharmacokinetic parameters, especially the parameters describing concentrations of *S*-verapamil (Cmax, Cmin, and AUC).

Also of interest to us was the observation that a simple regression analysis of the relationship between the nonstereospecific assay results and prolongation of the PR interval indicated that the concentration-effect relationship identified for the immediate release formulation differed from the relationships identified for each of the sustained-release formulations (Fig. 6). The difference approached statistical significance for all comparisons ($p = 0.1$) and exceeded it for some ($p < 0.05$). A similar finding has been reported recently by others (13). This formulation dependence disappeared once the effect was regressed against either of the individual enantiomer concentrations (data not shown). This pattern is directly analogous to the paradoxical difference in potency observed with verapamil when administered intravenously vs. orally that was discussed earlier in this chapter. The current example differs from the intravenous-oral case only in the magnitude of the effect. That is because the two types of oral formulations (sustained-release and immediate release) do not differ in their enantiomer ratio by as much as they do in a comparison of the intravenous and oral routes (Fig. 4).

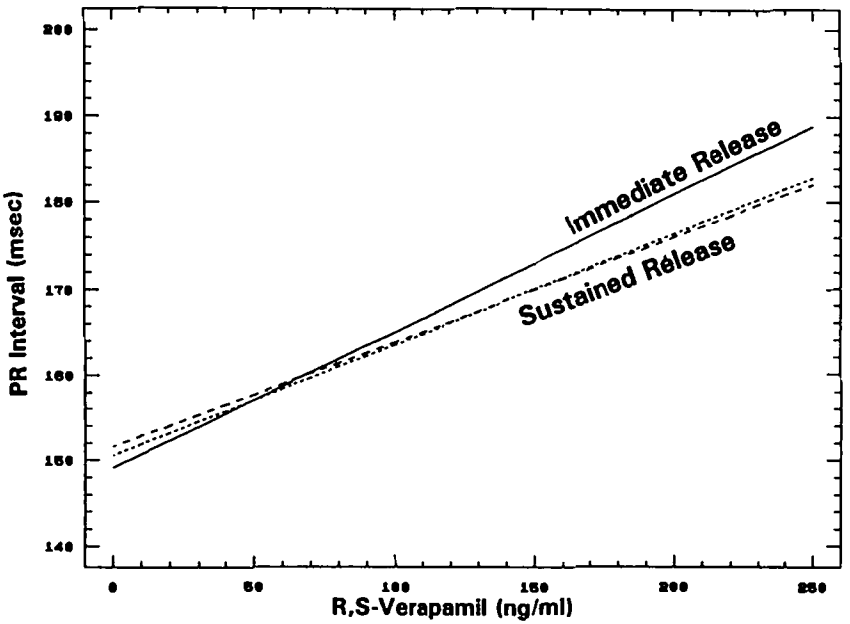


FIGURE 6 Mean relationship observed between prolongation of the PR interval and verapamil concentration measured in 24 normal volunteers using a nonstereospecific verapamil assay. The immediate release formulation was given 80 mg every 8 hr; both sustained-release formulations were given 240 mg once daily. Concentrations and PR intervals were measured at steady state.

Evaluation of the apparently conflicting results from the nonstereospecific and stereospecific assays in this pilot study forced us to recognize two pitfalls commonly overlooked in interpreting nonstereospecific data for chiral compounds. First, it is possible to conclude, using nonstereospecific data, that two formulations have no significant bioavailability differences, while concluding the opposite on the basis of data from a chiral assay, namely, that the formulations do show differences in bioavailability. Second, use of a nonstereospecific assay tends to reduce the discriminating power of a study to identify differences if they do exist. The observation identified in the first point is just one direct result of the process identified as the second point.

The nature of the pitfalls can be illustrated with an example. If we examine the simplest possible case, that is, consider a drug in which only one of the enantiomers has pharmacological activity, then inclusion of an

inactive enantiomer in the measurement of the "active" entity just serves to add noise to the measurement. As with any measurement, this noise will make it a bit more difficult to identify slight changes in the concentrations of the active enantiomer if, or when, they occur. The noise factor has the least impact when the active enantiomer is a large fraction of the assay signal and the largest impact when the active enantiomer is a small fraction of the total signal.

For instance, if the active enantiomer constitutes 90% of the total drug on the average, then a 10% change in the concentration of the active enantiomer will be manifested as approximately a 9% change in total drug concentration (if we assume that the enantiomer concentrations do not change in opposite directions). In this case, there is little effect from the noise because most of the measurement is due to the active enantiomer. However, if 90% of the drug is inactive enantiomer, then the 10% fraction that is active could entirely disappear without being detected. Just normal assay variability can be great enough to mask such a systematic loss if the 90% of the assay measurement that is noise has 10% assay variability associated with it. In this example, a 100% decrease, or increase, in bioavailability (and pharmacological activity) could occur without the measured achiral concentration showing a detectable difference. Only in pharmacokinetic environments where there are coincidental, approximately equivalent fractional changes in both the active and inactive enantiomers can there be reliable detection of changes in the concentration of an active enantiomer that constitutes only a small fraction of the total drug.

This masking or "dilution" of sensitivity to change and loss in discriminating power is a well-known and widely lamented consequence of combining random noise with a signal of interest in many fields. The detrimental impact of this interpretational pitfall increases as the fraction of the total drug that is the enantiomer of interest decreases. However, the consequences of this pitfall for properly interpreting pharmacokinetic comparisons derived from nonstereospecific assays of chiral drugs are not yet widely appreciated.

This pitfall is especially relevant to studies attempting to compare the rates and extent of drug absorption of two dosage formulations of a chiral drug where both data sets are generated from nonstereospecific assay results. Such exercises raise the prospect of comparing one noise-laden signal to a second noise-laden signal. As long as the noise contribution is relatively uniform, the probability of finding a favorable conclusion (e.g., bioequivalence) is increased. However, the double dose of noise also increases the likelihood that the favorable result is a false finding.

As we have discussed, the interpretational complexities that can be

encountered when using a nonstereospecific assay for a chiral compound can range from trivial to overwhelming when one of the enantiomers is inactive. Interpretation becomes almost impossible when both enantiomers have similar types of activities but different potencies (e.g., verapamil), or when the changes in the concentrations of the two enantiomers do not closely track each other in terms of direction or magnitude of change (e.g., verapamil).

The pilot study described above indicates that a conclusion of bioequivalence based on nonstereospecific data can be contradicted once chiral data are available. Although the shortcomings of using nonstereospecific data are clear, it is not as clear what other standard can replace it in actual practice. The obvious option, for all active enantiomers to simultaneously meet traditional bioequivalence guidelines, may be unrealistic. A study addressing this issue was conducted in our laboratory. Forty-four healthy male volunteers were studied in a two-way cross-over investigation comparing two sustained-release formulations from different manufacturing sites. The formulations were administered once daily to steady state. Plasma concentrations of the enantiomers of verapamil and norverapamil were determined on the final three days of each treatment period (days 6–8). On days 1–7, verapamil was administered with food, and on day 8, it was administered under fasting conditions.

The mean concentration-time profiles for each of the four analytes, and for each of the two formulations, generated by this study are shown in Fig. 7. The pharmacokinetic comparisons derived from this study for all four analytes are summarized in Table 4. As can be seen from Fig. 7 and Table 4, a four-way bioequivalence assessment proved both feasible and practical. This study also demonstrated that similar formulations will produce similar concentration-time profiles for all the enantiomers in the plasma (even given lot-to-lot variability, manufacturing site-to-site variability, and shelf-time variability).

Subsequent studies in our laboratory have reproduced the above results and demonstrated that similar formulations will produce similar *R*:*S* ratio vs. time profiles (plots C in Fig. 4), whereas dissimilar formulations produce *R*:*S* ratio vs. time profiles that appear to differ in subtle but discernible ways (plots B in Fig. 4). Since differences in the *R*:*S* ratio vs. time profiles were seen even as the formulations met the rigorous bioequivalency criteria for each of the four analytes, the *R*:*S* ratio may be a powerful tool in helping to identify and understand the interaction between absorption rate and differential saturation of metabolic enzymes.

Figure 8 illustrates another pharmacokinetic issue that needs to be considered when selecting a study design or interpreting nonstereospecific data for a chiral drug. In Fig. 8, the mean *R*:*S* ratios from 12

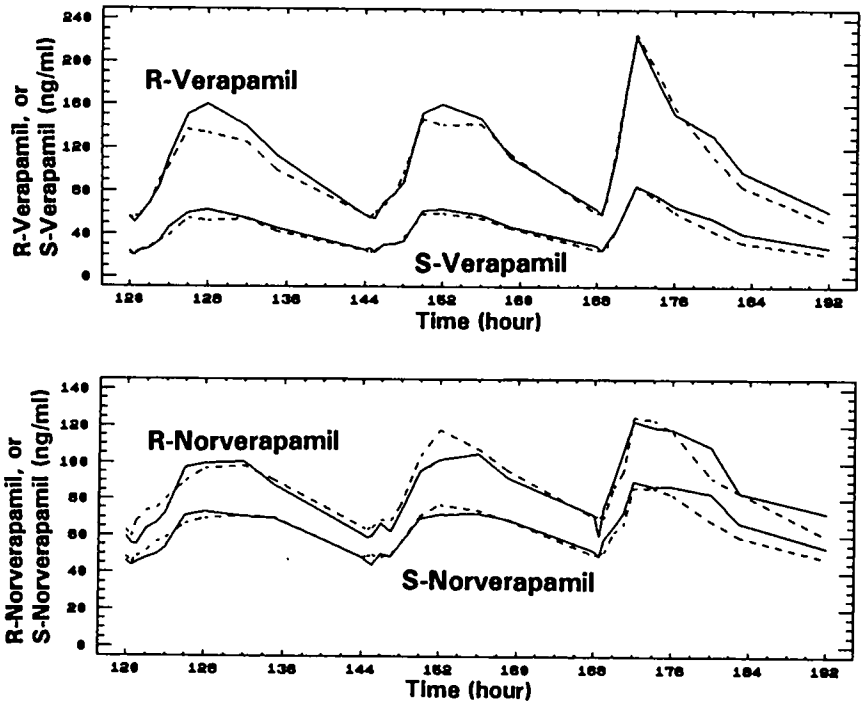


FIGURE 7 Stereospecific comparison of concentration-time profiles of verapamil and norverapamil obtained from two lots of sustained-release verapamil manufactured at different manufacturing sites (---, new manufacturing site; —, reference manufacturing site). Sustained-release racemic verapamil was administered 240 mg once daily to the 44 normal volunteers in a cross-over design study; plasma samples were collected at steady state on days 7–8. (On days 6 and 7 verapamil was administered with food, and on day 8 it was administered while fasting.)

subjects are shown following a single dose of sustained-release verapamil. The pattern shown in Fig. 8 differs substantially from all *R*:*S* profiles acquired following verapamil doses at steady state (e.g., plots B and C in Fig. 4 and both profiles in Fig. 5). Part of the differences may be related to the tendency for verapamil to decrease its clearance with chronic dosing (18), and part of them may be related to there being some "initial" enantiomer ratio present during steady-state dosing that reduces the impact of the freshly absorbed drug. The relative roles of these and other processes may become apparent as more sophisticated models of verapamil kinetics are developed. However, for now, the data indicate that care must be taken when using either single-dose or steady-state results to

TABLE 4 Verapamil and Norverapamil Chiral Bioequivalence Comparisons for Two Lots of Sustained-Release Racemic Verapamil Tablets Manufactured at Different Sites

	R-verapamil ratio (90% CI) power	*S*-verapamil ratio (90% CI) power	*R*-norverapamil ratio (90% CI) power	*S*-norverapamil ratio (90% CI) power
AUC	94.6 (100.6, 87.3)	94.4 (102.9, 85.7)	105.6 (113.2, 96.0)	97.7 (108.0, 94.2)
	0.95	0.97	0.99	0.99
Cmax	92.7 (101.5, 83.1)	103.6 (106.7, 85.8)	106.7 (115.3, 97.6)	103.9 (111.1, 96.4)
	0.94	0.87	0.96	0.99
Tmax	101.2 (112.0, 90.7)	96.4 (107.5, 86.5)	98.9 (111.4, 84.4)	91.0 (104.7, 77.5)
	0.97	0.87	0.66	0.67
Cmin	98.2 (106.5, 88.4)	92.2 (104.3, 79.8)	103.9 (116.6, 92.2)	96.9 (106.6, 86.9)
	0.94	0.75	0.76	0.90

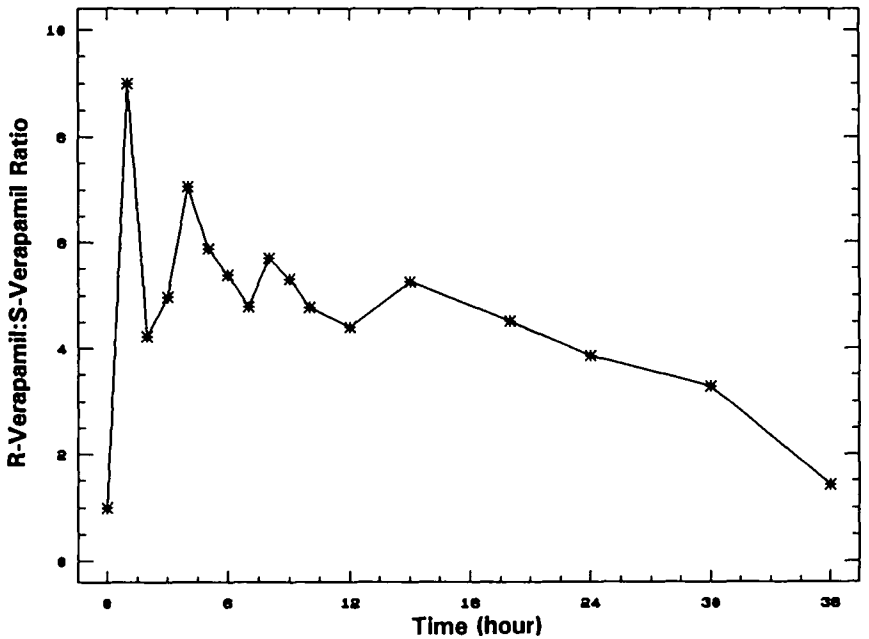


FIGURE 8 *R*-verapamil:*S*-verapamil ratio observed following a single 180-mg dose of sustained-release racemic verapamil. (The comparable relationship for the same formulation, at the same dose, but at steady state is shown as curves (C) in Fig. 3.)

extrapolate beyond their particular study conditions. This cautionary message certainly applies to both the pharmacokinetics and pharmacodynamics of verapamil, where not only the overall kinetics but also the relative contributions of the enantiomers are altered. It is not known whether other chiral drugs display similar phenomena.

Use of a pharmacodynamic endpoint to show therapeutic equivalence of two formulations as an alternative method of establishing bioequivalence has been suggested in many settings. Verapamil is a candidate for this approach because it has the readily detectable effect of prolonging the PR interval. Although each case for a pharmacodynamic endpoint must be argued on its own merits, our laboratory has identified several difficulties in using the PR interval as a surrogate for chiral assays of verapamil and norverapamil. First, study subjects are generally selected from a healthy young population whose PR intervals should not be particularly sensitive to verapamil concentrations. Second, in most such subjects the dynamic

range in PR interval due to verapamil is generally limited to between 150 and 220 msec. The maximum permitted effect for this parameter is only about 50% greater than the baseline. Third, the baseline PR interval varies from subject to subject and fluctuates within a subject from day to day; the additional circadian variation in the PR interval requires that a standardized daily regimen be established and maintained. Fourth, prolongation of the PR interval is a surrogate endpoint; it is neither a measure of the therapeutic antihypertensive effect of the drug, nor a measure of the principal toxicities found in the patient population. Fifth, we have found that prolongation of the PR interval shows a saturable response (Fig. 9), indicating that it is least sensitive to differences in the enantiomer concentrations of verapamil in the region of peak concentration, the region of primary interest in terms of both safety and efficacy. Finally, neither the

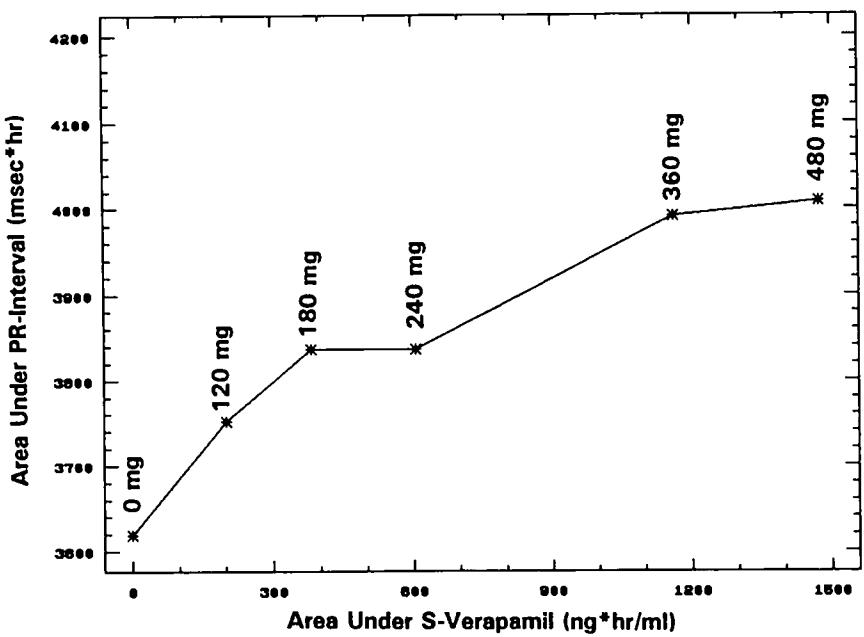


FIGURE 9 Pharmacodynamic relationship observed at steady state in 24 subjects for various doses of a sustained-release (once daily) verapamil formulation. The concentration measure used is the area under the *S*-verapamil concentration over the 24-hr dosing interval; the effect measure used is the area under the PR interval curve (measured with Holter monitor) over the 24-hr dosing interval. The doses of sustained-release verapamil that resulted in each concentration-effect pair are also shown.

appropriate size for confidence intervals nor what constitutes a clinically relevant difference has been generally agreed on for prolongation of the PR interval.

VI. CONCLUSIONS

Verapamil is a chiral drug that undergoes stereoselective metabolism in the liver. The relative bioavailabilities of the enantiomers of verapamil following oral administration of the racemic drug are dependent on the rate of presentation of verapamil to the liver by the portal vein, and hence the rate of absorption of verapamil from the gastrointestinal tract. The difference in the systemic concentrations of the enantiomers of verapamil appears to be explained by their different bioavailabilities; the different bioavailabilities are apparently a consequence of the relative affinities of the metabolizing enzymes for the two enantiomers (as reflected in their K_m values). Once absorption is completed and during chronic dosing, other stereoselective pharmacokinetic processes (i.e., protein binding, distribution volume, and plasma clearance) appear to control the relative concentrations of *R*- and *S*-verapamil. Additional research is required to understand these interactions.

The important paradoxical finding that verapamil potency depends on route of administration (intravenous vs. oral) also holds when dissimilar oral formulations are compared. The paradox is avoided by using a stereospecific assay to follow enantiomer concentrations and to associate the effect with concentrations of the appropriate enantiomer(s). Both the route of administration and type of oral formulation are important influences on the relative systemic concentrations of the two enantiomers. In addition, the amount of time elapsed post-dose, the amount of verapamil administered, and whether a single dose or chronic dosing is involved also have a major impact on the enantiomer ratio.

It is feasible and practical to establish the bioequivalence of oral verapamil products using traditional comparisons of the four active entities (the enantiomers of verapamil and those of norverapamil). The results from such a comparison may not be in agreement with comparisons based on a nonstereospecific assay, especially because of the reduced power of the nonstereospecific assay to detect differences in the lower-concentration analytes (*S*-verapamil and *S*-norverapamil). Using PR interval prolongation as a surrogate endpoint is not a suitable alternative to assaying enantiomer concentrations because of its lack of specificity as a safety or efficacy parameter, its limited dynamic range, and its decreased sensitivity as concentration increases.

Verapamil is a useful model for evaluating the relevance of stereo-

specific assays and enantiospecific data for racemic drugs in the clinical and regulatory environment. However, verapamil is just one of many racemic drugs, a specific example with high hepatic clearance pathways that are saturable and stereoselective. Generalizing these findings to other chiral drugs will require additional animal and clinical investigations and sophisticated simulation studies.

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12

ENANTIOSELECTIVE BINDING OF DRUGS TO PLASMA PROTEINS

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I. INTRODUCTION

After systemic introduction, most drugs undergo some degree of reversible binding to plasma proteins. Two plasma proteins are responsible for the majority of drug binding: serum albumin (that will be abbreviated to HSA in the case of human serum albumin and BSA for bovine serum albumin), which accounts for the bulk of drug binding and is especially important in the binding of acidic solutes, and α_1-acid glycoprotein (AGP), to which weakly basic drugs preferentially bind. As, in general, only the free or unbound fraction of a drug is able to traverse cell membranes to exert a therapeutic effect, or to undergo enzymatic metabolism, factors influencing the binding of a drug to plasma proteins may significantly affect its pharmacokinetics and pharmacodynamics (1). This is especially true when the drug is highly bound or has low intrinsic clearance (in which case, total hepatic clearance is proportional to the free fraction). A clear understanding of the plasma protein binding behavior of a particular therapeutic agent is therefore fundamental to its safe and rational use.

In the case of chiral drugs, the possibility arises that the individual stereoisomers may bind to plasma proteins with differing affinities, resulting in differing free fractions. As over 60% of the drugs in current clinical use are chiral (2) and the majority of synthetically derived chiral drugs are administered as mixtures of the constituent stereoisomers (most commonly the racemate), this potentiality is significant.

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A. Drug Binding to Plasma Proteins

First reports of drug-plasma protein binding tended to describe the process as rather nonspecific, similar to the way in which charcoal is able to adsorb various compounds (3). However, such beliefs quickly gave way to the growing realization that the addition to plasma of one of a very small number of "competitor" compounds was able to cause the displacement of the majority of compounds studied. This led to the postulation of the existence of a small number of drug binding sites within albumin (4–7) and AGP (8). The exact number of these sites remains the subject of some debate, but most workers agree on the existence of two major binding sites for HSA, the warfarin-azapropazone and indole-benzodiazepine sites (also known as sites I and II, respectively), and a single site within AGP (8). A small number of compounds that bind to HSA, such as digitoxin and tamoxifen, appear not to bind to either of the major sites, necessitating the postulation of further, minor binding sites (9).

Although the site-oriented approach to drug binding may successfully describe the majority of experimental findings, it largely ignores the considerable conformational mobility of the proteins involved, particularly serum albumin. In the case of HSA, a growing number of observations are not explained or appear contradictory, when viewed from a site-binding perspective. For instance, in displacement studies, the drugs warfarin, phenylbutazone, and azapropazone behave exactly as if they bound to the same site. However, the chemical modification of the single tryptophan residue of HSA causes a clear decrease in the binding of warfarin, but has no effect on the binding of phenylbutazone and azapropazone (10). Observations such as these have been explained by the postulation that binding occurs not at a particular, receptor-like site, but over a larger, less localized area on the protein (11). The actual existence of preformed binding sites has been disputed (12,13). What is apparent is that while the site-oriented binding model suffices to describe many empirical findings, the actual mechanism of binding appears to be rather more complex, with allosterically mediated displacement processes causing many of the observations previously assumed to be due to direct competition.

B. Early Reports of Enantioselective Binding to Plasma Proteins

In the early 1950s, Karush reported on the differential binding of the enantiomers of an anionic azo-dye, first to bovine (14) and then human (15) serum albumins. Although not of direct clinical significance, the work of Karush created an awareness of the potential of enantioselectivity in binding to plasma proteins and marks the first reports of species differ-

ences in the binding of enantiomers: A twofold difference in the binding affinity of the enantiomers of the dye to BSA was observed, whereas the difference in binding to HSA was almost insignificant.

Of greater biochemical consequence was the work of McMenamy and Oncley (16), who, in 1958, reported that the binding of the enantiomers of tryptophan to serum albumin (actually bovine mercaptoalbumin) was enantiospecific: The L-isomer bound to a specific site, while the D-form was apparently bound in a nonspecific manner. Based on Scatchard analyses, it was proposed that the binding affinity of L-tryptophan was approximately 100 times greater than that of its antipode. However, subsequent studies on native albumins from other species showed that although L-tryptophan generally binds with greater avidity than the D-form, the enantiomeric binding affinity ratio may be quite low, or even inverted, as has been described for certain avian albumins (17). Using various approaches, several groups (18–20) have shown the actual enantioselectivity in the binding of tryptophan to human serum albumin to be rather lower than in certain other species and that both enantiomers appear to bind at the same site, with differing affinities. Although Sébille, employing a high-performance chromatographic (HPLC) approach, could detect no binding of D-tryptophan to HSA (18), Lagercrantz et al. (19), using immobilized albumin as an affinity chromatographic support, were able to determine that the ratio of the association constants of the enantiomers of tryptophan was approximately eight, the binding affinity of the L-isomer being $1.1 \times 10^4\ M^{-1}$. Nuclear magnetic resonance studies (21) revealed that the binding of either enantiomer of tryptophan to HSA resulted in qualitatively similar changes in the nmr spectrum of the protein. This was attributed (21) to the enantiomers differentially binding to a common site on the albumin molecule. These observations were later confirmed by Fitos and Simonyi (20), who, in addition to Scatchard-type analyses, demonstrated that the free fraction of L-tryptophan could be increased by the addition of the D-enantiomer to the incubation medium.

These early studies clearly indicated the possibility of enantioselective binding of ligands to plasma proteins. However, until relatively recently, the examination of small concentrations of enantiomers, particularly in the biological milieu, was severely limited by the analytical techniques available. This, in turn, made the determination of the free fractions of enantiomers in physiological samples rather difficult, if not impossible, without recourse to the use of individual enantiomers or pseudoracemates. The technological breakthrough that rendered the determination of chiral species virtually a routine analytical task was the commercial introduction, beginning in the early 1980s, of a rapidly growing series of chiral stationary phases for HPLC (22). The current availability of over 80 such

phases (23), along with the ready availability of a range of chiral derivatizing reagents, means that the vast majority of small chiral molecules may be directly, and rapidly, resolved into their enantiomers and therefore quantified. The plasma protein binding, as well as all other pharmacokinetic parameters, of the individual stereoisomers of a chiral drug may now usually be routinely determined.

The recent development of new stationary phases for HPLC based on immobilized plasma proteins, particularly a phase based on HSA, offers a new approach to the examination of the binding of enantiomers to proteins (24–26). Such phases will be discussed in greater detail below.

II. ENANTIOSELECTIVE BINDING OF DRUGS TO SERUM ALBUMIN

The major component of plasma proteins responsible for the binding of drugs in humans is serum albumin (HSA), a globular protein of molecular weight 66,500. The plasma concentration of HSA is approximately 40 g/L, a level that remains relatively constant in healthy individuals. Weak acids tend to bind strongly to albumin, although both aprotic and weakly basic compounds may also bind. Basic drugs generally tend to associate with HSA in a low-affinity, but high-capacity, manner. For instance, approximately 50% of the propranolol found in plasma is loosely bound to albumin (27).

As has been mentioned above, the binding of the majority of drugs to HSA may be explained in terms of attachment to one, or both, of two major specific binding sites on the albumin molecule (6,28). Although the existence of these sites has been disputed by some (12,13), the site-based model of drug binding to albumin has been useful in interpreting the majority of experimental findings. If we assume the existence of drug binding sites I and II, then enantioselectivity in binding to both of these sites may be demonstrated (however, the demonstration of enantioselective binding should not necessarily be construed as evidence of the existence of preformed sites, as a wide range of biopolymers, such as cellulose, that do not contain any "receptor-like" features exhibit differential binding of enantiomers).

The enantioselective binding of tryptophan (16) is a result of differential binding at site II, which is held to be the more enantioselective of the major drug binding sites (29). However, enantioselective binding at site I has been proposed for chiral drugs binding there, such as warfarin (19,30–33).

Enantioselective binding to other drug binding sites on HSA (9), should they exist, has not been demonstrated. The enantioselective binding of drugs to isolated HSA, as determined in studies that did not consider the actual site of binding, is presented in Tables 1 and 2.

TABLE 1 Free Fractions of Drug Enantiomers in Isolated HSA Solutions

Compound	Observation	(% Unbound)	Ref.
Propranolol	(+):60.7	(−):64.9	(42)
Verapamil	(+):40.0	(−):57.2	(43)
Indoprofen	(−):0.12	(+):0.21	(44)
Chloroquine	(+):54.1	(−):64.7	(45)
Mephobarbital	(−):32.0	(+):42.0	(46)
N-methyl-5-cyclohexenyl-5-ethylbarbital	(−):48.0	(+):60.0	(46)
Omeprazole[a]	Ratio of binding affinities of enantiomers = 1.43	(unidentified)	(47)
Carprofen[b]	(*S*):1.7	(*R*):2.2	(48)
Carprofen glucuronide[b]	(*S*):8.0	(*R*):14.2	(48)
Etodolac	(*S*):1.9	(*R*):3.7	(49)

[a]Determined by HPLC, using HSA as a mobile-phase additive.
[b]Fatty-acid-free albumin.

A. Enantioselective Binding of Drugs to Binding Site I of Serum Albumin

The prototypical compound binding to site I is perhaps warfarin (Fig. 1). Enantioselectivity in the binding of the enantiomers of warfarin to HSA has been reported by several authors (19,24,25,30–33), whereas other workers have been unable to detect any differential binding of the enantiomers of warfarin to albumin (34–36). It is interesting to note the studies that resulted in the reporting of no difference in the binding of warfarin enantiomers all employed spectroscopic techniques. It is possible that the quantitative precision of such methods is not great enough to detect the perhaps 0.3% numerical difference in binding reported using techniques that are directly dependent on the actual free fractions of the enantiomers. Reported differences in the binding of the enantiomers of warfarin to HSA are relatively small; for example, Yacobi and Levy (32) determined that *R*(+)-warfarin had a free fraction of 1.2% in human plasma, compared to 0.9% for the *S*(−)-enantiomer. Two studies reporting different binding association constants for the enantiomers of warfarin have also appeared. Fitos et al. (37), using immobilized serum albumin affinity chromatography, reported association constants for (*R*)- and (*S*)-warfarin of 1.5×10^5 M^{-1} and 2.4×10^5 M^{-1}, respectively. Lagercrantz et al. (19), using a similar approach, placed these values at 3.3×10^5 M^{-1} for (*R*)-warfarin and at 4.4×10^5 M^{-1} for its antipode. It is therefore clear that although there is a demonstrable difference in the binding of the enantiomers of warfarin to HSA, this difference is small.

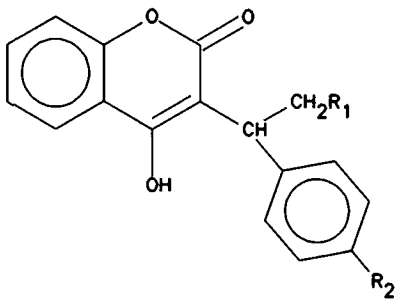
TABLE 2 Drugs Exhibiting Enantioselective Binding to Isolated HSA

Drug	n_1k_1 ($\times 10^{-3}$ M^{-1})[a]	Ref.
(*R*)-warfarin	429	(50)
	330	(19)
	228	(31)
	150	(37)
(*S*)-warfarin	730	(50)
	440	(19)
	496	(31)
	240	(37)
(*R*)-oxazepam hemisuccinate	7	
(*S*)-oxazepam hemisuccinate	257	(29)
(*R*)-oxazepam acetate	11	
(*S*)-oxazepam acetate	55	(58)
(−)-Ketoprofen	620	
(+)-Ketoprofen	1140	(51)
(*R*)-oxazepam glucuronide	ND	
(*S*)-oxazepam glucuronide	55	(52)
S-(+)-MK 571	1910	
R-(−)-MK 571	1222	(53)
(*S*)-α-(4-Chlorophenoxy)-propionic acid	31	
(*R*)-α-(4-Chlorophenoxy)-propionic acid	27	(54)
(*R*)-flurbiprofen	193	
(*S*)-flurbiprofen	344	(55)
(+)-Pirprofen	38	(56)
	78	(57)
(−)-Pirprofen	37	(56)
	136	(57)

[a] = binding affinity at primary binding site.
ND = not determined.

The source of the enantioselectivity in binding of warfarin to HSA is assumed to be the primary site to which it binds, that is, site I. However, Veronich et al. (38) proposed that the enantioselectivity in binding of warfarin to HSA arose from differential binding at secondary sites. In contrast to this, a recent study performed in this laboratory (39) has determined that the enantioselective binding site for warfarin on HSA accounts for over 70% of the total binding of the drug to albumin. This observation would suggest that it is indeed binding site I at which the enantioselectivity in the binding of warfarin enantiomers is expressed.

Other chiral coumarin anticoagulant drugs have been determined to exhibit higher enantioselectivity in binding to site I of HSA than warfarin.



Drug	R_1	R_2
Warfarin	$COCH_3$	H
Acenocoumarol	$COCH_3$	NO_2
Phenprocoumon	CH_2CH_3	H

FIGURE 1 Coumarin anticoagulants binding to site I of human serum albumin.

The *R*-enantiomer of acenocoumarol (Fig. 1) has an association constant ($1.6 \times 10^5\ M^{-1}$) some 2.5 times greater than its antipode, determined by affinity chromatography on an HSA-sepharose filled column (37). Phenprocoumon (Fig. 1), another coumarin anticoagulant thought to bind at site I, also exhibits enantioselective binding to HSA (34), with the *S*(−)-enantiomer exhibiting an approximately twofold higher affinity than its antipode. This leads to free fractions of the *S*(−)- and *R*(+)-enantiomers of 0.0072 and 0.0107, respectively (40).

Brown et al. (34) have attempted to explain the higher enantioselectivity in the binding of phenprocoumon, compared to warfarin, using circular dichroism (CD) studies. The binding of the enantiomers of phenprocoumon differed in the orientation of the bulky substituents (side chains) attached to the chiral center, which were proposed to contribute significantly to the overall binding of coumarins to HSA. On the other hand, the side chains of the enantiomers of warfarin were found to bind in the same fashion, with the coumarin nuclei binding in differing orientations.

More recently, Fitos and Simonyi (41) used the work of Brown et al. to attempt to explain the observation that the *S*-enantiomer of warfarin is able to induce an allosteric enhancement in the binding of certain (*S*)-benzodiazepines at site II, whereas its antipode apparently is not. Their hypothesis was that a particular orientation of binding of the coumarin nucleus was responsible for the allosteric coupling between the two binding

sites. However, the enantiomers of phenprocoumon behaved chromatographically in the same way as those of warfarin; the *S*-enantiomer alone exerted an allosteric increase in the binding of certain benzodiazepines. These results indicate that the coupling between binding sites is dependent on rather more than simply the orientation of the coumarin nucleus, or side chains.

Recent work by this group (39), using a stationary phase for HPLC based on HSA, has suggested that the enantioselectivity in binding to HSA of certain nonsteroidal antiinflammatory drugs (NSAIDs), namely, ketoprofen and suprofen, is expressed at site I. This hypothesis is based on the observation that although octanoic acid is able to displace approximately 70% of NSAID binding to HSA, this occurs without any concomitant loss of enantioselectivity. It is therefore apparent that the site which is responsible for the quantitative majority of binding of the NSAIDs [site II according to Sjöholm et al. (7)] is not the site responsible for the enantioselectivity in binding of these compounds. The minor binding site of ketoprofen and other NSAIDs is site I (7,9), which would therefore suggest that this is the site at which the enantioselective binding of the NSAIDs is expressed.

B. Enantioselective Binding of Drugs to Binding Site II of Serum Albumin

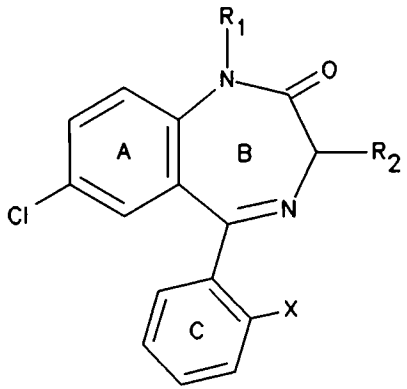
The occasional high enantioselectivity of the second major drug binding site of serum albumin (the indole-benzodiazepine site, site II) has led Müller (4) to describe HSA as a "silent receptor." Compounds thought to bind to site II include tryptophan, diazepam, and octanoate (59). The enantioselective binding of tryptophan to albumin is thought to arise from differential attachment of the isomers to this site (60).

The major binding site of the NSAID etodolac on HSA is site I, which accounts for only a small fraction of the total enantioselectivity in binding exhibited by this drug. The bulk of the enantioselectivity in binding of etodolac to HSA is apparently due to an almost enantiospecific attachment of the *S*-enantiomer to site II (49).

However, the most striking examples of enantioselective drug binding to albumin have arisen from the interaction between chiral 1,4-benzodiazepines and site II. Accordingly, a large body of research has addressed this topic.

1. *Enantioselective Binding of Chiral 1,4-Benzodiazepines to Site II of Serum Albumin*

Initial studies on the stereochemical aspects of the interaction between 1,4-benzodiazepin-2-ones (BDZs; see Fig. 2) and HSA were reported by Müller and Wollert (29,60), who first speculated on the site of the high



	R_1	R_2	X
Oxazepam	H	OH	H
Oxazepam Hemisuccinate	H	$OCO(CH_2)_2COO^-$	H
Lorazepam	H	OH	Cl
Lorazepam Acetate	H	$OCOCH_3$	Cl
Diazepam	CH_3	H	H

FIGURE 2 1,4-benzodiazepin-2-one drugs binding to site II of human serum albumin.

enantioselectivities often observed for the binding of these compounds. Interestingly, Müller and Wollert discovered that even prochiral benzodiazepines (e.g., diazepam; see Fig. 2) could exhibit characteristic extrinsic Cotton effects on binding to site II, emphasizing the high degree of stereoselectivity of the site: A particularly favored conformation of the ligand was selectively bound, or induced, by the protein. Subsequently, many reports of enantioselectivity in the binding of 1,4-benzodiazepines have appeared (e.g., 61–70). The various aspects of enantioselective binding of BDZs to serum albumin were expertly reviewed in 1979 by Alebic-Kolbah et al. (70).

The binding of the chiral BDZ oxazepam hemisuccinate (OXH; see Fig. 2) to HSA is a particularly striking example of enantioselective protein binding (29). As indicated in Table 2, the ratios of the affinity constants of the two enantiomers when binding to HSA may be as high as 50, depend-

ing on the conditions of observation. BSA, on the other hand, exhibits only a very small enantioselectivity in comparison to HSA, the ratio of the binding constants of *d*- and *l*-OXH being only in the region of 0.8 (29).

Studies employing CD spectroscopy of HSA-BDZ complexes have been used in an attempt to characterize the nature of the enantioselectivities observed. All BDZs studied, chiral or achiral, exhibited similar, biphasic CD spectra. The position of the bands in the CD spectra of the HSA-bound chiral compounds was only slightly shifted from those observed with the free ligands (29). These data were interpreted as supporting an extrinsically derived Cotton effect (69,70). Based on their chiroptical studies, Müller and Wollert formed several conclusions: First, both achiral and chiral BDZs bind to a common site; second, BDZ binding does not influence the protein conformation; third, both enantiomers of chiral BDZs bind in similar molecular conformations; and, finally, the primary point of attachment to albumin within the BDZ structure is the phenyl ring fused to the heptaatomic heterocycle (ring A, Fig. 2). Subsequent studies have shown that, in fact, HSA preferentially binds one of the two possible conformations of achiral BDZs (the *M* form), which is that adopted by the *S*-enantiomers of 3-OR substituted compounds, which invariably display higher binding affinities (68–70). Both enantiomers bind with the 3-substituent oriented in a quasiequatorial position, however; for the *R*-enantiomers, this necessitates adoption of the lesser favored *P* conformation. Other conclusions of Müller and Wollert have since been challenged: Binding of BDZs does apparently affect protein conformation (q.v.) (72); binding to other sites on HSA (for both *M* and *P* conformers) is revealed by closer examination of the CD data (66); and, finally, the importance of ring A in protein binding has been contested, nmr studies having indicated that the nitrogen atoms of the heptaatomic ring B play a more vital role (70).

It is clear that the binding of BDZs to HSA is not a simple, unimodular process. Recent studies using immobilized HSA indicate that although many BDZs share a common binding site, this accounts for only approximately 50% of the total binding observed, and that the remaining binding occurs at a number of disparate sites, determined by the molecular structure of the particular BDZ (73). However, the marked enantioselectivity in the binding of many BDZs to albumin remains an impressive example of this phenomenon.

C. Enantioselective Binding to Other Drug Binding Sites on Serum Albumin

The binding of several compounds to albumin may not be satisfactorily described by binding to either sites I or II. In addition to these sites, Fehske (6) proposes three further specific binding areas: for digitoxin, bilirubin,

and fatty acids. Sjöholm (7,9) would add to this list a specific site for the binding of tamoxifen.

Enantioselectivity in binding to minor binding sites has not yet been investigated. This is primarily due to a lack of specific chiral markers for the new sites. Should they actually exist, there is no reason to assume that the potential for enantioselectivity in ligand binding does not occur.

D. Enantioselective Interactions Between Drug Binding Sites on Serum Albumin

One of the striking characteristics of serum albumin is its large conformational mobility. Indeed, this factor has prevented the accurate determination of the tertiary structure of the protein until very recently (74), due to the consequent difficulty in obtaining good x-ray crystallographic data. Many solutes that bind to albumin have been demonstrated to induce reciprocal conformational changes in the protein [e.g., bilirubin; see (75)]. It is therefore not surprising that drugs that bind at one distinct area of the protein are able to influence the binding of second compounds at other, apparently remote sites. In this respect, the work of Simonyi and coworkers (41,71,76–79), on the enantioselective aspects of such interactions, is prominent. The most studied enantioselective allosteric interaction, between binding sites I and II, involves the *S*-enantiomers of warfarin and certain 3-substituted 1,4-benzodiazepin-2-ones (Fig. 2). Mutual enhancements or decreases in binding are observed, the actual effect being critically controlled by the molecular structure of the BDZ. The presence of an o-chlorine atom on the ring C seems to intensify the enhancements in binding caused by the addition of (*S*)-warfarin. Conversely, alkyl substitution at N(1) eliminates the phenomenon. The nature of the C(3) substituent may augment or diminish the effect. (*R*)-warfarin has a much reduced effect on the binding of BDZs, compared to its antipode (78).

To date, the most pronounced allosteric effect observed is the enhancement in the binding of (*S*)-lorazepam acetate (Fig. 2) induced by (*S*)-warfarin (76). The *S*-enantiomer of phenprocoumon has a similar influence on the binding of BDZs to (*S*)-warfarin, with which it shares a binding site (site I) (41).

We have also been able to detect allosteric interactions between the two major drug binding sites of HSA, using an immobilized protein-based stationary phase for HPLC (26,39,73).

An unresolved question, which is difficult to explain using the site-oriented model of drug binding to albumin, is why these effects *are* enantioselective, when the enantiomers of warfarin (for instance) are thought to occupy the same site. Also, it is not apparent why other drugs that bind to sites I and II do not display mutual allosteric enhancements

in binding. Developments in the methodologies available for the study of such phenomena (affinity chromatography, improved spectroscopic techniques) or a revision of current drug-HSA binding models (11) may provide the answers to these puzzles.

E. Immobilized HSA as a Tool in Protein Binding Studies

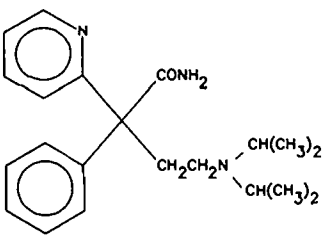
We have recently reported on the synthesis and characterization of a chiral stationary phase (CSP) for HPLC, based on immobilized HSA (the HSA-CSP) (24–26,39,73,80). This phase, because it broadly retains the binding characteristics and conformational mobility of free HSA, provides a powerful tool for the examination enantioselective aspects of ligand binding to albumin (80). Due to the inherent enantioselectivity of HSA, many chiral ligands are separated into their isomeric forms when injected onto the HSA-CSP. The basis therefore exists, by using specific modifiers added to the column mobile phase, to probe the source of enantioselectivity in the binding of drugs to HSA. For instance, the effect of one enantiomer added to the mobile phase on the chromatographic retention of its antipode may be examined. In the case of oxazepam hemisuccinate, the enantiomers were found to bind independently (25), whereas the enantiomers of ibuprofen competed for the same site(s) (24). Application of mathematical treatments to the chromatographic data can quickly provide quantitative data on the binding of individual enantiomers (39). The HSA-CSP promises to be a useful agent in the examination of enantioselective binding to HSA in the future.

III. ENANTIOSELECTIVE BINDING OF DRUGS TO α_1-ACID GLYCOPROTEIN

α_1-acid glycoprotein (AGP), which has a molecular weight of 40,000, is normally present in plasma at concentrations approximately 100 times lower than those of HSA. The actual levels of AGP found in plasma are subject to a very large variation in health, as well as disease. The primary physiological role of AGP is uncertain, but levels are increased in many acute conditions, suggesting an immunological or tissue repair function (81). AGP is an acidic protein due to its high content of sialic acid residues, although binding of basic drugs appears to be driven by hydrophobic rather than electrostatic forces. Removal of the sialic acid residues of AGP does not significantly reduce drug binding (82). At therapeutic concentrations, it is rare for drugs to bind solely to AGP (81).

Drug binding to AGP is thought to occur at a single hydrophobic pocket, or cleft, within the protein section of the molecule (8,83). The enantioselectivity of drug binding at this site was examined in 1987 by

Brunner and Müller (84). Various drugs have been found to bind enantioselectively to AGP. The laevorotatory enantiomer of propranolol (Fig. 3B) is preferentially bound to AGP (42,85), the ratio of the free fractions (−:+) being 0.79. This enantioselectivity is apparent over a range of AGP concentrations (85,86). Interestingly, the enantioselectivity in binding of propranolol to HSA is inverted, compared to that of AGP (42). The significant difference in the tissue distribution of the enantiomers of



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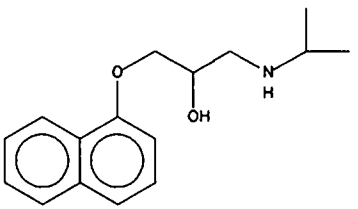

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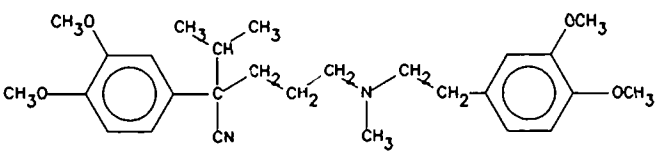

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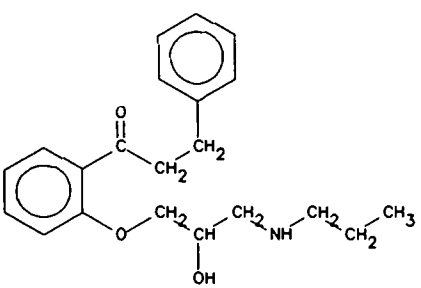

IV

FIGURE 3 Drugs that display enantioselective binding to α_1-acid glycoprotein. I, disopyramide; II, propranolol; III, verapamil; and IV, propafenone.

propranolol in the rat is due to enantioselective binding to AGP (87). The enantioselective binding of β-adrenoreceptor antagonists, in general, significantly affects their actions (88).

The *S*-enantiomer of the antiarrhythmic drug propafenone (Fig. 3D) was found to bind to a single class of binding sites on AGP with greater affinity than its antipode (89). Both enantiomers were also significantly bound in a nonspecific fashion.

Another antiarrhythmic drug, disopyramide (Fig. 3A), is also enantioselectively bound to AGP (90,91). Disopyramide is a rare example, in that AGP almost exclusively accounts for the binding of the drug in plasma (92). The *S*-enantiomer of disopyramide was found to be the form more highly bound to human AGP and plasma. Again, both enantiomers appear to compete for a common binding site (93). The enantiomers of the major metabolite of disopyramide, mono-*N*-dealkyldisopyramide, are able to displace the enantiomers of the parent drug from this binding site in a competitive fashion (94). The plasma concentration of AGP in humans was found to have an enantioselective influence on the unbound fraction of the isomers of disopyramide, and consequently on other pharmacokinetic parameters, such as nonrenal clearance (95).

The more pharmacologically potent enantiomer of verapamil (Fig. 3C), the (−)-form, is bound more loosely to AGP than (+)-verapamil (43,96). The free fractions of the (+)- and (−)-enantiomers, in a 0.55-g/L solution of AGP, were 0.079 and 0.142, respectively (43). The free fractions of the enantiomers of verapamil in plasma samples from human patients were higher than those anticipated from in vitro studies, after oral dosage only. Samples from patients who had been administered the drug intravenously showed similar enantiomeric free fractions to those observed in solution studies on AGP. This apparent inconsistency was explained as being due to competition for the binding site(s) of the enantiomers of verapamil by drug metabolites. These attain higher concentrations following oral dosage, due to an enantioselective first-pass metabolism effect (43).

The concentration of AGP in the plasma of healthy volunteers correlated significantly with the binding ratio (bound/free) of the enantiomers, as well as the racemic form, of methadone. The average free fractions for (+)- and (−)-methadone in plasma were 0.100 and 0.142, respectively (97). Two variants of AGP, orosomucoids 2A and F1, were also found to play important determining roles in the binding of methadone enantiomers (97). The authors of the study point out that the levels of AGP variants should be considered in protein binding studies.

Other drugs reported to bind enantioselectively to AGP include the local anesthetics prilocaine, mepivacaine, and bupivicaine (3); chloroquine and desethylchloroquine (45); quinine/quinidine (3); isoprotenerol (98); vinca alkaloids (99); and acenocoumarol (37).

Although AGP is the most quantitatively important protein in the plasma binding of many basic drugs, it usually exhibits only low enantioselectivity. This may have measurable pharmacokinetic and pharmacodynamic consequences, as is the case with, for instance, disopyramide, verapamil, and the β-blockers. More generally, however, clinically important effects due to enantioselective binding of drugs to AGP are much less frequently observed than for HSA.

IV. ENANTIOSELECTIVE BINDING TO OTHER PLASMA PROTEINS

Although serum albumin and α_1-acid glycoprotein account for the majority of the drug binding capacity of the plasma proteins, other minor proteins may be important in the binding of certain drugs. The possibility of enantioselective binding to these minor proteins has not, in general, been explored. Usually, the clinical importance of binding to such proteins is negligible, but occasionally it may be pharmacologically significant. Lipoproteins, for instance, have the capability to bind basic, or neutral, lipophilic compounds, such as amitryptyline and nortryptyline (100). Although the plasma levels of lipoproteins are small, relative to those of HSA and AGP, they may be the major binding component of plasma for certain drugs, such as probucol, cyclosporin A, and ticlodipine (101). The importance of lipoproteins in the binding of a wider range of drugs may be enhanced in disease states such as hyperlipoproteinemia. To date, no work has been published that considers the possibility of enantioselectivity in binding to lipoproteins. The binding of drugs to lipoproteins closely resembles the partition between an aqueous buffer and chloroform, which may suggest that the process is essentially dissolution of the drug in a lipoprotein core, rather than specific attachment to a classical binding site. Perhaps then enantioselective binding would be unexpected in this instance.

Complement C3 appears to be important in the plasma binding of imipramine (102). Again, no systematic studies on the potential differential binding of enantiomers to this protein have appeared.

V. ENANTIOSELECTIVE DRUG BINDING TO PLASMA

Although it is important to understand the contributions of the individual components of plasma to drug binding, in the clinical situation, the interplay between plasma proteins, endogenous ligands, and drugs often results in behavior far different from in vitro observations using isolated proteins. For this reason, many studies consider the binding of enantiomers to whole plasma, rather than to isolated proteins. In such cases,

TABLE 3 Free Fractions of Drug Enantiomers in Human Plasma at Therapeutic Drug Levels

Drug	(% unbound)		Ref.
Acenocoumarol	(*S*):2.0	(*R*):1.8	(103)
Chloroquine	(−):51.5	(+):33.4	(104)
Disopyramide	(*R*):34.0	(*S*):22.2	(105)
	(*R*):36.6	(*S*):10.9	(106)
Fenfluramine	(−):2.9	(+):2.8	(107)
Glifumide	(*R*)	(*S*)	(108)
Ibuprofen	(*S*):0.643	(*R*):0.419	(109)
Indacrinone	(*R*):0.9	(*S*):0.3	(110)
Methadone	(−):12.4	(+):9.2	(111)
Mexiletine	(*S*):28.3	(*R*):19.8	(112)
Moxalatam	(*R*):53.0	(*S*):33.0	(113)
Nilvadipine	(−):0.9	(+):1.0	(114)
Oxazepam glucuronide	(*R*):45.0	(*S*):13.0	(52)
Pentobarbital	(*R*):36.6	(*S*):26.5	(115)
Phenprocoumon	(*R*):1.07	(*S*):0.72	(40)
Pindolol	(*R*)45.0	(*S*)45.0	(116)
Propoxyphene	(+):1.8	(−):1.89	(117)
Propranolol	(+)12:2	(−):10.9	(85)
	(+):20.3	(−):17.6	(88)
Quinidine	Quinine:14.0	Quinidine:13.0	(118)
Tocainide	(−):86–91	(+):83–89	(119)
Verapamil	(−):11.0	(+):6.4	(120)
	(−):22.4	(+):16.3	(121)
	(−):13.6	(+):9.6	(122)
Warfarin	(*R*):1.2	(*S*):0.9	(32)

the observed behavior may be due to the (possibly opposing) contributions of several proteins and is often modified by the presence of endogenous materials, particularly fatty acids and bilirubin. Tables 3 and 4 contain examples of enantioselectivity in the binding of drugs to plasma.

VI. EFFECT OF DISEASE STATE ON ENANTIOSELECTIVE PLASMA PROTEIN BINDING

The vast majority of studies on drug-protein binding have employed solutions of isolated plasma proteins or serum samples from healthy volunteers. Few data are currently available on the potential of enantioselective disease-induced changes in plasma protein binding. This is the

TABLE 4 Drugs Exhibiting Enantioselective Binding to Human Plasma at Therapeutic Concentrations

Drug	Dissociation constant ($\times 10^6 M^{-1}$)	Ref.
(−)-Alprenolol	0.7	
(+)-Alprenolol	2.1	(98)
(+)-Disopyramide	0.610	
(−)-Disopyramide	0.990	(94)
(*S*)-glifumide	0.4	
(*R*)-glifumide	4.4	(108)
(−)-Isoproterenol	0.9	
(+)-Isoproterenol	4.0	(98)
(+)-Propranolol	0.026	(87)
	1.4	(98)
(−)-Propranolol	0.140	(87)
	0.4	(98)
	4.0	(98)

case despite the fact that the patient treated in the clinic may present a very different environment to the drug than that existing in the rather idealized situation of in vitro experiments.

Although the level of serum albumin tends to remain relatively constant in healthy individuals, the concentration of the other major plasma protein, AGP, may vary significantly. In disease states, the levels of either or both major proteins may be vastly diminished or elevated. Particularly significant are those conditions in which the relative levels of these proteins are greatly modified. Clearly, drugs such as acenocoumarol (37), chloroquine (45), and propranolol (42), which exhibit inverse enantioselectivities in their binding to HSA and AGP, as well as significantly different enantiomeric pharmacologies, will demonstrate the most dramatic effects in this context.

Hyperalbuminemia is a relatively rare condition, but hypoalbuminemia occurs frequently in severe hepatic and renal disorders. In disorders such as these, there may also be relatively elevated serum concentrations of free fatty acids, which may compete with drugs for binding sites on HSA. The *S*-enantiomer of the glucuronide conjugate of the 1,4-benzodiazepin-2-one drug oxazepam binds to site II on HSA, whereas its antipode binds in an apparently nonspecific manner. The elevated levels of fatty acids found in renally impaired patients can therefore cause a displacement of oxazepam glucuronide (52), which may be enantioselective.

Rabbits that had been rendered uremic, by administration of uranyl nitrate, displayed an enantioselective decrease in the plasma protein binding of 2-phenylpropionic acid, the effect being more pronounced for the highly bound *R*-enantiomer (123). This behavior was repeated in a small group of uremic patients administered racemic flurbiprofen. The enantiomeric ratio of free (*R*):(*S*) flurbiprofen increased from 1.1 in healthy individuals to 1.4 in the uremic group (55).

Pathophysiology may have an indirect influence on plasma protein binding. For instance, high levels of the major metabolite of disopyramide, mono-*N*-dealkyldisopyramide, relative to the parent compound, occur in patients with renal insufficiency and also in those with elevated hepatic function (94). Enantioselective competition between the parent drug and metabolite for the same binding site on AGP were postulated to give rise to relatively higher levels of free (+)-disopyramide than in normal subjects (94).

Although the physiology of the patient may remain largely unchanged by his or her pathological condition, other coadministered agents may interfere with the enantioselective protein binding of a particular drug. This interference may be mediated directly, through competition for binding sites, or via allosteric interactions. The result may be supranormal free concentrations of the displaced compound. When the displaced drug has a small therapeutic index, the consequences of this may be clinically important. A typical example of this relatively unexamined phenomenon is the greater displacement of (*S*)-warfarin, compared to its enantiomer, by phenylbutazone, which leads to a significantly greater volume of distribution for (*S*)-warfarin (124). However, of greater clinical importance in this case is the enantioselective inhibition of the metabolism of (*S*)-warfarin by phenylbutazone (124).

Several studies have been reported (e.g., 125,126) concerning enantioselective drug interactions with highly protein-bound species, such as warfarin. As the free fractions of the individual enantiomers of the displaced compound were not determined, it is impossible to evaluate the full clinical significance of enantioselective displacement from plasma proteins. However, it may be anticipated that the enantioselective displacement of highly protein-bound drugs, with high eudismic ratios, will be clinically significant.

VII. INTERETHNIC DIFFERENCES IN ENANTIOSELECTIVE PLASMA PROTEIN BINDING OF DRUGS

The importance of genetic factors in pharmacokinetic and pharmacodynamic processes is becoming more widely recognized, particularly in

the field of drug biotransformation (127). Recently, it has been determined that interethnic differences in response to drugs may be related to racial differences in plasma protein concentrations (128). For instance, Chinese subjects are more sensitive to the β-antagonist effects of propranolol than Caucasians, in part due to lower plasma protein binding in the former group (129). Zhou et al. (130) recently compared the free fractions of the enantiomers of propranolol in Chinese and Caucasian subjects. Among the Chinese, the binding of both enantiomers was significantly lower than in Caucasians, there being a greater proportion of free (−)-propranolol (the eutomer) in the Chinese subjects. This, the authors surmise, could account, in part, for the greater sensitivity of Chinese to propranolol. The lower plasma protein binding in Chinese subjects can be ascribed directly to lower plasma concentrations of AGP (128). Other chiral drugs that bind primarily to AGP (other β-blockers, lignocaine, verapamil) have higher free concentrations in Chinese subjects (128), although enantioselective aspects have yet to be explored.

VIII. SPECIES DIFFERENCES BETWEEN ENANTIOSELECTIVITY IN PLASMA PROTEIN BINDING

This chapter has addressed primarily the enantioselective plasma protein binding of drugs in humans. However, proteins from other species are sometimes used in binding studies, and the conclusions made extended to the human situation. Although this extrapolation may occasionally be valid, quantitative, and indeed qualitative, differences in enantioselective plasma protein binding may occur between species. For instance, rat plasma binds benzodiazepines (131) and warfarin (32) with similar enantioselectivities to those observed for human plasma, but displays no enantioselectivity toward disopyramide (105). The *S*-enantiomer of disopyramide was more highly bound than the *R*-isomer to serum protein in humans, the gorilla, and the pig. The reverse was true in bovine and ovine serum. These enantioselectivities were due to differences in association constants. No enantioselective binding was observed for disopyramide with serum from horses and goats (106).

The binding of a new, potent leukotriene D4 antagonist, MK-571, in the plasmas of 12 different species was recently examined (53). The species studied were subdivided into those in which the *S*-enantiomer bound more tightly (e.g., human, baboon, cow, dog), those that displayed greater binding of the *R*-enantiomer (rat, guinea pig, sheep), and those that displayed no enantioselective binding (rabbit, hamster, mouse).

Differences have also been observed between plasma proteins isolated from different species. Bovine albumin is non-enantioselective for phen-

procoumon. HSA displays a marked preference for the *S*-enantiomer, whereas rodent albumins seem to bind (*R*)-phenprocoumon more strongly (40). A similar qualitative difference exists between HSA and BSA in the binding of warfarin enantiomers. HSA preferentially binds (*S*)-warfarin, whereas BSA binds the *R*-form more tightly (19).

It is clear that despite apparently small differences in the primary structure of serum albumins from different species and gross similarities in the binding of racemates, the examination of the binding behavior of enantiomers reveals many dissimilarities. This observation would imply that the molecular processes involved in the binding of small ligands to serum proteins are highly dependent on the structure and conformation of both participants in the interaction.

IX. CONCLUSION

The present chapter has sought to illustrate the growing awareness of the potential for enantiomers of drugs to be bound to plasma proteins to different extents. In general, the clinical consequences of enantioselectivity in the plasma protein binding of drugs will be minor, but in certain cases, the effects are significant. The examination of enantioselective binding to proteins has been facilitated by recent advances in chiral analysis. In addition, the use of immobilized plasma proteins as stationary phases for HPLC provides a subtle, rapid, and precise means to probe drug enantiomer-biopolymer interactions. In turn, the systematized study of enantioselective binding offers a particularly delicate insight into the molecular processes involved in the binding of drug ligands to proteins.

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13

FDA Perspective on the Development of New Stereoisomeric Drugs: Chemistry, Manufacturing, and Control Issues

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One of the most technically challenging aspects of the manufacturing and control of chiral drug substances and drug products is the detection and quantitation of one enantiomer of a chiral drug substance in the presence of the other. This is true for both racemic and enantiopure drug substances, as well as the drug products manufactured from them. The fact that diastereomers have differing physical and chemical properties renders the regulatory considerations related to their analytical controls, manufacturing processes, and stability fundamentally similar to those for nonchiral drugs.

The relevance of this analytical methodology to the development of new stereoisomeric drugs has been recently addressed by the U.S. Food and Drug Administration (FDA) in all areas of development, ranging from manufacturing control through pharmacology to clinical evaluation (1). It is stated therein "[t]his policy statement is intended to address these issues thereby assisting manufacturers in the development of new stereoisomeric drugs." The relevance of these same concerns to the generic duplicate versions of marketed drug products is reflected in a statement reflecting awareness that the standards for identity of the active ingredient in such drugs must take stereochemistry into consideration (2 at 17958–9).

I. DEFINITIONS

A few of the finer points of technical language are worthy of special emphasis, if only as a reminder. The term *chiral* is used to refer to both racemates and pure enantiomers. From a regulatory viewpoint, the standards for identity, strength, quality, and purity of both the drug substance and drug product are the same for both chiral and nonchiral drugs. It is only in specific technical details, such as the choice of a regulatory method or the establishment of regulatory specifications, that our approaches may diverge.

The term *racemate* refers to any mixture of equal or nearly equal amounts of enantiomeric molecules. In reference to solid state properties, the most common type of racemate is a racemic compound, in which each crystal, no matter how small, is racemic. The racemic conglomerate or mixture, such as Pasteur resolved, is much less commonly encountered. In this case, each crystal is composed entirely of the same enantiomer, but the bulk sample approaches a 1:1 ratio.

To describe a bulk material that is composed of one enantiomer with no significant amount of the other, the term *enantiopure* has been recently proposed by Eliel and Wilen (3). For reasons of clarity, this term is preferable to older usages, such as "optically pure".

The terms *eutomer* and *distomer* have been coined to refer to the members of an enantiomeric pair that have, respectively, the greater and lesser physiological activities. It is generally understood, but often unstated, that this reference is necessarily related to the single activity being studied. The eutomer for one effect may well be the distomer when another is studied. For purposes of clarity, these terms should be avoided in regulatory writing, though they may be quite technically appropriate in other circumstances.

II. GENERAL POLICY

The central regulatory objective for a chiral drug is knowledge of its composition. Whether racemic, enantiopure, or intermediate, the quantitative isomeric composition must be known for the drug supplies used in studies of its pharmacology, toxicology, and clinical effectiveness. Since these findings lead to the development of specifications for a marketed product, they establish the stereochemically sensitive analytical method as the nexus of chiral drug development.

The justification for the development of analytical methodology for quantitation of one enantiomer in the presence of the other is not derived solely from its importance as a control on the manufacturing process. It is equally essential for those investigations of absorption, distribution, bio-

transformation, and excretion that constitute the pharmacokinetic profile of the chiral drug (1,4).

Because diastereomeric impurities and degradation products, as well as geometric isomers, present an analytical challenge that is substantially similar to that of other impurities, the FDA policy statement specifically excludes these stereochemical problems. The considerations here are limited to those that are the direct consequence of the analytical problem of simultaneously quantitating molecules that are identical in their physical (except for the rotation of the plane of polarization of light) and most chemical properties.

Although the policy statement primarily addresses the development of new stereoisomeric drugs, its significance is not limited to this phase. The manufacturing controls developed during this time form the basis for demonstrating the batch-to-batch consistency of the manufacturing process. They are, therefore, a key part of compliance with good manufacturing practices (GMPs).

III. ANALYTICAL CONTROL METHODS AND SPECIFICATIONS FOR CHIRAL DRUGS

A. Drug Substances

The specifications for both enantiopure and racemic chiral drug substances should be sufficient to establish both chemical and stereochemical aspects of identity, strength, quality and purity. This implies both that the identity test use a stereochemically specific method and that the assay method be stereochemically selective.

B. Specifications for Stereochemical Identity or Purity

Regulatory specifications for the identity of a bulk drug substance have two major applications. First, they are used as controls on the manufacture of the bulk drug substance. Second, they document compliance with GMPs by verifying the identity of the drug component before its release for further manufacturing. Such identity tests as the infrared spectrum or chromatographic retention time are unlikely to be capable of discriminating between stereochemical forms of a drug in most cases. An IR spectrum may be suitable for establishing stereochemical identity if there are obvious differences between the racemic and enantiopure material, but this may be difficult to demonstrate. Melting ranges may also be suitable, but there appears to be a trend toward categorically eliminating these specifications from compendial monographs in favor of instrumental methods. In any event, a conclusion that an identity test is stereochemically sensi-

tive should be supported by test results on the other stereochemical form(s).

Many chiral compounds are known by the chemist to be racemic because of the lack of stereoselective influences on the synthesis or to be enantiopure because of natural origin. Such knowledge, while based on a sound technical foundation, may not be suitable for regulatory purposes. For example, the commercial availability of a racemate or the "opposite" enantiomer may make its substitution for the approved component conceivable. Therefore, it may be necessary to bring other factors (e.g., synthetic feasibility or commercial sources) into consideration to establish whether a stereochemically specific identity test is necessary.

Certain identity tests intended to establish the stereochemical identity of a drug are known. Their applicability to a specific drug substance may vary depending upon the magnitude of differences between the values to be determined. These are discussed below in the following order (which does not suggest the suitability of the technique for regulatory purposes):

1. Optical rotation
2. Melting range
3. Infrared spectrum
4. X-ray powder diffraction
5. Optical rotatory dispersion
6. Chiral chromatography (including liquid, gas, and thin-layer, as well as chiral capillary electrophoresis)

Optical rotation has the dual advantages of historical use and widespread recognition in the compendia. For an enantiopure material, it defines its configuration when used in conjunction with other valid chemical tests. However, optical rotation has been used ineffectively when the primary analytical goal is the determination of stereochemical purity. The limits selected for the specification seem to be unrelated to the purity required by other methods. For example, the compendial monograph for naproxen requires that the drug substance meet a specification of "between +63.0 and +68.5°" in a chloroform solution. Based on the published specific rotation, this corresponds to a stereochemical purity of 95.5 to 103.7%, compared to the assay limits of 98.5 to 100.5%, determined by titration with sodium hydroxide (5).

The traditional sodium D wavelength was (historically) chosen for practical reasons associated with the use of visual polarimeters rather than for any technical advantage. Current technology makes the photoelectric polarimeter the instrument of choice, permitting the use of several lines in the mercury spectrum, in addition to the classical choice of the sodium D wavelength. Chafetz (6) has provided a variety of examples of the higher

specific rotations (and resulting increased sensitivity) obtained at the shorter wavelengths. Thus, specific rotation should be determined at several wavelengths, and the choice of which to use should be justified.

A major disadvantage of optical rotation as a measure of stereochemical purity is that interpretation of the single value lacks significance if any impurities are chiral. To insure the validity of an optical rotation specification, it should be supported by specific rotation measurements on any known chiral impurity or degradation product. This is, of course, in addition to the usual studies of the effects of solvent, concentration, and temperature on the specific rotation of the principal analyte.

A relatively common use of optical rotation is as an identity test for a racemate with specification limits that are symmetrical around zero. Such a specification has little if any regulatory significance. Its validation necessarily depends upon knowledge of the specific rotation and thus requires the resolution of the racemate on a laboratory scale. Furthermore, even with such supporting data, the method is dependent upon the accuracy of the sample preparation, since a solvent blank would also show a rotation of zero. Other analytical methods are far more appropriate for the stereochemically specific identification of racemates.

As a stereochemically specific identity test, a measurement of melting range is complementary to optical rotation. It can suffice to identify the racemic form but cannot distinguish enantiopure materials of opposite handedness. The melting range of the unwanted stereochemical form must also be known if such a test is to be valid. Chafetz (6) has cited examples of melting ranges of official drugs, some of which have received compendial recognition, even though they are of questionable validity. However, it should not be assumed that nothing is learned from these measurements.

For example, racemic pseudoephedrine melts at 118°C, while the pure enantiomer melts at 119°C (7). This near identity of melting ranges, while of little regulatory value, suggests that the racemate may exist as a crystalline conglomerate, rather than as a racemic compound. Because of such situations, prudence in the development of analytical specifications suggests that the minimum difference in the melting ranges of racemic and enantiopure material should be at least twenty degrees Celsius for use as a regulatory identity specification for a chiral drug. Such a difference will, in general, be sufficient to distinguish between a crystalline racemic compound and racemic conglomerate (8).

Infrared spectra, where they are used as regulatory specifications, generally make use of a reference standard. The specification is usually written to require that the sample spectrum "exhibits maxima only at the same wavelengths" as the reference spectrum. This type of specification

may be suitable for achiral drugs or where only chemical structure information is wanted. But, for a stereochemically specific identity test for a chiral drug substance, different wording may be more appropriate. Although many absorption peaks will be different for the racemate and the enantiomer, some will be identical. Thus, the specification should be written to define more precisely what is meant by "exhibits maxima only at the same wavelengths". It may be necessary to specify wavelength ranges, numbers of peaks in a certain range, or even peak absorbance ratios to guarantee that the analytical data are sufficient to identify the stereochemical form correctly. In any case, it must be shown that the unwanted stereochemical form will not pass the specification.

Few drugs make use of X-ray powder diffraction as a regulatory test. However, this method has a unique advantage as a stereochemically specific identity test for chiral drugs. The crystal structure, and therefore the powder diffraction pattern, are necessarily different between the racemate and the enantiomer, except in the case of a racemic conglomerate. Furthermore, published reference data are readily available. In combination with the invariance of the d-spacing measurements, this may make X-ray diffraction more attractive to the regulatory scientist.

For identification of racemates, optical rotatory dispersion (ORD) appears to be of great potential use. However, there appears to be no regulatory use of this method by any of the compendia. ORD measurements are made over a range of wavelengths, which makes the significance of a flat spectrum over a range of wavelengths much greater than a single optical rotation measurement of zero.

The ultimate stereochemical identity test is, of course, the direct resolution of the enantiomers using chiral liquid or gas chromatography (9). When compared to a reference standard of the racemate, and under experimental conditions that will resolve the peaks of both enantiomers, the occurrence of two equal peaks will identify the racemate, and one peak will signify an enantiopure material. A proof of the stereochemical identity of the analyte can be provided, based on a match of retention times with a reference standard of known stereochemistry. Inequality between the peaks is a measure of enantiomeric enrichment. Therefore, it is conceivable that both stereochemical identity and purity can be established from a single experiment.

Some chiral drug substances either occur naturally or are synthetic derivatives of natural products. In these cases, the controls on the raw materials are often assumed to guarantee the stereochemical identity of the finished bulk drug substance product without the use of a specific stereochemically sensitive test. Such an assertion is of little significance for regulatory purposes. First, determination of the source species for an

isolated natural product is often very difficult. Second, syntheses of the racemic equivalent of an enantiopure natural product are frequently well known, even for highly complex molecules such as the opiates. Finally, compendial monographs are intended as analytical controls on the result of a process and do not mandate the process itself (10). Therefore, a statement of natural origin is only informative and does not serve as a confirmatory specification for identity. Even for natural products, a stereochemically specific identity test is needed.

The FDA's *Guideline for the Manufacture and Control of Drug Substances* (11) addresses the relationship between identity and assay test methods. A less specific identity test may be paired with a more specific assay, and vice versa. This position is equally applicable to chiral drugs in that identification and assay methods need not be equally stereospecific. However, there are additional regulatory considerations for chiral drugs. For a manufacturing facility to be in compliance with the FDA's Good Manufacturing Practice Regulations, each component of a drug product must be tested by at least one identity test (12). This test must be as specific as possible. Thus, for a chiral drug, identity testing must include confirmation of its stereoisomeric identity, even if this requires multiple tests.

C. Drug Products

As with the bulk drug substance, specifications for both enantiopure and racemic chiral drug products should include both a stereochemically specific identity test and stereochemically selective assay method. The analytical method to be used should not be arbitrarily chosen to be the same as for the bulk drug but should be chosen on the basis of the composition, method of manufacture, and stability characteristics of the formulation.

The stereochemical identity of the active ingredient has, presumably, been established by the testing of the bulk drug before its release for manufacturing. The chiral drug product, in turn, should be shown to have the intended stereochemical quality. It should, therefore, be assayed by a method which is capable of detecting other stereoisomers, including, for the drug with a single stereogenic center, its enantiomer. For racemic chiral drug products, or for products where the active ingredient is of natural origin and has multiple stereogenic centers, a stereoselective assay method may not be necessary. Our laboratories have, for example, published data describing an HPLC method capable of detecting (*S*)-(+)-desoxyephedrine (the controlled substance, methamphetamine) contamination in a commercial nasal preparation of (*R*)-(−)-desoxyephedrine (13). This contaminating enantiomeric enrichment was found to have been caused by unexpected variations in the manufacturing process.

D. Stability

Questions of the stability of the bulk drug are just as important for chiral drug substances and products as for any others. However, stereoisomeric stability should be considered as well. The protocol for stability testing should include one or more methods that will establish the stereochemical integrity of the drug substance and product. If, however, testing of the drug product has established that racemization does not occur under the labeled conditions for storage and use within the proposed shelf life, stereochemically sensitive testing may not need to be routinely included in the stability protocol. It may, however, need to be occasionally re-evaluated, especially if the recommended storage and use conditions or expiration dating for the product are to be changed.

Most mechanisms for racemization are chemical in nature, since the breaking and reforming of chemical bonds is necessarily involved. For an enantiopure drug substance, the potential for inversion should be investigated during the early stages of drug development. Such studies should include exposure of the drug substance to the full range of conditions likely to be encountered in manufacturing and storage, as well as possible chemical interactions that may result in racemization. This may involve consideration of possible reactions with inactive ingredients in the formulation. For example, an enantiopure beta-blocker might be quite stable in solid oral formulations, but subject to racemization in solution at the pH range typical of ophthalmic products. Even if the latter type of product is not anticipated, racemization under these conditions should be studied for the drug substance. Finally, it should be noted that information about racemization conditions may also be a significant factor in the validation of a resolution method that involves racemization of the "unwanted" isomer to increase the overall yield.

As an analytical problem, the demonstration of stereochemical integrity of a drug product is a simpler task than it may seem. Only the drug with a single stereogenic center routinely presents an analytical problem that cannot be resolved with nonchiral methods. For the drug with multiple stereogenic centers, it would be quite unusual to find that a single degradative process acted simultaneously at all stereogenic centers. For such a drug substance, normal degradative processes would produce a mixture of diastereomers, which would likely be able to be analyzed by nonchiral chromatographic methods.

E. Systematic Nomenclature

The labeling of a drug product is required to include its systematic chemical name. This name must include the correct relative or absolute

stereochemical descriptors (14). The development of a chiral drug substance, therefore, requires its unequivocal structural identification (see Section F, which follows) in order that the labeling is technically correct.

Furthermore, the interpretation of all the reports of developmental chemistry depends upon a knowledge of the drug being studied. Yet, it is not unusual to find a marketing application to be deficient on this point. For example, in its early development, a candidate drug may be referred to by a code name or number, without identification of its chirality. At later stages, the correct stereochemical name may be used without complete correlation to the earlier code.

The FDA is represented on the U.S. Adopted Names Council, in cooperation with the American Medical Association, the American Pharmaceutical Association, and the U.S. Pharmacopeia (USP). This group has adopted a variety of consistent rules for established names. Proposals for the adoption of nonproprietary names submitted undergo routine review for stereochemical accuracy in both systematic names and structural formulas. As part of the ongoing review of the compendial literature, the USP has a project in process that will result in the correction of systematic names which are incomplete with regard to stereochemical descriptors.

However, nomenclature information is considered by the USP to be an unofficial part of the compendial monograph in that it is not a regulatory requirement that must be satisfied for a drug substance to be of compendial quality. It is, however, of major technical significance, particularly for information retrieval.

F. Proof of Stereochemical Structure

For any chiral drug, establishment of its stereochemical identity is not an insurmountable technical problem, even for enantiopure material. The techniques of X-ray crystallography and NMR spectroscopy will, in general, lead to conclusions of high certainty, at least for the material studied. However, for enantiopure material, it is critical that the relevance of conclusions based on these techniques be correlated to larger batches of material through chiral properties, such as optical rotation. Of the thousands of published determinations of absolute configuration based on X-ray studies, most are unsupported by such data on solutions of the specific crop of crystals from which a few were selected for study.

IV. CONCLUSION

Regulatory policy must provide a level of manufacturing control that is adequate to assure consistency in the identity, strength, quality, and purity of a marketed drug. This is no less true for drug products manufac-

tured from chiral drug substances. The additional analytical questions that chiral drugs present require that the scientific work supporting the policy be based on the use of technology that is not only current but also appropriately validated.

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14

Stereochemically Pure Drugs

An Overview

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Recently a great deal of attention has been focused on the separation of stereochemically pure enantiomers for the purpose of marketing drugs as single active moieties. The motive behind such attempts is twofold: First, stereochemically pure drugs may provide safer and more efficacious alternatives to their corresponding racemates, and second, such a product, once developed, may be eligible for a period of market exclusivity. Hence, some of the more popular racemic drugs such as ibuprofen (1,2) have been patented as single isomers and are being investigated as new medicinal agents.

In the last decade, the stereochemically pure compounds were thought to be just a refined form of their respective racemates, and the inactive or less active enantiomer was considered an isomeric "ballast" (3). Although this view is still valid in some instances, it cannot be extrapolated to the entire class of racemic drugs. It is now realized that changes in the enantiomeric composition of chiral drugs may result in alteration of their pharmacologic and toxicologic profiles; that is, the "inactive" enantiomer may somehow, directly or indirectly, contribute to the overall therapeutic profile of the racemate. Hence, at least for existing racemic drugs with satisfactory safety records, the stereochemically pure drug does not necessarily provide better medication. However, dismissing the potential advantages of single enantiomers based on what is known about the racemate is also unwarranted as single enantiomers may provide new therapeutic opportunities. Therefore, stereochemically pure compounds must be considered new drugs, and from research and development viewpoints they must be investigated thoroughly for possible additional indications and side-effects, as compared to their respective racemic formulations. It is also important to mention that the issue of enantiomer vs. racemate must be

discussed on the basis of individual drugs, since one cannot provide a general guideline for the entire class. Nevertheless, the concept offers new opportunities in the field of drug development that must be explored and wisely exploited.

The decision to develop a stereochemically pure compound must be based on pharmacodynamic, safety, pharmacokinetic and pharmaceutic considerations. Therefore, attempts will be made to discuss the issue of racemate vs. enantiomer from these viewpoints, as well as those of regulatory affairs. This chapter is not intended to be exhaustive, hence, many more examples may exist than those to which we refer.

I. PHARMACODYNAMIC AND TOXICITY CONSIDERATIONS

From the therapeutic point of view, racemic drugs are divided into three groups (a detailed listing can be found in Ref. 4): (1) racemates with equipotent enantiomers, (2) those with one enantiomer possessing the majority or all of the beneficial properties, (3) racemates with properties superior to those of their stereoisomers, and (4) racemates with enantiomers possessing stereospecific effects (i.e., enantiomers having entirely different pharmacodynamic properties). For the latter group of racemic drugs, there appears to be little controversy as to the need for separation of the enantiomers. Propoxyphene is an example of a drug with stereospecific effects [(−)-enantiomer analgesic while (+)-enantiomer antitussive].

It must be pointed out that many drugs may belong to more than one of the above categories, and with the ever-growing knowledge of stereochemistry of drugs' action and disposition, they may be more appropriately classified into different categories. For example, the β-adrenoceptor blocker, propranolol, may be classified under the second category with respect to its cardiovascular effects, whereas as a spermicidal agent, it belongs in the first category (5).

A. Equipotent Enantiomers

There are not many known racemic drugs with equipotent enantiomers (4). Examples include the antiasthmatic drug proxyphylline, the antineoplastic cyclophosphamide, the antimalarial agent primaquine, and the antiarrhythmic agents propafenone, flecainide, and disopyramide. For these drugs there seems to be little rationale for separating the two enantiomers, as the known target receptors do not distinguish between the two chemical entities. However, although the enantiomers may be qualitatively and quantitatively similar with respect to the main therapeutic

properties for which they are indicated, they may have subtle and sometimes unknown differences. For example, although enantiomers of propranolol have equal potencies in reducing sperm motility, the β-adrenoceptor property of the drug is attributed mainly to its *S* enantiomer (4). Hence, as a spermicide, *R*-propranolol may be a safer drug as it reduces sperm motility without much of an effect on the cardiovascular system. A similar scenario has been observed for another β-blocker, timolol: Both enantiomers appear to be effective in the treatment of open-angle glaucoma (6). However, topical use of the available *S*-timolol has been associated with some systemic side-effects such as asthmatic attacks (7). On the other hand, the *R* enantiomer that only has weak β-blocking activity increases retinal/choroidal blood flow that can improve the treatment (8). Therefore, the availability of an optical *R*-timolol seems to be timely.

Enantiomers of propafenone, a potent antiarrhythmic drug, are equipotent with respect to their sodium channel-blocking activity. However, *R*(−)-propafenone has significantly less β-blocking potency. Hence, in patients who do not need β-blocking activities, the stereochemical pure *R*(−)-propafenone may be a safer alternative to the available racemate as it is expected to produce less nonspecific peripheral vasodilation (9). However, this remains to be proven. Nevertheless, the pharmacokinetics of propafenone are complicated due to its extensive stereoselectivity, first-pass metabolism, and genetically controlled metabolism (9). The time course of the drug is also altered by the presence of many other drugs (e.g., 10) and by food (11). Although the stereoselective nature of these alterations is not fully explored, a less complicated formulation, that is, a stereochemically pure product, may demonstrate a more predictable pharmacokinetic/dynamic pattern than the racemate.

Interestingly, the secondary effects of enantiomers may be masked by the presence of their antipodes and only surface after the therapeutic and toxicity properties of the enantiomers are compared with their respective racemate. For example, enantiomers of disopyramide illicit equal antiarrhythmic effects, but the *S* enantiomer possesses a greater extent of anticholinergic side-effects (12). Intuitively, a formulation composed of the *R* enantiomer would seem to be a safer alternative. However, it appears that the single enantiomer of disopyramide possesses additional side-effects that are minimal after administration of the racemic drug.

B. One Enantiomer with All or Most Activity

Many racemic drugs belong to this group for which one enantiomer is known to possess the majority or all the beneficial properties; for example, important drugs in this group are many of the chiral nonsteroidal anti-

inflammatory drugs (NSAID), some β-adrenoceptor antagonists, and some calcium channel blockers (4). Among these, the chiral NSAIDs have received the most attention.

The antiinflammatory and analgesic effects of NSAIDs are attributed almost exclusively to the *S* enantiomer (13).* The *R* enantiomer, therefore, may be considered a useless and potentially harmful ingredient. However, for some NSAIDs with a 2-arylpropionic structure (profens), the inactive *R* enantiomer acts as a prodrug because it is biotransformed to the active antipode. Hence, administration of the inactive enantiomer results in in vivo formation of the active antipode, thereby eliciting both antiinflammatory and analgesic effects. In such cases, then, there would seem to be little justification for the use of the single enantiomer. The prodrug nature of the *R* enantiomers of profens, however, has been overgeneralized based on data generated from laboratory animals (13). Indeed, in humans, among the available profens, the metabolic chiral inversion is known to be therapeutically significant only for fenoprofen and ibuprofen, for which 100 and 60%, respectively, of the *R* enantiomer is inverted (4). Nevertheless, stereochemically pure chiral NSAIDs may prove beneficial as compared to their respective racemates for the following reasons: Although the *R* enantiomers may be devoid of antiinflammatory activities, concerns have been raised with respect to their contribution to the toxicity of NSAIDs. For example, incorporation of the *R* enantiomers of profens into the triglycerides, observed during the process of inversion in the rat, has been proposed to contribute to the side-effects of the drugs at the level of the central nervous system (14). Furthermore, a recent report (15) indicates that in the rat, the pharmacologically "inactive" enantiomer of ibuprofen is also able to reduce renal function, similar to *S*-ibuprofen but to a smaller degree. Although the proposed toxicities of the *R*-profens are either speculative or yet to be substantiated by more studies, its presence in a formulation does not provide any obvious benefit. Furthermore, for those profens that undergo chiral inversion, the erratic nature of the process may contribute to the variability in plasma concentration of the active enantiomer (16).

It has been reported that the clearance of both isomers of ketoprofen glucuronide, the main metabolite of ketoprofen, another racemic profen, is reduced in elderly arthritic patients due, perhaps to an age-induced reduced renal function (17). As the therapeutic benefits of this drug are ascribed mainly to its *S* enantiomer and *R*-ketoprofen is not inverted to a

*Recently, data have been provided indicating that *R*-flurbiprofen may have analgesic activities similar to those of *S*-flurbiprofen (35).

significant extent (18), administration of the single enantiomer may be associated with a reduced renal burden in the elderly. Therefore, *S*-ketoprofen may be a safer NSAID than racemic ketoprofen.

The therapeutic advantages of β-adrenoceptor antagonists in the treatment of hypertension and angina are ascribed mainly to their *S* enantiomers. The *R* enantiomers, however, are not totally devoid of cardiovascular activities as properties like antiarrhythmias have been attributed to them. Hence, single enantiomers of these compounds may offer β-adrenoceptor blockers with different overall therapeutic profiles. The risk:benefit ratio of using a single enantiomer of β-blockers, therefore, remains to be explored.

Labetalol is a β-blocker with nonselective β- and selective α_1-adrenoreceptor antagonist properties. With relatively high doses, labetalol elicits postural hypotension, an undesirable side-effect. Dilevalol is one of the four isomers (*R*, *R*) of labetalol that is almost devoid of α-adrenoreceptor activity. Hence, the reduction in peripheral vascular resistance observed for dilevalol occurs via its β_2-agonist activity, which is not associated with postural hypotension. This renders dilevalol suitable for physically active hypertensive patients and those who complain of cold extremities (19). However, administration of dilevalol has been reported to be associated with a few reversible cases of hepatitis and jaundice (20). Hence, it is unlikely that this drug will become available as a new β-blocker.

C. Racemate Superior to Enantiomers

The examples provided above indicate that single enantiomers are not always better alternatives to their respective racemates. Another interesting example is 5-dimethyl-sulphamoyl-6,7-dichloro-2,3-dihydrobenzofuran-2-carboxilic acid, a racemic diuretic whose diuretic activity is attributed to the *S*(−)-enantiomer, while the *R*(+)-enantiomer has uricosuric properties that suppress the *S*-enantiomer-induced side-effect of elevated blood uric acid (21). It is not known if manipulation of the *S*:*R* ratio of this drug, as is seen for indocrinone (below), results in a better diuretic agent.

The diuretic properties of indocrinone have been reported to improve by manipulation of the enantiomers' ratio (22): Although the diuretic property is predominantly ascribed to *R*(−)-indocrinone, both enantiomers possess urisocuric activity. The uricosuric effect is desirable as it counteracts the diuretic-induced rise in plasma uric acid concentration. However, this property of racemic indocrinone is transient. Examination of the overall pharmacodynamic profile of various formulations of indocrinone suggests that the patients remain isouricemic during a 7-day treatment period with an *R*:*S* ratio of 10:40.

It is clear that at least for the two diuretics discussed above, as well as

labetalol and disopyramide cited earlier, the patient does not benefit more from treatment with stereochemically pure isomers as compared with the available formulations.

II. PHARMACOKINETIC CONSIDERATIONS

In general, for drugs with complex pharmacokinetics such as verapamil, propranolol, propafenone, and ibuprofen, stereochemically pure compounds may require less complicated assays and simpler pharmacokinetic and pharmacodynamic data analysis. These advantages, however, are becoming less significant due to the recent advent of convenient and cost-effective stereospecific assays and the growing awareness of the possibility of stereoselectivity in the pharmacokinetics of chiral drugs. Nevertheless, there are other possibilities that render the development of stereochemically pure drugs desirable based on pharmacokinetics grounds.

There may be cases in which a stereochemically pure drug is found preferable on the basis of its stereoselective pharmacokinetics: For example, even when enantiomers are equipotent, they may be different from one another in their pathways of elimination; for instance, one enantiomer may depend on renal pathways, whereas the other is extensively metabolized. Hence in subjects with reduced renal function such as elderly patients, administration of the racemate may result in an excessive accumulation of one of the enantiomers. Consequently, the single enantiomer that does not depend on the kidneys for its elimination may prove beneficial as compared to the racemate.

The *S* enantiomer of hexobarbital possesses three- to fourfold greater hypnotic activity than its antipode (4). However, in the elderly population, the clearance of *R*-hexobarbital, but not that of *S*-hexobarbital, is substantially reduced (23). Administration of the *S* enantiomer, therefore, will produce a more predictable clearance than that of the racemate. Also, as the safety profile of the less active enantiomer is unknown, one cannot rule out the possibility of its involvement in the overall toxicity of hexobarbital. For a related barbiturate, pentobarbital, it has been suggested that despite its weaker pharmacological activities, sedation with the *R* enantiomer is accompanied by symptoms of hyperexcitability (24).

The relative concentration of the pharmacologically active *S* enantiomer of ibuprofen (*S*:*R* ratio) increases with prolongation of the gastrointestinal transit time of racemic formulations due to a corresponding increase in chiral inversion of the *R* to *S* enantiomer in the gut (25). Administration of *S*-ibuprofen, therefore, reduces the formulation-dependent variability in the concentration of the active enantiomer in the body.

In the absence of its antipode, an enantiomer may exhibit greater pharmacological activity. This is possible when a less active enantiomer acts as an antagonist or one accelerates clearance of the other. For example, in the rat, the analgesic effects and plasma concentration of *d*-propoxyphene are enhanced in the presence of *l*-propoxyphene (26). Conversely, as compared to the racemate, plasma concentrations of flurbiprofen enantiomer seem to increase when either enantiomer is given alone to both humans and rats (27,28). An opposite observation has been reported for indobufen in the rat and mouse (29).

III. PHARMACEUTIC CONSIDERATIONS

From physicochemical viewpoints, enantiomers and racemates are often very different from one another. For example, it has been shown that individual enantiomers of ibuprofen have greater water solubility and faster dissolution than their racemates (30). A formulation of *S*-ibuprofen, therefore, may have more rapid absorption and consequently a shorter onset of analgesia. This is important, as evidence exists in the literature in support of a meaningful relationship between ibuprofen serum concentrations and its analgesic effects (31). Hence, a more rapidly absorbed formulation may provide shorter onset of action. Therefore, as a pain reliever, stereochemically pure NSAIDs may be preferable to their respective racemates. Furthermore, one may take advantage of the single enantiomer's greater solubility to prepare various soluble formulations of NSAIDs and products with an accelerated dissolution rate.

The relatively unknown dependency of the physicochemical properties of chiral drugs is not exclusive to NSAIDs because similar observations have been made for other compounds (32).

IV. REGULATORY CONCERNS

The regulatory requirement for the development of chiral drugs, in general, and stereochemically pure compounds, in particular, has puzzled many of the parties involved in the field of research and development. De Camp (33) suggests that the U.S. guidelines for the development of stereoisomers of existing racemic drugs is clear. He stated that stereochemically pure compounds derived from available racemates will be treated as new drugs. However, they will be treated as a chemical type-2 compound, hence requiring less investigation. The European Economic Market guideline appears to be more stringent than that cited by De Camp since it suggests submission of full data on the stereoisomer (34). Never-

theless, it is likely that a decision regarding the depth of required data should depend on the potency and toxicity of the drug of interest and the nature of the target patient population.

V. CONCLUSION

1. The therapeutic and economic risk/benefit aspects of enantiomer vs. racemate must be judged for each drug separately.
2. From a pharmaceutical developmental viewpoint, enantiomers must be considered new therapeutic agents that may offer exciting and useful therapeutic opportunities.

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Stereoisomeric Drugs in Therapeutics

Clinical Perspectives

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I. INTRODUCTION

The therapeutic revolution of the 20th century has been due to a number of interrelated phenomena, the sum result of which has been the effective treatment of human disease with potent and specific organic molecules. Rapid development of organic chemical syntheses at a time of tremendous advances in the understanding of physiology, pathology, biochemistry, cell biology, and most recently molecular biology has permitted screening of large numbers of compounds for specific pharmacodynamic effects. In some cases, planned drug design has been based on knowledge of the molecular structure of the desired site of drug action.

Biological systems have long been known to exhibit chirality, although the reasons for this have not been fully explained. In any case, this results in the chirality of drug receptor areas and confers the requirement of chiral specificity on drugs that have asymmetric centers and resulting mirror-image stereoisomers. Unfortunately, until very recently, the advances in chemical organic synthesis included stereospecific synthesis in a very limited number of instances. Therefore, a large number of potent and effective therapeutic agents are administered as racemic mixtures, although in most instances only one of the stereoisomers exhibits the desired pharmacological effect. This chapter is developed as a clinical perspective of the present, a review of areas not well defined and requiring further clinical and basic investigation, comment on clinical research in the

drug development process, and finally an optimistic view of the future for development of isomerically pure therapeutic agents.

II. THE PRESENT

Although the pharmacodynamics of all enantiomers of drugs that are administered as racemic mixtures have not been determined, enough data are available to classify racemic drug mixtures into four general groups (modified from Birkett) (1). These are (1) mixtures of an active and a relatively inert isomer, (2) mixtures of isomers with different and unique pharmacodynamic activities, (3) mixtures of isomers with opposing pharmacodynamic activities, and (4) mixtures of isomers with similar pharmacodynamic effects, although often of different potency. Examples of each will be discussed and placed into a clinical context.

A. Mixtures of an Active and Inert Isomer

This circumstance has been characterized by Ariëns as a mixture of active drug and "pharmacological ballast" (2). Propranolol is a prototype β-adrenoceptor blocking drug that is administered as a racemic mixture, however, one isomer, (−)-propranolol, is approximately 100-fold more potent than (+)-propranolol (3). Although this pharmacodynamic difference has been recognized for nearly 25 yr, there are little data to suggest this has limited the clinical usefulness of the drug. As a drug with a wide therapeutic index that is administered to a desired pharmacodynamic endpoint, the presence of a relatively inactive isomer has been of limited significance in therapeutics. However, this does confuse interpretation of findings when plasma drug concentrations are reported as the racemic mixture or pharmacokinetic studies of the racemic mixture are presented in the literature.

Another variant on this theme is ibuprofen, a prototype arylpropionic acid nonsteroidal antiinflammatory drug, that is administered as active (+)-ibuprofen and inactive (−)-ibuprofen (4). In this case, however, the inactive (−)-enantiomer undergoes chiral inversion to the active (+)-enantiomer, resulting in the pharmacological ballast becoming pharmacologically active in vivo.

For the clinical pharmacologist, neither of these racemic drug mixtures is problematic for drug therapy in the clinic if a pharmacodynamic endpoint (e.g., decrease in blood pressure with propranolol or improvement in arthritic pain with ibuprofen) is used to establish drug dose. However, to effectively characterize the pharmacokinetics of the active isomer, an endeavor that may be useful during drug development, administration, and/or specific determination of the active isomer is required. Such data

would subsequently be essential to develop relationships between the drug effect and plasma concentration of a specific isomer and/or racemic mixture.

B. Mixtures of Isomers with Different and Unique Pharmacodynamic Activity

An example of this circumstance is labetalol. This is a drug with two assymetric centers, therefore four isomers (5). One isomer (*R,R'*-labetalol) is a nonselective β-adrenoceptor blocker. A second isomer (*S,R*-labetalol) is an α_1-adrenoceptor blocker, whereas (*R,S*)- and (*S,S*)-labetalol have little pharmacological effect. Development of the mixture of four isomers has provided an effective antihypertensive drug with both α-1 and β-adrenoceptor blocking effects (6). One of the isomers (*R,R'*-labetalol) was extensively evaluated as a β-adrenoceptor antagonist devoid of α-adrenergic activity (7) and is no longer in clinical development. This isomer, dilevalol, was withdrawn from development due to hepatotoxicity that has been suggested to be isomer-specific (8). However, hepatotoxic reactions have now been described with the four-isomer mixture (9). Therefore, it is unlikely that the adrenergic pharmacodynamic specificity of the isomers extends to isomer-specific toxicity.

In contrast to labetalol, in which isomer-specific pharmacodynamic endpoints may be associated with expected adrenergic pharmacological activity, examples of isomer-specific toxicity raise a more pressing concern for racemic mixtures of drugs in development. Thalidomide has been suggested as such an example, however, this is probably not illustrative of the concept, as each individual isomer may be equally teratogenic (10). A much more clearly documented example is that of ketamine, an anesthetic used as a racemic mixture. An elegant study by White et al. (11) demonstrated that the more potent anesthetic (+)-ketamine was less associated with clinical toxicities, including psychic disturbance and agitation, than either the (−)-isomer or clinically used racemic mixture. A mechanism for this finding is uncertain, but it suggests that these toxicities are specific chiral (possibly receptor-mediated?) drug effects. If we consider the number of drugs used clinically as racemic mixtures, it appears that isomer-specific toxicity is an unusual occurrence. A requirement would be a stereoselective component of the events leading to the toxic event. Such an event could have a pharmacodynamic basis with no pharmacokinetic contribution as in the case of ketamine, or one could postulate a pharmacokinetic mechanism in which a toxic metabolite of one isomer only was formed due to stereoselective biotransformation by the appropriate drug-metabolizing enzyme system. Alternative possibilities would be stereoselective absorption of a toxic isomer or stereoselective renal excretion of a

nontoxic isomer. These hypothetical situations have not been demonstrated in clinical practice, although enantiomer-selective absorption clearly occurs with dihydroxyphenylalanine (dopa) (12) and methotrexate (13) and enantiomer-selective renal drug elimination has been demonstrated for the β-adrenoceptor antagonist, pindolol (14).

C. Mixtures of Isomers with Opposing Pharmacodynamic Activities

Dihydropyridine calcium antagonist drugs, with the exception of nifedipine that lacks an assymetric center, have been uniformly developed as racemic mixtures. Blockade of the potential operated calcium channel has been consistently associated with one enantiomer, with the other generally considered inert. Hof et al. demonstrated rather conclusively for one dihydropyridine, isopropyl 4-(2,1,3,-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridinecarboxylate, that the (−)-isomer inhibited calcium flux through the potential operated calcium channel, while the (+)-isomer enhanced calcium flux and these effects resulted in the inhibition and enhancement, respectively, of rabbit aortic ring contraction (15). In this case, when the racemic mixture was studied at low concentrations, aortic ring contraction was enhanced, whereas at higher concentrations, contraction was inhibited. Interpretation of the pharmacodynamic activity of the compound was only possible by study of the enantiomers separately, and obviously development of such a compound as a therapeutically useful drug would require development of the enantiomer with the desired pharmacodynamic effect.

An interesting variation on this theme is the rather conclusive demonstration that each of the enantiomers of a number of opiates including methadone, codeine, and morphine bind selectively to different opiate receptors in mouse brain, with high binding stereoselectivity by each enantiomer for the specific receptor (16). The possible clinical correlate is unique pharmacodynamic activity attributable to occupation of the respective receptors with *l*-pentazocine, a more potent analgesic drug, and *d*-pentazocine associated with dysphoric anxiety (17). Again, administration of the racemic mixture of the synthetic opiates, the drugs available for therapeutic use, leads to a complex summation of the pharmacodynamic effects of each of the enantiomers (18). Among the opiate and synthetic narcotics, characterization of the various central and peripheral receptors supports the likelihood that differing pharmacodynamics of each isomer are related to occupation of specific and different receptors by the enantiomers. Development of racemic mixtures of these drugs may provide an example in which therapeutic usefulness was impaired, and development of specific isomers that occupy specific opiate receptors would provide

drugs with a different pharmacodynamic effect or an improved therapeutic index.

D. Mixtures of Isomers with Similar Pharmacodynamic Effects, Although Often with Different Potency and/or Pharmacokinetic Characteristics

Coumarin-derivative anticoagulants provide an instructive example of isomers with similar pharmacodynamic effects. However, it is an example in which one isomer is much more potent [in the case of *S*- and *R*-warfarin an 8:1 potency ratio (19)] and the isomers have different pharmacokinetic characteristics (20). In clinical practice, when therapy is administered to a pharmacodynamic endpoint such as prolongation of plasma prothrombin time, the use of a pure isomer has no advantage over the racemic mixture. However, to understand drug–drug interactions involving warfarin, the use of pure isomers is critical. In the case of a recently reported diltiazem-warfarin pharmacokinetic interaction, although the clearance of racemic warfarin was markedly impaired, no pharmacodynamic effect was noted due to diltiazem-induced inhibition of the clearance of the relatively inactive *R*-warfarin (20). In contrast, in the pharmacokinetic interaction between phenylbutazone and warfarin, in which the clearance of racemic warfarin is similarly impaired, a striking pharmacodynamic effect was noted as a result of phenylbutazone-induced inhibition of *S*-warfarin (21).

Although warfarin and many other drugs that are extensively bound to plasma proteins exhibit stereoselectivity in this binding (19,22), the transient nature of stereoselective protein binding interactions in the absence of changes in drug clearance leads to few demonstrable clinical consequences when a racemic drug is administered in contrast to a pure isomer. However, the stereoselectivity of plasma protein binding may dictate the extent of tissue distribution of each isomer, as in the case of propranolol (23). Here, if the isomers displace each other from binding, one could envision the potency or tissue-selective toxicity of an active isomer in a racemic drug mixture to be different when the active isomer is administered alone. However, no examples of such a situation have been reported. A related example is the case of disopyramide, in which plasma protein binding of the isomers exhibits stereoselectivity, and binding of each isomer is concentration-dependent (nonlinear) (24). When administered separately, the *d* and *l* isomers of disopyramide had similar clearance, distribution, and elimination half-life. However, when administered as the racemic mixture, they had lower total clearance and a smaller apparent volume of distribution was noted for *l*-disopyramide. Due to the nonlinear protein binding of each isomer, with the increased free fraction of each at higher concentrations, one would anticipate that the difference in clearance

(and steady-state concentration during chronic dosing) would diminish at higher doses and concentrations of racemic disopyramide. This example is explained in some detail to outline potential pharmacokinetic complexity. However, clinically if a pharmacodynamic measure (such as arrhythmia suppression) is used to determine dose, the usefulness of a racemic drug is not significantly impaired. In contrast, during drug development a clear understanding of such pharmacokinetic complexity is important to establish dose interval and dose-concentration-effect relationships.

III. AREAS REQUIRING INVESTIGATION

A. Stereoselective Pharmacokinetics in Disease States

During drug development, pharmacokinetics in patients with liver disease, renal disease, and when appropriate cardiac disease are routinely studied. At the present time, little information is available for drugs administered as racemic mixtures to determine if the stereoselective disposition observed in healthy volunteers remains or changes in the presence of these states. In the absence of data, one can only speculate. However, these diseases do have marked effects on a number of drug disposition processes that exhibit marked stereoselectivity. With severe liver disease, it seems unlikely that each of the stereoselective oxidative processes will decline in parallel. At present no data are available to determine if racemic plasma drug concentrations after single or multiple drug doses are composed of the same ratios of drug isomers.

Similarly, in the case of renal disease, Giacomini and others have clearly demonstrated the stereoselectivity of renal drug excretory processes (14,27). Again with decreased renal function, including glomerular filtration, proximal reabsorption, and secretion, no information is available to determine whether the stereoselective processes decline in parallel with nonstereoselective mechanisms, such that after the administration of a racemic drug, the isomeric ratio in plasma remains the same.

Finally, absorption and first-pass hepatic extraction of racemic mixtures of high-clearance drugs have been demonstrated to have marked stereoselectivity when studied in healthy volunteers. Examples are verapamil (28) and tertbutaline (29). In the case of verapamil, the pharmacologically more active isomer, *l*-verapamil, undergoes much more extensive first-pass hepatic extraction after oral administration. Thus, when racemic-mixture plasma verapamil concentrations are measured, a markedly less pharmacodynamic effect at a given plasma drug concentration is observed after oral drug administration. For tertbutaline, in which (−)-tertbutaline

is the pharmacologically active isomer, the oral bioavailability of (−)-tertbutaline was reported to be 14.8% and that of (+)-tertbutaline 7.5%. Since tertbutaline is predominantly excreted unchanged (55%) in the urine, the authors interpreted this stereoselectivity in bioavailability to be predominantly at the level of drug absorption. In the case of patients with liver disease, in which first-pass hepatic extraction is diminished (30), there are no data available to predict whether or not the stereoselectivity of first-pass extraction of drugs remains constant as the extraction ratio is diminished due to disease. Congestive heart failure in patients has been demonstrated to alter both first-pass hepatic drug extraction and the apparent volume of distribution of selected drugs such as lidocaine (31). However, again there are no data to allow the prediction of stereoselectivity of either hepatic extraction or drug distribution when racemic mixtures of drugs are administered to patients with congestive heart failure.

B. Stereoselective Pharmacokinetics in Aging

It is now generally accepted that drug pharmacokinetics are altered in aging humans (32). Preliminary reports indicate that otherwise healthy aging individuals experience altered stereoselective disposition of the barbiturate derivatives hexobarbital (33) and mephobarbital (34). For mephobarbital, *R*-mephobarbital had much higher clearance than *S*-mephobarbital in young subjects, whereas in the elderly, the clearance of *R*-mephobarbital was decreased and *S*-mephobarbital clearance was not age-related. Such findings support the likelihood that the stereoselective drug disposition for other racemic drugs may change with age as well.

C. Stereoselective Pharmacodynamics in Disease States and Aging

Levy and others (35,36) have developed an extensive literature which demonstrates in animal models of disease that the pharmacodynamics of a number of drugs are altered, even after controlling for pharmacokinetic changes. Studies with drugs administered as racemic mixtures in humans with renal dysfunction, hepatic dysfunction, and other disease states that address the issue of stereoselective pharmacodynamics are lacking. However, the cited animal studies suggest that such a line of investigation would be fruitful.

IV. RACEMIC DRUGS AND THERAPEUTIC DRUG MONITORING

The development of consistent and reproducible relationships between plasma drug concentration and therapeutic and/or toxic drug effect across

patient populations is an important requirement before considering the use of plasma drug concentrations to guide pharmacotherapy. This approach is considered less desirable than the use of clinical and/or pharmacodynamic endpoints to establish drug dose and the presence of therapeutic effects or toxicity if these parameters are clinically obvious, predictable, or measurable by a simple laboratory test (e.g. plasma prothrombin time). However, to properly use drugs with a low therapeutic index, for example, digoxin, theophylline, phenytoin, phenobarbital, aminoglycosides, and a number of antiarrhythmic drugs, plasma drug concentration monitoring is essential to properly treat patients. Fortunately for the clinician, the antiarrhythmic agents are the only major group of drugs administered in most instances as racemic mixtures and for which plasma drug concentration monitoring is routinely used to guide therapy. As has been suggested by Reidenberg (37) and others for these selected few occasions, the availability of either concentrations of the active isomer, or preferably administration of the active isomer only, is necessary for optimal therapeutic drug monitoring. In contrast, for most drugs administered as racemic mixtures, such as warfarin, calcium antagonists, β-adrenoceptor blockers, and tricyclic antidepressants, therapy is most appropriately guided by the use of pharmacodynamic and clinical endpoints. Therefore, to suggest that the use of racemic drugs markedly impairs the usefulness of therapeutic drug monitoring is not justified.

V. CLINICAL RESEARCH IN DRUG DEVELOPMENT

Much has been written regarding a proper approach to the development of racemic mixtures of drugs, and whether such drugs are even candidates for development (1,8,22,37–42). As clinical pharmacologists and medical practitioners, we shall advance a point of view that represents a compromise among the various camps, but one that we believe can effectively serve medical therapeutics now and in the future. The previous material in this paper represents a summary, not a comprehensive review, of the database from which attitudes, opinions, and therapeutic approaches can be formulated.

As has been observed on repeated occasions, many drugs in therapeutic use are administered as racemic mixtures. The therapeutic efficacy of these drugs has generally been demonstrated and the clinical practitioner is familiar with their use. Particularly for drugs in this group with a wide therapeutic index such as the β-adrenoceptor blockers, effective therapeutics would only be hampered by taking issue simply because they are administered as racemic mixtures. Therefore, we adopt the view that such drugs should be "grandfathered" and fully accepted as racemic mixtures.

That is not to say continued research with pure isomers of such drugs should be hampered. Instead, many important pharmacological and physiological questions can be explored using such pure isomers. In addition, such investigations may enhance the therapeutic usefulness of the drug racemic mixture.

How best to serve therapeutics for racemic mixtures of drugs in development or being considered for clinical development? It seems a desirable objective to characterize the pharmacokinetics and pharmacodynamics of pure isomers of drug candidates and to select a pure isomer on the basis of these studies for further development. This tactic would prevent the various difficulties as summarized earlier in this chapter when attempts are made to characterize the disposition, efficacy, and toxicity of drug candidates. The sponsor of a drug candidate should have the opportunity to request an exception from such a policy, and a number of situations can be envisioned in which such a request should be viewed favorably. They are the following:

1. Development of a pure isomer (in contrast to a racemic mixture) would markedly delay the development of a drug that may represent a breakthrough in the treatment of a life-threatening disease. The drug sponsor would then be expected to develop pharmacokinetic and pharmacodynamic data on both the racemic mixture and the individual isomers concurrent with the regulatory evaluation and approval process.
2. Synthesis and/or resolution of isomers in commercial quantities for a drug candidate viewed as a significant advance for a therapeutic indication is prohibitively expensive, thereby in fact preventing development of the putative therapeutic advance.

In contrast, the more common situation is the proposed development of the racemic mixture of a drug that is an extension of a chemical or therapeutic class of drugs. Examples are β-adrenoceptor blockers, calcium antagonists, and nonsteroidal antiinflammatory drugs. The development of such drugs has been recognized as important, as often changes in pharmacokinetic properties may be therapeutically advantageous, the therapeutic index may be improved, and on occasion individual patients will have an improved therapeutic response to one member of a drug class. Such drug candidates, however, do not generally lead to therapeutic breakthroughs in the treatment of disease. Therefore, we believe in such cases drug sponsors should be required to develop an isomerically pure drug.

The future is bright for the development of single stereoisomers of drugs for two reasons. First, the present situation in which large numbers

of drug racemic mixtures are in therapeutic use simply represents a lack of synchrony in scientific progress. The synthesis of complex organic molecules with both asymmetry and potent pharmacological activity moved ahead of chiral synthesis. At the present time, important advances in chiral synthesis (43) suggest that the synthesis of pure isomers of drug candidates is rapidly coming abreast of the identification of drug targets for which a candidate may be effective. Second, the rapid development of drugs that are prepared by biological systems synthesizing only one isomer of a complex molecule, and in commercial quantities, obviates the racemic mixture issue for an increasingly large number of drug candidates.

VI. CONCLUSION

At the present time, the clinical pharmacologist and practitioner utilize a number of racemic mixtures of drugs to optimally provide pharmacotherapy to patients. Unfortunately, this is in effect administering drug mixtures, each component of which may have unique pharmacokinetics and pharmacodynamics. The efficacy of these drug mixtures has generally been established, and acceptance of their continued use seems the appropriate stance. In contrast, chiral drug candidates either in development or being considered for such should generally be developed only as pure isomers.

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16

DEVELOPMENT OF STEREOISOMERIC DRUGS

An Industrial Perspective

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I. INTRODUCTION

In previous chapters, analytical, biological, and other scientific aspects of drug stereochemistry were described. The purpose of this chapter is to discuss pharmaceutical drug development for those drugs containing a stereogenic center. It is currently estimated that it takes approximately 12 yr to bring a new chemical entity (NCE) to regulatory clearance in the United States, at a cost (including discovery and development) averaging $231 million (1). With such a major resource commitment by the pharmaceutical industry to research and development, it is critical that NCEs be subjected to a rational development program to appropriately evaluate their safety and efficacy. The development of drugs with chiral centers presents specific challenges that must be addressed at various stages from discovery through clinical evaluation and finally to market.

II. DRUG DEVELOPMENT PROGRAM

A typical drug development program is outlined in Fig. 1. NCEs with a chiral center are typically synthesized and initially tested for desired pharmacological activity in animal models and/or in vitro screens as a racemic mixture. Should no pharmacological activity be demonstrated, the NCE is no longer considered a viable candidate drug. However, should some activity emerge, the first decision related to the NCE's chirality is required. Subsequent biological tests are usually more specific regarding drug activity and often require more material for evaluation. What should

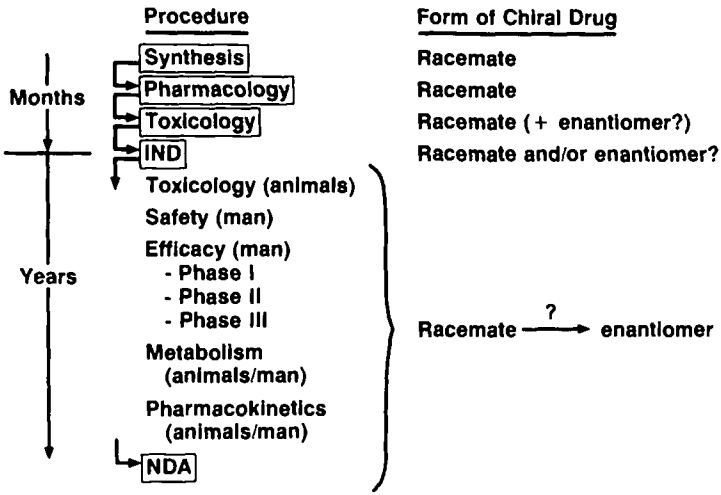

FIGURE 1 Typical drug development scheme (simplified).

be tested in these followup measurements: the racemic mixture or each of the component enantiomers? Depending on the chemistry of what is now a drug candidate, the synthesis/resolution of the individual enantiomers may be somewhat difficult and thus time-consuming, thereby delaying further testing. Since time is a critical resource in drug development and, at this stage, sufficient data are required for a decision on whether or not toxicity studies should be initiated, delays in synthesis of sufficient amounts of the pure enantiomers may be contraindicated. After all, should the racemate not be sufficiently active in the subsequent biological tests, the candidate drug would not be further evaluated. The earlier such information is available, the more efficient the overall process. However, should the drug product still demonstrate desirable activity, the next step would be initiation of the safety evaluation studies. Again, a critical decision is required. What should be tested? It would certainly be desirable to know whether the desired pharmacological activity resides in a single enantiomer (which is more common), or whether both enantiomers are required (rare). Such information may indeed be available as a result of synthesis of sufficient amounts of enantiomers on a laboratory scale. However, should activity be due to a single enantiomer, such small-scale synthesis may not be adequate for the large amounts required to initiate toxicological evaluations. Numerous techniques are emerging for the large-scale preparation of single enantiomers (2) and the fact that such entities need to be rapidly available for appropriate testing has provided

the stimulus for the explosive development of efficient synthetic techniques over the past few years. Ideally, only the pharmacologically active moiety should be tested in the toxicity studies, so that subsequent evaluation of the data is not subject to potentially complex interpretations.

The IND (investigational new drug) application is submitted to regulatory agencies upon completion of sufficient animal safety and efficacy studies to support initiation of clinical trials. Should the sponsor decide to perform the early clinical studies with the racemic mixture, such a decision would have to be appropriately justified to the respective regulatory authority. The justification could include life-threatening indication, novel therapeutic approach, or difficulty in large-scale synthesis of the single enantiomer. Occasionally, the drug company may wish to obtain early positive clinical efficacy data before committing resources to follow up with the active enantiomer. It is appreciated that the later the decision is delayed as to whether to develop the racemate or a single enantiomer, the greater the "gamble" the sponsor is making insofar that tests made with the racemate may have to be repeated with the single stereoisomer.

III. CHIRAL DRUG DEVELOPMENT: TECHNICAL REQUIREMENTS

When Professor Ariens shook up the scientific community in 1984 by using such terms as "isomeric ballast" and "sophisticated nonsense in pharmacokinetics and clinical pharmacology" to describe the neglect of stereochemical factors for chiral compounds (3), this stimulated an explosive revival in the state of the science. The long-known basic scientific principles began to be applied to drug development, and today the pharmaceutical industry is very cognizant of the relevant issues (4–6), some of which are listed in Table 1.

Should the decision be made to proceed with a racemic mixture, the drug sponsor in today's climate must vastly expand its development

TABLE 1 Development of Racemic Mixtures or Single Enantiomers: Technical Requirements

—Development of enantiomeric assay
—Synthesis/resolution of individual enantiomers
—Safety evaluation of individual enantiomers
—Pharmacokinetics of individual enantiomers
—Bulk drug: enantiomeric composition/purity
—Chiral inversion

program. Since it is well known that enantioselective metabolism and pharmacokinetics are more the rule than the exception (7–8) and that such selectivity can have profound toxicological (9–10) and therapeutic (11–12) implications, it is critical that a sufficiently sensitive and specific stereoselective assay be developed to measure the concentration of individual enantiomers in biological fluids. As discussed in this and other texts (13), the technology in this area has rapidly exploded in recent years such that, in most instances, the development and validation of a chiral assay today should not be substantially more difficult than that of a "conventional" assay. As with any analytical technique, the earlier the chiral assay is developed, the sooner an evaluation can be made as to the "exposure," pharmacokinetics, and metabolism of the individual enantiomers after racemate administration to laboratory animals and man.

If a sponsor proceeds to develop a racemate, it is necessary to study not only the pharmacological activity, but also the relative safety of the individual enantiomers. Such information is critical in the overall decision scheme as to whether a racemate or active enantiomer should ultimately be marketed (14). Bulk material is required for such testing (15), and chemical development groups within the industry work rapidly to introduce synthetic and/or resolution procedures so that sufficient amounts of individual enantiomers are available for testing. Delays in the availability of such material naturally result in delays in the overall program.

Pharmaceutical development and purification of a bulk drug involve the establishment of specifications for purity of the material within the dosage form. A racemate is normally a 50:50 mixture of the component enantiomers. However, during the development of a single enantiomer, specifications need to be determined on the maximum amount of allowable impurities, which would include other stereoisomers. Thus, the enantiomeric purity of the bulk drug as well as a drug within the dosage form needs to be established. Depending on the chemistry of the drug and on the dosage form vehicle, the absence of chiral inversion may need to be evaluated. Likewise, although metabolic chiral inversion is known to occur with nonsteroidal antiinflammatory 2-arylpropionic acid drugs (16), it probably does not occur with other compounds. It is, therefore, debatable whether metabolic chiral inversion should be evaluated for all chiral drugs (whether developed as an enantiomer or racemate); this should be addressed on a case-by-case basis using common-sense science. Indeed, if a single enantiomer is being developed and the chemistry of the NCE is such that inversion is most unlikely, it is questionable as to whether a chiral assay needs be developed and validated only to assess metabolic chiral inversion in the toxicity species and man.

In summary, the technical requirements in the development of drugs

with a chiral center comprise the cost-effective and rapid availability of appropriate analytical techniques and bulk drug, as is the case with conventional NCEs. However, the pharmaceutical industry is well aware of the specific problems and resources involved when chirality becomes a factor.

IV. CHIRAL DRUG DEVELOPMENT: REGULATORY ASPECTS

Drug development can be (or at least should be) viewed as a team effort between scientists in sponsoring companies and appropriate officials at regulatory agencies. In the field of chiral drug submissions, the attitudes of regulatory bodies have more or less been a reflection of the state of the science. Simply put, prior to the 1980s, the issue of chirality was virtually ignored both as part of drug development within the pharmaceutical industry and by regulatory agencies (17). Currently, there is a flurry of activity among regulatory authorities worldwide to address the issue of chiral drug submission and registration.

In anticipation of such guidelines from the Center of Drug Evaluation and Research (CDER) within the U.S. FDA, the U.S. Pharmaceutical Manufacturers Association (PMA) issued in 1989 a position paper entitled, "Comments on Enantiomerism in the Drug Development Process" (18). The authors discussed characteristics of racemates and enantiomers as they relate to drug development, concluding that the choice of a racemate or single enantiomer should be made on a case-by-case basis and not "mandated by regulation." At this writing, the CDER's Stereoisomer Policy Committee has prepared a draft policy statement that is undergoing review within the agency. The statement indeed does not mandate what should be developed. It covers the chemical (analytical control methods, nomenclature, manufacture of bulk drug, manufacture of finished product) and biological (analytical assay, pharmacokinetics, metabolism, pharmacodynamics) factors that need to be described and justified if the decision is made to develop a racemic mixture. Current published papers by FDA representatives on the chemical (19) and biological (17) considerations for stereoisomers are basically discussions on points to consider and possibly reflect the FDA thinking that the forthcoming policy statement will not be a checklist guideline.

In Japan, the Pharmaceutical Affairs Bureau (PAB) within the Ministry of Health and Welfare (MOHW) (see Ref. 20 for a description of its organization and functions) has also not yet issued formal guidelines on the approval of racemic drugs. Indeed, no formal guidelines may be prepared on this issue. As recently discussed by Shindo and Caldwell (21),

the 1989 Japanese requirements for drug manufacturing approval (22) contain two relevant sections on "points to consider when preparing data." Recommendations contained therein as well as others require that when "the drug concerned is a racemate, it is recommended to investigate the absorption, distribution, metabolism and excretion of each optical isomer." In keeping with Japanese principles for drug submissions in general, this would require greater focus on nonclinical radiolabeled ADME studies than would be desired in other countries. Additionally, although there are no binding regulations on the marketing of chiral drugs in Japan, and applications are stated to be considered on a case-by-case basis, Japanese expectations are such that substantial efficacy, pharmacology, toxicology, and pharmacokinetic studies would be recommended for each isomer, as well as the possible occurrence of in vivo inversion.

As with other countries, there are no detailed guidelines on racemic drug registration within the European Economic Community (EEC). In the 1989 edition of the Committee for Proprietary Medicinal Products (CPMP) Notice to Applicants, the topic is briefly discussed in the section dealing with the preparation of expert reports (23). Relevant preclinical and clinical information to be discussed in the expert report should include toxicological issues, pharmacological aspects (pharmacodynamic activity of each stereoisomer), pharmacokinetics (including information on the relative metabolism of each isomer), extrapolation of the preclinical data (species differences in enantiomer exposure), and relevant clinical issues. It is also stated that "where a mixture of stereoisomers has previously been marketed, and it is now proposed to market a product containing only one isomer, *full data* on this isomer should be provided."

Outside the EEC, the Swiss regulatory agency (Interkantonale Kontrollstelle fur Heilmittel) has issued a brief statement on its approach to evaluating submissions on chiral drugs. The Swiss state that, for a mixture of isomers, data for each component should be available on the respective pharmacodynamics, pharmacokinetics, toxicity, and metabolism. Pharmacokinetic data, based on nonspecific analytical methods, will be assessed very critically. For pure isomers, other isomers, if present, are regarded as impurities. A new application for a pure isomer of a substance that is already registered as a racemate will be treated as an NCE application.

In Sweden, the Medical Products Agency (MPA) recently (Oct. 1990) issued its own discussion entitled, "Some Views from the Medical Products Agency on the Documentation for Chiral Durgs" (24). This request for discussion and comment covers four scenarios: (1) new enantiomer (evaluate enantiomeric purity using a chiral assay; also, address in vivo inversion; if present, contralateral stereoisomer should be regarded as a metabolite);

(2) new mixture of stereoisomers (standard biological differences and/or potential interactions between component stereoisomers); (3) enantiomer of marketed mixture of stereoisomers (if distomer active, document eutomer as NCE; if distomer inactive, eutomer submitted as generic application and all claimed advantages should be verified); and (4) generic application of marketed mixture (appropriate bioequivalence studies for *both* enantiomers).

It can be seen that the current positions of the different regulatory agencies are not comprehensive and, in some instances, not particularly clear. This can create problems for both sponsoring companies (who generally register drugs internationally and must, therefore, develop programs to satisfy all major regulatory agencies), as well as reviewers within agencies who must evaluate submitted data on chiral drugs. It would appear that the Japanese are not working on formal guidelines, and that the FDA has decided to issue a policy statement rather than an actual guideline. The CPMP is the only major regulatory body working on formal guidelines for chiral drugs. Current work on international harmonization is focusing on drug safety evaluation studies, and there has been no initiative to harmonize chiral drug submissions among the major regulatory bodies. The challenge rests with the drug sponsors to be cognizant of the regulatory atmosphere and expectations in the absence of formal guidelines.

V. INDUSTRIAL ISSUES IN DRUG DEVELOPMENT

Although this chapter focuses on chirality in drug development, the racemate vs. enantiomer "debate" also impacts on racemic drugs already in therapeutic use. Lennard (25) has recently argued that, for many marketed racemic drugs, such as the β-adrenoceptor antagonists, available evidence indicates that the therapeutically inactive enantiomer is not harmful, and that the patient would not benefit from receiving the pure active enantiomer. On the other hand, there is no doubt about the compelling reasons for chiral drugs that are used as single enantiomers, such as dopa [the (−)-form is less toxic], methotrexate [the (−)-form is more active], propoxyphene [the (+)-form is the analgesic, and the (−)-form an antitussive], and pentazocine [the (−)-form is more analgesic, the (+)-form causes a greater degree of anxiety].

From the drug development perspective, to state dogmatically that all chiral drugs should be developed as single enantiomers is simplistic and unrealistic. There is no doubt that there are strong compelling reasons to develop single enantiomers (5,14,26), as listed in Table 2. The principal reason is that, in most instances, the desired therapeutic activity resides

TABLE 2 Reasons to *Develop a Single* Enantiomer

1. Therapeutic activity resides principally in one enantiomer
2. Low therapeutic index
3. Toxicity associated with distomer
4. No chiral inversion
5. Economic feasibility

principally in a single isomer and, therefore, there is no clinical advantage to proceed with the racemate. This becomes even more acute if the active enantiomer exhibits a low therapeutic index or there is clinically significant toxicity associated with the distomer. The absence of chiral inversion assures enantiomeric purity within the dosage form and of the administered drug within the body. Finally, the bulk production of the single enantiomer both for testing and marketing should be sufficiently economically feasible so as to support reasonable cost structure to the patient.

Although the list of reasons to justify development of a racemate is numerically longer than that for the enantiomer (Table 3), the justifications for such a decision need to be convincing. In those relatively rare instances when there is additive or synergistic activities with both isomers, then the racemate is potentially the better drug, provided there is no enantioselective toxicity. If there is a high therapeutic ratio, this could support racemate development. The principal safety concern is the liability of the distomer insofar that the less active enantiomer should not impart significant toxicity to the racemate. Should chiral inversion occur to give rise to a fixed ratio of the two enantiomers and depending on the route/reversibility of the inversion, this could support a decision to proceed with a racemate.

TABLE 3 Justification for the Development of a Racemate

1. Additive or synergistic therapeutic activities of both enantiomers
2. High therapeutic index
3. Negligible/low toxicity of the distomer
4. Chiral inversion yields fixed ratio of enantiomers
5. Physicochemical characteristics
6. Novelty of drug
7. Indication for life-threatening disease
8. Cost-ineffective to manufacture active enantiomer on a large scale

Occasionally, the physicochemical characteristics (which determine dissolution and solubility characteristics) of the racemate may impart higher and/or less variable bioavailability than after administration of the single enantiomer; solubility is also a determining factor for intravenous or other routes of administration. If racemate development is perceived to be more rapid than that of the eutomer, then the novelty of the drug product or indication for life-threatening disease may be attempted as a justification for marketing the racemate. Cost-ineffective bulk synthesis has also been listed; however, although this could be proposed as an "excuse" a decade ago because the technology to manufacture bulk drug economically had not been extensively developed, such is rarely the case today. Process chemists have made significant advances in recent years to improve the technology such that cost-ineffective large-scale production would now need to be convincingly justified.

VI. RACEMATE OR ENANTIOMER: FACTORS TO CONSIDER DURING DRUG DEVELOPMENT

When a chiral compound synthesized as a racemate is found to have desirable pharmacological activity in initial tests, the decision-making process is begun as to whether the development should continue as a racemate or single enantiomer. Some issues to be addressed in this process are listed in Table 4. Since it is the rule rather than the exception that enantiomers exhibit different responses in the chiral environment of biological systems, such pharmacokinetic and pharmacodynamic differences should be assessed as early as possible. Often, delays can occur in such evaluations due to the unavailability of sufficient quantities for testing. Pharmacokinetic differences are assessed using a stereospecific assay, the development and validation of which may be time-consuming.

Should an early decision be made to develop the eutomer, then the drug development program would be the same as for conventional NCEs, with the possible exception that assessment of in vitro and/or in vivo chiral inversion may be desirable. However, if development continues with the racemate, time, cost, and staff resource commitments become magnified. For example, a very important variable to consider is species differences in enantiomer exposure. Appropriate toxicokinetic studies are advisable in order to assure that, at toxicological doses, the animal species tested have attained sufficient plasma concentrations of *each* enantiomer to support clinical evaluation at therapeutic doses in humans. The enantiomeric ratio (based on maximum drug concentrations [Cmax] and/or area under the plasma drug concentration-time curve [AUC]) should be evaluated, and

TABLE 4 Racemate or Enantiomer: Factors to Consider During Drug Development

1. Similarity/differences between enantiomers
   Pharmacodynamics (pharmacology and toxicity)
   Metabolic disposition
   Pharmacokinetics
2. Biological factors
   Chiral inversion
   Species differences in exposure to enantiomers
   Therapeutic index of the eutomer
   Liability of the distomer
3. Technical factors
   Development of stereospecific assay
   Timing: how much information available for IND?
   Physicochemical properties
   Difficulty/expense of bulk production
4. Therapeutic need/novelty
5. How is the patient best served?

any differences observed between animals and humans should be addressed in the overall safety evaluation of the drug.

The therapeutic index of the eutomer will also impact on the decision. Because the distomer provides no therapeutic advantage to the patient, the toxicity/liability/side-effects of this stereoisomer need to be evaluated. It is, therefore, probably wise to conduct at least some safety evaluation study(ies) with the distomer if the racemate is being considered for development.

Technical and other practical factors also merit consideration. Are there indeed technical difficulties in the manufacture of a bulk drug that would render marketing of the eutomer cost-prohibitive? Accumulation of relevant data for each stereoisomer is a time-consuming process. Are these delays justifiable therapeutically? When an IND is submitted for a racemate, there may be limited or negligible safety and/or pharmacokinetic data on the individual enantiomers. Should this delay initiation of phase I clinical trials?

The issue centers around how the patient is best served. The goal of both drug sponsors and drug regulators is to develop safe and effective drugs. The practical consideration with the high costs involved is the optimum utilization of time and cost resources. Each drug is an individual entity, and the decision must be based on the respective characteristics of the drug in question.

VII. CONCLUDING COMMENTS: WHERE DO WE STAND?

The enantiomer vs. racemate issue is still being termed a "debate." It is the author's view that a debate no longer exists. The science is well recognized. The chemical and analytical technology has seen dramatic advances in the past decade. Basic scientists, pharmaceutical developers, and regulatory authorities are well aware of the issues. Development of a single enantiomer is wise and prudent. Patients would not be exposed to a drug mixture. Pharmaceutical companies are very much aware that, in the 1990s, racemate development will be cost-ineffective and time-consuming. Regulatory agencies are asking the right questions and taking a very hard look at racemate NCE's; the justification must be convincing. Therefore, why ask for trouble?

It is relatively simple dogma to state that only single enantiomers should be candidates for future drugs. As discussed, there are and will be scenarios whereby the marketing of a racemic mixture will better serve the patient. The right questions need to be asked, the appropriate well-designed studies should be conducted and interpreted, and a balanced view needs to be taken. It is apparent that few new drugs will be marketed as racemates. However, our responsibility is to use good science and common sense in the decision-making process, and to keep in mind that dogma and prudence may not result in the best attainable therapeutic agent.

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