HORMONAL STEROIDS

Biochemistry, Pharmacology, and Therapeutics

PROCEEDINGS OF THE FIRST INTERNATIONAL CONGRESS ON HORMONAL STEROIDS

Edited by

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VOLUME 1

1964

ACADEMIC PRESS · NEW YORK AND LONDON

ACADEMIC PRESS INC.
111 FIFTH AVENUE
NEW YORK, NEW YORK, 10003

United Kingdom Edition Published by
ACADEMIC PRESS INC. (LONDON) LTD
BERKELEY SQUARE HOUSE
LONDON, W. 1.

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Library of Congress Catalog Card Number: 64-23201

Printed in Hungary

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Foreword

It is over thirty years since the isolation and chemical identification of the first hormonal steroid (estrone). There followed in the 1930's a brilliant series of investigations which led to the indentification of most of the major steroids secreted by the ovaries, the testes, the adrenal cortex, and the placenta. Since those days, when these steroids were available at best in milligram amounts, there has been an exponential increase in the amounts of steroid hormones preparable by partial synthesis and in the investigation of their pharmacological, physiological, and biochemical properties. Keeping pace with, and, indeed, often anteceding our knowledge of the steroid hormones, has been the production of derivatives, analogues, and homologues of the hormones. In order to encompass the rich chemical, biochemical, and medical knowledge in the steroid hormone field, the inclusion of work with these steroid relatives led to the "International Congress on Hormonal Steroids." Our knowledge of the regulatory activity of the steroid hormones at the organ, tissue, and cellular levels has been aided and enlarged by studies with the synthetics. Their contribution to the sharpened insight is obvious in numerous papers presented to this Congress, and included in this volume.

As we review the subject matter presented in the symposia and round-table conferences, the major areas of research with steroids are sharply illuminated. These major areas may be listed as:

(1) Steroid chemistry, ranging from the natural product isolation to organic syntheses and including studies of reaction mechanisms.

(2) The biogenesis and metabolic transformations of the naturally occurring hormones.

(3) The secretion, transport, conjugation, and excretion of the steroid hormones and of synthetic chemical relatives.

(4) The control of steroid hormone secretion by endogenous humoral and neurohumoral agents and by diverse exogenous agents.

(5) The relationship between chemical structure and physiological function of hormonal steroids of all types.

(6) The regulatory effects of hormonal steroids on specific target tissues.

(7) The repercussive, secondary or indirect actions of the hormonal steroids upon diverse processes.

(8) The role of hormonal steroids in endocrine and neuroendocrine pathology, including steroid therapy.

(9) Hormonal adjustments to physical and psychological stresses and to the aging process.

Among the papers presented to the Congress are accounts of data extremely pertinent to the foregoing topics. These papers illustrate the enormous amount of research activity devoted to the steroids and their biological and medical involvements. Indeed, they confirm the impression that there is scarcely a vital process, an aspect of behavior or personality, or organic pathological lesion which is not in one way or another affected by steroids. From the crowns of our heads to the tips of our toes, the steroid flux is incessantly at work. In this volume, the reader will find able presentations by experienced and sagacious investigators of the what, how, and why of this multivarious steroid activity.

We wish to thank all the people who have contributed to the organization of the Congress and to the publication of these volumes and made them a successful reality: Drs. F. Fraschini, G. Gavazzi, G. Giuliani, G. Mangili, M. Motta, E. Müller, L. Martini, and A. Pecile.

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Inverted Steroids, Their Preparation and Biological Activities

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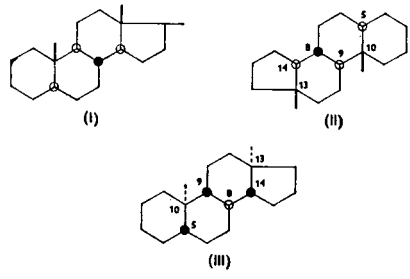
The preparation of a number of new steroid derivatives, which we have called "inverted steroids," has already been reported in a series of publications. In the present paper, we describe some new compounds of the same type and we shall try to draw a picture of the biological activities of these "inverted steroids."

By "inverted steroids" we mean "optical antipodes of modified steroids" (Kagan, 1960): if one draws the structural formula of (5α)-androstane (I) and rotates it 180 degrees in the plane of the paper (II), it is apparent that the configuration of carbon atoms 14, 13, 8, 9, 10, and 5 in (II) is identical to that of carbon atoms 5, 10, 9, 8, 13, and 14 of *enantio*-(5α)-androstane (III). On this basis, we have prepared the "inverted isomers" of several steroid models, the biological activities of which are well known, by introducing at position 3 the structural components normally present at position 17.



1. Several derivatives of (5α)-androstane with hydroxyethynyl group in position 3 were prepared from the corresponding ketones. The stereochemistry of these compounds has been established (Marquet *et al.*, 1960). The same reactions were also carried out on compounds with an oxygenated function at position 7 (corresponding to position 11 in the normal steroid structure) (Kagan and Jacques, 1960).

2. The above-mentioned ethynyl carbinols were transformed by

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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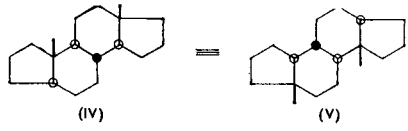
Rupe's reaction into derivatives of 3-acetylandrost-2-ene and then by hydrogenation into derivatives of 3β-acetylandrostane (Dvolaitzky *et al.*, 1961). After conversion of the ketone function into the dioxolane, the end of the chain was brominated (by phenyltrimethylammonium tribromide in tetrahydrofurane), and the compounds obtained were transformed by acetoxylation into substances possessing a chain of the deoxycorticosterone type at position 3.

3. The same ethynyl carbinols were also hydrated by mercuric acetate or mercuric chloride in piperidine. In the former reaction, ketol acetates are obtained, whereas the latter leads to the formation of ketols (Kagan *et al.*, 1960). These reactions were carried out on androstane derivatives with an oxygen function at position 17.

All the above compounds belong to the (5α)-androstane series. In order to imitate more closely the *enantio*-(5α)-androstane (III) skeleton, we have undertaken the preparation of some derivatives of A-nor-(5α)-androstane (IV).

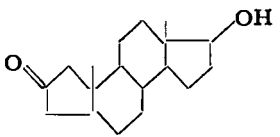


For reasons of convenience, we first prepared the 2-ethynyl and 2-chlorethynyl carbinols; these are easily obtained from the known 2-oxo-17β-hydroxy-A-nor-(5α)-androstane (V) (Marker *et al.*, 1937).

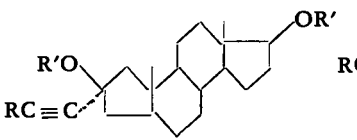
The reaction of acetylene with this compound in the presence of potassium *tert*-amylate affords an excellent yield of 2β,17β-dihydroxy-2α-ethynyl-A-nor-(5α)-androstane (VIa) (m.p. = 180–182°C, $[\alpha]_D^{19}$ + +23° ± 4°,[1] and a small quantity of the 2-epimer (VIIa) (m.p. = 210°, $[\alpha]_D^{20}$ —4° ± 2°). We have also prepared the corresponding diesters: diacetates (VIb) (m.p. = 219–220°, $[\alpha]_D^{20}$ + 14° ± 2°) and (VIIb) (m.p. = 172–175°, $[\alpha]_D^{22}$ —8° ± 1°); dipropionate (VIc) (m.p. = 159–160°, $[\alpha]_D^{20}$ + 11.5° ± 1.5°) and dibenzoate (VId) (m.p. = 194–195°, $[\alpha]_D^{26}$ + + 26.5 ± 1).

By oxidation with CrO_3-pyridine complex, we obtained, from (VIa) and (VIIa), the corresponding 17-ketones: (VIII) (m.p. = 208–210°, $[\alpha]_D^{20}$ + 98° ± 1°) and (IX) (m.p. = 236–238°, $[\alpha]_D^{20}$ + 58° ± 1.5°). The chlorethynylation of ketone (V), under the conditions described by Viehe (1959), yields two isomers: (X) (m.p. = 168°, $[\alpha]_D$ + 36° ± 2°) and (XI) (m.p. = 228°, $[\alpha]_D$ —23°), the latter being far less abundant. Catalytic hydrogenation of both (VIa) and (X) (Pd/C) affords 2β,17β-dihydroxy-2α-ethyl-A-nor-(5α)-androstane (XII); this result shows that, in both cases, the more abundant product has the same configuration.

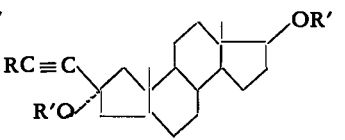
[1]All the optical rotational measurements were made on 1% solutions in dioxane. The compounds mentioned gave satisfactory analyses.



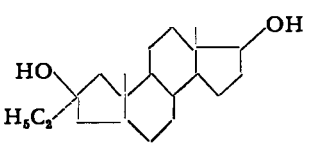
V



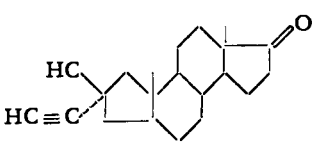
VIa R=R=H
VIb R=H, R=Ac
VIc R=H, R=Pr
VId R=H, R=Bz
X R=Cl, R=H



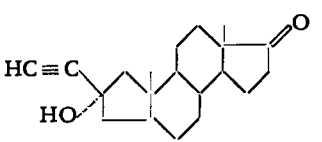
VIIa R=R=H
VIIb R=H, R=Ac
XI R=Cl, R=H



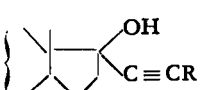
XII



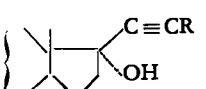
VIII



IX



XIII R=H, C_6H_5 or Cl



XIV R=H or C_6H_5

It has not yet been possible to bring a final proof of the stereochemistry of these different compounds; a nucleophilic attack of the carbonyl group at position 2 from the α face seems highly probable according to what we know of the reduction of an A-nor ketone of this type (V) (Dauben *et al.*, 1961) with sodium borohydride.

We have previously observed that 17-acetylenic carbinols (partial formula XIII) exhibit negative plain rotatory dispersion (RD) curves; the curves of the corresponding epimers (XIV) are positive and quasi enantiomeric to those of the above compounds (Mamlok *et al.*, 1961). The RD curves of the 17-epimeric benzoates (17α negative; 17β positive) are also quasi enantiomeric (Jones and Klyne, 1960). It is to be noted that in this particular case, the sign of the RD curves is the same for compounds where the chromophore (benzoate, —C≡C— group) has the same configuration. We have compared the curves of the A-nor-2-ethynyl and- 2-chlorethynyl compounds with those of the epimeric benzoates in the same position (and of known configuration). Here again the curves are of opposite sign for each pair of epimers: the 2α-benzoate exhibits a positive curve, as do the compounds (VI) and (X) (the more abundant ones),[2] which are assumed to possess a 2α-acetylenic side chain.

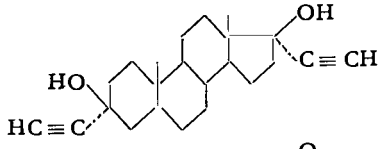
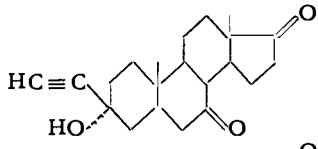
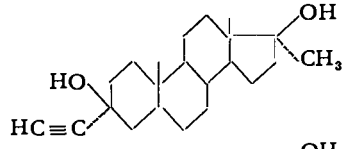
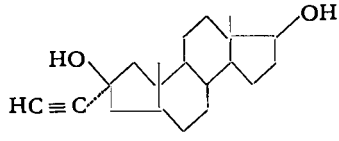
These speculations regarding configuration at the 2 position obviously require further confirmation, and this is being undertaken.

About thirty new compounds obtained in the course of our work (either prepared previously or described for the first time in this paper) have been tested in bioassay as inhibitors of standard "normal" hormones. The results of these tests are summarized in Tables I, II, and III. The most potent antiprogestin E-1182 and the esters E-1199 and E-1596 have been studied *in extenso* for various biological activities. E-1182 itself has proved to be weakly uterotropic in the mouse (at 1 mg compared to 0.02 μg of estrone), completely inactive as a progestin in the Clauberg assay in the rabbit, and nonandrogenic in the rat and chick. These three antiprogesterones have been tested in both rats and mice as possible inhibitors of implantation. The data on these experiments are presented in Table IV. They demonstrate that when these substances are administered in divided doses on days 1, 2, and 3 of an established pregnancy, significant inhibition of implantation may be induced by each of them in the mouse and by E-1182 and E-1199 in the rat. Superficially E-1182 appears to be the most potent of the three, but much more data are required for exact comparative study. It should be noted that E-1182 is clearly active when administered to the rat by mouth as well as by injection.

[2]These results were obtained in collaboration with Miss M. Minssen and will be published in detail later.

TABLE I

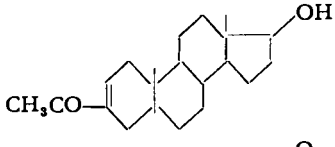
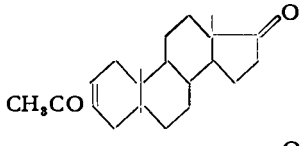
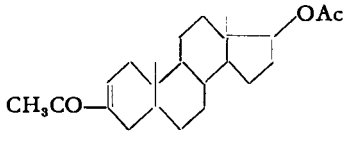
Activity of Inverted Steroids as Antiestrogens: Inhibition of the Uterotropic Effect of Estrone in Immature Mice

Compounds	Activity[a]
	1250 : 1
	2500 : 1
	2500 : 1
	2000 : 1

[a]Ratio of the minimum effective inhibiting dose to the inhibiting dose of the standard.

TABLE II

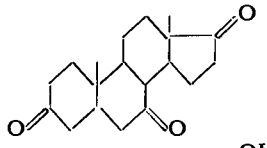
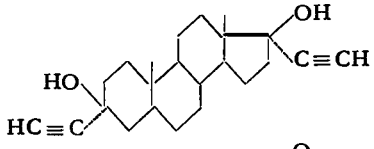
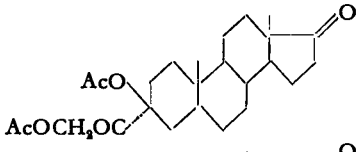
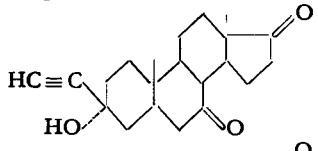
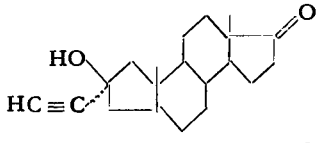
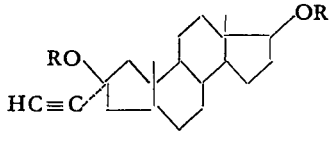
Activity of Inverted Steroids as Antiandrogens: Inhibition of the Effect of Testosterone Enanthate on Chick Comb Weight

Compounds	Activity[a]
	4 : 1
	4 : 1
	6 : 1

[a]Ratio of the minimum effective inhibiting dose to the inhibiting dose of the standard.

TABLE III

Activity of Inverted Steroids as Antiprogestins: Inhibition of the Carbonic Anhydrase Stimulating Effect of Progesterone in the Rabbit Uterus

Compounds	Activity[a]
	5 : 1
	(?) 8 : 1
	10 : 1
	10 : 1
	10 : 1
	R = H 1 : 1 (E—1182) R = Ac 2 : 1 (E—1199) R = Pr 3 : 1 (E—1596) R = Bz 10 : 1

[a]Ratio of the minimum effective inhibiting dose to the effective dose of the standard.

TABLE IV

Implantation Inhibition by Compounds Administered on Days 1, 2, and 3 of Pregnancy

Compoud	Subcutaneous implantation inhibiting[a] doses (mg/day)		Oral implantation inhibiting[a] doses (mg/day)	
	Rat	Mouse	Rat	Mouse
E-1182	0.02, 0.1 **0.5 , 2.5**	**0.02, 0.1** **0.5 , 2.5**	**5.0, 15.0**	2.5
E-1199	0.5 , **2.5**	**0.5 , 2.5**	NT[b]	NT
E-1596	0.5 , 2.5	**0.5**	NT	NT

[a]Boldface values are significantly active.

[b]NT, not tested.

In Table V, we present the data on the effect of E-1182 administration on the number of embryos found in mice or rats in which implantation was not completely inhibited. Table V shows that in control mice

TABLE V

Effects of a Neutral Antiprogestin (E-1182) on Number of Embryos

Treatment[a]	Mean number of embryos in mice	Treatment	Mean number of embryos in rats
Control	11.7–12.6	Control	11.6–11.7
E–1182 SC[b] **0.02** mg	11.0	E–1182 SC 0.02 mg	11.0
E–1182 SC **0.1** mg	8.0	E–1182 SC 0.1 mg	9.2
E–1182 SC **0.5** mg	3.0	E–1182 Oral 5.0 mg	6.0
E–1132 Oral 2.5 mg	7.5	E–1182 Oral 15.0 mg	5.5

[a]Boldface values show significantly activity.

[b]SC, subcutaneous administration.

the average number of embryos per mouse ranges from 11.7 to 12.6. In those animals in which embryos were implanted, the average number is, in every instance, less than those given for the controls. Similarly, in the rat we have found mean embryo numbers in control females to range between 11.6 and 11.7; again, in treated animals which carry some young, the average embryo number is always less than that seen in the controls. There is, indeed, a suggestion of a quantitative relationship between the dosage and the mean number of implants found.

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Ether Derivatives of Steroidal Hormones

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I. INTRODUCTION

There is no doubt that steroidal hormones, as isolated from natural sources, in practice are not suitable for therapeutic needs. First, most of them, like testosterone and estradiol, display an activity that is too weak and transient when they are injected in their original form. Hence, it would not be possible to get an adequate therapeutic effectiveness except by resorting to frequent injections or to some devices no longer in use, such as subcutaneous implants. Secondly, a serious shortcoming shared by all C_{18} and C_{19} steroidal hormones, as well as by progesterone, is the lack of oral activity. A third reason depends on the frequent need of modifying the biological characteristics inherent in each hormone. As a matter of fact, in many cases it would be desirable to dissociate these biological properties in order to be able to take advantage of some of them without having the drawbacks of the others. The above considerations make manipulations of steroidal molecules both justifiable and advisable.

Now, the modifications that can be brought into steroid molecules for a better therapeutic utilization may be divided into two groups: reversible and irreversible modifications. To the first group belong the derivatives which, in the body, regenerate the parent hormones or are transformed into products of the normal steroidal metabolism. Therefore, sooner or later, they become enmeshed with the metabolic processes of the endogenous hormones. Many esters of natural hormones are an example of this group. They are particularly useful in parenteral therapy, allowing total dosage to be greatly reduced and injections to be spaced out. Actually, through esterification, it was possible to bring testosterone, estradiol and other compounds into practical use.

To the second group, which is characterized by irreversible alterations of the original steroidal structure, belong, for instance, the derivatives obtained by introducing into different points of the molecule substituents that cannot be removed by the organism. In this way,

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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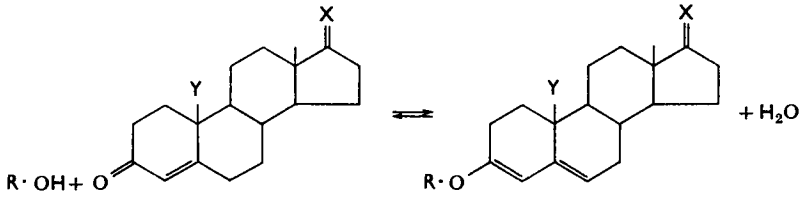
one can succeed in giving oral activity to steroids which are deprived of it or in shifting in the desired way the biological properties of natural hormones. However, some untoward effects may show up as a consequence of the structural anomalies artificially introduced into the molecule.

It should indeed be desirable to reach the two aforementioned goals by means of reversible derivatives of natural steroids. This is what we have just achieved in part through a systematic study of the derivatives obtained by etherification of the different oxygenated functions normally present in steroidal hormones, a field to which previous authors have paid only scant attention. The present paper is an attempt to demonstrate the unforeseen value of these compounds.

II. Enol Ethers of Δ^4-3-Ketosteroids

The first ether derivatives studied in our laboratories were enol ethers of Δ^4-3-ketosteroids. The schematic reaction which leads to their formation from an alcohol and a Δ^4-3-ketone with elimination of a water molecule is as follows:



Δ^4-3-ketosteroid Enol ether

This reaction is reversible, and the parent ketone can be easily regenerated in diluted alcoholic medium by slight acidification. The main Δ^4-3-ketosteroids which have been employed in our laboratories as starting compounds are the following: androst-4-ene-3,17-dione (androstenedione), 17β-hydroxyandrost-4-en-3-one (testosterone), 17β-hydroxy-17α-methylandrost-4-en-3-one (methyltestosterone), 17β-hydroxy-19-norandrost-4-en-3-one (19-nortestosterone), 21-hydroxypregn-4-ene-3,20-dione (deoxycorticosterone), 17,21-dihydroxypregn-4-ene-3,11,20-trione (cortisone), 11β,17,21-trihydroxypregn-4-ene-3,20-dione (hydrocortisone), pregn-4-ene-3,20-dione (progesterone), 17α-hydroxypregn-4-ene-3,20-dione (17α-hydroxyprogesterone), 17α-ethynyl-17β-hydroxyandrost-4-en-3-one (ethisterone), 17α-ethynyl-17β-hydroxy-19-norandrost-4-en-3-one (norethindrone), 6α-methyl-17α-acetoxypregn-4-ene-3,20-dione, and many others substituted Δ^4-3-ketones.

The alcohols mainly employed to prepare steroidal enol ethers are listed in Table I.

TABLE I

Alcohols Mainly Employed to Form Enol Ethers of Δ^4-3-Ketosteroids

Number of carbon atoms	Name	Symbol
1	Methyl	-1
2	Ethyl	-2
3	*n*-Propyl	n-3
3	Isopropyl	iso-3
4	*n*-Butyl	n-4
4	Isobutyl	iso-4
4	*sec*-Butyl	sec-4
5	*n*-Pentyl	n-5
5	Isopentyl	iso-5
5	Cyclopentyl	c-5
6	*n*-Hexyl	n-6
6	Isohexyl	iso-6
6	Cyclohexyl	c-6
7	*n*-Heptyl	n-7
8	*n*-Octyl	n-8
9	*n*-Nonyl	n-9
10	*n*-Decyl	n-10
12	*n*-Dodecyl	n-12

A. ORAL ACTIVITY

A phenomenon that struck us at the beginning of our studies on these compounds was the possibility of obtaining an enhancement of oral activity in respect to the parent compounds (Ercoli and Gardi,

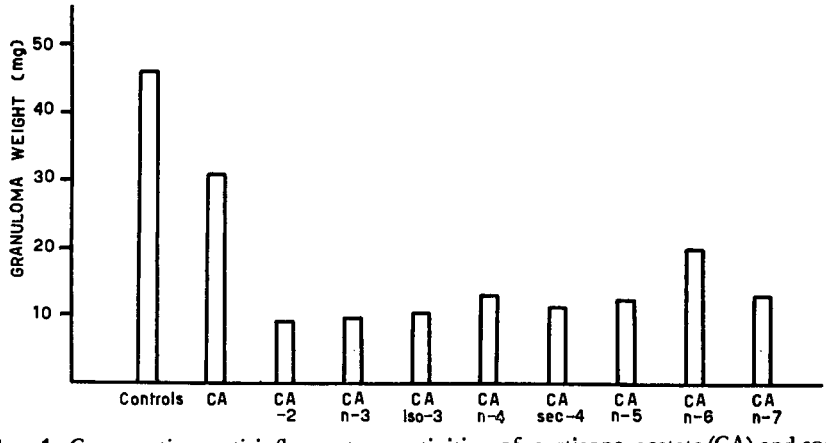


FIG. 1. Comparative anti-inflammatory activities of cortisone acetate (CA) and some of its enol ethers, given orally. Each bar represents the mean of values for 10 rats. Each rat was given 5 μmoles of steroid daily for 6 days.

1960). That holds true for a great number of etherifying alcohols and is particularly evident for progestational (Falconi *et al.*, 1961a; Ercoli and Falconi, 1962) and corticoid hormones (Falconi *et al.*, 1961b), as well as for methyltestosterone (Ercoli *et al.*, 1960a,b).

Figure 1 illustrates the anti-inflammatory activity of several enol ethers of cortisone acetate, given by mouth, as compared with cortisone acetate itself. As end point, the weight of granuloma induced by subcutaneous implantation of an agar pellet, was chosen. All the assayed enol ethers, from the ethyl to *n*-heptyl, proved to be more effective than the parent cortisone acetate.

The same influence of etherification may also be seen in the case of mineralocorticoids such as deoxycorticosterone acetate (DOCA) (Fig. 2). In this experiment adrenalectomized rats were given orally either DOCA or one of its enol ethers, starting from the day of operation. On the tenth day the DOCA-treated animals showed a survival rate of 20%. Equimolar doses of several enol ethers of DOCA led to a far greater percentage of survivors, from 40 to 90%.

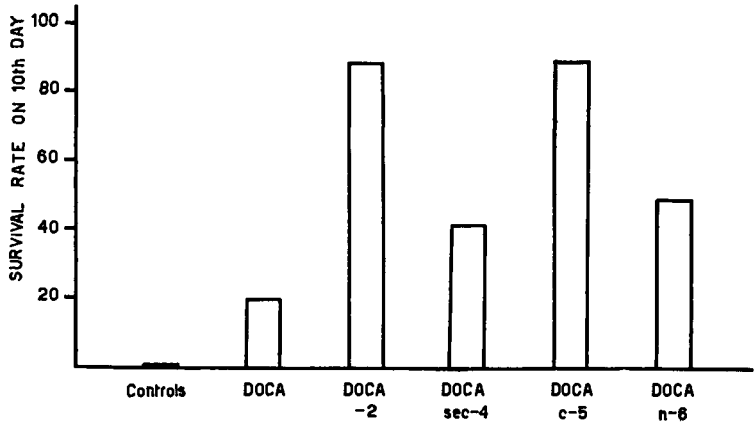


FIG. 2. Comparative life-maintaining activities of deoxycorticosterone acetate (DOCA) and some of its enol ethers, given orally to adrenalectomized rats. Each bar represents the mean of values for 10 rats. Each rat was given 1.6 μmoles of steroid daily for 10 days.

Another striking example of the greater oral activity of enol ethers as compared with the parent compounds can be seen in the case of methyltestosterone (Fig. 3).

In the field of progestational compounds, we found that 17α-acetoxyprogesterone amyl and cyclopentyl enol ethers were highly active as oral progestational agents, displaying in the Clauberg test about the

same activity as parenterally administered progesterone. I am not giving experimental data about this subject; it has been dealt with in previous publications. Also progesterone can be transformed into an orally active compound by condensation with cyclopentyl alcohol. The resulting enol ether, which is probably more effective in human beings than in laboratory animals, is the only oral progestational agent so far known that will exactly mimic the physiological actions of parenterally administered progesterone (Andreoli, 1962; Andreoli and Vischi, 1962; Hecht-Lucari and Scarpellini, 1961; Klopper, 1961; Vischi, 1961).

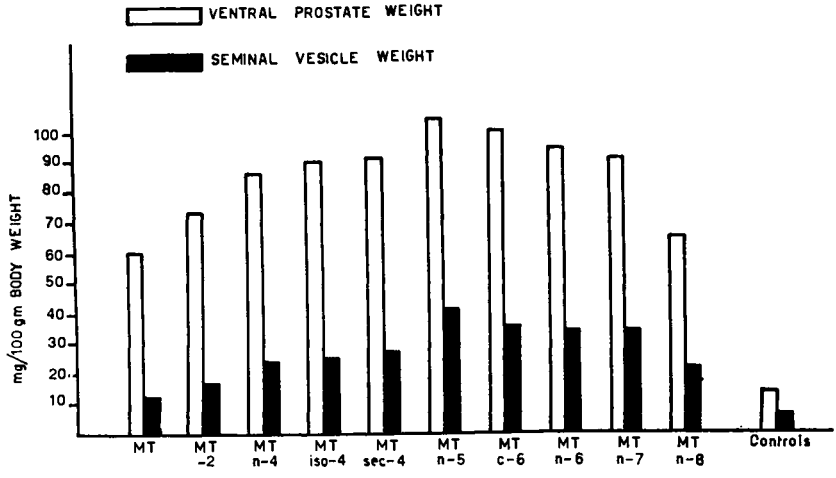


FIG. 3. Comparative androgenic activities of methyltestosterone (MT) and some of its enol ethers, given orally to immature castrate rats. Each bar represents the mean of values for 10 rats. Each rat was given 1 μmole of steroid daily for 7 days.

As far as testosterone and its esters are concerned, the oral activity of these compounds also may be enhanced through etherification (Ercoli *et al.*, 1960b). However unquestionable this potentiation may be, the degree of effectiveness reached in this way is too slight for therapeutic utilization of these compounds. It will be seen later how it was possible to reach such a goal by means of ethers of other kinds.

It appears from the data reported up to now that the nature of the etherifying alcohol has a great influence in determining the degree of oral activation of the parent steroid. Enhancement is very high with certain alcohols whereas it is very low with others. Sometimes even a reduction of activity may be found. Moreover, the alcohols most suitable for increasing the activity of Δ^4-3-ketosteroids are not always the same for the different compounds. Thus, for many corticosteroids

the highest degree of activation appears to be reached by alcohols with 2 or 3 carbon atoms, whereas for both progestational substances and methyltestosterone the most suitable alcohols have a chain consisting of 5–7 carbon atoms (in a straight or cyclic chain).

A second general point is that the increase of oral activity brought about by etherification is not necessarily shared to the same extent by the various hormonal properties inherent in the parent Δ^4-3-ketones. Thus, though the main activity of a compound may remain unchanged, its secondary effects may be enhanced and vice versa. For instance (Fig. 4) antiestrous and contraceptive activities of 17α-ethynyl-19-nortestosterone acetate are strongly enhanced in the rat by etherification with cyclopentyl alcohol. On the contrary, these properties are reduced

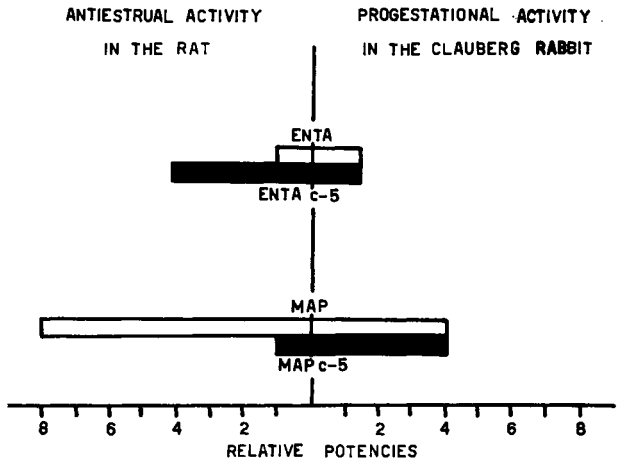


FIG. 4. Effect of enolic etherification with cyclopentyl alcohol on the relative antiestrual and progestational potencies of 17α-ethynyl-19-nortestosterone acetate (ENTA) and 6α-methyl-17α-acetoxyprogesterone (MAP), given orally.

when the same process is applied to 6α -methyl-17α-acetoxyprogesterone. It is to be noted, however, that the potencies of both compounds in the Clauberg test remain unchanged after etherification (Falconi and Ercoli, 1961). Recent studies have also shown that there is no correlation between antiestrous activity and pituitary-inhibiting power of those compounds (Falconi and Bruni, 1962). This is just an example of the opportunity provided by the study of steroidal ethers for a better knowledge of some endocrinological problems.

B. PARENTERAL ACTIVITY

When enol ethers of Δ^4-3-ketosteroids are given parenterally, we observe exactly the opposite we saw following oral administration, i.e., they show reduced or even abolished hormonal properties in comparison with the unetherified steroids (Ercoli, 1958a,b; Ercoli and Gardi, 1960; Ercoli *et al.*, 1960a,b).

The same loss of activity found in steroidal enol ethers when they are injected subcutaneously also appears in local application, i.e., by direct contact with target organs. Thus, cyclopentyl enol ether of 17α-acetoxyprogesterone, which is orally endowed with a high progestational effect on rabbit endometrium, elicits but scarce secretive changes when applied locally in the McGinty test (Falconi *et al.*, 1961a). This finding suggests that the above derivative is scarcely active per se, but is transformed into a more effective compound after oral administration.

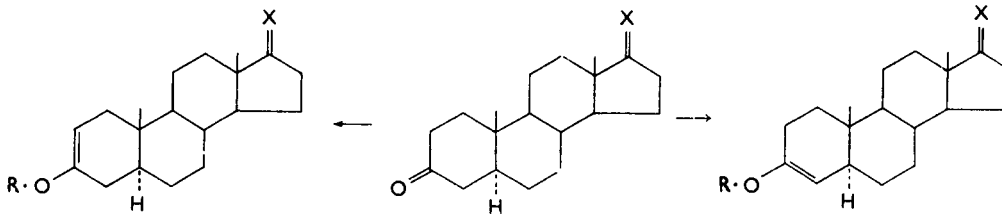
Although the specific hormonal activity of a Δ^4-3-ketosteroid administered by parenteral route can be lost by etherification, this does not necessarily hold true for other biological properties of the parent steroid. Some years ago we had already the opportunity (Ercoli and Koller, 1956) to comment on antitumoral activity in human breast cancer of a nonandrogenic testosterone derivative, i.e., the benzyl enol ether of testosterone phenylpropionate, administered parenterally (Gardini, 1956). Such a result was the first demonstration of a separation, in an androgenic derivative, of the action against mammary tumors from the specific sexual effects. Only a few years ago, this result was considered almost unobtainable by cancerologists and endocrinologists, on the basis both of negative experiments and theoretical reasons. In this field, however, the most remarkable results were obtained, as will be shown below, with enol ethers of saturated 5α,3-ketosteroids.

III. ENOL ETHERS OF 3-KETO-5α-STEROIDS

Just a few years ago, it was thought that only Δ^2-derivatives could be formed from saturated 3-ketones of cholestane series. In our laboratories we have observed, instead, that under particular conditions it is also possible to obtain Δ^3-derivatives (Gardi *et al.*, 1962).

The biological behavior of both Δ^2 and Δ^3 compounds is similar to that seen in enol ethers of Δ^4-3-ketosteroids: i.e., increase of the activity of parent compounds following oral administration and loss of activity following parenteral administration.

It is interesting to take advantage of the latter phenomenon in order to separate the different properties of the parent steroid. For instance, some Δ^2-enol ethers of dihydrotestosterone, which are devoid of androgenic activity when administered parenterally, exhibit against 3-methyl-



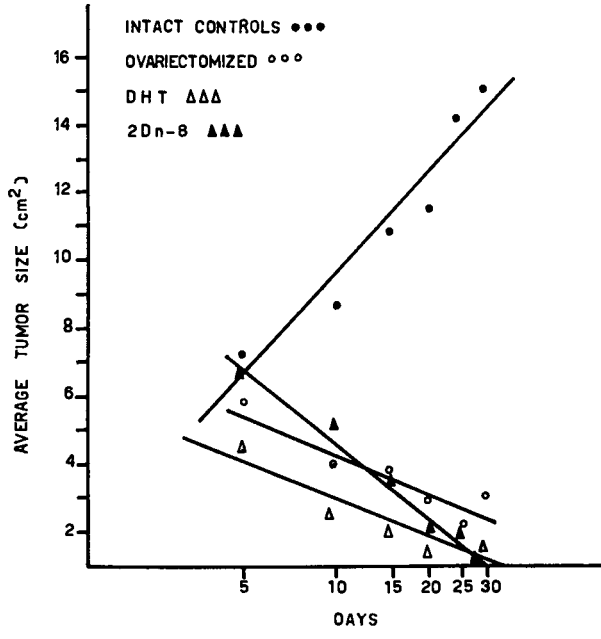


FIG. 5. Effect of various treatments, i.e., ovariectomy, intramuscular administration of dihydrotestosterone (DHT) and of its *n*-octyl enol ether (2D n-8), on the growth of 3-methylcholanthrene-induced rat mammary carcinoma. Conditions: Twelve rats per group. Mammary cancer was induced by the intragastric administration of 10 mg of 3-methylcholanthrene 6 days a week for 50 days. The tumors were allowed to grow for 1 month. Then the controls were injected with sesame oil; another group was ovariectomized; steroid-treated rats received 2 mg of compound daily for 30 days.

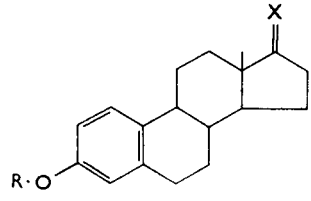
cholanthrene-induced mammary cancer in the rat an antitumoral effect fully comparable to that of dihydrotestosterone (Ercoli and Briziarelli, 1961). Figure 5 shows the changes of tumor size induced by various treatments: i.e., ovariectomy, administration of dihydrotestosterone or *n*-octyl enol ether of dihydrotestosterone. It is evident that the last compound diminishes tumor size to a degree equal to, or even greater than, that observed after ovariectomy or after administration of dihydrotestosterone. For details of these and other experiments concerned with steroidal treatment of mammary tumors, described by my associate Dr. Briziarelli, see the Round Table Discussion on steroids and tumors (Volume II). Δ^3-Derivatives of dihydrotestosterone retain a certain degree of androgenic activity when administered by injection and, therefore, seem to be less useful for the aforementioned purpose.

IV. ACETALS OF 3-KETOSTEROIDS AND ETHERS OF 3α- OR 3β-HYDROXYSTEROIDS

In our laboratories a few acetals of dihydrotestosterone and some ethers of 3-hydroxy steroids, such as androsterone, androstenediol, and methylandrostenediol, have been prepared. In this case too, it was possible to observe, in comparison with the parent steroids, an enhancement (albeit limited) of oral potency accompanied by a loss of activity by the parenteral route.

V. 3-ETHERS OF ESTROGENIC PHENOL STEROIDS

The general structure of these compounds is the following:



Many 3-ethers of different estrogenic steroids, such as estrone, estradiol and its esters, estriol, epiestriol, 17α-ethynylestradiol, and 17α-methylestradiol, have been prepared and biologically tested in our laby oratory. Following oral administration, an enhancement of activity could be observed also in this series of derivatives, as compared to free phenols (Ercoli and Gardi, 1961; Ercoli *et al.*, 1962b). Figure 6 shows the uterotropic activity of several ethers of estrone. It must be pointed out that the activation is limited to a rather small number of etherifying alcohols, namely those having a chain of 4 or 5 carbon atoms. More-

over, the degree of activation is generally low. Only cyclopentyl ethers exhibited a strong enhancement of activity for all the estrogens tested (Ercoli *et al.*, 1961; Falconi, 1962a). This is shown in Fig. 7, where the uterotropic activities of 3-cyclopentyl ethers are reported in comparison with those of the parent steroids. If the dose-response curves for each pair of steroids are compared, it can be seen that the cyclopentyl ether

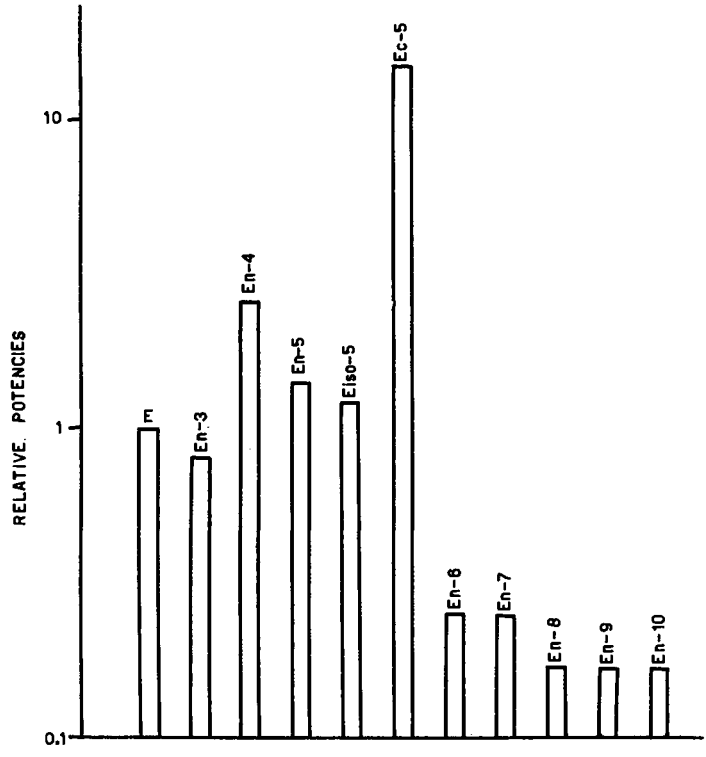


FIG. 6. Comparative uterotrophic activities of estrone (E) and several estrone ethers given orally to immature mice.

dose-response curve is shifted to the left, has a steeper slope, and far exceeds the plateau reached by the curve of the parent estrogen. According to our findings, the cyclopentyl ether of 17α-ethynylestradiol seems to be the most potent oral estrogenic agent described up to now, closely followed, however, by the cyclopentyl ether of 17α-methylestradiol.

After parenteral administration, also 3-ethers of estrogenic steroids lose their activity almost completely (Ercoli *et al.*, 1961, 1962b), with the remarkable exception of several cyclopentyl ethers (Falconi, 1962b). In particular, by injection, the cyclopentyl ethers of estriol and epiestriol appear to be much more active than their respective phenols (Falconi, unpublished data).

As to the possibility of obtaining, by means of etherification, some dissociation of activity also in the estrogenic steroid series, it is to be noted that some estrone ethers, namely estrone *n*-heptyl and *n*-nonyl ether, which are almost devoid of uterotropic effect both by mouth and injection, are as potent as estrone in lowering the serum cholester-

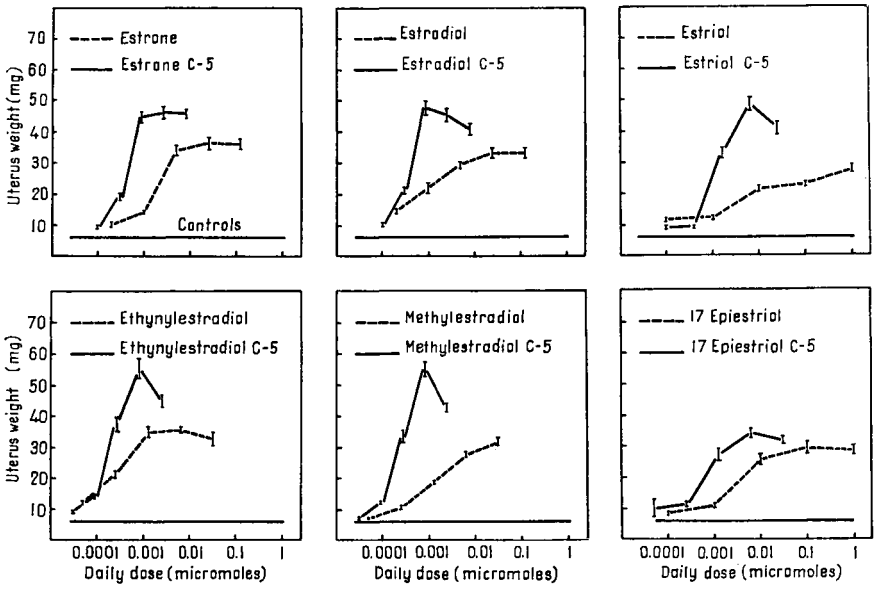


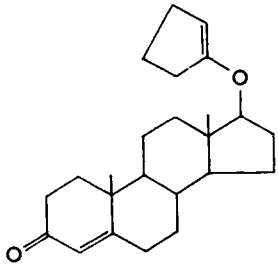
FIG. 7. Comparative uterotropic activities of some estrogens and their 3-cyclopentyl ethers, given orally to immature mice. Ten mice per group. Steroids were administered daily for 3 days.

ol : phospholipid ratio in chicks fed on hypercholesterolemic diet (Galletti *et al.*, 1961; Ercoli *et al.*, 1962b). I can add that some estrone ethers obtained by condensation with high alcohols exhibit a remarkable activity also against experimental mammary cancer.

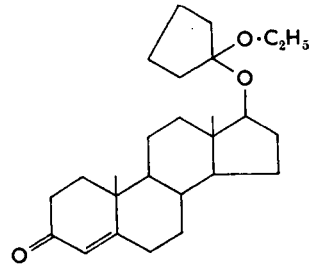
VI. ETHERS OF 17β-HYDROXYSTEROIDS

We have already said that some enol ethers of testosterone, 19-nortestosterone, or their esters, display an oral activity higher than that of the parent compound, but not enough to warrant their clinical use. Our studies shifted therefore to the effects of etherification at 17β-hydroxyl group (Ercoli *et al.*, 1962a). In this case too, we thought that it might be convenient to prepare ether derivatives easily hydrolyzable in acidic medium, a property which already characterizes enol ethers of 3-ketosteroids. Thus, in the manner in which we had prepared alkyl

enol ethers of testosterone, Dr. Gardi and I began to prepare testosteronyl enol ethers of several ketones. Mixed acetals were prepared as well.



Cyclopentanone testosteronyl enol ether



Cyclopentanone ethyl testosteronyl acetal

The biological results were very promising, especially with regard to the compound represented in the right-hand formula, which proved to have by mouth an androgenic activity double of that of methyltestosterone (Table II). Subsequently our studies were extended to Δ^1-dehydro

TABLE II

Oral Activities of Some Steroid-17β-yl Enol Ethers and Acetals

Compound	Oral activity	
	Androgenic[a]	Anabolic[b]
5α-Androstan-17β-ol-3-one 17-(1′-ethoxy)-cyclopentyl ether	2.7	3.0
Testosterone 17-(1′-ethoxy)cyclopentyl ether	1.9	2.1
Testosterone 17-($\Delta^{1'}$-cyclopentenyl) ether	1.3	1.1
Testosterone 17-(1′-ethoxy)cyclohexyl ether	1.0	2.2
Δ^4-Androstene-3β, 17β-diol 17-(1′-ethoxy)cyclopentyl ether	2.3	2.0
19-Nortestosterone 17-($\Delta^{1'}$-cyclopentenyl) ether	0.4	4.4
	Uterotropic[c]	
Estradiol 3-benzoate 17-(1′-ethoxy)cyclopentyl ether	2–4	
Estradiol 3-benzoate 17-($\Delta^{1'}$-cyclopentenyl) ether	2–4	

[a]Calculated on seminal vesicle weight, methyltestosterone = 1

[b]Calculated on levator ani weight, methyltestosterone = 1

[c]Immature mice 3-day assay, estradiol per os = 1

derivatives, to saturated derivatives of the 5α-series, and to 19-norandrostanes. The findings turned out to be very favorable: for details, described by Dr. Falconi, see the Round Table Discussion (Volume II) on new anabolic steroids.

I would like to add here that it has thus been possible to solve a problem which had remained unresolved since the beginning of the researches on steroidal hormones: that is, how to achieve androgenic, estrogenic, and anabolic compounds endowed with a high oral activity without resorting to 17α-alkylation or to alkylations of other kinds. Moreover, our 17-ether derivatives belonging to the androstane and norandrostane series do not display either the hepatotoxic effect or the progestational properties developed by the corresponding 17-alkylated derivatives.

Unlike the enol ethers of 3-ketosteroids, these new compounds are remarkably active even if administered by injection, showing in this way a very high and prolonged activity, comparable to that of some of the most potent esters of C_{18} and C_{19} hormones.

Let us say, finally, that, whatever the route of administration, the new compounds originate urinary metabolites identical with those derived from the unetherified parent steroids. This fact holds true for all the classes of ether examined up to now. Judged from chemical and biological standpoints they constitute, therefore, reversible modifications of steroid hormones.

VII. OTHERS

Many cyclic diether derivatives are formed through exchange reactions between the dihydroxyacetone chain of corticosteroids and different aldehydes and ketones as well as alkyl orthoesters. The very interesting chemistry of these compounds is described in detail by Dr. Gardi in the Round Table Discussion on recent advances in steroid chemistry (Volume II). Some compounds of this series are of great interest since they have a high biological activity, both general (as in acetals) and topical (as in orthoesters).

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DISCUSSION

The Influence of Enol Etherification on the Biological Activity of Δ^4-3-Ketosteroids

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Enol etherification enhances the oral activity of steroids such as methyltestosterone and 17-acetoxyprogesterone (Ercoli *et al.*, 1960a; Falconi *et al.*, 1961). The oral effectiveness of testosterone or progesterone may also be increased by enol etherification, but the specific activity is still very low relative to subcutaneous injection of the parent ketones (Ercoli, 1961). With highly active steroids such as 6-methyl-17-acetoxyprogesterone or 17-ethynyl-19-nortestosterone, oral progestational potency is not further enhanced by enol ether formation (Falconi and Ercoli, 1961). It would thus appear that the intrinsic oral activity of the parent Δ^4-3-ketone determines the degree of influence of enol etherification.

An interesting point for consideration is that enol ethers of Δ^4-3-ketosteroids are less active than their parent compounds when injected subcutaneously or, in some cases, when applied directly to the target organs (Ercoli *et al.*, 1960b). Falconi *et al.* (1961) state that the enol ether structure is essentially inactive per se, but when administered orally is probably transformed to an active form (possibly the parent kctone?). The time, and the circuitous metabolic pathway required for such a transformation are said to prolong duration of action, thus accounting for enhanced potency. This interpretation would be strengthened if direct evidence were available concerning the rate of absorption, the biological half-life, and the excretion rate, in feces as well as urine, of steroid enol ethers in comparison with their parent ketones.

Enol ethers of progesterone, acetoxyprogesterone, and methyltestosterone are very unstable chemically and readily undergo decomposition unless stored under nitrogen in crystalline form or as solutions in sesame oil, also made and stored under nitrogen. This is not a problem for the clinician, since the properly formulated doses are sealed under nitrogen in gelatin capsules and are quite stable. The instability can be a real problem for the biologist who must make solutions and dilutions for daily injections. For example, Meli *et al.* (1963) found that a degraded sample as well as a chemically pure sample of methyltestosterone cyclopentyl enol ether were significantly more active orally than was pure methyltestosterone. Even a residue obtained from the mother liquor following recrystallization of naturally decomposed methyltestosterone cyclopentyl enol ether was found to be more active orally than methyltestosterone. Infrared analysis indicated that the residue contained approximately 40% methyltestosterone cyclopentyl enol ether, 25% methyltestosterone, and 35% unknown substances, the

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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latter by difference (Meli *et al.* 1963). Such results raise the question whether adequate chemical analyses were conducted or considered necessary during the course of earlier endocrine evaluations of these compounds, since a large chemical alteration may not be reflected by a major loss of biological activity. In view of chemical instability, information is also required concerning the possible degradation of steroid enol ethers in the small intestine. For example, Meli (1963) found that methyltestosterone cyclopentyl enol ether is equally androgenic by oral or intraperitoneal administration in rats, whereas testosterone cyclopentyl enol ether was 3—4 times more androgenic intraperitoneally then orally. Thus, passage through the gastrointestinal tract reduced activity of the testosterone derivative without influencing the methyltestosterone enol ether. It would, therefore, seem premature to offer a generalized hypothesis regarding conversion, or site of conversion, of enol ethers to active forms (see Falconi *et al.*, 1961). Meli's (1963) results suggest that the nature of the parent ketone, not enol etherification per se, governs the metabolic fate and thus the biological activity of a given compound.

The relative lack of activity of subcutaneously injected steroid enol ethers might be due to their high lipid solubility rather than to failure of transformation to an "active form." Thus, highly lipophilic steroids might not be released from the subcutaneous fat depot (Reed *et al.*, 1930) in sufficient concentration to exert systemic biological activity. This question could be answered directly by analyses of steroid concentrations in body fat, or indirectly by subcutaneous or intramuscular injection of steroid enol ethers in doses sufficiently high to ensure an adequate rate of release. According to Falconi *et al.* (1961) 17-acetoxyprogesterone cyclopentyl enol ether was inactive in the McGinty test (local application to the uterine endometrium of the rabbit). It would be important to know whether this is true of enol ethers of other gestagens (progesterone, 6-methyl-17-acetoxyprogesterone, and 17-ethynyl-19-nortestosterone). If any of these enol ethers were active locally, it would be hard to defend the thesis of "conversion to an active form" (Falconi *et al.*, 1961).

Finally, it has been proposed that the metabolites of enol ethers of naturally occurring steroids are similar to those of their parent ketones (Ercoli, 1961; Klopper, 1961). Thus, administration of progesterone cyclopentyl enol ether in the human is reported to increase pregnandiol excretion (Klopper, 1961). It is not clear why twice as much pregnandiol was excreted following administration of progesterone as following a comparable dose of progesterone cyclopentyl enol ether (Klopper, 1961). Lacking factual metabolic evidence, the following possibilities must be considered: (1) A portion of the enol ether is transformed to progesterone (and unknown compounds?) by the gut and/or by the liver. The progesterone thus formed is responsible for the pregnandiol excretion, but not necessarily for the biological activity, (2) The enol ether is absorbed as such and stored in body fat, from which it is released at a slower rate than the parent ketone.

It is concluded that careful metabolic studies must be conducted to elucidate the effects of the small intestine and/or the liver on each of the Δ^4-3-ketosteroid enol ethers. The possibility of differential storage of steroid enol ethers in body fat must also be investigated. Such information could resolve the question of the "active form."

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ADDENDUM

Since submission of this manuscript, the following pertinent observations have been made:

1. The 3-cyclopentyl enol ether of progesterone is active in the McGinty test at doses of 15—30 μg intraluminally (Meli, unpublished results).
2. Whereas small daily subcutaneous doses of methyltestosterone–3–cyclopentyl enol ether were only slightly effective, the single subcutaneous administration of relatively large doses (1 or 5 mg) resulted in a more prolonged and intense stimulation of the sex accessories in castrated male rats than that obtained with comparable doses of methyltestosterone (Meli, Endocrinology **72**, 715, 1963)
3. Unlike its parent ketone, methyltestosterone–3–cyclopentyl enol ether is stored in body fat following oral administration (Meli, Honrath and Wolff, Endocrinology, in press).

These observations are in disagreement with the "conversion theory" and support the view that storage in and release from body fat depots is the mechanism responsible for the increased oral activity brought about bu 3-enol etherification.

New Steroids with Hormone Like Activities C-6 Substituted Hormone Analogs[1]

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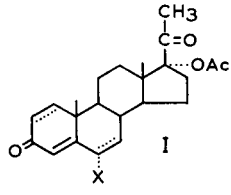
I. INTRODUCTION

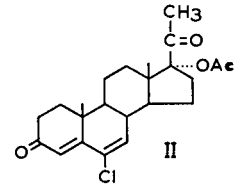
During the last decade, major efforts have been made to modify the biological properties of the naturally occurring hormones and in the field of progestational agents an important goal has been the preparation of orally active compounds, since the naturally occurring hormone progesterone is only weakly active by mouth.

II. MODIFICATIONS OF 17α-ACETOXYPROGESTERONE

Following the discovery that 17α-acetoxyprogesterone was active orally, a wide variety of C-6 substituted and dehydro analogs of this compound were made by our group in Mexico (Bowers *et al.*, 1959 a,b,c; Ringold *et al.*, 1959a,b).



X= H, CH_3, Br, Cl or F



6-Chloro-6-Dehydro-17α-Acetoxyprogesterone

CHLORMADINONE

Initially the effect of introducing a methyl group (Ringold *et al.*, 1959b) and the halogen atoms bromine, chlorine, or fluorine at C-6 (Ringold *et al.*, 1959a; Bowers *et al.*, 1959c) was investigated. In every case there was a marked enhancement of biological activity with respect to 17α-acetoxyprogesterone. It was then found that a further increase in activity was brought about by the introduction of additional double

[1]This paper is considered to be Steroids CCXVII in the series from these laboratories.

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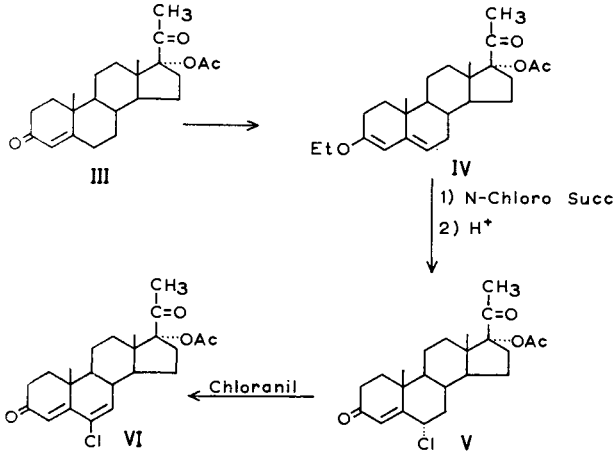
bonds at C-1 and C-6 (Ringold *et al.*, 1959a; Bowers *et al.*, 1959c). From a study of the biological activity of approximately twenty compounds of this type (I), one compound was selected for a detailed study; this compound was 6-chloro-6-dehydro-17α-acetoxyprogesterone (II), chlormadinone.

III. Synthesis and Properties of Chlormadinone

The synthesis of chlormadinone, from 17α-acetoxyprogesterone (III) proceeded via its enol ether (IV) which was then chlorinated with *N*-chlorosuccinimide. Acid treatment of the resulting 6β-chloro compound afforded 6α-choro-17α-acetoxyprogesterone (V), which underwent dehydrogenation with chloranil to afford chlormadinone (VI).



Its oral progestational activity was assayed by the McPhail-Clauberg rabbit assay (McPhail, 1934) and compared to related compounds. Norethisterone was added as a reference compound; the results are recorded in Table I.

TABLE I

Relative Potencies of Chlormadinone and Other Progestational Agents

Steroid	Relative potency
17[a]–Acetoxyprogesterone	1
Norethisterone	10
6[a]–Chloro–17[a]–acetoxyprogesterone	20
6[a]–Methyl–17[a]–acetoxyprogesterone (medroxyprogesterone)	20
6–Chloro–6–dehydro–17[a]–acetoxyprogesterone (chlormadinone)	350

[a]Oral activity assayed by McPhail–Clauberg rabbit assay.

Chlormadinone was by far the most active of all the compounds we have investigated. However, it is a long way from data such as this to the development of a drug of proved ability which is known to be free of undesirable side effects.

The steps necessary for the development of a progestational agent are listed below:

1. Synthesis
2. Progestational assays
3. Lack of side effects in animals
   a. Estrogenicity
   b. Androgenicity
   c. Fetal masculinization
   d. Antipituitary effects
4. Tolerance studies in animals
5. Clinical evaluation

We have already discussed the synthesis and the progestational activity of chlormadinone; the next step was to study various parameters of its biological activity in animals.

In the past few years much attention has been called to undesirable side effects of some synthetic progestational agents, such as the inhibi-

TABLE II
Properties of Chlormadinone[2]

Test	Result	Details of assay
Estrogenic activity	Negative	Orally: Up to 1 mg in female mice had no effect
Antiestrogenic activity	Positive	Orally: At 100 μg good response against 0.4 μg of estrone
FSH inhibition	Negative	Orally: Up to 40 mg in the parabiotic rat had no effect on the ovarian weight
ACTH inhibition	Negative	Clinical study: At 50 mg daily for 5 days. No depression of blood or urinary 17α–OH corticoids.
Androgenicity	Negative	Orally: Immature male rats. Up to 20 mg no effect as compared to control on seminal vesicle or levator ari muscle
Anti androgenicity	Positive	At 50 mg effective in blocking androgenic effect produced by 2.0 mg of testosterone in male rats

[2]The details of the biological assays are summarized by R. I. Dorfman, ed. (1962). "Methods in Hormone Research," Vol. II. Academic Press, New York.

tion of certain pituitary hormones and also fetal masculinization due to the inherent androgenicity of the compound. Table II summarizes the results of estrogenic, antiestrogenic, androgenic, antiandrogenic assays and its effect on the production of follicle-stimulating hormone (FHS) and corticotropin (ACTH).

It can be seen from these results that chlormadinone is nonestrogenic and nonandrogenic; in fact, it has some antiestrogenic and antiandrogenic activities. In addition it does not appear to effect FSH or ACTH production.

A study was then carried out to investigate the effect of chlormadinone, 6α-chloro- and 6α-methyl-17α-acetoxyprogesterone, on fetal masculinization in rats (Kincl and Dorfman, 1962). The results are summarized in Table III.

TABLE III

Properties of Chlormadinone: Influence on the Female Rat Fetus

Steroid	Daily dose[a] (mg)	Vaginal orifice	Vagina	Prostate
Chlormadinone	0.15–7.5	Normal	Normal	Normal
6α–Chloro–17α–acetoxy-progesterone	2.5–7.5	Normal	Normal	Normal
6α–Methyl–17α acetoxy progesterone	0.15	Normal	Normal	Enlarged
	1.5	Absent (62%)	Blind (62%)	Enlarged (100%)
	2.5	Absent (100%)	Blind (100%)	Enlarged (92%)
Testosterone	0.5	Absent (100%)	Blind (100%)	Enlarged (100%)

[a]Oral; 6 days.

Even at high dose levels, chlormadinone did not have any effect on the female rat fetus. It is interesting to note that there is an appreciable difference in this respect between 6α-chloro- and 6α-methyl-17α-acetoxyprogesterone, the latter compound having a marked masculinizing effect on the female rat fetus.

Animal tolerance studies were then carried out with chlormadinone in rats for 6 months at dose levels up to 30 times the expected therapeutic dosage.

The animals remained in good condition throughout the treatment, and a complete histological study of all the organs showed that chlormadinone was nontoxic and caused no abnormalities.

In addition to the rat studies, chlormadinone was administered orally to dogs at a dose of 10 mg per kilogram body weight. A total of 25 pups (14 female) were born to dogs so treated; all showed normal development, including the sexual organs.

A considerable amount of clinical work has now been carried out with this compound which has shown that a complete progestational effect is brought about at 1.5 to 2.0 mg daily administered orally. Thus it is the most active oral progestational agent known with a proved record of safety and efficacy.

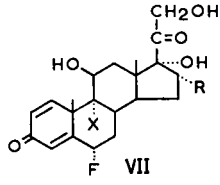
It has been used very successfully in all cases of progestational deficiency, e.g., amenorrhea, dysmenorrhea, dysfunctional bleeding.

IV. 6-HALO CORTICAL HORMONES

Parallel with our work on 6-halo progestational agents, we prepared a wide variety of 6-halo cortical hormones. However, in this series the C-6 fluoro compounds were superior to the corresponding chloro compounds.

A. SYSTEMATICALLY ACTIVE CORTICOIDS

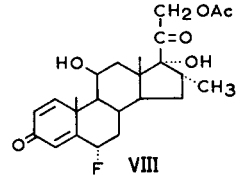
Structural formula (VII) shows the range of the 6-fluoro compounds that were investigated in detail. From the results of biological assays and preliminary clinical work, one compound was selected for intensive



VII

X= H or F

R= H, OH or CH3



VIII

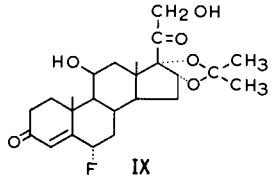
PARAMETHASONE ACETATE

study. This compound was 6α-fluoro-16α-methylprednisolone acetate (VIII), paramethasone acetate (Edwards *et al.*, 1959a,b). Time does not permit a detailed discussion of the development of this compound. Animal studies showed it to be approximately 65 times more active than hydrocortisone in the rat anti-inflammatory assay. In addition it does not retain sodium; in fact it is a mild sodium excretor with little effect on potassium. Clinical metabolic studies show that at 5 times the therapeutic dose level, catabolic effects such as nitrogen and calcium loss are minimal. (Irwin *et al.*, 1961).

This compound has been studied intensively in several countries throughout the world; it has proved to be a very valuable systematically active cortical hormone.

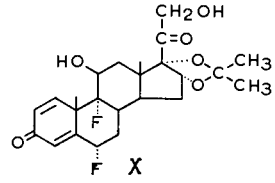
B. TOPICALLY ACTIVE CORTICOIDS

Several 6-fluoro cortical hormones were evaluated as topically active agents; of these two proved to be very effective. They are 6α-fluoro-16α-hydroxyhydrocortisone acetonide (IX), flurandrenolone, and 6α, 9α-difluoro-16α-hydroxyprednisolone acetonide (X), fluocinolone acetonide (Mills *et al.*, 1958).



IX

FLURANDRENOLONE



X

FLUOCINOLONE ACETONIDE

In clinical studies fluorandrenolone was shown to be 20 times more active than 1% hydrocortisone cream; and fluocinolone acetonide, 40–100 times more active. Both compounds have been evaluated in several thousand patients; in double-blind studies they have been shown to be equal or superior to existing agents (Robinson, 1961; Scholtz, 1961a; Holtzmann and Korting, 1960; Bruner, 1962; Cahn and Levy, 1961; Jamman and Beer, 1962; Fujita and Minami, 1961; Ahumada, 1961; Barba Rubio, 1961). In particular, fluocinolone acetonide represents a major breakthrough in dermatological therapy, for it has proved effective in the treatment of a variety of dermatoses which had been resistant to other systemic and topically active corticoids (Scher, 1961). Remarkable advances have been made in the treatment of psoriasis with fluocinolone acetonide (Scholtz, 1961b); this disease hitherto had not responded well to corticoid therapy.

In conclusion, it can be seen that the introduction of chlorine and fluorine atoms at C-6 into progestational agents, systemic, and topically active cortical hormones has brought about some major clinical advances.

ACNOWLEDGMENTS

The investigations described in this paper represent the concerted efforts of a great many people; in particular, major contributions have been made by Drs. Anderson, Djerassi, Dorfman, Edwards, Irwin, Kincl, Kraay, Mills, Ridolfo, Ringold, and Rudel.

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DISCUSSION

Pharmacological Aspects of the Study of Chlormadinone

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Of particular interest with regard to the pharmacological aspects of the study of chlormadinone are its activity pattern and side effects. From the data presented, there can be no doubt that this new compound is extraordinarily active in the test of McPhail-Clauberg. However, this is only a part of the progestational pattern. Among the synthetic progestational agents many are found that are active in this test but do not maintain pregnancy in spayed animals. This is demonstrated by the data of Table A, which led us to introduce the term *pregestagens* for substances that only induce a secretory endometrium, reserving the term *gestagens* for those that also maintain pregnancy. I would like to know whether chlormadinone is a gestagen or a pregestagen.

TABLE A

Progestagens and Gestagens

Compound	Oral administration	
	Clauberg	Pregnancy
Progesterone	0	0
Medroxyprogesterone acetate	+	±
Dimethisterone	+	0
Methylestrenolone	+	+
Ethynylestrenolone	+	0[b]
Methylestrenol	+	+
Ethynylestrenol	+	0
Ethylestrenol	+	±
Allylestrenol	+	+
Propylestrenol	+	0

[a]Criteria: Clauberg test, 1.0 mg total dose activity at least ± Pregnancy, 10.0 mg/day, at least 20% survival of fetuses.

[b]Dosage: 5 mg/day/rat.

Important in the study of side effects is the reported absence of masculinization of fetuses from pregnant rats treated with the substance. Low androgenic activity does not mean that masculinization of the fetus will not occur. We found several substances having very little effect on seminal vesicles and prostate of castrated rats that strongly masculinized fetuses.

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The period of treatment is of extreme importance. Even strongly masculinizing substances were, in our hands, effective only in rats treated during the last 5–10 days of pregnancy, whereas they could be administered during the first 10 days without causing masculinization.

The histological studies are extremely tricky. The presence of a solid vaginal cord, instead of a tube with a lumen, adhering to the urethra is usually considered as masculinization. We found this to be true when it occurs in transverse sections at the level of the pubic symphysis. At lower levels, where the cavernous bodies can be seen, such a situation is normal in newborn female rats.

We think the best proof for the absence of masculinization is a normal capacity for reproduction in offspring of treated mothers. This we showed to be the case after treatment with the nonmasculinizing allylestrenol, whereas after treatment with masculinizing compounds the vagina still ended blind at maturity.

A. BOWERS: I should like to emphasize that, using Dr. Overbeek's nomenclature, chlormadinone is certainly a gestagen. In studies in which it was administered both orally and by injection, it was more active than progesterone by injection in maintaining pregnancy in spayed rabbits.

We are also in full agreement that the precise period of treatment during pregnancy has an important effect on whether or not masculinization of fetuses takes place. In our studies the rats were treated with chlormadinone by oral administration twice daily from the 15th through the 20th day of pregnancy (6 days of treatment). If normal birth did not occur by the 23rd day of pregnancy, the young were delivered by cesarean section and given to foster mothers. Anogenital distances and body weights of the young were obtained at day post-partum. At autopsy on the 21st to 24th day post-partum, the body weight and anogenital distance were measured and anatomical findings were recorded for the clitoris, perineum, vaginal orifice, vagina, and the uterus.

By these criteria no evidence of fetal masculinization was noted with chlormadinone even at dose levels as high as 7.5 mg per day for 6 days. Testosterone, however, causes a very marked fetal masculinization as determined by all the above parameters at a dose evel of 0.5 mg per day.

Antiandrogenic, Antiestrogenic, and Antiovulatory Compounds[1]

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I. INTRODUCTION

The interaction of steroid hormones and related compounds has been known for many years, but it is only within the past few years that this field, particularly that portion which deals with inhibitory actions at the peripheral level, has been developed to a reasonable state. This paper will deal specifically with some of the more established methods and compounds which can interfere with the peripheral action of androgens and estrogens and also with the antiovulatory action of steroids in the mated rabbit. Of particular importance is the fact that these forms of biological activity are not necessarily related to other more classical activities of the steroids. They seem to have an independent existence and may have an important regulatory function in normal biological processes.

II. METHODS

A. ANTIANDROGEN ASSAY METHODS

White leghorn chicks, obtained from Hall Bros. Hatchery in Wallingford, Connecticut, arrived in the laboratory within 1 day of hatching. The various test materials were injected in sesame oil or an aqueous suspension consisting of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxymethylcellulose (0.5%), and benzyl alcohol (0.9%) or inuncted directly on the comb in a sesame oil solution. The detailed procedures are recorded elsewhere (Dorfman, 1962a).

Male albino rats were obtained from the Charles River Breeding Laboratories; they arrived in the laboratory at 21 days of age and were used 4–7 days later. The test compounds, suspended in the same aqueous vehicle as that used for the chick experiments, were injected subcutaneously. If two compounds were administered simultaneously by injection, separate sites were used in order to avoid problems of interaction or absorption delays at the site (Dorfman, 1962a).

[1]Supported in part by Grant CY-2193 of the United States Public Health Service.

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B. ANTIESTROGEN ASSAY METHODS

Swiss albino mice 20–22 days old were injected subcutaneously once daily with estrone for 3 days. The total dose of 0.4 μg was contained in 0.3 ml of sesame oil, 0.1 ml was injected daily. Control groups of mice received only sesame oil. The test substance was injected subcutaneously or by gavage daily in 0.2 ml of aqueous suspending vehicle at a different site from that used for the estrone injections. Separate sites were used for the injection of the estrogen and the test compound to prevent the possibility that a lowered estrogenic effect might be due to interference of estrogen absorption from the injection site. The test compounds were administered in an aqueous suspending medium since many of these substances at the dose levels studied were insoluble in oil. The aqueous suspending medium consisted of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxymethylcellulose (0.5%), and benzyl alcohol (0.9%). One day after the last injections the animals were sacrificed and the uterine weights and body weights were determined.

The assays were usually performed in groups of 132 mice which were divided into 14 groups. One group of 12 mice served as sesame oil-injected control animals, another group of 12 mice received a total dose of 0.4 μg estrone. The remaining 12 groups of 9 mice each received the test compounds. Usually a single compound was studied at three or more concentrations in any specific assay. Finally, in order to arrive at the relative activity of a given compound, all the data for a specific compound at a specific dose were combined. To determine the minimum dose required to produce inhibition, a response was chosen which, when calculated statistically, gave a value of P of less than 0.02 (Dorfman *et al.*, 1961a,b).

C. ANTIOVULATION ASSAY METHODS

Adult estrous female rabbits were injected subcutaneously (or administered by gavage) once with 1 ml of the test compound suspended in the aqueous medium composed of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxymethyl cellulose (0.5%), and benzyl alcohol (0.9%). Eighteen to 24 hours after treatment, the female rabbits were bred. The ovaries were examined for ovulation points 18–24 hours after mating. The results of each treatment group are recorded as the mean number of ovulations per rabbit. The assays were performed under code by Dr. Elva G. Shipley at the Endocrine Laboratories, Madison, Wisconsin, and the λ value for estimation of precision was of the order of 0.25.

III. RESULTS

A. ANTIANDROGENS

1. Antiandrogens Active by Injection in the Chick

Table I summarizes the antiandrogenic activity of various compounds injected into chicks stimulated with a single dose of 0.5 mg of testosterone enanthate (Dorfman, 1962a). In this table the mean comb inhibition is listed as a percentage. For example, norethisterone (17α-ethynyl-19-nortestosterone) was studied five times at the 0.5 mg level with groups of chiks ranging in number from twenty-five to twenty-seven. The percentage inhibitions were 50, 56, 78, 52, and 55, giving a mean

TABLE I

The Antiandrogenic Activity of Various Compounds Administered by Injection to Testosterone Enanthate (o.s.) Stimulated Chicks

Compound	Total Dose (mg)	Number of chicks	Comb inhibition (%)
Norethisterone	0.1	57	38
	0.25	56	40
	0.5	129	58
	1.0	55	63
	1.5	73	45
	2.0	55	66
	4.0	55	67
	4.5	71	50
Ro-2-7239	0.1	56	24
	0.25	56	16
	0.5	127	27
	1.0	56	38
	1.5	72	34
	2.0	28	61
	4.0	56	54
	4.5	74	43
Cortisol	0.5	73	20
	1.5	76	22
	4.5	73	30
Mer-25	0.5	74	6
	4.5	73	16
Progesterone	0.5	76	29
	1.5	74	35
	4.5	74	40

value of 58% for the five groups, representing a total of one hundred and twenty-nine (129) observations.

All compounds under the conditions studied showed antiandrogenicactivity. Mer-25 [1-(*p*-2-diethylaminoethoxyphenyl)-1-phenyl-2-*p*-methoxyphenylethanol], although inactive at the 0.5 mg dose, exhibited minimum activity at 4.5 mg. This makes Mer-25 the least active of the group studied. The test using the compounds herein described, does not permit quantitative evaluations. No true dose response relationships could be established. Norethisterone appears to be the most active antiandrogen studied by this method. At the range of 0.1–0.5 mg comb inhibitions of 38–58% were recorded, whereas Ro-2-7239 (2-acetyl-7-oxo-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydrophenanthrene) gave a lower inhibition range of 16–27% and cortisol and progesterone were 20% and 29%, respectively, at the 0.5-mg dose level.

2. *Antiandrogens Active by Inunction in the Chick*

The comb growth of testosterone enanthate-stimulated chicks may be inhibited by various compounds including Ro-2-7239 and 19-norprogesterone (Table II) and A-norprogesterone, norethisterone. As a rule progestationally active compounds including progesterone and 6-chloro-Δ^6-dehydro-17-acetoxyprogesterone are active in this assay. A set of typical results are presented in Table II.

TABLE II

The Antiandrogen Activity of Ro–2–7239 and 19–Norprogesterone (19 NP) in the Chick

Test material		Testosterone enanthate total dose (mg)	Number of chicks	Mean comb ratio ± S. E.
Designation	Total Dose (mg)			
0	0	0	8	0.25 ± 0.015
0	0	0.5	8	0.64 ± 0.098
Ro 2–7239	1	0.5	10	0.37 ± 0.029
19 NP	3	0.5	10	0.40 ± 0.032

3. *Antiandrogens Active in the Castrated Rat*

Table III presents a typical set of data obtained by this method and illustrates that Ro-2-7239 may inhibit the action of the androgens testosterone and Δ^4-androstene-3,17-dione on both the seminal vesicles and prostate.

TABLE III
Rat Antiandrogen Assay[a]

Material administered	Total dose (mg)	Number of rats	Mean tissue ratio ± S. E		
			Seminal vesicle	Prostate	Levator ani
0	0	7	0.05±0.005	0.07±0.007	0.24±0.031
A	8	6	0.50±0.049	0.72±0.052	0.46±0.027
A R	8 50	7	0.30±0.020	0.42±0.026	0.48±0.053
T	2	7	0.94±0.058	0.96±0.072	0.67±0.045
T R	2 50	7	0.68±0.053	0.65±0.031	0.57±0.026

[a]Conditions of assay: 26–27–day–old castrated rats were injected subcutaneously with 0.5 ml/day for 7 days. Compounds were suspended in aqueous medium. T = testosterone; A = Δ^4–androstene–3, 17–dione; R = Ro–2–7239.

Thirty-two compounds (Table IV), mostly steroids, have been studied for possible antiandrogenic activity in the testosterone-stimulated immature castrated rat using the indicators of prostate, seminal vesicles, and levator ani. Twenty-one compounds were active on the basis of the seminal vesicle response, thirteen compounds inhibited the action of testosterone on the prostate, and seventeen compounds prevented the action of testosterone on the levator ani muscle (Table V). The inactive compounds are listed in Table VI.

TABLE IV
Compounds Studied in a Rat Antiandrogen Assay

I	4,17 (20)–*cis*–Pregnadien–21–oic acid,3,11–diketomethyl ester
II	17α–Hydroxyprogesterone hexanoate
III	17α–Hydroxy–Δ^4–pregnene–3,11,20–trione
IV	2α–Methyl–17β–hydroxy–19–nor–Δ^4–androsten–3–one
V	$\Delta^{4,16}$–Pregnadiene–3,11,20–trione
VI	9α–Chloro–17β–hydroxy–17α–methyl–Δ^4–androstene–3,17–dione
VII	21–Fluoro–Δ^4–pregnene–3,20–dione
VIII	21–Fluoro–11β–hydroxy–Δ^4–pregnene–3,20–dione
IX	17α–Acetoxyprogesterone
X	6α–Methyl–17α–acetoxyprogesterone
XI	Chloresterol
XII	11α–Hydroxyprogesterone
XIII	16,17–Epoxyprogesterone
XIV	19–Norprogesterone
XV	Progesterone

TABLE IV

Compounds Studied in a Rat Antiandrogen Assay

XVI	Pregnenolone
XVII	16,17-Epoxy-21-acetoxy-Δ^4-pregnene-3,20-dione
XVIII	16,17-Epoxy-3β-acetoxy-Δ^5-pregnen-20-one
XIX	Civetone
XX	3β-Hydroxy-Δ^4-pregnen-20-one-20-ethylene ketol
XXI	6-Chloro-Δ^6-dehydro-17-acetoxyprogesterone
XXII	6-Chloro-Δ^6-dehydro-17-hydroxyprogesterone
XXIII	Ro 2-7239
XXIV	A-Norprogesterone
XXV	17α-Hydroxyprogesterone
XXVI	Clomiphene
XXVII	6β-Hydroxytestosterone
XXVIII	Civetolone
XXIX	6α-Chloro-17-acetoxyprogesterone
XXX	Cinophen
XXXI	Aminopyrine
XXXII	Neomycin sulfate

TABLE V

Relative Potency of Various Anti-androgenic Compounds (Relative potency listed in order of decreasing activity)

On basis of prostate			On basis of seminal vesicles			On basis of levator ani		
Compound no.	Minimum dose (% inhibition)		Compound no.	Minimum dose (% inhibition)		Compound no.	Minimum dose (% inhibition)	
XXIII	20	(38)	XXIII	20	(38)	XV	50	(33)
XXI	50	(31)	IV	25	(33)	VII	50	(27)
VI	50	(30)	V	25	(31)	XXI	50	(27)
VII	50	(29)	VI	50	(30)	X	50	(22)
III	50	(27)	XXI	50	(30)	III	50	(20)
VIII	50	(20)	XIV	50	(29)	VIII	50	(20)
V	50	(19)	VIII	50	(29)	II	50	(15)
I	50	(15)	XXII	50	(28)	XXIII	50	(10)
II	50	(14)	VII	50	(26)	XIV	110	(25)
XIII	100	(26)	X	50	(21)	XXIV	150	(59)
XXIV	100	(23)	XV	50	(21)	XII	150	(44)
XII	150	(37)	III	50	(19)	XXXI	150	(38)
XIV	150	(36)	IX	50	(15)	XXII	150	(37)
			XIII	100	(43)	V	150	(33)
			XXIV	100	(31)	XXXII	150	(27)
			XII	150	(49)	I	150	(9)
			XXXI	150	(30)	XIII	200	(18)
			XXXII	150	(30)			
			II	150	(29)			
			XXX	150	(28)			
			I	150	(16)			

TABLE VI

Compounds Inactive in a Rat Antiandrogen Assay

On basis of prostate		On basis of seminal vesicles		On basis of levator ani	
Compound	Dose (mg)	Compound	Dose (mg)	Compound	Dose (mg)
XVI	500	XVI	500	XVI	500
XI	250	XI	250	XI	250
XXV	200	XXV	200	XXV	200
XX	150	XX	150	IV	150
XXII	150	XXIX	150	VI	150
XXIX	150	XVII	125	IX	150
XXX	150	XVIII	125	XXIX	150
XXXI	150	XXVIII	100	XXX	150
IV	150	XXVII	40	XX	150
IX	150	XXVI	25	XVII	125
XXXII	150	XIX	25	XVIII	125
XVII	125			XXVIII	100
XVIII	125			XXVII	40
XXVIII	100			XXVI	25
XXVII	40			XIX	25
XXVI	25				
XIX	25				

Antiandrogenic activity has been described for all three indicators (seminal vesicle, prostate, and levator ani) for Ro-2-7239, 6-chloro-Δ^6-dehydro-17-acetoxyprogesterone, A-norprogesterone, 19-norprogesterone, 11α-hydroxyprogesterone, 9α-chloro-17α-methyltestosterone, 21-fluoroprogesterone, 11-keto-17α-hydroxyprogesterone, 11β-hydroxy-21-fluoroprogesterone, 11-keto-Δ^{16}-dehydroprogesterone, $\Delta^{4,17(20)}$(-*cis*-pregnadien-21-oic acid 3,11-ketodimethyl ester, 17α-hydroxy hexanoate, 16,17-epoxyprogesterone, 6α-methyl-17-acetoxyprogesterone, and progesterone (Dorfman, 1962b).

B. ANTIESTROGENS

1. Immature Mice

Antiestrogenic activity of C_{19} modified steroids has been studied both by injection (Dorfman *et al.*, 1961a) and by gavage (Dorfman *et al.*, 1961b). In this group the antiestrogenic activity, with one exception, was significantly greater when administered by subcutaneous injection than by the oral route. Some of these differences are indeed striking (Table VII). For example, 17β-hydroxyandrostan-3-one was 40 times more active by injection and 17α-methyl-19-nor-17β-hydroxyandrostan-3-one was 20 times more active by the same route. One exception

was 17α-methyl-19-nortestosterone, which produced a minimum effect at 32 μg subcutaneously and a similar effect at 40 μg by gavage. This

TABLE VII

The Comparative Antiestrogenic Activity of Various Steroids by Subcutaneous Injection SI and by Gavage

Steroid	Minimum dose to produce inhibition μg		Maximum inhibition %	
	SI	G	SI	G
17α-Ethyl-19-nortestosterone	8	40	54	43
17α-Ethynyl-19-nortestosterone	16	32	56	40
17α-Methyl-19-nortestosterone	32	40	46	44
17α-Methyltestosterone	32	250	35	35
17α-Methyl-19-nor-17β-hydroxyandrostan-3-one	50	1,000	38	24
17β-Hydroxyandrostan-3-one	100	4,000	30	24
17α-Ethyl-19-nor-17β-hydroxyandrostan-3-one	250	—	27	—
Androstane-3β, 17β-diol	250	1,000	22	36
Testosterone	500	4,000	36	10
19-Nor-17β-hydroxyandrostan-3-one	500	4,000	32	36
19-Nortestosterone	500	1,000	33	17
17α-Ethynyl-19-nor-17β-hydroxyandrostan-3-one	500	4,000	27	12
17α-Vinyl-19-nor-17β-hydroxandrostan-3-one	500	>10,000	20	—
17-Ethynyltestosterone	500	>4,000	22	—
17α-Ethynyl-19-norandrostan-3β, 17β-diol	1,000	500	14	34
19-Norandrostane-3β, 17β-diol	> 4,000	4,000	—	20

difference in dosage is not significant. This may also be true for 17α-ethynyl-19-nortestosterone, the subcutaneous dose of 16 μg and gavage dose of 32 μg being perhaps not significantly different. On the other hand, the relative potency of 17-ethyl-19-nortestosterone by the two routes shows a ratio of 5 for subcutaneous to gavage administration efficiency.

When enhancing ratios for certain structural modifications were compared for the subcutaneous and gavage routes, interesting and surprising differences were observed. As indicated in Table VIII, the dihydro (5α) derivative of testosterone is equal to that of testosterone by gavage, but by subcutaneous injection the dihydro derivative was five times more active.

TABLE VIII

Comparative Enhancing Ratios as a Result of Δ^4–Reduction (5α) by Subcutaneous Injection (SI) and Gavage (G)

Steroid	Enhancing ratio	
	SI	G
Testosterone	5	1.0
19–Nortestosterone	1	<1
17α–Methyltestosterone	—	0.13
17α–Enthynyltestosterone	—	—
17α–Methyl–19–nortestosterone	0.5	—
17α–Ethynyl–19–nortestosterone	0.03	—

Excellent agreement for the two routes is seen for structural changes involving loss of carbon 19 for 17α-ethynyltestosterone and 17α-ethyltestosterone. The enhancing ratios by the two routes were quite similar for these compounds (Table IX). This was not the case for the other

TABLE IX

Comparative Enhancing Ratios as a Result of Loss of C–19 (Formation of 19–Nor Derivative) by Subcutaneous Injection (SI) and Gavage (G)

Steroid	Enhancing ratio	
	SI	G
Testosterone	0.25	4
17α–Methyltestosterone	1.0	6.5
17α–Ethyltestosterone	62	>100
17α–Ethynyltestosterone	31	40
Androstane–3β, 17β–diol	0.03	0.25
17β–Hydroxyandrostan–3–one	0.2	1.0

four compounds and their corresponding 19-nor derivatives. In all other instances a significantly higher enhancing ratio was found by gavage. The enhancing ratios were as high as 16 for testosterone in favor of gavage, and the smallest advantage was found for androstane-3β,17β-diol, which was as high as five.

Essentially similar enhancing ratios for both the subcutaneous and oral routes were found for all the 17α-alkyl substituted derivatives of 19-nortestosterone and for 17α-methyltestosterone (Table X). These compounds can also be characterized by the high individual enhancing ratios per se. Contrariwise, no change could be detected for the 17α-ethynyl derivative of 19-norhydroxyandrostan-3-one. Values of unity for both the injection and gavage studies were recorded. Striking differences between the oral and injection routes were found in a number

TABLE X

Comparative Enhancing Ratios as a Result of 17α-Methyl, 17α-Ethyl, and 17α-Ethynyl Substitution Studied by Subcutaneous Injection (SI) and Gavage (G)

Steroid	17α-Methyl		17α-Ethyl		17α-Ethynyl	
	SI	G	SI	G	SI	G
Testosterone	7.8	16	1	<1	1	1
19-Nortestosterone	15.6	25	62	25	31	31
19-Nor-17β-Hydroxyandrostan-3-one	7.8	—	2	—	1.0	1.0
17β-Hydroxyandrostan-3-one	0.2	2	—	—	—	—
Δ^5-Androstene-3β, 17β-diol	>2	<0.25	—	—	—	—
19-Norandrostane-3β, 17β-diol	>1	0.1	—	—	>4	2.0

of instances, with usually the higher enhancing ratio found for the injection route. Tenfold differences in favor of the injection route were observed for the 17α-methyl derivatives of the following steroids: 17β-hydroxyandrostan-3-one, Δ^5-androstene-3β,17β-diol, and 19-noran-

TABLE XI

The Antiestrogenic Activity of Derivatives of Progesterone

Derivative of progesterone	Total number of mice	Dosage range studies (μg)	Minimum dose to produce inhibition (μg)	Maximum inhibition (%)
Progesterone	159	10–4000	250	56
6α, 16α-Dimethyl-	117	10–500	10	60
6α-Fluoro-16α-methyl	95	5–2000	10	58
6β, 16α-Dimethyl-	138	5–2000	20	53
17α-Acetoxyprogesterone	102	1–3000	25	39
6β, 16α-Dimethyl-(5α)-hydroxy-	27	10–100	30	18
6β-Fluoro-16α-methyl-	53	25–2000	50	44
6α-Chloro-	54	1–500	100	46
6α, 16-Dimethyl-	52	10–500	100	32
6α-Methyl-	27	5–100	100	20
4-Hydroxy-5α-fluoro-	27	5–100	100	22
6α, 17α-Dichloro-	67	50–2000	125	45
19-Norprogesterone	94	5–1000	180	45
4-Methyl-	39	10–500	Negative	
11, 12-Dibromo-	16	50–1000	Negative	

drostane-3β,17β-diol. A similar but less intense effect was recorded for 19-norandrostane-3β,17β-diol and its 17α-ethynyl derivative.

The antiestrogenic activity of progesterone derivatives by subcutaneous injection is illustrated in Table XI. Among the most potent antiestrogen derivatives of progesterone were 6-; 6-and 16-; and 6-and 17-substituted variants where the substituting group was chlorine, fluorine, or methyl. 17α-Acetoxyprogesterone was a highly active antiestrogen. 19-Norprogesterone was reasonably active.

2. *Antiestrogen and Antimammary Tumor Activity*

Seventeen steroids which were previously shown to have antimammary tumor activity in one or more systems in rats or mice were studied as to their ability to inhibit estrone-induced uterine growth in the immature mouse. Antiestrogenic activity was not a prerequisite for antitumor activity in either the testosterone propionate-sensitive or testosterone propionate-resistant rat mammary tumor systems. A relatively highly active antiestrogenic compound was inactive in the mouse mammary adrenocarcinoma test. It is concluded from these studies that there is no necessary correlation between antiestrogenic activity of a steroid and its antimammary tumor activity.

C. ANTIOVULATORY COMPOUNDS

Thirty-six steroids were studied by subcutaneous injection in a rabbit antiovulatory test which depends upon the suppression of luteinizing hormones at the pituitary level, the direct interference with the peripheral action of the luteinizing hormone on the ovary, or a combination of both.

The steroid 19-norprogesterone was the most active antiovulatory steroid found. It was judged to be about 150 times more active than progesterone. The highly active progestational agents, 6-chloro-Δ^6-dehydro-17α-acetoxyprogesterone, 6-chloro-Δ^{16}-bisdehydro-17α-acetoxyprogesterone, 6α-chloro-Δ^1-dehydro-17α-acetoxyprogesterone, and Δ^6-dehydro-17α-acetoxyprogesterone were highly active antiovulatory agents having potency ratios compared to progesterone of 30, 8, 5, 2.8, $\geqq$ 2, and 2. The antiovulatory activity of subcutaneously administered steroids was not necessarily related to its progestational activity or its antigonadotropic activity as determined in a parabiosis assay.

Thirty-seven steroids were studied by oral administration using essentially the same assay conditions as those previously described for the injection assay involving the mated estrous rabbit. Norethisterone served as the reference standard and a dose response curve was established between 0.31 and 1.25 mg doses of the compound. Nine highly active antiovulatory compounds have been found and are listed in decreasing order of potency, norethisterone having the arbitrary value of 1: 6-

chloro-Δ^6-17α-acetoxyprogesterone (35), 6α-methyl-Δ^1-dehydro-17α-acetoxyprogesterone ($\geq$ 10), 6-fluoro-Δ^6-dehydro-17α-acetoxyprogesterone (5), Δ^6-dehydro-17α-acetoxyprogesterone ($\geq$ 3), 6α-methyl-17α-acetoxyprogesterone (2.6), 6-chloro-Δ^{16}-bisdehydro-17α-acetoxyprogesterone ($\geq$ 2), 2-hydroxymethyl-17α-methyl-17β-hydroxyandrostan-3-one ($\geq$ 2), and 6α-fluoro-16α-methyl-17α-acetoxyprogesterone ($\geq$ 1.25).

The antiovulatory activity of orally administered steroids was not related necessarily to the progestational activity of a compound nor to the antigonadotropic activity as measured in parabiotic rats.

IV. SUMMARY AND CONCLUSIONS

Reliable bioassay procedures for the detection of antiestrogenic, antiandrogenic, and antiovulatory activity of steroids and related compounds have been developed and are herein described. The nature of the antiestrogenic and antiandrogenic assays are such that it seems likely that the inhibitors act by blocking the stimulating action of the hormones at the target tissue level. A start has been made on the relationship between high activity of antiestrogens and structure in the steroid series. Similar initial and crude relationships have been described for antiandrogens. Of particular importance is the fact that both antiandrogenic and antiestrogenic activity of steroids are not necessarily related to any known classical steroid activity of this group of compounds.

High antiovulatory activity was studied principally in progestationally active steroids, but the results presented in this paper strongly indicate that progestational activity is not necessarily correlated with antiovulatory activity. The test employed in the studies reported here would rate as biologically active those compounds which interfere with the suppression of the luteinizing hormone at the pituitary level, the direct interference with the peripheral action of the luteinizing hormone on the ovary, or a combination of both effects.

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DISCUSSION

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In respect to the structure-activity relations of steroidal compounds, we have tried to find some correlation which might exist between hormonal and feedback activity. The results of our experiments with parabiotic mice indicate that the antigonadotropic activity of natural sex steroids can be listed in the order estrogen, androgen, and progestin. When synthetic steroids are tested, their hormonal activities do not necessarily reflect their antigonadotropic potencies. Furthermore, the anesthetic activity of natural steroids are in the order progestin, cortexone, androgen, and estrogen. This is quite the reverse of the antigonadotropic potency. Progestational activity, however, is not related with either anesthetic or hypnotic activity. For example, an extremely potent progestin, 6-chloro-Δ^6-dehydro-17α-acetoxyprogesterone, is about 40 times as active as norethisterone by mouth in the Clauberg assay and 4 times as active as progesterone by subcutaneous route in the mouse deciduoma test, but it produces no anesthetic activity at all in mice, and its antigonadotropic activity in our parabiotic mice is much lower than that of norethisterone and almost equal to that of progesterone. Nevertheless, this compound is markedly active in the rabbit antiovulation test, 35 times as active as norethisterone in the data of Dr. Dorfman. Hence the steroid activities on the central nervous system, including the hypothalamopituitary level, may be independent from their hormonal activities on the target organs. These findings are in accord with the conclusion of Dr. Dorfman, who emphasized that the antiovulatory activity was not necessarily related to the progestational activity nor to the antigonadotropic activity in the parabiotic rats. This illustrates troublesomeness and complexity of the bioassay program for the characterization of steroidal compounds; on the other hand, it gives us hope that further dissociation of the steroid activities can be achieved by suitable modification of the steroid molecule.

The mechanism of gonadotropin secretion is considered to be the manifestation of a complex sequence of neurohumoral transmissions regulating the production, storage, and release of each active component, FSH and LH. Further, the ovarian reactivity to gonadotropin also is an important step in the ovulation mechanism. These many factors may be affected by certain steroids, selectively or nonselectively. It is urged, therefore, that analytical determination of antiovulatory activity is necessary for better characterization of steroidal compounds.

Another point to be noted is that antiestrogenicity of the steroid is not related with its antimammary tumor activity. We have been informed that the case of Δ^1-testololactone is similar; this compound, although neither androgenic nor antiandrogenic and almost devoid of any hormonal activity, seems to be effective on a certain type of human prostatic cancer.

May I ask Dr. Dorfman a few questions on the antiandrogen and antiestrogen. Pregnandiol is reported to have antideciduomatogenic activity in rats, and it is slightly antiprogestational when administered locally into the rabbit uterus, according to our recent study. Thus pregnandiol may be a natural antiprogestin, besides estrogen. Es-

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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triol is widely known as an antiestrogen, besides progestin. I wonder whether you could suggest any androgen metabolite with antiandrogenic activity.

The second question is this: In your assay, you get only partial inhibition without regular dose-response relations. According to our experience, as well as to data of other investigators, it is difficult to determine the antiestrogenicity of steroidal compounds because some of them have antiestrogenicity along with estrogenicity and their manifestations depend upon the experimental conditions. However, in our tests, a nonsteroidal compound, Mer-25, has so far been consistent in its antiestrogenic effect and has exerted 100% inhibition when the dosage is increased. Have you also found this to be the case? If this is true, how do you explain this difference between Mer-25 and steroidal antiestrogen.

RALPH I. DORFMAN: We have not yet found an androgen metabolite which has significant antiandrogenic activity in the chick or rat assay.

It is true that Mer-25 has produced 100% antiestrogenic effects whereas our steroid antiestrogens usually produce maximum effects of about 60%. However, in many instances we have not used as high doses of the steroid as we have of Mer-25; we have not yet studied this point thoroughly.

The Actions of "Retro" Progestagens

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In a previous communication (Reerink *et al.*, 1960) a new kind of steroid hormone analogue was announced, the so-called "retro" steroid. Reerink and Westerhof synthesized steroids in which the stereochemical configuration was altered by reversing the spatial position of the substituents at carbon atom 9 (C-9) and carbon atom 10 (C-10). The angular methyl group at C-10 is in the α-position, whereas the hydrogen atom attached to C-9 is in the β-position. One of the compounds of the retro series, 6-dehydro-retro-progesterone (Fig. 1), is commercially available as an orally active progestational compound.

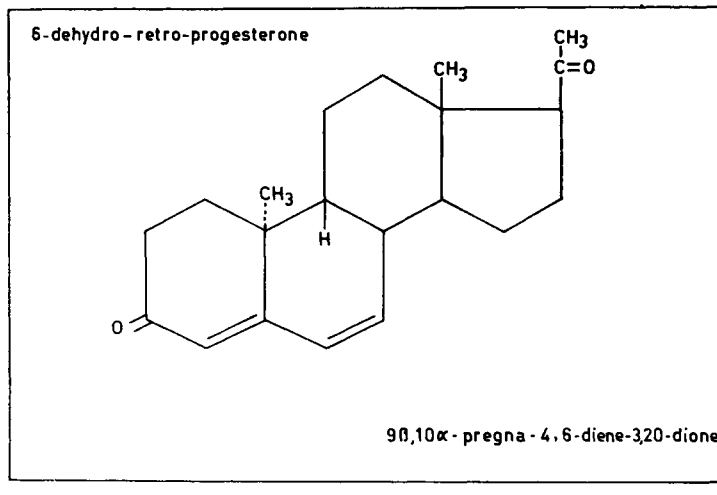

FIG. 1. Structural formula of 6-dehydro-retro-progesterone.

First a description is given of one of the outstanding features of this compound: the pregnancy-maintaining effect after ovariectomy in rats.

In the rat, bilateral ovariectomy performed during the first half of pregnancy results in termination of gestation. When castration is per-

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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formed during the second half of pregnancy, abortion occasionally does not occur because of the capacity of placentas to produce some progesterone and estrogen.

Abortion or resorption, which generally follows castration, is accepted to be caused by inadequate production of progesterone and estrogen.

It has been shown by Stucki (1958) (see Table I) that, in rats castrated on the 8th day of pregnancy, the pregnancy can be successfully maintained by subcutaneous injections of sufficient quantities of progesterone and estrone.

TABLE I

Maintenance of Pregnancy in Rats Castrated on 8th Day of Pregnancy[a]

Compound	Daily doses: Compound (mg)	Daily doses: Estrone (μg)	Successful pregnancies per number of "tries"	Live young per successful pregnancy	Net success index
Progesterone	8		5/5	4	36
	4		4/5	2.5	18
	2		1/5	2	4
Progesterone	8	1	4/5	5.5	40
	4	1	5/5	6.8	62
	2	1	5/5	3.6	33
	1	1	2/5	4.5	16

[a]From Stucki (1958)

Subcutaneous injections of 0.625 mg of 6-dehydro-retro-progesterone twice daily in the same set-up of the experiment resulted in complete protection of young and maintenance of pregnancy.

Table II shows that, just as in the experiments with progesterone performed by Stucki, the pregnancy-maintaining activity of the compound showed considerable improvement after concomitant administration of estradiol, a substance inactive by itself.

TABLE II

Maintenance of Pregnancy in Rats Castrated on 10th Day of Pregnancy

Combination administered[a]: Estradiol monobenzoate[a]	Combination administered[a]: 6-Dehydro-retro-progesterone[a]	Number of females with live young	% Live young
0	625	4/5	86
0.5	4	0/3	0
0.5	40	3/3	70

[a]Values are number of micrograms administered subcutaneously twice daily.

TABLE III

Maintenance of Pregnancy in Ovariectomized Rats after Oral Administration of Various Combinations of 6–Dehydro–retro–progesterone and Ethynylestradiol

Combination administered[a]		Number of females with live young	% Live young	Combination administered[a]		Number of females with live young	% Live young
Ethynyl-estradiol	6–Dehydro–retro-progesterone			Ethynyl-estradiol	6–Dehydro–retro-progesterone		
0	0	1/10	1	2	78	1/4	13
0.5	0	0/6	0	2	156.25	2/5	25
0	5000	0/6	0	2	312.5	3/4	75
0	40000	0/3	0	2	625	4/5	72
0.125	2500	1/5	3	2	1250	5/5	98
0.25	156.25	1/4	20	4	78	1/4	12
0.25	312.5	5/5	36	4	156.25	4/6	45
0.25	625	4/5	36	4	312.5	5/5	81
0.25	1250	2/5	33	4	625	4/4	100
0.25	2500	3/5	41	4	2500	2/2	100
0.5	78	1/4	19	8	156.25	1/3	3
0.5	156.25	3/6	47	8	312.5	3/4	24
0.5	312.5	7/7	89	8	625	4/4	100
0.5	625	6/6	72	8	1250	5/5	97
0.5	1250	5/5	91	16	312.5	2/5	17
0.5	2500	5/5	86	16	625	3/4	46
1	78	3/5	16	16	1250	2/3	52
1	156.25	2/6	26	16	10000	2/3	55
1	312.5	5/5	85	32	625	3/4	49
1	625	7/7	90	32	1250	4/5	66
1	1250	5/5	93	32	2500	4/4	90
1	2500	5/5	95	64	1250	2/4	30
				64	2500	3/5	37
				64	40000	2/3	55

[a]Values are number of micrograms administered orally twice daily.

Oral administration of 6-dehydro-retro-progesterone in dosages up to 40 mg daily failed to maintain pregnancy in spayed rats. When, however, ethynylestradiol was added to the regimen, extremely low dosages could maintain pregnancy when drug administration was started the day before castration on the 10th day of pregnancy, as shown in Table III.

A graphic representation of these data is shown in Fig. 2. Curves were fitted to these data according to preliminary statistical calculations (see below). The lines represent the percentages of living fetuses after oral

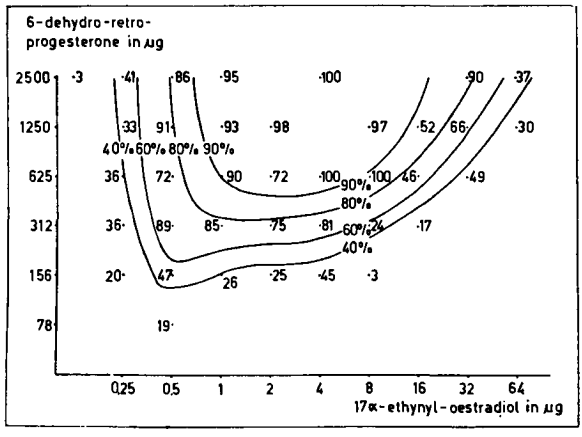

FIG. 2. Percentages of live fetuses after oral administration of 6-dehydro-retro-progesterone and after addition of 17α-ethynylestradiol to the regimen of spayed rats.

administration of various dosages of ethynylestradiol and 6-dehydro-retro-progesterone. As this figure shows, addition of a larger quantity of ethynylestradiol than the favorable ratio results in interference with the pregnancy-maintaining effect.

A number of steroids have been defined as progestins on the basis of their ability to produce a secretory endometrium in the uterus of the estrogen-primed immature rabbit. Although development of a secretory endometrium appears to be associated with the process of implantation, it cannot be assumed a priori that such compounds are able to maintain pregnancy. 17α-Ethynyl-19-nortestosterone, for example, shows high potency in the so-called Clauberg test, but has, as Stucki reported, no pregnancy-maintaining effect in spayed rats. Neither could the addition of ethynylestradiol produce any better effect. The hypothesis was tested that in this compound the built-in estrogenic effect destroys the pregnancy-maintaining ability according to the following principles, if the pregnancy-maintaining effect of a certain combination of doses of two compounds, one progestational and one estrogenic is measured on the basis of the (average) percentage of living young (after

sectio in uterus), this percentage can be related quantitatively to the amounts actually given (cf. Table III, Fig. 2.). The relation between the percentages alive, transformed to probits, and the doses of 6-dehydro-retro-progesterone and ethynylestradiol can be described adequately by a mathematical function

$$Y = f(x_1, x_2) \tag{1}$$

where Y = probit value corresponding to percentage living young, x_1 = dose of 6-dehydro-retro-progesterone, and x_2 = dose of ethynylestradiol.

The results obtained in a series of experiments with various combinations of doses of both compounds were analyzed statistically in order to determine the explicit form of the function $f(x_1, x_2)$. Various forms were tried, and it turned out that a reasonably close approximation was obtained with an equation of the form

$$Y = a_0 + \frac{a_1}{x_1} + a_2 \log x_2 + a_3 (\log x_2)^2 + a_4 \frac{\log x_2}{x_1} + a_5 \frac{(\log x_2)^2}{x_1} + a_6 \frac{x_2}{x_1^2}$$

The coefficients a_i were estimated by the method of maximum likelihood, allotting proper weights to the probit values (cf. Finney, 1952). From this preliminary equation (Eq. 2) the contour lines in Fig. 2 could be constructed.

In addition to describing the relation between percentage of living young and doses of 6-dehydro-retro-progesterone and ethynylestradiol, Eq. 2 can be used to estimate the pregnancy-maintaining potency as well as the estrogenic activity of progestational compounds, from suitable experiments. If the pregnancy-maintaining potency of a certain compound is denoted by θ_1 (in terms of 6-dehydro-retro-progesterone on a weight basis) and its estrogenic activity by θ_2 (in terms of ethynylestradiol, also on a weight basis), the values of θ_1 and θ_2 are estimated by iterative computations as follows.

The percentages of living young are observed at various doses of the test-compound as such, and in combination with a number of known doses of 6-dehydro-retro-progesterone and ethynylestradiol.

These experiments are concentrated preferably within the region of percentage responses between 10 and 90%. As a running check on the applicability of Eq. 2, these percentages are observed simultaneously at predetermined dose combinations of 6-dehydro-retro-progesterone and ethynylestradiol. Starting with initial guesses of θ_1 and θ_2, preferably close to the "true" values, improved estimates are obtained iteratively according to the principle of weighted least squares as applied to the (working) probit values corresponding to the observed percentages of living young (cf. Finney, 1952).

A more detailed account of these methods of computation will be submitted for publication elsewhere by R. van Strik. Table IV shows the data based on addition of 6-dehydro-retro-progesterone to 17α-et-

TABLE IV

Maintenance of Pregnancy after Ovariectomy in Rats with Orally Administered 17α–Ethynyl–19–nortestosterone (I) and 6–Dehydro–retro–progesterone (II)

Dosis (2 × daily)		Observed percentage	Calculated percentage[a]
I (μg)	II (μg)		
625	312.5	79.55	72.24
1250	—	0.00	0.00
1250	156.25	5.00	57.93
1250	312.5	76.67	82.38
2500	—	0.00	0.00
2500	156.25	0.00	52.79
2500	312.5	72.32	82.64
5000	312,5	11.54	78.52
5000	1250	97.92	94.95

[a]Calculated with: estrogenic activity = 0·0008 × ethynylestradiol, pregnancy–maintaining activity = 0·01 × 6–dehydro–retro–progesterone.

hynyl-19-nortestosterone. It is clear that the combined administration was able to maintain pregnancy. This can only mean that in 0.625 mg of 17α-ethynyl-19-nortestosterone the estrogenic activity is sufficient to produce a favorable ratio for 6-dehydro-retro-progesterone. But it also means that the pregnancy-maintaining activity of 17α-ethynyl-19-nortestosterone was very low, since it was not able to increase the pregnancy-maintaining effect of 6-dehydro-retro-progesterone. Calculations based on the mathematical function of which the graphical representation was shown, gave the following results: 1.25 mg 17α-ethynyl-19-nortestosterone is approximately equal to 1 μg ethynylestradiol and about 1/100 of the pregnancy-maintaining activity of 6-dehydro-retro-progesterone.

In order to check this test on another compound, we also tested 3-deoxy-17α-allyl-19-nortestosterone, a compound which is able to maintain pregnancy by itself if administered in high dosages. Table V shows that this compound also has an inbuilt estrogenic activity, 2.5 mg being approximately equal to 1 μg ethynylestradiol. The pregnancy-maintaining effect of this compound has approximately one-tenth the activity of 6-dehydro-retro-progesterone. The results of our test and the statistical calculation based on it showed that dosages of 5 mg/daily and more of 3-deoxy-17α-allyl-19-nortestosterone would be able to maintain pregnancy after ovariectomy, a finding that is in agreement with the data published in the literature. The conclusion may be drawn from these test data that 6-dehydro-retro-progesterone is one of the strongest pregnancy-maintaining compounds available.

Finally the pharmacological effect of a new retro steroid, 17α-(2′-me-

TABLE V

Maintenance of Pregnancy after Ovariectomy in Rats with Orally Administered 3–Desoxy–17α–allyl–19–nortestosterone (I), 6–Dehydro–retro–progesterone (II), and Ethynylestradiol (III)

Dosis (2×daily)			Observed percentage	Calculated[a] percentage
I (μg)	II (μg)	III (μg)		
156.25	312.5	0.5	66.25	75.80
312.5	—	—	0.00	0.00
312.5	312.5	—	0.00	0.78
312.5	—	0.5	0.00	0.00
315.5	312.5	0.5	43.06	78.52
625	—	—	10.00	2.56
625	156.25	—	23.12	30.50
625	312.5	—	31.25	38.97
625	—	0.5	0.00	1.54
625	312.5	0.5	62.43	82.64
1250	—	—	26.64	38.59
1250	156.25	—	61.18	69.85
1250	312.5	—	59.58	77.04
1250	—	0.5	0.00	36.69
2500	—	—	41.32	75.80
40000	—	—	100.00	91.62

[a]Calculated with: estrogenic activity = 0·0004 × ethynylestradiol; pregnancy maintaining activity = 0·1 × 6–dehydro–retro–progesterone.

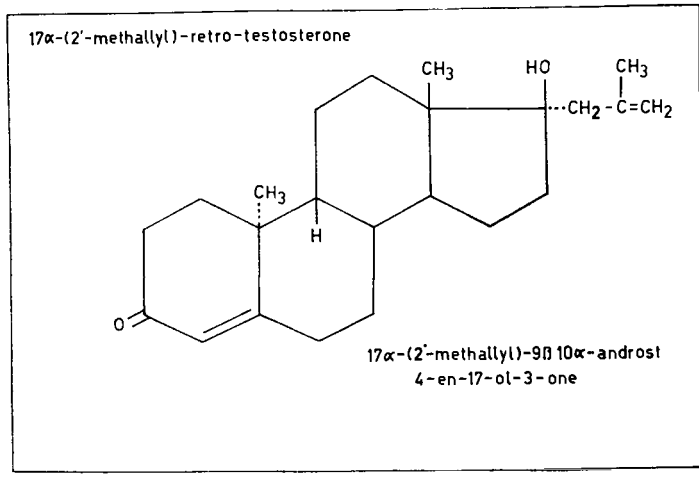

FIG. 3. Structural formula of 17α-(2'-methallyl)-retro-testosterone.

thallyl)-retro-testosterone, is discussed; the structural formula is shown in Fig. 3.

In the estrogen-primed infantile female rabbit in the Clauberg test, this compound shows a marked progestational effect as well after subcutaneous as after oral administration. However, the secretory transformation was not comparable to that in the normal pregnant rabbit or to that in rabbits injected with progesterone or 6-dehydro-retroprogesterone. The endometrium was more decidua-like in appearance. It was therefore not surprising that 17α-(2′-methallyl)-retro-testosterone was highly active in deciduomata formation (see Table VI). No

TABLE VI

Deciduoma test on Castrated Female Mice[a]

Substance	Mean weight in (gm)		Traumatized horn (mg)	Control horn (mg)	Increase over control horn (mg)
	Begin	End			
Peanut oil	16.0	18.4	9.6	5.2	4.4
17α-(2′-methallyl)-retro-testosterone[b] 2 mg	17.4	18.8	41.5	9.1	32.4
1 mg	15.9	16.7	31.2	8.9	22.3
0·5 mg	15.8	18.4	29.4	10.2	19.2
0·25 mg	19.1	17.5	32.8	11.8	21.0

[a]Means of 8 animals.
[b]Twice daily.

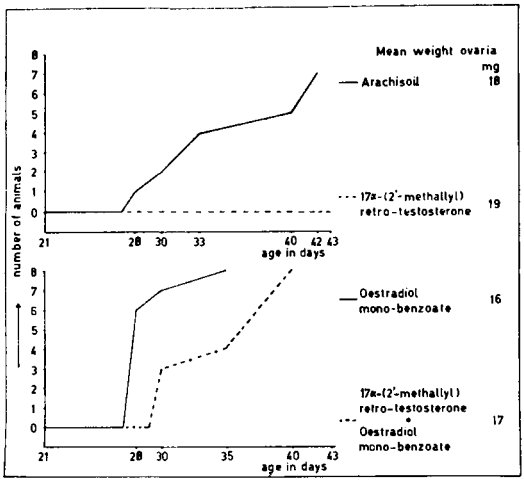

FIG. 4. Effect on time of vaginal opening in infantile female rats of 17α-(2'-methallyl)retro-testosterone as compared with effect produced by estrogens.

estrogenic activity was found in this compound, but a rather strong antiestrogenic effect could be observed. This was the case not only in the antiuterotropic test (all progestational compounds have such an effect), but also in the vaginal opening test on infantile female rats.

Figure 4 shows the data obtained in a test on infantile female rats 21 days old. Normally the vagina of our infantile rats opens at approxi-

mately 6 weeks of age. In this case, 7 out of 8 rats in the control group had an open vagina at 42 days of age. None of the 8 rats injected with 2 mg of 17α-(2′-methallyl)-retro-testosterone every other day had an open vagina at 42 day. A histological inspection of the ovaries showed no formation of corpora lutea.

After combined injections of 17α-(2′-methallyl)-retro-testosterone and estradiol monobenzoate every other day, opening of the vagina was delayed as compared to the time of opening in rats injected with estradiol monobenzoate alone. These results indicate that 17α-(2′-methallyl)-

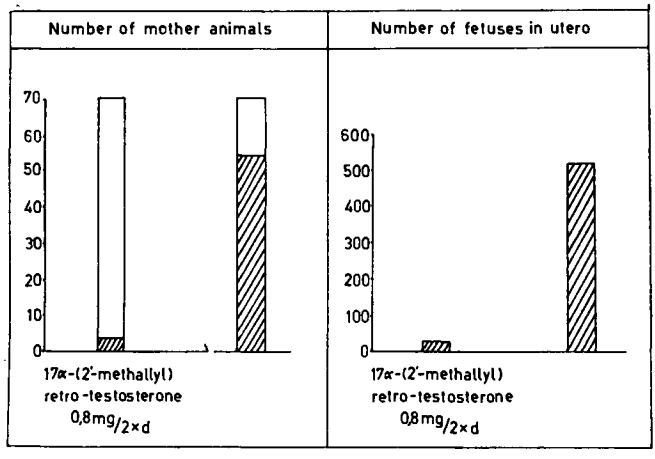

FIG. 5. Fertility-inhibiting effect of 17α-(2'-methallyl)-retro-testosterone in normally mature female rats.

retro-testosterone has an antagonizing effect on the production of endogenous estrogens in the rat and also that injected estrogens are antagonized in their effect. In rabbits this compound also has an ovulation-inhibiting effect if injected 10 hours before copulation takes place. The effect on fertile matings in rats showed this compound to be a strong inhibitor of fertility, as shown in Fig. 5. Female rats, 72 in each group, were injected once daily with 0.8 mg of 17α-(2′-methallyl)-retro-testosterone, or with the blank solution. After the third day, one male rat was admitted to two female rats. This male rat was not injected, but injections of the female rats were continued. After the 9th day the male rat was removed; the female rats received injections for another 3 days.

In the group receiving 17α-(2′-methallyl)-retro-testosterone, 3 female rats out of 72 had normal pregnancies with rather small litters, whereas in the control group, 53 out of the 72 became pregnant. Rather to our surprise, this compound proved to have no effect on the nidation of the fertilized ovum of the rat.

In a test in which 17α-(2′-methallyl)-retro-testosterone was injected in a dosage of 1 mg in female rats with sperm in the vaginal smear from day 1 to day 3 of pregnancy, or from day 2 to day 4, day 3 to day 5, or day 4 to day 6, no effect could be observed on the percentage of living fetuses.

In a preliminary study it was shown that, after subcutaneous injection, 17α-(2′-methallyl)-retro-testosterone has a pregnancy-maintaining effect after ovariectomy at a dosage of at least 1 mg/daily.

No androgenic effect on seminal vesicles and ventral prostates could be observed in infantile or castrated male rats (Table VII). Nor could we

TABLE VII

Test on Androgenic Activity[a]

Substance	Weight in (gm)		Testicles (mg)		Seminal vesicle (mg)	Ventral prostate (mg)	Levator ani (mg)
	Beginning	End	L.	R			
			Infantile ♂ rats				
Peanut oil	54	78	373	367	18,7	28,2	16,8
Testosterone, 100 μg	50	75	300	298	69,2	53,8	31,7
17α (2′–methallyl) retro–testosterone, 2 mg	53	71	344	343	14,9	21,2	14,9
17α–(2′–methallyl) retro–testosterone + testosterone, 2 mg + 100 μg	48	71	203	199	49,9	41,9	29,0
			Castrated ♂ rats				
Peanut oil	138	153	—	—	11,2	6,5	24,5
Testosterone, 50 μg	135	162	—	—	42,2	21,9	45,0
17α–(2′–methallyl) retro–testosterone, 2 mg	134	155	—	—	11,5	4,5	25,5

[a]Substances were administered twice daily to each animal at the dosage shown in columm

TABLE VIII

Teston masculinization of female fetus with, 17α–(2′–Methallyl)–retro–testosterone[a]

Total number of mother animals	Total daily dose (mg)	Anogenital distance (mm)			Total number of live fetuses
		< 2	2–3	> 3	
6	10	35	0	26	61

[a]After histological inspection no masculinizing effect on clitoris or tractus genitalis.

detect any masculinizing effect on the female fetuses (Table VIII). Further studies with this compound are in progress, but from the data presented it is clear that also with this member of the retro-steroid series very interesting and promising results are obtained.

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DISCUSSION

Possible Uses of Retrosteroid Derivatives in Humans

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It is difficult to say how far it is justifiable to extrapolate the pharmacological propreties of a steroid compound, as determined on experimental animals, to the human subject. For instance, Dr. Schöler has shown that 6-dehydroretroprogesterone inhibits ovulation in rabbits. My Swedish colleagues, E. Diczfalusy, K. G. Tillinger, and U. Borell, and I have found that it does not inhibit ovulation in women, though it does prevent dysmenorrhea. It will be desirable, therefore, to determine whether methallylretrotestosterone inhibits ovulation in women.

Dr. Schöler has emphasized that this compound is strongly antiestrogenic, as shown by the prevention of vaginal opening in infantile rats. It should be easily possible to test this property by studying its effect on the vaginal cytology in women.

If it is found to be antiestrogenic in women, how can we best employ this property therapeutically? I would suggest that: (1) it might be found to be able to arrest uterine hemorrhage in a metropathic bleeding episode by lowering the level of circulating estrogen; (2) it might prevent the painful premenstrual breast swelling in cases of mammary fibroadenosis; (3) it might prevent the development of the typical properties of the "ovulatory" cervical mucus, and that this may be useful as a contraceptive procedure, especially if it is found not to inhibit ovulation.

Dr. Schöler has shown that methallylretrotestosterone maintains pregnancy by producing a secretory endometrium, by inducing deciduomata, and by preventing absorption or abortion of the fetus in the castrated pregnant rat. He has shown that combination with estrogen in the correct ratio improves the pregnancy-maintaining properties of 6-dehydroretroprogesterone. Does this suggest that the ideal physical pregnancy maintaining compound in the human female should be a combination of an estrogen and a progestogen in the correct ratio?

If one believes that lack of endogenous progesterone is a fundamental factor in habitual and threatened abortion, how important is it to employ a "rat pregnancy-maintaining gestogen" in the prophylaxis or treatment of these conditions? If it is important, then this compound might have an advantage over other synthetic progestogens.

Finally it is fascinating to a clinician that these retrosteroid derivatives appear to be active progestogens with no androgenic properties.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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Biological Properties of 4-Hydroxy-3-Keto-Δ^4-Steroids

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The important role of the Δ^4-3-keto group in the steroid molecule makes very interesting, the study of the effect of introducing a hydroxyl group at C-4. This substitution at C-4 may:

1. Modify the steric configuration of a highly specific region of the steroid molecule; C-4 is a key position which conditions the biological response of many target organs and regulates the interactions between the steroid and cellular enzymes and coenzymes.
2. Change the metabolic fate of the steroid by inhibiting, favoring, or in some way changing the reactions that occur at this region, which are usually degradation reactions (as more frequently occurs with steroids having corticoid or progestational properties).
3. Change the metabolic fate of functional groups not in the Δ^4-3-keto region, which are still fundamental for the biological action; for instance, this occurs when an atom of chlorine is introduced at C-4 in testosterone with consequent decrease in the production of 17-ketosteroids *in vivo* (Sala and Castegnaro, 1953) and inhibition of dehydrogenation at C-17 *in vitro* (Castegnaro and Sala, 1961).

Let us now examine the biological effects originating from the introduction of a hydroxyl group into testosterone, 17-α-alkyltestosterone, and progesterone derivatives; this change can modify in many respects the biological activity of the parent steroids and may be recorded among the substitutions undertaken with the objective of obtaining new therapeutically useful steroids.

The study of the relationships between chemical structure and biological activity of synthetic steroids differently substituted may help in discovering the mechanism of action and the biological meaning of the different functional groups of the steroid molecule. This research appears particularly promising when the structural changes lead to a dissociation of different biological effects, inasmuch as one can identify the biochemical reactions responsible for the biological responses.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics:
Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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I. TESTOSTERONE DERIVATIVES

The introduction of a hydroxyl group at C-4 in testosterone derivatives employed subcutaneously definitely reduces the androgenic effect and moderately reduces the myotropic effect (Hershberger *et al.*, 1953), thus increasing the therapeutic index. This phenomenon occurs both in testosterone alcohol and in esterified testosterone.

Even in testosterone esters having a delayed activity, 4-hydroxylation favors the dissociation between the myotropic and the androgenic effect. Table I reports the behavior of the therapeutic index as calculated from the ratio:

$$\frac{\textit{weight of levator ani muscle of treated rat—weight of levator ani muscle of controls}}{\textit{weight of prostate of treated rat—weight of prostate of controls}}$$

TABLE I

Influence of 4-Hydroxylation on the Therapeutic Index of Testosterone Derivatives

Compound	Therapeutic index after day			
	7	14	21	28
Testosterone CPP.	0.28	0.32	0.31	0.29
4–Hydroxytestosterone 17–CPP.	0.86	0.75	0.46	1.08
4–Hydroxy–19-nortestosterone 17–CPP.	0.85	1.61	1.45	2.67

at different times of two testosterone derivatives injected in castrated rats at a single dose equivalent to 2 mg of testosterone. 4-Hydroxytestosterone 17-cyclopentylpropionate (CPP) shows an evident increase in the therapeutic index compared with testosterone CPP; an even greater increase is observed in 4-hydroxy-19-nortestosterone CPP. This finding clearly shows that the double structural modification (4-hydroxylation and 19-nor configuration) increases the dissociation between the two biological effects of testosterone.

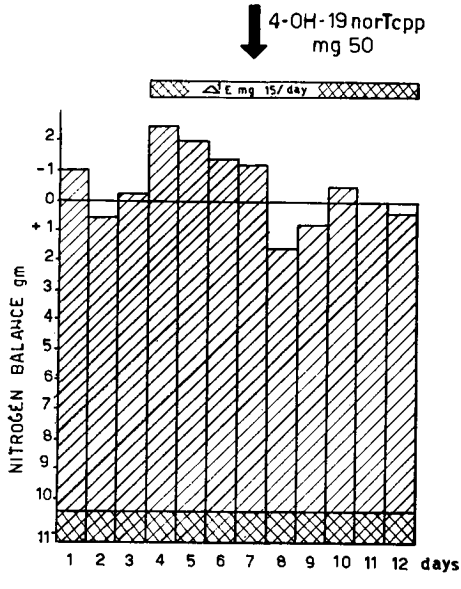

FIG. 1. Effect of 4-hydroxy-19-nortestosterone 17-cyclopenthylpropionate on nitrogen balance in one patient affected by rheumatoid arthritis and treated with prednisone.

In regard to endocrine side effects, 4-hydroxylation inhibits the progestational activity (McPhail, 1934) and reduces the antioestrogenic (Edgren and Calhoun, 1957) and the antigonadotropic properties (Hertz and Mayer, 1937) of 19-nortestosterone.

Being capable of reducing the androgenic properties and of abolishing or reducing other endocrine side effects of 19-nortestosterone, 4-hydroxylation can be considered to be a useful change for therapeutic purposes.

Figure 1 shows the effect of 4-hydroxy-19-nortestosterone 17-CPP on the nitrogen balance of a patient affected by rheumatoid arthritis: the administration of a maintenance dose of prednisone (15 mg daily) brings about a negativization of the nitrogen balance; the administration of a single dose of 50 mg of the anabolic steroid counteracts this effect, at least for 6 days.

II. 17α-ALKYLTESTOSTERONE DERIVATIVES

The introduction of a hydroxyl group at C-4 in these oral active derivatives modifies the biological activities of the parent steroid depending on the nature of the alkyl group.

In the case of 17α-methyl group, the C-4 hydroxylation remarkably increases the myotropic effect, both of testosterone and of 19-nortestosterone. 4-Hydroxy-17α-methyltestosterone is in fact, characterized by an increase of the myotropic and a decrease of the androgenic effect, with a definite increase of the therapeutic index, as compared with 17α-methyltestosterone. 4-Hydroxy-17α-methyl-19-nortestosterone presents a strong increase of the myotropic and of the androgenic effect, with a moderate increment of the therapeutic index, as compared with 17α-methyl-19-nortestosterone (Table II). The potentiation of the myo-

TABLE II

Biological Effects of 17α-Methyltestosterone Derivatives

Derivative	Myotropic effect	Androgenic effect
4-Hydroxy-17α-methyltestosterone		
Oral	3.63	0.46
SC	1.65	0.52
	× 17α-methyltestosterone	
4-Hydroxy-17α-methyl-19-nortestosterone		
Oral	13.04	10.24
SC	0.71	1.05
	× 17α-methyl-19-nortestosterone	

tropic and androgenic effects is a characteristic of oral administration; by subcutaneous route, the potentiation is much less evident or it does not exist at all. This different behavior, associated with the route of administration, attributes to the hydroxylation at C-4 a role which facilitates intestinal absorption of 17α-methyltestosterone derivatives, besides favoring dissociation between myotropic and androgenic activity.

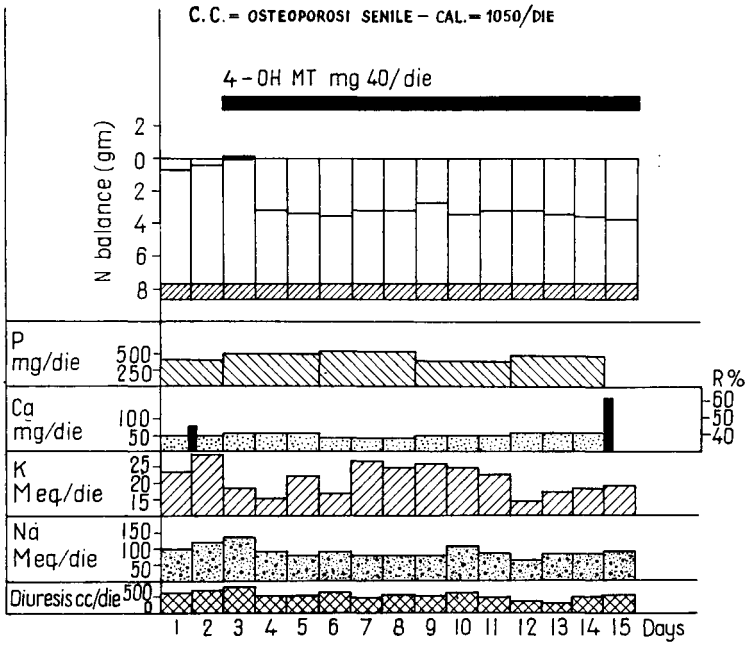

FIG. 2. Metabolic effect of 4-hydroxy-17α-methyltestosterone in one patient affected by senile osteoporosis.

The high myotropic and the low androgenic properties of oral 4-hydroxy-17α-methyltestosterone, evidenced in experimental animals, has been confirmed in humans. This steroid is indicated in the treatment of protein depletion states and of leanness. It leads to positivization of the nitrogen balance, without exerting, at the usual dose (20–40 mg per day), androgenic, progestational, antiestrogenic, or antigonadotropic effects. Figure 2 illustrates the metabolic effect of 4-hydroxy-17α-methyltestosterone in a patient affected by senile osteoporosis (positivization of nitrogen balance and increase of calcium retention).

The biological properties of 4-hydroxy-17α-methyl-19-nortestosterone, summarized in Table III, clearly differentiate this new derivative both from 17α-methyltestosterone and from 17α-methyl-19-nortestosterone.

TABLE III

Biological Properties of 4-Hydroxy-17α-methyl-19-nortestosterone

Property	Route	Activity
Myotropic	Oral	13.04 × Methyltestosterone (oral)
Androgenic	Oral	2.81 × Methyltestosterone (oral)
Progestational	Oral	0
Antiestrogenic	SC	2.66 × Testosterone propionate (SC)
Antigonadotropic	SC	2.18 × Testosterone propionate (SC)

In the case of a 17α-ethyl or 17α-ethynyl group, the addition of a hydroxyl group at C-4 does not lead to an increase in biological activity: 4-hydroxy-17α-ethyltestosterone does not possess myotropic, androgenic, or progestional properties, although it keeps a low degree of antiestrogenic activity. 4-Hydroxy-17α-ethynyltestosterone is devoid of myotropic and androgenic effects and presents only a weak progestational and antiestrogenic activity. As for 19-nortestosterone derivatives, the androgenic and myotropic effects are reduced in 4-hydroxy-17α-ethyl and in 4-hydroxy-17α-ethynyl derivatives; the other endocrine effects will be mentioned below.

If we now consider Ringold's theory (1961) on the function of steric factors in the reduction of the androgenic activity turning from 17α-methyl- to 17α-ethyltestosterone, we may interpret the different behavior of 4-hydroxylation with regard to the substituting group at C-17 alpha as a consequence of the nonabsorption of 17-ethyl derivatives on the cell surface; consequently, the hydroxyl group at C-4 cannot interfere—as in the case of 17α-methyltestosterone or 17α-methyl-19-nortestosterone—on the interaction between the α-side of the steroid and the cell proteins.

The side effects of 4-hydroxy derivatives of 17α-alkyltestosterone are summarized in Table IV.

In regard to the progestational properties, it is known that 17α-methyltestosterone is not active, whereas a definite, although weak, activity is possessed by 17α-ethynyltestosterone. More active are the alkyl derivatives of the 19-nortestosterone series: the morphologic pattern achievable in the uterine mucosa of the female rabbit previously treated with estrogens is of the progestational type, if not completely superposable to the effect of progesterone, owing to the predominant stimulation of the stroma rather than to the arborization of the mucosa.

The introduction of a hydroxyl group at C-4 reduces notably, the progestational effect of 17α-ethynyltestosterone; moreover, in 19-nortestosterone derivatives, progestational changes are lacking. Figure 3 shows the response of the uterus of the female rabbit to 17α-ethyl-19-

TABLE IV

Secondary Endocrine Effects of 17α–Alkyltestosterone Derivatives

	Progestational effect[a]	Antiestrogenic effect[b]	Antigonadotropic effect[b]
17α–Methyltestosterone	0	0.74	1.57
4–Hydroxy–17α–methyltestosterone	0	0.65	2.20
17α–Ethynyltestosterone	1	1.17	—
4–Hydroxy–17α–Ethynyltestosterone	0.3	0.20	—
17α–Methyl–19–nortestosterone	3.2	4.55	3.33
4–Hydroxy–17α–methyl–19–nortestosterone	0 (up to 5 mg)	2.66	2.18
17α–Ethyl–19–nortestosterone	2.0	2.52	3.75
4–Hydroxy–17α–ethyl–19–nortestosterone	0 (up to 10 mg)	1.80	0.88
17α–Ethynyl–19–nortestosterone	2.2	1.66	0.51
4–Hydroxy–17α–ethynyl–19–nortestosterone	0 (up to 5 mg)	0.40	0.73

[a]17α–Ethynyltestosterone (oral) = 1.
[b]Testosterone propionate (SC) = 1.

nortestosterone and to 4-hydroxy-17α-ethyl-19-nortestosterone in oral total doses of 5 mg. The progestational effect of the ethyl derivative of 19-nortestosterone is evident; 4-hydroxy-17α-ethyl-19-nortestosterone is on the contrary inactive.

The antiestrogenic effect is always reduced by the introduction of a hydroxyl at C-4, both in the compounds of the normal series and in 19-nortestosterone derivatives. Noteworthy is the progressive reduction of this effect when turning from the methyl to the ethyl and ethynyl derivatives of 19-nortestosterone. As to the antigonadotropic action, no definite and constant influence of the hydroxyl group is observed.

By the analysis of the results reported concerning the effects of the introduction of a hydroxyl group at C-4 in testosterone and its derivatives, we realize that we are dealing with a new series of biologically active and therapeutically interesting steroids. It is never advisable to express, on the basis of experimental results, deductions concerning the relations between structural modifications and biological activity, so many are the possible exceptions and the risks of generalization. However, it is justifiable, in our opinion, to point out that the introduction of a hydroxyl group at C-4 changes the biological properties

of the parent compounds as follows: (1) It favors the dissociation between myothropic and androgenic effects with a consequent increase of the therapeutic index. (2) It favors the intestinal absorption of the derivatives of 17α-methyltestosterone and of 17α-methyl-19-nortestosterone and thus increases their biological activities. (3) It abolishes the

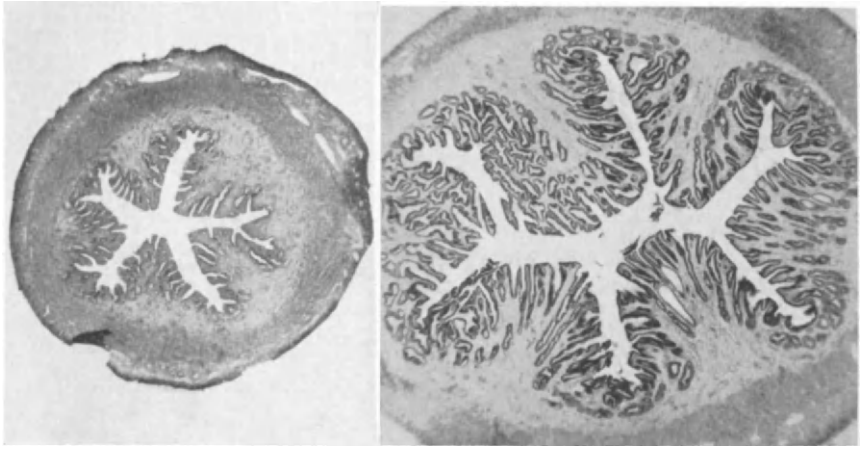

FIG. 3. Effect of 17α-ethyl-19-nortestosterone (left) and of 4-hydroxy-17α-ethyl-19-nortestosterone (right) on uterine mucosa of estrogen-primed rabbits.

progestational and reduces the antiestrogenic and, in part, the antigonadotropic effects.

III. PROGESTERONE DERIVATIVES

The introduction of a hydroxyl group at C-4 reduces the progestational activity of compounds subcutaneously injected, but the influence of this modification is different in orally active products. This interpretation is deducible from the analysis reported in Table V, which represents the progestational properties of 4-hydroxyprogesterone as compared with progesterone and of 4-hydroxy-17α-acetoxyprogester-

TABLE V

Progestional Properties of 4-Hydroxy Derivatives of Progesterone

Compound	Progestational effects	
	SC	oral
Progesterone	1.00	—
4–Hydroxyprogesterone	0.44	—
17α–Acetoxyprogesterone	9.71	1
4–Hydroxy–17α–Acetoxyprogesterone	5.44	8.2

one, compared with 17α-acetoxyprogesterone both by subcutaneous and by oral route. It is clear that there is a reduction of the progestational effect in these steroids employed subcutaneously, whereas a remarkable increase by oral route is observed; in fact 4-hydroxy-17α-acetoxyprogesterone is 8.2 times more active than 17α-acetoxyprogesterone.

Evidently, as happens with the derivatives of 17α-methyltestosterone, we have to conclude that the addition of a hydroxyl group at C-4 favors intestinal absorption of the progestational steroid. The progestational effect of 4-hydroxy-17α-acetoxyprogesterone both by subcutaneous and by oral route is typical; it occurs also for local introduction into a uterine horn and is complete, being characterized by the maintenance of pregnancy in ovariectomized animals (Sala *et al.*, 1959) (Table VI) and by a definite deciduomagenic effect on the castrated rat (Chambon, 1952). It has a moderate antiestrogenic effect, no antigonadotropic and no corticoid properties.

TABLE VI

Effect of 4-Hydroxy-17α-Acetoxyprogesterone on the Maintenance of Pregnancy in Mice

Treatment	Dose (mg/kg/day)	% Fetuses alive
Controls	—	0
Progesterone	1	65
Progesterone	5	98
4-Hydroxy-17α-Acetoxyprogesterone	5	97

Particular attention has been given to the androgenic effect and to the virilizing activity of 4-hydroxy-17α-acetoxyprogesterone in fetuses (Revesz *et al.*, 1960), in connection with the virilizing activity of steroids endowed with progestational action, even in the absence of the typical androgenic affect.

The new derivative does not present androgenic properties (stimulation of the ventral prostate and of seminal vesicles in rat, weight increase of chick's comb). The effect on fetuses is summarized in Fig. 4, which reports the anogenital distances of female and male fetuses born from rats which had been treated during pregnancy with different doses of various progestins. The virilizing effect of 17α-methyl-19-nortestosterone and, to a lesser degree, of MAP appears evident through the increase of anogenital distances of female fetuses; on the contrary, progesterone and 4-hydroxy-17α-acetoxyprogesterone, employed at doses 10 times higher than those of virilizing progestins, are completely inactive.

With the aim of establishing whether the virilization of fetuses is bound to progestational activity or just to particular structures present

in some progestins, we have employed different dosages of different compounds in order to have an equivalent progestational activity.

The nonvirilizing activity of progesterone and 4-hydroxy-17α-acetoxyprogesterone, injected at the very high doses of 50 mg per day per rat, points out the second possibility and identifies as virilizing the

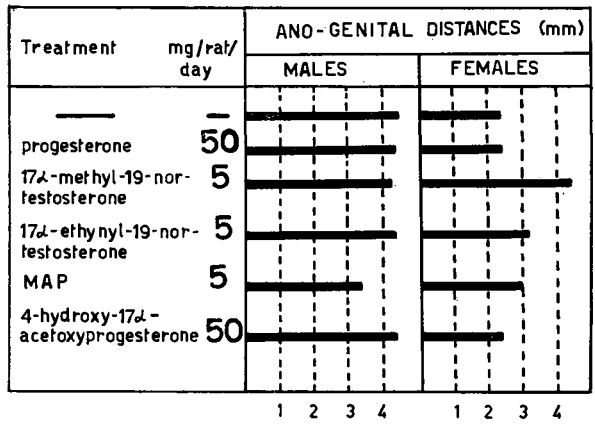

FIG. 4. Virilizing effect on fetuses of several progestins.

19-nortestosterone structure and, in a minor degree, the introduction of a 6α-methyl group in 17α-acetoxyprogesterone.

In conclusion, the introduction of a hydroxyl group at C-4 increases the progestational effect of 17α-acetoxyprogesterone by the oral route, favoring its gastrointestinal absorption; such potentiation concerns only the progestational properties, all present in a typical way in the new derivative, and does not regard other endocrine activities such as the virilizing effect on fetuses (completely absent even with very high doses), the antiestrogenic and the antigonadotropic properties.

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DISCUSSION

Applications of Ring A Modifications of 4-Hydroxy-3-keto-Δ^4-Steroids

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Sala has shown that modifications in ring A, e.g., substitution at C_4, lead to steroids which are different from the parent compounds.

Thus it is possible to increase or decrease some of their activities. 4-Hydroxy-17α-methyl-19-nortestosterone, for example, shows a pronounced dissociation of the anabolic-androgenic potency. In 17α-methyl-19-nortestosterone androgenic activity prevails. On the other hand, the progestational activity of 17α-hydroxyprogesterone 17α-acetate is increased by substitution at C-4; the other potencies are not influenced.

Similar modifications are observed after introduction of an additional double bond at Δ^1 or an alkyl group at C-1 of derivatives of androstane.

All these observations have practical importance in regard to decreased androgenic action, especially in the treatment of female patients. The risk of masculinization becomes unimportant even when the treatment lasts for a long period of time, since the doses proposed for clinical use are based on the dissociation of the two potencies.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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Some Aspects of Steroid Dynamics

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I. Introduction

By following the concentration of radioactivity (measured specifically as the hormone) in plasma after a single injection of the labeled steroid,

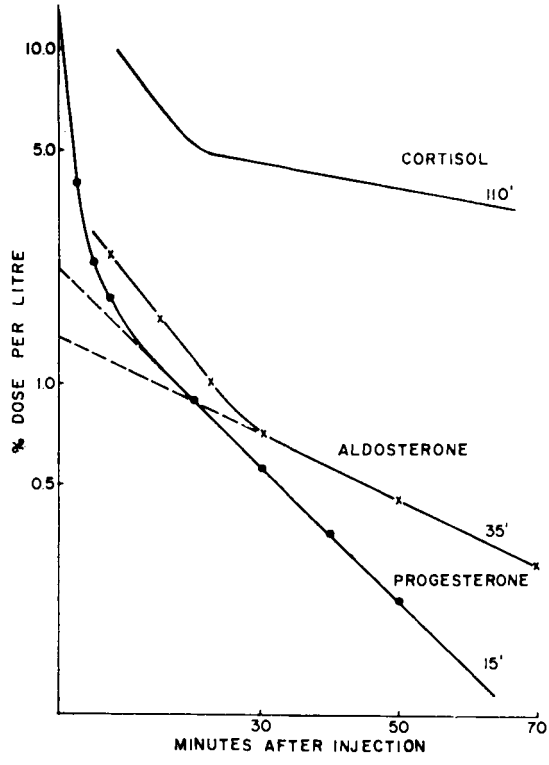

Fig. 1. Comparison of the disappearance curves after a single-injection of progesterone, aldosterone, and cortisol.

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it is found that the disappearance curve of many steroids can be represented satisfactorily by two exponentials and a two-compartmental model is then suitable to describe their metabolism and transport. Figure 1 shows disappearance curves for cortisol, progesterone, and aldosterone. The curve for cortisol was calculated from the data of Peterson (1959). Those for progesterone and aldosterone were estimated from the combined data, in each case, for six individuals after the in-

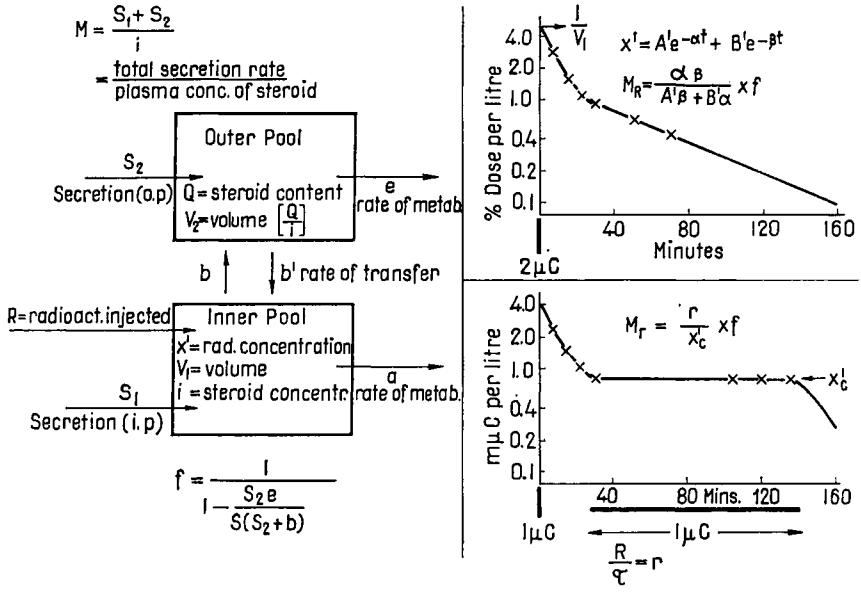

FIG. 2. The metabolic clearance rate of aldosterone estimated by the single injection and constant infusion methods. On the left is shown the appropriate two-compartmental model whereby the transport and metabolism can be described.

jection of about 0.1 μg (7-H^3) steroid. Figure 2 shows a suitable two-compartmental model.

In certain conditions the volumes of distribution of the steroids can be obtained (Tait *et al.*, 1961; Ayers *et al.*, 1962). However, perhaps the most interesting parameter that can be calculated from the plasma radioactive concentrations after a single injection of labeled steroids is the "metabolic clearance rate (M.C.R.)" defined as the secretion rate divided by the plasma concentration of the steroid. This was previously referred to as a turnover rate (Tait *et al.*, 1961), but the above term is more appropriate. If this parameter is estimated, then the plasma concentration can be calculated knowing the secretion rate, conversely, given the plasma concentration and M.C.R. values, the secretion rate can be obtained. One of the general aims of the study of steroid metabolism should be the prediction of the metabolic clearance rate of an individual steroid from a knowledge of its metabolism by tissue enzymatic systems

and its binding by proteins of plasma and tissues and hence perhaps eventually from a knowledge of its chemical structure alone.

The metabolic clearance rate can also be calculated from the constant radioactive concentrations in plasma (measured specifically as the hormone) after a prolonged infusion of the labeled steroid. Usually a single injection is given before the start of the infusion to shorten the time necessary to attain constant plasma concentrations.

The two methods (single injection and single injection followed by infusion), as applied to the estimation of the metabolic clearance rate of aldosterone, are illustrated in Fig. 2.

II. THEORY

A. SYMBOLS

The metabolism and transport of the steroids can be described by two compartments (Fig. 2). The radioactive steroid is introduced into the inner pool (including the plasma), and metabolism and secretion of the hormone occurs in both pools.

Let S_1 and S_2 be the secretion rate into the inner and outer pool respectively; $S_1 + S_2 = S$, the total secretion rate, let I and Q be the steroid contents of the inner and outer pool, i = steroid concentration in inner pool (including plasma), a and e be the steroid metabolized in unit time in the inner and outer pool, let b = rate of steroid transferred from inner to outer pool, and b^1 = rate of steroid transferred from outer to inner pool, let the corresponding fractional rate constants be

$$\theta_1 = b/I;\ \theta_2 = b^1/Q;\ \theta_3 = a/Q \text{ and } \theta_4 = e/Q$$

let x and z = fraction of injected radioactivity (measured specifically as the hormone) in the total inner and outer pool at time t, let x^1 = fraction of injected radioactivity as concentration in inner pool (equal to that in plasma) at time t, let V_1 = the volume of the inner pool (I/i) and V_2 = volume of outer pool (Q/i) which is defined arbitrarily, then the following equations apply:

1. Dynamic Equations

Assuming that individual reactions are first order and that the pools are homogeneous

$$\frac{-\,dx}{dt} = \theta_1 x - \theta_2 z + \theta_3 x$$

$$\frac{dz}{dt} = \theta_1 x - \theta_2 z - \theta_4 z$$

2. Steady State Equations

Assuming that total rates of secretion $(S_1 + S_2)$ and metabolism $(a + e)$ are equal at all times or that the content of each pool is constant then

$$Q(\theta_2 + \theta_4) - \theta_1 I = S_2 = b^1 + e - b$$
$$(\theta_3 + \theta_1) I - \theta_2 Q = S_1 = a + b - b^1$$
$$\theta_4 Q + \theta_3 I = S_1 + S_2 = S = a + e$$

These comprise five basic equations from which the metabolic clearance rate can be estimated by either the single injection or infusion methods.

B. SINGLE INJECTION

If the plasma disappearance curve can be represented by $x^1 = A_e^{1-\alpha t} + B_e^{1-\beta t}$

$$\alpha + \beta = \theta_1 + \theta_2 + \theta_3 + \theta_4$$
$$\alpha\beta = \theta_2\theta_3 + \theta_3\theta_4 + \theta_4\theta_1$$
$$A^1\alpha + B^1\beta = \theta_1 + \theta_2$$
$$A^1\beta + B^1\alpha = \theta_2 + \theta_4$$

Although V_1 can be calculated from the curve as $V_1 = 1/(A^1 + B^1)$ all θ values and also V_2, the volume of the outer pool, cannot be, calculated from the available data unless θ_3 or θ_4 (metabolism in either pool) can be assumed to be zero. However, it can be shown (Tait *et al.*, 1962) that the M.C.R. (metabolic clearance rate) =

$$\frac{S}{i} = \frac{\alpha\beta}{A^1\beta + B^1\alpha}\left[\frac{1}{1 - \dfrac{S_2 e}{S(S_2 + b)}}\right]$$

in the general case with metabolism in both pools.

The estimated metabolic clearence rate $\text{M.R.C.}_E = \dfrac{\alpha\beta}{A^1\beta + B^1\alpha}$ can be calculated from the experimental data, then the true metabolic clearance rate (cf. Fig. 2) = $\text{M.C.R.}_E \times f$ where

$$f = \frac{1}{1 - \dfrac{S_2 e}{S(S_2 + b)}}$$

C. INFUSION

Similarly it can be shown that if r = rate of infusion of radioactive steroid (fraction of total infused radioactivity in unit time) and x^1_c = final constant radioactivity in plasma (measured specifically as the hormone) then

$\text{M.C.R.}_E = \frac{r}{x^1_c}$ and $\text{M.C.R.} = \text{M.C.R.}_E \times f$ where again

$$f = \frac{1}{1 - \frac{S_2 e}{S(S_2 + b)}}$$

As previously mentioned, usually a single injection of radioactivity is given before the infusion to obtain constant plasma radioactive concentrations more quickly, but this does not alter the calculation.

D. COMPARISON OF INFUSION AND INJECTION METHODS

Therefore in all circumstances, if a two compartmental model is appropriate, theoretically the two methods should give the same result. If $f = 1$ both estimates will equal the true metabolic clearance rate = secretion rate/plasma concentration. This will be so if S_2 or e is zero, i.e., when secretion or metabolism is negligible in the outer pool. These criteria could be stated alternately that if there is metabolism in both pools then the radioactive and secreted hormone must enter the same pool. In practice, these criteria are likely to be met except in certain restricted circumstances, e.g., secretion and metabolism of a steroid by both the maternal organism and the fetus in studies on pregnancy and certain studies on the biosynthesis of steroids *in vivo* (Burstein and Dorfman, 1962).

In general therefore a comparison of estimates by the two methods will give no indication whether secretion and/or metabolism occurs in both pools. It may, however, be a valuable test for the validity of the model, i.e., whether two or more compartments are necessary, as well as the more technical aspects of the methods, e.g., whether the radioactivity is being measured specifically as the hormone.

III. RESULTS AND DISCUSSION

A. PLASMA METABOLIC CLEARANCE RATES BY THE INFUSION AND INJECTION METHODS

1. Aldosterone and Progesterone Estimates

A comparison of the estimated plasma metabolic clearance rates for aldosterone by the single injection and infusion methods, in pregnant and tnonpregnant women, are shown in Fig. 3. The experimental me-

thods will be described in detail elsewhere (Tait *et al.*, 1962). They are similar to those of Tait *et al.*, 1961. There is no significant difference in the results for the same group of subjects by the two methods.

Figure 4 shows a similar comparison for the estimated metabolic clearance rate of progesterone. The experimental methods will be de-

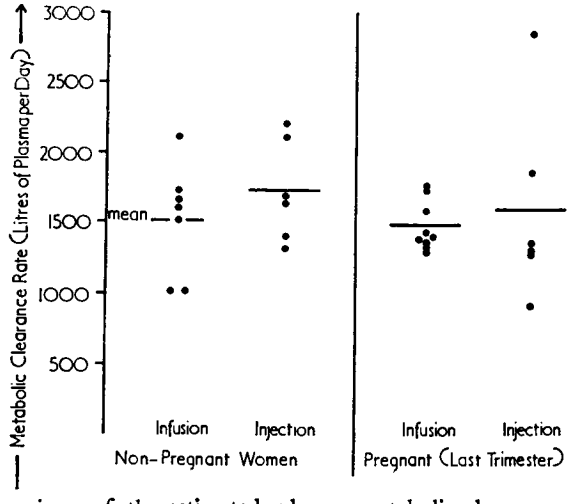

FIG. 3. A comparison of the estimated plasma metabolic clearance rates for aldosterone by the single injection and infusion methods in pregnant and nonpregnant women.

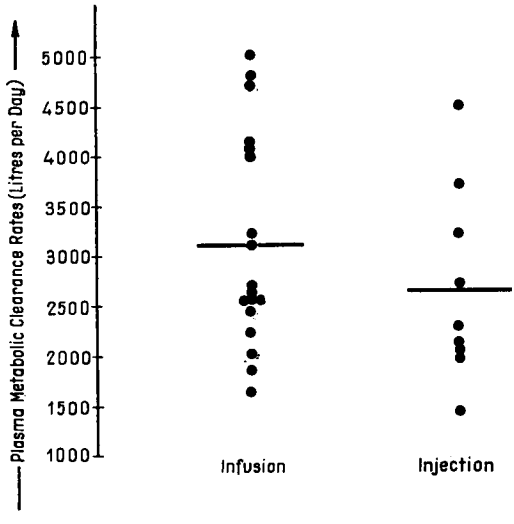

FIG. 4. A comparison of the metabolic clearance rate of progesterone estimated by the single injection and constant infusion methods.

scribed in detail elsewhere, but they are similar in principle to those used for aldosterone. The subjects included males, ovariectomized, and ovariectomized-adrenalectomized females. No significant difference has as yet been found between these different groups as regards estimates of metabolic clearance rate of progesterone. Again there is no significant difference in the mean estimates for M.C.R. by the two methods. The combined value was 2950 liters of plasma per day (27 subjects).

2. Method of Choice

The method of choice is indicated by the theoretical considerations and experimental results described above:

a. If the initial and total volumes of distribution and also the individual metabolic and transport constants are required, then the single injection method will give the information[1] but not the infusion method, at least as the latter is carried out at the present time.

b. If the metabolic clearance rate is required, then the infusion method is likely to give the most reliable and precise results. Thus, when the disappearance of a steroid in plasma after a single injection is very rapid as for progesterone, there are considerable difficulties in accurate sampling at known time intervals in order to obtain the characteristics of the first exponential ($A_e^{1-\alpha t}$). Of more importance, because of the low plasma radioactive concentrations, the characteristics of the second exponential ($B_e^{1-\beta t}$) are also difficult to estimate and it is impossible to test critically whether the final slope (plotted semilogarithmically, Fig. 2) is a straight line. With the infusion method, the plasma radioactivity can be measured for a much longer period of time (with the same total administration of radioactivity) and the criterion for equilibrium, i.e., constant plasma radioactive concentration, can be rigorously tested by comparing three values taken at intervals, during the latter part of the infusion. Also, from the variation of these values, the error of every estimate of metabolic clearance rate can be easily calculated. The corresponding calculation for the single injection method may require a computor (Ayers *et al.*, 1962).

As there is no significant difference between the estimates obtained by the two methods for the same group of subjects, they will be pooled for future discussion.

[1] The initial volume can be calculated in all circumstances; total volume and the individual metabolic and transport constants, only if metabolism is assumed to be zero in the inner or outer pool.

B. ESTIMATE OF WHOLE BLOOD CLEARANCE RATES

1. Aldosterone

The plasma metabolic clearance rate for aldosterone in nonpregnant women was 1631±106 (SE) liters of plasma per day (13 subjects) and 1543±113 (SE) liters of plasma per day (15 subjects) for pregnant (last trimester) women. There was therefore no significant difference between the two groups of subjects.

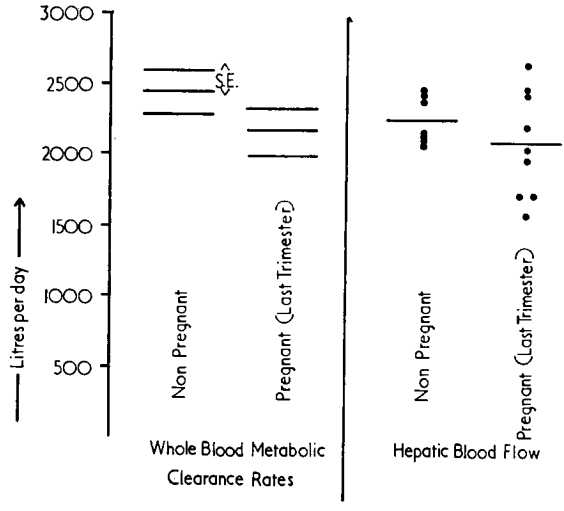

FIG. 5. A comparison of whole blood metabolic clearance rate of aldosterone and hepatic blood flow in pregnant and nonpregnant subjects.

Although technically it is easier to measure the plasma rather than the whole blood clearance rates, the latter may be more physiologically meaningful. In three pregnant and three nonpregnant women the plasma and whole blood clearance rates were determined concomitantly and the ratio M.C.R. whole blood : M.C.R. plasma found to be 1.51, 1.37, and 1.61 (mean 1.50) for the nonpregnant and 1.39, 1.41, and 1.42 (mean 1.41) for the pregnant subjects. The appropriate plasma clearances have been converted to whole blood values by using these mean ratios and are shown in Fig. 5. These values are similar to estimated hepatic blood flows for the same group of subjects also shown in Fig. 5. The values for the hepatic blood flow of nonpregnant subjects are the mean estimates by various methods (Sheldon *et al.*, 1961; Halpern *et al.*, 1959; Caesar *et al.*, 1961; Reemtsma *et al.*, 1960; Bradley *et al.*, 1945, 1953), which are in reasonable agreement with one another. The values for the pregnant subjects are estimates for individual subjects (last trimester) taken from the data of Munnell and Taylor (1947). For both the

estimated metabolic clearance rate and hepatic blood flow, the values for the pregnant subjects are slightly lower than the nonpregnant, although the differences are not significant.

These results suggest that the hepatic extraction of aldosterone in normal subjects is about 100% and extrahepatic metabolism is small. In that case, the metabolic clearance rate will be equal to the hepatic blood flow. This is supported by the data of Ayers *et al.* (1962), who found greatly reduced metabolism of aldosterone in dogs with a bypassed liver and also by the work of Coppage and co-workers (1961) in man who found that aldosterone was less than 10% as effective orally as intramuscularly, although the steroid was effectively absorbed (and presumably passed entirely through the portal vein to the liver) from the gastrointestinal tract. In one subject the peripheral and hepatic venous blood concentrations of radioactive aldosterone have been measured after a constant infusion of the labeled hormone. The peripheral concentration was then constant. The concentration in the hepatic plasma and whole blood was 5% of the corresponding peripheral values (Little *et al.*, 1962).

These quantitative considerations suggest that it may be useful to express the metabolic clearance rate of a steroid (whole blood) as

$$\text{M.C.R.} = \text{H.B.F.} \times \text{H.E.} + \text{E.H.C.R.}$$

where H.B.F. is hepatic blood flow, H.E. is hepatic extraction, and E.H.C.R. is the extrahepatic clearance rate. If H.B.F. (which can be determined by the usual methods) and H.E. (which could be determined by hepatic venous sampling of radioactive steroid after the infusion[2]) are known then the proportion of extrahepatic metabolism may be quantitatively expressed as

$$\frac{\text{E.H.C.R.}}{\text{M.C.R.}} = \frac{\text{M.C.R.} - \text{H.B.F.} \times \text{H.E.}}{\text{M.C.R.}}$$

It should be noted that these expressions will apply only if the radioactive steroid, like the steroid secreted by the normal gland, is mixed with the total blood volume before entering the liver. The relationship between peripheral blood concentration and secretion rate is clearly not applicable in general for oral or direct hepatic arterial administration.

The general approach may be illustrated by the preliminary results on aldosterone and progesterone. The M.C.R. for aldosterone (nonpregnant) is 2446 liters per day, the hepatic blood flow 2250 liters per day,

[2]As in the aldosterone experiment described, the extraction so measured would be actually the total in the hepatolienal circulation, and strictly the terms enterohepatic and extraenterohepatic extraction should then be used.

and, from the results of Coppage *et al.* (1961) and Little *et al.* (1962), the hepatic extraction is 90–100%. The proportion of extrahepatic to hepatic metabolism, E.H.C.R./M.C.R. will therefore be 8–15%.

2. Progesterone

In the case of progesterone, the whole blood clearance rate is about 5000 liters whole blood per day (whole blood : plasma ratio 1.7). There is yet no information on the hepatic extraction of progesterone, but its rate of metabolism by hepatic tissues *in vitro* suggests that, unless its high binding to albumin prevents its transport into the liver cells *in vivo*, its extraction will be 100%. In this case, the proportion of extrahepatic to total metabolism will be about 50%. If the hepatic extraction is actually less than 100% this figure will be greater. This relatively high value for the proportion of extrahepatic metabolism for this steroid corresponds with its known rapid metabolism by extrahepatic tissues (Berliner and Wiest, 1956).

3. Cortisol and Corticosterone

In the case of cortisol and corticosterone, the plasma metabolic clearance rate can be calculated from the data of Peterson (1961) to be about 200 liters per day. Assuming little penetration of these steroids into the red cells (Slater, 1961) the whole blood clearance rate will be about 340 liters per day, and if extrahepatic metabolism is zero then the hepatic extraction of these steroids will be about 15%. If the extrahepatic metabolism is significant this estimate should be even lower.

C. EXPLANATION AND PREDICTION OF WHOLE BLOOD CLEARANCE RATES OF STEROIDS IN GENERAL

The plasma binding of cortisol and corticosterone to proteins other than albumin (henceforward termed relative binding) is known to be high (Slaunwhite and Sandberg, 1959) whereas that of aldosterone is very low. This suggests that the binding of steroids to plasma proteins, other than albumin, may be important in determining their hepatic extraction. This is also supported by the finding that the relative binding of cortisol is increased by estrogen treatment (Mills *et al.*, 1960) or in pregnancy (Sandberg and Slaunwhite, 1959). The metabolic clearance rate is reduced under these conditions, as shown by the decrease of the slope of the disappearance curve (Migeon *et al.*, 1957; Christy *et al.*, 1959). In the case of aldosterone, the relative binding is not increased in pregnancy (Meyer *et al.*, 1961) and neither is the metabolic clearance rate. These considerations are illustrated in Fig. 6.

Thus, the binding of steroids to plasma proteins other than albumin may have an influence in deciding their hepatic extraction and hence

the value of the major component of the metabolic clearance rate, the hepatic clearance rate (H.B.F. × H.E.). It may even be that the intrinsic hepatic extraction of most natural steroid hormones if they reach the metabolic liver cells is about 100% and their actual hepatic extraction will be mainly determined by transport considerations.[3] Unfortunately the situation in the liver is probably a dynamic one with a concentration gradient of unbound steroid across the membrane of the metabolic cell

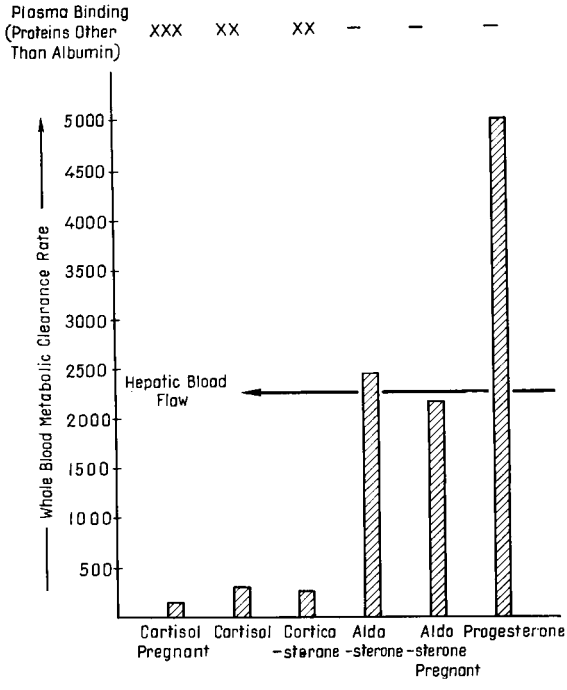

FIG. 6. A comparison of the whole blood metabolic clearance rate of cortisol, corticosterone, aldosterone, and progesterone to show the influence of relative protein binding on these values.

whereas the nearest corresponding *in vitro* situation used for determining nonprotein-bound steroid is that of equilibrium dialysis. However, it is of interest that the estimated hepatic extraction of cortisol, corticosterone, and aldosterone is of the same order of magnitude as the value for preparation of nonprotein-bound steroid (other than to albu-

[3] However, if the hepatic extraction is low as with cortisol, rate of transport and hence overall extraction may be determined by rate of metabolism of individual cells, i.e., concentration gradient of steroid across the cell. The effects of estrogen administration on the metabolic clearance rate may yet prove to involve a direct action on the metabolic activity of the liver cells in addition to the effects of increased plasma binding.

min) determined in undiluted plasma at 37°C by equilibrium dialysis. The influence of binding to albumin remains to be investigated, but the high metabolic clearance of progesterone which is strongly bound to plasma albumin suggests that this may not be an important factor for steroids which are rapidly metabolized by tissue.

Although it may actually be shown that the intrinsic hepatic extraction of many steroids (the extraction of steroids entering the metabolic liver cells) in the normal subject is nearly 100%, in pathological situations, particularly cirrhosis, the situation may be different. Coppage *et al.* (1961), and Kono *et al.* (1961) have shown that for aldosterone, whose hepatic extraction in normal subjects is about 100%, in cirrhosis the disappearance curve after a single injection may be considerably flattened. From their data it can be calculated that for such subjects, the metabolic clearance rate may be reduced at least threefold. As the hepatic blood flow is not necessarily reduced in such subjects, the hepatic extraction must be considerably under 100%.

The intrinsic hepatic extraction (the extraction of steroids entering the liver metabolic cells) may eventually be quantitatively expressed as the actual extraction (measured by hepatic venous sampling after infusion) divided by some function of the dialyzable fraction of the steroid as measured *in vitro*. The intrinsic hepatic extraction probably correlates with the rate of metabolism of steroids of different structures by liver tissue and enzymatic systems *in vitro*.

For some steroids, the extrahepatic clearance rate will be appreciable and its prediction will be essential for explanations of total metabolic clearance rate. At the present time, quantitative studies are few in this field and much work requires to be done. However, for the steroids discussed in this paper it appears that the degree of extrahepatic metabolism is to some extent correlated with the molecular environment of the 20-ketone group, which is known to be reduced by extrahepatic tissue (Gold, 1961), and this may provide a useful preliminary working hypothesis.

1. The Overall Total Body Extraction of Steroids

It would seem that the maximum possible metabolic clearance rate of a steroid is the cardiac output. The validity of this conclusion would depend on there being negligible metabolism of the steroid by blood and little diffusion of steroid from the secreting gland to metabolizing tissues. Both assumptions are likely to be relatively correct. If this is so, then the maximum ratio of the secretion rate to the blood concentration of a steroid (M.C.R.) will be about 8 liters per day for the women studied. The actual M.C.R. : cardiac output ratio will therefore give a figure which could be termed the overall extraction factor. For progesterone,

this factor would be about 60%, for aldosterone 30%, and for cortisol it would be about 5%. It can be seen that the body is remarkably efficient in metabolizing progesterone.

D. ESTIMATION OF PLASMA CONCENTRATIONS FROM METABOLIC CLEARANCE AND SECRETION RATES

The secretion rate of a hormone can be estimated after an injection of radioactive steroid by measuring the specific activity of a urinary metabolite. All the radioactivity excreted as that metabolite must be collected, and all the metabolite must arise from the hormone. Then if R = radioactivity injected, τ = specific activity of the metabolite in time T, then it can be shown using the model in Fig. 2 (Laumas *et al.*, 1961) that

$$\frac{R}{T} = S\left[\frac{1+\dfrac{a}{a+e}\left(\dfrac{S_2}{b}\right)}{1+\dfrac{\varepsilon_1}{\varepsilon_1+\varepsilon_2}\left(\dfrac{S_2}{b}\right)}\right]$$

where ε_1 and ε_1 are the rates of excretion (combination of rate of formation and renal clearancel) of a particular metabolite from the inner and outer pools, respectively. Thus again, as for the metabolic clearance rate estimates, S_2 or e must be zero for the secretion rate calculation to be valid. It can also be shown that the expression is valid whether the radioactivity is given as a single injection or a constant infusion. Hence the secretion rate can be determined at the same time as the M.C.R. value and therefore the plasma concentration of the hormone can be calculated (mean value during urine collection). This calculation has already been discussed for aldosterone (Tait *et al.*, 1961). For progesterone, the secretion rate has been estimated by determining the specific activity of urinary pregnandiol (Pearlman, 1957). The specific activity was measured by correlating the radioactivity and sulfuric acid chromogen in the fractions from a high resolution partition column. The purity of the fractions was checked by infrared spectroscopy. The urine was collected for 4 days. The following values were obtained (milligrams per day): males (14 subjects) 4.2, range 1.1–6.5, and ovariectomized females (5 subjects) 1.7, range 0.9–2.5. From the clearance rate of progesterone (2950 liters plasma per day), the plasma concentration can be calculated (micrograms per 100 ml plasma): males, mean 0.14, range 0.04–0.21 and ovariectomized females, mean 0.06, range 0.03–0.08. Values determined directly by the use of a S^{35}-thiosemicarbazide, H^3 indicator method (Riondel *et al.*, 1962) are in the same range of values for these groups of subjects (mean 0.10, range 0.03–0.31 μg/100 ml, 11 males; mean 0.06, range 0.05–0.07 μg/100 ml, 6 ovariectomized females). However, larger numbers of subjects and diurnal and day-to-day variation of secretion and M.C.R. remain to be investigated.

E. ALTERATIONS IN THE METABOLIC CLEARANCE RATE OF ALDOSTERONE

As discussed, there is now evidence that the hepatic extraction of aldosterone is nearly 100% in normal subjects. If this is so, it is to be expected that the metabolic clearance rate will be proportional to the hepatic blood flow in various physiological conditions. Thus for a compound such as bromosulfalein, with a hepatic extraction of only 50%, the clearance may be relatively independent of hepatic blood flow

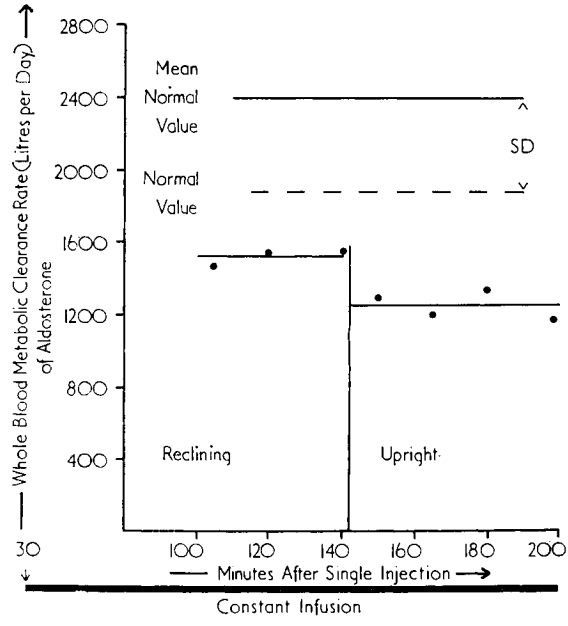

FIG. 7. Metabolic clearance rate of aldosterone estimated by the infusion method in a subject placed in upright and reclining positions.

(Bradley *et al.*, 1953) as the extraction may vary inversely with the flow rate. However, for a compound with 100% extraction, it is likely that its clearance rate will be proportional to the hepatic blood flow particularly when it is lowered from normal.

Figure 7 shows one investigation of this possibility. The subject was infused continuously with (7-H^3) aldosterone in the reclining position until the plasma radioactivity as aldosterone was constant. She was then placed in an upright position and the plasma radioactivity was again measured at intervals. It can be seen that there was a highly significant lowering of the metabolic clearance rate in the upright position. This has been found in for out of five subjects investigated. Culbertson *et al.* (1951) have shown a marked lowering of the hepatic blood flow in

similar conditions. This alteration in metabolic clearance rate would be expected to raise the aldosterone plasma concentration (even if the secretion rate remained constant) and perhaps cause some salt retention.

There are undoubtedly other mechanisms for causing salt retention in this situation. As shown by Muller and co-workers (1958) and also by Gowanlock *et al.* (1959), there is probably an increase in aldosterone secretion which could not be due to a decrease in metabolic clearance rate. Also the rapid onset of salt retention from the reclining to the upright position makes it doubtful whether aldosterone causes the immediate response (Pitts, 1959). Renal hemodynamics may also play a part (Gowanlock *et al.*, 1959). Nevertheless it seems possible that this alteration in metabolic clearance rate, if confirmed in other subjects, and the consequent increase in plasma concentration of aldosterone may play some part in the complex mechanism of salt retention in this situation. In general, it throws some doubt on the interpretation of experiments which completely dismiss the role of aldosterone in a situation by showing electrolyte responses of adrenalectomized animals or subjects with Addison's disease maintained on a constant intake of steroids although some glucucorticoid secretion may be necessary for a full response (Rosenbaum *et al.*, 1955). It may also be that the lowered metabolic clearance rate of aldosterone in some vena cavally constricted dogs (Ayers *et al.*, 1961) may be due to lowered hepatic blood flow as well as to alterations in intrinsic hepatic extraction caused by congestion (Ayers *et al.*, 1961).

It is of interest that in many of the conditions, such as vena caval constriction and severe sodium depletion, known to result in stimulation of aldosterone secretion, it might be expected that there would be a lowered hepatic blood flow and also therefore a decreased metabolic rate of aldosterone. These two mechanisms would be expected to be additive in raising the peripheral plasma concentration of aldosterone, although quantitatively the increase in secretion would be expected to be the major factor in most situations. It may be that in chronic experiments the lowering in M.C.R. would decrease the secretion rate by causing salt retention. Dobson (1957) suggested that alterations in hepatic blood flow may be a factor in the control of peripheral plasma concentrations of aldosterone. This does not now seem likely to be the only factor (Dobson, 1957) as it is well established that there is a marked increase in aldosterone secretion rate in sodium-depleted experimental animals (Denton, 1961) and in normal man (Ayres *et al.*, 1957; Peterson, 1959). Studies on the simultaneous alterations in the metabolic clearance rate of aldosterone-stimulating hormone remain to be carried out as one possible connecting link between the two mechanisms, which may still operate even though variations in output of ASH should prove to be an important factor in the control of aldosterone secretion (Davis *et al.*,

1962). *Note added in proof.* For a more recent treatment of the definition and meaning of the M.C.R. of a steroid, see Tait, J. F. (1963). *J. Clin. Endocrinol. and Metabolism* (in press) and Tait, J. F. and Burstein, S. (1963) *in* "The Hormones", vol. 5 (G. Pincus, K. V. Thimann and E. B. Astwood, eds), Academic Press, New York.

ACKNOWLEDGEMENTS

This work was supported by United States Public Health Grants A-3179, A-3598, and A-3490, Josiah Macy, Jr. Foundation Grant and Institutional Grant IN-56, American Cancer Society (Massachusetts Division).

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DISCUSSION

Ring A and Side-Chain Reduction Processes in Cortisol Metabolism

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The metabolic transformation of cortisol may be performed through different processes. Gold *et al.* (1959), working on this subject, came to the following conclusions: about 80% of cortisol (F) was transformed into its tetrahydro derivatives (THF, allo-THF, THE) by means of successive ring A reduction processes, the first of which (Δ^4-bond reduction) yielded the dihydro derivative DHF and was rate limiting, so that DHF neither accumulated nor appeared in the urine (Schneider, 1952); a fraction of THF and THE was converted into cortol and cortolone by means of a further side-chain reduction process and into 11-oxy-17-ketosteroid after removal of the side-chain; 20% of the cortisol metabolites did not pass through the phase of its tetrahydro derivatives: cortisol first underwent a side-chain reduction process yielding its 20-hydroxy derivatives, which in turn might be further converted into cortols and cortolones (α and β) by a ring A reduction process (Fig. 1).

It is well known that the cortisol metabolites largely undergo a process of conjugation and that for the most part they are conjugated with glucuronic acid (Gallagher, 1954; Peterson *et al.*, 1955; Migeon *et al.*, 1956).

Studies on the urinary metabolites of F carried out by means jo 4–C^{14}-cortisol have yielded information as to the quantitative relationships between the above-mentioned metabolic pathways of cortisol (Hellman *et al.*, 1954; Plager *et al.*, 1954, 1957; Gallagher, 1954; Peterson *et al.*, 1955). A small percentage (3%) of F metabolites was excreted in a free form consisting mainly of free THF, THE, and F. Another fraction, the glucurone-conjugated one, accounted for 50–55% of the total amount. In this fraction the Porter–Silber nonreacting metabolites accounted for 17–18%. It is known that they form the so-called 17-hydroxycorticosteroids (17-OHCS) which mainly consist of the glucuronosides of THF, THE, and allo-

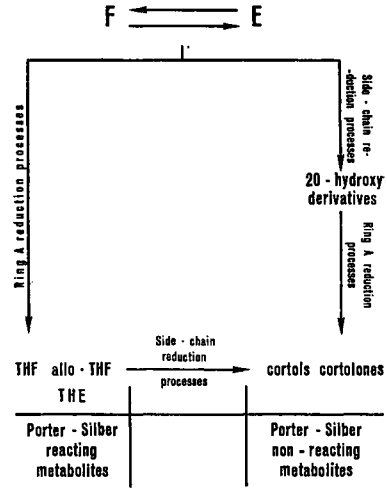

Fig. 1. Ring A and side-chain (C^{20}) reduction processes in cortisol metabolism.

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THF: to these must be added the small fraction (3%) of the free compounds mentioned above. In total 17-OHCS account for about 20% of the total amount of the cortisol metabolites (Peterson *et al.*, 1955). Therefore, 17-OHCS may be considered as the metabolic products of a well-defined transformation pathway leading to the tetrahydro derivatives of F through the ring A reduction processes. On the other hand, the formation of the Porter–Silber nonreacting glucurono-conjugated metabolites of F (20-hydroxy derivatives, cortols, cortolones) is due to a side-chain reduction process yielding the 20-hydroxy derivatives of F: this process may be either preceded or followed by ring A reduction, so that cortols and cortolones are formed.

Using a personal method for the contemporary dosage in plasma both of free and glucurono-conjugated 17-OHCS (Ceresa and Cravetto, 1958), we were able to follow the curves of these compounds after intravenous injection of cortisol in normal subjects and in patients suffering from various conditions (Ceresa, 1959, 1961, Fig. 2; Ceresa *et al.*, 1960a, 1960b).

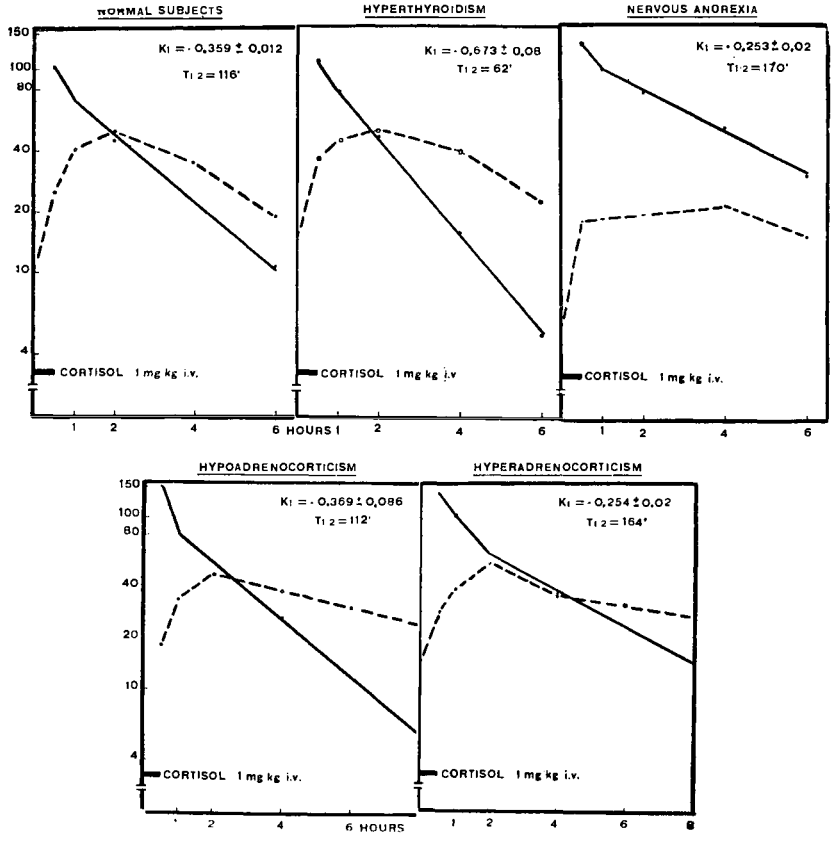

FIG. 2. Free (straight line) and glucurono-conjugated (dotted line) 17-hydroxycorticosteroids of plasma (μg/100 ml) after intravenous injection of cortisol in normal subjects and in patients suffering from various conditions.

In order to evaluate the possibility that the ring A reduction processes yielding the 17-OHCS are involved to a different extent in normal subjects and under different conditions, an attempt was made to calculate the rate of formation of glucurono-conjugated 17-OHCS from free cortisol. This formation constant was then compared with the free cortisol removal constant from plasma.

The mathematical model for this system was

$$\frac{dx}{dt} = -K_1 x$$

$$\frac{dy}{dt} = K_{1a} x - K_2 y$$

where x = plasma level of free cortisol (μg/100 ml); y = plasma level of glucurono-conjugated 17-OHCS; K_1 = removal constant of x; K_{1a} = formation constant of y (that is, that part of K_1 yielding y); K_2 = removal constant of y. In this model x is a direct precursor of y.

The value of K_1 was determined by the usual least squares method from the experimental data of $x(t)$ plotted on semilogarithmic paper. In order to calculate K_{1a} the method of the analysis of the precursor-product relationship (Rescigno and Segre, 1961) was applied according to the suggestions of Segre (1962). For this purpose the first value of the weighting function between $y(t)$ and $x(t)$ was calculated for decreasing values of Δt.

The results of this analysis are shown in Table I. The removal constants of the in-

TABLE I

Subjects	K_1	K_{1a}	$K_{1a} : K_1$
Normal subjects	0.359 ± 0 012[a]	0.071 ± 0.007	0.20
Hyperthyroidism	0.673 ± 0.08	0.132 ± 0.023	0.19
Nervous anorexia	0.253 ± 0.02	0.050 ± 0.008	0.20
Hypoadrenocorticism	0.369 ± 0.08	0.065 ± 0.008	0.18
Hyperadrenocorticism	0.254 ± 0.02	0.125 ± 0.014	0.50

[a]Standard error of the mean.

jected cortisol (K_1) were found to differ under the various conditions considered: higher and respectively lower than normal where higher (hyperthyroidism) and respectively lower (nervous anorexia) metabolic rates were registered. Also the constants at which the injected cortisol was removed as glucurono-conjugated 17-OHCS (K_{1a}) were found to differ in the five groups. This means that the intensity of the metabolic processes yielding the glucurono-conjugated 17-OHCS may change according to the different rates at which free F is removed from plasma under the various conditions examined.

The ratio $K_{1a} : K_1$ gives the fraction of total removal of F due to the formation of glucurono-conjugated 17-OHCS, that is, the relative involvement of the metabolic pathway represented by the ring A reduction processes. In the normal group the ratio was 0.2; this value agrees with the findings of Peterson *et al.* (1955), according to whom 17-OHCS account for one-fifth of the total amount of cortisol metabolites.

The ratios were practically identical in hyperthyroidism, nervous anorexia, hypoadrenocorticism. On the contrary, in patients suffering from hyperadrenocorticism the $K_{1a} : K_1$ ratio was considerably higher. This means that in these patients, as compared with the other conditions considered, the ring A reduction processes play a greater role in cortisol degradation. Therefore in hyperadrenocorticism the metabolism of F seems to differ qualitatively with respect to the other conditions examined.

The values for the $K_{1a} : K_1$ ratios in hyperthyroidism, nervous anorexia, and hypoadrenocorticism did not give any evidence of a different involvement of the ring A reduction processes in the metabolism of F in comparison with normal subjects.

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Interaction of Thyroid with Steroid Hormone Metabolism[1]

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Thyroid hormone is a major factor in steroid hormone metabolism. From the results thus far, it would be fair to presume that the biochemical processes involved in the transformation of every steroid hormone can be materially altered by change in the level of thyroid hormone. These steroid-transforming enzyme systems, therefore, are important targets upon which thyroid hormone acts and are sensitive indicators of this influence. The purpose of this discussion is to summarize the results of studies with five different steroid hormones.

The interrelationship between the level of thyroid hormone and the biotransformation of steroids was first investigated with the androgens (Bradlow *et al.*, 1956). It can be illustrated by comparison of the endogenous androgen metabolites of normal subjects with a group of myxedematous patients matched as far as possible with respect to age and sex. It is convenient to consider these in terms of the androsterone fraction, that is, the percentage of the total quantity of androsterone (A) and etiocholanolone (E) that is comprised by androsterone alone. From Fig. 1 it can be seen that the normal subjects had an androsterone fraction of approximately 40%. In contrast the androsterone fraction of the patients with spontaneous myxedema was approximately 15%. It is now known that these two metabolites in normal subjects are derived almost exclusively from the adrenal hormone dehydroisoandrosterone (Vande Wiele and Lieberman, 1960). Therefore, deficiency in thyroid

[1] This investigation was supported in part by a grant from the American Cancer Society and a research grant (CY-3207) from the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

The work described is the joint contribution of a group of investigators. These are Drs. Bradlow, Fishman, Fukushima, Hellman, and Zumoff, to whom I am indebted for their cooperation and persistence in exploration of the areas reported as well as many others which could not be included in this brief summary. We, in turn, are indebted to our assistants who have carried out much of the experimental work. These include: V. Bankers, M. Bobrovich, S. Carey, M. Champen, K. Daley, S. Dobriner, M. Dunham, T. Freeman, A. Gallagher, B. Gallagher, N. Hellinger, R. Jandorek, D. Kreling, R. Lehman, D. Leskin, J. Liang, A. Moore, S. Nicodemus, M. Pardon, T. Salumaa, M. Smulowitz, S. Teller.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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hormone caused a smaller portion of dehydroisoandrosterone to be converted to androsterone. In addition to this alteration in peripheral transformation, there is a sharply decreased production of both metabolites in myxedema. Thus, a low level of thyroid hormone is associated with decreased production of the hormone and a metabolic alteration that leads to greater production of etiocholanolone and smaller formation of androsterone.

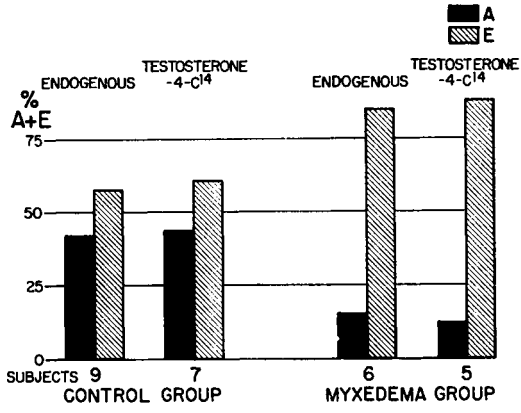

FIG. 1. Comparison of the metabolites derived from endogenous androgen (dehydroisoandrosterone) and exogenous testosterone in euthyroid control subjects and patients with myxedema.

The same changes in metabolism were demonstrated with testosterone. For this study, testosterone-4-C^{14} was administered intravenously and the urinary steroids in the ensuing 48 hours were examined. The seven normal subjects were part of the control group and it is of interest to note how closely the biotransformation of exogenous testosterone resembled the metabolic fate of the endogenous dehydroisoandrosterone. The androsterone fraction after the tracer hormone was again about 40%, in excellent agreement with the endogenously produced metabolites. A comparable study was made of five patients in the myxedema group who received an intravenous tracer dose of testosterone. Here again there was a sharp difference between these patients and the control group in that 15% or less of the exogenous hormone was transformed to androsterone. There was a concomitant increase in the amount of etiocholanolone produced.

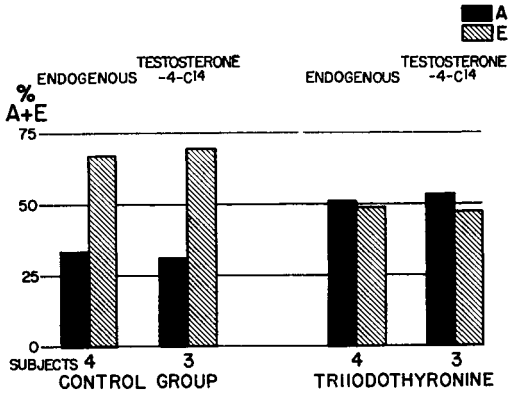

FIG. 2. Comparison of the metabolites derived from endogenous androgen (dehydroisoandrosterone) and exogenous testosterone in euthyroid control subjects and the same people after treatment, with 3,5,3′-triiodothyronine.

Figure 2 demonstrates the effect of triiodothyronine administration on and rogen metabolism both from glandular production and after testosterone-4-C^{14} in the same subjects. It can be noted that triiodothyronine brought about the reverse of the alteration in metabolism found with myxedema. This was true for both the endogenously produced hormone and the exogenous tracer. The androsterone fraction increased from 30% to over 50% when thyroid hormone was elevated. Even more striking changes in the androsterone fraction were observed in spontaneous hyperthyroidism, where the androsterone fraction amounted to almost 75% (Gallagher *et al.*, 1960).

While effective treatment of myxedema increases dehydroisoandrosterone production, an elevated level of thyroid hormone is not necessarily associated with heightened secretion of this steroid. In fact, most euthyroid subjects when given triiodothyronine show the alteration in metabolism that has been described without change in the total production of hormone. However, occasional euthyroid patients who receive triiodothyronine do show evidence of increased secretion as well. In spontaneous hyperthyroidism most of the patients examined in our series exhibited androgen metabolites in the lower range of normal values and only one of ten produced amounts in the higher level of normal. In this respect the interrelationship of androgen and thyroid hormone is different for that found with hydrocortisone.

The peripheral metabolism of hydrocortisone was altered by the level of thyroid hormone, but at quite a different molecular site. Rather than the stereochemistry of carbon 5, thyroid-hydrocortisone interacted on the processes that determine the state of oxidation or reduction of the carbon 11 hydroxyl group. In this discussion three metabolites of hydrocortisone with the intact side chain will be considered. These are tetra-

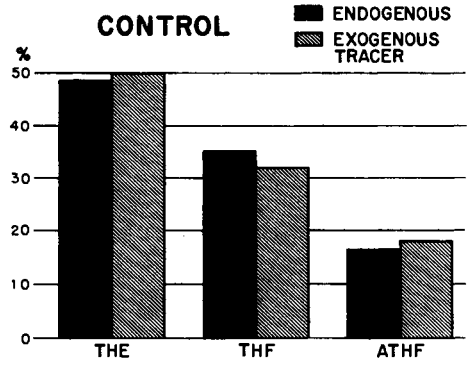

FIG. 3. Reducing metabolites formed from endogenous and exogenous hydrocortisone by normal euthyroid subjects.

hydrocortisone (THE), tetrahydrohydrocortisone (THF), and allotetrahydrohydrocortisone (ATHF), which together comprise about 50% of the urinary metabolites derived from hydrocortisone. Analogous results were obtained with the other metabolites (Hellman *et al.*, 1961).

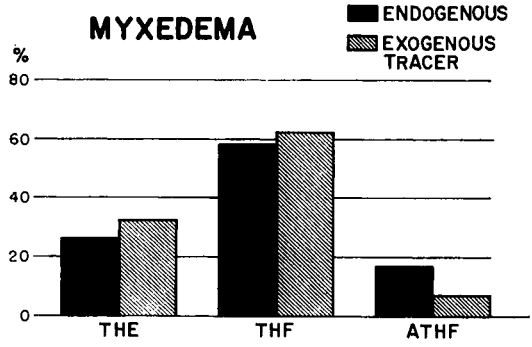

FIG. 4. Reducing metabolites formed from endogenous and exogenous hydrocortisone by patients with myxedema.

In Fig. 3 the composite results from a series of euthyroid subjects under control conditions are shown. The values charted for the endogenously produced metabolites are the percentage of the total which each compound comprised; the tracer results are in terms of the fraction of radioactivity found in each compound after the intravenous administration of a small dose of hydrocortisone-4-C^{14}. The tracer hormone faithfully mirrored the endogenous production so that either the quantity of endogenous metabolites or the amount of radioactivity in each substance could be used to measure the thyroid effect. The major metabolite in these euthyroid subjects was THE, which represented about 50% of the total of the three compounds. Under control conditions THF was the next largest and comprised about 35% of the total. The smallest component was ATHF at the level of about 17% of these three end products. These results

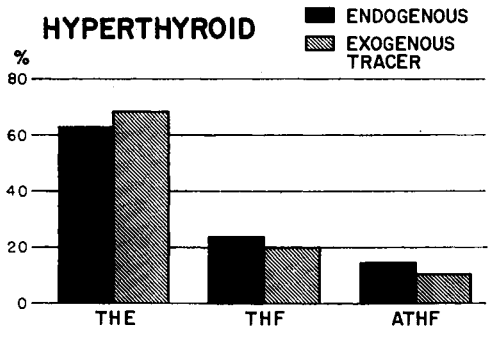

FIG. 5. Reducing metabolites formed from endogenous and exogenous hydrocortisone by patients with hyperthyroidism.

are in excellent agreement with the extended investigations of normal subjects reported from the Worcester Foundation (Romanoff *et al.*, 1959).

A group of patients with myxedema was examined in the same manner, both with respect to their endogenous production of these three metabolites and the way in which they transformed a tracer dose of hydrocortisone-4-C^{14}. There is a great contrast in hydrocortisone metabolism between the euthyroid subjects and patients with a deficiency of thyroid hormone, as shown in Fig. 4. The myxedema patients had 60% of the total of these three metabolites as THF. The amount of THE was materially diminished as compared to the euthyroid group and comprised approximately 30% of the total. The amount of ATHF was changed only slightly, if at all.

The reverse of this situation was characteristic of spontaneous hyperthyroidism where THE represented more than 60% of the total, while the amount of THF corresponded to about 20% (Fig. 5). As in myxedema the ATHF fraction was essentially unaltered from that characteristic of the euthyroid subjects. Comparable findings were obtained in euthyroid subjects treated with triiodothyronine and studied both under control conditions as well as during the induced hyperthyroid state. These manifested the same change in the peripheral metabolism of hydrocortisone that was characteristic of spontaneous hyperthyroidism.

It is noteworthy that the amount of hydrocortisone converted to the two pregnane derivatives, THE and THF, remained essentially constant and there was no increase or decrease in the metabolite of the allopregnane series, ATHF. Therefore, there was no alteration by thyroid hormone of the mechanisms that saturated ring A; rather the level of thyroid hormone was predominant on those processes that regulated oxidation or reduction of the hydroxyl group at C-11.

The physiological consequences of these alterations in the peripheral metabolism of hydrocortisone are profound. In the hyperthyroid state a large fraction of hydrocortisone is oxidized to the biologically inactive cortisone. Cortisone per se is very probably incapable of suppressing the pituitary. Therefore, there is an increased secretion of corticotropin (ACTH) and further outpouring of hydrocortisone. This mechanism operates until the absolute level of hydrocortisone (and perhaps its related 11-hydroxy metabolites) is sufficient to bring the pituitary adrenal system into a new level of homeostasis. As a consequence there is an increased production of hydrocortisone in hyperthyroidism although the circulating effective adrenal hormone is essentially within normal limits. This effective level is reflected in the amount of THF found in the urinary metabolites. In hypothyroidism, oxidation of the 11-hydroxyl group is diminished, so that the pituitary can be suppressed at a low level of hydrocortisone production. This explanation is in harmony with the fact that in hyperthyroidism the total output of hydro-

cortisone metabolites is greatly increased over normal, whereas in hypothyroidism the total production of hydrocortisone is materially decreased (Fig. 6). The glandular output, therefore, is determined by the transformations which the hormone undergoes in the peripheral tissue, and these in turn, are regulated by the effective level of thyroid hormone.

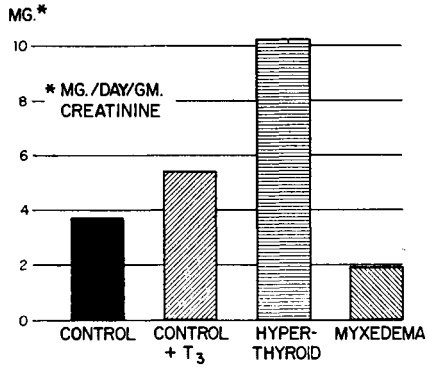

FIG. 6. Comparison of endogenously produced reducing metabolites of hydrocortiosne at various levels of thyroid hormone. Sum of THF, THE, and ATHF.

Bearing in mind that thyroid hormone affected the stereochemical course of the reduction of the double bond in androgens and the state of oxidation of the 11-hydroxyl group of hydrocortisone, it seemed of interest to us to study a hormone where both effects might possibly occur in the same molecule. The substance chosen was the adrenal hormone 11β-hydroxyandrost-4-ene-3,17-dione. As shown in Fig. 7, in the euthyroid individual the principal metabolite of this hormone is 11β-hydroxy-5α-androstan-17-one (OH-A), which accounts for more than 60% of the total products that have been identified. The two oxygenated metabolites of the etiocholane series are small by comparison and together account for approximately 15% of the remainder.

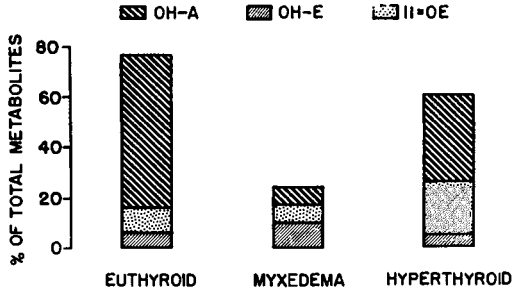

FIG. 7. Comparison of three metabolites formed from 11β-hydroxyandrost-4-ene-3,17-dione at different levels of thyroid hormone.

The metabolism of this hormone was greatly altered at different levels of thyroid activity, but not in the manner we had expected. In myxedema the amount of this hormone transformed to OH-A was greatly diminished. This finding might seem consistent with the results with

dehydroisoandrosterone or testosterone. However, the hormone did not go to the usual products of the 5β-androstane series but instead to other as yet unidentified compounds. This is work in progress which we hope to complete in the near future. In hyperthyroidism the amount of OH-A obtained was less than in the euthyroid individual but not quite so low as in myxedema. On the other hand, there was an increase in the 11-keto derivative of the 5β-androstane series which might possibly be a reflection of the effect seen with hydrocortisone metabolism. These puzzling results require much further work. It is evident, however, that here are also a group of enzyme systems that metabolize steroid hormones, which in turn are affected by the thyroid.

The biotransformation of the phenolic hormone estradiol is also controlled by the thyroid hormone (Fishman *et al.*, 1962). This problem was studied in a group of euthyroid subjects who received a tracer dose of radioactive estradiol while they were untreated and at a later time, after administration of triiodothyronine. Patients with myxedema and hyperthyroidism were similarly studied before and after their disorders were corrected. For technical reasons, only three metabolites are considered in this discussion. These are estrone, estriol, and 2-methoxyestrone. The results are given as percentages of the radioactivity recovered in the form of each compound. There was an inconsistent effect upon estrone which increased sightly as the level of thyroid hormone was elevated. There was, however, a considerable change in the pro-

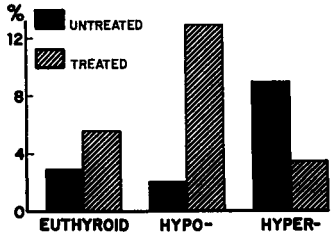

FIG. 8. Changes in transformation of exogenous estradiol to estriol induced by thyroid hormone; "treated" with euthyroid and hypothyroid subjects refers to administration of thyroid hormone; "treated" with hyperthyroid subjects refers to administration of I^{131}.

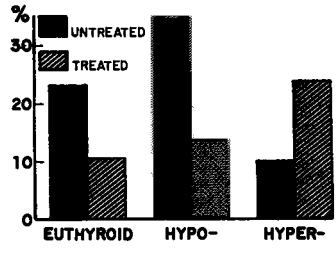

FIG. 9. Changes in transformation of exogenous estradiol to 2-methoxyestrone induced by thyroid hormone; "treated" has the same meanings as in Fig. 8.

duction of estriol. In every instance when thyroid hormone was increased, either spontaneously or by the administration of triiodothyronine, the estriol derived from the tracer hormone was decreased to less than half that found during the control study (Fig. 8).

An even greater change was found with 2-methoxyestrone (Fig. 9). In every individual studied, the amount of 2-methoxyestrone was increased as thyroid hormone was elevated. In fact, 2-methoxyestrone was over 250% of the control level if all the subjects studied are considered as one group.

That there was only a minor change in estrone is probably very significant since the central role of this compound in estrogen metabolism has been established. It seems evident that this precursor was available in adequate amount and thyroid hormone exerted its action on the stages beyond oxidation to estrone. At least two enzyme systems distinct and different from those already reported for neutral steroids were very materially affected by thyroid hormone. This influence of the thyroid may very well have important physiological consequences since estrogens produce such profound changes at minute concentration.

In summary then, the level of thyroid hormone is one of the principal determinants in the direction of metabolism of steroid hormones. The enzyme systems concerned affect many sites in different steroid hormones. The physiological implications of some of these alterations have been established and those remaining provide a fertile ground for further exploration.

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DISCUSSION

Thyroid and Steroid Hormone Metabolism

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It is common knowledge that hypofunction of the thyroid is generally accompanied by a decrease of the urinary excretion of 17-hydroxysteroids and of 17-ketosteroids, without apparent signs of adrenocortical insufficiency. In hyperthyroidism, a normal or increased excretion of corticoids is found, although signs of hypercorticism are generally absent, and even relative adrenocortical insufficiency may be present, as is suggested by the favorable influence of corticoid treatment of thyroid storm.

Although these facts have been known for some time, their physiopathologic mechanisms were, until recently, incompletely understood despite several studies devoted to these problems. Levin and Daughaday in 1955 observed normal plasma corticoid levels both in hyper- and hypothyroidism and showed that exogenous cortisol disappears much faster from the plasma in hyperthyroidism than in normal controls whereas in hypothyroidism, the disappearance rate is decreased. These authors also observed that the increment of urinary excretion of Porter and Silber chromogens after cortisol load (15 mg/m^2) was lower in hyperthyroidism than in normal patients. On the other hand, administration of thyroxine to guinea pigs resulted in an increased excretion of 17-OHCS before the increase in basal metabolism was maximal. As an explanation for the lack of consistent evidence of adrenocortical hyperfunction in clinical hyperthyroidism these authors suggested: (1) an altered degradation of cortisol, resulting in less Porter–Silber chromogens, (2) an antagonism between thyrotropin (TSH) secretion and corticotropin (ACTH) secretion: in spontaneous hyperthyroidism, characterized by increased TSH secretion, ACTH secretion would be decreased; this would explain the relative adrenal insufficiency of the thyrotoxic crisis, characterized by a normal or low secretion of cortisol together with an increased utilization. Administration of thyroxine would result in an inhibition of TSH secretion with a concomitant increased ACTH secretion.

Attractive as this hypothesis may be, it seems difficult to explain the increased cortisol production observed in hyperthyroidism by Peterson (1958). This increased production was interpreted as being secondary to the accelerated cortisol metabolism. The latter was shown by Garren and Lipsett (1961) to be a specific effect of the thyroid hormone as in euthyroid hypermetabolism these authors found a normal or diminished plasma removal rate. It is, however, the great merit of Dr. Gallagher's group (Hellman et al 1959, 1961) to have shown that the level of thyroid hormone not only changes the total production and inactivation of hydrocortisone, but also influences

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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steroid metabolism in an qualitative manner. In a series of beautiful experiments, which Dr. Gallagher succinctly reviewed in the preceding paper, he showed that, when thyroid hormone level is high, testosterone and dehydroisoandrosterone are converted predominantly to metabolites of the androsterone series whereas etiocholanolone is the main metabolite when thyroid hormone level is low. As moreover androsterone was shown to lower plasma cholesterol levels, this leads to the most important and highly stimulating conclusions that a hormone can influence enzymes controlling the metabolism of another hormone and that metabolites of hormones may have an independent physiological function; it can be expected that in the near future other hormone metabolites will be shown to have a physiological function.

Another stimulating finding is the fact that hyperthyroidism or triiodothyronine administration shifts cortisol metabolism toward the formation of 11-oxo metabolites. This leads to the hypothesis that the increased production of hydrocortisone found in hyperthyroidism is at least partly a consequence of the efficient oxidation of the 11β-hydroxyl group. In hypothyroidism, on the other hand, the slower oxidation of the 11β-hydroxyl group would result in a longer pituitary suppression with decreased cortisol production.

In this connection the results of the *in vitro* experiments of McGuire and Tomkins (1959, 1960) should be recalled: these authors found an accelerated reduction of the Δ^4-3 keto group in thyroxine-treated rats; this in itself would lead to an increased excretion of 11β-hydroxytetrahydrocorticoids, but the *in vivo* experiments show that thyroxine accelerates the oxidation of the 11-hydroxyl group even more than the reduction of the Δ^4-3-keto group.

If thyroxine deficiency is responsible for the decreased oxidation of the 11β-hydroxyl group, then in panhypopituitarism a similar distribution of metabolites should be found as in primary hypothyroidism. We studied this in three patients and compared their excretion pattern to the pattern in four patients with Cushing's syndrome, two patients with hyperthyroidism, and one patient with primary myxedema.

Table I shows the results. Whereas in normal subjects the ratio of 11-hydroxy to 11-oxo metabolites is about 1.2, it can be seen that in Cushing's syndrome the 11β-hydroxy metabolites greatly predominate over the 11-oxo metabolites. Paradoxically, the same phenomenon is observed in panhypopituitarism and in myxedema. The altered metabolism in Cushing's syndrome as well as after ACTH stimulation may be related to changes in protein binding, but the alteration observed in both primary and secondary hypothyroidism points to the direct influence of thyroxine. These results confirm those of Gallagher's group. We also measured the cortisol production by the isotope dilution method. In normal controls the mean production was 21.0 mg, whereas in hypothyroidism, we found a mean cortisol production of 14.0 mg agaist 23.6 mg in hyperthyroidism. The latter figure is hardly above the upper limit of normals, and in fact, several hyperthyroid patients had a cortisol production of less than 20 mg. This is at variance with the results obtained by Peterson. However, when one compares Peterson's results obtained both by the plasma method and the isotope dilution method, one is struck by the discrepancy between the results: by the plasma method production values were respectively, 51, 65, and 65 mg against 15.8, 30.5, and 30 mg by the isotope dilution method; the latter values are only slightly above the upper normal limit. Although we all know that plasma half-life studies may not be extrapolated to the 24-hour production, this important discrepancy observed in hyperthyroidism might suggest that the diurnal variations in plasma cortisol levels or in the cortisol pool might be greater in hyperthyroidism than in normal subjects. **Added in proof:* This has since been observed by

TABLE I

Excretion Pattern of Croticoids in Different Groups of Patients[a, b]

Diagnosis	Patient	Sex	Age	20α-Hydroxy-cortisol	20β-Hydroxy-cortisol	20β-Hydroxy-cortisone	Cortisol	Cortisone	THE	THF	ATHF (3α, 5α)	11β-OH / 11-oxo
Adrenocortical hyperplasia	D. C.	F	35	950	1.840	300	3.460	170	1.000	1.470	8.000	10.7
Adrenocortical hyperplasia	A.	M	33	60	380	—	2.800	—	3.500	3.800	1.100	2.3
Adrenocortical hyperplasia	D. P.	M	23	60	—	—	1.450	40	1.600	2.300	—	2.3
Adrenocortical carcinoma	M.	F	29	—	—	200	1.200	80	2.040	2.680	2.440	1.5
Graves' disease	D. C.	F	38	—	48	—	120	60	6.200	2.600	1.400	0.6
	V. K.	F	33	—	24	20	180	48	4.800	1.600	1.800	0.75
Hypothyroidism	D. C.	M	44	—	—	—	28	12	500	880	400	3.9
Panhypopituitarism	CAU	M	52	—	—	—	10	5	140	280	100	2.7
	FRE	F	49	—	—	—	12	—	500	950	440	2.8
	MAR	F	3	—	—	—	12	6	200	750	300	5.3

[a] All values given as micrograms per 24 hours.

[b] THE, tetrahydrocortisone; THF, tetrahydrohydrocortisone; ATHF, allotetrahydrohydrocortisone.

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The demonstration of an altered estradiol metabolism in hyperthyroidism with increased formation of estrone and 2-methoxyestrone is another example of the thyroid hormone action on the enzyme systems that metabolize steroid hormones. This mutual influence of hormones, together with the concept that hormone metabolites may have an independent physiological function, seems to us the most important acquisition of the experiments reported by Dr. Gallagher.

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The Metabolism and Localization in Tissues of Tritium-Labeled Steroid Sex Hormones[1]

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I. INTRODUCTION

An insight into the biochemical mechanisms underlying the biological action of the steroid sex hormones might be gained by determining the precise nature and molecular concentration of these hormones and their metabolites in systemic blood plasma and in target sexual tissues. Since the concentration of endogenous hormones in target tissues is much too low to be detected by conventional methods, indirect methods are resorted to which require (1) the preparation of steroid sex hormones of extraordinarily high specific activity, and (2) their administration to women with breast cancer and also to normal intact rats of both sexes. The radioactive hormone is infused intravenously at a constant rate over a prolonged period of time in order to approach a steady state such as might exist in the living organism under physiological conditions. It seemed more advantageous to determine radioactivity concentrations in the various tissues at dynamic equilibrium than to follow the rapid and variable rates of disappearance of the hormonal radiometabolites.

II. STUDIES WITH LABELED ANDROGEN

Our studies on the metabolism *in vivo* of Δ^4-androstene-3,17-dione-7-H^3 and its localization in the ventral prostate and other tissues of the rat have recently been reported (Pearlman and Pearlman, 1961). The free, or unconjugated, radioactive metabolites which were isolated from the plasma and the ventral prostate and quantitatively estimated, included testosterone, 5α- and 5β-androstan-3α-ol-17-one (androsterone

[1] This investigation was supported by grants from the National Cancer Institute, United States Public Health Service; the National Science Foundation; and the Jane Coffin Childs Memorial Fund for Medical Research.

[2] Recipient of a Research Career Development Award, United States Public Health Service.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics1
Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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and etiocholanolone), Δ^4-androstene-3,17-dione, and 5α-androstane-3,17-dione. The pattern of radiometabolites in the ventral prostate resembled that in the plasma, but the concentration of radioactivity in that sexual organ was significantly higher than that in plasma or muscle, a finding suggestive of a selective process in operation in the prostate. The molecular concentration of total radiometabolites, consisting of both nonandrogenic and androgenic steroids, remained at catalytic levels (10^{-6} M) in the ventral prostate despite the continuous infusion of Δ^4-androstene-3,17-dione-7-H^3 at a rate which undoubtedly exceeded that of endogenous hormone.

III. STUDIES WITH LABELED PROGESTERONE

Studies similarly with estrone-6,7-H^3 and progesterone-7-H^3 are in progress. Methods for labeling these hormones (and also Δ^4-androstene-3,17-dione) with tritium have been described (Pearlman, 1961; O'Donnell and Pearlman, 1958). Only those studies on patients with breast cancer will be presented herein. The experimental design is based on the following considerations.

The physiological secretion of the endogenous hormone may be simulated by infusing the radioactive hormone intravenously at a constant rate; the radioconcentration in the plasma during the course of the infusion is determined. One may then apply the following set of equations (Eqs. 1 and 2) to determine the turnover time (T) of endogenous hormone:

$$c/r = c'/r' \tag{1}$$

where c = plasma concentration of infused radioactive hormone (or metabolites), c' = plasma concentration of endogenous hormone (or metabolites), r = infusion rate of radioactive hormone, and r' = secretion rate of endogenous hormone. If the infusion is carried out over a sufficiently long period of time, the plasma radioactivity will ultimately attain a maximal and constant level, and a steady state may be said to ensue.

$$T = c/r = c'/r' \tag{2}$$

at the steady state, where T = turnover time (as minutes per liter of plasma), c = the plasma radioconcentration (as microcuries per liter of plasma), and r = the infusion rate (as microcuries per minute). Additional criteria of the steady state in experiments of this sort require that the composition of the plasma radioactive material remain constant and that the ratio c/r remain very small relative to the infusion period.

The concept of turnover time of steroid sex hormones may be a particularly useful one since, as was pointed out (Pearlman, 1957a), it defines the quantitative relationship between the amount of hormone which the organism produces and the amount which circulates in the

blood stream. As the response of the target sexual organs is dependent on the plasma hormone concentration, it is important to understand not only the factors controlling the endogenous hormone secretion rate, but also those factors modifying its turnover time in the plasma pool under various physiological conditions.

Progesterone-7-H^3, specific activity 1.72 $\mu c/\mu g$, was infused intravenously into an oophorectomized subject (E.S.) at a constant rate of

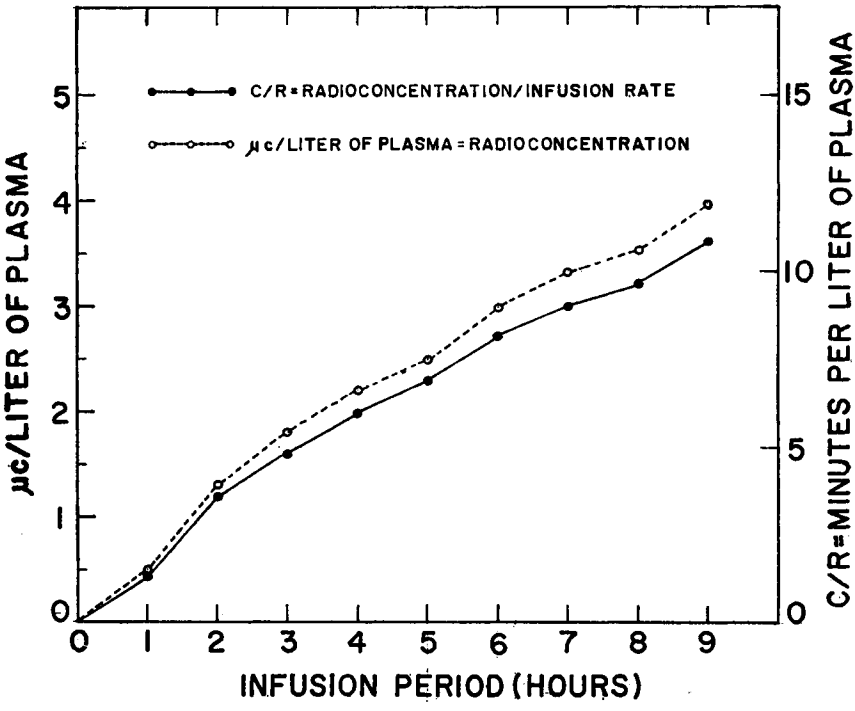

FIG. 1. Plasma concentration of acetone-soluble radioactivity during the intravenous infusion of progesterone-7-H^3 into an oophorectomized subject at a constant rate of 0.302 μc/minute.

0.302 μc/minute (or 0.175 μg/minute) for 9 hours. The plasma level of acetone-soluble radioactivity rose steadily during the infusion period (see Fig. 1). Although a steady state was not reached, it may have been approached because the ratio c/r at the ninth hour was very small (11.85 minutes) relative to the total infusion period, and the composition of the plasma radiometabolites at the sixth and ninth hour was essentially constant, as will be shown presently. It appears that the radioactive hormone is very rapidly removed from the blood stream and maintained at very low circulating levels despite the continuous infusion of progesterone-7-H^3.

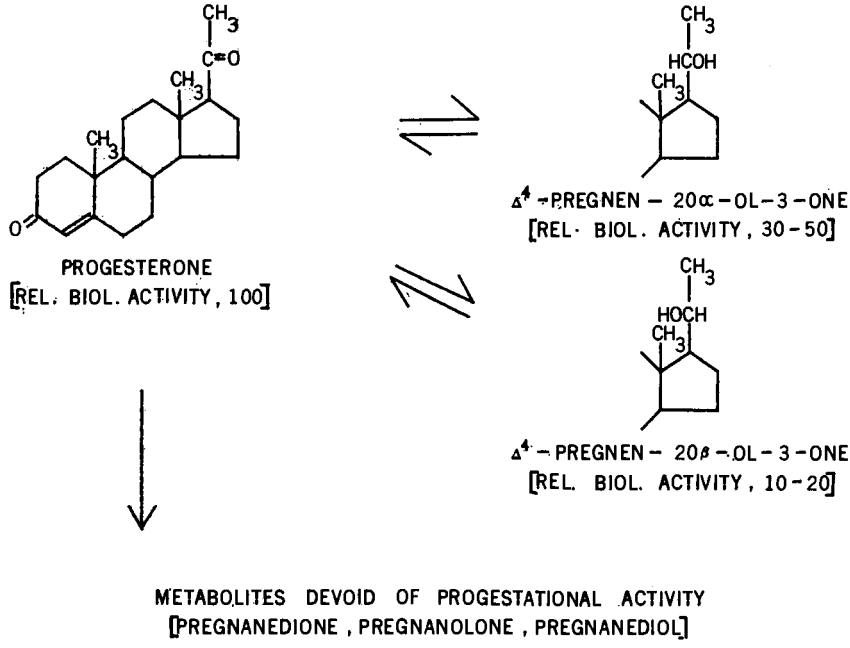

FIG. 2. The metabolism of progesterone with respect to its biological inactivation.

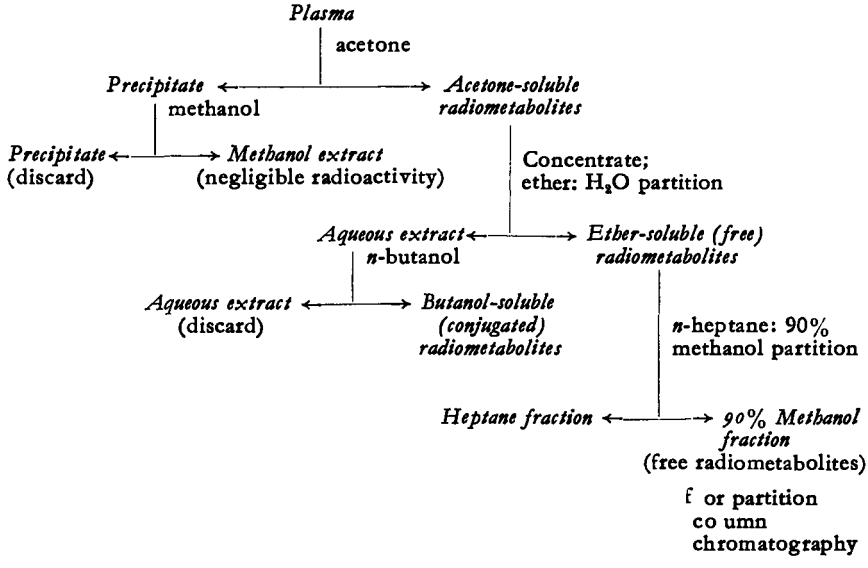

Fig. 3. Extraction of plasma and preliminary fractionation of plasma extracts; progesterone-7-H^3 and estrone-6, 7-H^3 infusion experiments.

What is the precise nature of the radioactive material in the plasma? Our knowledge regarding the metabolism of progesterone, particularly with respect to its biological inactivation, is summarized in Fig. 2. The addition of hydrogen at carbons 4 and 5 of the progesterone molecule to form pregnanedione is irreversible and results in complete loss of biological activity; subsequent metabolic transformations may therefore be regarded as inconsequential from a biological point of view. Thus, one may expect the progestational activity of the plasma to reside in the free (ether-soluble) fraction, and to be altogether lacking in the conjugated (butanol-soluble) fraction; our primary interest, for reasons previously mentioned, is in the biologically active metabolites. With this in mind, the plasma radioactive extracts were fractionated as outlined in Fig. 3. The percentage of radioactivity recovered in the free (ether-soluble) fraction is given in Table I. It is at once

TABLE I

Partitioning of Acetone-Soluble Extracts of Plasma into Free (Ether-Soluble) and Conjugated (Butanol-Soluble) Fractions

Plasma sample[a] at hour	Radioactivity recovered (%) in fraction		
	Free (A)	Conjugated (B)	(A+B)
6	16.9	78.1	95.0
8	19.6	70.3	89.9
9	13.1	79.9	93.0

[a] Plasma samples were obtained during the intravenous infusion of progesterone-7-H^3

apparent that only a small fraction of the total plasma radioactivity could possibly represent progestationally active compounds. Moreover, subsequent examination of the free fraction revealed that only 36.1–45.0% of its radioactivity could be attributed to progesterone, and 6.1—8.4 % to Δ^4-pregnen-20α-ol-3-one; very little, if any, of the 20β-epimer of the latter compound was present. This was established by partition column chromatography on siliconized Celite of the individual free fractions derived from the plasma collections at 6, 8, and 9 hours.

The results in each case were similar, and these are summarized in Table II; a typical chromatogram (8-hour plasma) is shown in Fig. 4. The radioactive material in the eluate fractions containing carrier progesterone were pooled (6 + 8 + 9 hour plasma collections), diluted further with carrier, and chromatographed over aluminum oxide; the eluate fractions were recrystallized to constant specific activity. Thus, it was shown by isotope dilution analysis that 91.1% of the radioactivity in the progesterone area seen in Fig. 4 was indeed attributable to progesterone. The eluate fractions in the Δ^4-pregnen-20α-ol-3-one area (Fig. 4) were pooled (see also Table II, 6 + 8 + 9 hour plasma

TABLE II

Identification and Estimation of Free Radiometabolites of Progesterone-7-H^3 in Plasma[a]

Pooled eluate fractions (ml): plasma sample			Total radioactivity (%) chromatographed: plasma sample			Steroid identified
6 Hr	8 Hr	9 Hr	6 Hr	8 Hr	9 Hr	
12–26	12–26	12–26	28.2	28.7	15.9	Polar
28–44	28–44	28–44	7.5	6.1	8.4	Δ^4-Pregnen-20α-ol-3-one
46–90	46–98	46–74	5.4	5.1	4.9	
92–140	100–152	76–128	45.0	37.6	36.1	Progesterone
Stripping	Stripping	Stripping	2.9	5.8	5.7	Nonpolar
Total recovered			90.0	83.3	71.0	

[a] Isotope dilution analysis was perfomed on the appropriate pooled eluate fractions obtained on partition column chromatography (see Fig. 4).

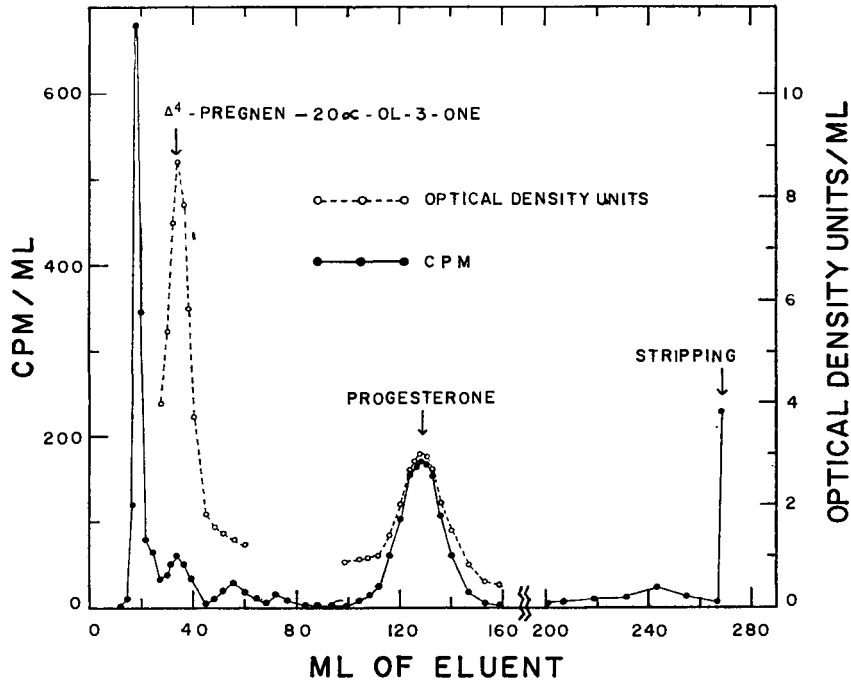

FIG. 4. Reverse phase partition column chromatography of free (ether-soluble) radioactive fraction (progesterone-7-H^3 infusion experiment). Solvent system, toluene-70% methanol. Progesterone and Δ^4-pregnen-20α-ol-3-one were added as carriers; optical density was determined at 240 mμ in ethanol.

collections) and rechromatographed on a partition column of Celite in another solvent system as described in Fig. 5; the eluate fractions in the peak area were pooled, diluted with carrier, and recrystallized to constant specific activity. Thus, 89.8% of the radioactivity in the Δ^4-pregnen-20α-ol-3-one area (Fig. 4) was shown to be due to this compound.

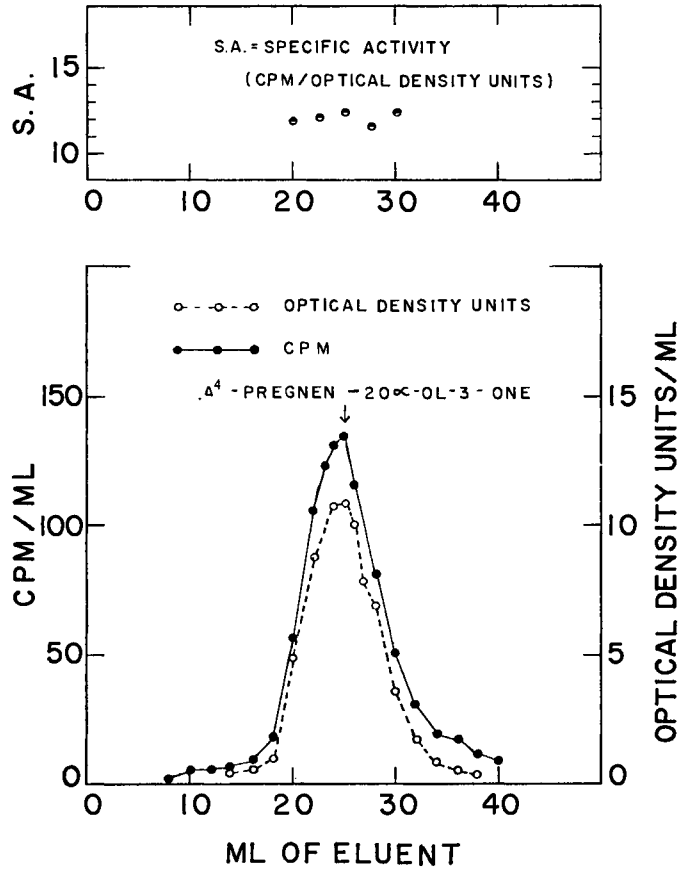

FIG. 5. Partition column chromatography of pooled eluate fractions (see Fig. 4 and Table II) containing Δ^4-pregnen-20α-ol-3-one as carrier. Solvent system, *n*-heptane-toluene (40 : 60)-80% methanol.

Of only incidental interest was the butanol-soluble fraction of the plasma. Purification of this material revealed that its radioactivity was due chiefly to pregnane-3α,20α-diol in a conjugated form, probably the glucuronide, which was completely hydrolyzed by refluxing for 10 minutes in 1 *N* hydrochloric acid.

From the above data, it may be calculated that radioac ive proges-

terone constitutes only 3.85% of the total plasma radioactivity at the ninth hour of hormone infusion, and that the turnover time of progesterone is about 0.46 minutes per liter of plasma. The turnover time of progesterone in the entire plasma pool (2.5 liters × 0.46 minutes per liter = 1.15 minutes) appears to be very short indeed in the oophorectomized subject under study. It is interesting to compare this figure (1.15 minutes) with that (about 3.3 minutes) found in advanced human pregnancy. The latter figure (Pearlman, 1957a) was derived from an endogenous secretion rate of 250 mg of progesterone per 24 hours in metabolism experiments with progesterone-16-H^3 (Pearlman, 1957b) and a circulating level of 80 μg of progesterone per liter of peripheral blood [physicochemical estimation by Zander (1955)]. It would appear therefore, that the turnover time of circulating progesterone is relatively independent of gross variations in the endogenous secretion rate of the hormone since, it will be recalled, the infusion rate of progesterone-7-H^3 in the study described today was only 0.175 μg/minute or 0.252 mg/24 hours. However, the endocrine status of the individual may be a factor modifying the turnover time of progesterone (and possibly of other steroid sex hormones).

IV. STUDIES WITH LABELED ESTROGEN

Estrone-6,7-H^3, specific activity 0.448 μc/μg, was infused into patients with breast cancer at a constant rate of 0.212, 0.179, and 0.185 μc/minute in experiments 3, 5, and 6, respectively; the plasma was extracted and fractionated as previously described (see Fig. 3). The results are shown in Figs. 6 and 7. The turnover time of the acetone-soluble radioactive metabolites was 4.95 and 10.6 minutes per liter of plasma in experiments 3 and 5, respectively (Fig. 6).

The subject, C. W., in experiment 6 was at a more advanced stage of the disease when extensive metastases to the liver had occurred and liver function was very poor. It is apparent from Fig. 6 (compare C.W. in experiments 3 and 6) that the removal of estrogen from the blood stream was greatly impeded in the latter condition; indeed, a steady state was not reached by the ninth hour of infusion as was the case in experiments 3 (C.W.) and 5 (E.R.).

As our primary interest is in the biologically active metabolites in plasma, it may be helpful at this point to review very briefly what is already known regarding the metabolism of estrone and the relative estrogenic potency of its metabolites (see Fig. 7). Next are presented the results (see Table III) obtained on partitioning the radioactive plasma extracts into free (ether-soluble) and conjugated (butanol-soluble) fractions: only one-third of the plasma radioactivity resides in the free fraction. It appears that not only is the hormone rapidly removed from the blood stream, but it is also rapidly metabolized to form conjugated

products. Preliminary data obtained in our laboratory by Dr. K. R. Laumas indicate that radioactive estrone accounts for less than one-half the total free radioactivity and that substantial amounts of more polar radiometabolites are present.

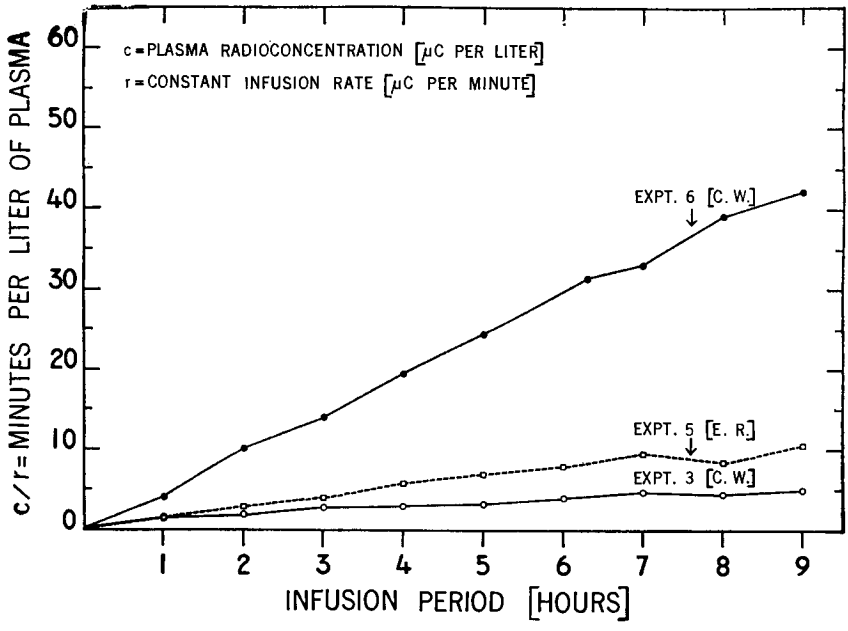

FIG. 6. Ratio of concentration *(c)* of acetone-soluble radioactivity in plasma to infusion rate *(r)* of estrone-6,7-H^3. The radioactivity in methanol extracts of the precipitate obtained following acetone extraction of the plasma was negligible in all experiments.

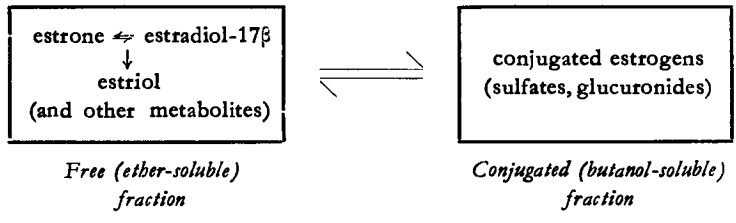

Fig. 7. The metabolism of estrone.

The relative order of estrogenic potency (high → low) is: estradiol-17β, estrone, estriol, conjugated estrogens.

TABLE III

Partitioning of Acetone-Soluble Extracts of Plasma into Free (Ether-Soluble) and Conjugated (Butanol-Soluble) Fractions

Expt. no.	Subject (endocrine status)	Plasma sample[a] (hour)	Radioactivity recovered % in fraction		
			Free (A)	Conjugated (B)	(A + B)
	C. W.				
3	(oophorectomized–	6	31.4	69.7	101
	hypophysectomized	9	36.5	37.9[b]	74.4
	E. R.				
5	(oophorectomized)	6	22.3	75.1	97.4
	adrenalectomized)	9	27.2	70.7	98.0

[a] Plasma samples were obtained during the intravenous infusion of estrone–6,7–H^3.
[b] Recovery appears to have been incomplete in this instance.

V. CONCLUDING REMARKS

Tritium-labeled steroid sex hormones (androgen, progesterone, and estrogen) of very high specific activity were prepared and administered to the living organism under conditions simulating the physiological secretion of endogenous hormone. It was observed that the organism is capable normally of maintaining circulating sex hormones at very low or catalytic levels despite the continuous infusion of the hormone over a prolonged period of time. This quantitative relationship between plasma hormone concentration and hormone secretion (or infusion) rate was determined as the turnover time.

The target sexual tissues appear to be exposed via the systemic circulation to a variety of biologically active metabolites arising from the single hormone originally administered. Localization of tritium-labeled androgen in the ventral prostate of the rat was also demonstrated.

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DISCUSSION

Comments on the Biological and Biochemical Aspects of the Experiments

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Dr. Pearlman has told us that a selective process is in operation in the prostate as far as the concentration of the radioactive androgens is concerned, although the amount does not rise above a catalytic level of 10^{-6} *M*. Would that mean that a specific mechanism exists by means of which androgen metabolites are transported and stored in the prostate or could one suppose that only one compound is selectively taken up by the gland and further metabolized *in situ*? In respect to the amount of these compounds in the prostate, I would like to ask Dr. Pearlman whether he has tried, by means of very high androgen dosage, to find out whether the prostate androgen content would than still remain at a low catalytic level. A parallel situation occurs with respect to the absorption of cholesterol by the intestinal wall. There, only free cholesterol is absorbed against a gradient and is further metabolized within the intestinal wall. Nevertheless, the concentration of cholesterol in the intestinal tissue does not exceed a certain level, irrespective of the amount present in the lumen.

It is obvious that steady state conditions are necessary to carry out this type of experiment. However, it is well known that, physiologically speaking, the endogenous secretion of corticosteroids fluctuates in a daily rhythm that precludes any "steady state" condition occurring normally. I wonder whether Dr. Pearlman has any data which may indicate that such a situation exists also as far as the compounds he has studied are concerned?

I would like now to turn again to the prostate and to point out that this organ is extremely sensitive to exogenously administered androgens. The same holds true for two other organs, the seminal vesicle and the levator ani muscle. In this connection I would like to ask two questions: (1) Does the administration of Δ^4-androstene-3,17-dione result in a selectively increased concentration of its metabolites in these two target organs also? (2) Does Dr. Pearlman have any experience with anabolic compounds in this respect? The basis for this question is the fact that there appears to be a close biological relationship between anabolic agents and androgens, especially in their effect on prostate, seminal vesicle, and levator ani. Of special practical interest is the fact that the turnover time of circulating progesterone was found to be relatively independent of gross variations in the endogenous secretion rate. Is this true also for estrone? It should be noted finally that the liver function evidently influences this value to a large degree. Even if normally the quantities of steroid metabolizing enzymes are sufficient,

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the estrone studies of Dr. Pearlman show that liver disease can greatly increase the turnover time. It would be interesting to know which of the steroid-metabolizing enzymes is affected first and which last and whether there seem to be any sex or species-specific differences in this connection.

W. H. PEARLMAN: It seems to me rather improbable that only one androgenic compound would be selectively taken up by the ventral prostrate; in fact, our experiments show that a variety of androgens enter prostate tissue via the systemic plasma.

We have not carried out experiments to determine whether the prostate androgen content would rise above catalytic levels after intravenous injection of androgens in excessively high amounts.

It is conceivable that the plasma androgen level may fluctuate from day to day or even during the course of a single day. However, physiological conditions of endogenous hormone secretion might be expected to resemble more closely the steady state approached in our experiments than the situation obtained in experiments in which the hormone is injected in a single, rapid dose. Our experiments attempt to supply information which is lacking regarding the precise nature and concentration of the estrogen and progesterone metabolites that circulate in plasma. We do not have proof that a steady state actually prevails under normal, physiological conditions, but this is not really the point. The experiments which I have just outlined provide, we believe, a useful approach to the problem.

We have not studied the seminal vesicles in our experiments, nor do we have any experience with anabolic compounds in this respect.

The turnover time of circulating estrogen likewise appears to be relatively independent of gross variations in the endogenous hormone secretion rate, but further evidence is required.

It would be indeed very interesting to find out which of the enzymes involved in steroid sex hormone metabolism are first affected when liver function is impaired. This may very well be a subject for future investigation, as might also be your query regarding the influence of sex or species differences in this connection.

In Vitro Conversion of Deoxycorticosterone to Some Acidic Metabolites[1]

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I. INTRODUCTION

Although there is no evidence that carboxylic acids are among the excreted metabolites of the adrenal steroids in man or animals, their formation has not been excluded since the average fractionation scheme includes one or more steps which serve to eliminate substances of this class. As an approach to this problem it seemed of interest to determine whether such metabolites are formed *in vitro*. Using deoxycorticosterone (I) as substrate and surviving guinea pig liver slices as the enzyme source, we have found that under aerobic conditions four steroid carboxylic acids are formed. These are 3-keto-4-etienic acid (Va), 3,20-diketo-4-pregnen-21-oic acid (VIa) and both possible 3-keto-20-hydroxy-4-pregnen-21-oic acids (IIIa and IVa). Approximately 78% of the etienic acid (which was the major acidic metabolite) was recovered as an ester glucuronide. It is the purpose of this paper to describe the isolation, identification, and preparation of the above carboxylic acids and to detail the purification and characterization of the glucuronide.

II. METHODS AND RESULTS

A. INCUBATION AND EXTRACTION PROCEDURES

In a representative experiment 12.5-ml volumes of 0.1 *M* Tris buffer, pH 7.6, containing 200 μg of free substrate (I) per milliliter were incubated with agitation for $1^1/_2$ hours at 38°C (gas phase O_2) with a sufficient number of guinea pig liver slices to maintain a steroid-to-tissue (wet weight) ratio of 1 : 1000 or more. The incubate was diluted with hot methanol and filtered; after adding tertiary-butanol to suppress foaming, the filtrate was concentrated to a small volume *in vacuo*. In this fashion a total of 500 mg of (I) were incubated over the course of 5 days with slices prepared from a total of 100 animals.

[1] This investigation was supported by research grant AM-01255 from the Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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The combined aqueous concentrate was freed of neutral lipid in the usual fashion and the neutral fraction was recovered by extraction with ethyl acetate. Reextraction following pH adjustment to 1.5 and the addition of salt gave a green-brown acidic fraction.[2]

B. PAPER CHROMATOGRAPHIC FRACTIONATION

Of the several fractionation techniques tried, the most effective employed paper chromatography solely. Figure 1 indicates the fractionation sequence in outline form.

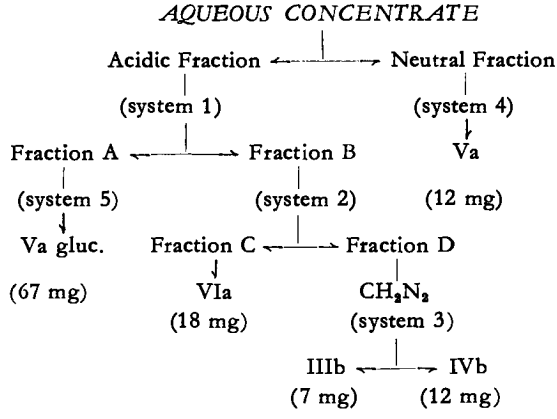

FIG. 1. Isolation sequence and yields of metabolites.

The crude acidic fraction was applied as 16-cm bands on thirty 19 × 60-cm sheets of Whatman No. 1 paper and chromatographed[3] for 3 hours using the system toluene, 170; *n*-butanol, 30; glacial acetic acid, 60; water, 140 ml (system 1). This served to divide the ultraviolet-absorbing metabolites into a major, polar component (fraction A) and a minor, mobile group (fraction B). Both were recovered by extracting the paper with methanol. Prior to rechromatography fraction B was to a degree

[2] Because of the known lability of the α-ketol side chain, several small-scale experiments (each repeated on three separate occasions) were carried out to determine whether the acids isolated were formed artifactually. The incubation of (I) in the complete system lacking only tissue or the addition of (I) to a substrate-free system at the conclusion of the incubation uniformly failed to give ultraviolet-absorbing [or soda-fluorescence (Bush, 1952) positive] areas on chromatograms of the acidic fractions.

[3] Equilibration and chromatography were carried out in uninsulated, paperlined glass tanks at a temperature of 25 ± 1° C. The absorption spectra were determined with a Zeiss PMQ II spectrophotometer and the optical rotations in a Zeiss 0.01° polarimeter. The melting points were taken with a Fisher-Johns apparatus and are recorded as read. The Huffman Microanalytical Laboratories, Wheatridge, Colorado, performed most of the elementary analyses.

freed of extraneous matter by distribution between ethyl ether and aqueous sodium bicarbonate. The recovered acidic fraction was applied as wide bands on twenty 19 × 60-cm sheets and chromatographed for 5 hours using the system toluene, 70; *n*-heptane, 130; methanol, 100; anhydrous formic acid, 30; water, 70 ml (system 2). This separated fraction B into a major, mobile, ultraviolet-absorbing component (fraction C) and a minor, polar moiety (fraction D).

Fraction C was suspended in water, freed of neutral material, and reextracted with ethyl acetate following acidification. The acidic extract was extracted with aqueous sodium bicarbonate from which the acid was recovered in the usual fashion. The residue was treated briefly with charcoal and crystallized twice from ethyl acetate to give 18 mg of plates which melted at 183–186°C; $[\alpha]_D^{27} = +173°$ ($c = 0.0836$ in methanol). Anal.: Found: C = 73.00; H = 8.27. The methyl ester, prepared with diazomethane, melted at 138–139°C; $[\alpha]_D^{26} = +181°$ ($c = 0.090$ in methanol). The isolated acid and its methyl ester were identified as 3,20-diketo-4-pregnen-21-oic acid (VIa) and its methyl ester (VIb) by direct comparisons [constants, mixture melting points, and paper chromatographic mobilities (four systems other than systems 2 and 3)] with authentic samples of (VIa) and (VIb).

Fraction D was recovered in the same fashion, treated with excess ethereal diazomethane and applied as wide bands on five 19 × 60-cm sheets. Chromatography for 9 hours using the system isooctane, 170; toluene, 30; methanol, 150; water, 50 ml (system 3) gave two well-separated ultraviolet-absorbing bands, namely a minor, mobile component and a major, polar constituent. Suspension of the methanol-soluble extracts in water followed by ethyl acetate extraction gave residues clean enough to permit crystallization from ethyl acetate: *n*-hexane, and then ethyl acetate. This gave, as nearly colorless crystals, 7 mg of a mobile ester (melting point 178–180°C) and 12 mg of a polar ester (melting point 187–188°C). The melting points were the same as the corresponding mobile (IIIb) and polar (IVb) esters of reference, and the melting points of the appropriate mixtures were not depressed. The paper chromatographic mobilities of the isolated mobile and polar esters were, within the limits of the method, the same as the corresponding synthetic mobile (IIIb) and polar (IVb) esters in four systems including system 3. This identified the isolated esters as the mobile methyl-3-keto-20-hydroxy-4-pregnen-21-oate (IIIb) and its polar C-20 epimer (IVb) but leaves the assignment of configuration at C-20 unsettled[4].

Initially we were unable to detect the etienic acid (Va) (or found only

[4] The assignment, generally, of configuration at C-20 in the steroid α-hydroxy acids has proved to be a more complex matter than anticipated. Originally (Lewbart, 1961) it was shown that, in the cases of the epimeric pairs of α-hydroxy acids derived from pregnan-3α-ol-11,20-dion-21-al and pregnane-3α,11β-diol-20-on-21-al, acetylation at

small amounts) in fraction B, but eventually it was found in the neutral fraction. [5]Once discovered, it was readily recovered by aqueous sodium bicarbonate extraction, applied as wide bands on four 19 × 60-cm sheets and chromatographed for 8 hours using the system *n*-heptane, 200; methanol, 50; glacial acetic acid, 60; water, 90 ml (system 4). Recovery in the usual fashion followed by crystallization from methanol gave 12 mg of needles, melting point 236–239°C, $[\alpha]_D^{28} = +138°$ ($c = 0.0090$ in methanol), $\lambda_{max.} = 240–241$ mμ, $\varepsilon = 16{,}400$. The methyl ester melted at 133–134°C. Direct comparisons (constants and the melting points of appropriate mixtures) of the isolated acid and its methyl ester with authentic specimens of (Va) and (Vb) fixed the identity of the isolated acid.

The major part of the etienic acid (Va) was recovered as the referred-to glucuronide. This conjugate was purified in the following fashion. Fraction A was suspended in dilute Tris buffer, pH 7.6. The pH was adjusted to 1.5 and, after the addition of some salt, the suspension was extracted with ethyl acetate. The combined extracts were extracted twice with 2 *M* Tris buffer, pH 7.6, once with more dilute buffer, and twice with water. Following the removal of neutral material, the pH was lowered and the glucuronide was recovered as indicated above. The residue was chromatographed on a 30 × 600-mm column in which the support was Celite and the system ethylene chloride, 190; tertiary-butanol, 10; glacial acetic acid, 60; water, 140 ml (system 5). Those fractions of the eluate containing the conjugate were evaporated *in vacuo* and freed of acetic acid. The residue was dissolved in aqueous methanol and, after adjustment of the pH to around 6 by the careful addition of dilute sodium hydroxide, the solution was treated briefly with charcoal, concentrated, and the sodium salt of the conjugate allowed to crystallize from aqueous methanolic acetone. Recrystallization from the same system gave 67 mg of nearly colorless needles.

The individual recoveries of the metabolites are indicated in Fig. 1. Together they total 91 mg or 18% of the deoxycorticosterone incubated.

C-20 uniformly resulted in a positive shift in M_D in one series to which the β configuration tentatively was assigned. Recently Dr. Mattox has, by an independent series of reactions, correlated one of the above α-hydroxy acids with a 20,21-diol of known configuration. This established that the configuration of the acids at C-20 is the reverse of that assigned on the basis of the M_D shift. At the present time the author and Dr. Mattox are attempting to establish the configuration at C-20 of the α-hydroxy acids described in this paper.

[5] The paper chromatographic mobility of the etienic acid (Va) is so much greater than that of the acids (IIIa), (IVa), and (VIa) that it was advantageous to recover it in this fashion and to rechromatograph it separately. In the control experiments (see footnote 2) the neutral fraction was recovered by extraction with ethyl ether and the acidic fraction by reextraction with ethyl acetate, a procedure designed to place all the acids in the acidic fraction.

C. SYNTHESIS OF ACIDS OF REFERENCE

Figure 2 indicates the methods used and the structures of the acids prepared. It is our intention to suggest, in formulas (IIIa), (IIIb) and (IVa), (IVb), that the configurations at C-20 have not been fixed. Details for the preparation of (II), (IIIa), and (IVa) were taken from the Ph.D. thesis of Dr. Marvin Lewbart (1961) and therefore are not detailed here.

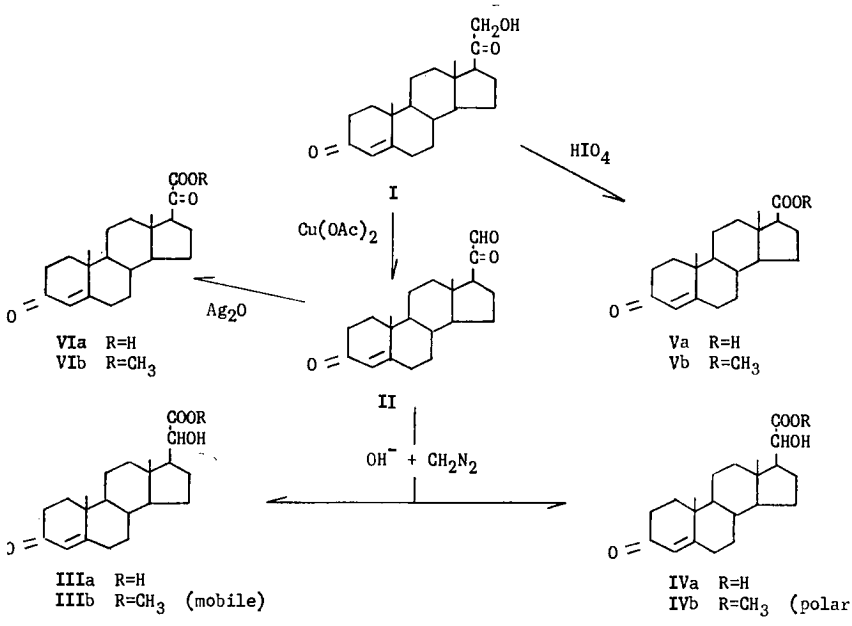

FIG. 2. Preparation of acids of reference.

The author wishes to thank Dr. Lewbart and Dr. Vernon Mattox for their generous advice in the preparation of these compounds.

Treatment of (I) with copper acetate in methanol in an oxygen atmosphere gave the glyoxal (II). It crystallized only from aqueous methanol as the hemiacetal. Our inability to obtain the glyoxalor its hydrate in pure crystalline form parallels the experience of Reich and Reichstein (1939).

Four experiments were carried out in which the hemiacetal of (II) was rearranged in aqueous sodium hydroxide to the epimeric α-hydroxy acids (IIIa) and (IVa). After treating the acidic fraction with diazomethane, separation of the epimeric esters was effected by paper chromatography using system 3. The yield of (IIIb), based on the weight of the hemiacetal, averaged 18% and that of (IVb) 35%. Both were crystallized from ethyl acetate. Constants for the mobile ester (IIIb) were: melting point, 181–182°C; $[\alpha]_D^{27} = +47.3°$ ($c = 0.0084$ in me-

thanol). Analysis: Calcd. for $C_{22}H_{32}O_4$ (360.5): C, 73.29; H, 8.94. Found: C, 73.41; H, 8.85. Constants for the polar ester (IVb) were: melting point, 187–188°C; $[\alpha]_D^{26} = +78.6°$ ($c = 0.0096$ in methanol). Analysis: Found: C, 72.93; H, 8.80.

The α-keto acid (VIa) readily was prepared in 60–65% yield from (II) by oxidation with silver oxide in benzene : pyridine. Crystallization from ethyl acetate gave plates melting at 183–186°C with some decomposition; $[\alpha]_D^{28} = +171°$ ($c = 0.0084$ in methanol). Analysis: Calcd. for $C_{21}H_2{}^8O_4$ (344.4): C, 73.02; H, 8.16. Found: C, 72.86; H, 8.03. $\lambda_{max} = 241–242$ mμ, $\varepsilon = 16{,}700$. The methyl ester melted sharply at 139–140°C. $[\alpha]_D^{24} = +181°$ ($c = 0.0082$ in methanol). Analysis: Calcd. for $C_{22}H_{30}O_4$ (358.5): C, 73.71; H, 8.43. Found: C, 73.69; H, 8.50.

Oxidation of (I) with aqueous periodic acid furnished a reference sample of 3-keto-4-etienic acid (Va). The product, crystallized from methanol, melted at 236–239°C with some decomposition. $[\alpha]_D^{26} = +140°$ ($c = 0.0085$ in methanol), $\lambda_{max.} = 240–241$ mμ, $\varepsilon = 16{,}700$. The methyl ester (Vb) was crystallized from aqueous acetone and melted at 133–134°C.

D. CHARACTERIZATION OF THE GLUCURONIDE

Incubation of an aqueous solution of the conjugate at pH 4.5 for 24 hours at 38°C with a commercial preparation of beef liver β-glucuronidase gave an acidic fraction from which pure 3-keto-4-etienic acid (Va) readily was recovered. Its identity was based on its melting point [234–238°C and not depressed on admixture with pure (Va)], its paper chromatographic mobilities [identical with that of pure (Va) in three systems including system 4], and the melting point of its methyl ester [132–134°C and not altered on mixing with pure (Vb)]. On the assumption that the conjugate is a monoglucuronide, the yield of the etienic acid (Va) as determined by direct ultraviolet spectroscopy at 240 mμ averaged, in three experiments, 91% of theory.

We were unable to crystallize the glucuronide free acid, but treatment with diazomethane gave a methyl ester which was crystallized from acetone: *n*-heptane. Constants: melting point indefinite owing to bubble formation. $[\alpha]_D^{26} = +113°$ ($c = 0.0086$ in methanol). Analysis: Calcd. for $C_{27}H_{38}O_9$ (506.5): C, 64.01; H, 7.56. Found: C, 63.91; H, 7.46. $\lambda_{max.} = 241–242$ mμ, $\varepsilon = 16{,}050$. With 2,4-dinitrophenylhydrazine in acid ethanol the glucuronide methyl ester gave a red hydrazone which was crystallized from chloroform : ethanol and then from ethyl acetate. Constants: melting point 222–224°C. Analysis: Calcd. for $C_{33}H_{42}O_{12}N_4$ (686.7): C, 57.71; H, 6.16; N, 8.15. Found: C, 57.34; H, 6.20; N, 8.23. $\lambda_{max.} = 392$ mμ, $\varepsilon = 28{,}000$.

This information is sufficient to establish the structure of the isolated conjugate as an ester glucuronide of the etienic acid (Va). In the sug-

gested structural formula (Fig. 3) esterification involves the C-1 hydroxyl group of the glucuronic acid moiety. This was assigned in conformity with the structures of other ester glucuronides [see Williams (1959) for references] rather than on additional evidence.

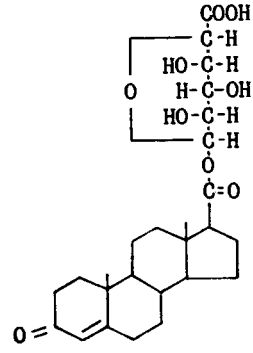

FIG. 3. Structure proposed for isolated glucuronide.

III. DISCUSSION

The few published studies on the *in vitro* conversion of adrenocortical steroids to acidic metabolites are limited to the isolation of 3-keto-4-etienic acid (Va) following the perfusion of deoxycorticosterone through rat liver (Picha and associates, 1952), human placenta (Romanoff, 1959), and bovine adrenal (Levy and Maloney, 1962).

Our belief that the acids recovered in our experiments are true metabolites of deoxycorticosterone rather than artifacts of manipulation, rests on the adequacy of our control studies, the avoidance of harsh technical maneuvers, and the variety and amounts of metabolites obtained. The conjugation of the etienic acid (Va) in our system does not necessarily serve as evidence that the acid was formed metabolically. Whether acids such as (Va) or (VIa) are formed *in vivo* in the guinea pig or other species remains unproved but surely merits investigation. Perhaps some of the steroid metabolites in human urine referred to as "unknown conjugates" or "highly water-soluble forms" are in fact carboxylic acids.

The recovery of the α-keto acid (VIa) provides the basis for an enzymatic mechanism accounting for the formation of the etienic acid (Va). Oxidative decarboxylation of the former would give the latter directly; simple decarboxylation would give the same product, presumably via the etienaldehyde. We regard the α-hydroxy acids (IIIa) and (IVa) as minor metabolites of the α-keto acid (VIa). Little evidence is available relative to the steps involved in the conversion of the C-21 hydroxymethyl group (of I) to the carboxyl group (of VIa). Cortisone-21-aldehyde rapidly is reduced to cortisone by a cell-free rat liver preparation

in the presence of DPNH (Schneider, 1953), and subsequently it was shown (Schneider, 1959) that liver alcohol dehydrogenase is equally effective. But we have been unable to demonstrate a reversal of this reaction. This would be far more interesting and useful in that, coupled with a suitable aldehyde dehydrogenase, the α-keto acid would be the product. Figure 4 illustrates the postulated metabolic sequence.

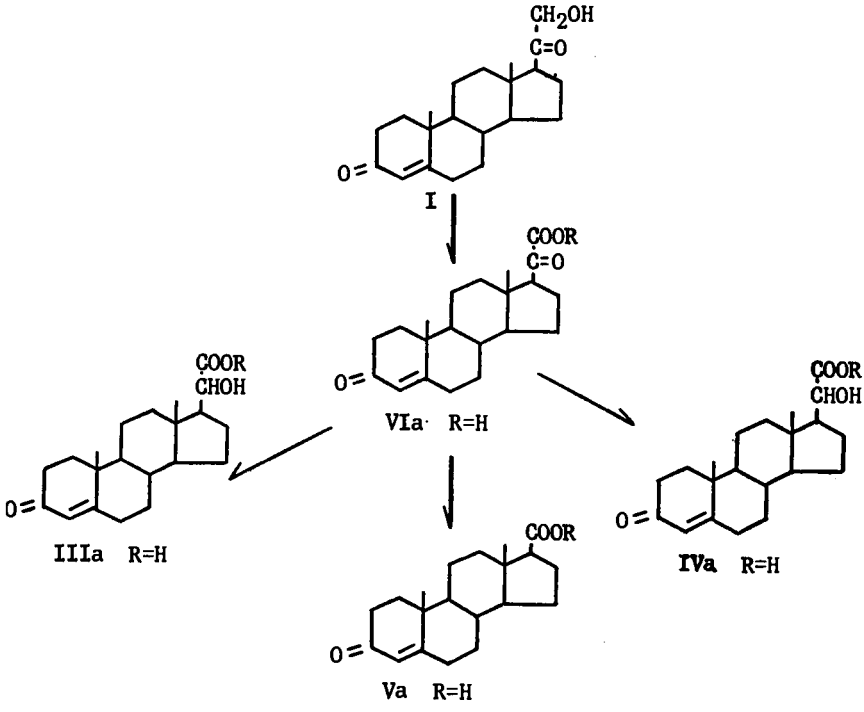

FIG. 4. Postulated metabolic sequence.

Disregarding differences in mechanism, the metabolic reactions here described are similar to those involved in the conversion of the cholesterol side chain to that characteristic of the bile acids to the extent that both are oxidative and degradative. A recent paper (Suld and associates, 1962) is to be consulted for references and a summary of the bile acid biogenesis work.

Williams' treatise (1959) lists a variety of ester glucuronides, none of which involve steroid carboxylic acids. The ester glucuronide which we have described appears to be the first such example in the steroid field. The enzymatic synthesis of a steroid glucuronide of the ether type (Isselbacher, 1956) also utilized guinea pig liver.

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DISCUSSION

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Dr. Schneider is to be complimented on his careful identification of these acidic substances formed from deoxycorticesterone (DOC) by guinea pig liver slices. It is unusual, these days, to find an investigator who takes the trouble to report melting points, optical rotations, and carbon and hydrogen analyses of new metabolic products. To obtain material for these measurements, however, one must incubate relatively large amounts of steroid, and in the present investigations, the steroid : tissue ratios used are so large as to be unphysiological. Dr. Schneider admits this. One wonders, however, if such conditions do not "force" the specificity of enzymes, leading to the formation of artifacts. The metabolic picture may be quite different when the steroid : tissue ratio is low. Cooke and Taylor, for example, at this Congress, report differences in progesterone metabolism *in vitro* when the steroid : tissue ratio is very low. Isotopic methods permit the use of such low ratios and the identity of products formed need be no less rigorous than by the methods used by Dr. Schneider.

I should also like to have seen corticosterone or cortisol incubated, rather than DOC, which is not known to be a hormone in the guinea pig.

I think that Dr. Schneider is being a little dogmatic in claiming that one of his metabolites is present as a glucuronide. His evidence would have been more convincing if he had shown that this β-glucuronidase hydrolysis was inhibited by the well-known specific inhibitor saccharolactone. The glucuronic acid liberated by enzymatic hydrolysis, if indeed it is such, may be oxidized to *D*-glucosaccharic acid, which may be identified as its dibenziminazole derivative (Grant and Marrian, 1950). This would undoubtedly be troublesome but, in my opinion, necessary before concluding that one was dealing with a glucuronate.

It is also of interest to note that Dr. Schneider added his steroid substrate in ethanol and that Cooke (1961) has shown that ethanol and propylene glycol inhibit glucuronide formation. The use of another vehicle, e.g., dioxane, might have given a different result.

Despite my reservations, I think we must accept these very interesting findings of Dr. Schneider, especially in view of the finding of these acidic substances by others in perfusion experiments. I wonder whether Dr. Schneider has any information on the biological activity of these acidic steroids.

J. J. SCHNEIDER. We believe that the utility of an *in vitro* preparation such as one employing liver slices should not be judged in terms of its approach to or deviation from the physiological reality Dr. Grant seeks. All such systems are necessarily unphysiological. We regard the liver slice preparation as a convenient and active enzyme source and have used it to determine which reactions *do* occur under wholly arbitrary conditions. We have never assumed that the same reactions occur or occur to the same

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extent in the intact animal or that a parallel series of metabolites would be obtained using another substrate. Our interest in physiology in this connection is limited solely to our concern that the reactions be enzyme induced. We believe that for the present the term "artifact" should be limited to substances formed nonenzymatically.

It is true that deoxycorticosterone is not secreted by the guinea pig adrenal, but this consideration has little bearing on the work in question.

Our dogmatism, relative to the composition of the glucuronide, reflects our faith in organic chemistry. But Dr. Grant's points are excellent and we shall carry out the procedures he suggests.

We were unaware that ethanol can inhibit glucuronide formation. Since 78% of the eticnie acid accounted for was conjugated, the effect in this case must have been slight. We noted, in agreement with Dr. W. Taylor, that the extent of conjugation was strongly influenced by the substrate : enzyme ratio.

We have not tested these acids for biological activity.

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Neurohumoral Factors from the Brainstem and Epiphysis[1]

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It seems appropriate to emphasize at the outset that the final answers to many aspects of this study are as yet unknown, and to review briefly some of the earlier experimental findings that led us to the present problems. For some time there has been a suspicion that the central nervous system is involved in the regulation of electrolyte balance; indeed, almost thirty years ago J. P. Peters postulated that this was the case. After the discovery of aldosterone, we began a series of studies aimed at determining whether central nervous control of this steroid was a factor in electrolyte balance. It soon became evident that ablation of an area in the posterior diencephalon and rostral midbrain results in marked depression in aldosterone output. In our experience, a very small lesion placed in the central region of the rostral dorsal midbrain, below the pineal gland, is sufficient to bring about this effect (Newman *et al.*, 1958; Taylor and Farrell, 1962).

The effects of similar lesions in man have been studied by Krieger *et al.* (1961, 1963). These investigators reported four patients with discrete tumors in the pretectal area who showed low urinary aldosterone levels or failed to show the expected increase in aldosterone output in response to sodium deprivation.

One should be careful not to conclude that brainstem regulation of aldosterone secretion involves only a stimulatory component whose destruction leads to diminished response. Following certain of our lesions, marked stimulation of steroidogenesis results (Newman *et al.*, 1958; Taylor and Farrell, 1962). In particular, damage to the pineal gland per se as well as the habenular area leads to elevation of steroid output, as though corticotropin (ACTH) secretion were enhanced. In

[1] Research supported by grants from the Cleveland Area Heart Society, Western Ohio Heart Chapter, and the United States Public Health Service.

[2] Work carried out during the tenure of an Established Investigatorship of the American Heart Association.

[3] Work carried out during the tenure of Fellowships of the United States Public Health Service and the American Heart Association.

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other instances, selective increases in aldosterone secretion without a corresponding change in cortisol output were seen. The conclusion appears inevitable, that inhibitory as well as stimulatory components of the aldosterone-regulating system, exist in the brainstem.

There have been other reports indicating that structures in the posterior diencephalon and rostral midbrain are important in water and electrolyte metabolism. Gilbert (1956, 1958) found dehydration to follow destruction of the subcommissural organ, and changes in the staining characteristics of the area to occur in response to alterations in electrolyte intake. Bradley Scheer and associates (1962) have also recently observed changes in the neurosecretory staining elements of the walls of the third ventricle in the epiphyseal area of frogs following changes in the salinity of their environmental medium. Panagiotis and Hungerford (1961) have found pineal lipid to be depleted following sodium restriction. At these meetings, Clementi and his co-workers have presented evidence that in the rat the osmic acid staining granules of the pineal gland are depleted as a consequence of aldosterone administration. Thus, several different lines of evidence point to the brainstem, the epiphyseal area in particular, as the locus of at least one aspect of central nervous system involvement in electrolyte regulation, probably mediated through changes in the rate of aldosterone secretion.

Some of our earlier experiments were designed to determine whether this region of the brain is the source of a humoral agent for aldosterone secretion. We began by the assay of saline extracts of brain tissue. The logical assay preparation for a humoral factor, which is elaborated by a structure within the brainstem, is an animal, with that part of the neuraxis removed. Therefore, we have used the decerebrate brain-removed hypophysectomized dog as an assay preparation. In dogs, anesthetized with sodium pentobarbital, the brain stem is sectioned just caudal to the superior colliculi dorsally and ventrally at the anterior border of the pons. The entire brain substance, rostral to the section (including the pituitary) is removed. The animal is allowed to rest for 3 hours, during which time respiration, blood pressure, and temperature are continuously monitored. The left lumboadrenal vein is then cannulated and adrenal venous blood is collected for a 1.5- to 2-hour period. The sample to be assayed is infused throughout the adrenal venous blood collection period. Aldosterone and cortisol are isolated from the adrenal venous effluent by paper chromatographic methods. Using this assay procedure, we found that extracts of posterior diencephalon, pineal gland in particular, contain a factor which stimulates aldosterone secretion. Saline, acetone, alcohol, or chloroform extracts of pineal, if carefully prepared, have all yielded activity (Farrell, 1959a,b, 1960).

Our recent efforts have been aimed at purifying this material which we called *adrenoglomerulotropin*. Other workers have also reported aldosterone stimulating activity in extracts of diencephalon and pineal. Lucis

et al. (1961) found stimulation of aldosterone synthesis *in vitro* by a freshly prepared saline extract of hog diencephalon. Kovács *et al.* (1960), using acetone extracts of human pineal tissue, demonstrated stimulation of aldosterone output in rat adrenals *in vitro*. The extensive investigations of Keller *et al.* (1961) are of particular interest. These workers found a selective action of a fraction from pineal extracts on the carboxylic acid esterase activity of the zona glomerulosa of the rat adrenal. By the use of this assay method, they purified the hormone to such a

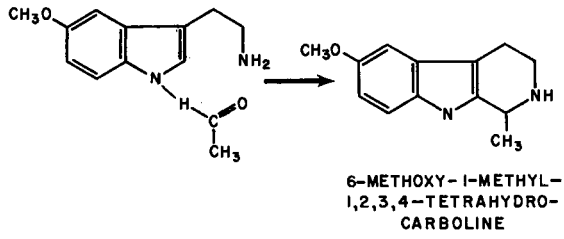

FIG. 1. 5-Methoxytryptamine can condense with acetaldehyde to form 1-methyl-6-methoxy-1,2,3,4-tetrahydro-β-carboline.

point that, in January 1961, they were able to assign to it a structure similar to that of melatonin.

At the same time, we were pursuing the isolation of adrenoglomerulotropin from pineal extracts. We have used a variety of techniques in an effort to obtain pure material. In general, the initial extraction is best carried out with an organic solvent, such as chloroform, ethanol, or acetone, since the active material is quite soluble in these. We have used flirosil columns eluted with hexane to purify the extracts with some success, but at present find that paper chromatography has certain advantages over the columns. The active material migrates with an R_f of less than 0.1 on benzene : ethyl acetate : water, 20 : 1 : 20; 0.9 on butanol : acetic acid : water, 4 : 1 : 5; 0.9 on propanol : NH_3, 7 : 3; and 0.7 on silicone-impregnated paper developed with chloroform : ethanol : water, 5 : 5 : 3. Two distinguishing features became apparent when the material was chromatographed on these various paper systems. Activity was associated with a spot which had a pale yellow fluorescence when activated by ultraviolet light and which gave an atypical (greenish) or negative reaction with Ehrlich's dimethylaminobenzaldehyde reagent. The ultraviolet absorption spectrum was difficult to define because of the minute quantity of material present, but an absorption maximum in the vicinity of 270 mμ was commonly seen. It was evident that the potency of the substance was extremely high, an effective dose being in the microgram range.

At this stage of development of the problem, we had very few clues as to the identity of the substance. The existence of high concentrations

of indoles (5-hydroxyindole-3-acetic acid, 5-methoxyindole-3-acetic acid, serotonin, and melatonin) in pineal (Giarman and Day, 1958; Lerner *et al.*, 1960) suggested that an indolic structure similar to one of these should be considered. On the other hand, the negative Ehrlich's reaction and high degree of lipid solubility of adrenoglomerulotropin seemed to make this an unlikely possibility. However, Dr. McIsaac had observed small amounts of material with the same general properties as adrenoglomerulotropin in the urine of animals treated with melatonin. He had postulated that melatonin can cyclize to yield the carboline structure, thus producing a lipid-soluble fluorescent Ehrlich-negative compound (McIsaac, 1961a). The same structure can result from condensation of tryptamine derivatives with aldehyde either *in vitro* or *in vivo* (McIsaac 1961b) (Fig. 1). To investigate the possibility that adrenoglomerulotropin is a carboline, a series of such compounds was synthesized for comparison of physical and biological properties. Of the synthetic materials tested, 1-methyl-6-methoxy-1,2,3,4-tetrahydro-β-carboline appeared to be a reasonable match for the adreno-

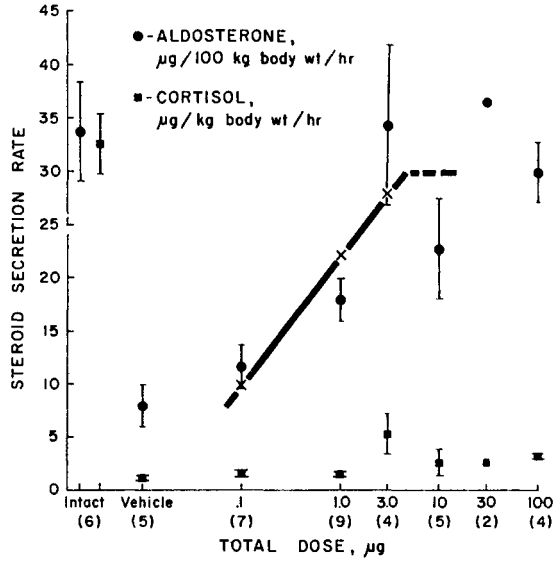

FIG. 2. Bioassay of 1-methyl-6-methoxy-1,2,3,4-tetrahydro-β-carboline in the decerebrate brain-removed hypophysectomized dog. Mean secretory rates and standard errors are shown for the assays (number indicated by figures in parentheses) at each dose level. A maximal response was obtained with 3 μg infused over the 1.5- to 2-hour period of adrenal venous blood collection. The solid black line was calculated by the method of least squares: $b = 12.25 \pm 2.16$; $r = 0.758$; significance of regression, $P < 0.001$. For comparison, the steroid secretion rates of intact dogs also subjected to laparotomy and the hemorrhage of adrenal venous blood collection are shown on the left.

glomerulotropin fraction isolated from pineal tissue. This carboline migrates with R_f values similar to those cited above for adrenoglomerulotropin in the four chromatographic systems studied (Farrell and McIsaac, 1961). Further, it was observed that adrenoglomerulotropin possesses the property of antagonizing the oxytocic action of serotonin, a property characteristic of the carboline series.

Bioassay of the methoxycarboline in the decerebrate preparation used for assay of adrenoglomerulotropin added further support to the possibility of identity of the two. Bioassay results of the synthetic 6-methoxytetrahydrocarboline are shown in Fig. 2. Decerebration results in marked depression in steroidogenesis as compared to that in intact animals subjected to laparotomy and adrenal venous blood collection. The administration of the tetrahydrocarboline induced a striking increase in aldosterone secretion. A linear relationship between dose and response was found between the 0.1 μg dose level and the maximal effective dose of 3 μg. The regression over this dose range, calculated by the method of least squares, had a highly significant slope with a probability value of less than 0.001. The aldosterone secretion level of the decerebrate preparations was restored to that of the intact dogs stimulated by laparotomy and hemorrhage. Doses larger than 3 μg did not bring about a further increase in response. There was a modest increase in cortisol output at some of the higher doses of carboline, though the effect failed to be significant except at the 100-μg dose level. In no instance did the cortisol output approach that seen in intact dogs.

The related compounds 10-methoxyharmalan (1-methyl-6-methoxy-3,4-dihydro-β-carboline), melatonin, 1-methyl-1,2,3,4-tetrahydro-β-carboline, and 5-methoxytryptamine were either inactive or very much less active than the 1-methyl-6-methoxy-1,2,3,4-tetrahydro form (Farrell and McIsaac, 1961). However, the 6-hydroxy analog of the tetrahydrocarboline was subsequently found to be as active as the 6-methoxy form (Fig. 3). Indeed, comparison of the doses required for maximal steroidogenesis suggested that the 6-hydroxy form is somewhat more active.

On the basis of this study, we are reasonably confident that adrenoglomerulotropin has physicochemical and physiological properties very similar to those of compounds of the carboline group. However, our information at this time does not permit a final statement as to the exact structure of adrenoglomerulotropin. We are currently continuing these studies and accumulating larger amounts of material in the hope of obtaining definitive identification.

Also remaining unanswered is the question of the mechanism of action of adrenoglomerulotropin. In intact animals the carbolines yield unpredictable results. We have occasionally observed what appeared to be profound stimulation of aldosterone secretion, but in other experiments no stimulation was observed, and in some cases there actually

appeared to be inhibition. This behavior in the intact animal suggests that compensatory mechanisms exist which may act to block the action of the carboline under certain as yet poorly understood physiological conditions. It should be pointed out that the steroidogenic action of these compounds may not be their only or even most important function. The carbolines possess some very interesting pharmacological properties. They are serotonin antagonists and monoamine oxidase inhibitors. They possess psychotomimetic properties similar to those of

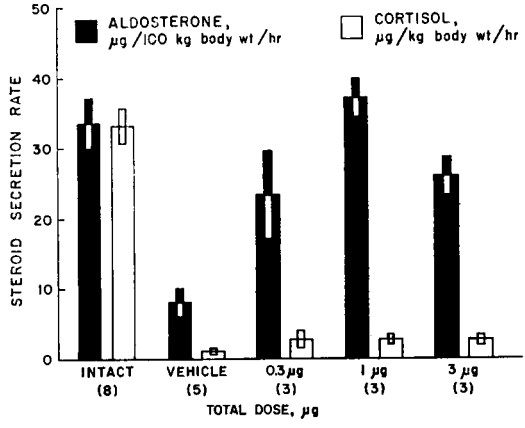

FIG. 3. Bioassay of 1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline in the decerebrate brain-removed hypophysectomized dog. The bars indicate the mean secretory rate and standard error for the assays (number indicated by figures in parentheses) at each dose level. A significant effect on aldosterone secretion was observed when 0.3 μg was infused over the 1.5- to 2-hour adrenal venous blood collection period with a maximal effect at 1 μg. Steroid secretion rates of intact dogs also subjected to laparotomy and the hemorrhage of adrenal venous blood collection are shown on the left for comparison.

lysergic acid diethylamine and they enhance the pressor action of the catechol amines. Thus, if these compounds or related substances with similar biological activity are indeed secreted by the brainstem, they may play a more fundamental role in body processes than is apparent at this time.

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ADDENDUM

Since the praparation of this manuscript, approximately 50 kg. of beef pineal has been processed in an effort to conclusively identify adrenoglomerulotropin At the present time, it is an opinion that the factor is in fact not the methoxy carboline, but rather a substance with certain similar physical and physiological properties.

DISCUSSION

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The recent findings suggest that the renin-angiotensin system plays an important role in the regulation of aldosterone secretion. Renin administration produces a marked hypertrophy of the glomerulosa zone of the adrenal cortex in the rat (Deane and Masson, 1951). Gross (1960) demonstrated that salt depletion or adrenalectomy increases the renin content of normal rat kidney, while additional salt or aldosterone reduces it.

In man, Genest *et al.* (1961) have shown a significant increase in urinary aldosterone, with concomitant sodium retention and a fall in the Na : K ratio, after the angiotensin infusion at subhypertensive levels and without changes in the glomerular filtrate rate. In studies on the adrenal secretion rate of aldosterone in normal human subjects Laragh *et al.* (1960a) confirmed that angiotensin caused an increased secretion rate of aldosterone.

These experiments indicate the high sensitivity of aldosterone secretion to angiotensin administration. It is worth noting that angiotensin does not affect shifts in sodium distribution in isolated toad's bladder (Leaf, (1960).

Infusion of angiotensin into hypophysectomized dogs, as well as into intact animals, increased the rate of secretion of aldosterone (Carpenter *et al.*, 1961).

It would be valuable to know if the central nervous system plays a role in the action of angiotensin upon adrenal function. Ross and McLean (1960) confirming previous findings of Orti *et al.* (1957), have shown that, in the urine of rats and of normal subjects on a restricted sodium intake, there is present an aldosterone-stimulating substance, probably released by the diencephalon. Fundamental progress in this field has been obtained in the studies of Farrell (1958, 1960) and his group, who have localized the site of production of adrenoglomerulotropin in the diencephalon and in the pineal tissue. Prof. Farrell's paper here gives the chromatographic, colorimetric, and fluorometric properties of this factor, identical with those of tetrahydrocarboline.

It would be interesting to elucidate whether angiotensin is an agent capable of influencing the central regulation of aldosterone secretion, or whether it is of a nonspecific or indirect nature. I think Prof. Farrell could give us valuable information and advices with his experience in this field of investigation. In this connection, I would like to ask Dr. Taylor two questions:

(1) Do we know the effect of angiotensin administration on the excretion of aldosterone-stimulating substance (adrenoglomerulotropin) in intact animal or man?

According to Davis *et al.* (1960), in the midbrain-transected dog, increased aldosterone secretion results secondary to thoracic vena cava constriction (a condition in which an increased production of renin by the kidney has been supposed) (Davis *et al.*, 1961). It does not fall if the venous pressure remains sufficiently elevated to continue to stim-

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ulate a very high rate of aldosterone production. These results, obtained in the dog with interruption of nervous pathways at the midbrain level, need verification and more penetrating study to gain acceptance.

With regard to this aspect of the problem the second thing I would like to ask is the following:

(2) Have you tested the effect of angiotensin administration on the excretion of aldosterone and of aldosterone-stimulating substance in animals with midbrain and caudal diencephalon lesions?

In summary, is the diencephalon related to angiotensin-aldosterone regulation?

At present, these investigations appear to be of special interest in assessing how the renin-angiotensin system plays a role in the regulation of aldosterone secretion in the normal physiological state, or at least in certain diseases. This approach may be, I think. a very profitable line of investigations for the future.

A. N. TAYLOR: In reply to the discerning questions raised by Dr. Romanelli, perhaps a few points of review should be raised. The interesting work of Deane, Gross, Genest, Laragh, and Davis and their respective collaborators is, of course, well known to us. There is, however, one persistent finding which has, to us, placed the whole proposition that angiotensin regulates aldosterone secretion in considerable doubt: namely, that in the clinical or experimental condition in which renin secretion is almost universally believed to be elevated (renal hypertension), there is by no means a consistent elevation of aldosterone secretion. This was apparently originally mentioned in an early report by Genest (1957). Recently, Carpenter and co-workers (1961) have repeated the observation in the dog. In our own experience, as well as in that of Cope *et al.* (1962), many if not most cases of proven renal hypertension in man are not accompanied by hyperaldosteronism. One wonders if the effects of acute administration of angiotensin on aldosterone secretion are of a transitory nature. Occasionally, in experimental malignant renal hypertension (Cope *et al.*, 1962; Laragh *et al.*, 1960b) or in malignant hypertension in man (Genest, 1957; Cope *et al.*, 1962; Laragh *et al.*, 1960b; Travis *et al.*, 1961; Genest *et al.*, 1958), one can observe an increase in the secretion rate of the steroid. However, the state of the subject with malignant hypertension is so far removed from the physiological that one would hesitate to accept any arguments based on the condition. In some instances the elevated aldosterone output seems to be more related to a renal sodium wasting tend- ency than to anything else (Genest *et al.*, 1958). For the present, then, we would prefer to maintain reservations as to the physiological significance of the angiotensin-aldos sterone scheme.

As to the possibility of central nervous system control of renin secretion, very little of a definitive nature can be said. It is intriguing to speculate that such might be the case. There are the interesting experiments of Romani and Keller, which indicate that their preparation of adrenoglomerulotropin does not haves its expected effect on adrenal histology (of rats) if the kidneys are removed 24 hours prior to injection of the hormone (Romani and Keller, 1962). Romani and Keller interpret the results to mean that aldosterone synthesis is reduced and they implicate the kidney as a necessary intermediate in the action of adrenoglomerulotropin. On the other hand, Sheppard and his co-workers (1962) have reported that the adrenals of rats 24 hours after nephrectomy synthesize aldosterone at a rate much *higher* than in controls, quite in contrast to Romani and Keller's conclusions from their histological observations. We should say that further speculation will serve little purpose, but the active work going on in the field should provide some final answers soon.

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Interrelationship of Renal and Adrenal Hormones

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Although the concept of the adrenal cortex as an endocrine gland producing at least three genuine steroid hormones is generally accepted, little is known about the internal secretion of the kidney and humoral factors of renal origin, to which, possibly, a hormonal function ought to be ascribed. Renin, assumed to be formed and stored in the macula densa of the distal tubules and the granules of the juxtaglomerular or epitheloid cells (Bing, 1962; Bing and Kazimierczak, 1961; Cook and Pickering, 1958), is a protein with the properties of an enzyme which liberates from a probably specific substrate (angiotensinogen) the decapeptide angiotensin I, which then is enzymatically converted to the octapeptide angiotensin II. Neither renin nor angiotensinogen, the latter belonging to the α_2-globulins of the plasma, is chemically defined or even available in a sufficiently purified state to allow of judging the specificity of the enzyme-substrate interrelationship. In contrast, the reaction product, either in the form of the decapeptide or the final, active octapeptide, and various derivatives have been synthesized mainly by two groups of investigators (Page and Bumpus, 1961; Schwyzer, 1962; Schwyzer and Turrian, 1960) and are now available for study in sufficient amounts. When discussing the possible actions of the renin-angiotensin system, it has to be considered that so far it cannot be decided whether the main action of these substances is restricted to the kidney, possibly near to the site of formation or storage, or whether the enzyme or the polypeptide influence other remote target organs. The search for the "physiological" action of a tissue hormone has to take into account that there may be fundamental differences between the activity which the substance exerts locally and the various effects to be observed after intravenous injection. No conclusions on the transmitter function of either acetylcholine or noradrenaline could be drawn from the variety of pharmacodynamic effects elicited after intravenous infusions, and the blood pressure-lowering effect of trypsin has no relation to its proteolytic activity in the duodenum. These examples should render us careful and prevent too far-reaching conclusions from observations made after systemic application of either renin or angiotensin.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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Various attempts have been made to detect and measure renin and angiotensin in the blood under different conditions, especially in experimental renal hypertension of the Goldblatt type and in corresponding clinical equivalents. Although numerous authors claim to have found a definite increase of both substances, either in the renal venous blood coming from the kidney with reduced blood flow or in the systemic circulation (Skeggs, 1960), others did not confirm these findings (Peart, 1959, 1960). Recently, Peart found reninlike activity in the renal lymph of dogs after clamping one renal artery, thus indicating how the enzyme may reach the systemic circulation under special pathological and perhaps also physiological conditions (Lever and Peart, 1961; Peart, 1962). It has, however, to be admitted that the methods of detecting renin and angiotensin in the blood are not sufficiently sensitive to permit determining the amounts which are possibly secreted by the kidney. In cross-circulation experiments, using the nephrectomized rat—which is highly sensitive to the pressor effect of renin—as an assay animal, we found in the normal rat a circulating humoral factor which elicited a reninlike reaction on blood pressure in the test animal (Gross *et al.* in press; Rondell and Gross, 1961) (Fig. 1). This gives a hint that under normal conditions, renin may appear outside the kidney, exert. ing an effect on specific target organs.

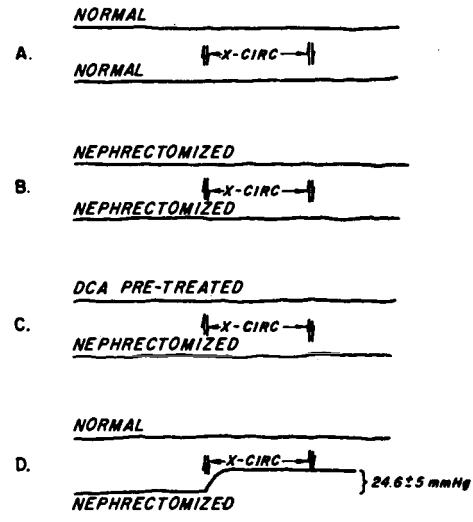

FIG. 1. Isovolemic cross circulation in the rat. In the nephrectomized rat, highly sensitive to renin, blood from normal rats leads to a pressor response, probably due to renin; the renin-depleted animal, however, pretreated with cortexone and salt, does not give a corresponding reaction. From Rondell (1961).

Investigations on experimental hypertension gave evidence which enabled us in 1958 to make the first claim that there is a relationship between the renin-angiotensin system of the kidney and the secretion of aldosterone (Gross, 1958, 1960; Gaunt *et al.*, 1959). There is an inverse relationship between the concentration of renin in the kidney and sodium balance (Fig. 2). The more sodium is retained, either owing to overdosage with cortexone or aldosterone and salt, or even salt alone, the more reduced is the renin concentration in the kidney, whereas a

state of sodium deficiency, provoked by adrenalectomy or by dietary restriction, enhances the renin content in the kidneys (Gross and Sulser, 1957; Pitcock *et al.*, 1959). Consequently, the sensitivity to exogenous renin is also inversely related to the concentration of endogenous renin, being most pronounced in the nephrectomized animal or when renin, in the state of positive sodium balance, disappears (Gross, 1958), and

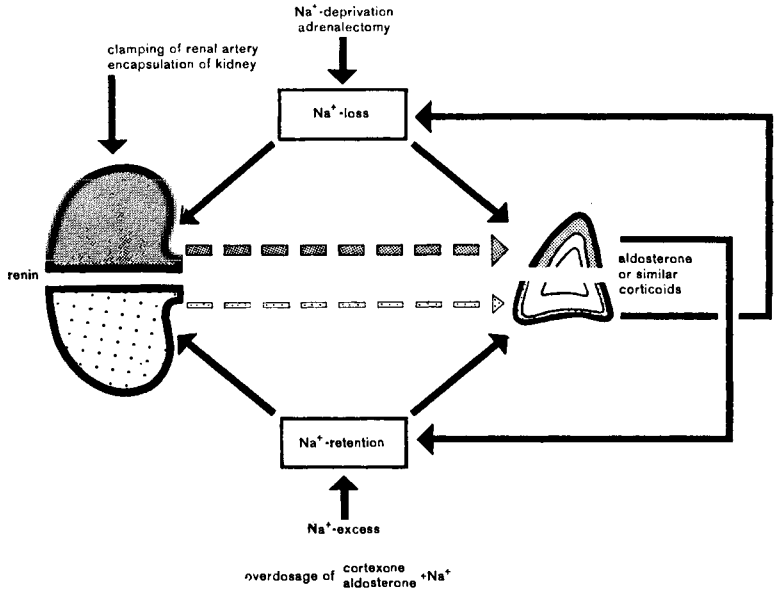

FIG. 2. Relationship between the secretory activity of the kidney (renin-angiotensin) and that of the adrenal cortex under various experimental conditions.

being diminished in the presence of an increased renin concentration (Gross and Sulser, 1957). This is in accord with the reduced pressor effect of angiotensin in the sodium-depleted dog (Carpenter *et al.*, 1961).

A histological equivalent of these variations is the coincidental alteration in the number of granules in the juxtaglomerular cells of the kidney (Hartroft and Hartroft, 1953, 1955; Hartroft and Edelman, 1960). Simultaneously, glucose-6-phosphate dehydrogenase, an energy-supplying enzyme, demonstrable especially in the macula densa part of the distal tubule, shows a similar change in activity to the renin concentration (Hess and Gross, 1959). This alteration of enzyme activity, however, is not restricted to the kidney: it is also found in the epithelial duct cells of the salivary glands, which are in addition flattened, resulting in an appreciably wider lumen, thus showing a similar morphological change under these conditions to that in the distal tubular system of the kidney (Gross and Hess, 1960).

The working hypothesis derived from these data that the renin-angiotensin system may influence the activity of the glomerular zone of the adrenal cortex was strongly supported by the finding of various authors that infusion of angiotensin stimulates aldosterone secretion in the dog as well as in the human being (Carpenter *et al.*, 1961; Genest *et al.*, 1960; Genest, 1961; Laragh *et al.*, 1960). Furthermore, it was demonstrated in dogs that after removal of the kidneys, stimuli such as blood loss, vena caval constriction or sodium depletion, normally leading to an increased aldosterone secretion, are no longer effective and that a previously elevated secretory rate disappears after nephrectomy (Davis *et al.*, 1961a; Mulrow and Ganong, 1962). On the basis of this additional evidence, an aldosterone-stimulating hormone of renal origin, probably the renin-angiotensin system, was postulated.

The main obstacles to proving this hypothesis are the inadequate methods for the determination of secretion rates and blood levels of either renin or angiotensin and the lack of knowledge about the nature of the appropriate stimulus for the secretion and/or liberation of renin/angiotensin.

To evaluate the role which humoral factors of renal origin may play as stimulators of adrenal cortical activity, acute changes have to be differentiated from chronic ones. In other words, the immediate response of adrenal cortical secretion to more or less specific stimuli acting under "physiological" conditions has to be distinguished from alterations which are the consequence of a chronic "pathogenic" stimulus provoked, for example, by reducing renal blood flow or establishing a positive sodium balance. In order to elucidate further the interrelationship between humoral factors of renal origin and adrenocortical hormones under acute and chronic conditions, the following approaches are possible: (1) to study the immediate and direct effects of injected renin and angiotensin on the activity of the adrenal cortex; (2) to investigate the role of the intact kidney in the hormonal production of the adrenal cortex; (3) to use pathological conditions, such as experimental renal hypertension or various forms of secondary hyperaldosteronism, as tools.

These possible avenues will now be discussed in more detail.

I. Immediate and Direct Effect of Renin-Angiotensin on the Adrenal Cortex

Angiotensin, given intravenously to dog or man, is a strong stimulator of aldosterone secretion at a dose level below that at which there is an effect on blood pressure (Genest, 1961; Mulrow and Ganong, 1961; Slater *et al.*, 1962). Similar observations in dogs were made with more or less purified kidney extracts or renin preparations (Davis *et al.*, 1962; Mulrow *et al.*, 1962). It has, however, also been observed that the secretion of 17-oxycorticoids and of corticosterone may be en-

hanced, especially in the hypophysectomized animal, although results are less uniform than for aldosterone. The response of both cortical hormones to angiotensin is dose dependent, that of aldosterone being claimed to be the more sensitive (Mulrow *et al.*, 1962), although not by all investigators (Bartter *et al.*, 1961; Slater *et al.*, 1962). In the rat, angiotensin seemed to sensitize the animal to the effect of ACTH on corticosterone secretion (Goldman and Cahill, 1962), but did not influence the ascorbic acid content of the adrenal cortex (Carr and Bartter, 1961). Hence, there must be a difference in the mode of action of the two stimulators.

A direct effect of renin on the adrenal cortex was demonstrated, as repeated injections were followed by an enlargement of the glomerular zone (Deane and Masson, 1951). No similar morphological response, however, has been described so far after continuous infusion of angiotensin, certainly not in doses that stimulate aldosterone secretion without influencing blood pressure. In spite of the stimulation of the 17-oxycorticoid secretion, no histological signs of increased activity were described in the zona fasciculata. In view of the fact that the renin preparations so far available are all contaminated with various other proteins, the specificity of their effect on the glomerular zone needs further investigation.

II. THE ROLE OF THE INTACT KIDNEY IN ADRENOCORTICAL SECRETION

Various investigators have shown that the increase of aldosterone secretion in response to acute blood loss is abolished by nephrectomy (Davis *et al.*, 1961b; Mulrow and Ganong, 1962) and also that the elevated secretion which occurs during sodium depletion is replaced by a subnormal secretion rate within 1–2 hours after removal of the kidneys. On the other hand, it was possible to restore high secretion rates of aldosterone after nephrectomy by the injection of kidney extracts (Davis *et al.*, 1961b). The response of aldosterone secretion not only to exogenous renin or angiotensin, but also to the removal of the endogenous source of renal humoral factors is as prompt as that obtained during variations of blood volume. It must be mentioned that increased sensitivity to exogenous renin, which is found after nephrectomy, takes appreciably longer, i.e., at least 6–8 hours, before it is fully established (Gross, 1958). The enhanced and prolonged pressor action of renin found in the nephrectomized animal is therefore not necessarily linked with the absence of a stimulatory effect on the adrenal cortex; further evidence that renin/angiotensin has different effects on various target organs.

Dietary sodium depletion or adrenalectomy is followed by an increase of renin concentration and glucose-6-phosphate dehydrogenase in the kidney (Hess and Gross, 1959), but it is not yet known whether removal

of the kidneys leads to significant hypoplasia of the adrenal cortex, especially the glomerular zone. Survival time after nephrectomy may be too short for the establishment of such an effect, and the lack of the excretory function of the kidney interferes too greatly with the electrolyte balance so that it is not possible to obtain a clear-cut picture of the isolated effect of the absence of the renin-angiotensin system. On the other hand, even when the adrenals are not present, renin concentration in the kidney may fall, provided that enough sodium is given.

III. Pathological Conditions as Experimental Tools

A. Experimental Renal Hypertension

Clamping one renal artery in the rat leads to an increase of renin concentration in the kidney, to which the blood flow is reduced, while renin disappears from the contralateral untouched kidney (Gross and Lichtlen, 1958). In respect to renin concentration, the clamped kidney therefore corresponds to a kidney in a sodium-depleted animal, while the unclamped kidney seems to behave in the same way as under conditions of sodium retention. Simultaneously, the activity of glucose-6-phosphate dehydrogenase in the macula densa is reduced in the unclamped kidney as well as in the epithelial cells of the salivary gland

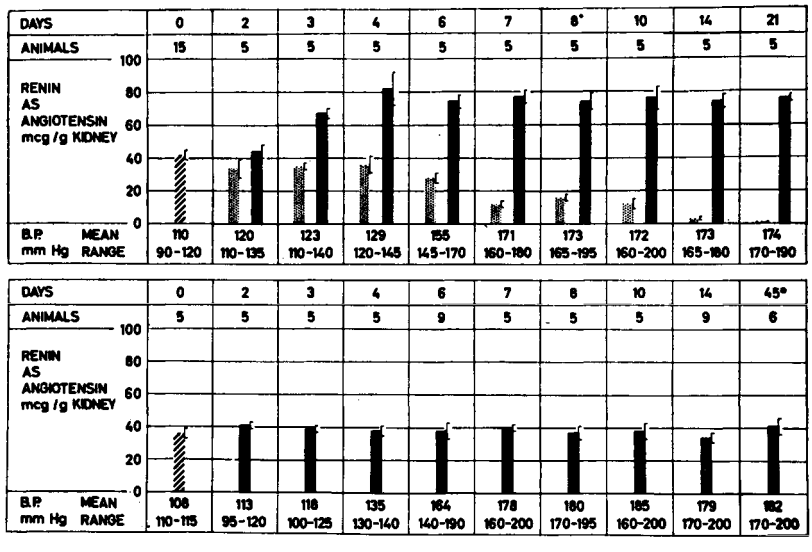

FIG. 3. Renin concentrations in kidneys of renal hypertensive rats (expressed as μg of angiotensin produced from rat plasma by 1 gm of fresh kidney tissue). Upper section: constriction of one renal artery. Lower section: unilateral nephrectomy and simultaneous constriction of one renal artery. Black columns: clamped kidneys. Stippled columns: unclamped kidneys. Striped columns: normal kidneys. From Regoli *et al.* (1962b).

ducts (Gross and Hess, 1960). Recently, we were able to demonstrate that the increase of renin concentration in the clamped kidney occurs 2–3 days after the intervention and precedes the decrease of renin content in the contralateral kidney by 3–4 days (Regoli *et al.*, 1962 b) (Fig. 3). It may therefore be argued that, as the first consequence of clamping one renal artery, renin secretion is stimulated in that kidney and leads to an increase of aldosterone secretion, which provokes in turn the sodium retention responsible for the reduction of renin concentration in the unclamped kidney and also the corresponding extrarenal changes. But this does not explain the fact that after initial removal of one kidney and clamping of the remaining one, renin concentration in the solitary kidney does not increase at all but stays constant during the whole duration of the experiment. There is, however, evidence that extrarenal changes in the epithelial cells of the salivary ducts develop similar alterations as in the presence of an unclamped kidney (Regoli *et al.*, 1962 b).

When interpreting these findings, one must first admit that variations in the renin content of the kidney do not necessarily reflect secretory activity. Although various findings from kidney grafting and cross-circulation experiments are available which indicate that an increased concentration of renin is accompanied by a high secretory rate (Gross *et al.* in press.; Omae *et al.*, 1960, 1961), this gives no insight into the details of regulation. The data obtained in animals having one renal artery clamped in the presence or absence of a contralateral unclamped kidney indicate that there may be a humoral interrelationship between both kidneys, the nature of which needs further investigation. This assumption is also supported by the fact that removal of the clamped kidney is followed within 2–3 weeks by the reappearance of renin in the unclamped kidney, whereas extirpation of the unclamped kidney leads to normalization of the increased renin content of the clamped kidney (Regoli *et al.*, 1962a). In the first case, the omission of the stimulator (renin) emanating from the clamped kidney, could be regarded as responsible, but no satisfactory explanation can be given so far for the decline in the secretion to normal after removal of one target organ, which may, however, perhaps only be an indicator organ.

It could be argued that, as a result of clamping one renal artery, renin/angiotensin secretion is increased and that consequently aldosterone secretion is stimulated, leading to sodium retention which is responsible for the enzymatic variations observed in the unclamped kidney or elsewhere. There is, however, no evidence that aldosterone secretion is enhanced beyond normal under these conditions, either in rats or in dogs. In dogs it was found that, in benign renal hypertension, aldosterone secretion is in the normal range (Carpenter *et al.*, 1961); in rats, even much higher doses of aldosterone than those secreted under conditions of maximum stimulation were not able to prevent the fall of blood pressure after adrenalectomy (Fig. 4). Peart has

recently shown that in the rabbit prolonged infusion of angiotensin over more than 100 days does not lead to sustained hypertension comparable to that observed if both renal arteries are clamped (Peart, 1962). This demonstrates that an increase of aldosterone secretion, probably elicited by the continuous infusion of angiotensin, is incapable of producing a chronic elevation of blood pressure. Thus, we conclude that aldosterone and sodium have a permissive action for the manifestation of experimental renal hypertension, but that there is no marked underlying hypersecretion in the sense of a primary or secondary hyperaldosteronism (Gross, 1962). The latter may, however, occur when more severe cardiovascular alterations become manifest during the malignant course of the disease in the human being (Laragh, 1962; Laragh *et al.*, 1960).

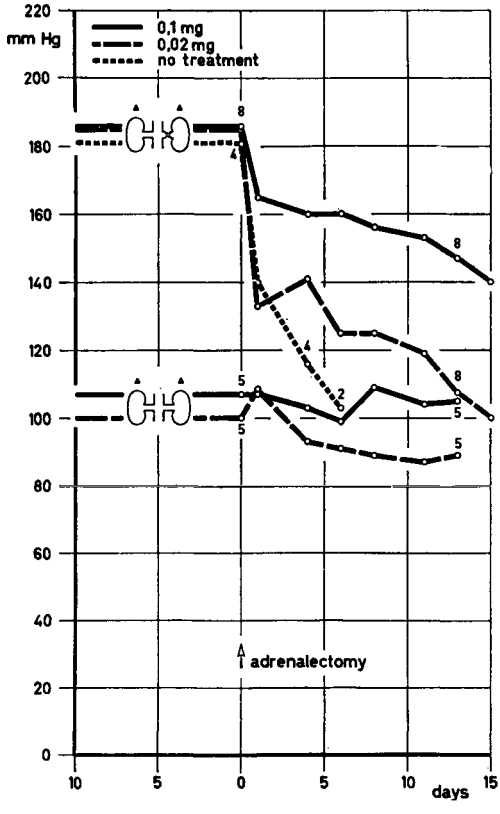

FIG. 4. Inability of aldosterone to maintain blood pressure in experimental renal hypertension. Even with much higher doses of aldosterone than those obtained under maximum stimulation of the zona glomerulosa, hypertension cannot be sustained after removal of the adrenals.

B. SECONDARY HYPERALDOSTERONISM

In dogs with thoracic caval constriction as well as with cardiac failure, an increase in the renin content of the kidneys, simultaneously with an increase of granules in the juxtaglomerular cells, has been found (Carpenter *et al.*, 1962; Davis *et al.*, 1962). Simultaneously, these animals also showed a reduced pressor response to injected angiotensin, thus indicating the presence of higher than normal amounts of renin and/or angiotensin in the organism and perhaps also in the circulating blood. A similar observation was reported by Laragh, who, in patients with secondary hyperaldosteronism due to cirrhosis of the liver, has described reduced sensitivity to angiotensin (Laragh, 1962). This cor-

responds well with the tachyphylactic action of angiotensin and with the cross-tachyphylaxis between renin and angiotensin found in the rat as well as in the dog (Bock and Gross, 1961; Gross and Bock, 1962; Gross Bock and Timian, 1961).

In the dog, constriction of the aorta above the renal arteries was followed by an increase of aldosterone as well as of corticosterone secretion (Davis *et al.*, 1962). From these findings it was concluded that reduction of arterial blood pressure and blood flow in the kidney is the stimulus for renin secretion. This corresponds to claims made by Tobian that the juxtaglomerular cells may act as stretch or volume receptors which respond to variations of pressure or volume in the afferent arterioles of the renal glomerula (Tobian, 1960, 1962). Increased stretch should lead to a diminished renin secretion and consequently to a reduction of juxtaglomerular granularity, while a decreased stretch stimulates renin production and secretion. There are, however, various discrepancies in the observations so far available which make it difficult to accept this attractive hypothesis:

1. It is improbable that sudden reduction of pressure and flow in the renal artery exerts the same stimulus on hypothetical stretch or volume receptors as a dietetic reduction of sodium, leading to much less drastic and slowly developing changes in blood volume and consequently to flow n the afferent arterioles. Conversely, the same holds true for sodiumiretention and increased volume or flow.

			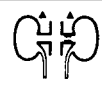		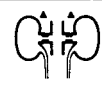			
ANGIOTENSIN γ/g kidney	40 ±6	40 ±6	1 ±0.1	80 ±7	20 ±10	100 ±20	40 ±5	40 ±4
BP mm Hg	105 ±2		180 ±2		185 ±4		105 ±4	195 ±4

FIG. 5. Renin content of the kidneys (expressed as micrograms of angiotensin per 1 gm kidney) under various experimental conditions. Clamping both renal arteries leads to a reduction of renin concentration in one (the heavier) kidney in spite of diminished pressure and flow. The solitary kidney shows no increase of renin concentration after clamping. From Regoli *et al.* (1962a).

2. Clamping of one renal artery in the absence of a contralateral kidney is not followed by an increase of renin concentration in that kidney, but it cannot be excluded that there may be a higher secretory rate than normal (Regoli *et al.*, 1962b) (Fig. 5).

3. Clamping of both renal arteries leads to a reduction in the renin content of both kidneys. There is always a slight difference in the degree

of blood flow reduction between the two kidneys, and consequently, there will be a certain difference in kidney weight. The lighter kidney regularly contains more renin than the heavier one which, in spite of definite reduction of pressure and flow, shows a marked diminution of renin concentration (Gross, 1960; Regoli *et al.*, 1962b) (Fig. 5).

On the other hand, the partial prevention of the disappearance of renin, which is obtained by clamping the renal artery in rats simultaneously given an overdosage of cortexone and salt, is a hint that the reduction of flow and pressure in the kidney has a stronger influence on renin concentration than sodium retention or other factors affected by the latter (Gross, 1960). Thus, one might argue that a diminution of renal arterial pressure and flow is one possible stimulus for renin secretion but does not exclude the existence of others that so far are either unknown or unrevealed as such.

Although there is no doubt that there exist humoral relationships between the kidneys and the adrenal cortex and that, under certain conditions, aldosterone secretion is stimulated by angiotensin, it is not established that this mechanism is mainly responsible for the regulation of aldosterone secretion. Many different humoral factors have already been claimed to be the aldosterone-stimulating hormone (Farrell, 1960; Farrell and McIsaac, 1961); for the renin-angiotensin system, too, it is not definitely proved that it has a direct stimulatory function outside of the kidney. It cannot be excluded that most of the effects observed hitherto are the consequences of events occurring simultaneously but independently of one another. The results obtained under acute conditions and in chronic experiments show only a partial conformity and have to be interpreted separately. In spite of the fact that many new and important observations have been made and data have been collected which support the view that the renin-angiotensin system has physiological functions and is probably closely related to the activity of the adrenal cortex, the situation has become very complicated, and there are still quite a few gaps to be filled in our knowledge before an exact definition of the renal-adrenal interrelationship can be given. In order to avoid disappointments, the established facts should be considered carefully and should be the basis for seeking the missing evidence rather than forming new hypotheses which may be quite attractive but have to be abandoned when new results are available. In order that the truth of today will not be the error of tomorrow, a substantial amount of further work is necessary to render the foundations from which we are trying to proceed more solid and safe.

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DISCUSSION

Interrelationship of Renal and Adrenal Hormones

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In the last few years we have been following with interest the fundamental work of Dr. Gross and his collaborators on the subject of steroids and hypertension. The study that he has just presented is particularly valuable in extending our insight into the interrelationships between renal and adrenal hormones and confirming previous work by Gross and Lichtlen (1958) Gross (1960) and Regoli *et al.* (1962) which clearly demonstrated these complex relationships. Recent experiments by Kaplan and Bartter (1962) have shown that renin will not, but angiotensin II will, consistently stimulate the secretion of aldosterone, corticosterone, and cortisol by isolated outer slices of beef adrenal cortex; these *in vitro* studies show clearly that the action of angiotensin II may be a direct one on the zona glomerulosa of the adrenal.

Various stimuli have been shown to influence the activity of the zona glomerulosa. (1) The importance of adrenoglomerulotropin, a humoral factor secreted by the midbrain is set forth in the preceding paper by Farrell and associates. The 1-methyl-6-methoxy-1,2,3,4-tetrahydro-2-carboline of Farrell and McIsaac appears to be inactive in the hypohysectomized nephrectomized dog (Bartter, 1962). As Farrell's preparation is a decerebrate (midcollicular section) animal with kidneys, this suggests that either (a) the brain exerts an inhibitory effect on the action of the carboline or (b) the kidney is necessary for its action. (2) We know the works that have shown (Gann *et al.*, 1960) that the diminished intracarotid pulse pressure leads to an increase in aldosterone secretion by reducing the impulse traffic in the nerve arising at the thyrocarotid arterial junction, thus releasing inhibition of a central center which may control release of a neurohormone mediating increase in aldosterone secretion. Until now no evidence has been reported on the relationship between the diminished intracarotid pulse pressure and the increased secretion of adrenoglomerulotropin. (3) It is generally accepted that ACTH can increase aldosterone secretion, even though its influence is considered of lesser physiological significance. (4) It has been shown that modifications in total body potassium are involved in the regulation of aldosterone secretion (Laragh and Stoerk, 1957); whatever its importance may be under normal physiological conditions, the influence of potassium in certain abnormal situations has proved even greater than the regulatory effect of intravascular volume. This phenomenon has been demonstrated in patients with primary aldosteronism, in whom aldosterone secretion does not respond to modifications of the intravascular volume but increases concurrently to the increment in body potassium (Bartter, 1960); also, there are patients with secondary aldosteronism whose intravascular volume diminishes following diuretic therapy, but their aldosterone

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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secretion instead of increasing, as it should, diminishes because of the frequently concurrent potassium depletion (Bartter *et al.*, 1962). All these data reveal that aldosterone secretion is regulated by not one but a diversity of stimuli. Perhaps the physiological importance of one or another stimulus may vary according to the circumstances. (5) Still another factor has been shown to influence aldosterone secretion; this is the renin-angiotensin system; the evidence for it has been beautifully put forward for us by Dr. Gross.

Some workers in the field believe that the renin-angiotensin system is responsible for the hyperaldosteronism that follows the constriction of the inferior vena cava (Davis *et al.*, 1962); I would like to draw attention, though, to one point that I believe stands against this hypothesis: Bartter *et al.* (1961) have shown that the infusion of angiotensin II increases aldosterone secretion but also produces larger percentage increments in the secretion of 17-hydroxycorticoids, cortisol and corticosterone; whereas the absolute values of cortisol and corticosterone so produced do not exceed those of nonstressed normal animals, similar increases are not seen with caval constriction or with adrenoglomerulotropin or with changes in body potassium.

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Hypothalamic Control of the Production of Corticosterone[1]

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I. INTRODUCTION

Almost every author agrees that the destruction of the median eminence (M.E.) modifies the functional behavior of the adrenal cortex and that it can also affect the weight of the gland (McCann and Brobeck, 1954; Slusher, 1958) and the adrenal content of ascorbic acid (McCann, 1953; Bouman *et al.*, 1957; McCann and Haberland, 1960) and of the corticosteroids (Hume and Nelson, 1955; Laqueur *et al.*, 1955; Ganong *et al.*, 1961). It also affects the occurrence of compensatory adrenal hypertrophy (McCann, 1953; Smelik *et al.*, 1959; Ganong and Hume, 1954; De Wied *et al.*, 1958; Ganong *et al.*, 1961) and the response to stress (De Groot and Harris, 1952; McCann, 1952; Ganong and Hume, 1954). These modifications are related to the extent of the lesions and to their location. The anterior and middle parts (more uncommonly the posterior part) of the hypothalamus have sometimes been held responsible for the control of the adrenal production of corticosterone.

Also the cerebral cortex (Porter, 1954; Soulairac *et al.*, 1956; Egdahl, 1961), the limbic system (Okinaka *et al.*, 1960), the mesencephalic structures (Moll, 1959; Martini *et al.*, 1960), the brainstem (Slusher, 1960), and the amygdala (Martin *et al.*, 1958; Mason, 1958) can influence the adrenal function, at least in particular situations. The action of every center of the cerebral cortex and of the hypothalamus which regulates the function of the adrenal cortex, occurs by means of the hypophysis.

Only a few authors admit a direct nervous control of the hypophysis by the hypothalamus (Hagen, 1954; Da Lage, 1955). The direct nervous pathway (hypothalamus-adrenal) does not seem to be important in regulating the hormone secretion of the adrenal gland, but it could affect the threshold of response of the hormones. If a purely nervous direct hypothalamoadenohypophyseal route exists, it would have a vasomotor function and would intervene in the regulation of the blood circulation of the pars distalis, thus combining its quantitative effects with the qualitative effects of the neurohumoral pathway (Benoit and Assenmacher, 1955).

[1] This work was supported by a grant of the Consiglio Nazionale delle Richerche.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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The numerous substances that can reach the adenohypophysis by means of the hypophyseal portal vessels (Green and Harris, 1947) were considered to be the hypothalamohypophyseal mediators. Saffran *et al.* (1955) found a corticotropin-releasing factor (CRF) in the neurohypophyseal tissue of the rat. Guillemin *et al.* (1957) isolated from the hypothalamus and the posterior part of the hypophysis, a substance (fraction D)—found also in other parts of the encephalon—capable of provoking the discharge of ACTH (Guillemin *et al.*, 1959). Royce and Sayers (1958) have observed that a pepsin-labile hypothalamic factor can stimulate ACTH release. A corticotropin-influencing factor (CIF) (Eik-Nes *et al.*, 1961) was also isolated; it acts like CRF and induces the increase of the ACTH secretion and probably inhibits a specific pituitary proteinase that favors the destruction of ACTH.

Because of the great uncertainty on the localization of the centers responsible for the regulation of the hypophyseal-adrenal activity on the chemical nature of the hypothalamic mediator(s), and on the different ways to effect control, the question is presently still under study and debate.

Therefore, it seemed opportune to conduct an accurate study on the nervous control of the adrenal cortex activity, also in the attempt to define the importance of certain areas of the hypothalamus in the regulation of the various adrenal functions.

II. MATERIALS AND METHODS

Male white albino rats of the Wistar strain, weighing 120–160 gm, were used for the functional exploration of the adrenal gland, and rats weighing ca. 90 gm, for karyometric research on the encephalon. The animals were kept at a temperature of about 21°C; the diet was ad libitum. Electrolytic lesions were made with electrodes localized by means of a Krieg-Johnson stereotaxic instrument. Currents of diverse intensities were used (0.5, 1.5, 2.5 mA) for periods ranging from 5 to 10 minutes. Afterward the locations of the lesions were histologically examined. In some of the rats, lesions of the cerebral cortex (area 10) were performed by means of a plate electrode, using a 5 mA current for 20 minutes.

Catheterization was done with a needle introduced into the left adrenal vein and directed through the renal vein. The animals were anesthetized with sodium pentobarbital (4 mg per 100 gm body weight) and with morphine (2 mg per 100 gm body weight) to prevent surgical stress (Munson and Briggs, 1955). Catheterization lasted no longer than 5 minutes. The hematocrit values of the blood collected in this manner were constant.

Corticosterone production was expressed in micrograms per minute per gram of the left adrenal gland (μg/min/gm). The ratio between

adrenal corticosterone production and peripheral hormone level was adopted in order to express indirectly the rate of the hormone metabolism (A : P ratio). Plasma corticosterone was evaluated as described by Saba and Hoet (1962).

Karyometric investigation was carried out in the right adrenal gland, fixed in Bouin's fluid and stained with hematoxylin-eosin. The mean nuclear diameter of 100 cells of the zona fasciculata was calculated. In some animals, the mean of the nuclear mass of the adrenal cortex cells was measured using the interference microscope (Baker type) (Baker, 1955).

Some of the normal or lesioned rats were treated with vasopressin or oxytocin (0.2 IU per 100 gm body weight) or with epinephrine (50 μg per 100 gm body weight); these substances were injected intraperitoneally 90 minutes before decapitation.

The diencephalic karyometric evaluation was performed both on normal animals and on those treated with hydrocortisone. Encephalons were fixed for 48 hours in formalin and stained with Carazzi's hemalum.

III. RESULTS

A. PRODUCTION OF CORTICOSTERONE BY THE ADRENAL CORTEX OF NORMAL RATS IN BASAL CONDITIONS

Corticosterone was evaluated in the blood collected from the left adrenal vein. Values obtained on rats of the Wistar strain (reported in Table I) indicate that sometimes important differences in the production and peripheral level of corticosterone exist at various hours of the day.

TABLE I

Adrenal Production and Plasma Level of Corticosterone in Normal Rats at Different Hours of the Day

Number of animals	Body weight (gm)	Hours	Corticosterone		
			Plasma level (μg/100 ml)	Adrenal production left side (μg/min/gm)	A : P ratio
6	133 ± 27	9–11	18.1 ± 0.9	7.1 ± 0.8	0.40 ± 0.04
6	137 ± 5	15–17	19.1 ± 0.8	5.3 ± 0.9	0.27 ± 0.04
6	134 ± 13	21–23	24.7 ± 0.9	4.3 ± 0.0	0.17 ± 0.02

[a]Values ± standard error of the mean.

Adrenal corticosterone production diminishes, although not significantly, in the afternoon, and still more so in the evening ($P < 0.02$) as compared to the morning. The peripheral corticosteronemia change, inversely; it remains pratically unchanged from morning until afternoon, and in the evening it increases significantly ($P < 0.001$).

The progressive decline of the A : P ratio from the morning to the evening ($P < 0.001$) indicates a uniform slowing of metabolism (Saba *et al.*, 1962). In fact, in the evening we have observed, besides the reduced production, an increase in the peripheral plasma hormone levels. This is an indirect sign of the lengthening of the biological half-time of the hormone and reduced turnover.

Significant variations were observed in corticosterone production and in peripheral hormone levels during a 24-hour period. In consideration of these phenomena, for the studies reported below we collected the blood in the morning.

B. CORTICOSTERONE PRODUCTION BY THE ADRENAL CORTEX IN RATS AFTER ELECTROLYTIC LESIONS OF THE HYPOTHALAMUS

The plasma corticosterone levels in the peripheral blood and in the blood collected from the adrenal vein was evaluated in animals with electrolytic lesions in the hypothalamus (Table II).

Animals examined 3 days after lesioning in the median eminence were found to have increased plasma corticosterone values in the peripheral blood, whereas in animals observed after 7 days the levels were notably lower. The adrenal production of corticosterone increased on the third day; on the seventh it had diminished. The A : P ratio increased on the third day; on the seventh it was inconstant, and normal or reduced values were found. The increase of hormone production on the third day could be due to a transitory irritative phenomenon at the site of the lesion.

The importance of the anterior part of the median eminence on the regulation of corticosterone is evident 7 days after lesioning, when a significant reduction of the peripheral levels and adrenal production of the hormone is observed.

The peripheral plasma corticosterone level rises with adrenal production only up to a certain limit; when adrenal production goes below a particular level there is no parallel reduction of the peripheral corticosterone. It is as though an attempt at conservation is made by means of a slowing down of catabolism. The hormone turnover, indirectly measured by the A : P ratio, is accelerated on the third day whereas on the seventh, it is sometimes normal and at other times (in rats whose production is lowered to minimum rate), clearly diminished.

TABLE II

Adrenal Production and Peripheral Plasma Level of Corticosterone in Normal Conditions and After Various Hypothalamic Lesions in the Rat

Rat. no.	Current (mA×sec)	Site of lesions [a]	Days after lesioning	Adrenal [b] weight (mg) L	Adrenal [b] weight (mg) L+R	Plasma corticosteroid levels [c] P (μg/100 ml)	Plasma corticosteroid levels [c] A (μg/min)	Plasma corticosteroid levels [c] A:P ratio
Controls ($N=6$)	—	—	—	10–18	26.8±2.1	18.1±0.9 (16.6–22.5)	7.1±0.8 (4.8–10.2)	0.40±0.04 (0.29–0.56)
61 L	0.5×5	M.E.	3	—	21	26.7	—	—
62 L	0.5×5	M.E.	3	—	22	31.6	—	—
63 L	0.5×5	M.E.	3	—	30	28.5	—	—
67 L	0.5×5	M.E.	3	—	28	33.3	—	—
50 CL	0.5×5	M.E.+V.M.	3	17	32	30.3	5.4	1.78
68 L	1.5×10	M.E.	3	—	35	29.8	—	—
69 L	1.5×10	M.E.	3	—	30	23.3	—	—
91 CL	1.5×10	M.E.	3	19	37	26.2	15.5	0.59
92 CL	1.5×10	M.E.	3	20	38	24.6	18.1	0.73
45 L	0.5×5	M.E.	7	—	25	5.2	—	—
47 L	0.5×5	M.E.	7	—	32	10.4	—	—
72 CL	1.5×10	M.E.	7	20	38	8.2	2.7	0.38
73 L	1.5×10	M.E.	7	—	30	5.5	—	—
74 CL	1.5×10	M.E.	7	14	28	9.6	3.7	0.39
75 CL	1.5×10	M.E.	7	14	27	9.6	2.1	0.21
76 L	1.5×10	M.E.	7	—	34	9.0	—	—
80 CL	1.5×10	M.E.	7	17	35	10.7	1.7	0.16
81 CL	1.5×10	M.E.	7	18	36	7.1	0.1	0.01
82 CL	1.5×10	M.E.	7	19	33	11.4	1.7	0.15
83 CL	1.5×10	M.E.	7	14	28	11.3	0.4	0.03
84 CL	1.5×10	M.E.	7	16	38	7.7	0.2	0.03
88 CL	1.5×10	M.E.	7	14	24.5	7.7	0.7	0.09
89 CL	1.5×10	M.E.	7	13.5	27	7.7	0.3	0.04
90 CL	1.5×10	M.E.	7	17	33	9.2	0.5	0.05
21 CL	0.5×5	A.H.+P.V.	7	15	30	16.4	4.4	0.27
30 CL	0.5×5	A.H.+P.V.	7	18	35	17.1	5.3	0.31
32 CL	0.5×5	A.H.+P.V.	7	17	34	16.7	4.7	0.28
34 L	0.5×5	A.H.+P.V.	7	—	27	19.0	—	—
35 CL	0.5×5	A.H.+P.V.	7	16	33	16.9	5.7	0.34
46 L	0.5×5	A.H.+P.V.	7	—	31	16.2	—	—
48 L	0.5×5	L.H.+V.M.	7	—	23	14.6	—	—
49 L	0.5×5	L.H.+V.M.	7	—	21	15.8	—	—
93 CL	1.5×10	P.M.	7	16	31	14.4	4.9	0.34
94 L	1.5×10	P.M.	7	—	30	16.0	—	—
95 CL	1.5×10	P.M.	7	16	33	19.5	4.2	0.21
96 CL	1.5×10	P.M.	7	17	34	16.0	3.6	0.23
97 CL	1.5×10	P.M.	7	16	32	18.4	5.0	0.27

[a] M.E. = median eminence; V.M. = ventromedial nucleus; A.H = anterior hypothalamic nucleus; P.V. = paraventricular nucleus; L.H. = lateral hypothalamic nucleus; P.M. = premammillary nucleus.

[b] L = left adrenal, catheterized; L + R = left plus right adrenal.

[c] P = peripheral hormone level; A = corticosterone production of left adrenal.

C. EFFECTS OF HYPOTHALAMIC LESIONS ON ADRENAL WEIGHT

Adrenal weight was measured at different times after lesions had been produced at various locations; these lesions differed according to the intensity and duration of the current used (Table III).

TABLE III

Weight Changes of the Adrenal Glands in Rats with Various Hypothalamic Lesions

Number of animals	Body weight (gm)	Current (mA × sec)	Site of lesions [a]	Days after lesioning	Adrenal weight (mg)
21	127	—	—	—	28,3±1,1
10	110	0,5×5″ 1,5×10″	A.H.	7	31,1±1,9
7	120	2,5×10″	A.H.	15	30,5±2,4
10	130	0.5×5″ 1,5×10″	E.M.	7	33,7±1,5
10	106	2,5×10″	E.M.+A.Z.	15	19,8±1,0
5	121	1,5×10″	P.H.	7	32,3±1,4
13	102	2,5×10″	P.H.	15	28,6±1,1

[a] A.H. = anterior hypothalamus; A.Z. = adjacent zones; P.H. = posterior hypothalamus.

Of the lesions that were not extensive and involved the anterior part of the median eminence or other formations of the anterior and posterior parts of the hypothalamus, the gland weight did not undergo significant changes. When the lesions destroyed extensive areas of the hypothalamus, the adrenal weight was reduced because of the lesions in the median eminence and in the adjacent zones (Tronchetti *et al.*, 1961). The weight was not modified, however, by lesions in the anterior and posterior parts of the hypothalamus.

D. EFFECTS OF HYPOTHALAMIC LESIONS ON THE MEAN NUCLEAR DIAMETERS OF THE ADRENAL CELLS OF THE ZONA FASCICULATA

The mean nuclear diameter (MND) of the adrenal cells of the zona fasciculata was measured in rats with lesions of the anterior and posterior parts of the hypothalamus and particularly of the median eminence (Table IV).

The MND is reduced only in the animals with lesions in the median eminence that were made with currents of 1.5 mA for 10 minutes. The reduction is significant ($P<0.001$) in the animals 7 days after lesioning and less evident, but still significant ($P<0.05$), 3 days after

TABLE IV

Mean Nuclear Diameter (MND) of the Adrenal Cells of the Zona Fasciculata after Hypothalamic Lesions

Number of animals	Site of lesions[a]	Current (mA × sec)	Days after lesioning	Adrenal weight (mg)	MND, cells of zona fasciculata (μ)
5	M.E.	0.5×5	3	26.6±2.0	5.58±0.05 ($P < 0.05$)
5	M.E.	0.5×5	7	31.6±0.5	5.31±0.02 ($P < 0.001$)
1	M.E.	1.5×10	3	34.5±2.3	5.46±0.03 ($P < 0.001$)
13	M.E.	1.5×10	7	31.7±1.2	5.07±0.04 ($P < 0.001$)
2	S.C.N.	0.5×5	7	30 to 33	5.71 to 5.80
5	A.H.N.+P.V.N.	0.5×5	7	31.8±3.3	5.71±0.04 (N.S.)
2	L.H.N.+V.M.N.	0.5×5	7	21 to 23	5.70 to 5.86
6	P.M.N.	1.5×10	7	31.7±0.3	5.73±0.05 (N.S.)
6 (Controls)	—	—	—	26.8±2.1	5.80±0.07

[a]M.E. = median eminence; S.C.N. = suprachiasmatic nucleus; A.H.N. = anterior hypothalamic nucleus; P.V.N. = paraventricular nucleus; L.H.N. = lateral hypothalamic nucleus; V.M.N. = ventromedial nucleus; P.M.N = premammillary nucleus.

lesioning. Even more extensive lesions, made in other areas of the hypothalamus, do not modify the MND.

E. MODIFICATIONS OF THE NUCLEAR MASS OF THE ADRENAL CELLS IN RATS LESIONED IN THE HYPOTHALAMUS

The behavior of the nuclear mass was measured both in normal rats and in those lesioned in the hypothalamus, particularly in the median eminence (Table V).

TABLE V

Nuclear Mass (NM) of the Adrenal Cells of the Zona Fasciculata in Animals with Hypothalamic Lesions.

Groups	Number of animals	Site of lesions[a]	Current (mA × sec)	Days after lesioning	NM (mμg)
I	6	—	—	—	23.84±0.71
II	5	M.E.	0.5×5	3	20.22±1.80
III	5	M.E.	0.5×5	7	23.71±0.92
IV	3	A.H.	0.5×5	7	23.33

[a]M.E. = median eminence; A.H. = anterior hypothalamus.

As shown in Table V, the mean nuclear weight undergoes some modifications in the animals lesioned in the median eminence, but not in those with lesions in other parts of the hypothalamus.

The reduction of the nuclear mass is evident, however, only at 3 days after lesioning, but not in animals lesioned 7 days earlier.

The percentage distribution of the nuclei was scarcely modified at 3 days after lesioning, and it was almost normal after 7 days.

F. EFFECTS OF EPINEPHRINE, OXYTOCIN AND VASOPRESSIN ADMINISTRATION ON THE ADRENAL FUNCTION OF RATS LESIONED IN THE MEDIAN EMINENCE

Three days after the small lesions in the anterior part of the median eminence had been made, epinephrine (50 μg per 100 gm body weight), oxytocin (0.2 U/100 gm) or vasopressin (0.2 U/100 gm) was injected intraperitoneally in four rats of each group. After 90 minutes, the animals were killed and then the peripheral plasma corticosterone and the MND of the adrenal cells of the zona fasciculata were measured (Table VI).

TABLE VI

Effects of Pharmacological Stimuli on the Mean Nuclear Diameter (MND) of the Cells of the Zona Fasciculata and the Plasma Corticosterone Values in Rats with Lesions in the Median Eminence (M.E.)

Site of lesions	Treatment	Adrenal weight (gm)	MND (μ)	Corticosterone (μg/min/gm)
—	—	21.0–33.5	5.79±0.10	18.1±0.9
M.E.	—	32.0	5.32	28.9
M.E.	Epinephrine	26.7	6.03	30.6
M.E.	Oxytocin	25.8	6.03	29.9
M.E.	Vasopressin	34.5	6.06	28.6

Three days after electrolysis of the median eminence, there was a clear increase of the corticosteronemia and the MND was reduced significantly ($P<0.05$). There were no adrenal weight changes.

The administration of epinephrine, oxytocin, or vasopressin in rats with lesions of the anterior part of the median eminence did not cause other modifications of the plasma corticosterone levels, which were already increased. But it prevented MND reduction, caused by the lesions, in the adrenal cells of the zona fasciculata. The MND of these cells was in fact only slightly larger than in normal animals and significantly increased in respect to the untreated lesioned animals.

G. ADRENAL CONTROL OF HYPOTHALAMIC ACTIVITY

The MND was measured in different structures of the anterior, middle, and posterior hypothalamus in normal rats and in rats treated, respectively, with 10, 50, and 100 μg of hydrocortisone subcutaneously daily for 10 days. The animals were decapitated on the eleventh day and a check of organ weights and of the encephalic histology was performed (Tables VII and VIII).

TABLE VII

Body and Endocrine Glands Weights in Hydrocortisone Treated Rats

Organ	Controls	Hydrocortisone treated rats		
		10 μg	50 μg	100 μg
Body weight, gm	98.0±3.0	96.0±4.0	103.0±5.0	96.0±4.0
Adrenals, mg%	32.5±3.0	27.7±2.3	25.8±1.7	25.7±2.0
			($P < 0.02$)	($P < 0.05$)
Testes, mg%	1810.0±25.0	1571.0±10.0	1610.0±75.0	1410.0±82.0
Hypophysis, mg%	4.6±0.1	4.7±0.2	4.8±0.1	4.3±0.8
Thyroid, mg%	20.5±1.3	20.2±1.4	20.6±2.5	19.1±3.2

TABLE VIII

Effects of Hydrocortisone Treatment on the Mean Nuclear Diameter (MND) of the Various Hypothalamic Nuclei in the Rat

Hypothalamic nuclei[a]	Controls	Hydrocortisone–treated rats					
		10 μg		50 μg		100 μg	
		MND	$P <$	MND	$P <$	MND	$P <$
P.V.	7.55±0.13	8.59±0.02	0.01	8.41±0.08	0.001	8.64±0.17	0.001
S.O.	6.29±0.09	6.34±0.10	N.S.[b]	6.73±0.10	0.01	7.24±0.04	0.001
V.M.	6.76±0.20	7.38±0.08	0.001	7.83±0.08	0.001	7.91±0.06	0.001
D.M.	6.47±0.20	6.62±0.19	N. S.	7.00±0.08	0.001	7.17±0.18	0.001
A.	6.08±0.26	6.19±0.06	N. S.	6.09±0.06	N. S.	6.14±0.04	N. S.
V.Pm.	6.52±0.12	6.67±0.08	N. S.	6.75±0.08	0.05	6.75±0.17	0.05
D.Pm.	6.27±0.25	6.61±0.08	0.05	6.93±0.19	0.01	6.94±0.19	0.01
M.M.	6.47±0.15	6.76±0.10	0.02	7.13±0.17	0.001	7.13±0.17	0.001
L.M.	6.42±0.20	6.53±0.10	N. S.	7.06±0.10	0.001	7.06±0.10	0.001

[a]P.V. = paraventricular; S.O. = supraoptic; V.M. = ventromedial. D.M. = dorsomedial; A. = arcuate; V.Pm. = ventral premammillary; D.Pm. = dorsal premammillary; M.M. = medial mammillary; L.M = lateral mammillary.

[b]N.S. = not significant.

Highly significant variations in diameter were noted only in the ventromedial nucleus ($P<0.001$) and in the paraventricular nucleus ($P<0.01$) of the animals treated with 10 μg of hydrocortisone. In rats

to which larger doses of hydrocortisone (both 50 and 100 μg) were administered, the variations in diameter of the diencephalic nuclei were always more significant in all the structures considered except the arcuate nucleus.

Significant modifications of the endocrine glands were not observed in these animals. On the contrary, there was a notewo rthy adrenal weight reduction only when larger doses of hydrocortisone were used ($P<0.02$).

IV. DISCUSSION

The central nervous system, which regulates the adrenal cortex activity principally through the hypophysis, seems to have the focal point of convergence of the nervous and neurohumoral paths in the median eminence. The cortical, amygdaloid, limbic, reticular, and hypothalamic regions are involved in the control and rhythm of adrenal activity (Saba *et al.*, 1962). However, it seems that it is precisely the median eminence, in particular its anterior part, that must intervene in this regulation. The cerebral cortex (Porter, 1954; Soulairac *et al.*, 1956; Carnicelli *et al.*, 1962), areas of the hippocampus (Porter, 1954), amygdaloid nuclei (Koikegami *et al.*, 1955; Mason, 1958; Okinaka *et al.*, 1960), reticular mesencephalic structures (Anderson *et al.*, 1957; Martini *et al.*, 1960) can interfere with the adrenal function, and opposite impulses can come from the various zones.

Extracts obtained from the different regions of cerebral tissue can stimulate different aspects of the adrenal cortical function (Slusher and Roberts, 1954). A greater and more specific activity in this sense was found in extracts from precise regions of the hypothalamus, such as the median eminence (Royce and Sayers, 1958; Brizzee and Eik-Nes, 1961), and also from zones whose destruction did not seem to modify the adrenal cortical function in a constant manner (Slusher and Roberts, 1954; De Wied, 1957).

A large part of encephalon, but especially the hypothalamic region, seems to be involved in the regulation of the ACTH production. Many authors have observed by direct and indirect methods, that the destruction of the anterior, middle, or posterior parts of the hypothalamus, especially if extensive, can provoke adrenal hypofunction and hypotrophy. In regard to the median eminence, most authors agree that extensive destruction of this region causes adrenal hypofunction (McCann and Brobeck, 1954; Ganong and Hume, 1954; De Wied *et al.*, 1958; Smelik *et al.*, 1959). But it was also observed that, after lesions limited to anterior or posterior parts, modifications appeared only in certain morphological or functional aspects of the adrenal gland. Some authors believe that the anterior part of the median eminence is involved only in the regulation of adrenal ascorbic acid contents, whereas areas behind it control corticosterone production (Slusher,

1958). Other authors have observed that lesions in the median eminence cause an increase in adrenal weight and prevent the release of corticosteroids (Krieger and Wagman, 1961).

We have observed that even minimal lesions (<600 μ) in the median eminence [smaller than lesions considered by others to be insufficient to modify the weight or to impede the compensatory adrenal hypertrophy (Moll, 1960)] are able to modify various aspects of adrenal activity (Fig. 1). Lesions localized in the anterior part of the median

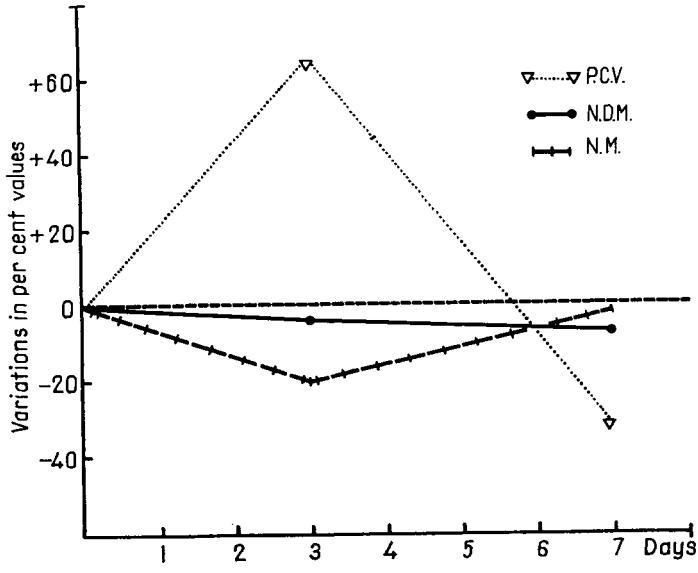

FIG. 1. Plasma corticosterone values (▼....▼), mean nuclear diameters (●—●), and nuclear mass (+++) in rats with lesions in the median eminence.

eminence induce a great reduction in the adrenal production of corticosterone with a consequent fall (sometimes compensated by a slower metabolism of the hormone) of the peripheral corticosterone. They also cause a reduction of the MND of the cells of the zona fasciculata and modify their nuclear mass although in a different manner. On the contrary, these same lesions have little influence (perhaps because of their limited size) on adrenal weight, which, at least after a week, remains unchanged. Lesions of the same intensity in other parts of hypothalamus do not significantly change the corticosterone production or the behavior of the MND or the adrenal weight.

The functional alterations of the adrenal cortex became apparent some days after electrolysis of the median eminence. Lesions of the anterior part produced, after 3 days, hyperactivity (in certain aspects)

of the adrenal cortical function. At the same time corticosterone production constantly increased and the MND of the cells of the zona fasciculata was already significantly reduced ($P<0.05$).

This dissociation of effects can in part be attributed to increased hypothalamic activity probably referable to a perilesional irritation.

However, these results furnish a proof that the same structure can affect, in a different way, the adrenal production of corticosterone and the volume and mass of the adrenal cells. We can also suppose that the center designed to control some aspects of nuclear trophism is more limited than the area responsible for the production of steroids. This functional dissociation is also confirmed by the different effect of epinephrine and hypophyseal neurohormones in regard to the production of corticosterone and of the MND of the adrenal cells. Three days after lesioning, a check was made by stimulation of the adrenal gland function before it could change its responsivity to ACTH (Dear and Guillemin, 1960). Since the stimuli used were capable of preventing reduction in diameter after lesions, but not of increasing the rate of adrenal production of corticosterone, it could be that they are not capable of further stimulating corticosterone production of the residual perilesional structures. But perhaps they can still act on the adrenal gland through an extrahypothalamic pathway.

From these data it is evident that a limited zone of the hypothalamus can regulate adrenal cortical activity. Some functions however, seem to evade rigid neural control and present an autoregulation within a certain time. The phenomenon that occurs for the nuclear mass can be interpreted as a process of autoregulation. In fact, the return of the nuclear mass to normal values can indicate that the synthesis of nuclear proteins becomes independent from every hypothalamic control and from the other adrenal functions.

A hypothalamic regulation of the adrenal cortical activity exists, and there seems to be, for certain functional aspects, an adrenal autoregulation. In the same way, the adrenal cortex can influence its hormonal control by hormononeural regulation. With the study of the behavior of the MND of the hypothalamic structures after hydrocortisone administration, histometric variations were observed that were significant also for physiologic doses of the hormone. From this research (as observed by Halász and Szentágothai, 1959) it seems, however, that the ventromedial nuclei are the first to be sensitive to humoral variations. It is almost as though they were designed to regulate the neurosecretory substances and/or to prepare the hypophyseal adrenal sensitivity to substances produced by the various structures of the hypothalamus involved in the production of corticosterone.

Studies by many authors, made in order to ascertain the localization and importance of the hypothalamic centers involved in the control of the adrenal cortical function, have given diverse results, probably

because many structures are involved, in different degrees, in this control. Therefore, the results can be different depending on the extent of the lesions and on the type and quantity of the cells involved.

The median eminence, which represents in a sense the point of convergence of different structures, is probably chiefly responsible for this control. It is possible that substances secreted by other centers become activated in this region, augmenting its importance. The finding that small lesions of a part of this region are sufficient to modify the production of adrenal hormone and its rhythm suggests the great importance that the median eminence assumes in the regulation of adrenal activity. In fact, there is a nychthemeral rhythm of the peripheral plasma corticosterone levels (McKarty *et al.*, 1960) and of the adrenal production of corticosterone (Saba *et al.*, 1962). This phasic behavior of adrenal activity seems to be directly dependent on a variation of the functional tone of superior structures.

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DISCUSSION

Effect of Hypothalamic Lesions

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Lesions in the median eminence of the hypothalamus cause a block of the release of ACTH from the anterior pituitary only if the lesion involves more than the entire median eminence and probably destroys the pituitary portal system.

If lesioned animals are exposed to the stress of ether anesthesia, as shown in Fig. A, corticoid production *in vitro*, of the left adrenal gland, serving as an index of ACTH, activity [van der Vies, J., Bakker, R. F. M., and De Wied, D. *Acta Endocrinol.* **34**, 513

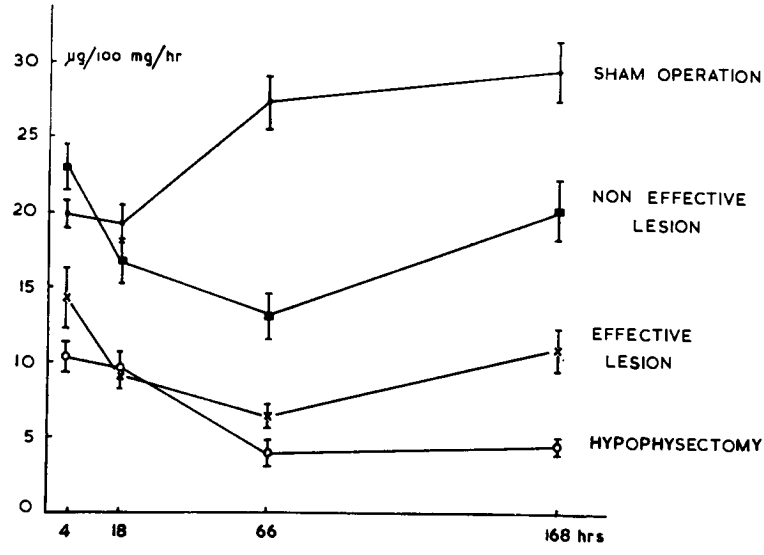

Fig. 1. Effect of sham operation, lesion of the median eminence, and hypophysectomy on corticoid production *in vitro* of left adrenal gland in response to ether anesthesia at various time intervals after surgery.

(1960)], is already depressed at 4 hours after placement of the lesion. It gradually declines further and it remains low for at least a week after the destruction of the median eminence. If the occurrence of CAH is used as an index of the effectiveness of the lesion in inhibiting the discharge of ACTH, 30% of the lesioned animals appear to be noneffectively blocked. Corticoid production *in vitro* of the left adrenal of these rats

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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rises significantly in response to the stress of ether anesthesia, although the increase in adrenocortical activity is less than in sham-operated rats. In the effectively lesioned rats adrenal atrophy occurs, whereas the weight of the gland of noneffectively lesioned animals is not essentially affected. These findings are in accord with those presented by Professor Tronchetti and co-workers.

That the anterior pituitary of effectively lesioned animals is still capable of releasing

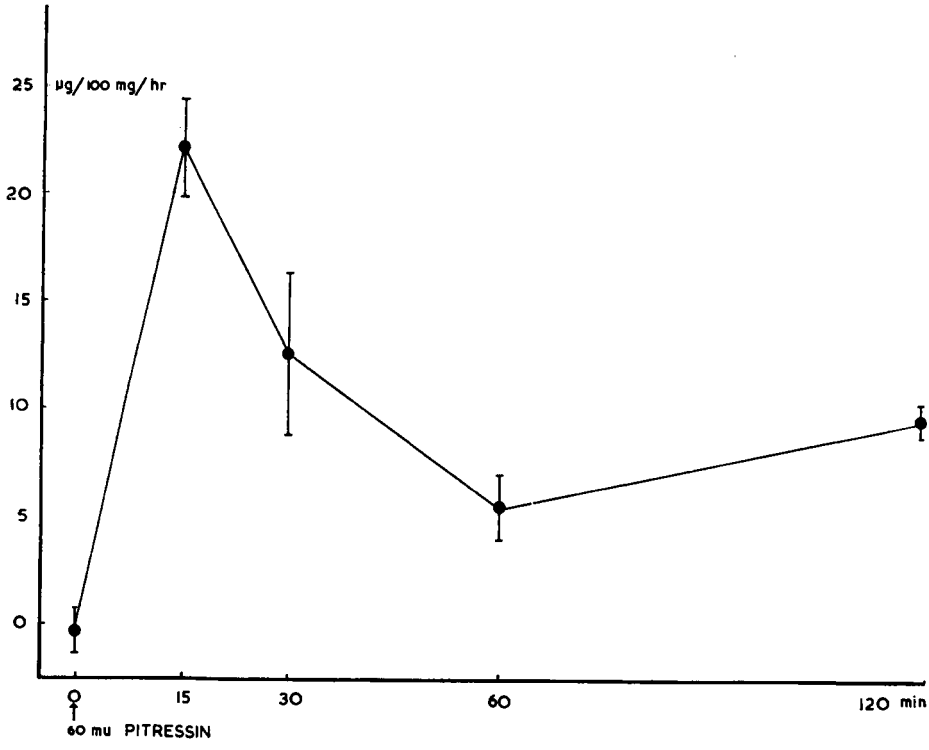

FIG. 2. Effect of 60 mU of Pitressin (Parke, Davis & Company) on corticoid production *in vitro* of effectively lesioned rats at various time intervals after intravenous administration.

ACTH following an adequate stimulus is shown in Fig. 2. The intravenous administration of 60 mU of Pitressin into rats lesioned 18 hours earlier induced an increase in the rate of steroid formation *in vitro*. It is pertinent to note that the effect was most marked at 15 minutes after the injection and that it lasted only for about 60 minutes. I would like to ask Professor Tronchetti why the effects of vasopressin, oxytocin, and epinephrine were measured 90 minutes after the injection into lesioned animals.

The experiments of Professor Tronchetti and co-workers on the adrenal control of hypothalamic activity are most interesting. The observation that daily injection of hydrocortisone in rats cause a highly significant diametric variation in the ventromedial and paraventricular nuclei may indicate that these structures are involved in the negative feedback action of adrenal steroids in the release of ACTH from the adenohypophysis.

To block the discharge of ACTH, rats were injected subcutaneously with graded doses of dexamethasone 16 hours prior to exposure to noxious stimuli or to the injections of either lysine vasopressin or USP ACTH. Figure 3 shows the results.

Corticoidogenesis *in vitro* elicited by handling of the animals was readily suppressed by the lowest dose of dexamethasone. In contrast, the more severe stress of unilateral adrenalectomy was inhibited by considerably higher doses of the steroid. At least

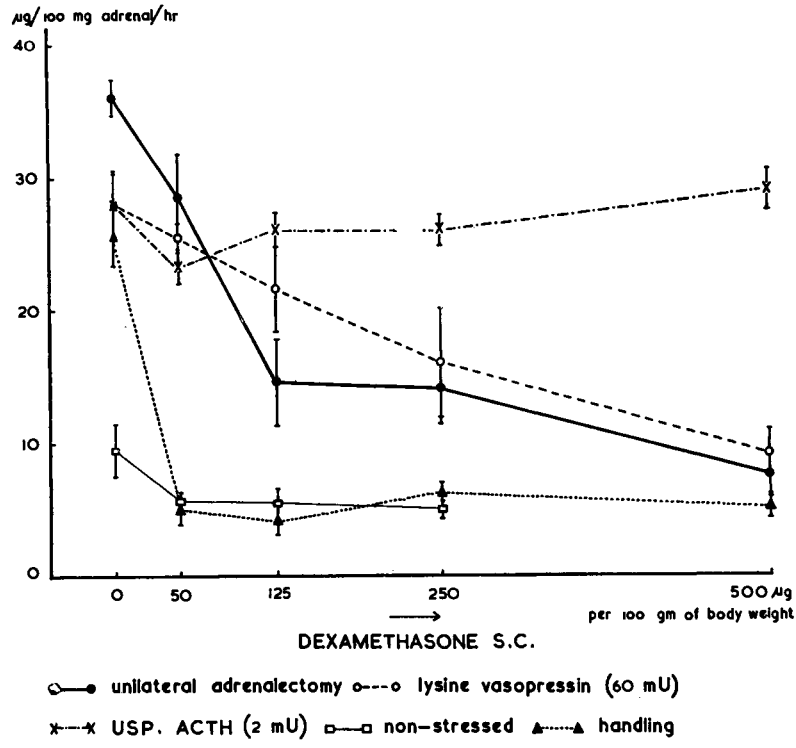

FIG. 3. Effect of graded doses of dexamethasone on corticoid production *in vitro* of adrenals of rats exposed to stressful stimuli or to lysine vasopressin or USP ACTH.

500 µg of dexamethasone had to be administered before a complete block of ACTH release was obtained. The adrenal response to the intravenous injection of 2 mU of USP ACTH was not affected by the same pretreatment with dexamethasone, since the rate of corticoid production *in vitro* was nearly similar 15 minutes after the injection of this hormone at all dose levels tested. The administration of graded doses of dexamethasone also gradually diminished the release of ACTH elicited by 60 mU of vasopressin, indicating a gradual decline of the responsiveness of the anterior pituitary to the octapeptide. Since the corticotropic effect of vasopressin is still present in rats with hypothalamic lesions, as shown before, these results are interpreted to indicate that dexamethasone suppresses the corticotropic effect of noxious stimuli by a direct blocking action on the anterior pituitary. I would appreciate Professor Tronchetti's comments on this observation in regard to the site of action of adrenal steroids in the release mechanism of ACTH.

F. TRONCHETTI: The adrenal production of corticosterone and the mean nuclear diameter (MND) of the adrenal cells was measured at 30, 60, and 90 minutes after the intraperitoneal injection of oxytocin, epinephrine, and vasopressin. No significant variation in corticosterone production, which already was increased after electrolytic lesions, or in adrenal cell size was observed at 30 and 60 minutes after drug administration. On the contrary, 90 minutes after the injection of these drugs, corticosterone production still showed no significant change, but we obtained a significant increase of the MND.

In view of these findings, in this paper we considered only the effects at 90 minutes after drug injection.

The cytohormonal response we obtained at 90 minutes seems have the same significance (adrenal stimulation) as that observed by Dr. De Wied; probably these responses are different aspects of the adrenal cortical function, which appear at different times, in part as a result of different ways of administering the drugs.

Actually, it is impossible to establish in a definitive manner the mechanism and the site of action of the adrenal steroids in regulating ACTH release.

Our study shows that administration of hydrocortisone induces morphological modification also in the hypothalamus; but further investigations are necessary in order to establish whether or not these effects are direct.

The Regulation of ACTH and Corticosteroid Secretion

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I. INTRODUCTION

Removal of a large portion (Egdahl, 1960, 1962), or all of the brain, leaving behind an isolated pituitary, leads to a high resting level of adrenal corticosteroid secretion, accompanied, in some cases, by an additional increase in secretion following trauma. It might be tempting to infer from these results that the brain is not normally essential to the initiation of increased adrenal corticosteroid secretion in trauma. It is the purpose of this paper to suggest that the brain is indeed vital to the initiation of ACTH release in trauma, to discuss some of the mechanisms involved in the regulation of corticosteroid output, and to report data in man bearing upon the control of ACTH, 17-hydroxycorticosteroid, and aldosterone secretion.

II. METHODS

Adrenal venous blood samples were obtained in dogs by the method of Hume and Nelson (1955). Timed samples of adrenal venous blood were obtained in patients undergoing abdominal laparotomy (Hume and Bell, 1959) by the method of Hardy and Turner (1957) and, later, in a few patients, by means of an indwelling cannula inserted through the femoral vein and allowed to remain in place for periods of up to 24 hours (Hume *et al.*, 1962). The samples were analyzed for 17-hydroxycorticosteroids (17-OHCS) by the method of Nelson and Samuels (1952) and were expressed in micrograms per minute. In some cases determinations were carried out for compounds F (cortisol), B (corticosterone), E (cortisone), and S (17-hydroxy-11-deoxycorticosterone) in the following manner: The plasma was extracted by the Nelson–Samuels method and the filtrate was spotted on chromatogram paper in the E_2B system. (Eberlein and Bongiovanni, 1955.) A photograph was taken in ultraviolet light, and with this as a guide the spots were eluted with 50% methanol and chloroform. The eluates were dried, and

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics:
Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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compounds E, F, and S were determined by the Porter–Silber reaction. The eluate from the B spot was oxidized with cupric acetate (Lewbart and Mattox, 1960) and the sample was then run by the Porter–Silber reaction. Some samples were run for aldosterone by a modification of the method of Kliman and Peterson (1960). Blood ACTH levels were determined by the method of Nelson and Hume (1955). Removal of the hypothalamus in dogs was carried out through a transbuccal approach. Large portions of the anterior hypothalamus were removed with the suction tip in one of two ways: (1) The suction tip was inserted directly behind the optic chiasm and suction was maintained until a large portion of the hypothalamus was removed, or (2) the optic chiasm was split and the suction tip was inserted through the optic chiasm and a more anterior portion of the hypothalamus was removed (Jackson and Hume). These animals did not usually survive more than a few days. The vascularity of the pituitary was proved in all cases by the injection of India ink into the carotid arteries followed by a study of microscopic sections of the pituitary and hypothalamus.

II. RESULTS AND DISCUSSION

A. FACTORS PRODUCING AN INCREASED SECRETION OF 17-OHCS IN TRAUMA

Certain anesthetic agents—particularly ether (Hume, 1958a)—psychic factors (Hume, 1958a, 1961), unreplaced blood loss (Hume, 1961), and trauma itself are all capable of producing ACTH release. Certain anterior hypothalamic lesions abolish the response to anesthesia, psychic factors, and trauma itself (Hume, 1958b; Hume and Egdahl, 1959). Unreplaced blood loss was not tested. The anesthetics now most commonly used clinically do not in themselves produce ACTH release, blood loss is replaced as it occurs, and psychic factors are inoperative during anesthesia. The intraoperative response is therefore due to operative trauma itself.

B. CHARACTERISTICS OF THE RESPONSE TO OPERATIVE TRAUMA

A series of dogs was studied in which the left adrenal gland had been removed 3 weeks before. Two days prior to the experiment a right adrenal vein cannulation was carried out. At the beginning of the experiment the animals were put to sleep under Nembutal anesthesia. After several control samples had been obtained, the snare was tightened around the right adrenal vein so that all adrenal effluent flowed out the catheter. During the time the snare was up the body was deprived of all adrenal secretion, since the secretion of the right gland was flowing out the cannula and the left gland had been previously removed. Ten consecutive 1-minute samples were collected; after this

the snare was released. It may be seen from Fig. 1 that the diversion of adrenal secretion over a 10-minute period did not lead to a release of ACTH. The experiment was then repeated in exactly the same fashion except that this time the animal was traumatized immediately after the snare had been pulled up. Adrenal secretion increased within 3 minutes and continued to rise throughout the period of observation. It is apparent that increased adrenal secretion begins very promptly after

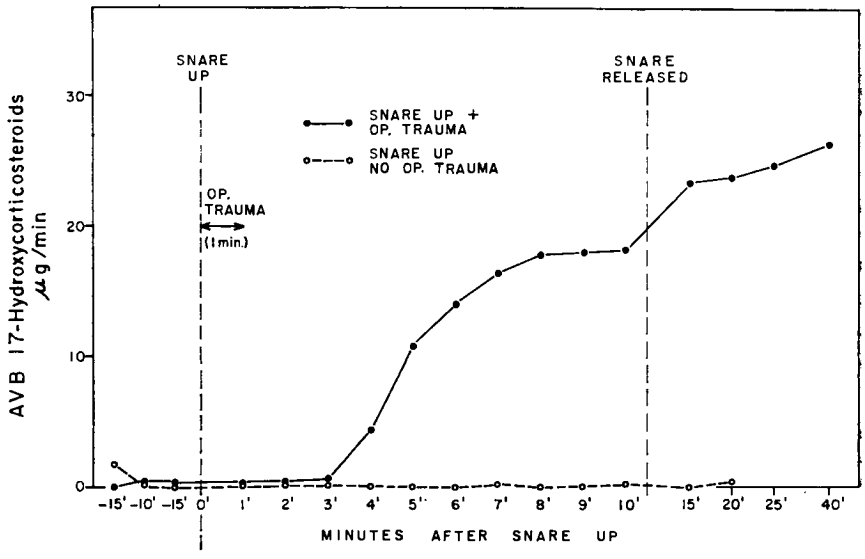

FIG. 1. Adrenal venous blood 17-OHCS in a dog having a previous left adrenalectomy and a chronic indwelling cannula in the right adrenal vein. The broken line shows the effect on corticosteroid output when the adrenal vein snare is pulled up, thereby diverting all the adrenal blood flow to the outside through the cannula and acutely depriving the dog of adrenal secretion. No increase in corticosteroid output occurred over a 10-minute period. Then the experiment was repeated, but this time the animal was traumatized while the snare was up and a marked increase in corticosteroid output began within 3 minutes of the trauma.

trauma and that this increase is not dependent upon acute changes in corticosteroid secretion. The intensity of the response is about equal to that seen with ACTH infusions.

C. THE EFFECT OF HYPOPHYSECTOMY

Hypophysectomy promptly stops all adrenal response to operative trauma. Within 2 or 3 days after hypophysectomy the adrenal is unresponsive to ACTH. A well-developed adrenal atrophy is present a month after hypophysectomy. Partial hypophysectomy is capable of producing a markedly atrophic adrenal which still retains secretory responsiveness.

D. EXTRAPITUITARY ADRENAL INFLUENCES

A variety of substances will produce increased ACTH secretion in the intact animal. Among these are Pitressin, mecholyl, and histamine (Hume, 1958a). We have shown that commercial Pitressin, purified oxytocin or vasopressin, or even synthetic oxytocin or vasopressin[1] are also capable of increasing corticosteroid secretion in the adrenal venous blood of the hypophysectomized dog (Hume, 1958a). This has been shown to be true for doses as small as 50 milliunits (mU). The effect of oxytocin is less marked than that of vasopressin. Mecholyl produced a modest increase in adrenal secretion in one out of two experiments. Serotonin in large doses produced a response in one of eight experiments. Histamine failed to produce an increased secretion in any of eight experiments. The effect of these substances is certainly pharmacological rather than physiological because, in general, large doses have to be used, hypophysectomy always stops the response to operative trauma completely whether or not the brain is present, and cord section abolishes the response to operative trauma even though the posterior pituitary is intact. Furthermore the effect of vasopressin (ADH) on the adrenal is a peculiar one, and differs from that of ACTH in three ways: (1) 17-OHCS secretion after vasopressin does not usually follow a log-dose response curve; (2) repeated doses of vasopressin given to an hypophysectomized ACTH assay animal may cause the adrenal cortex to continue to secrete all during the control period, instead of returning promptly to basal levels as seen with ACTH; and (3) if the animal is maintained for as long as 4 or 5 hours with repeated injections of vasopressin, but without ACTH, it was usually found that the response to ACTH at the end of this time was either decreased or absent, a result suggesting that the vasopressin had not had an ACTH-like effect in maintaining adrenal reactivity to ACTH. This did not occur if small doses of ACTH (1.0–5.0 mU) were interspersed among the ADH injections.

E. THE EFFECT OF CORD SECTION OR NERVE SECTION

Following transection of the spinal cord in the dog, trauma to the denervated area fails to produce an increased 17-OHCS secretion. If the trauma is applied above the level of cord section a normal response occurs (Hume and Egdahl, 1959). When a hind leg is isolated so that it is attached to the body simply by an artery, a vein, and a nerve, trauma to the leg still produces the expected increase in adrenal corticosteroid output. As soon as the nerve is cut, however, this increased output ceases (Egdahl, 1959). In several paraplegic patients undergoing various operative procedures it was shown that although the adrenal

[1] Kindly supplied by Dr. v. du Vigneand.

was normally reactive to ACTH there was no significant rise in peripheral corticosteroids with operative trauma (Hume, 1958a; Hume and Egdahl, 1959). In one patient (Hume *et al.*, 1962) samples of adrenal venous blood were obtained during abdominal laparotomy, and it was found that there was no significant adrenal response to this trauma. A single injection of ACTH was followed by an immediate and very marked outpouring of 17-OHCS. The absence of increased adrenal secretion in response to major operative trauma suggested that in man, as in the experimental animal, an intact nervous pathway from the injured area to the brain was essential for ACTH release. Adrenal reactivity is presumably maintained by cyclic secretion of ACTH from the pituitary, triggered off by the hypothalamus, and by an additional periodic secretion superimposed upon the diurnal mechanism by events that are capable of stimulating the pituitary through emotional surges and daily activities.

F. THE EFFECT OF HYPOTHALAMIC LESIONS

Hypothalamic lesions were made either as small electrolytic lesions or operative removals of large sections of the hypothalamus. The small lesion had no *acute* effect upon adrenal corticosteroid secretion in response to trauma (Hume and Egdahl, 1959). However, a month after the lesion was made the dog was found to have normal-sized adrenal glands which had lost most of their responsiveness to ACTH. The combination of the small but responsive adrenal seen with partial hypophysectomy and the normal-sized but unresponsive adrenal seen with hypothalamic lesions led us to postulate that the secretion of a "growth-type" ACTH was under pituitary autoregulation, whereas a "corticosteroidogenic" ACTH was released from the pituitary through hypothalamic control. One might postulate that the entire effect of the hypothalamic lesion had been simply to render the adrenal unresponsive to ACTH without altering the ability of the pituitary to secrete ACTH in response to trauma. This possibility was ruled out by measuring blood ACTH levels in animals with anterior hypothalamic lesions traumatized under ether anesthesia. Although we could always detect plasma ACTH levels in normal dogs under these circumstances, we were never able to detect ACTH in dogs with lesions in the anterior hypothalamus (Hume, 1958a; Hume and Egdahl, 1959). Under normal circumstances, lack of adrenal secretion would have led to an increased tendency to secrete ACTH by the pituitary, and the adrenalectomized animal secretes increased amounts of ACTH following trauma (Sydnor and Sayers, 1959). The results obtained when larger hypothalamic lesions were created are in marked contrast to the results obtained with the small localized lesions. One of these experiments is shown in Fig. 2. The adrenal cannula had been placed in the animal

the day before, and at the beginning of the experiment the hypothalamus was removed through an opening made just behind the optic chiasm. It may be seen that no significant response to operative trauma or to intense nerve stimulation occurred. A good response to ACTH was present. By contrast to this when the hypothalamic removal in-

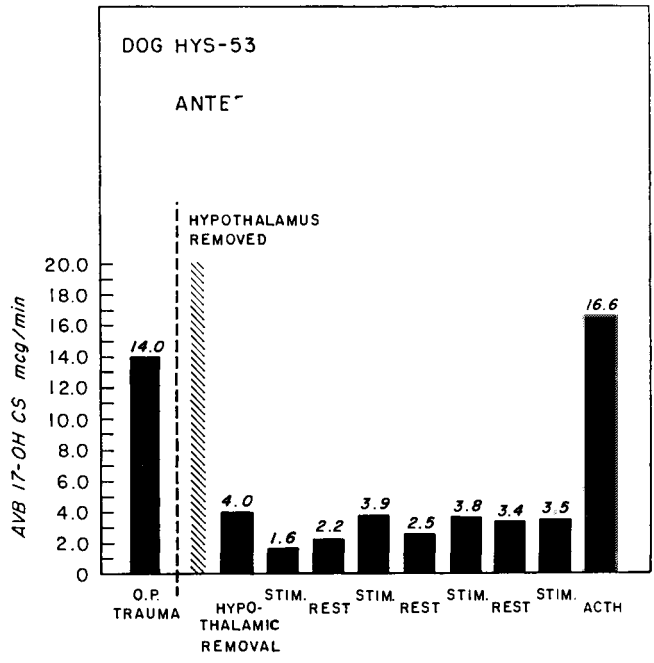

FIG. 2. The adrenal response to operative trauma is shown in the first vertical column. On the following day the anterior hypothalamus was acutely removed by suction just *behind* the optic chiasm. After this the secretion of the adrenal cortex remained at rather low rates in spite of repeated prolonged electrical nerve stimulation. An injection of ACTH produced a prompt adrenal response.

cluded an additional more anterior area, there was a marked tendency for high resting levels to occur. Only a slight further increase was noted with ACTH. (Fig. 3)

The amount of ACTH ordinarily released from the pituitary in response to trauma is usually greater than that needed to produce a maximal response from the adrenal cortex. One may therefore encounter a situation where there is a significantly reduced release of ACTH, but in which the adrenal response still appears to be nearly normal. ACTH secretion during trauma under Nembutal anesthesia is consistently less than that seen with ether, although good adrenal responses are present with both anesthetics. It is quite possible that animals after removal of

the brain or of large areas of the anterior hypothalamus have a somewhat decreased capacity for peak ACTH secretion. It is also possible that if they were capable of surviving for long periods of time the adrenal would ultimately become unresponsive. The increased levels of corticosteroid output seen after hypothalamic or brain removal prob-

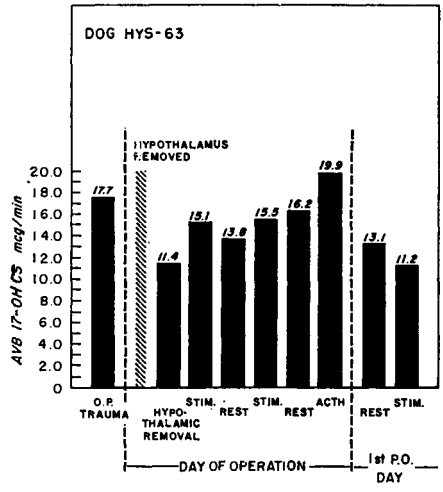

FIG. 3. The effect on adrenal secretion of acute removal of the anterior portion of the hypothalamus *through* the optic chiasm. Operative trauma during placement of the cannula the day before produced a good response. The levels of corticosteroid secretion remained high following hypothalamic removal and did not rise higher with nerve stimulation. ACTH produced only a slight increase in secretion. On the following day the values were again high but did not go higher with stimulation.

ably depend on ACTH secretion rather than on some unnatural adrenal stimulant like vasopressin, because the adrenal remains responsive for several days, which it presumably would not do without ACTH. It seems likely that the brain provides inhibitory as well as stimulatory control for the pituitary. (Egdahl, 1960; 1962; Mason, 1958; Taylor and Farrell, 1962). In the absence of pituitary inhibition the pituitary may respond to previously ineffective stimuli.

G. ADRENAL SECRETION IN MAN

Measurement of adrenal venous blood corticosteroid secretion in man revealed a mean value during operative trauma of 33.1 μg/min. This was not further increased with ACTH since it was already maximal. The resting secretion averaged 3.1 μg/min. A second injection of ACTH produced a mean value of 37.4 μg/min. The average aldosterone output was 0.072 μg/min during operative trauma, and this did not normally further increase with ACTH. In the resting period it was 0.024 μg/min,

and this promptly increased to 0.055 with an injection of ACTH (Hume *et al.*, 1962). The major corticosteroid secreted by the adrenal was compound, F, compound B being present in about one-third the amount of F. Small amounts of compounds E and S were present. The F : B ratio varied from 3.3 to 1 during operative trauma to 2.5 to 1 in the resting state. With the injection of ACTH in the resting state it promptly jumped to 4.7 to 1. A paraplegic patient undergoing abdominal laparotomy showed almost no detectable secretion of 17-OHCS but a good secretion of aldosterone. The secretion of 17-OHCS increased greatly with ACTH and there was also an increase in aldosterone output. In the resting state aldosterone output fell to low levels, but it again increased rapidly when ACTH was given. It would thus appear that in man operative trauma led to increased aldosterone output even in the absence of ACTH, and the output was increased still further when ACTH was given. The resting output of aldosterone was also increased by ACTH, but not to the levels seen during operative trauma. An infusion of potassium increased aldosterone output without altering 17-OHCS secretion.

H. ACTH SECRETION IN MAN

It is stated by Williams *et al.* (1961) that patients who have been adrenalectomized for Cushing's syndrome have definitely higher plasma ACTH concentrations on maintenance doses of cortisol than do patients with Addison's disease. This finding was not confirmed by Vance *et al.* (1962), who stated that patients with Cushing's syndrome did not have elevated levels preoperatively and that in certain cases the secretion of ACTH by pituitary tumors was easily suppressed by an intravenous infusion of cortisol. These authors found elevated levels of ACTH in the blood of Addisonian patients 24 hours after the last dose of steroid had been administered. The elevated levels increased when medication had been withheld for 48 hours and were almost completely suppressible with 5 mg of hydrocortisone intravenously. By contrast the adrenalectomized patients tended to have less elevation of the ACTH level than the Addisonian patients, and only one of six had a markedly elevated level. Extremely elevated levels were seen in the plasma of patients with ACTH-producing tumors of the pituitary. Suppression was attempted with an intravenous infusion of 20 mg of cortisol. This treatment failed to suppress the secretion in one patient but did suppress it in two others. This was four times the dose of cortisol that had been used in the Addisonian patients, however.

Our own findings are in accord with those of Williams *et al.* (1961) (see Fig. 4). By the technique used we failed to detect any plasma ACTH level in patients who had been adrenalectomized for carcinoma of the breast and maintained on 37.5 to 50 mg a day of cortisone. The cortisone was stopped for 20 hours prior to the sampling. In preoperative

Cushing's disease we likewise failed to detect any plasma ACTH. In three patients with total adrenalectomy for Cushing's disease, however, there were markedly elevated plasma levels, and only one of these patients (M. M.) appears to have an ACTH-secreting tumor of the pituitary. Furthermore, in two other patients with Cushing's disease who underwent subtotal adrenalectomy, no detectable level was found in

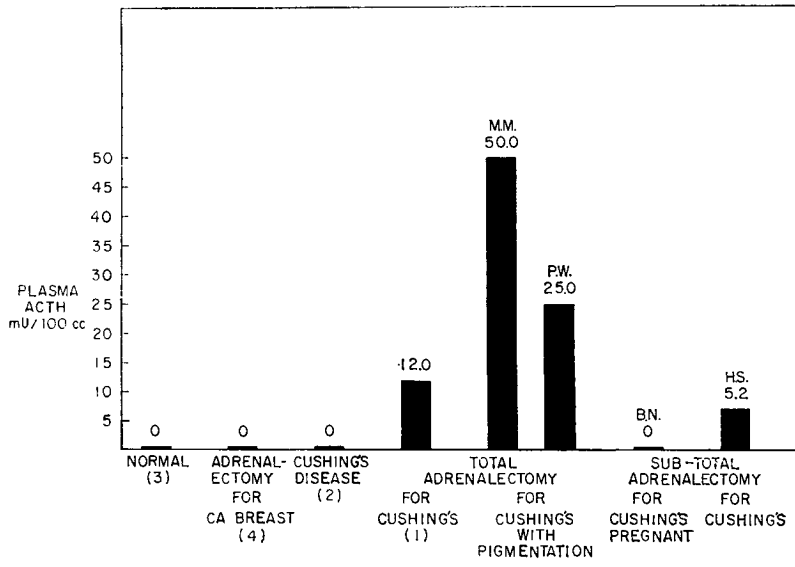

FIG. 4. No blood ACTH was detectable in two normal patients with the technique used. No ACTH was detected in four patients after adrenalectomy for carcinoma of the breast. These subjects had been receiving 37.5–50 mg of cortisone per day, and the cortisone had been stopped for 20 hours before the sample was taken. Two patients with Cushing's disease failed to show any measurable plasma ACTH. Following total adrenalectomy for Cushing's diseaee, easily detectable elevated levels of ACTH were present in the blood. This was most pronounced in the patient with an ACTH-secreting pituitary tumor (M. M.) and was also pronounced in patient P. W., who had considerable pigmentation. In one patient with subtotal adrenalectomy for Cushing's disease there was no detectable levels of ACTH, but this patient was pregnant. In another patient with a subtotal adrenalectomy for Cushing's disease, without symptoms or pigmentation, easily detectable amounts of ACTH were present in the blood. All these patients except those with subtotal adrenalectomies were maintained on the same dosage range of cortisone. ACTH secretion seemed to be suppressed with this dosage in adrenalectomized patients not having Cushing's disease, whereas patients with Cushing's disease usually failed to show suppression on this dose after adrenalectomy.

one, who was pregnant at the time, but a significantly elevated level was found in the other.

Further studies in one of the patients with Cushing's disease are shown in Fig. 5. Eight months after total adrenalectomy for Cushing's

disease, the patient began to show a little discoloration of the elbows and periorbital area. The plasma ACTH level at this time was 5 mU/100 ml. Two years postoperatively she showed definite generalized darkening of the skin. The plasma ACTH level had risen to 25–30 mU/100 ml. An infusion of 20 mg of cortisol given over a 4-hour period produced a fall in plasma ACTH levels from 30 mU/100 ml to 10 mU/100 ml.

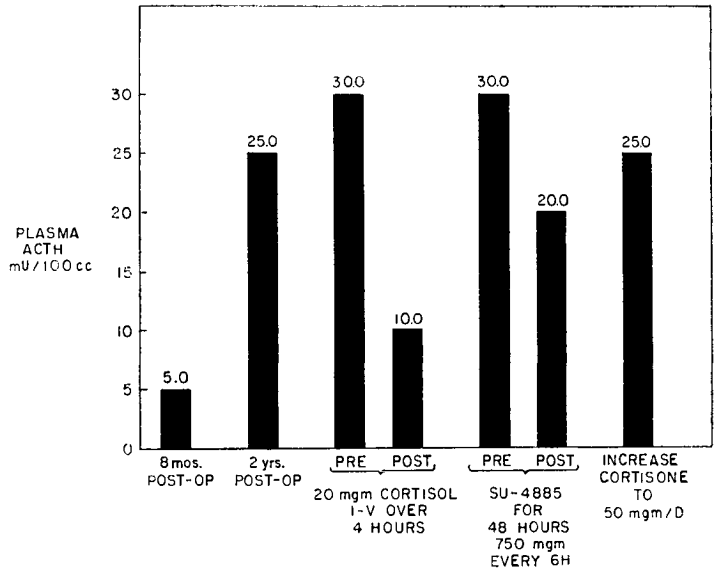

FIG. 5. Patient P. W. underwent a total adrenalectomy for Cushing's disease. About 8 months postoperatively she had a little pigmentation of her elbows and the area around her eyes, and began to run an elevated ACTH level. About two years post operatively she had developed more pigmentation and a considerably higher level of ACTH. Definite suppression of ACTH secretion was produced by 20 mg of cortisone given intravenously over a 4-hour period. This produced a blood level of 39 μg%, which is the same blood level noted in this patient before her adrenalectomy for Cushing's disease. Su-4885 failed to produce any increase in ACTH secretion. Increasing the daily dose of cortisone to 50 mg a day raised the blood cortisone level to 8.5 μg%, but failed to suppress ACTH secretion.

(This is a less significant depression than that seen by Vance *et al.* (1962) in a patient with Addison's disease on a much smaller dose.) The patient had been on 30 mg per day previously; the dose of cortisone was increased to 50 mg per day without detectable suppression of ACTH secretion. The plasma level during the infusion was 39 μg%, and on 50 mg per day it was 8.5 μg%. There is some suggestion that subtotal adrenalectomy effected a better suppression of ACTH secretion than total adrenalectomy with cortisone replacement (Hume and Rosen,

1962). The cases are too few to warrant drawing any conclusions, however.

Vance *et al.* gave SU-4885 to four normal patients, three patients with Cushing's disease, and two patients with hypoadrenalcorticism. There was no increase in blood ACTH concentration, in contrast to the observation of Ganong and Gold (1960) that plasma ACTH increased in the adrenalectomized dog after infusion of SU-4885. We administered this drug to two patients with Cushing's disease who had had total adrenalectomies and who had elevated levels of plasma ACTH. The results in one of the patients is shown in Fig. 5. No increase in plasma ACTH occurred. It would seem highly unlikely that this substance would produce an increase in plasma ACTH in the absence of the adrenals, since its action is to reduce circulating cortisol and thus produce an increased ACTH secretion by reducing feedback. It would be highly coincidental if such a substance also had a direct effect on the pituitary.

It appears likely that in patients with Cushing's syndrome and bilateral adrenal hyperplasia there is an increased secretion of ACTH which in turn produces an increased secretion of cortisol leading to a reduced ACTH output. On the basis of plasma cortisol levels it seems likely that the diurnal rhythm is lost in Cushing's disease, and the net effect of these opposing forces is to provide secretion of normal amounts of ACTH around the clock, rather than intermittently, leading to increased adrenal secretion and increased adrenal reactivity. When the adrenals are removed there is a marked fall in plasma cortisol levels; the hypersecretion of the pituitary is then unmasked and becomes easily detectable by increased levels of ACTH in the blood. Adrenalectomy in patients with normal pituitaries receiving maintenance amounts of cortisol is not accompanied by elevated plasma ACTH levels of the order seen in Cushing's disease after adrenalectomy. It is quite likely that the increased drive for ACTH secretion in Cushing's disease comes from the hypothalamus, both because the diurnal variation is lost and because of evidence suggesting that the corticosteroid feedback mechanism works at a brain, rather than pituitary, level (Egdahl, 1963).

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DISCUSSION

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Dr. Hume has made several points in his paper, but my discussion will center around one of these–the role of the brain in the pituitary-adrenal response to stress. He has illustrated beautifully that the afferent pathways of the spinal cord are needed for the stimulus of trauma to reach the brain. The afferent stimulus, under ordinary conditions, is probably a chemical agent that travels from the brain to the adenohypophysis to stimulate the release of ACTH. However, Dr. Hume has pointed out that a neurohormone, vasopressin, apparently promotes corticosteroidogenesis in the hypophysectomized animal. Perhaps this effect of vasopressin is still exerted via ACTH. There is now ample evidence for the presence of ACTH, or ACTH-like activity, in the neurohypophysis (Mialhe-Voloss, 1952; Mialhe-Voloss, 1954) and this ACTH is depleted by stimuli, especially by the so-called neurogenic stimuli, such as sound or emotion (Mialhe-Voloss, 1956; Rochefort *et al.*, 1959). More recently, Schally *et al.* (1962) have found ACTH activity in the hypothalamus of the dog. Perhaps a further search with sensitive techniques will reveal ACTH activity in other tissues. The extrahypophysieal ACTH may be detached from its stores by vasopressin, given to an hypophysectomized animal, and so stimulate the adrenal.

In another species, the rat, we have carried out literally hundreds of experiments, in which vasopressin and other neurohypophyseal peptides have been added to adrenal glands *in vitro*, without a sign of direct stimulation of steroid production.

In contrast, vasopressin is a powerful ACTH releaser when added to rat pituitary tissue *in vitro*. It is only 5–10 times less active, on a weight basis, than the most potent preparation of CRF so far reported (Schally *et al.*, 1958). Lysine vasopressin, arginine vasopressin, and arginine vasotocin are of the same order of activity. Oxytocin, on the other hand, has approximately one-hundredth the activity of the vasopressins (Saffran, 1962).

Several workers have presented evidence that the ACTH-releasing activity of posterior pituitary extracts can be accounted for entirely by the vasopressin content (McCann and Fruit, 1957; Rumsfeld and Porter, 1962). In our laboratory, experiments performed during the summer of 1961 with Dr. D. de Wied have shown that percolates of posterior pituitary powder (Saffran *et al.*, 1962) contain an excess of ACTH releasing activity over the pressor activity; in some fractions the excess is as great as 5 to 1. In these experiments the ACTH-releasing activity was estimated by both *in vitro* (Saffran, 1961) and *in vivo* (de Wied, 1961) methods, with identical results, with vasopressin used as a standard in both assays. The percolates are being used for the further purification of the corticotropin-releasing material.

One direct question to Dr. Hume: Did vasopressin stimulate corticosteroidogenesis in isolated adrenal tissue as well as in the hypophysectomized dog?

DR. HUME. I should like to thank Dr. Saffran for his interesting comments.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics:
Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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I do not believe that the effect of vasopressin on corticosteroid release from the adrenal in the hypophysectomized dog is due to the release of extrapituitary ACTH as suggested by Dr. Saffran. In the first place, in the hypophysectomized animals tested the neurohypophysis has been removed. Secondly, it has recently been shown that the threshold dose of vasopressin that is capable of producing corticosteroid release from the adrenal when it is injected into the adrenal artery does not produce corticosteroid release when it is injected into the carotid artery. This indicates that the effect of the vasopressin on the adrenal was not exerted by ACTH release but by a direct action upon the adrenal itself (R. H. Egdahl, unpublished material).

Although the *in vitro* experiment cited by Dr. Saffran seems to offer evidence against the direct effect of vasopressin on the rat adrenal, it may be that there are differences between the rat adrenal and the dog adrenal. Furthermore, *in vitro* experiments are not necessarily equivalent to *in vivo* experiments, particularly when a vasopressor substance is being tested. We have not, therefore, in answer to Dr. Saffran's question, tested vasopressin in isolated adrenal tissue.

There is perhaps one other variable that might account for the differential results between isolated adrenal tissue and the activity of the adrenal gland *in vivo* in the hypophysectomized dog. In the absence of ACTH itself the adrenal gland rather rapidly loses its ability to respond to a single acute exposure to ACTH. In the *in vivo* experiments mentioned, the vasopressin has been tested at a time when the adrenal remains still maximally responsive to ACTH. It is possible that vasopressin simply produces a release of corticosteroid from the adrenal rather than stimulating the process of corticosteroidogenesis itself. It the *in vitro* fragments had been depleted of corticosteroids this might account for the differential results.

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The Inhibiting Action of Adrenocortical Steroids on ACTH Secretion

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I. INTRODUCTION

The concept of a pituitary-adrenal homeostatic mechanism is based on the assumption that there are receptors which react to environmental concentration of adrenocortical hormones in such a way that ACTH secretion is increased during adrenal insufficiency and lowered during adrenal hyperfunction (Martini *et al.*, 1960a). Neither the nature, nor the location of these receptors is known. It should, however, be pointed out that they do not seem rigidly specific because they can be blocked also by synthetic analogs of adrenocortical hormones (Fochi *et al.*, 1960; Gavazzi *et al.*, 1962).

The evidence favoring the anterior pituitary as the receptor area of the feedback control of ACTH secretion is rather poor and includes the observation that the injection of hydrocortisone into the pituitary fossa causes adrenal atrophy (Rose and Nelson, 1956) and the fact that the addition of cortisone depresses oxygen consumption in pituitary tissue cultures (Roberts and Keller, 1955).

The possibility that receptors are located in the central nervous system (CNS) rather than in the pituitary was considered after it had been shown that lesions in the median eminence block the compensatory adrenal hypertrophy (Ganong and Hume, 1954; Bachrach and Kordon, 1958) and the adrenal ascorbic acid depletion induced by unilateral adrenalectomy (Fulford and McCann, 1955; Smelik, 1959) and the increase in blood and pituitary ACTH that follows bilateral adrenalectomy (McCann and Sydnor, 1954; Fortier and De Groot, 1959). The hypothesis of a nervous site of action of adrenocortical hormones was strenghtened by the evidence derived from experiments in animals with pituitary tissue grafted far from the sella turcica and lacking any direct connection with the hypothalamus. In these animals it was shown that unilateral adrenalectomy was not followed as usual, by compensatory hypertrophy and by adrenal ascorbic acid depletion in the remaining gland (Nikitovitch-Winer and Everett, 1958; Martini *et al.*, 1959).

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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There is also indirect evidence that high corticoid levels inhibit the release of ACTH by an action on the CNS: corticoids inhibit the release of vasopressin (Dingman and Despointes, 1960; Martini *et al.*, 1960a) and of other ACTH-releasing substances (Schapiro *et al.*, 1958) but do not prevent the release of ACTH which follows vasopressin administration (Martini *et al.*, 1960a; Leeman *et al.*, 1962) or the injection of hypothalamic extracts (Leeman *et al.*, 1962); radioactive hydrocortisone and cortisone are concentrated by the posterior pituitary, but not by the pars distalis (Courrier and Zizine, 1956; Schapiro and Katz, 1959); cortisone induces chromatolysis and vacuolation of hypothalamic cells (Castor *et al.*, 1951; Halász and Szentágothai, 1959; Szentágothai *et al.*, 1960); dexamethasone (a synthetic analog of hydrocortisone) fails to inhibit ACTH release after brain removal (Egdahl, personal communication). A most direct approach to the problem of the locus of corticoid action has been that of Endrőczi *et al.* (1961), who have shown that the injection of cortisone acetate into the tuberal region of the hypothalamus of cats and rats inhibits adrenal function.

In recent years attempts have been made in a number of laboratories to elucidate whether parts of the brain other than the hypothalamus could be involved in the homeostatic control of the pituitary–adrenal axis. Martini *et al.* (1960b) have shown that the adrenal ascorbic acid depletion which follows unilateral adrenalectomy is blocked in rats subjected to midbrain transection. This result was interpreted at the time as meaning that "the feed-back mechanism elicited by the fall in the blood levels of corticosteroids did not take place either at the hypophyseal or hypothalamic level," and a center sensitive to low blood levels of corticosteroids situated, under the sectioned area, was postulated.

In the present study the effect of midbrain transection on ACTH release was further investigated, using the determination of blood corticosterone levels as indicator of the activity of the pituitary–adrenal axis. After it was observed that midbrain transection is followed by high blood levels of corticosterone, it was thought that an attempt to modify these elevated titers by means of one of the synthetic analogs of hydrocortisone (dexamethasone or Δ^1-16-α-methyl-19-α-fluorohydrocortisone) which are known to induce pituitary adrenal suppression in normal animals (Fochi *et al.*, 1960; Gavazzi *et al.*, 1962) could help in elucidating the possible site of the blocking action of adrenocortical steroids.

II. MATERIALS AND METHODS

Female rats of the Sprague–Dawley strain, weighing 150–200 gm, were used throughout these experiments.

Midbrain transection was performed as follows: rats were anesthetized with ether, a sagittal incision was made in the scalp, and the mus-

cles were removed from the occipital bone; a hole was then made by means of a little drill, the hole was enlarged to about 0.8–1 cm in diameter, the dura was opened, and the supratentorial part of the midbrain was exposed; midbrain incision was made at the tentorial level with a small scalpel; the level and the plane of transection varied somewhat in different animals but the transection was always complete (Martini *et al.*, 1960b). After midbrain transection the animals were kept in single cages at 25°C and given penicillin subcutaneously once daily; they were fed by stomach tube with milk plus amino acids, glucose, salts, and vitamins.

Dexamethasone (Δ^1-16-α-methyl-9-α-fluorohydrocortisone) was administered intraperitoneally as a suspension in 0.3 ml of an aqueous system of 0.9% NaCl; 0.4% Tween 80; 0.5% carboxymethylcellulose; 0.9% benzyl alcohol. Animals were killed 4 hours after treatment.

Plasma-free corticosterone determinations have been carried out according to the method of Silber *et al.* (1958) using the fluorescence medium proposed by Zenker and Bernstein (1958) and a Farrand model B fluorometer; administration of dexamethasone has been previously shown not to interfere with corticosterone blood determinations (Fochi *et al.*, 1960; Gavazzi *et al.*, 1962).

In a few experiments it was felt necessary artificially to elevate corticosterone blood levels using a stress procedure. 0.5 ml/100 gm of body weight of a 10% solution of formalin was used for this purpose.

III. RESULTS

Table I shows the values of blood corticosterone obtained after midbrain transection. It is evident that high blood levels of corticosterone are observed starting at 1 hour after the operation. These very high titers last for many days. The value of corticosterone obtained in the

TABLE I

Blood Corticosterone in Female Rats after Midbrain Section

Hours after treatment	Number of rats	Blood corticosterone (μg/100 ml)
Controls	96	22 ± 3
1	4	79 ± 5
2	4	68 ± 6
4	4	111 ± 8
12	11	72 ± 6
24	35	73 ± 6
48	14	77 ± 5
72	18	61 ± 4
96	2	75 ± 15

control female animals not submitted to operation is similar to values reported by Kitay (1961).

The question arose whether these high levels represented an acute stress reaction or were due to impairment of some nervous mechanism controlling the secretion of ACTH. To elucidate this point it was felt useful to submit normal animals to a severe stressing procedure; formalin was chosen because its stimulating effect may be considered to be rather prolonged. Table II shows the results obtained in normal female animals given formalin. It is quite evident that formalin stimulates the adrenal cortex to release corticosterone at a much higher

TABLE II

Action of Formalin[a] on Blood Corticosterone in Normal Female Rats

Hours after treatment	Number of rats	Blood corticosterone (μg/100 ml)
Controls	96	22 ± 3
1	5	129 ± 8
2	4	106 ± 2
4	29	98 ± 6
8	14	71 ± 7
12	4	36 ± 3
16	5	26 ± 6
24	14	41 ± 7
36	5	26 ± 5
48	8	35 ± 7

[a]Dosage: 0.5 ml per 100 gm body weight, 10% solution.

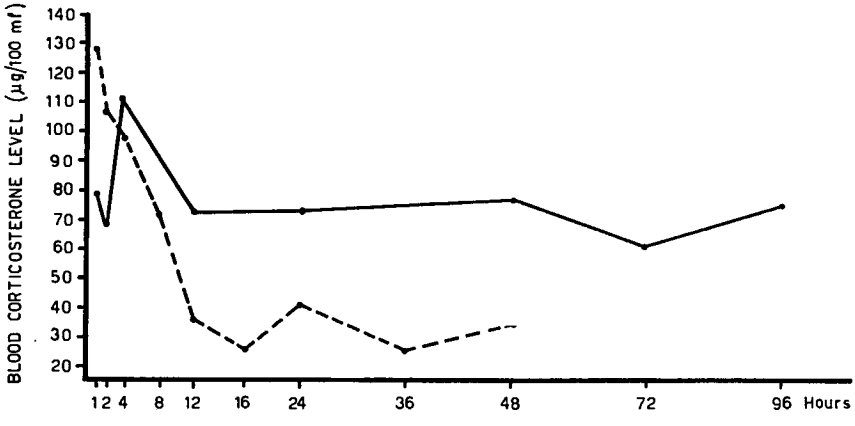

FIG. 1. Corticosterone levels after midbrain transection (solid line) and after injection of formalin (dashed line)

rate than midbrain transection. After a peak which is reached very quickly (see the results obtained 1 and 2 hours after injection) the corticosterone blood levels drop rapidly to near normal ranges. The behavior of corticosterone levels after midbrain transection and after formalin injection is then quite different (Fig. 1).

If it was possible that the high levels of corticosterone observed after midbrain sectioning were related to the impairment of a nervous mechanism controlling the pituitary–adrenal axis, it was relevant to study the action of a blocking steroid after midbrain transection. Fochi *et al.* (1960) and Gavazzi *et al.* (1962) have shown that dexamethasone administration induces in normal animals a drop of corticosterone blood levels which is proportional to the injected dose of the steroid; a maximum drop was obtained with 25 μg of dexamethasone per 100 gm body weight. When 25 μg of dexamethasone per 100 gm body weight was administered to female rats submitted to midbrain transection (Table III), it became apparent that only an insignificant drop

TABLE III

Action of Dexamethasone on Blood Corticosterone in Midbrain-Sectioned Female Rats at 72 hours*

Treatment	Number of rats	Blood corticosterone (μg/100 ml)	P
Controls	96	22 ± 3	
Controls plus dexamethasone (25 μg/100 gm)	57	3 ± 0.6	< 0.001
Midbrain sectioned	18	61 ± 4	
Midbrain sectioned plus dexamethasone (25 μg/100 gm)	13	48 ± 8	> 0.1
Midbrain sectioned plus dexamethasone (100 μg/100 gm)	5	24 ± 2	< 0.001

*Administered 4 hours before killing the animals.

of corticosterone blood levels could be obtained. Only a dose four times higher of the injected steroid proved effective in inducing a significant drop of corticosterone blood levels. Therefore it seemed evident that the hypophyseal-adrenal axis of these animals was much less sensitive to adrenal steroid than that of normal animals. That this lack of sensitivity was not an artifact related to the high departure levels of corticosterone in the operated animals is shown in Table IV. In formalin-treated animals which have blood titers of corticosterone even higher than those of midbrain-sectioned animals, 25 μg of dexamethasone per 100 gm body weight was highly effective in inducing a significant decrease of plasma corticosterone.

TABLE IV

Action of Formalin[a] and of Dexamethasone[b] on Blood Corticosterone in Normal Female Rats

Treatment	Number of rats	Blood corticosterone (μg/100 ml)	P
Controls	96	22 ± 3	
Dexamethasone (25 μg/100 gm)	57	3 ± 0.6	
Formalin (4 hr)	15	83 ± 4	
Formalin plus dexamethasone (25 μg/100 gm)	4	40 ± 15	< 0.01
Formalin (8 hr)	14	77 ± 5	
Formalin plus dexamethasone (25 μg/100 gm)	14	46 ± 5	< 0.001

[a]Dosage: 0.5 ml per 100 gm body weight, 10% solution.

[b]Administered 4 hours before killing the animals.

IV. DISCUSSION

The experiments, here presented, have shown that female rats submitted to midbrain transection have very high blood levels of corticosterone. This result agrees with those of Anderson *et al.* (1957) who reported that urinary steroid excretion in midbrain-sectioned dogs in the absence of any stimulation were rather high, and with results of Egdahl (1960), who reported elevated values of 17-OH-corticoids in the adrenal vein of dogs after midbrain transection. These results could be interpreted as meaning that midbrain transection disrupts an inhibiting mechanism that controls ACTH secretion. The possibility of an inhibiting mechanism controlling ACTH secretion was first suggested by Slusher and Critchlow (1959) and by Slusher and Hyde (1961), who observed an increased corticosterone recovery after lesions involving the posterior part of the midbrain and an area at the level of rostral pons and conversely a decrease in adrenal vein corticosteroid after stimulation of the same area. This hypothesis does not, however, explain the lack of inhibiting response observed after dexamethasone administration in the operated animals.

The lack of sensitivity to dexamethasone could be interpreted as meaning (1) that the receptors for the blocking action of the steroid are situated under the sectioned area, or (2) that the receptors are situated above the lesion but need the participation of a modulating system probably situated in mesencephalic structures. If the results we have presented had to be interpreted on the basis of the hypothesis that receptors sensitive to changing levels of adrenal steroids are located in some site under the sectioned area, we would expect to have maximal corticosterone blood levels in the sectioned animals. This was

not the case in the experiments here described, in which even greater stimulation of the adrenal cortex was obtained with formalin. Moreover, if this hypothesis were correct no inhibition at all of the hypophyseal–adrenal axis would be obtained even with large doses of corticoids. We think that the most obvious explanation for all the results here reported is that mesencephalic lesions disrupt a modulating mechanism which influences the hypothalamic nuclei controlling ACTH secretion and regulate the sensitivity of the "receptor" area for the feedback control of ACTH secretion probably located in the hypothalamus. In agreement with this interpretation Endrőczi *et al.* (1961) have observed that microinjections of cortisone into the hypothalamus decrease ACTH secretion in direct proportion to the dose of the steroid used, thus stressing the existence of a receptor center in this area; when cortisone was injected into mesencephalic structures, blood corticoid levels were also affected but to a lesser degree.

It is interesting to compare the results here reported with those obtained in previous experiments in which it was shown that unilateral adrenalectomy was not followed by any adrenal ascorbic acid depletion in the remaining adrenal after midbrain transection (Martini *et al.*, 1960a,b). It is now evident that liberation of ACTH, which normally takes place after unilateral adrenalectomy because of a drop of circulating levels of adrenal steroids, could not happen after midbrain lesion because of the very high blood levels of corticosterone after the operation. The high levels of corticosterone in the blood of the sectioned animals also explain why these animals showed a reduced response to exogenous ACTH; it is known that ACTH is much less effective when given concurrently with adrenal steroids (Martini *et al.*, 1960a).

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DISCUSSION

Neurosecretion and Control of the Pituitary Gland

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Professor Martini's communication further reemphasizes the importance of the mesencephalon and of the hypothalamus in the control of adenohypophyseal secretion. Thus far, however, the evidence for this control has been derived mostly from neurophysiological, electrophysiological, and transplantation studies (Hume, 1958; Sawyer, 1959; Harris, 1955; Everett, 1956; Nikitovitch-Winer and Everett, 1957; Bogdanove, 1957). As a result, specific anatomical structures which could act as mediators still remain to be directly implicated.

Because of their inability to demonstrate profuse nervous connections between the diencephalon and the anterior pituitary, Green and Harris in 1947 suggested that whatever influence the hypothalamus might have must be in the form of a neurohumor acting via the hypophyseal portal circulation. Since then, many investigators have presented evidence which seems to bear out this hypothesis.

With the proximity of the supraopticohypophyseal tract to the hypophyseal portal circulation, it was quite natural that the neurosecretory complex as originally described by Scharrer (see Scharrer & Scharrer, 1954) should be looked to for the controlling influence. This was particularly attractive when it was found that extracts of the neurohypophysis as well as synthetic posterior lobe hormone preparations appeared to influence the secretory processes of the adenohypophysis (Saffran *et al.*, 1955; Guillemin and Rosenberg, 1955; Martini *et al.*, 1959).

It is indeed quite possible that this neurosecretory mechanism is responsible for the control of all the secretory activity of the anterior lobe. On the other hand, it is also not beyond possibility that other sites of neurohumoral activity exist.

In 1960 and 1961, Leveque and Hofkin described two such possible sites; one associated with the ependymal lining located at the base and part of the sides of the supraoptic recess and the other also similarly associated with the ependyma of the infundibular recess in the rat. The substance(s) that we demonstrated could be stained with periodic acid-Schiff (PAS) reagent after the sections had been treated so as to remove the chrome alum-hematoxylin-phloxin stainable neurosecretory material.

Since the PAS-reactive sites we described were similar, if not identical, in location with those implicated by many investigators (Hume, 1958; Sawyer, 1959; Greer, 1957; Harris, 1955) in the mechanism of adenohypophyseal secretory control, we attempted to determine whether experimental procedures would result in quantitative changes in the amount of PAS substance. One hundred and eight male albino rats were used in the following experiments. One group of animals served as controls. The experi-

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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mental animals were either thyroidectomized, gonadectomized, adrenalectomized, or hypophysectomized; or were administered 25 mg propythiouracil or 15 mg cortisone acetate per kilogram body weight; or were cold stressed at 6^0 centigrade for hour; or were administered cortisone and then cold stressed; or were given 2.5% sodium chloride as a drinking solution.

Although our evaluation of the material is not completed, three patterns in the amount of PAS-positive material have so far emerged with respect to the infundibular recess: (1) the gonadectomized, adrenalectomized, hypophysectomized, and cortisone-injected animals all have an amount of the material equivalent to that found in control animals; (2) the cold-stressed animals the animals injected with cortisone and then cold stressed, and those given sodium chloride have a much greater amount of reactive material than the controls; (3) the propythiouracil-injected animals have less reactive substance than the controls.

From our preliminary observations, we have concluded that the PAS-positive material in the infundibular recess is, at least in part, involved in thyrotropic hormone secretion. However, we cannot as yet completely ignore the fact that some of our more recent results seem to imply a further involvement of this area in adrenocorticotropic hormone release.

Moreover, Flerkó (1963) reports that gonadotropes remain normal in pituitaries transplanted in the "hypophysiotropic" area of the hypothalamus. Examination of his preparations reveals that his implantation sites correspond very well with the PAS-reactive areas we have described.

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Adrenal Cortical Function in Dogs with Central Nervous System Ablations

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It has previously been established by Hume (1949), De Groot and Harris (1950) and confirmed by many others that lesions in the hypothalamus will markedly diminish or prevent ACTH secretion in response to various stimuli. These experiments do not indicate what role, if any, higher central system structures play in the control of ACTH secretion. The rather startling finding (Egdahl, 1960) that dogs whose central nervous system has been removed have elevated "resting" corticosteroid secretion makes it necessary to consider the possibility that there are in the brain both excitatory and inhibitory areas controlling ACTH secretion. This paper describes experiments designed to determine the role of various central nervous system areas in the control of pituitary–adrenal activity.

I. METHODOLOGY

A. GENERAL

Although the protocols vary, there are many technical features common to all experiments that are described in this paper. Adult mongrel dogs ranging in weight from 8 to 20 kg were used; operations were carried out under intravenous Nembutal anesthesia, using sterile technique in most cases. Cannulation of the lumboadrenal vein was carried out according to the technique of Hume and Nelson (1955) and adrenal venous 17-hydroxycorticosteroids were measured according to the method of Nelson and Samuels (1952). The various central nervous system lesions which are discussed below were carried out using standard neurosurgical techniques. Suction removal of the desired areas was readily performed, and hemostasis was obtained by the use of oxycel and silver clips.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics:
Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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B. ISOLATED PITUITARY PREPARATION

Pituitaries were isolated in a series of dogs by suction removal of the entire brain down to the diaphragm of the sella and the level of the anterior pons. These findings have been previously reported in detail (Egdahl, 1960). The animals exhibit a rapid depression of corticosteroid secretion to basal levels following brain removal, with grad-

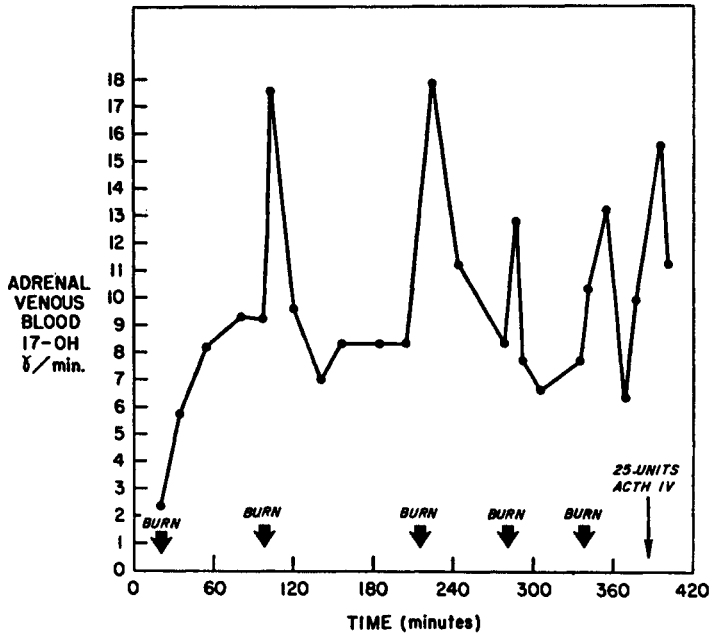

FIG. 1. Dog with isolated pituitary. It can be seen that corticosteroid secretion increased following burning on each of five occasions.

ual drift upward in the next 2–6 hours to elevated secretion levels. At this time the animals respond to the stimulus of inferior vena caval constriction with a further increase of steroid output (Egdahl, 1961). When the extremities of these animals are burned, a significant increase in adrenal cortical secretion occurs in many of them. Figure 1 illustrates the pattern of adrenal cortical secretion following repeated burning. It can be seen that the animal responded to burning each time with an increase in secretion. Elevated corticosteroid secretions are observed as long as dogs with isolated pituitaries survive (5 days is the longest thus far), although this hypersecretory state is usually not maximal as shown by a further increase following the administration of exogenous ACTH.

C. BILATERAL DECORTICATION

Dogs were bilaterally decorticated by suction removal of both cerebral cortices. On the day following operation, such animals revealed an adrenal cortical hypersecretion. The administration of Nembutal anesthesia resulted in the rapid suppression of adrenal cortical output to basal levels. The animal continued to remain responsive to peripheral

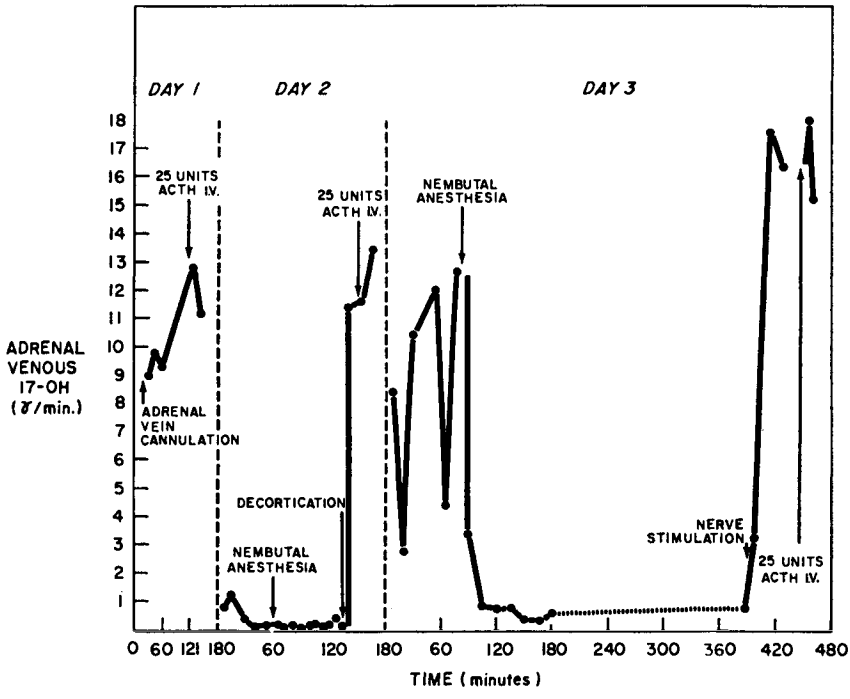

FIG. 2. Dog with bilateral decortication. Basal corticosteroid output prior to decortication, but adrenal hypersecretion following this procedure. Secretory rate depressed by Nembutal anesthesia, but normal pituitary-adrenal response occurred in response to nerve stimulation.

trauma, however, as shown by an immediate corticosteroid response following burning or nerve-stimulation. In Fig. 2 may be seen the pattern of adrenal cortical response in such an animal. It is significant that this dog had low corticosteroid levels on the day following adrenal vein cannulation prior to anesthesia, although the levels were high on the day following decortication. Dogs with craniotomy, but without brain operations have, "basal" adrenal cortical secretion. The findings are in marked contrast to the studies in dogs with isolated pituitaries in which it has been found that the administration of Nembutal anesthesia does not result in decline of adrenal cortical hypersecretion, but often leads to a significant increase.

D. MIDBRAIN TRANSECTION AND HYPOTHALAMIC ISLANDS

A series of dogs had been prepared in which complete midbrain transection at the level of the anterior pons, or midbrain transection combined with removal of cerebral cortex and thalamus in the rostral segment of brain (hypothalamic island) was carried out. Both groups of animals behaved similarly to dogs with isolated pituitaries in that

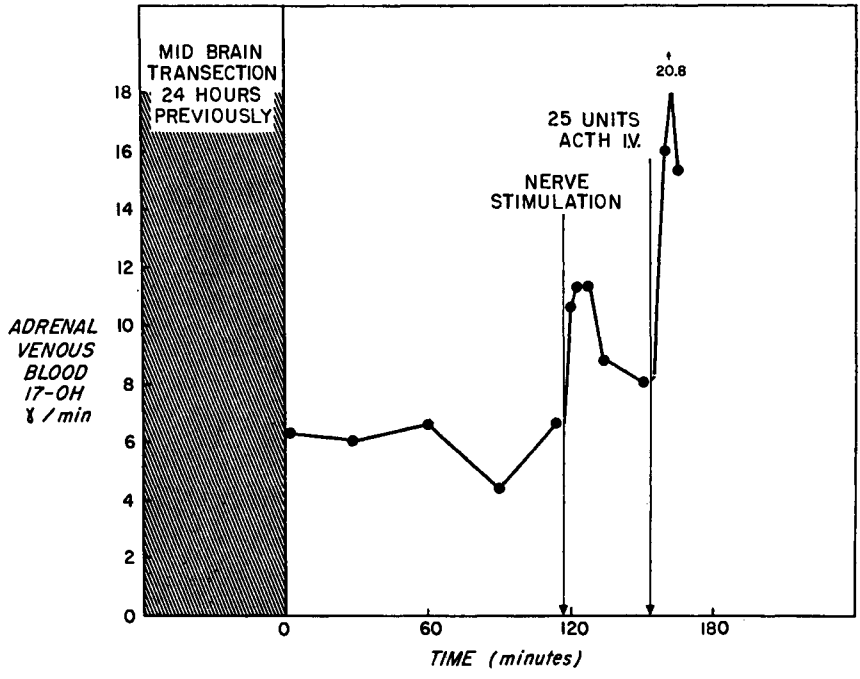

FIG. 3. Dog with midbrain transection. Shows elevated adrenal secretion 24 hours after midbrain transection, with further significant increase following nerve stimulation.

they exhibited adrenal cortical hypersecretion in the postoperative period. Many animals exhibited responses to nerve stimulation or burns. A curious paradox in these groups of animals is the fact that approximately half of them failed to respond to burns or nerve stimulation with a further increase in corticosteroid secretion. All animals of this series had complete transection as revealed by necropsy findings, and we are currently unable to explain the variations in experimental results in these animals.

E. ISOLATED ANTERIOR PITUITARY

Studies of a series of dogs with brain removal down to the pituitary and the level of the anterior pons have been carried out. In addition, the posterior pituitary and pituitary stalk were removed in these animals after excision of the diaphragm of the sella. Such animals have completely adequate vascularization of the residual anterior pituitary

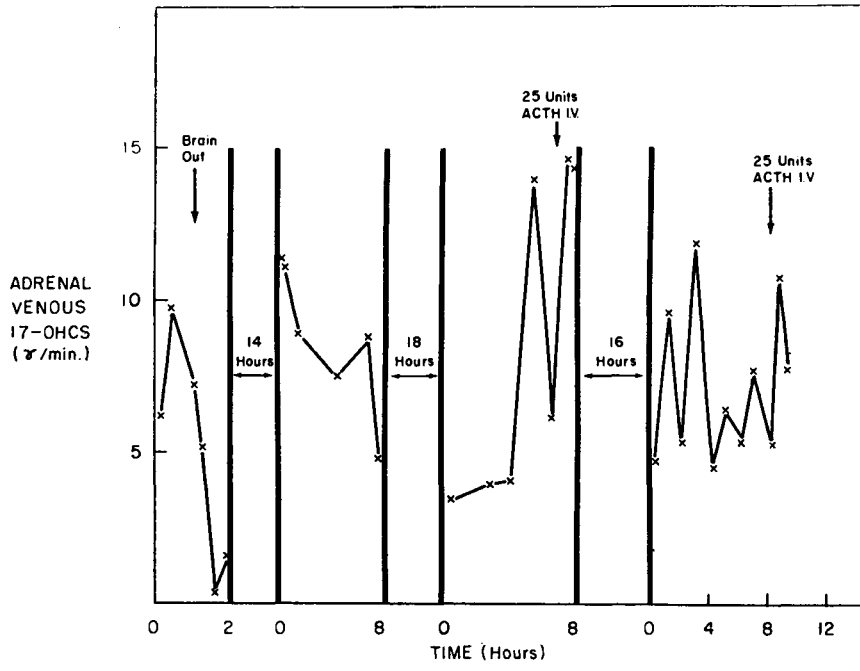

FIG. 4. Dog with isolated anterior pituitary and brain removal down to the pons. This animal revealed adrenal cortical hypersecretion for 2 days following brain removal. Histology revealed normal anterior pituitary anatomy with no evidence of ischemia.

as shown by India ink injection studies and gross visualization of the gland itself. There is no difference in adrenal secretory behavior of this group of animals and those subjected to brain removal in which the posterior pituitary is left *in situ*. Figure 4 shows a representative experiment in which hypersecretion of adrenal cortex was present during the 2-day survival period of the dog after brain removal and removal of the posterior pituitary.

F. TOTAL BRAIN AND SPINAL CORD REMOVAL: NEPHRECTOMY AND EVISCERATION

A series of experiments was carried out in which the total brain was removed down to the spinal cord, leaving an isolated pituitary. These animals revealed adrenal cortical hypersecretion, and in two instances a response occurred following nerve stimulation. In addition, one ani-

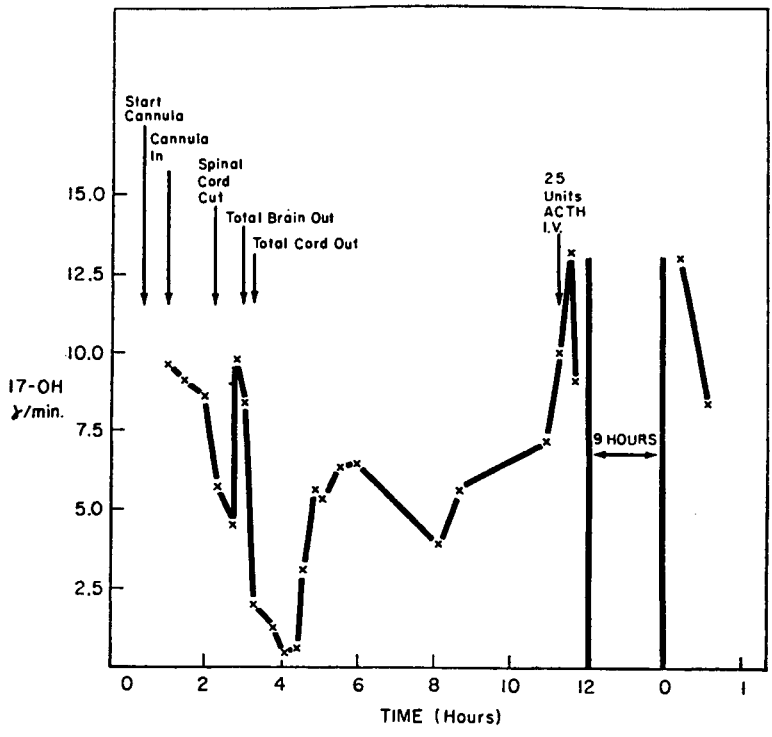

FIG. 5. Dog subjected to total brain removal and total spinal cord removal, in addition. Note low period of steroid secretion immediately following brain removal and gradual return to hypersecretory state.

mal subjected to removal of the total brain and also of the total spinal cord had an adrenal cortical secretory pattern similar to that described for dogs with the less complete ablations. This total central nervous system ablation experiment is illustrated in Fig. 5. It can be seen that this animal demonstrated all the characteristic features of dogs with isolated pituitaries, including the postoperative depression of corticosteroid output, gradual elevation to high levels, and continued hypersecretion into the day following surgery. Unfortunately, it was impossible to maintain this animal for more than 22 hours, but hypersecretion persisted during this period of time.

An additional series of experiments were carried out in which either bilateral nephrectomy or complete abdominal evisceration was performed in dogs with isolated pituitaries prepared as described under Section 2, above. In these animals adrenal cortical hypersecretion was observed as described above and responses to stimuli occurred in some animals.

II. DISCUSSION

The findings of this study indicate that an intact hypothalamus is not necessary for ACTH secretion. In an earlier paper (Hume, 1949) it was speculated that the hypersecretion observed in dogs with isolated pituitaries was due to the production of a neurohumor in the hindbrain. However, the total central nervous system ablation studies rule out this as the primary explanation. It has been suggested by others (Ganong, 1961) that pituitary ischemia or leak of an ACTH-releasing substance from the posterior pituitary to the anterior pituitary may explain the hypersecretion. The pituitaries are well vascularized even when the posterior gland has been removed, and removal of the posterior pituitary does not prevent the hypersecretion. Accordingly, it is felt that these are unlikely explanations for the observed findings.

Recently, confirmatory evidence concerning the ability of animals with isolated pituitaries to secrete ACTH has become available. Wise and co-workers (Wise *et al.*, 1962) have demonstrated that dogs with the brain removed down to the medulla, leaving an isolated pituitary, have elevated corticosteroid secretion when adrenal vein cannulations are carried out 4 hours after brain removal. They interpreted these experiments as indicating an adrenal cortical response to trauma in these animals, but it is apparent from our studies that an equally attractive explanation would be based on the gradual elevation of corticosteroid secretion following the "low period" which is observed postoperatively in dogs with isolated pituitaries.

There are two explanations for the behavior of the preparation described above which appear to deserve more attention than other possibilities. First, the high central nervous system may produce an inhibitory substance which counteracts the effect of ACTH-releasing substances. According to this concept, brain removal would take away the source of this inhibitory hormone and allow the pituitary to secrete ACTH in amounts not released in intact animals without specific stimuli. The findings in dogs with midbrain transection or hypothalamic islands could be explained on the basis of interruption of ascending impulses from the spinal cord that act to cause release of inhibitory substances from the higher brain. Therefore, midbrain transection would de-afferent the inhibitory center. It is difficult to explain the increased secretion from the adrenal cortex following burns and nerve stimulation in these animals without postulating the production of a

neurohumor, produced in the stimulated area or at nerve endings, which has ACTH-releasing abilities. Although previous experiments from our laboratories have demonstrated that burns in denervated extremities do not result in ACTH secretion, it is possible that dogs after brain removal have pituitaries that are more sensitive to substances which can cause ACTH secretion than is the intact animal. One other idea makes the concept of an inhibitory substance or inhibitory areas unnecessary. According to this hypothesis, brain removal results in the production of a substance that causes ACTH secretion. Further amounts of this substance are released with peripheral trauma.

There is at present little evidence on which to base further speculation concerning these mechanisms. Experiments are in progress in our laboratories designed to demonstrate an inhibitory substance in peripheral blood or central nervous system tissue, if present. In addition, we are extracting and analyzing for ACTH-releasing ability extracts of peripheral muscles and heart, as well as peripheral blood in normal dogs and in dogs with isolated pituitaries. A fundamental question which remains unanswered is that relating to pituitary ACTH secretion. Inasmuch as it is difficult to get dogs to survive more than 5 days after brain removals, we have no assurance that ACTH is synthesized in the dogs after brain removal. There is good evidence for ACTH secretion, but until more chronic survival of these animals is obtained the possibility of a gradual exhaustion of ACTH stores from an unstable pituitary must be considered.

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DISCUSSION

Hypothalamus and Release of Ovulating Hormone

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Dr. Egdahl's work on isolated pituitaries in dogs has brought forth very interesting results concerning the control of ACTH secretion. As a consequence of this work we must change our dogmatic views that the adenohypophysis cannot secrete normal or elevated amounts of ACTH when it is not stimulated by the diencephalon. Dr. Egdahl's experiments which have been recently confirmed (Wise *et al.*, 1962) show conclusively that following decerebration in dogs, there is an increase of corticosteroid outputs. Unfortunately, a satisfactory explanation of the results has not as yet been reached.

According to the present views of Dr. Egdahl, the most rational explanation of his results is that the rise of blood cortical levels observed following decerebration is due to a release of a cortical inhibition of ACTH secretion. However, one wonders whether it is the ablation of the brain itself or the numerous changes in the organism induced as the result of this removal that cause the increase in ACTH secretion. Dr. Egdahl suggested briefly the latter possibility when he hypothesized that brain removal results in the production of a substance which causes ACTH secretion. If this hypothesis is correct, then Dr. Egdahl's experiments indicate that under very abnormal conditions the adenohypophysis can secrete ACTH even though it is not in contact with the diencephalon. Whether this finding plays an important role in the control of ACTH secretion under physiological conditions remains to be demonstrated.

As we have seen, Dr. Egdahl's work suggests that the integrity of the central nervous system is not absolutely necessary for the secretory function of the adenohypophysis. My own investigations have been oriented in the opposite direction; that is, they have been mainly concerned with experiments designed to establish the prime importance of the central nervous system in the control of hypophyseal secretion and in particular of gonadotropin hormone secretion.

In recent years, experiments performed on rats (Nikitovitch-Winer, 1962; McCann, 1962) and rabbits (Campbell *et al.*, 1961) indicate the presence of a specific substance in hypotahlamic tissue capable of releasing ovulating hormone from the adenohypophysis. In my own work the animals used were "proestrous blocked" rats (twice injected with 30 mg/kg of pentobarbital) in which bovine and rat median eminence extracts were infused directly into the anterior lobe of the pituitary and their effect on ovulation studied. Out of 34 animals which received an equivalent of 0.9–2.4 mg wet weight of median eminence tissue, 24 ovulated following the infusion. Cortical and hypothalamic extracts, arginine-vasopressin, epinephrine, histamine, and extraction solvent were infused in 28 animals. Only one of these animals ovulated after the

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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infusion. When the median eminence extracts were given intravenously, the equivalent of tissue wet weight had to be increased to 11 mg for unboiled extracts and to 48 mg for boiled extracts in order to induce ovulation.

This work performed in 1959–1960 (Nikitovitch-Winer, 1962) was recently fully repeated. In the new series of experiments two changes were made: (1) Atropine (350 or 700 mg/kg) and pentobarbital (30 mg/kg) were used in combination to block ovulation instead of the two injections of pentobarbital, since the latter treatment caused a rather high mortality. (2) The bovine median eminence and cortical extracts were prepared with 0.2 *N* acetic acid instead of 0.5% acetic acid as was done in the previous work, since the extraction with the higher concentration of acid was found to be more effective. After the acid extraction of the brain tissue, the extract was centrifuged at 15,000 rpm; the supernatant was boiled for 15 minutes and then recentrifuged. At this point, in the case of an aliquot of one extract, 24-hour dialysis against water was performed.

Five preparations of median eminence extracts were tested for their ability to induce ovulation in 19 rats. Sixteen animals ovulated following the intrapituitary infusion of the extract. In contrast, none of the 8 animals which received the dialyzed extract ovulated. The control experiments comprised 32 animals which received the active extracts, but in which the hypophyseal cannula was displaced (i.e., it was outside the pituitary), 17 animals which were injected only with atropine and Nembutal, 10 animals which received the extraction solvent, and 5 which received extracts of cerebral cortex. None of these animals ovulated after the treatment. In addition, the effect of synthetic oxytocin (Synthocinon, kindly supplied by Sandoz & Co.) was tested in 12 animals. Doses varying from 25 mU to 100 mU were used. Only 1 animal which received 100 mU ovulated after the treatment.

Recently, experiments were begun in an attempt to determine whether the ACTH-releasing factor and the ovulating hormone-releasing factor are one and the same substance. This work is conducted in collaboration with Dr. S. E. Leeman from Brandeis University. Two different extracts prepared and tested for ACTH-releasing activity by Dr. Leeman were used in this preliminary study. The extracts were prepared with 0.1 *N* HCl and were not subsequently boiled.

1. *Calf hypothalamic extract* (20 mg wet weight per milliliter). Of this extract, 0.2 ml per 100 gm body weight produced a fall in ascorbic acid of approximately 100 mg% in pentobarbital or pentobarbital-morphine anesthetized rats, whereas they had no effect in hypophysectomized animals. This extract was ineffective in inducing ovulation in "proestrous blocked" rats when administered intravenously at dosages of 1–2 ml per 100 gm body weight. This volume corresponds to 50–100 mg equivalent wet weight of hypothalamic tissue per animal.

2. *Sheep hypothalamic extract* (12 mg wet weight per milliliter). Per 100 gm body weight, 1 ml of extract produced an increment in adrenal corticosteroid plasma levels of 25.4 ± 2.3 μg% in pentobarbital anesthetized animals, 27.7 ± 4.3 μg% in pentobarbital-morphine anesthetized rats, and 0.0 ± 0.7 μg% in hypophysectomized animals. This extract was also ineffective in inducing ovulation when administered intravenously in a dose of 2 ml per 100 gm body weight in 4 animals. This volume corresponds to 60 mg equivalent wet weight of hypothalamic tissue per animal.

The above findings show that the hypothalamic extracts with proved ACTH-releasing activity, did not have an effect on the ovulating hormone release even though they were administered in amounts much greater than those necessary to induce ACTH release.

The presented results confirm earlier findings on the existence of a hypothalamic substance capable of releasing the ovulating hormone from the rat adenohypophysis. The fact that the median eminence extracts retain activity after boiling and that they lose their activity on dialysis suggests that the substance responsible for ovulating hormone release is probably a polypeptide. The results also suggest that this substance is not identical with the ACTH-releasing factor.

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Neural Aspects of the Feedback Action of Ovarian Steroids

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An analysis of the effects of ovarian steroids (OS) on the secretion of gonadotropins reveals two successive phases: (1) a "positive" feedback mechanism appears to be involved in cyclic ovarian function, as evidenced by the triggering role of OS on luteinizing hormone (LH) release; (2) on the other hand, the fact that prolonged treatment with estrogen inhibits secretion of follicle-stimulating hormone (FSH) as well as LH points to the "negative" or "degenerative" feedback effects of OS on gonadotropin secretion.

Until recently the opinion was generally held that the peripheral hormonal milieu influences production and release of gonadotropic hormones exclusively by direct action on the adenohypophysis. It was suggested some years ago, however, by Hohlweg and Junkmann (1932) that the gonadal hormones might influence gonadotropic functions through a hypothetical "sexual center" located somewhere in the brain. In the light of experimental data accumulated in the last decade, it now appears highly probable that this feedback action is mediated indirectly but by multiple neural elements located in different parts of the hypothalamus and related regions of the brain rather than by a distinct sex center.

I. Localization of the Neural Mechanisms Controlling Gonadotropin Secretion

A tentative schema of the mechanism of hypothalamic control of FSH and LH secretion suggested by recent experimental results is shown in Fig. 1.

A. Median Eminence and Basal Tuberal Region

Since electrical stimulation of the tuber cinereum elicits ovulation and, on the other hand, electrolytic destruction of this region brings about atrophy of the gonads and different parts of the genital tract, the conclusion is obvious that this region plays a decisive role in gon-

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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adotropin discharge. Experiments of Harris and Jacobsohn (1952) and Nikitovitch-Winer and Everett (1957, 1958) leave little room for doubt that lack or serious impairment of gonadotropin secretion is caused not simply by defective blood supply of the anterior pituitary, but by dis-

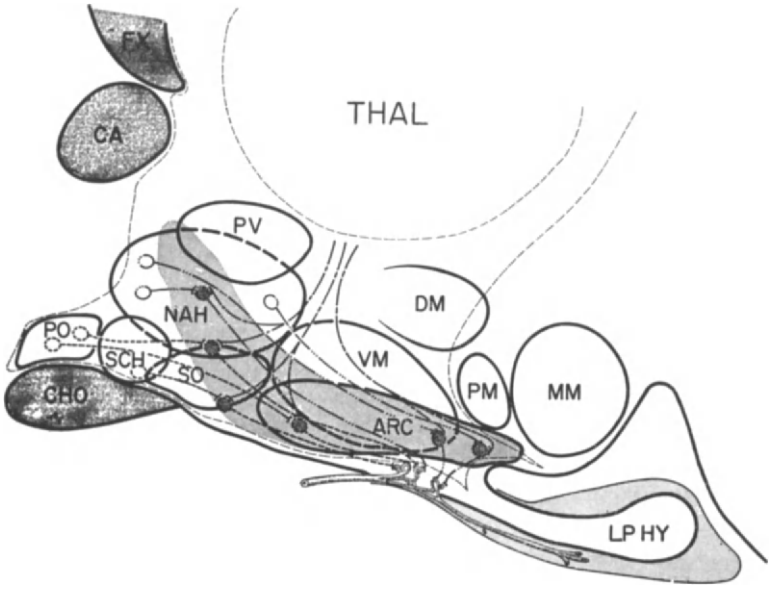

FIG. 1. Schematic representation of the hypothalamic control mechanisms for gonadotropin secretion. The pars distalis and pars tuberalis of the pituitary gland are represented by the black-dotted area. Red arrows show the direction of the blood flow in the hypothalamohypophyseal portal system, the special capillary loops of which penetrate the median eminence. Neural elements of the FSH control mechanism are indicated in green, those of the LH-mechanism in blue. The neurons belonging to the "tropic-neurosecretory" system, situated in the yellow "hypophyseotropic" area, are indicated by solid soma of appropriate color. Their nerve endings terminate on the capillary loops of the portal system. Elements of the "release regulating" system are represented by the neural units with open soma located in the preoptic (LH) and anterior hypothalamic (FSH) areas. Extrahypothalamic influences conveyed to the neurosecretory system are symbolized by the red axons. Abbreviations used: *ARC*, arcuate nucleus; *CA*, anterior commissure; *CHO*, optic chiasm; *DM*, dorsomedial nucleus; *FX*, fornix; *LPHY* lobus posterior of the hypophysis; *MM*, mammillary complex; *NAH*, anterior hypothalamic nucleus; *PO*, preoptic area; *PM*, premammillary nuclei; *PV*, paraventricular nucleus; *SCH* suprachiasmatic nucleus, *SO*, supraoptic nucleus; *THAL*, thalamus; *VM*, ventromedial nucleus.

ruption of a specific humoral influence, exerted by the hypothalamus on the adenohypophysis. This is demonstrated most clearly by recent experiments of Halász (1962) carried out in our laboratory.

Normal periodic acid-Schiff reactive basophilic cells appear exclu-

sively in pituitary grafts situated in a rather narrow "hypophyseotropic" area of the hypothalamus extending from the paraventricular nuclei downward to the optic chiasm and posteriorly as far as the mammi lary nuclei. Connection of the graft with the capillary loop system of the median eminence is, however, not essential for producing this effect. Hypophysectomized rats with pituitary grafts in the "hypophyseotropic" area resumed normal vaginal cycles and formation of corpora lutea within a few months; those with pituitary grafts in other parts of the hypothalamus or of the brain failed to do so consistently.

Preservation of the basophiles in intrahypothalamic pituitary grafts and maintenance of the normal weight and histological structure of the testes in hypophysectomized rats with intrahypothalamic pituitary implants has been found also by Knigge (1962).

This hypothalamic tropic influence appears to be of a true neurosecretory nature, as first suggested by Benoit and Assenmacher (1953). Data are available suggesting that the neurosecretory system of the so-called "hypophyseotropic" area stimulates the production of FSH and LH and in addition maintains the release of these hormones at a *basal level*. This system is, however, unable to enhance or inhibit the release of these hormones by itself.

B. PREOPTIC AND ANTERIOR PART OF THE HYPOTHALAMUS

"Release regulating" mechanisms for FSH and LH might be localized in the anterior hypothalamic and preoptic area, respectively. Lesions of the areas mentioned may disturb the release of FSH and/or LH, depending on the site of the injury, without, or only with slight, impairment of hormone production. If the "hypophyseotropic" area is involved, the impairment of the production of gonadotropins may perhaps be related to the extent of the destruction of this area, i.e., to the loss of cells synthetizing the neurosecretory tropic agent(s). Experimental findings suggesting the hypothesis that the "hypophyseotropic neurosecretory system"—mentioned in Section I, A—might be controlled by the so-called "release regulating mechanisms" of the preoptic and anterior hypothalamus are discussed in detail elsewhere (Flerkó, 1962, 1963).

It would be very difficult to imagine, of course, that all the neural elements of the hypothalamic areas mentioned above are involved exclusively in the control of gonadotropin secretion. In the integrative apparatus of the hypothalamus the same elements might be involved as links in completely different functional chains. The convenient simplification of the so-called gonadotropic, or thyreotropic center, etc., can evidently have no real anatomical meaning. It appears to be better to speak of neural "mechanisms" which may have certain key points in well-defined anatomical regions, which do not necessarily corre-

spond, however, to the individual hypothalamic nuclei and which are by no means independent, but interlaced and connected with each other.

II. Functional Pattern of the Sex-Steroid Feedback

It seems to be fairly probable that secretion of gonadotropins might be influenced by the feedback actions of ovarian steroids at three different levels.

A. Anterior Pituitary Gland

Continuous action of large amounts of estrogen (25–50 μg/day) does inhibit gonadotropin secretion, apparently by direct action on the adenohypophysis, and this direct inhibiting action of massive estrogen doses cannot be eliminated by hypothalamic lesions (Flerkó, 1962).

Furthermore, it has been shown by Flerkó and Bárdos (1960) that constant action of estrogen following lesions of the anterior hypothalamus—thus within the physiological range—brought about a significant increase in pituitary weight ($P < 0.01$) as well as cytological changes similar to those observed after prolonged estradiol treatment (2 μg/day). In intact estrogen-treated animals the ovaries were atrophied and contained only small atretic follicles indicating the absence of FSH release. On the other hand, ovaries of rats with anterior hypothalamic lesions contained normally developing follicles indicative of FSH output, although these lesioned rats were also under constant estrogen action as revealed by the constant vaginal cornification and by the presence of cystically enlarged endometrial glands.

These experimental data clearly show that although the morpohlogical response of the hypophyses to estrogen were the same, both in intact and lesioned rats, their gonadotropic activities were markedly different. In order to explain this discrepancy, we have to assume that estrogen acts upon the hypophysis in two ways, i.e., in a direct and indirect manner. The hypophyseal tissue reactions (enlargement of the pituitary gland and cytological changes) might be the result of a direct action, whereas the functional reaction (in this case the inhibition of FSH secretion) could be due to an indirect effect of estrogen mediated by the anterior hypothalamus. These observations also clearly show that conclusions about hypophyseal gonadotropic activity cannot be drawn from pituitary cytological data alone without taking into account the state of the gonads and accessory reproductive organs.

B. PREOPTIC AND ANTERIOR HYPOTHALAMIC RELEASE REGULATING MECHANISMS

1. *Positive Feedback*

It was demonstrated by Sawyer *et al.* (1949) and by Everett and Sawyer (1949) that the effects of estrogen and progesterone, respectively, in advancing spontaneous LH release could be eliminated by neural blocking agents. This clearly shows the neural character of the "positive" feedback action of OS on gonadotropin secretion. Concerning progesterone Ralph and Fraps (1960) and Barraclough and Yrarrazaval (1961) suggested that the anterior and ventral hypothalamus in the hen, and the medial preoptic area in the rat, respectively, are specific sites at which progesterone facilitates ovulation.

2. *Negative Feedback*

There is, furthermore, fairly good evidence that the "negative" feedback control of gonadotropic function exerted by *physiological* amounts of OS is exerted on neural structures located mainly in the anterior and preoptic area of the hypothalamus.

The inhibitory effect of estrogen (1 μg/day) on the castration-induced rise in gonadotropin output can be greatly diminished by electrolytic lesions placed in the anterior hypothalamus of juvenile rats, as revealed in parabiosis experiments (Flerkó, 1956, 1957).

Estrogen released from small fragments of ovarian tissue autotransplanted into the anterior hypothalamus inhibited gonadotropin secretion. Similar ovarian inplants in the adenohypophysis or liver tissue grafts in the anterior hypothalamus did not produce this effect (Flerkó and Szentágothai, 1957).

In Hohlweg and Daume's (1959) experiment, estrogen, when injected into the anterior hypothalamus of rats, had an antigonadotropic effect, 125 times larger than if administered subcutaneously.

Destruction of gonadal feedback mechanisms in the anterior hypothalamus of infantile rats and anestrous ferrets in the winter time has been assumed by Donovan and van der Werff ten Bosch (1956a,b; 1959a,b) to initiate the secretion of gonadotropins in amounts sufficient to promote follicular development and estrogen production similar to the mature and estrous animals, respectively.

Experimental data listed in this subsection appear to support the hypothesis that in the rat, nervous elements of the anterior hypothalamus are indispensable for the feedback mechanism by which gonadotropin release is inhibited by a slight (physiological) elevation of the sex steroid level in the blood. On the other hand, absence of compensatory gonadal hypertrophy following hemicastration in animals with anterior hypothalamic lesions (Assenmacher, 1957; D'Angelo and Kra-

vatz, 1960; Flerkó and Bárdos, 1961a) supports the idea that some neural elements of this region might also play a role in the mechanism by which a decrease in the sex steroid level enhances gonadotropin release. FSH assays of D'Angelo and Kravatz (1960) suggest that under conditions of enhanced demand—provoked by unilateral ovariectomy —rats bearing anterior hypothalamic lesions failed to release FSH, although their hypophyses contained an amount of FSH at least equal to, if not larger than, those of rats without hypothalamic lesions.

C. TUBERAL HYPOPHYSEOTROPIC NEUROSECRETORY SYSTEM

Recent data also suggest the tuberal hypophyseotropic neurosecretory system—located mainly in the arcuate-basal ventromedial nuclei region of the median eminence—is influenced by OS in the sense of a negative feedback action.

When Lisk (1960) implanted fine needles containing estradiol into the arcuate nucleus, estrus cycles ceased and after 30 days the uterus resembled that of a castrated animal. The ovaries contained no large follicles and the interstitial tissue was atrophic.

In Davidson and Sawyer's (1961) experiment, implantation of minute amounts of estradiol into posterior median eminence–basal tuberal region of the hypothalamus of the rabbit was followed by failure of copulation-induced ovulation, and eventually by ovarian atrophy. Similar implants into the adenohypophysis, mammillary body, and other parts of the brain did not produce these results.

Most recently McCann (1961), using the ovarian ascorbic acid depletion test (Parlov, 1958), has found that single dose of estrogen (1–50 μg of estradiol) caused detectable LH to disappear from the plasma within 3 days and that pretreatment with ineffective doses of estrogen plus small doses of progesterone has a similar inhibitory effect on LH release. In such animals, however, an injection of stalk–median eminence extract results in the reappearance of LH in the plasma within 10 minutes. From this finding it may be inferred that constant estrogen action prohibits hypothalamic cellular activity which normally produces some neurosecretory LH-releasing factor, and then, when this factor is replaced, there is a bypass of the estrogen block that renders possible the release of the stored LH from the anterior pituitary.

McCann's finding clearly explains why reduction or total elimination of the estrogen feedback by resection of ovaries or by ovariectomy followed by reimplantation of one of the ovaries into the spleen, respectively, has been followed by luteinization in constant-estrous rats produced by anterior hypothalamic lesion (Flerkó and Bárdos, 1961b).

Anterior hypothalamic lesions destroy the nervous structures indispensable for the FSH release regulating (inhibiting) action of small amounts of estrogen. Depending on the localization and extent of the

injury, these lesions also can seriously damage the neighboring LH release mechanism at the same time. If so, FSH and LH secretion are maintained at *basal levels* by the hypophyseotropic neurosecretory mechanism. Consequently FSH and LH are continuously secreted in amounts sufficient to maintain follicular development and continuous estrogen secretion. In this case constant vaginal estrus (cornification) takes place immediately after the operation.

Actually in the majority of the rats with anterior hypothalamic lesions, the complete absence of formation of new corpora lutea, indicated by the constant vaginal cornification, takes place only 2–3 weeks after placement of the lesion (Flerkó and Bárdos, 1959). This suggests that the hypothalamic LH-releasing mechanism, mainly preoptic in localization, is usually spared by anterior hypothalamic lesions. Because the ovaries of these animals are under persistent FSH-influence and regardless of the more or less disturbed hypothalamic LH-releasing stimulus, their secretion of estrogen becomes more and more continuous. The estrogen in turn, by its feedback action on the preoptic LH-releasing and tuberal neurosecretory mechanisms, inhibits the release of LH sufficiently to induce formation of corpora lutea. If, however, the estrogen feedback is greatly reduced or eliminated by excessive resection of ovarian tissue or more effectively with intrasplenic grafting, the preoptic LH-releasing and tuberal neurosecretory mechanisms having been delivered from the inhibitory feedback action of estrogen, can stimulate the adenohypophysis to discharge LH in quantities sufficient to elicit formation of corpora lutea.

A similar mechanism is possibly involved in the production of corpora lutea following the injection of progesterone into rats with persistent vaginal estrus produced by hypothalamic lesions in the experiments of Greer (1953). One might thus assume that, in the experiments of Greer, progesterone injected into the lesioned rats would antagonize the constant estrogenic action.

Cystic ovaries of rats made constant estrus by anterior hypothalamic lesion are similar to the polycystic ovaries in the Stein–Leventhal syndrome. Therefore, it is conceivable that the frequent beneficial therapeutic effect of the so-called "wedge resection" of the ovaries of patients with this syndrome might be due simply to the diminution of the blood estrogen level. This interpretation, however, cannot easily be reconciled with the findings of Ingersoll and McArthur (1959) that frequent and erretic release of LH may occur in women with Stein–Leventhal syndrome.

Results of the experiments mentioned above suggest that neural structures involved in the feedback control of gonadotropin secretion are localized within the preoptic, anterior, and tuberal part of the hypothalamus. The function of the whole system is influenced, in addition, by afferent impulses arising from other parts of the brain: the rhinen-

cephalic and midbrain reticular structures must be considered in particular.

Alternating phases of positive (stimulatory) and negative (inhibitory) feedback actions of the OS—in addition to extero- and interoceptive neural signals—seem to be important factors in regulating the release of FSH as well as LH. It would appear that both the facilitatory and the inhibitory effects of OS—within the physiological range—on the adenohypophysis may be mediated by influences on the discussed hypothalamic mechanisms. The sequential phases of positive and negative feedback control of release of pituitary ovulating hormone by progesterone in the rabbit (Sawyer and Everett, 1959) could be very well correlated with a lowering and raising, respectively, of thresholds of electrical activity in the hypothalamus (Kawakami and Sawyer, 1959). Thus, the feedback action of OS acting through the hypothalamus appears to be the decisive factor in the maintenance of the cyclic character of gonadotropin discharge in the spontaneously ovulating animals.

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DISCUSSION

Neural Aspects of the Feedback Action of Ovarian Steroids: the Role of Progesterone

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Although the task of dealing adequately with the studies of Flerkó is rather daunting, it remains a pleasure to take up one or two points raised in his thoughtful and succinct paper, even though the full details of the work described by Flerkó (1962, 1963) and Halász (1962) are not yet available. Among these the existence of a hypophysiotropic area and the emphasis on the controlling action of estrogen acting on the anterior hypothalamus are of particular interest.

The hypophysiotropic area is a region of the hypothalamus extending from the paraventricular nuclei to the optic chiasma and posteriorly to the mammillary nuclei, within which pituitary grafts (in hypophysectomized rats) permit the resumption of normal vaginal cycles and corpus luteum formation. If the existence of this area is established, then a variety of questions are raised. One concerns the nature and mode of action of the postulated transmitter agents normally discharged into the hypophyseal portal vessels to control the secretion of gonadotropin. Once the diffusion of active substance from the median eminence is excluded, then it appears that at least one active agent is produced by the cells of the posterior hypothalamus, or by nerve fibers projecting there. But since there must be release of FSH and LH in sequence from the graft in the course of an estrous cycle, must it also be presumed that two transmitting agents are produced by the neural constituents of that part of the hypothalamus? Are the substances produced at different times by the same nervous structures, or does the graft lie amid a mixed population of nerve cells or fibers, only part of which is active on occasion? Can it be that the rhythmic release of FSH and LH simply reflects different intensities of stimulation by the hypothalamus, FSH being released by relatively weak excitation and LH being added as the intensity increases, perhaps as a consequence of the feedback action of gonadal steroids? It may also be asked whether the posterior hypothalamus is subject to control by the anterior hypothalamus, or whether the whole region acts in concert. In this connection a study of the effect of anterior hypothalamic lesions in hypophysectomized rats with functional pituitary grafts near the mammillary nuclei might well be most revealing.

An alternative explanation of the results of the experiments with intrahypothalamic pituitary grafts could simply be that gonadal hormones exert a feedback action on the posterior, as well as the anterior, hypothalamus. This suggestion has experimental support. To mention one finding quoted by Flerkó, Lisk (1960) showed that in the rat estrogen applied to the arcuate nuclei or mammillary nuclei caused long periods of

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diestrus, loss of ovarian weight, and the appearance of wheel figures in the nuclei of the interstitial cells. This last feature is of great interest, for this transformation is associated with an insufficiency of LH in hypophysectomized rats and has been described in the ovaries of animals displaying a spontaneous persistent estrus (Everett, 1940). It may imply that a feedback action of ovarian hormones on the secretion of LH is effected at this point.

The emphasis on the feedback action of estrogen would seem to have arisen from consideration of the great potency of estrogens, as compared to progesterone, in this regard. This naturally led to the use of estrogen in most studies and to a relative neglect of progesterone. In order to restore some balance I shall stress a different viewpoint. Largely through the work of Everett and of Sawyer in rats and rabbits (see Sawyer and Everett, 1959), it is now established that progesterone can facilitate, as well as prevent, ovulation. In the rabbit it was shown that a dose of 2 mg of progesterone in oil given subcutaneously enhanced the ovulatory response to mechanical stimulation of the vagina 1–4 hours after administration, although the hormone alone was not highly active in causing ovulation (Sawyer and Everett, 1959). However, in the light of the study of Hilliard *et al.* (1961) it now seems that the treatment may have been inappropriate. This group found that following mating, a rise in the output of progesterone from the rabbit ovary could be measured within minutes, and long before ovulation some 10 hours later. The observations given indicate that within an hour or so of coitus, progesterone is secreted at a rate of nearly 1 mg per ovary per hour. Progesterone secretion was invariably associated with the later occurrence of ovulation, whether produced by hormone injection or coitus. Now, according to current views (see Young, 1961), the nervous stimulus for ovulation directly causes a release of LH which acts on the ovarian follicle to induce rupture, corpus luteum formation, and progesterone secretion. However, an equally adequate explanation might be that the triggering stimulus discharges gonadotropin (not necessarily LH) which causes a sudden release of progesterone (and estrogen?) from the ovary, and that it is the progesterone feedback that alters pituitary function toward LH release for ovulation. This concept is supported by the findings of Hilliard *et al.* (1961) that progesterone secretion, and ovulation, was evoked by FSH, and not only by LH.

On similar lines it may be argued that persistent estrus in rats, as induced in a variety of ways (Takewaki, 1962) is caused by a lack of progesterone, not by interference with a feedback action of estrogen. Everett (1940) was the first to show that progesterone administration caused a resumption of ovulation in rats with a "spontaneous" constant vaginal cornification, and Greer (1953) demonstrated that progesterone also elicited ovulation in rats in which persistent estrus was induced by hypothalamic lesions. The luteinization seen in the ovarian remnant of hypothalamic-lesioned persistent-estrus rats after removal of one and a half ovaries (Flerkó and Bárdos, 1961) could well be accounted for by the resulting surge of gonadotropin from the pituitary, which promotes progesterone formation. In studies of the hormonal control of ovarian function in the ferret (Donovan unpublished) it has become clear that ovulation may follow estrogen administration or hemicastration. This is probably due to a rebound release of gonadotropin.

Because of the prominence given to the effects of changes in the blood level of estrogen in the control of pituitary function, it is pertinent to consider recent studies made on spayed rats in which a piece of ovary was grafted into the spleen. This technique is said to have the advantage that the steroids produced by the graft are inactivated in the liver and so cannot act upon the pituitary gland. Accordingly, the luteinization and

corpus luteum formation seen in grafts prepared in rats made persistently estrous (Noumura, 1958; Flerkó and Bárdos, 1961; Desclin *et al.* 1962) is attributed to removal of the inhibitory action of estrogen on LH release. However, an alternative explanation is available, for the investigations of Kullander (1954, 1956) indicate that progesterone escapes from the graft into the general circulation, whence it can promote LH release as indicated earlier. This view receives further support from the finding of Kempf (1950) that progesterone causes corpus luteum formation in ovarian grafts made into the ears of male rats. Such grafts are normally purely follicular in structure.

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Electrolytic Irritative Lesions in the Hypothalamus and Other Forebrain Areas: Effects on Luteinizing Hormone Release and the Ovarian Cycle[1]

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I. INTRODUCTION

The investigations to be summarized here are outgrowths and elaborations of those described at the Harvard Conference on Ovulation in 1960 (Everett, 1961). They stem from the fact that in rats maintained under controlled lighting of 14 hours daily, regularly cyclic females release "spontaneously" from their pituitary glands the ovulating dose of luteinizing hormone (LH) during a critical period of 2 hours on the afternoon of proestrus (Everett, 1956). The stimulus from the brain responsible for this release can be blocked at will of the experimenter by intraperitoneal injection of pentobarbital shortly before 2:00 P.M. (Everett and Sawyer, 1950). A number of other drugs are also effective, among them morphine and, most notably, atropine in massive dosage. It has been suggested by Sawyer and associates (1955) that all three drugs exert their blocking action on LH release in a comparable manner, by elevation of threshold in the reticular formation of the brain. Both atropine and pentobarbital have been exceedingly useful in studying the time relationships of LH release in this species, either drug possessing certain attributes appropriate to certain purposes. Thus, atropine in massive subcutaneous dosage, by virtue of its prolonged action, served in acute studies to sharply define the "critical period" of LH release (Everett *et al.*, 1949), whereas pentobarbital with its shorter action and less severe side effects could be used repeatedly on successive days once the time limits of the "critical period" were evident (Everett and Sawyer, 1950). By such means we were able

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Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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to demonstrate that not only is the time of LH release closely limited to the 2-hour interval on the day of proestrus, but that the neural apparatus which controls it includes a 24-hour clock. For if the stimulus is blocked on one day it will occur on the following day during the same 2-hour period, or if blocked on these 2 days it will take place on the third day at that time.

Our broad problem is to determine, if possible, what components of the central nervous system participate in this close timing of the stimulus for LH release. More immediately, we have been concerned with (1) what parts of the neural mechanism are blocked out by pentobarbital, (2) whether there is evidence of threshold changes at different times in the cycle, and (3) whether one can experimentally induce ovulation by stimulation of the brain without at the same time inducing a pseudopregnancy. The initial step lay in Critchlow's discovery (1958) that, in rats "blocked" with pentobarbital in the manner described above, ovulation can be induced by electrical stimulation of the tuber cinereum immediately above the median eminence. In our own studies (Everett, 1961), we found that similar results can easily be obtained with electrodes placed in the preoptic region, although it now appears that the effect observed in that area is actually caused by chemical irritation consequent to electrolysis.

II. Stimulation of Brain Tissue by Electrolytic Deposits from Stainless Steel

Subsequent analysis of our stimulation procedures has led to the demonstration that the induction of ovulation by preoptic stimulation is brought about, not through electrical stimulation per se, but by the electrolytic deposition of iron and perhaps other metals from the stainless steel electrodes routinely employed (Everett and Radford, 1961). In some way not yet understood, the metallic compounds thus deposited produce a zone of sterile inflammation radiating from the electrode tip well beyond the limited spot of coagulation (Fig. 1). The result is a spheroidal focus of chemical irritation which continues to act long after the electric current has ceased to flow.

Platinum electrodes proved to be singularly ineffective by contrast with stainless steel or nichrome. On the other hand, with stainless steel, any form of electric current can be effective if it deposits iron, including, as we shall see, anodic direct current as little as 10 μA for 20 seconds (2×10^{-4} coulombs). The mere insertion and withdrawal of electrodes, implantation of inert materials (glass, copper wire, platinum wire, pellet of Fe_2O_3), lesioning by radiofrequency, or lesioning by electrolysis through platinum electrodes, all have given essentially negative results. All told, among a list of 71 such preparations ovulation failed to occur in 63, and only 4 rats ovulated the full set of eggs.

The remaining 4 rats had minor evidence of LH action, with only a single follicle being activated or ruptured. On the other hand, microinjection of 5% $FeCl_3$ into the preoptic tissue was nearly uniformly effective in causing full ovulation. Parenthetically, it should also be mentioned that the implantation of $CuCl_2$ crystals in the preoptic tissue ovulated each of three rats (unpublished). This is of theoretical interest

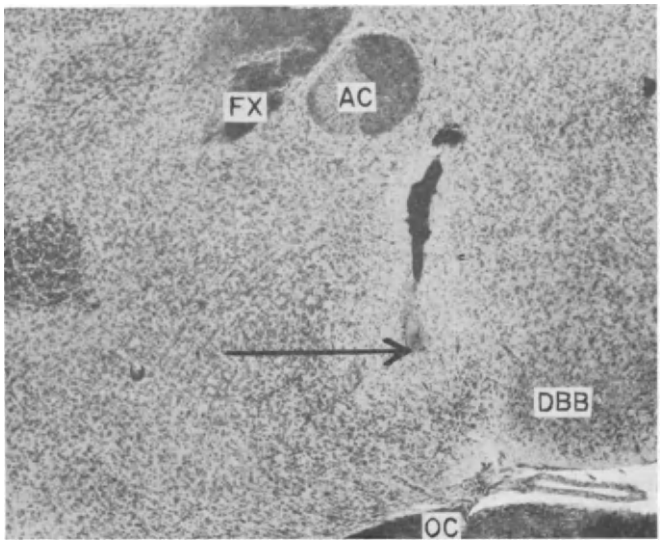

FIG. 1. Parasagittal section of preoptic and anterior hypothalamic areas of a rat brain, showing characteristic tissue reaction to metal deposited electrolytically from stainless steel. Electrode tip (arrow) rested in medial preoptic area. Ovulation of seven ova was induced by this irritant focus, which was formed by monophasic, rectangular 1-msec 150-μA pulses, 100/sec for 60 seconds. Although not mentioned in the text, this rat was hypophysectomized 50 minutes after passage of current. The brain was perfused on the following day. *AC*, anterior commissure; *DBB*, diagonal band; *FX*, fornix; *OC*, optic chiasm. Luxol Fast Blue-Cresyl Fast Violet stain. Magnification: 40 X.

in view of the well-known fact that systemic administration of copper salts will induce ovulation in the rabbit (Fevold *et al.*, 1936; Harris, 1941).

III. MAPPING STUDIES

Without knowing precisely how the electrolytic focus acts to produce the stimulus, one can nevertheless make use of it in a variety of ways. We have undertaken, for example, to explore the brain extensively by means of standardized foci variously placed in different individuals.

At this juncture, the routine procedures should be outlined. Unless otherwise specified, the standard subjects were adult cyclic female rats from our colony, each with a history of regular 4-day cycles and clearly

proestrous on the day of experimentation. Pentobarbital (35 mg/kg body weight) was injected intraperitoneally shortly before 2:00 P.M. During the resulting anesthesia the rat was placed in the stereotaxic apparatus, the electrode was introduced according to the desired coordinates, current was passed, the electrode was removed, and the skin incision was repaired. The result of each experiment was deter-

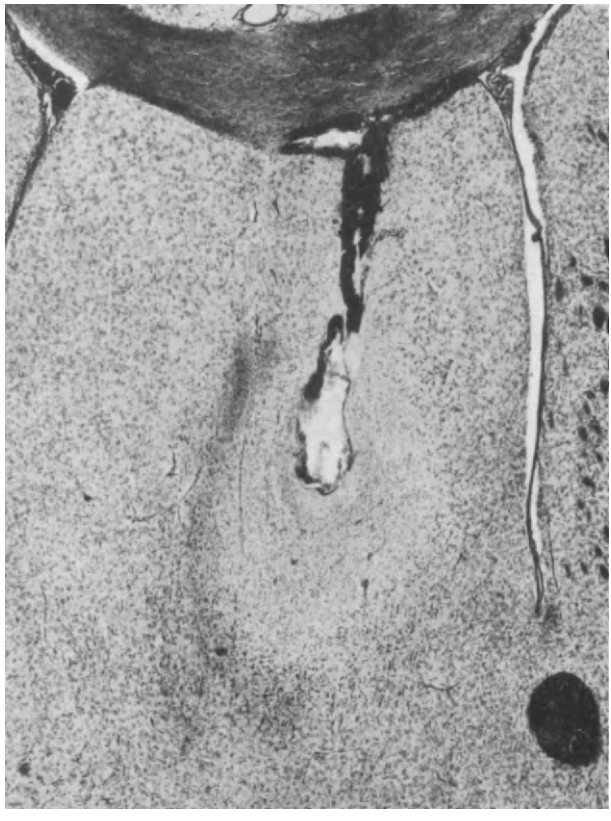

FIG. 2. Locations of *centers* of small irritative lesions in the exploratory study of hypothalamus and medial preoptic areas, sagittal diagram. Filled symbols: cases of induced ovulation. Open symbols: negative results. Circles: 1.0 mm from midline. Triangles: 1.0–2.5 mm laterally. Lesion produced in each case by 100-μA 1-msec pulses for 60 seconds (see text). *AC*, anterior commissure; *FX*, fornix; *MI*, massa intermedia; *MBR*, midbrain; *MMB*, mammillary body; *OC*, optic chiasm; *ON*, optic nerve; *SEPT*, *septum*. One-millimeter scale divisions at left and below.

mined on the following morning by examining the oviducts for tubal ova and the ovaries for ruptured and unruptured follicles. The electrode position was later established by histologic examination of serial sections of the brain. The electrodes were concentric 0.4-mm bipolar

stainless steel units, the center wire protruding slightly from the end of the sheath, and each element insulated to the tip. The center wire was anodic. Electric current was delivered in one of two standard forms, depending on whether a large or small focal lesion was desired. Small foci approximately 0.5 mm in diameter were produced by a 60-second train of monophasic rectangular 1-msec pulses at 100/sec with

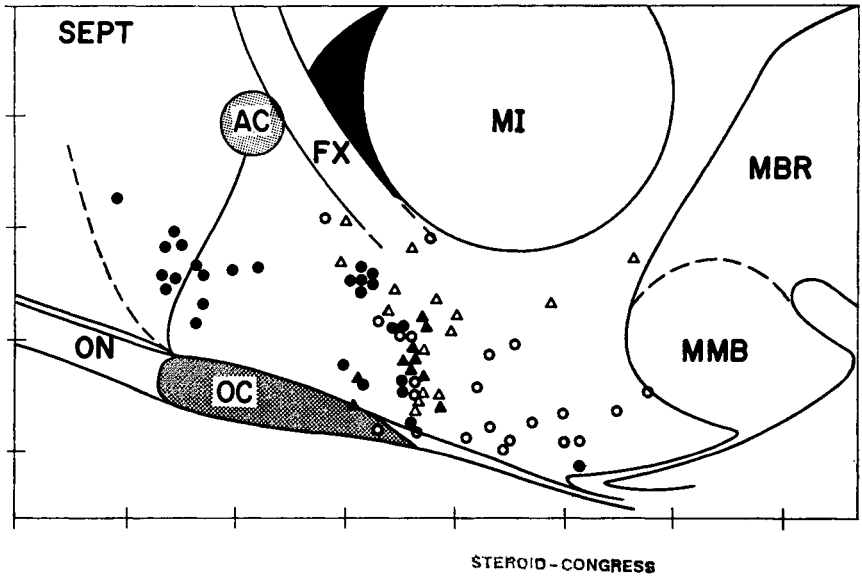

FIG. 3. Frontal section of rat brain, showing a large electrolytic focus in the septum produced by 100 μA, direct current, for 30 seconds. There were nine tubal ova on the day after stimulation. Luxol Fast Blue–Cresyl Fast Violet stain. Magnification: 40 x.

a current of 100 μA in each pulse. Although direct current at low level could have been used instead of the monophasic pulse train, the latter was chosen on the basis of previous experience with its effects. The pulse was constantly monitored by a calibrated oscilloscope for width (duration) and height (current), and thus the total amount of electricity delivered was maintained very close to 6×10^{-4} coulombs. Large foci approximately 1.0–1.5 mm in diameter were formed by 100 μA direct current passed for 30 seconds (30×10^{-4} coulombs), the current again being monitored by a calibrated oscilloscope. Only one focal lesion was placed in any given rat.

Results from small foci in various locations in the medial hypothalamus and medial preoptic area are partially represented in Fig. 2. The filled circles and triangles represent the locations of electrode tips in rats that ovulated, and the open figures represent negative results. The circles are locations less than 1.0 mm from the midline, whereas

triangles represent locations from 1.0 to 2.5 mm lateral to the midline. Electrodes in the medial preoptic region were consistently effective. However, more caudally, in the region above the caudal margin of the optic chiasm the frequency of positive results became greatly reduced. In the tuberal region itself, negative results were the rule; only the one positive case was found, the electrode resting near the origin of the pituitary stalk. The extreme basal level between this point and the posterior margin of the chiasm must be more completely explored before one can say whether results from this method of stimulation differ significantly from those of Critchlow (1958).

FIG. 4. Diagrammatic sagittal view of the rat brain, showing the postulated course of fibers concerned with ovulation (see text). Widely dispersed origins are indicated in the septal complex (left), where relatively large irritative lesions (broken outlines) are required to activate the number of fibers that can be affected by small lesions in the preoptic area.

An interesting development was the finding that, rostral and dorsal to the preoptic region, positive results could be obtained by means of the large foci placed in the medial or lateral septal nuclei (Fig. 3), in the nucleus accumbens, or in the medial parolfactory area. Small foci similarly placed were not effective. This observation has led us to postulate that in these rostral locations the particular nerve fibers involved in the ovulation phenomenon are so dispersed that only the larger foci can affect sufficient numbers of them. Even in the preoptic region and anterior hypothalamus it would appear that the unique effectiveness of the electrolytic foci relates directly to the bulk of brain tissue involved as well as to the prolonged period of action. We postulate that the irritant effect spreads to an array of fibers of passage about the periphery of the lesion, that the fibers in question are still relatively diffuse in the anterior hypothalamus, but that they converge basally as they move into the tuber. In that region it thus becomes more difficult to reach them.

The diagram in Fig. 4 illustrates in a general way our concept of the course of these fibers from the septal complex through the medial preoptic and anterior hypothalamic areas to the region of the median eminence. It is important to recall the experiments of Hillarp (1949), who observed that constant estrus could be induced in rats by either the

bilateral destruction of large areas in the anterior hypothalamic area or very restricted basal areas in the anterior tuber cinereum. Our findings are in agreement with the implications of Hillarp's results and supplement them by indicating that fibers controlling ovulation originate as a diffuse system widely dispersed in the septal complex. The fact that this system remains responsive under pentobarbital anesthesia implies that few synapses are included en route to the tuber cinereum.

We have thus far been unable to elicit ovulation by placing large or small irritative foci in the caudate-putamen, the anterior olfactory area, the olfactory tubercle, the far lateral preoptic area, or dorsal thalamus adjacent to the foramen of Munro. The same is true for the lateral hypothalamus caudal to the optic tract and most of the medial hypothalamus posterior to the optic chiasm. It may be supposed that, if there are any connections from or through these areas into the septal complex and medial preoptic areas which normally play a role in LH release, they are either (1) blocked out by the barbiturate, (2) insensitive to the kind of stimulus employed, or (3) not accessible in sufficient numbers in any one location.

It is significant that only a portion of the fiber array in either the septal complex or the preoptic area need be activated to bring about LH release in sufficient quantity for ovulation. Thus, a large focus may be placed high in the medial or lateral septal nuclei, in the nucleus accumbens, or in the medial parolfactory area. In the medial preoptic area, a focus no larger than 0.5 mm diameter may be placed almost anywhere, with equivalent result. Furthermore, the focus need be on only one side of the brain.

How are these facts to be interpreted? On the assumption that any given fiber of the system depicted in Fig. 4 plays upon only one point in the median eminence, either directly or through a relay nucleus, need only a fraction of such points be activated to evoke release of an ovulating dose of LH? Is it immaterial which fraction? Or is there an extensive multiple innervation of the various points with the result that a stimulus restricted to a relatively few fibers will bring all the median eminence into play? If only one zone of the median eminence is concerned with gonadotropin release, the same questions would apply specifically to that zone.

IV. VARIATION OF PREOPTIC THRESHOLD

Directly related to these questions is the observation that a difference in "threshold" exists between a 4-day cyclic rat in proestrus and a 5-day cyclic rat on the third day of diestrus, with respect to the amount of electricity that must be delivered through a preoptic electrode to invoke ovulation. This was indicated previously (Everett, 1961) on the basis of two experimental series conducted before the electrochemical

nature of the stimulus was appreciated. To make doubly sure of the existence of the threshold difference, we recently carried out a third series, this time employing *direct current* of low level (Table I). After

TABLE I

Comparative Effects of Low Levels of Direct Current during Late Diestrus and Proestrus

Direct current (μA)	Time (sec)	10^{-4} coulombs	Results[a]	
			Diestrus	Proestrus
10	60	6	+	
	50	5	+	
	40	4	++++	
	30	3	00	+
	20	2	0000	+++++
	15	1.5		++00
	10	1		+0000
7.5	10	0.75		00000

[a]Each symbol represents an individual rat (+ = ovulated, 0 = not ovulated).

certain preliminary trials, we determined to use a constant current level of 10 μA and to vary the time. The proestrous rats were uniformly ovulated by 10 μA passed for 20 seconds (2×10^{-4} coulombs), and a few rats responded when the time was even shorter. The diestrous rats, on the other hand, required about twice that duration (hence 4×10^{-4} coulombs), in agreement with the previous observations made when pulses were used rather than direct current. It seems clear, therefore, that a proportionately greater amount of electrode metal is required in the diestrous rat and, hence, supposedly a larger lesion is made and a greater number of nerve fibers are activated.

What bearing do these observations have upon questions raised in Section III? In the diestrous rat, is it necessary that more of the median eminence be activated than in the proestrous rat? Or, assuming multiple innervation to all the gonadotropic zone of the median eminence, is the threshold of that zone relatively high in diestrus, so that greater numbers of fibers must act to stimulate it? Or, is the threshold difference really within the pars distalis, which may thus require a greater quantity of chemotransmitter to liberate a given amount of LH? I will not attempt to provide answers.

V. EFFECTS OF PREOPTIC STIMULATION ON THE ESTROUS CYCLE. ABSENCE OF PSEUDOPREGNANCY

To this point we have dealt with acute experiments exclusively. In long-term experiments not yet complete, we have encountered several phenomena that are especially appropriate to this symposium.

In 25 rats an irritative focus was introduced in the preoptic area under pentobarbital anesthesia during either proestrus (4-day cycle) or the third day of diestrus (5-day cycle). Direct current was passed through the standard stainless steel electrode in amount well above threshold for ovulation (either 10 μA for 60 seconds or 100 μA for 30 seconds).

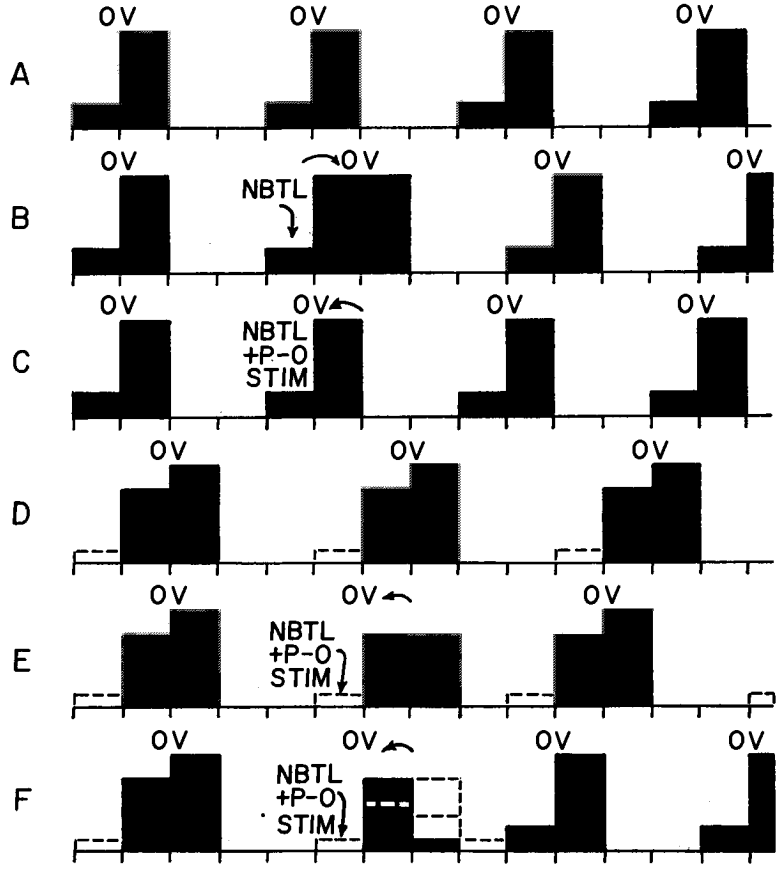

FIG. 5. Diagrams of characteristic vaginal smear sequences, showing effects of electrolytic stimulation of the preoptic area *(P-O STIM)* under pentobarbital anesthesia *(NBTL)*. Full height of the dark blocks represents full cornification, the shorter blocks indicating proestrous or metestrous stages. Broken lines indicate variability. Scale marks show 24-hour intervals. The presumptive time of ovulation is shown by *OV*, delay or advance being indicated by arrows.

Daily vaginal smears were continued and most animals were followed in this way for 1–4 weeks.

Figures 5A and 5D, respectively, represent normal 4-day and 5-day vaginal cycles. Experiments with 4-day cyclic rats will be considered first since they are somewhat simpler than the others.

When pentobarbital is administered to a 4-day cyclic rat in proestrus (Fig. 5B), not only is spontaneous release of LH blocked for 24 hours, but if the experiment continues one finds that the vaginal smear remains cornified an additional 24 hours, becoming diestrous on the following day (Everett and Sawyer, 1950). In succeeding cycles the onset of proestrus will consistently be 1 day later than it would have been had the experimental cycle not been prolonged. If, however, during the period of pentobarbital anesthesia in the experimental cycle, one induces LH release by preoptic stimulation (Fig. 5C) in the manner earlier described, ovulation will occur at approximately the normal time, the extra day of cornification will not be added to the vaginal smear sequence, and subsequent cycles will occur precisely at the time expected had there been no experimental intervention.

In the 5-day cyclic rat stimulated on the third day of diestrus (Fig. 5E and F), one characteristically finds that on the following day the vaginal smear is proestrous, apparently unaffected by the early release of LH and the resultant ovulation during the preceding night (Everett, 1961). Subsequently, however, distinct modifications of the vaginal smear sequence are encountered. While the vagina supposedly goes on to full cornification later that second day, by the morning of the third day, when full cornification would have been a normal occurrence, the smear contains a rich admixture of leucocytes. Functionally, this day now corresponds to the first day of the interval before the next estrus. Thus the new cycle dates from the time of the preceding ovulation and its attendant events, a relationship that perhaps one should have expected.

It is noteworthy in passing that 7 of 10 rats changed from 5-day to 4-day cycles immediately following the experimental procedure. However, without larger numbers of cases no great significance can be attached to this fact, for such changeovers take place spontaneously on occasion.

It is *highly* significant, on the other hand, that pseudopregnancy-like periods of diestrus rarely happened in rats stimulated in either late diestrus or proestrus. Nearly all continued to have cycles, as illustrated in Fig. 5C, E, and F. Only four rats became "pseudopregnant." I am inclined to regard all four pseudopregnancies as having occurred "spontaneously," or at least as not being caused in any specific way by the preoptic stimulation. The frequency of spontaneous pseudopregnancy in our colony is such that from 5 to 10% of the nonexperimental stock may be in that state during any 2-week period.

Conceivably the pentobarbital anesthesia blocked the pseudopregnancy mechanism in spite of the fact that it does not interfere with the induction of LH release. To examine this possibility, indwelling electrodes were employed in a few rats for stimulation in the absence of an anesthetic. In each of 3 rats a standard electrode was stereotaxically

placed in the preoptic region and affixed to the skull together with an appropriate subminiature Winchester connector. After two or more subsequent cycles, an electrolytic stimulus was effected during the afternoon of proestrus by means of either 10 μA direct current for 60 seconds (2 rats) or a 60-second train of 100-μA 1-msec pulses at 100/sec (1 rat). No agent of any kind was administered to block the animals' spontaneous ovulation. We were concerned only with the presence or absence of a pseudopregnancy-like period of diestrus in the days immediately following the stimulation. All animals continued to have short cycles.

Certain questions immediately occur to one on learning this. Is the stimulation procedure by its very nature in some way antagonistic to pseudopregnancy-inducing mechanisms? What would be the result if the preoptic stimulus were administered during estrus rather than proestrus? Or, must one stimulate quite different pathways in order to bring about the long period of corpus luteum secretion? These are questions for the future; no doubt there will be many more.

For the present it is sufficient to note the clear demonstration of the one basic fact that by stimulation of the brain one can experimentally induce ovulation in rats without at the same time inducing pseudopregnancy. The rat, of course, has known how to do this all along.

VI. SUMMARY AND CONCLUSIONS

Ovulation can be induced in pentobarbital-blocked proestrous rats by stimulation of the anterior hypothalamic area, the medial preoptic region, or the septal complex with irritative focal lesions formed by electrolytic deposition of iron from stainless steel electrodes. The unique effectiveness of the electrolytic foci relates not only to the persistence of stimulative action long after passage of electric current, but also to the bulk of brain tissue involved. It is postulated that the irritant effect spreads to an array of fibers of passage at the periphery of the lesion.

Results of mapping studies suggest that the fibers in question originate in a highly diffuse system throughout the septal complex, converge only moderately, and are still diffuse as they course through the preoptic region and anterior hypothalamic area, and finally as they enter the tuberal region come close together basally. Only a part of the diffuse array need be activated, and one part is as adequate as another.

In 5-day cyclic rats, ovulation can be advanced 24 hours by preoptic electrolytic stimulation during late diestrus, but such rats require twice the amount of electricity required in proestrus, suggesting that more of the special nerve fibers must be activated. Early release of LH and early ovulation are not accompanied by rapid acceleration

of the vaginal smear sequence, for on the following morning one finds tubal ova in the presence of a proestrous smear.

Pseudopregnancy is not ordinarily a sequel to ovulation induced by preoptic electrolytic stimulation, either when the electrolysis is carried out under pentobarbital anesthesia or in an unanesthetized animal through an indwelling electrode.

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DISCUSSION

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In their latest work on the mechanism associated with the timing of ovulation in the rat, Dr. Everett and his collaborators have shown that the block to ovulation induced by pentobarbital can be overcome by an electrochemical stimulus to the preoptic region. The fact that the stimulus to ovulation appears to be due not to the electrical stimulus per se, but to deposition of metallic ions from the electrode tip, seems to me to be of considerable interest, since it brings to mind earlier studies dealing with the effects of a variety of pharmacological agents on ovulation in the rabbit. These were reviewed in 1955 by Professor Harris in his monograph. It has been suggested for example, that the ovulatory stimulus initiated by the administration of copper acetate intravenously, or directly into the third ventricle, may be attributable to a direct effect of copper acetate on pituitary cells, and indeed it has been further demonstrated that copper acetate, when injected directly into the anterior-pituitary gland in small quantities, stimulated ovulation in the majority of rabbits so treated. Of course Dr. Everett's group is dealing here with an animal exhibiting a different type of sexual cycle, "spontaneously" ovulating as compared to "reflexly" ovulating, but the possibility that their results could be attributed to the transmission of metallic ions via the portal system to the anterior pituitary should perhaps be borne in mind. The positive effects observed after microinjection of 5% ferric chloride and of cupric chloride might lend support here, but, on the other hand, direct electrical stimulation of the anterior pituitary is apparently ineffective (Harris, 1955).

It may well be, however, that such a possibility does not accord with the findings of Dr. Everett's group that the relevant nerve fibers, which they believe to be associated with the ovulatory response, converge basally into the tuber cinereum, thus accounting for the larger lesions necessary in the anterior hypothalamus, where it is suggested the fibers are more diffuse.

If we assume that the ovulatory response is in fact due to the irritation of the appropriate nerve fibers, we have again to consider the nature of the precise triggering mechanism. I take it that most of us would not envisage a direct relationship—a direct nervous connection between the median eminence and the anterior lobe—and an alternative possibility that ovulation is brought about via direct nervous pathways between the central nervous system and the ovaries has been discounted over the years by a number of workers. For example, Christian and Markee (1958) showed that in female rabbits electrical stimulation of the hypothalamus resulted in ovulation in 5 out of 10 animals with high-thoracic spinal cord transections. We must thus turn our thoughts inevitably to the question of an intermediary releasing factor between the stimulated fibers and the luteinizing hormone (LH) in the anterior pituitary. It may be pertinent to mention that a previous co-worker of Dr. Everett, Dr. Nikitovitch-Winer, has recently published (1962) some observations made while working in Professor Harris's laboratory. She has demonstrated that median eminence extracts taken from proestrous rats and from bovine sources caused ovulation when infused by cannula directly into

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Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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the anterior pituitaries of "pentobarbital blocked" proestrous rats. Control substances, such as vasopressin, epinephrine, and histamine, were ineffective. Much larger quantities of the extract were required to initiate ovulation when the extract was administered intravenously, results which would contraindicate the possibility that the extracts themselves contained LH. A similar piece of evidence relating to the effect of median eminence extracts on the release of LH in rabbits, has been published by Campbell *et al.* (1961) McCann (1962) has also recently published evidence for an LH releasor derived from rat stalk-median eminence tissue.

This recent evidence, must be added to the formidable array of available data supporting the thesis that the median eminence contains one or more substances which are the immediate cause of LH release.

As yet, we are unable to identify these substances, although I would point out that some workers are inclined to the view that the posterior lobe hormones may by the agents responsible for LH release (Martini *et al.*, 1959).

We must also bear in mind the further complication that there may be two hypothalamic systems controlling LH release, as postulated by Barraclough and Gorski (1961), and supported by Desclin *et al.* (1962).

One of these systems is thought to be situated in the preoptic area and to be concerned with cyclic LH release; the other is believed to be in the region of the arcuate nuclei and to have a tonic activity which appears to be controlled by the level of estrogen circulating in the blood. I would like to ask Dr. Everett whether, in view of his finding regarding the different thresholds for stimulation in the proestrous and late diestrous rats, he has tried to initiate ovulation by stimulating rats at an earlier stage of diestrus. One would assume that the threshold levels would be considerably higher in this case. As a corollary to this, would it be possible to stimulate the release of LH in pseudopregnant rats and in this way terminate the pseudopregnancy? Finally, with regard to his point that no pseudopregnancy appears to supervene in his stimulated rats, it would appear that no luteotropic hormone (LTH) release is stimulated by his procedure and it would seem that any systems regulating LTH release must be distinct from those concerned with LH release. In this respect, it might be interesting to study the effects of stimulating rats whose cycles have been blocked with reserpine as reported by Barraclough and Sawyer (1957), since reserpine appears to facilitate the release of LTH in rats (Benson, 1958; Desclin, 1957).

J. W. EVERETT: Dr. Benson has raised several points that require comment. When it became evident that we were, in fact, producing a chemical rather than an electrical stimulus, we tested the use of copper, not only in the form of the crystals stereotaxically implanted, as mentioned in the paper, but also as electrode material. The latter experiments were unexpectedly complicated by the peculiar electrochemistry of copper, which very rapidly polarized at the low values of current used. A variety of tricks were employed to counteract the polarization, but we never arrived at a uniformly effective procedure. Strangely, although the electrodes were well placed and although there was a pronounced inflammatory zone around the electrode tip in each case, only 3 out of 13 rats ovulated. This low ratio, in contrast to the result of implanting the $CuCl_3$ crystals, cannot be explained at this time.

The possibility seems to be ruled out that the electrochemical stimulus produces its effect by diffusion of the irritant substance(s) into the pituitary portal circulation and thence to the anterior lobe itself. Recall the many negative results from electrodes placed in the tuberal region. In this region even the large direct-current lesions were

ineffective. Negative results were also produced by large foci placed, like the effective septal lesions, close to the lateral ventricle, but on the opposite side of it in the caudate nucleus. In two cases, large foci were deliberately placed close to the foramen of Monro, again with negative results.

We have very little information about the effects of stimulation on the second day of diestrus. In the few trials that we made, our interest was to learn whether the irritative effect of the metallic compounds would carry over until the next day (proestrus) and produce LH release on that day. Apparently not, but it is difficult to design a clean experiment to test either that question or the one which Dr. Benson raised. It seems doubtful that the follicles are competent on the second day of diestrus to respond to the ovulating dose of LH.

With respect to the question of induction of ovulation during pseudopregnancy, significant data are available from unpublished experiments (Riley and Everett). Of 20 rats that were stimulated on the 5th, 6th, or 8th day of leucocytic vaginal smears, 5 rats ovulated and 2 others had preovulatory changes in their ovaries. Of 7 rats stimulated on the 10th day, all ovulated. With only one exception, the vaginal smear remained leucocytic on the day after ovulation. The exception was an animal stimulated on the 5th day; she was fully cornified on the day after stimulation and warrants some scepticism. As for terminating pseudopregnancy by producing new corpora lutea during its course, I am not sure that would terminate it. There is little I can say about the threshold for preoptic stimulation during pseudopregnancy, other than mention one significant fact. We have never been able to induce ovulation by stimulation on the third day, although this is chronologically comparable with the 3rd day of diestrus in the 5-day cycle. The fact is clear, but the explanation is not yet evident.

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Regulation of Luteinizing Hormone (LH) Secretion by a Hypothalamic LH-Releasing Factor[1]

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There is now little doubt that the hypothalamus regulates the secretion of gonadotropins by the pars distalis of the hypophysis. The literature on this subject has been ably reviewed by Harris (1960). An investigation of the factors that regulate luteinizing hormone secretion has been undertaken in this laboratory utilizing a new, highly sensitive assay for LH, the ovarian ascorbic acid depletion test of Parlow (1961). In this test female rats are pretreated with pregnant mare's serum gonadotropin and then with human chorionic gonadotropin to produce heavily luteinized ovaries. After removal of the first ovary to obtain a control value for ascorbic acid concentration, the test substance is injected intravenously. One hour later the second ovary is removed and the percentage ovarian ascorbic acid depletion can then be determined. Either immature or adult animals can be used for this test. The immature rat is more sensitive to LH, as might be expected from its smaller size. Of anterior lobe hormones tested, only LH has appreciable activity in the test. Vasopressin can evoke ovarian ascorbic acid depletion also, but only at doses which appear to be outside the physiological range encountered in body fluids. This effect of vasopressin appears to be a direct one on the ovary since it persists in the hypophysectomized rat and occurs unilaterally after retrograde injection of vasopressin into the ovary via the ovarian vein (McCann and Taleisnik, 1960).

A beginning has now been made in defining the factors that influence the secretion of LH. The ovarian ascorbic acid depletion technique is not sufficiently sensitive to measure LH in plasma from the normal rat. However, within a week following ovariectomy, detectable levels of LH appear which persist for at least 6 months. Pituitary LH also is elevated in the ovariectomized rat (Parlow, 1959; Taleisnik and

[1] This investigation was supported by research grants from the U. S. Air Force, AF 49(638)-685,S/A 3, and the U. S. Public Health Service, A1236C5.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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McCann, 1961). If the rate of disappearance of LH from the circulation is unaltered in the spayed animal, these findings mean that castration is followed by increased rates of synthesis and discharge of LH. The ovarian steroids estradiol and progesterone are capable of reducing the titer of LH in the oophorectomized female, but progesterone has a relatively feeble effect unless the animal has been pretreated with estrogen (McCann and Taleisnik, 1961; McCann, 1962b; Giuliani *et al.*, 1961). The rise in plasma and hypophyseal LH that follows ovariectomy is prevented by lactation, a finding which suggests that suckling may reflexly inhibit LH discharge (Parlow and Rothchild, 1960; McCann *et al.*, 1961).

Most of our attention has been devoted to an investigation of the neural regulation of LH secretion. Hypothalamic lesions in the median eminence of the tuber cinereum prevent the rise in plasma LH which occurs following ovariectomy and decrease the already elevated titers found in previously ovariectomized animals. Two to three months after such lesions, hypophyseal LH stores are markedly reduced, and no rise occurs following ovariectomy, in contrast to the situation in the normal animal. Consequently, it appears that destruction of the median eminence markedly interferes with both the secretion and storage of LH. Lesser effects on LH secretion and storage are found in animals with constant vaginal estrus following hypothalamic lesions (Taleisnik and McCann, 1961).

Since lesions in the median eminence inhibit LH secretion, it was of interest to determine the effects of stimulation of this area on LH discharge. In adult females, pretreated with gonadotropins as described above, the mere insertion of a 26-gauge needle bilaterally into the median eminence was found to evoke ovarian ascorbic acid depletion (OAAD). We have called this technique hypothalamic piqure and have made an extensive exploration of the rat brain to determine the distribution of active sites. Administration of ether anesthesia, placement

TABLE I

Effect of Central Nervous System Piqure on Ovarian Ascorbic Acid Depletion (OAAD) *(Loci Other Than A 6)*

Locus[b]	% OAAD
Sham operation	3 ± 1 (23)[a]
A 4, L 1.5, U 0	15 ± 4 (12)
A 4.5, L 0.5, U 0	9.5 ± 4.5 (9)
A 7, L 0.5, U 0	11 ± 3 (9)
A 7.5, L 0.5, U 0.5	12 ± 2 (9)
A 7.5, L 2.5, U 0	11 ± 7 (5)

[a]Mean ± SE; number of observations gives in parentheses.

[b]Symbols refer to stereotaxic coordinates: A = distance anterior to ear bar (mm); L = distance lateral to midline (mm); U = distance from base of brain (mm).

of the rat in the stereotaxic instrument, and drilling of small bur holes bilaterally was without effect on ovarian ascorbic acid (Table I). Surprisingly, the insertion of the needle into the cerebral cortex was a sufficient stimulus to evoke OAAD in two of three loci tested. Small depletions which were not significantly greater than those obtained on piqure of the cortex alone were obtained from a variety of loci in

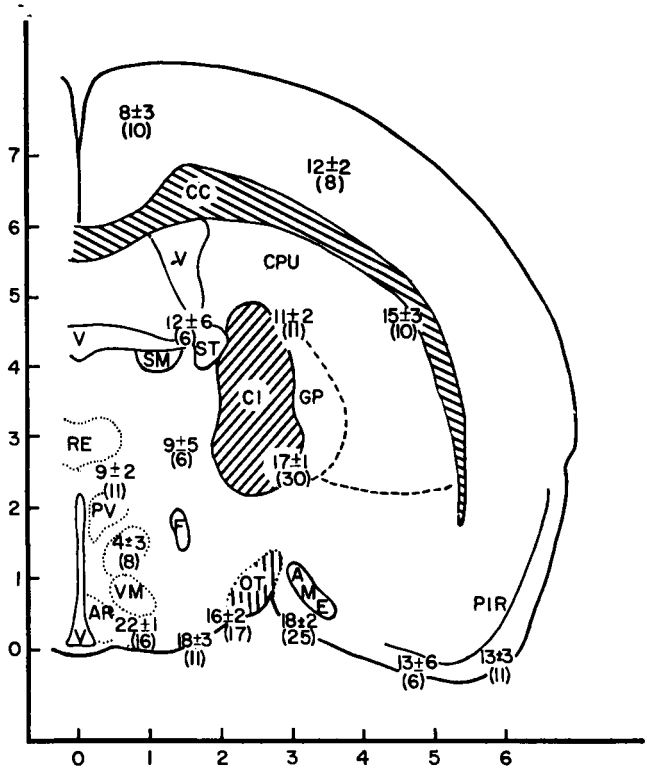

FIG. 1. Transverse section through hypothalamus, 6 mm anterior to the ear bar illustrating ovarian ascorbic acid depletion from piqure of various loci. Numbers on axes refer to stereotaxic coordinates Results are given as mean ± SE (number of rats tested). Abbreviations: *V*, ventricle; *CC*, corpus callosum; *CPU*, nucleus caudatus-putamen; *SM*, stria medullaris thalami; *ST*, stria terminalis; *CI*, capsula interna; *GP*, globus pallidus; *RE*, nucleus reuniens thalami; *PV*, necleus paraventricularis; *F*, fornix; *VM*, nucleus ventromedialis; *AR*, nucleus arcuatus; *OT*, tractus opticus; *AME*, nucleus amygdaloideus medialis; *PIR*, cortex piriformis. Loci were confirmed by histologic examination of 140 brains.

the hypothalamus (Fig. 1, Table I). By contrast, piqure of the median eminence evoked an OAAD which was significantly greater than could be accounted for by piqure of the overlying structures (Fig. 1). Piqures anterior or posterior to this structure likewise produced significantly

smaller responses than piqure of the median eminence (Table I). By contrast, piqure of the hypothalamic regions lateral to it evoked responses which were almost as great as those obtained in the median eminence (Fig. 1). This active region extended laterally into subcortical structures. Insertion of the needle into the amygdala was found to evoke a marked response which was not significantly less than that obtained in the median eminence. If the needle was inserted but stopped just dorsal to the amygdala (Fig. 1) a marked response was still present. There appears to be an active focus in this general region. However, it was difficult to proceed further with localization using this approach since piqure of adjacent structures produced responses which in some instances were not significantly less than those seen in this globus pallidus-amygdala region.

These results indicate that piqure of a variety of loci in the brain can evoke LH secretion as judged by OAAD. The median eminence appears to be the most active focus. However, an active area seems to extend laterally from this site into subcortical structures. The mechanism by which piqure evokes LH discharge is not known, but it may operate by mechanical stimulation of the neurons involved. Apparently wide areas of the forebrain have connections with the LH regulating mechanisms in the hypothalamus.

Since hypothalamic lesions were effective in blocking LH secretion, which in turn could be induced by hypothalamic piqure, a hypothalamic influence over LH secretion was established. It was important to elucidate the mechanism by which the hypothalamus regulates this tropin. Reasoning by analogy with the regulatory system for ACTH, it appeared likely that a hypothetical LH-releasing factor (LH–RF) might stimulate secretion of LH.

Crude acidic extracts of rat stalk–median eminence tissue have ACTH-releasing activity (McCann and Haberland, 1959). They also evoke OAAD on intravenous injection into immature females pretreated with gonadotropins as described above (McCann *et al.*, 1960). This effect could not be accounted for by possible LH contamination of the extracts since injection of the extract into hypophysectomized rats that were normally sensitive to LH produced a smaller effect on ovarian ascorbic acid. Further evidence that LH was not responsible for the activity was provided by the demonstration that rat hypophyseal LH activity was completely eliminated by heating for 10 minutes in a boiling water bath, whereas the activity in the hypothalamic extract was unaffected (Fig. 2). Thus, it appeared that stalk–median eminence extract had evoked a release of LH from the hypophysis of the test rats.

A variety of pharmacologically active agents present in hypothalamus were then screened for activity. The activity in the hypothalamic extract could not be accounted for by its content of vasopressin, oxytocin, or of these two substances mixed together. Nor could it be ex-

plained by the quantity of serotonin, substance P, histamine, or epinephrine known to be present. Since the activity was partially eliminated by peptic, and completely eliminated by tryptic, digestion, it appears that the active principle may be a polypeptide.

Extracts from several portions of the brain were tested to determine whether the activity was localized to any region. Extracts of cerebral

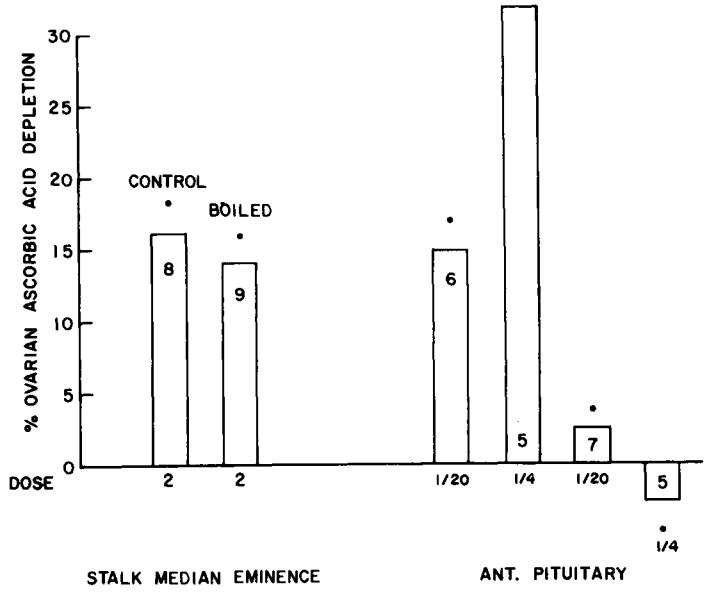

FIG. 2. Effect to boiling on the activity of extracts. Numbers on abscissa refer to number of stalk-median eminences or anterior pituitaries used. Numbers at top of each column indicate number of rats injected, and dots indicate standard error of the mean. From McCann (1962a).

cortex and of posterior, lateral, or caudal hypothalamus were devoid of activity. Activity, but much less than that found in the stalk–median eminence region, was found in extracts prepared from the ventral hypothalamus immediately overlying the median eminence and from the chiasmatic region. This latter piece of tissue included the supraoptic and suprachiasmatic nuclei.

The effect of intravenous injection of stalk–median eminence extract on plasma LH activity was also evaluated (McCann, 1962b). Ten minutes after intravenous injection of the extract into a given type of experimental animal, the rats were bled and 2 ml of the heparinized plasma injected into immature rats for determination of OAAD. This method of testing has been used in all experiments described below. The stalk–median eminence extract evoked a significant elevation in plasma LH levels in normal rats and in ovariectomized rats in which LH secretion had been inhibited, either by prior treatment with single

injections of estrogen, or by lesions in the median eminence. No effect on plasma LH was produced in hypophysectomized rats, again indicating the absence of LH in the extract. Surprisingly, hypothalamic extract had no effect on plasma LH in ovariectomized rats. An explanation of this finding is that the pituitaries of such animals are already responding maximally to the release of endogenous LH–RF. Consequently, exogenous LH–RF has no additional effect. Conversely, the response to LH–RF has not been blocked by large doses of estrogen

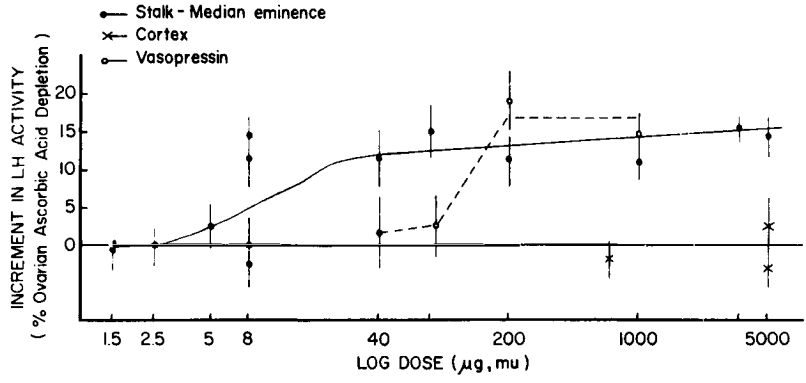

FIG. 3. Increment in plasma LH activity produced by injection of various substances in ovariectomized, estrogen-progesterone blocked rats. Vertical lines represent SEM.

or estrogen plus progesterone. This suggests that the inhibitory effects of ovarian steroids are mediated at some point proximal to the pituitary, presumably at the hypothalamic level (McCann, 1962a).

Recently we have investigated the ovariectomized estrogen–progesterone blocked rat as a possible assay animal for LH-releasing substances. Single subcutaneous injections of 50 μg estradiol benzoate and 25 mg progesterone were administered to chronically ovariectomized rats 3 days prior to use. The substance to be tested was injected intravenously, and 10 minutes later blood was taken for study as described above. The OAAD produced by 2 ml of plasma from rats given the test substance was evaluated and plasma from control donors was tested simultaneously. The results were expressed as the increment in plasma LH activity produced by the test substance (Fig. 3).

This system is a highly sensitive method for demonstrating LH-releasing activity. Stalk–median eminence extract was active at a dose of 40 μg wet weight of tissue. This dose is about twenty to thirty times less than the minimal effective dose obtained when extract was infused directly into immature assay rats. Activity was demonstrated in two of four experiments with a dose of only 8 μg. By contrast, cerebral cortex extract was inactive at a dose of 5000 μg. For the first time with this system, vasopressin possessed significant activity; the mini-

mal effective dose was 200 mU. Since hypothalamic extract was active at a dose estimated to contain no more than 0.4 mU, it is clear that vasopressin cannot account for the activity of the hypothalamic extract. One interesting feature about this assay system is the fact that under the present conditions the response appears to be all or none. Once significant LH release has been induced, additional release does not appear to occur on elevating the dose of extract.

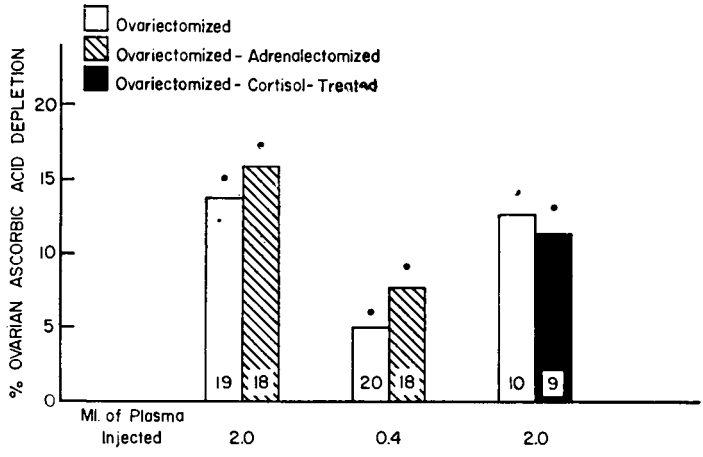

FIG. 4. Lack of effect of adrenalectomy or cortisol on plasma LH activity of ovariectomized rats. Numbers at base of columns indicate number of test animals injected; dots give the standard error of the mean.

These results indicate quite clearly that crude extracts of rat stalk–median eminence have a LH-releasing action. It is crucial to determine whether this LH–RF is distinct from the corticotropin-releasing factor (CRF) also found in these extracts. As a first step in answering this question, the effect of alterations in ACTH secretion on LH discharge has been evaluated. The LH activity in plasma of etherized, ovariectomized rats was compared with that in plasma of etherized, adrenalectomized–ovariectomized rats. No difference in LH activity was found (Fig. 4). Conversely, pretreatment of ovariectomized rats with 8 mg of hydrocortisone per 100 gm body weight intraperitoneally 4 hours before bleeding failed to alter LH titers in ovariectomized animals (Fig. 4). Adrenalectomy and hydrocortisone treatment are known to elevate and suppress, respectively, the release of ACTH, and yet no alteration in LH secretion occurred. Consequently, it appears that the mechanism of control of the two tropins is discrete, a result consistent with the hypothesis that CRF and LH–RF are separate entities.

Additional evidence for a hypothalamic gonadotropin-releasing factor has been obtained independently in Harris' laboratory by Campbell *et al.* (1961) and Nikitovitch-Winer (1962). They have shown in

rabbits and rats, respectively, that injection of stalk–median eminence extracts directly into the hypophysis will evoke ovulation. Extracts from other regions of the brain and pharmacologically active agents known to be present in hypothalamus were inactive in their test system. Their work employing an entirely different method leads to the same conclusions as those reached in this laboratory.

Summary and Conclusions

1. The ovarian ascorbic acid depletion method has been used to study the factors regulating the secretion of LH.

2. LH secretion is subject to negative feedback control by the ovarian steroids estrogen and progesterone. It is also inhibited by lactation, possibly acting via the suckling stimulus.

3. Hypothalamic lesions interfere with the secretion and storage of LH; conversely, LH secretion is induced by hypothalamic piqure.

4. Crude extracts of the stalk–median eminence region evoke LH secretion in a variety of test situations. This activity is not due to LH or to contamination with a variety of known pharmacologically active agents. It is trypsin labile, a fact suggesting that the active substance may be a polypeptide. The most sensitive test system so far described for this LH–RF is the ovariectomized estrogen–progesterone blocked rat.

5. Since conditions which markedly alter ACTH secretion have little or no influence on LH secretion, it is postulated that the LH–RF is not identical with CRF.

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DISCUSSION

Control of Ovulating Hormone Release

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I. The Background

Dr. McCann's excellent paper is very welcome to us as a quite emphatic contribution to a problem we have been approaching by a different method. Studies involving pituitary stalk section (Brown-Grant *et al.*, 1957) and electrical stimulation of the hypothalamus (Harris and Woods, 1958; Campbell *et al.*, 1960) lent support to the concept that the median eminence liberates a substance into the pituitary portal vessels which evokes the release of adenohypophyseal hormones. We began, in 1957, to study the problem of extracting from median eminence tissue a substance that would cause the release of luteinizing hormone (LH). At that time the Parlow assay was not available, and we were led to establish a method similar to one that we later discovered had been employed by von Euler and Holmgren (1956) for a different purpose. Our aim was to avoid intravenous administration of brain extracts in order (1) to conserve precious material by using small doses, and (2) to bring a high concentration of extract directly in contact with anterior pituitary cells. A brief account has been published (Campbell *et al.*, 1961).

II. The Method

A. pituitary cannulas

Accordingly we developed, after many trials and as many errors, a cannula which could be implanted in the head of a rabbit so that one end was in the anterior pituitary gland and the other end was available for injections. Essentially, the device is a length of platinum tubing held rigidly in a brass support. This is implanted into the rabbit with the aid of a stereotaxic instrument and lateral radiographs to guide the exact localization of the cannula tip in the anterior lobe. The device is held in place by a mound of acrylic cement which is anchored to the skull by stainless steel screws. A screw cap protects the protruding end of the cannula. Figure 1 shows a cannula in position (the acrylic cement is transparent to X-rays).

During intrapituitary infusions in conscious, unrestrained rabbits, the screw cap is removed and a length of polythene tubing is connected to the free end of the cannula. The other end of the tubing is connected to a small syringe held in a slow-injection machine. Pilot infusions followed by sectioning of the pituitary indicated that with an infusion rate of 1.1 μl per minute continued for 2 hours (total volume infused, 0.134 ml) very little pituitary damage could be detected. Such 2-hour infusions were made stan-

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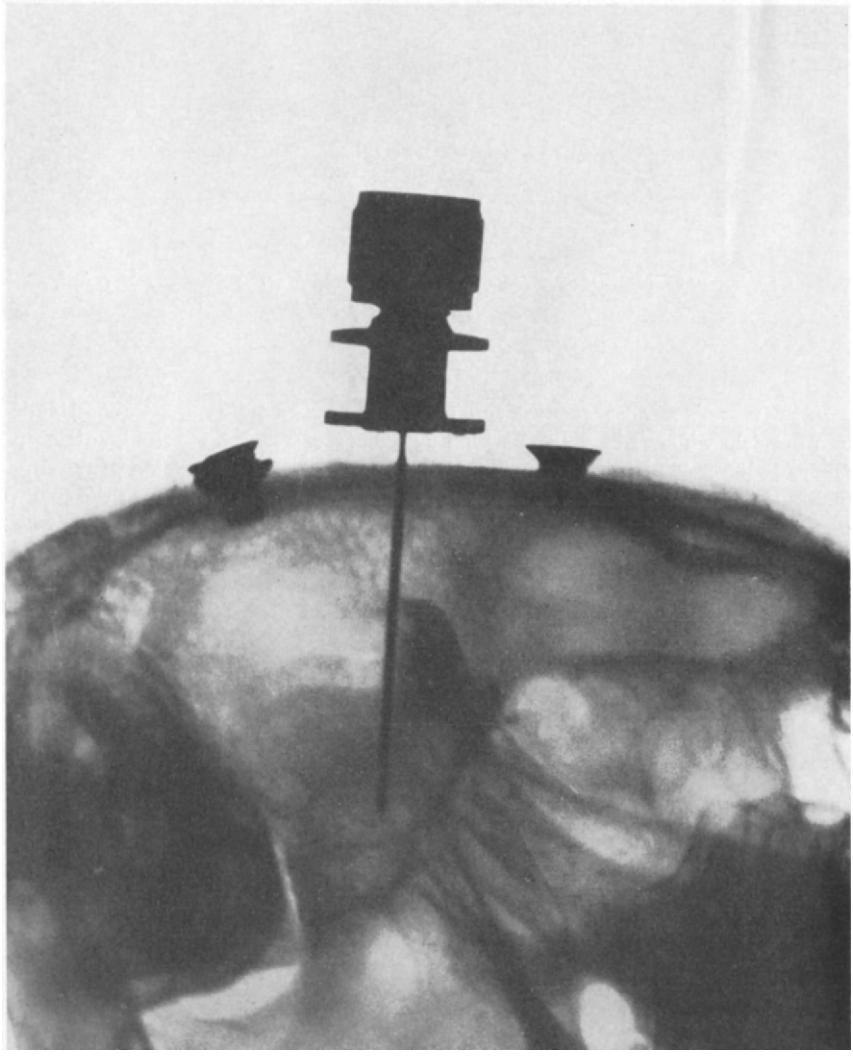

FIG. 1. Lateral radiograph of rabbit head with cannula in place. Tip of cannula is in the anterior lobe of the pituitary gland.

dard with a variety of substances and were controlled by a series of intravenous infusions (2.10 ml over 2 hours into a marginal ear vein). Every pituitary gland was subsequently sectioned to check the location of the cannula tip.

B. EXTRACTS

Intrapituitary infusions made with crude extracts of median eminence tissue from normal estrous rabbits proved promising in that a high proportion of animals were found to have ovulated when the ovaries were examined 48 hours after the infusions.

In view of the small size of the rabbit median eminence (approximately 7 mg), material was collected from slaughtered cattle, in which the median eminence is appreciably larger (approx, 100 mg). Extracts were made very simply by homogenizing the tissue in 0.5% acetic or hydrochloric acid, a procedure followed by neutralization, adjustment of tonicity, and centrifugation. An interesting point is that the activity of the extract was unaffected by exposure for 10 minutes to boiling water—a treatment that would effectively destroy any LH contaminant. Similar extracts were made from other parts of the cattle brains and infused under the same conditions. More recently we have prepared such extracts from normal male and female rhesus and macaca monkeys.

III. RESULTS

A. BRAIN EXTRACTS

Table I gives some of the results obtained with cattle material at a dose level of 20 mg

TABLE I

Effect of Intrapituitary and Intravenous Infusions of Cattle Brain Extracts (20 mg dose) *on Ovulation in the Rabbit*

Source of extract	Intrapituitary		Intravenous	
	Number of rabbits	Ovulation	Number of rabbits	Ovulation
Cerebral cortex	13	0	—	—
Caudate nucleus	6	1	—	—
Corpus callosum	12	0	—	—
Median eminence	28	18	9	0

equivalent of fresh tissue. It may be seen that 64% of the rabbits given intrapituitary infusions of median eminence extract responded by ovulating, whereas ovulation was virtually absent in rabbits given similar infusions of control brain extracts. A significant proportion (11 out of 22) of ovulations was obtained with intrapituitary doses of 2.5 mg equivalent of fresh tissue. But the *intravenous* infusion of 100 mg equivalent led to ovulation in only 1 out of 7 animals. Results comparable to these were obtained with extracts from the monkey median eminences. We are pleased that Dr. McCann has found activity in the median eminence of the rat, and Dr. Guillemin in the sheep. Species specificity has been unequivocally ruled out.

B. CONTROL SUBSTANCES

It seemed likely, then, that an LH-releasing substance was present specifically in the median eminence. Standard intrapituitary infusions were made with various compounds known to be present in this region, to see whether some familiar substance was responsible—especially as reports had begun to appear about the control of the anterior pituitary gland by materials traditionally associated with neurohypophyseal function.

Table II gives the results obtained with control substances. It may be seen that, under the conditions of these experiments, there is little scope for assigning an LH-releasing role to neurohypophyseal principles. This conclusion has since been reached by Dr.

TABLE II

Effect of Intrapituitary Infusions of Control Substances on Ovulation in the Rabbit

Substance	Dose	Number of rabbits	Ovulation
Synthetic lysine-vasopressin	1.34 and 2.68 IU	9	0
Synthetic oxytocin	0.40 IU	8	0
Lysine-vasotocin	2.7 and 4.8 IU	9	0
Substance P	20 IU	8	0
Epinephrine	120 μg	8	1
Histamine	120 μg	8	1
Serotonin	1 mg	4	0

McCann, Dr. Guillemin's group (Courrier *et al.*, 1961), and others. Table II also indicates that the active substance, which has been designated LRF, is probably an unknown compound.

Our work is now proceeding along more chemical lines, with the help of Dr. D. Elliott and Dr. P. Fawcett at the National Institute for Medical Research, Mill Hill, London. These workers are patiently separating the many unidentified polypeptide-like compounds present in the median eminence, and we are patiently testing the as yet crude mixtures for LH-releasing activity. At the present there is little to be said about this investigation except that we are having no difficulty in finding inactive fractions.

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Synthesis and Release of Progestin by Rabbit Ovary in Vivo[1]

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I. INTRODUCTION

Since the classic work of Corner and Allen (1929), progesterone has been defined as "the hormone of the corpus luteum," and its functon has been directly associated with pregnancy. A hypothesis has evolved which proposes that the ovary first secretes estrogen to bring an animal into behavioral estrus and then, following ovulation, the ovary secretes progesterone to prepare the uterus for implantation and maintenance of the fertilized ova. Evidence has been accumulating, however, that progesterone may be secreted prior to ovulation. Moreover it has been shown that, in addition to its action on the uterus, progesterone synergizes with estrogen to exert profound effects on the central nervous system.

In ovariectomized guinea pigs, rats, and mice, sexual activity is heightened if estrogen is supplemented by progesterone (Dempsey *et al.*, 1939; Boling and Blandau, 1939; Ring, 1944). In normal rabbits, progesterone will initially facilitate and later inhibit mating activity as well as ovulation to mechanical stimulation of the vagina or intravenous injection of copper acetate (Sawyer and Everett, 1959). The latter results were found to be attributable to the sequential facilitatory and inhibitory effects of progesterone on the release of ovulating hormone from the pituitary, and both effects have been correlated with altered thresholds of cerebral activity (Kawakami and Sawyer, 1959).

In the rabbit, ovulation is triggered by the coital stimulus, follicle rupture occurring not less than 10 hours and usually about 12 hours later. However, Verly (1951) has measured increased urinary excretion of pregnanediol in female rabbits shortly after mating; and Forbes (1953), using the mouse intrauterine bioassay, has detected significant elevations in progestin activity of periph eral blood drawn from does

[1]Supported in part by United States Public Health Service grants, AM-06106-02 and B 2983

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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within 100 minutes following coitus or 60 minutes after the intravenous administration of gonadotropin. Although the ovarian source of this activity has been questioned because the high values obtained with the Hooker–Forbes bioassay could not be corroborated by physicochemical methods of progesterone assay, the possibility has remained

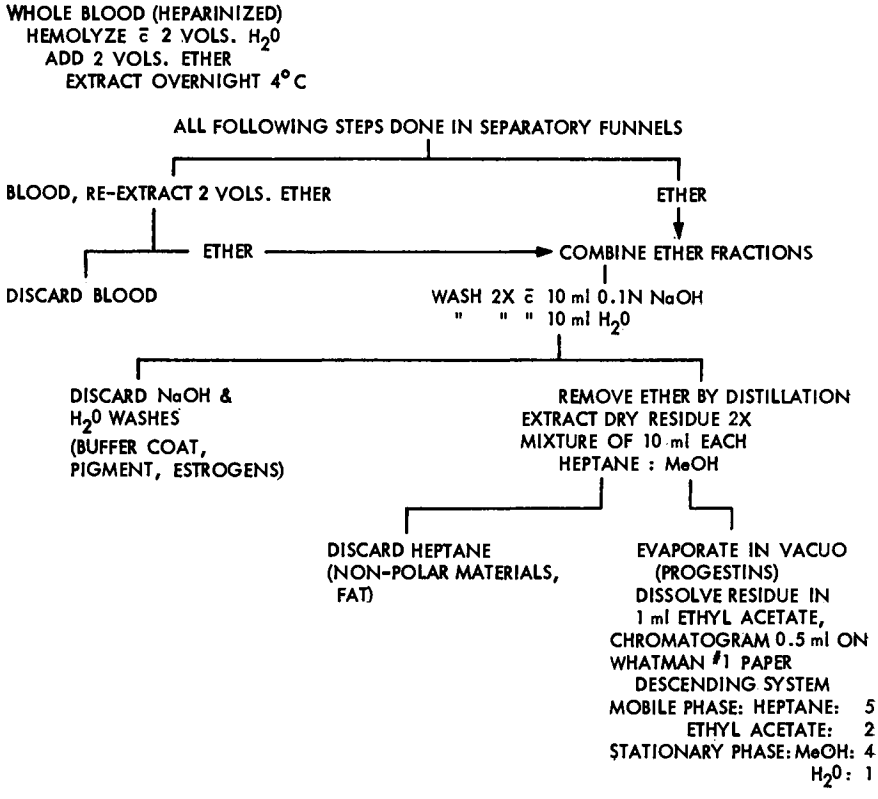

FIG. 1. Extraction and chromatographic system for progestin.

that the progestin activity detected by Forbes could be due to some ovarian gestogen other than progesterone. This possibility has been strengthened by the isolation from ovarian tissue of two steroid isomers closely related to progesterone in structure, which show progestin activity in the Hooker–Forbes mouse and the Clauberg rabbit bioassays (Zander *et al.*, 1958; Short and Moore, 1959; Kristofferson, 1960). Although neither isomer had been reported previously in rabbits, our studies (Hilliard *et al.*, 1961) indicated that the α-isomer, Δ^4-3-ketopregnen-20α-ol,[2] is the principal ovarian progestin produced

[2] After Zander *et al.* (1958). This steroid is identical with 20α-hydroxypregnen-4-en-3-one. IUPAC. "Nomenclature of Organic Chemistry." Butterworths, London, 1957.

by this species and that it is present in and secreted by ovaries in amounts approximately tenfold greater than progesterone. Moreover, following mating or single intravenous doses of gonadotropins that induce ovulation, both progesterone and the 20α-ol steroid are rapidly synthesized and released whereas moderate increases in synthesis and release may follow chronic administration of lactogenic hormone.

II. METHODS

More than 200 New Zealand white rabbits (3.5–5.0 kg in weight) were used. Progestin content of ovarian vein blood and ovaries were assayed according to a chromatographic procedure (Fig. 1) previously described (Hilliard *et al.*, 1962). Blood was collected under Nembutal anesthesia at estrus, during pregnancy and pseudopregnancy, and immediately following mating or intravenous administration of pituitary hormones and other ovulatory agents such as human chorionic gonadotropin (HCG), pregnant mare's serum (PMS), and copper acetate.

III. RESULTS AND DISCUSSION

A. RELEASE OF PROGESTIN FROM OVARIES WITH AND WITHOUT CORPORA LUTEA

In addition to traces of progesterone, substantial amounts of Δ^4-3-ketopregnen-20α-ol,[3] ranging from nondetectable quantities to 250 μg/hr per gland were released from estrous ovaries without corpora lutea as well as from those in which functioning corpora lutea were present. In general, the highest output of both progestins was obtained from the largest ovaries containing corpora lutea, but no correlation could be made with the number of functioning corpora lutea present.

Within 10 to 25 minutes after successful mating or the injection of a gonadotropin which induced ovulation—i.e., pituitary luteinizing hormone (LH) 1.0–4.0 Armour units; follicle-stimulating hormone (FSH) 1.0–2.0 mg NIH–FSH or 2.25–5.0 Armour units; as well as gonadotropins of extrapituitary origin, PMS 250 IU or HCG 25–250 IU—levels of progestin in the ovarian blood rose abruptly, as shown in Figs. 2 and 3, and reached a maximum in approximately 1 hour. Subovulatory doses of LH ($^1/_3$–$^2/_3$ Armour unit) or NIH–FSH (0.3 or 0.5

[3]We are indebted to Dr. Hans Simmer, Department of Gynecology and Obstetrics, UCLA Medical School, for further identification of this steroid in our extracts of rabbit ovarian vein blood. Dr. Simmer's criteria of identification were (1) paper chromatography in three different systems; (2) acetylation; (3) oxidation; (4) ultraviolet spectrum; (5) spectrum in H_2SO^3; and (6) infrared spectrum (Endocrinology **72**,:67, 1963).

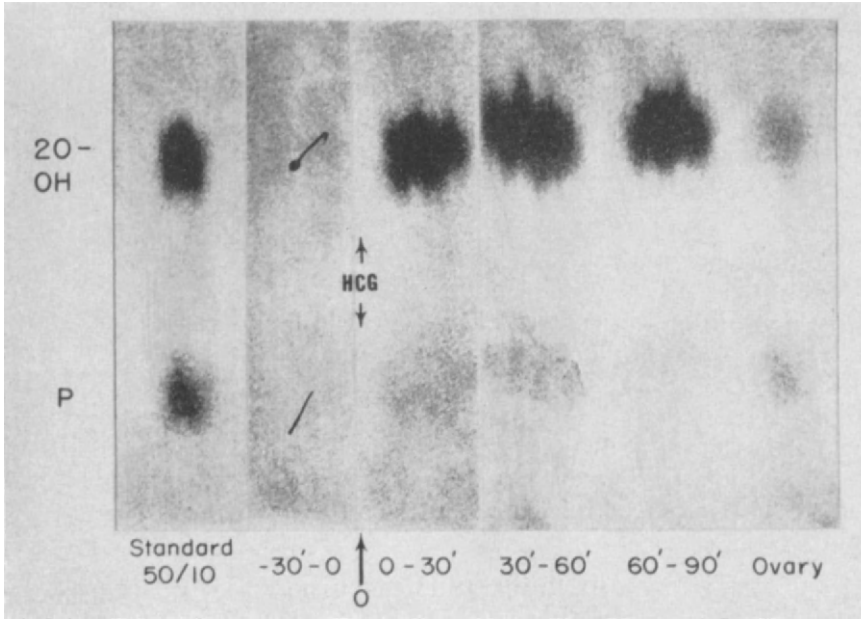

FIG. 2. Chromatogram of ovarian effluent before and immediately after intravenous administration of gonadotropin. Blood samples were collected for 30-minute periods and one-half of each sample was chromatogrammed. Left-hand column shows the standard: 50 μg Δ^4-3-ketopregnen-20α-ol and 10 μg progesterone. Right-hand column is chromatogram obtained from half of the ovary which was removed at the end of collections. From Hilliard *et al.* (1962).

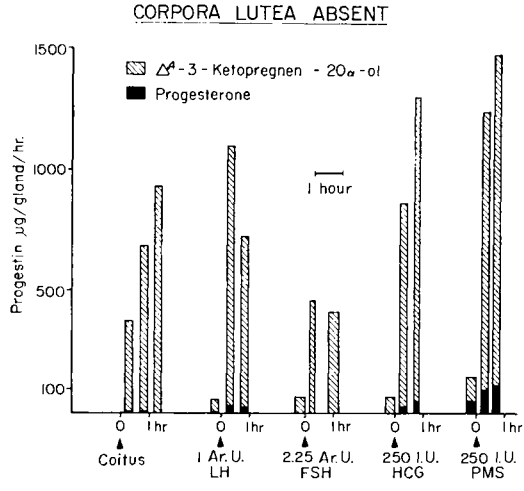

FIG. 3. Progestin release from nonluteinized rabbit ovary following coitus and treatment with gonadotropins which induced ovulation. From Hilliard *et al.* (1961).

mg) elicited small responses. The already elevated output of steroid within a few minutes postcoitum (Fig. 3) is good indirect evidence that the coital stimulus exerts an almost immediate effect on the pituitary gland and that discharge of ovulating hormone begins within minutes.

Ovulating doses of copper acetate (3 mg/kg, i.v.) in primed animals also induced an increase in progestin output, but the rise did not occur immediately, rather it was delayed for about 2 hours. It was necessary to inject the copper salt some time before surgery. Both the elevation in output of progestin and ovulation following copper failed to occur under certain conditions in which exogenous gonadotropin was effective: if pentobarbital (approximately 25 mg/kg) was given either before or as much as 30 minutes after the copper, the combination of the anesthetic and the surgical trauma of cannulation appeared to block activation of the pituitary gland. This result suggested that copper acts slowly in stimulating the release of pituitary ovulatory hormone and probably exerts its stimulating effect indirectly via the central nervous system. In all instances, however, the increased output of progestin was independent of follicular rupture since ovulation in our rabbits occurs 10–12 hours after the ovulating stimulus.

On the other hand, no significant changes in progestin release were observed following intravenous injection of hormones which failed to induce ovulation in primed animals, i.e., adrenocorticotropin (ACTH), 4 IU; thyrotropin (TSH), 2–5 USP units[4]; lactogenic hormone (LGH), 200 IU; Syntocinon,[5] 10 U; or Pitressin, 2 U.

Growth hormone proved to be an exception. Although never inducing ovulation in dosage up to 10 mg (10 Armour units), intravenous injections of 5 mg of either Armour STH (5 units) or NIH–GH were followed by significant elevations in progestin output from large ovaries containing either corpora lutea or corpora albicantes and which were often releasing substantial amounts of progestin prior to treatment with growth hormone. This suggested the possibility that such ovaries had received some stimulus before the experimental procedures were initiated and that the changes induced by growth hormone were of a synergistic nature. Since many of these animals showed evidence of lactation, in addition to having stimulated ovaries, lactogenic hormone was suspected as the possible synergist.

[4]A highly purified TSH preparation (15–20 USP units/mg) supplied through the courtesy of Drs. John Pierce and Mary Carsten, Department of Physiological Chemistry, UCLA Medical School. Commercial TSH preparations proved to be highly contaminated with gonadotropin and induced both ovulation and significant elevations in progestin output at these dose levels.

[5]Supplied by Sandoz Pharmaceuticals, Hanover, New Jersey, courtesy of Dr. H. Althouse.

B. RESPONSE OF OVARIES TO CHRONIC TREATMENT WITH LGH, ESTROGEN, AND STH

Although single injections of lactogenic hormone had failed to stimulate progestin release from ovaries with corpora lutea (Fig. 4), prolonged intraperitoneal injection of LGH (100 IU twice daily, 2 weeks) to four pseudopregnant rabbits increased the basal release of progestin. This was in contrast to the effect of prolonged subcutaneous injection

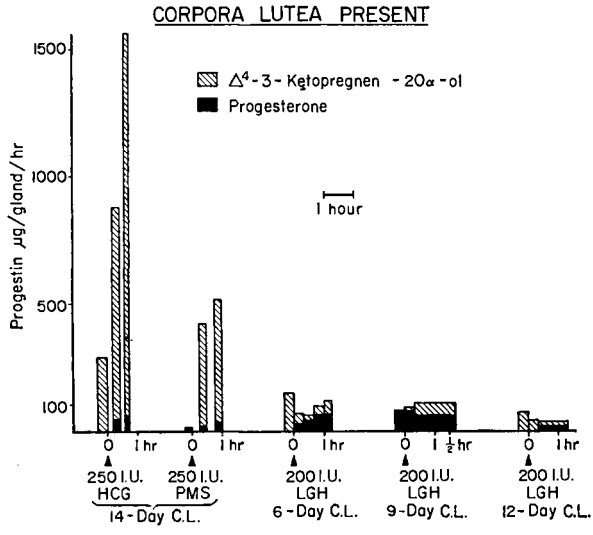

FIG. 4. Typical effects of treatment with placental gonadotropins and lactogenic hormone (LGH) on progestin release from rabbit ovary during pregnancy or pseudopregnancy. C. L., corpus luteum. From Hilliard *et al.* (1961).

of estradiol benzoate (0.1 mg in oil alternate days, 2 weeks), and a 2-week treatment with growth hormone (2.5 mg twice daily, i.p.) failed to affect progestin release significantly (Fig. 5). Following these respective chronic treatments, i.e., LGH, estrogen, and STH, ovaries still responded to acute doses of gonadotropin with further increase in progestin output.

Although lactogenic hormone has been shown to be luteotropic in hypophysectomized rats (Evans *et al.*, 1941), its luteotropic activity has not been demonstrated in hypophysectomized rabbits (Mayer, 1951). Injections of exogenous estrogen or estrogen crystal implants within corpora lutea have luteotropic activity in rabbits independent of the hypophysis (Robson, 1937; Hammond and Robson, 1951), and operative reduction of ovarian interstitial tissue in pseudopregnant rabbits results in corpus luteum regression which can be prevented by estrogen (Westman, 1934). Therefore, the theory has been proposed by Everett

(1961) that, in rabbits, FSH and LH released from the hypophysis liberates estrogen from the interstitial tissue, and this steroid in turn stimulates the corpora lutea to secrete progesterone. The present results are of a preliminary nature, but they suggest that lactogenic hor-

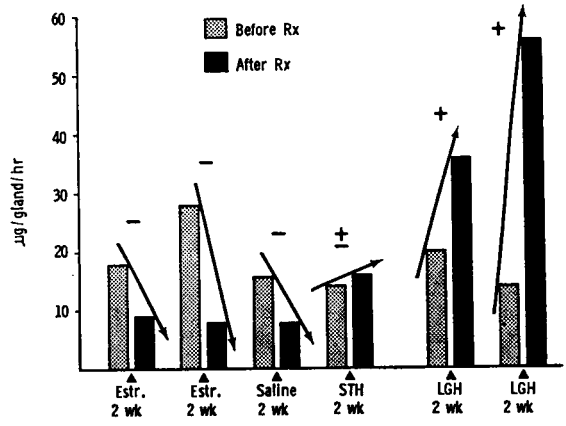

FIG. 5. Effect of prolonged treatment with estrogen, growth hormone (STH), and lactogenic hormone (LGH) on basal progestin release from pseudopregnant rabbit ovaries.

mone may exert luteotropic effects, possibly of a synergistic nature inasmuch as our rabbits were not hypophysectomized.

C. OUTPUT OF PROGESTIN FROM OVARIES WITHOUT FOLLICLES

Since estrous ovaries without corpora lutea released large amounts of progestin following gonadotropic stimulation, the possible participation of follicles in the elaboration and release of progestin in estrous ovaries was tested by destroying the mature follicles. In this experiment the follicles were cauterized from one ovary just prior to cannulation, and the normal ovary served as a control. Blood was collected from both ovaries simultaneously. Table I shows the results obtained on the normal and cauterized ovary of one animal before and after gonadotropic stimulation (200 IU HCG). At the end of the experiment the two ovaries were removed, weighed, and assayed for progestin content. Similar results were obtained on three additional animals. Considering the possible damage to circulation and interstitial tissue, the differences in progestin output and content of the normal and cauterized ovaries did not appear to be significant.

TABLE I

Progestin Content and Output from Ovary with Cauterized Follicles[a,b]

Sample	Collection time (min)	Blood (ml)		Progestin released (μg)	
		Control	Caut.	Control	Caut.
A	–30–0	35		13.5	
A^1			30		17.5
		250 I. U. HCG			
B	0–30	34		89.2	
B^1			38		91.2
C	30–60	25		87.8	
C^1			29		106.0
D	60–90	22		143.8	
D^1			28		108.2
		116	125	334.3	322.9
Terminal ovarian content (μg/100 mg ovary)				5.5	8.6

[a]Comparison of blood output and progestin release from the normal ovary and the cauterized ovary (Caut) of one animal. Samples A, B, C, and D were collected from the normal ovary. Samples A^1, B^1, C^1, and D^1 were collected simultaneously from the opposite ovary from wich the follicles had been removed by cauterization. After blood collections, both ovaries were removed and assayed for progestin content.

[b]From Hilliard *et al.* (1962).

D. PROGESTIN CONTENT OF OVARIES BEFORE AND AFTER GONADOTROPIC STIMULATION

Neither progesterone nor the 20α-ol steroid was measurable in unstimulated, immature ovaries in which interstitial tissue was pink and follicles were small and opalescent. In estrous ovaries weighing more than 200 mg apiece and having white interstitial tissue and clear, raised follicles but lacking corpora lutea, measurable amounts of the 20α-ol steroid and traces of progesterone were usually detectable. If one ovary was removed immediately before the administration of gonadotropin (LH and HCG) and the second ovary was removed $1^1/_2$ to 2 hours afterward, the ovary removed *after* gonadotropin administration invariably contained the greater amount of progestin. The increase in content as well as the increase in output which follows gonadotropic stimulation must, therefore, be attributed to immediate synthesis rather than to a release of preformed hormone stored in the gland. In contrast, the amount of total cholesterol in the stimulated ovary dropped after administration of gonadotropin (Table II).

II), and if ovarian cholesterol was prelabeled by intravenous injection of 40 μc of 4-C^{14}-cholesterol 24 hours before cannulation, some of the label was incorporated by both progestins (Hilliard, Meier, and Sawyer, unpublished 1962).

TABLE II

Effect of Gonadotropic Stimulation on Ovarian Content of Progestin and Total Cholesterol[a]

Rabbit no.	Ovary		Time after HGG (hr)	Steroid content	
	Side	Weight (mg)		Progestin (μg)	Cholesterol (mg)
I	Control	377	0	8.3	7.58
	Control	420	0	8.3	7.49
II	Control	378	0	7.7	7.97
	Stim.	392	2	23.0	3.17
III	Control	640	0	41.5	2.09
	Stim.	756	2	45.5	0.91
IV	Control	314	0	14.0	2.40
	Stim.	313	2	32.6	1.40
V	Control	459	0	Trace	3.28
	Stim.	505	2	27.2	2.13
VI	Control	610	0	49.0	1.27
	Stim.	606	1.5	72.0	1.03

[a]Results obtained on six rabbits. The first received no gonadotropin. The control ovary of rabbits II–VI was removed just prior to HCG administration, and the second ovary was removed $1^1/_2$–2 hours later. Progestin content of the stimulated ovary increased whereas total cholesterol content decreased (Hilliard, Meier, and Sawyer, unpublished 1962).

A marked fall in the esterified cholesterol of rabbit ovarian interstitial tissue following administration of gonadotropin has been reported by Claesson *et al.* (1953), who concluded that cholesterol must be the principal precursor of estrogen. Our results imply, however, that the rapid change in ovarian cholesterol may also be correlated with progestin synthesis.

E. ROLE OF OVARIAN INTERSTITIAL TISSUE IN PROGESTIN SYNTHESIS AND RELEASE

Since it is possible to stimulate the release of progestin from ovaries without corpora lutea and following destruction of the Graafian follicles, the interstitial tissue appears to serve as the site for at least the immediate synthesis which follows acute dosage with gonadotropins.

In the estrous rabbit ovary, this tissue is so profuse as to be called "the interstitial gland" and the cellular elements bear a marked resemblance to luteal tissue. There is also a further development of the interstitium during pregnancy and pseudopregnancy which appears to be maintained throughout lactation. It is appropriate that the pituitary hormone which most readily stimulates the rapid synthesis of progestin from the interstitial tissue is LH, otherwise known as ICSH (interstitial cell-stimulating hormone). The rapid postcoital release of this hormone has already received comment above.

IV. SUMMARY AND CONCLUSIONS

In the rabbit, the principal progestin present in and released by the ovary is Δ^4-3-ketopregnen-20α-ol.

This progestin is released in basal amounts from mature ovaries without corpora lutea as well as from ovaries in which corpora lutea are present.

It is rapidly synthesized and released from the ovary in response to mating or single, minimal ovulatory doses of gonadotropin, but not in response to other pituitary tropins. Under certain circumstances growth hormone appears to be an exception.

Ovarian interstitial tissue appears to be the site of rapid synthesis following acute dosage with gonadotropin.

Chronically progestin is synthesized and released in moderate amounts in response to prolonged treatment with lactogenic hormone, and following such treatment, the ovaries can still respond to gonadotropic stimulation.

ACKNOWLEDGMENTS

Progestin assays were performed by Miss Donna Archibald, for whose inspired technical assistance we are deeply grateful.

We are indebted to Dr. Jacob Stucki, The Upjohn Co., Kalamazoo, Michigan, for supplying our Δ^4-3-ketopregnen-20α-ol standard. All Armour hormones were generously supplied by J. D. Fisher, Armour Pharmaceutical Co., Kankakee, Illinois, and NIH preparations were furnished by the NIH Endocrine Section, Bethesda, Maryland.

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Effects of Hypothalamic Deconnection on the Pituitary Control of Testosterone Secretion

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I. INTRODUCTION

The control exerted by the central nervous system on the pituitary regulation of sexual steroid release in the male remains a very complex problem. So far, it has been investigated mainly by means of hypothalamic lesions and deconnection techniques, and various reasons account for its complexity: (1) The literature concerned with this problem refers nearly always to maintenance or atrophy of the whole gonad, without involving a more careful study of the endocrine activity of the testes. Moreover, data on the activity of the interstitial gland generally lack a proper complementary analysis of pituitary gonadotropic function. (2) Besides, and this does not help to solve the question, the hypophyseal control of gonadal activity itself appears to be better known in the female than in the male.

The classical view involving a ICSH (LH) action on the interstitial tissue and a FSH action on the seminal tubes no longer accounts for testicular activity, and a degree of synergism of FSH and LH has to be postulated today to explain the secretion of male hormone. Nor must the action of prolactin be underestimated. After the classical research of Riddle and Bates (1933), prolactin was to a certain extent considered to be an antigonadotropic hormone in the male. However, more recent works point out that this hormone might well have a stimulating effect in the male, either as a third gonadotropic factor acting synergistically with FSH and LH on both seminal and interstitial development, or perhaps synergistically with testosterone on the primary and secondary sex characters.

Segaloff *et al.* (1956) observed a synergistic action of LH and prolactin on the development of the ventral prostate and the seminal vesicles in hypophysectomized male rats. A similar synergism with LH and FSH on the maintenance of the interstitial gland and of the accessory glands of the male genital tract was shown by Woods and Simpson (1961) after injections of purified hormones in hypophysectomized rats. Moreover, the study of the cycle of pituitary gonado-

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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tropic cell activity in the male duck reveals that prolactin-cell activation is synchronized with FSH and LH cell activity in the naturally occurring cycle (Herlant *et al.*, 1960a; Tixier-Vidal *et al.*, 1962) as well as in the experimental photosexual reflex (Tixier-Vidal and Assenmacher, 1961). However, experiments on gonadectomized or hypophysectomized and castrated rats and guinea pigs indicated that prolactin may act synergistically with testosterone on the primary sex characters (Pasqualini, 1953; Chase *et al.*, 1957; Antliff *et al.*, 1960).

One should remember this new conception of the possible role of prolactin when interpreting some of the data to be reviewed below. In spite of this, many observations imply the existence of a stimulating control of the central nervous system over male hormone release, through hypothalamohypophyseal activation.

II. HYPOTHALAMIC DECONNECTION AND SECRETION OF MALE HORMONE

A. AMPHIBIANS

Destruction of the basal hypothalamus, or severance of the hypothalamohypophyseal tract, abolishes the thermosexual reflex in *Triturus cristatus*: i.e., the onset of testicular endocrine activity is no longer brought about by an increase of environmental temperature (Mazzi, 1951).

Ectopic pituitary grafts (at a distance from the median eminence) block sexual differentiation in the larvae of *Pleurodeles waltlii* (Pasteels, 1957, 1960) and in the axolotl (Vunder, 1935) and abolish male hormonal activity in *Rana temporaria* and *Triturus cristatus* (Vivien and Schott, 1957, 1958; Schott, 1960). However, these authors could show that when a pituitary is grafted under the median eminence, the secretion of male hormone remains normal.

B. BIRDS

Quite similar results have been obtained in birds. We noted a total inhibition of testicular endocrine function in the duck *(Anas)*, as well as blockade of its physiological activation by natural or artificial light after various types of surgery.

Testicular hormonal activity was determined by histological examination of the interstitial gland stained by means of the Benoit–Staubli (1951) technique (combining the classical Masson trichromic stain and a mitochondrial staining with Altmann's fuchsin) and by examination of a typical primary sex organ, the epididymis, which is very sensitive to the levels of circulating male hormone (Benoit, 1936).

The interstitial gland and the epididymis show a very characteristic involution, indicative of a total suppression of testosterone secretion,

similar to the changes consecutive to hypophysectomy, after the following operations: (Fig. 1) (1) transection of the hypophyseal portal vessels without nervous lesion (Assenmacher and Benoit, 1953); (2) pituitary graft in the anterior chamber of the eye or in the neck muscles (Assenmacher and Benoit, 1958; Assenmacher, 1958); (3) destruction of the anterior median eminence (Benoit and Assenmacher, 1952); (4) bilateral lesion in the anterior hypothalamus involving the supraoptic nuc-

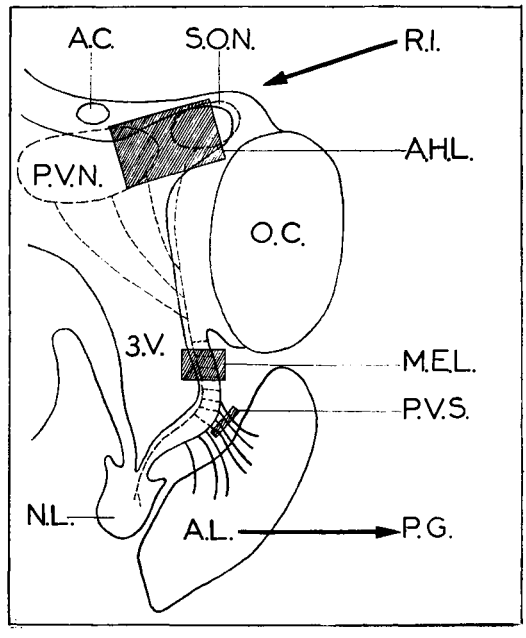

FIG. 1. Hypothalamohypophyseal deconnections which block the endocrine function of the testis in the duck: (1) *P.G.*, Pituitary graft into the anterior chamber of the eye; (2) *P.V.S.*, portal vessels section; (3) *M.E.L.*, median eminence lesion; (4) *A.H.L.*, anterior hypothalamus lesion; (5) *R.I.*, reserpine injections (25 μg/100 gm); *A.C.*, anterior commissure; *A.L.*, anterior lobe of hypophysis; *N.L.*, neural lobe of hypophysis; *O.C.*, optic chiasm; *P.V.N.*, paraventricular nucleus; *S.O.N.*, supraoptic nucleus; 3.*V.*, third ventricle.

leus and the anterior portion of the paraventricular nucleus (Assenmacher, 1957, 1958).

Besides, and without entering into the problem of the primary site of action of tranquilizers, we may add that chronic administration of reserpine at a medium dosage, for example, results in a strong inhibition of the interstitial gland in the male pigeon (Khazan *et al.*, 1960) and the duck (Tixier-Vidal and Assenmacher, 1962) and in a blockade of the light-induced stimulation of hormonal function in the duck (Assenmacher *et al.*, 1961).

C. MAMMALS

In Mammals most experimental results are in accordance with the above-mentioned data referring to other vertebrates.

1. Transection of the Hypophyseal Stalk

Transection of the hypophyseal stalk and of its portal veins results, whenever no vascular regeneration occurs: (1) in an absence of puberty in immature animals (Dott, 1923, in the dog); (2) in a sex hormone insufficiency revealed by the involution of primary and secondary sex characters [Crowe *et al.* (1910), Mahoney and Sheehan (1936), in the dog; Harris (1937), Brooks *et al.* (1940), in the rabbit; Westmann and Jacobsohn (1938), Brolin (1945), Barrnett and Greep (1951), in the rat] or in a severe decrease of urinary 17-ketosteroids in man (Linquette *et al.*, 1961). These effects appear to be absolutely constant.

2. Lesion of the Median Eminence

Destruction of the median eminence always leads to similar results. Young rabbits remain immature (Gaupp and Spatz, 1955); testosterone secretion in adult animals is abolished; the accessory glands of the genital tract resemble those of castrates in the rat (McCann, 1952) and in the dog (Davidson and Ganong, 1960).

3. Administration of Tranquilizers

Chronic administration of reserpine always blocks the endocrine activity of the testes in rats (Gaunt *et al.*, 1954; Tuchmann-Duplessis and Mercier-Parot, 1956; Verne *et al.*, 1957; Eränkö *et al.*, 1957; Khazan *et al.*, 1960) and in mice (Adams and Fudge, 1959). Chronic treatment with morphine leads to the same result (Knappe *et al.*, 1960) in the rat.

But the techniques of ectopic pituitary grafts and of hypothalamic lesions have yielded results which at times appear contradictory.

4. Pituitary Grafts

All authors agree that when the pituitary is grafted in the sella turcica or under the median eminence, a normal secretion of male hormone is maintained [Greep, 1936; Cutuly (1940), after grafts in the sella turcica of the rat; Harris and Jacobsohn (1952), under the median eminence of the rat; Jacobsohn (1954), after grafts under the median eminence of the rabbit]. Such a graft is even capable of stimulating atrophic testes and restoring fertility when performed 2–5 months after hypophysectomy, as could be shown by Smith (1961) in the rat.

On the contrary, several observations led to the classical view that

an ectopic graft is unable to carry on a proper conditioning of the testicular endocrine gland in the rat (Westmann and Jacobsohn, 1940; Cheng *et al.*, 1949; Fortier, 1952; Martini *et al.*, 1959).

Goldberg *et al.* (1955) note that in young rats bearing a graft in the anterior chamber of the eye the testes descend in 44% of the cases; the androgenic secretion, however, is always extremely low. Similarly and under the same conditions, the prostate and seminal vesicles undergo atrophy in adult animals, although their development is slightly greater than in hypophysectomized subjects (Goldberg and Knobil, 1957).

Some authors, however, have observed very different results in this respect.

Fry and Long (1956) noted a normal sexual development of the interstitial gland and of accessory glands after pituitary grafts in the anterior chamber of the eye or under the renal capsule of young rats. Sexual function in such animal is then normal. In the same way, maintenance of the interstitial tissue and of the sex characters was claimed by Cutuly (1940, 1941) after an ectopic graft in the anterior chamber of the eye and by Courrier (1956), Courrier and Collonge (1957, 1959), Courrier *et al.* (1961) after grafting a pituitary in the thyroidal region. Similar observations were described by Petrovic and Aron (1958) and Aron and Petrovic (1959, 1962) in male guinea pigs, but in these experiments the interstitial gland does not seem to have remained as functional as the seminal tubes.

5. *Hypothalamic Lesions*

As a rule, bilateral lesions of the hypothalamus result in an involution of the interstitial gland and, hence, of sex characters. Most of the lesions accounting for these symptoms are located within the basal part of the tuberal hypothalamus (ventromedial and infundibular nuclei). This was shown in the dog (Aschner, 1912; Bailey and Bremer, 1920; Camus and Roussy, 1920); in the rabbit (Bustamante *et al.*, 1942; Bustamante, 1943; Gaupp, 1950); in the rat (Cahane and Cahane, 1935; Hetherington and Ranson, 1940; Mess, 1952; Fülöp, 1952; McCann, 1952; Bogdanove and Halmi, 1952; Bogdanove, 1953; Bogdanove *et al.*, 1955).

Effective lesions were described also in the anterior hypothalamus [Biggart and Alexander (1939), in the dog] and in the posterior hypothalamus [Hume (1958), in the dog; Soulairac (1957, 1962), Soulairac and Soulairac (1956), in the rat]. It may, however, be questioned whether, particularly in this case, a severance of extrahypothalamic, especially rhinencephalic, afferent pathways to the hypothalamic centers would not rather account for the observed disturbances.

But we have to admit that hypothalamic lesions do not always block

the endocrine function of the testes. Cases of advanced puberty with precocious secretion of male hormone were described in human pathology; the primary lesions are generally of a tumoral nature and are electively located in the ventral part of the tuberal hypothalamus. The first case in the literature was reported by Le Marquand and Russel (1934); in 1951, Lange-Cosack reviewed 12 cases concerning male children.

In this respect, and contrary to what happens in the female, experimentation has been of little help up to now. Gaupp (1950) describes cases of precocious puberty in young rabbits bearing basal tuberal lesions, and Bogdanove (1957) reports the unusual case of normal or hypotrophic testes together with a marked hypertrophy of the accessory glands in rats and guinea pigs bearing anterior hypothalamic lesions.

III. GONADOTROPIC HORMONES AFTER HYPOTHALAMIC DECONNECTION

We have seen that hypothalamic deconnection consistently blocks the secretion of male hormone in nonmammalian vertebrates. On the contrary, experiments on mammals yielded some contradictory results. Total inhibition of the testicular endocrine function is consistently induced by transection of the portal veins or lesion of the median eminence; however, ectopic grafts frequently seem to maintain testosterone secretion, and certain types of lesions would even enhance it.

A full understanding of all these observations would require a careful analysis of pituitary gonadotropic function after hypothalamic deconnection, but this study has not been developed very much in the male.

A. DOSAGE OF PITUITARY GONADOTROPINS

Dosage by techniques of implantation of adenohypophysis to immature or hypophysectomized female rats, reveals that an important fall in gonadotropic content of the pituitary generally parallels the inhibition of testicular secretion. This was found after hypothalamic lesions [Bogdanove (1953), in the rat], after chronic treatment with morphine [Knappe *et al.* (1960), in the rat] and after lesion of the median eminence [Davidson *et al.* (1960), in the dog]. The last authors could demonstrate that both FSH and LH content was affected by this decreased gonadotropic secretion.

Little is known in this respect when hypothalamic deconnection does not affect the hormonal function of the testes. Only one attempt reported by Bogdanove (1956) in the rat, indicates that testes can look normal after hypothalamic lesion even when the pituitary gonadotropic content is strongly decreased.

B. CYTOLOGICAL AND HISTOCHEMICAL METHODS

These methods have given some complementary results.

Using the trichromic and histochemical methods devised by Herlant for birds' pituitary, which allow characterization of FSH, LH, and prolactin cells, we obtained the following results in the duck: (1) After transection of the portal veins, FSH, LH, and prolactin cells appear to be in a complete resting state, paralleling the total inhibition of testicular function (Assenmacher and Tixier-Vidal, 1960). (2) After chronic treatment with reserpine, which also inhibits the endocrine function of the testes, secretion of the FSH cells and excretion of the LH cells appear to be blocked (Tixier-Vidal and Assenmacher, 1962) whereas prolactin cells appear to be stimulated (Tixier-Vidal *et al.*, 1962; Tixier-Vidal and Assenmacher, 1962).

In mammals, the use of classic histological techniques mainly gave evidence of a pituitary chromophobia following hypothalamic deconnection (see a review of the literature in Benoit and Assenmacher, 1955). However, more selective cytological techniques recently yielded the following observations in male rats: (1) Chronic treatment with reserpine, which inhibits testosterone secretion, mainly induces involution of LH cells; FSH cells also undergo involution, but prolactin cells are stimulated (Pasteels, 1961). (2) Well-developed prolactin cells are consistently found in ectopic pituitary grafts. But LH cells are always scarce, even when the sexual characters are maintained (Herlant *et al.*, 1960b; Courrier *et al.*, 1961).

C. CONCLUSIONS

The following conclusions may be drawn from these observations:

(1) Hypothalamic deconnection mainly inhibits testosterone secretion. (2) The pituitary gonadotropic function is always severely affected by hypothalamic deconnection, even when the appearance of the interstitial gland and of the target organs of male hormone suggest an apparently normal endocrine function of the testes. Even in such cases, a fall in the gonadotropic content of the pituitary and the appearance of LH cells give evidence of LH deficiency. (3) However, as in the female, hypothalamic deconnection often stimulates prolactin release. This was described in ectopic pituitary grafts and after chronic treatment with reserpine. The hypothalamic lesions of Bogdanove (1957), which resulted in hypertrophy of the accessory glands of the genital tract in male rats, may similarly have induced hypersecretion of prolactin, such as was at times observed in the female.

This throws a particular light on the problem of a synergism of prolactin with the other gonadotropic hormones, particularly LH, or with testosterone. In fact, hypersecretion of prolactin paralleling a complete blockade of LH secretion (or release) seems to have no effect on

the hormonal activity of the testes. But some data strongly suggest that, when an enhanced prolactin release is present together with a residual secretion of LH, the male hormonal function is maintained or even potentiated.

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DISCUSSION

Effects of Pituitary transplantation in Birds

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I would like to comment on some points of agreement as well as of disagreement and to supply new data which complement the data so ably discussed by Dr. Assenmacher.

I agree with Dr. Assenmacher that full spermatogenesis in the male requires the presence of both FSH and LH, the role of the latter being that of causing androgen secretion which, in a manner as yet unexplained, aids in the process of spermatogenesis.

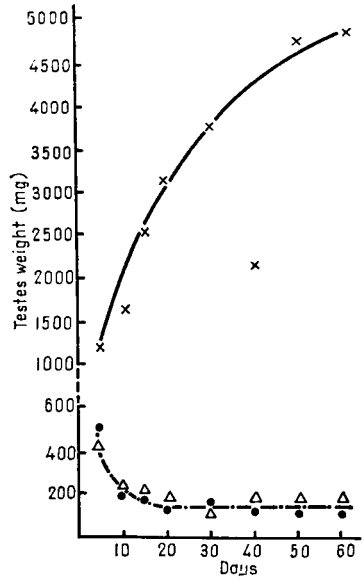

Fig. 1. Testes weight of control (x), hypophysectomized males (·) and those with hypophyseal autotransplants (△).

However, I cannot agree with the suggestion that prolactin may play a role in this process. The incompatibility of prolactin and at least the LH portion of the gonadotropic complex was well illustrated in the study on the effect of prolactin on the maternal instinct of cocks (Nalbandov, 1945). Here it was found that the injection of

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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prolactin drastically reduces testes size, tubule diameter, and comb area and, if continued long enough, stops spermatogenesis. It was further shown that this effect is exerted through the pituitary gland because when FSH and LH are injected simultaneously with prolactin, all these detrimental effects of prolactin are prevented.

The pioneering studies of Assenmacher and Benoit are too well known to need restatement. However, I should like to add some recent information which indicates that there is a distinct difference between mammals and birds in the function of dis-

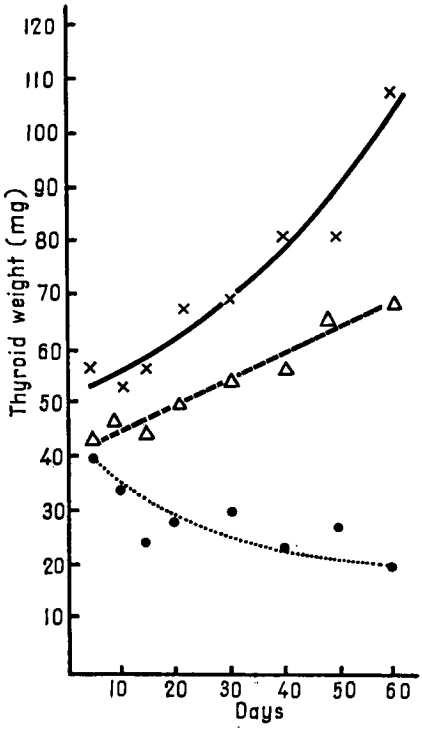

FIG. 2. Thyroid weights (for key to symbols, see Fig. 1.).

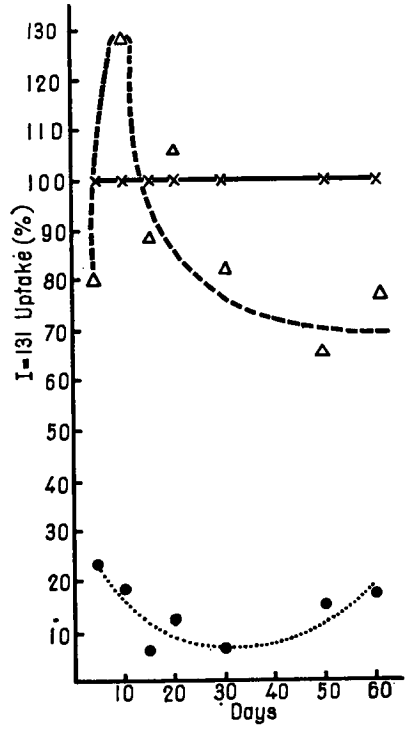

FIG. 3. Iodine 131 uptake by thyroids after hypophysectomy or autotransplantation.

placed pituitary glands. If the anterior lobe is autotransplanted to the kidney capsule of growing cocks, it is found that the production of gonadotropic hormone is completely absent, its absence causing atrophy of the testes and the combs (Ma, 1960). In fact, as far as these two criteria are concerned, the birds with hypophyseal autotransplants are indistinguishable from hypophysectomized males (Fig. 1). It is inter esting to note that starvation of hens causes an immediate cessation of laying which is caused by a deficiency of gonadotropic hormone production and which can be compensated for by the injection of exogenous gonadotropin (pregnant mare serum, PMS) (Morris and Nalbandov, 1961). Daily injection with PMS leads to continuous laying of eggs for as long as 11 days in the complete absence of feed intake. This observation emphasizes the dependence of the secretion of gonadotropins on optimal nutritive state of birds, which, however, apparently affects the production of the other

hypophyseal hormones little or not at all. The weight and histology of thyroids and adrenals are unaffected; injection of PMS alone, which is free from ACTH and TSH, is perfectly able to restore normal ovulation in starving hens. Since, according to the evidence to be presented shortly, the gonadotropic hormone complex is the only one so drastically affected in birds with autotransplanted pituitary glands, the question arises whether there is something unique about the synthesis of gonadotropic hormone molecules which require the singular vascular network of the sella turcica which may be especially well supplied with the substrate nutrients essential in gonadotropin for-

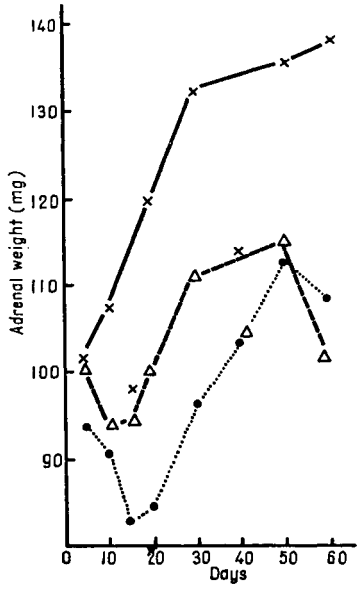

FIG. 4. Adrenal weights after hypophysectomy or autotransplantation.

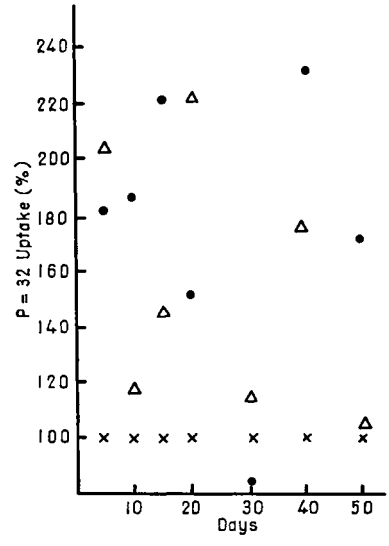

FIG. 5. P-32 uptake by adrenal glands after hypophysectomy or autotransplantation.

mation. This, of course, is not meant to imply that the hypothalamus does not also exert the well-established humoral control over the anterior pituitary. We should also reflect on the fact that the gonadotropic hormone complex is most easily inhibited by endogenous and especially by all exogenous steroid hormones whereas the other hypophyseal hormones are inhibited only by very high levels of steroids or not at all.

Ma (1960) further found that the autotransplanted pituitary continued to secrete TSH and ACTH. This can be seen from the fact that the thyroid weights of birds with autotransplants are insignificantly less than the thyroids of normal intact controls (Fig. 2). The weights and the I-131 uptake by the thyroids of intact controls and birds with hypophyseal autotransplants are very significantly higher than those of thyroids of hypophysectomized birds (Fig. 3).

A more puzzling situation was found in the case of the adrenal gland: the adrenals of all three experimental groups continued to increase in weight after an initial post-surgical decline in the two groups in which surgery was performed (Fig. 4). Also surprising was the finding that the adrenals of hypophysectomized birds, as well as those with autotransplants, took up significantly more P-32 than did the adrenal glands of

unoperated controls (Fig. 5). These observations suggest that the adrenals of both experimental groups are not inactive and continue to be stimulated by a tropic hormone. In the case of hypophysectomized birds, the question arises whether there may be an extrahypophyseal source of ACTH or of an ACTH-like hormone which continues to exert a tropic effect on the adrenals. This possibility is further supported by the finding that the amount of cortical tissue of the adrenals of hypophysectomized chickens does not atrophy but actually increases (Fig. 6).

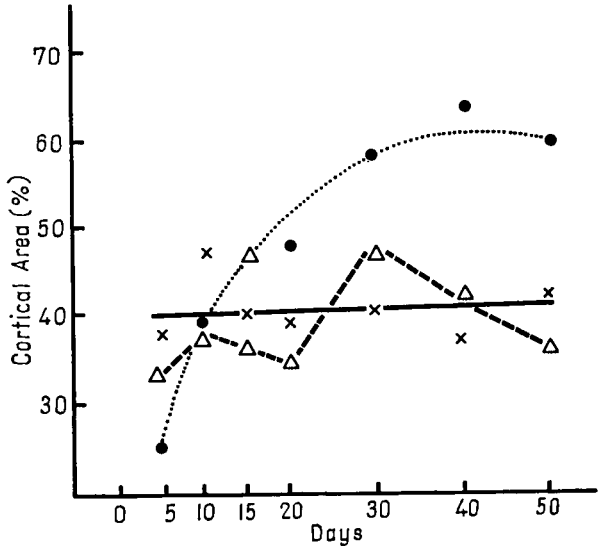

FIG. 6. Comparison of adrenal cortical areas of control birds (x), hypophysectomized birds (Δ) and those hypophyseal autotransplants (·).

TABLE I

Effect of Hypophyseal Autotransplants or Hypophysectomy on Weight and Ascorbic Acid Content of Adrenals

Days after operation	Treatment[b]	Adrenal weight (mg)	Ascorbic acid content per 100 mg adrenal	Difference
5	C	117.5	188.9 ± 4.7	0.00
	APX	106.0	222.4 ± 21.6	33.43[a]
	APX-Tr	101.3	212.5 ± 10.0	23.58[a]
10	C	124.8	174.2 ± 17.4	0.00
	APX	103.3	205.5 ± 14.8	31.27[a]
	APX-Tr	90.0	211.9 ± 32.8	37.72
15	C	135.8	192.7 ± 12.2	0.00
	APX	88.8	195.9 ± 7.7	3.22
	APX-Tr	108.0	205.8 ± 30.5	13.08

[a] $P < 0.05$.

[b] C = control; APX, hypophysectomy; APX-Tr, hypophyseal autotransplantation.

Of some interest is the fate of the ascorbic acid in the adrenal. It was found (Table I) that 5 days after surgery the adrenals of both groups subjected to operation contained significantly more ascorbic acid than did the adrenals of control birds. However, by day 20 the ascorbic acid level in the three groups was no longer significantly different. Since depletion of adrenal ascorbic acid is known to occur under the influence of ACTH, the assumption seems logical that in both operated groups an ACTH of hypophyseal or extrahypophyseal origin begins to act on the adrenal glands by day 20 after hypophysectomy or pituitary transplantation.

Work is now in progress to determine whether the adrenal, under the experimental conditions described, actually continues to function and retain the ability to produce steroid hormones. The observation made suggests that this is the case, but that there may be a difference in the quantity or quality of adrenal steroids elaborated. A distinct difference is noted in the reaction to stress of hypophysectomized birds and hypophysectomized birds with autotransplanted anterior pituitary glands. Hypophysectomized birds cannot withstand nocturnal fasts or sudden drops in environmental temperature. In contrast, birds with hypophyseal autotransplants withstand these stresses as well as do unoperated controls. Thus, in one experiment 12.5% of 48 males with hypophyseal autotransplants died within the first 5 days after surgery, all these deaths being attributed to surgical shock which involved two separate and rather severe surgical insults. In contrast, the mortality of 52 birds subjected only to hypophysectomy was 34.5% and continued throughout the experimental period of 55 days. Thus, even though by several criteria the adrenal gland of hypophysectomized chickens apparently continues to function, its steroid output is qualitatively or quantitatively distinctly different from that of hypophysectomized birds with autotransplanted pituitaries, or that of intact control animals.

If the stalk to the anterior lobe of laying hens was cut, we noted a drastic loss of body weight which was not regained during the 70-day observation period (Shirley and Nalbandov, 1956b). Ovarian weight, and hence oviducal weight, were also reduced almost to the size in hypophysectomized hens and did not recover during the experimental period. But section of the stalk did not significantly affect either thyroid or adrenal weight or histology. Neurohypophysectomy caused a cessation of laying and reduction of the steroid-dependent end organs, but all these returned to normal after a period of 36.5 ± 22.2 days, and normal laying frequency was resumed (Shirley and Nalbandov, 1956a). No effect was noted on the weight or histology of adrenals or thyroids after this operation.

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The Role of Molecular Configuration in the Interaction of Steroid Hormones with Coenzymes and Enzymes[1]

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The subject of the mechanism of action of steroid hormones has excited interest since the early days of steroid hormone biochemistry and physiology. In those times it was possible to define the mechanism of action only by the spectrum of physiological effects elicited when the steroid hormones were administered into suitable experimental animals. These effects were by and large anatomical changes, either gross or microscopic, or readily measurable changes in metabolic state related in magnitude to administered dose of hormone. With the deepening of our understanding of the patterns of intermediary metabolism and the detailed reactions undergone in the synthesis and breakdown of tissue constituents, it became possible to consider mechanism of hormone action in a fashion that is more readily definable in biochemical terms.

Our own present concept of the mechanism of hormone action in biochemical terms is based upon the definition of a chemical control mechanism derived by Britton Chance (1960) from a consideration of the control of carbohydrate metabolism.

For purposes of this discussion a hormone may broadly be considered to have the following properties: (1) It acts to affect a rate-limiting metabolic process. (2) The affinity for the site of action must be high. (3) A large response must result from a small change in hormone concentration. (4) In order to maintain an effective dose-response relation the rates of formation and removal of the hormone must be finely balanced.

[1]This work was supported by United States Public Health Service grants CY-1393 and C-2833, and by American Cancer Society, Inc., grant P-190.

This is Publication No. 1133 of the Cancer Commission of Harvard University.

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Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics:
Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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It is implicit in our working hypothesis that the sites that bind steroids in a specific fashion must be structurally related to one another regardless of the purpose for which the hormone is bound.

This concept of hormone action seemed to us a particularly fruitful one since from it one may derive a number of experimental approaches to the general problem of steroid hormone action. One may postulate that in order for a hormone to affect a rate-limiting process, it must be bound in a specific fashion influenced by the complementary chemical, physicochemical, and stereochemical properties of the hormone and its receptor site. Since the receptor site may be a coenzyme or an enzyme it seemed reasonable to study the structural relations that influence the interaction of steroid hormones with such substances. In the case of the interaction of steroid hormones with the coenzymes, a direct correlation may be obtained. In the case of the enzyme protein, the structure of the receptor site may be inferred from the correlation between the structure of the steroid and its affinity for the enzyme.

The study of interactions of steroid hormones with compounds related to some coenzymes was developed in collaboration with Dr. Allan Munck. These studies (Scott and Engel, 1957; Munck *et al.*, 1957) dealt with the formation of complexes between steroid hormones and purine bases, purine nucleosides and purine-containing nucleotides. The structural requirements for interaction were studied for both partners in the reaction, the steroid and the nonsteroid component. It was demonstrated that steroid hormones formed complexes with purine-containing substances. The interaction with purines was observed with adenosine, a typical purine nucleoside, and with diphosphopyridine nucleotide, an important coenzyme. A careful study of the structural requirements for interaction revealed that the α-side of the steroid was involved. This was demonstrated by measurement of the association constants for adenosine and a series of steroids in which the substituents were varied in a systematic fashion. Substances bearing substituents in the β-configuration at C-17 have similar association constants. Compounds with α-oriented substituents at the same position have lower association constants. Thus, the value for testosterone is 36 liters per mole, whereas that of epitestosterone is less than half this value. Substituents in ring C at C-11, whether α- or β-oriented, tend to reduce the association constant. Substituents in rings A and B have little or no effect. These results and considerations of the geometry of the molecules involved led to the conclusion that the relatively planar α-surface presented by the axial or pseudoaxial hydrogen atoms in rings C and D and part of ring B are involved in the interaction. It was our conclusion that the energy stabilizing the complexes formed was derived from interactions of the London–Van der Waals type. It is interesting in this connection to note that Abelson and Depatie (1960) have

studied the formation of complexes between amino acids and testosterone. Of eighteen amino acids studied, only two, tyrosine ethyl ester and tryptophan (14 liters per mole), form complexes; tryptophan has a higher association constant. The similarity in geometry between tryptophan and purine is noteworthy.

Studies by Munck (1957) on the interfacial activity of some steroid hormones shed further light on the nature of the complexes formed between steroid hormones and purines. It seemed appropriate to inquire whether the associations studied might not be a reflection of the interfacial activity of the steroids. When certain simplifying assumptions are made, one may calculate the energy (positive) involved in removing bound water from the α-surface of the steroid and from one side of the purine ring. These energies are added to the sum of the energies released (negative) when the hydrophobic surfaces of purine and steroid come together and when the displaced water forms a hydrogen-bonded structure. A value of —6 kcal per mole is arrived at. This is in remarkably good, though perhaps fortuitous, agreement with the energy of association that could be calculated from the data on complex formation (Scott and Engel, 1957).

These results revealed the type of specificity of the interaction of steroid hormones with other biologically important substances that gave us some hope that our working hypothesis might be a fruitful one. These hopes were further fortified by the studies of Marcus and Talalay (1955) on the effects of substrate structure on the reaction rates and affinity constants of the bacterial hydroxysteroid dehydrogenases. Similar results with the estradiol-dependent human placental transhydrogenase studied by Hagerman and Villee (1961), Talalay and Williams-Ashman (1958), Talalay *et al.* (1958), Hollander *et al.* (1958, 1959), and Jarabak *et al.* (1962) and with the human placental diphosphopyridine nucleotide-linked estradiol dehydrogenase, studied in our own laboratory (Langer and Engel, 1958; Langer *et al.*, 1959), added evidence for the highly specific nature of the interaction of steroid hormones with enzyme systems.

From our earlier studies on the substrate specificity of the DPN-linked human placental estradiol dehydrogenase, the importance of substituents in rings B, C, and D was evident (Table I). The results obtained with variously substituted derivatives of estrone and estradiol and with their stereoisomers supported the view that it is the α-side of the steroid that interacts with the enzyme–coenzyme complex and that α-oriented substituents in rings C and D or an α-oriented angular methyl group depress reactivity.

Our attention was then directed toward the effect of substitution in the aromatic ring or rings of estrone and equilenin. From our earlier data it was already evident that either ring A or ring B or both had to be aromatic in order for the steroid to be utilized as a substrate by

TABLE I

The Effect of Structural Alterations on the Reactivities of Steroids toward Estradiol Dehydrogenase[a, b]

Compound	$S \times 10^5 M$	V
7α-Hydroxyestrone	10.0	90
7β-Hydroxyestrone	10.0	65
11α-Hydroxyestradiol	3.3	29
11β-Hydroxyestradiol	3.3	20
Estriol	10.0	0
16-Epiestriol	10.0	1
Estradiol-3,17α	10.0	0
D-8-Isoestradiol	10.0	200
Lumiestrone 3-methyl ether	10.0	0

[a]Data from Langer *et al.* (1959).

[b]In the tables S is substrate concentration and V is maximum velocity relative to estrone or estradiol.

this enzyme. Neutral steroids, e.g., testosterone and 19-nortestosterone, were not utilized effectively, their reaction rates being of the order of 1% that of estradiol. The fact that $\Delta^{5(10),6,8}$-estratriene-3β,17β-diol was well utilized as substrate supported the view that planarity of rings A and B was an important feature of reactive molecules and that the entire α-surface was probably involved in the interaction of the steroid substrates with the enzyme.

The first group of compounds investigated was the isomeric nitroderivatives of estrone and their methyl ethers. These substances have been prepared by other investigators (Niederl and Vogel, 1949; Werbin and Holoway, 1956; Kraychy, 1959), but no systematic examination of their biological or enzymological properties has been reported. When the two nitroestrones were studied as substrates for placental estradiol dehydrogenase, it was found that their Michaelis constants were similar in magnitude to the Michaelis constant for estrone, but that the maximum velocities of their reduction were very considerably greater than that of estrone. Since it had been observed that the formation of methyl ethers served to convert estradiol or estrone to a more reactive substrate than the parent phenol, the methyl ethers of the isomeric nitro compounds were prepared. We were surprised to find that this did not hold for the nitro compounds (Table II). A distinct difference between the methyl ether of 2-nitroestrone and that of 4-nitroestrone was observed. The Michaelis constant for 4-nitroestrone methyl ether was one-tenth that of 2-nitroestrone methyl ether, and the 4-nitroestrone methyl ether reacted at less than one-quarter the rate of the isomeric 2-nitroestrone methyl ether.

TABLE II

Properties of Some Estrone Derivatives Substituted in Ring A[a]

Compound	$K_M \times 10^5 M$	V	$K_i \times 10^5 M$
2-Nitroestrone	1.5	194	5.2
2-Nitroestrone methyl ether	2.5	188	2.2
4-Nitroestrone	1.8	150	—
4-Nitroestrone methyl ether	0.25	40	0.54
Estradiol	1.8	—	—

[a]K_M is Michaelis constant and K_i is inhibitor constant. These values were computed either from the slopes and intercepts of double reciprocal plots (Lineweaver and Burk, 1934) by the method of least squares or by the statistical procedure of Wilkinson (1961).

The next experiments dealt with the possibility that 4-nitroestrone methyl ether, which has a Michaelis constant one order of magnitude smaller than that of estradiol, might function as an inhibitor if it were added to the reaction mixture in which estradiol is oxidized enzymatically in the presence of diphosphopyridine nucleotide. That this is indeed the case is shown in Fig. 1, in which the double reciprocal plot of Lineweaver and Burk (1934) is shown. It is evident that this substance functions as a competitive inhibitor of estradiol dehydrogenase;

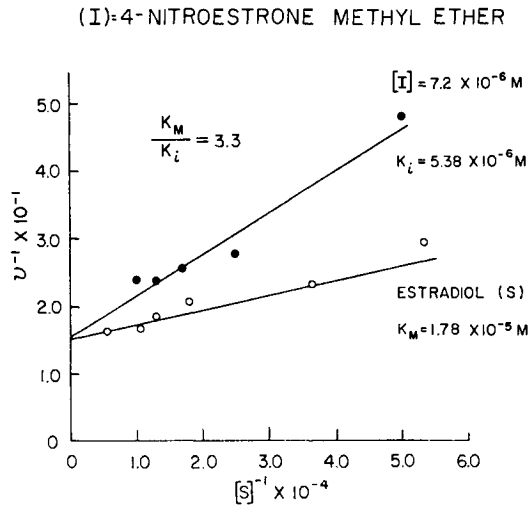

FIG. 1. The effect of 4-nitroestrone methyl ether on the enzymatic oxidation of estradiol to estrone. v is reaction velocity (change in absorbancy per minute). *(S)* is substrate (estradiol) concentration *(M)*; *(I)* is inhibitor (4-nitroestrone methyl ether) concentration; K_M and K_I are Michaelis and inhibitor constants, respectively. The lines were calculated by the method of least squares.

limited solubility prevented the examination of its properties at more than one concentration. The ratio of Michaelis constant for estradiol to inhibitor constant for 4-nitroestrone methyl ether shows that the inhibitor is bound to the enzyme about three times as avidly as is the substrate, estradiol.

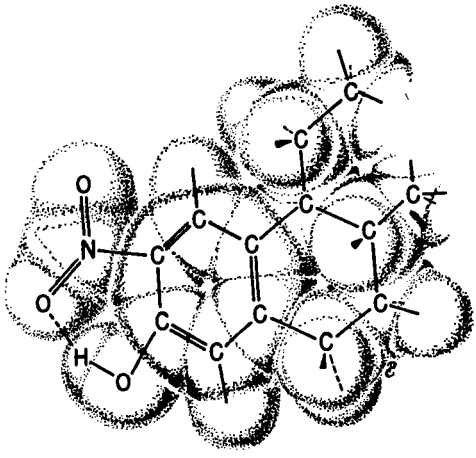

FIG. 2. Partial model of 2-nitroestrone.

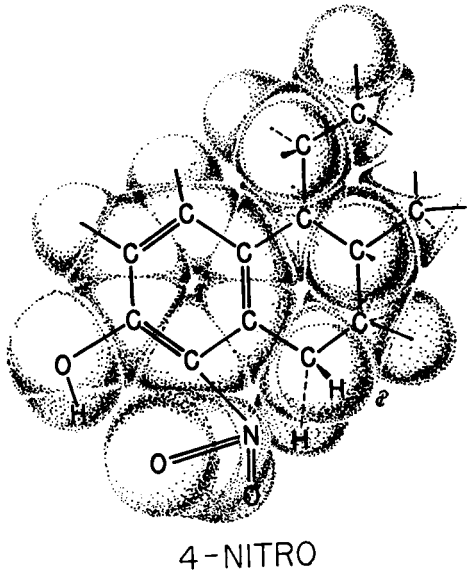

FIG. 3. Partial model of 4-nitroestrone.

This surprising observation and the marked difference in behavior of the isomeric nitroestrone methyl ethers led us to consider the structures of these compounds in some detail. Examination of a model of 2-nitroestrone (Fig. 2) shows that hydrogen bonding between the nitro group and the phenolic hydroxyl is readily achieved. This conclusion was also drawn from infrared spectrographic studies by Pickering and Werbin (1958). The situation in the case of 4-nitroestrone is quite different, as is evident from the examination of Figs. 3 and 4. These figures show that hydrogen bonding between the 4-nitro group and the phenolic hydroxyl is prevented by the out-of-plane conformation of the 4-nitro group. The 6α-hydrogen is so oriented as to prevent coplanarity of the nitro group and the aromatic ring. The infrared spectrum of this substance in the solid state gives evidence that the hydroxyl group and the nitro group are engaged in intermolecular rather than intramolecular hydrogen bonding.

Turning now to the methyl ethers, it may be seen from

the examination of the models that in 2-nitroestrone methyl ether (Fig. 5) there is some interference with free rotation of the nitro group about the carbon–nitrogen bond but that this interference is limited by the ability of the methyl group to rotate about the aromatic carbon–oxygen

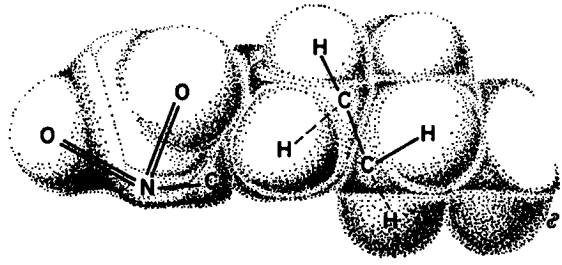

FIG. 4. Partial model of 4-nitroestrone.

bond. In the case of 4-nitroestrone methyl ether (Fig. 6), the blocking effect of the 6α-hydrogen is reinforced by the bulky methyl group of the methyl ether and the nitro group is now more likely to lie in a plane perpendicular to that of the aromatic ring.

If one considers now the behavior of the two nitroestrone methyl ethers with respect to possible interacting groups on the surface of the enzyme molecule, one can speculate that somewhere in the neighborhood of the enzyme site that binds the A or B ring of the steroid, there will be an aromatic group, possibly a tyrosyl residue, that can engage in either π–π or hydrophobic interaction with the aromatic ring of the steroid or both. Examination of the pH-activity curve for the oxidation of estradiol and its methyl ether and the relative rates of the reactions (Fig. 7) shows that ionization of the steroid hydroxyl group cannot play a role in determining the suitability of the substance as a substrate. Furthermore, in the reductive direction, i.e., from estrone to estradiol, the substrate hydroxyl group is wholly undissociated at pH 6, the activity maximum. This is understandable since hydrogen ion is a reagent in the total reaction. The notion that a tyro-

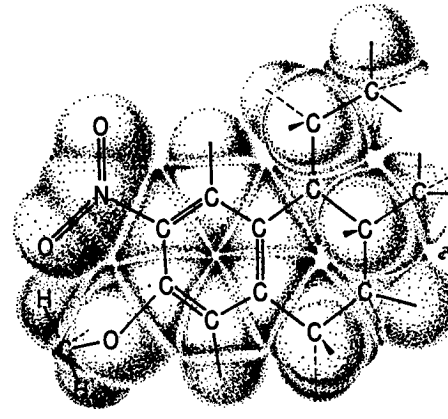

FIG. 5. Partial model of 2-nitroestrone methyl ether.

syl residue may be involved at or near the site of binding of the aromatic end of the steroid molecule is supported by the sharp decline in activity of the enzyme at pH values higher than the pK values of tyrosyl residues in peptide linkage. These values range from 9.8 to 10.3.

The role of the nitro group in 4-nitroestrone may now be understood somewhat better. By being constrained to the vicinity of a plane perpendicular to the aromatic ring of the steroid, it now becomes avail-

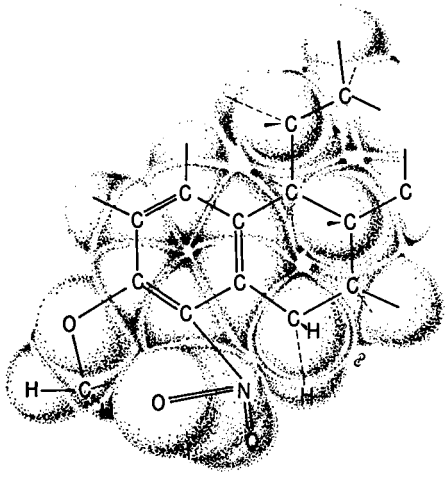

FIG. 6.
Partial model of 4-nitroestrone methyl ether.

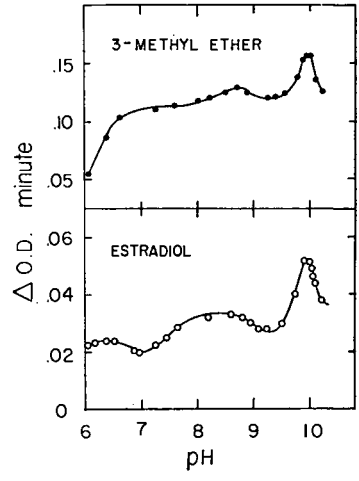

FIG. 7. The relation between pH and reaction rate for estradiol (lower curve) and estradiol 3-methyl ether (upper curve). From Langer and Engel (1958).

able for hydrogen bonding with the undissociated hydroxyl group of the postulated tyrosyl residue and thus makes a significant contribution to increasing the affinity of the enzyme for the substrate. This effect may indeed be augmented if the methyl group of the methyl ether is also engaged in hydrophobic bonding, possibly with alkyl residues on the protein surface. This would fix the methyl group below the plane of the aromatic steroid ring and constitute a further barrier to the free rotation of the nitro group about the carbon–nitrogen bond. These are, of course, purely speculative considerations, but they are not in conflict with the data that we have presented.

Studies of the structural requirements for the interaction of steroid hormones with purine compounds have revealed a high degree of structural specificity for the interaction. Examination of the substrate specificitv of human placental estradiol-17beta-dehydrogenase discloses a similarly high structural specificity. These findings have made

possible the construction of a tentative model of the chemical nature of the complementary site on the enzyme. From these data it may be inferred that similarly complementary chemical structures are present at the receptors site at which the steroid hormone exerts its specific physiological effect.

ACKNOWLEDGMENT

This work would not have been completed without the enthusiastic and skilful assistance of Mrs. D. Duncan and Miss G. Lanman which we wish to acknowledge with gratitude.

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DISCUSSION

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First, the action of a hormone is said to require a high affinity for its site of activation which results in an effect on a single rate-limiting process. Second, a small change in hormone concentration produces a disproportionately large response, and in order to maintain an effective dose-response relation, the rates of formation and removal of the hormone must be finely balanced.

It is clear that the elegant experimental work reported by Dr. Engel and his associates does not bear on the second part of the statement, but has been inspired by the first part. On the first point, Dr. Engel's thesis is clearly stated as follows: "In attempting to develop model systems for the study of steroid hormone action at the molecular level, we have adopted the working hypothesis that these structural features of the hormone molecule that are involved in the interaction of the hormone with enzymes, coenzymes, and other biologically important substances are *also* involved in the highly specific interaction that must occur when the hormonal steroid interacts with the sites at which it exerts its specific biological effect."

The model system that has been examined is DPN-linked estradiol dehydrogenase. To me, the features that parallel the biological effects are most meaningful. Thus, there is a requirement for an aromatic ring A or ring B and for the 17β-hydroxyl group. Also α-oriented substituents depress reactivity, a fact which, with other data, supports the view that the entire α-side of the steroid interacts with the enzyme–coenzyme complex.

How important is this concept of the α-face interactions? Is it the determining feature of the phenomenon of hormone action? It may be informative to examine another enzyme system with respect to α-face, β-face, and their substituents I am re-

TABLE I

Methylation at Positions 2, 6, and 17

Steroid	α-CH_3 at			Kidney β-glucuronidase (U/G)
	C-2	C-6	C-17	
Testosterone	—	—	—	9,050
Testosterone	—	—	+	25,500
Testosterone	+	—	—	25,000
Testosterone	—	+	—	14,800
Testosterone	—	+β	—	15,500

It is clear that in this system the nature of the substitution on the α-face is significant. Polar hydroxyl groups reduce potency, methyl groups increase it. On the other hand halogen atoms in the α-face as in chlorotestosterone enhance activity.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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ferring to the renal β-glucuronidase response to androgens in the intact mouse, which in turn is a reflection of the well-known renotropic action of the male hormones.

Taking testosterone as the reference compound, it is established that in the naturally occurring steroids, the configuration of substituents on ring D is especially important. Thus, the 17β–OH epimer is more potent than the 17α–OH or 17β derivatives. Among the synthetic androgens, esterification of testosterone, the substitution of a 17α–CH_3, the removal of the angular methyl at C–19, the reduction of the double bond in ring A, all produce more active compounds. On the other hand, the introduction of hydroxyl groups into the α side of the molecule at position 6, 11, or 14 markedly reduced activity. Does this mean that α-face substitution always diminishes potency? Not necessarily, as shown in the Table I, where methylation at positions 2, 6, or 17 is seen to enhance potency. Note that the 6α–CH_3 and 6β–CH_3 compounds have similar activity. Refer also to "Renal-Glucuronidase Response to Steroids of the Androgen Series" in *Mechanism of Action of Steroid Hormones* (C. A. Villee and L. L. Engel, eds.), Pergamon Press, New York, 1961.

TABLE II
Comparison of 5α Versus 5β Series

Series	Androstane positions				Kidney β-glucuronidase (U/G)
	C-3	C-11	C-9	C-17	
5β	= 0	= 0	αF	—OH, —CH_3	3100
5α	= 0	= 0	αF	—OH, —CH_3	10500
5β	= 0	β—OH	αF	—OH, —CH_3	3100
5α	= 0	β—OH	αF	—OH, —CH_3	36000

Table II shows the effect of altering the shape of the molecule without changing the substituents, i.e., a comparison of the 5α versus the 5β series. Note particularly the pair of halotestins (9α-F, 11β-OH, 17α-CH_3 testosterones). Obviously, the reduction in activity of the 5β compounds is in some way related to the distortion of the α- as well as the β-face of the molecule.

Returning now to Dr. Engel's working hypothesis, the question may be asked whether it is helped or hindered by the fact that complexes form readily between many steroid hormones and purine bases, purine nucleosides, purine nucleotides, DPN, tyrosine ethyl ester, and tryptophan? Does this combination occur only with the biologically active steroids? In what way are such combinations diferent from protein-bound catalytically inactive steroids that circulate in the blood?

Next, let us accept the evidence that for DPN-linked estradiol dehydrogenase a high affinity for estrogenic steroids has been demonstrated. Is this enzyme the single rate-limiting one referred to in Dr. Engel's definition of hormone action? Is it one which governs hormone action or hormone metabolism or both?

With regard to the interesting experiments on the methyl ethers of 2- and 4-nitroestrones, do these substances interfere *in vivo* with the biological activity of estrogens?

The test of a good working hypothesis is the number of questions which arise from it that can be put to experimental trial. Thus, this is a good hypothesis. It seems to me also that Dr. Engel has made a real contribution in directing our thinking toward examining more precisely the interaction of hormones and enzymes. I also predict that he will remain in the forefront of the battle to solve this most important problem.

L. L. ENGEL. We certainly do not wish to leave the impression that we believe α-face interaction to be necessarily a determining feature of hormone action, although it is a characteristic of the systems we have studied. β-Face interaction, clearly must also be considered, and indeed there is evidence that its role in the action of progestational compounds may be a highly significant one.

While a correlation between the behavior of a steroid hormone in an *in vivo* assay and its behavior in a simple *in vitro* system is gratifying, it is not necessarily to be expected. *In vivo* action of a hormone is modified by many factors, such as absorption, transport, and metabolism so that its *in vivo* behavior may bear little relation to its action in an *in vitro* system in which the presumed target system is isolated. These differences distinguish the problem of hormone action from the equally important different problem of drug design. It is conceivable that when we have *bona fide* isolated *in vitro* systems in which we can study the specific receptor sites of hormones, we may find compounds that are highly potent *in vitro* but wholly inactive *in vivo* because they are modified or destroyed before they can reach the target site. Conversely, a substance feebly active in an *in vitro* system may be highly potent *in vivo* because of its resistance to metabolic destruction prior to delivery to the target site. These factors serve to render less clearcut any correlation between *in vivo* and *in vitro* activities.

Steroid Metabolism and Steroid Action[1]

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I. INTRODUCTION

During the past several years a great number of synthetic hormone analogs bearing unnatural substituents in rings A or B have become available for biological, and in many cases clinical, evaluation. Some of these compound types which are pictured in Fig. 1 have led to

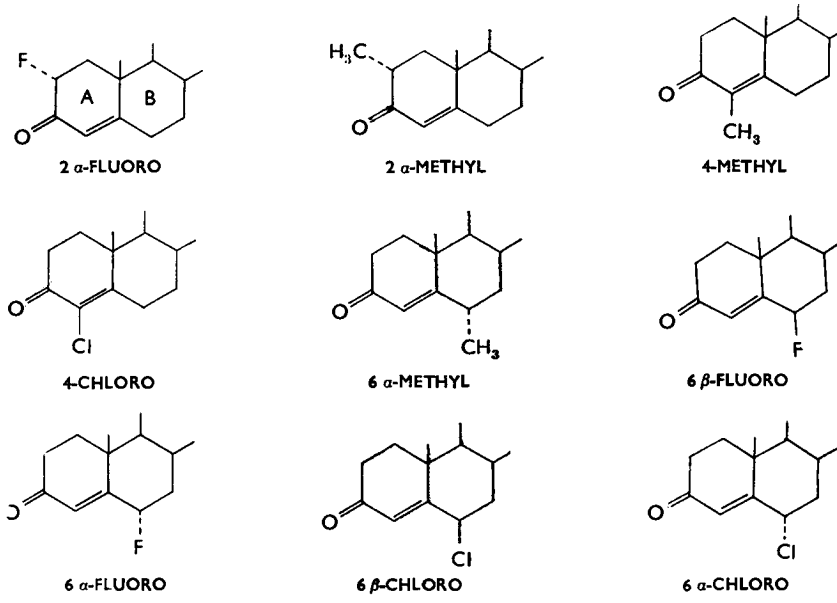

FIG. 1. Hormone analogs substituted in ring A or B.

dramatic increases or decreases in hormonal activity and have been the subject of a number of correlations of structure and activity. In collaboration with Drs. E. Forchielli and S. Ramachandran we have

[1]Supported in part by Grants A-4044 and A-2672 from the United States Public Health Service.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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been in the process of investigating a series of simple testosterone derivatives in order to determine the influence of alkyl substituents and of electronegative substituents on reductive enzymatic processes *in vitro* (Ringold *et al.*, 1962). Our interest has been directed toward the mechanism of the reduction as well as toward a possible correlation of reduction rate with biological activity. Unfortunately, in our system, generally we have not been able to relate the ease of reduction of these compounds with their biological activity. We have, however, established that abnormal substituents may force an abnormal metabolic pathway. The effect of electronic modification of the steroid nucleus on the rate of reduction and on reduction path will be discussed, and the possible biological role of Δ^4-3-ols will also be considered.

II. REDUCTION OF TESTOSTERONE

Male rat liver homogenates were chosen for our reductive studies; the general pattern for unsubstituted α,β-unsaturated steroidal ketones, as illustrated by the testosterone case, is seen in Fig. 2. Owing pri-

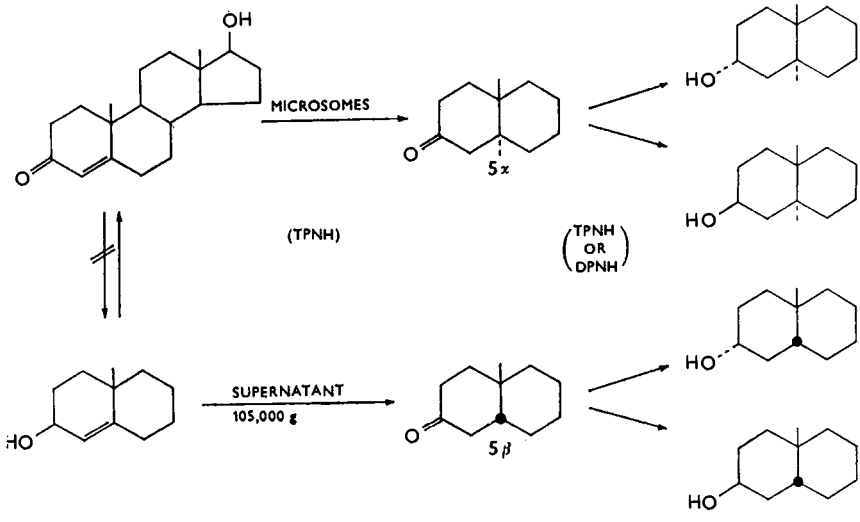

FIG. 2. Enzymatic reduction of testosterone *in vitro* with male rat liver enzymes.

marily to the work of Tomkins (1957) and Forchielli and Dorfman (1956), it has been shown that there are two general enzymes or sets of enzymes in male rat liver that reduce Δ^4-3-ketosteroids. When homogenates are centrifuged at 105,000 *g* the supernatant fraction, in the presence of TPNH or of a suitable TPNH-generating system, catalyzes the reduction of Δ^4-3-ones to the corresponding saturated 3-keto-5β-compound. The microsomal fraction, in the presence of the same coenzyme system, produces the dihydro compound with the 5α-con-

figuration. Also present in both the microsomal and supernatant fractions are saturated 3-keto reductases that convert compounds of either the 5α- or 5β-configuration to the saturated 3α- and 3β-alcohols. These latter enzymes may utilize either DPNH or TPNH as added cofactor (Tomkins, 1956). A path that has not been found with the natural hormones or their metabolites is the enzymatic conversion of a Δ^4-3-ketone to the corresponding Δ^4-3-ol. Ungar *et al.* (1957) investigated the possible intermediacy of such metabolites without being able to establish their presence although very rapid enzymatic oxidation of Δ^4-androstene-3,17β-diol to testosterone was observed.

III. MECHANISM OF REDUCTION

Extensive studies by investigators such as Westheimer *et al.* (1951) and Talalay (1957), who worked with model systems as well as enzymatic processes, have shown that reduced pyridine nucleotides, in general, operate by direct hydride transfer. McGuire and Tomkins (1960) demonstrated by tritium incorporation studies that this also appears

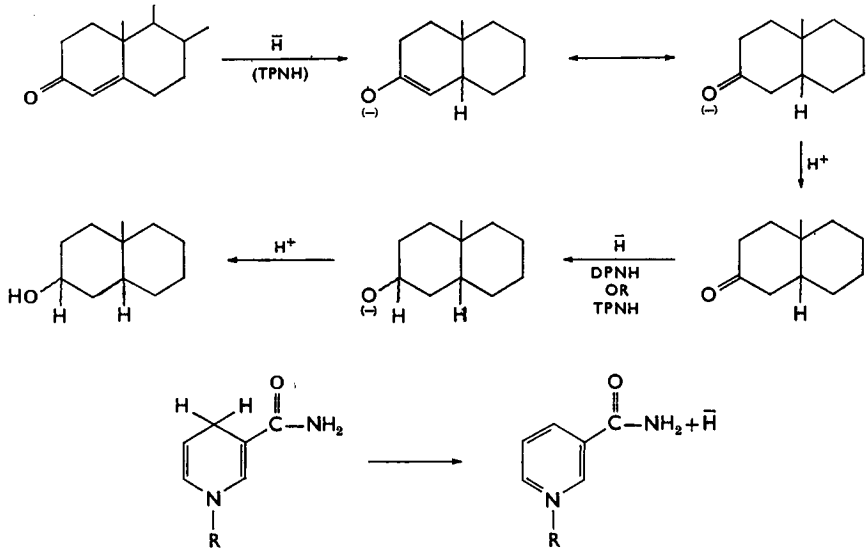

FIG. 3. Probable mechanism of enzymatic reduction.

to be the case with Δ^4-3-keto reductases. The probable mechanism of the nucleotide-dependent reduction of steroids is shown in Fig. 3. Direct hydride transfer to C-5 yields the anion of the saturated ketone, which is then protonated by medium giving the saturated 3-ketone. Then hydride attack on the carbonyl group, as with sodium borohydride reductions, leads to the alkoxide, which is again protonated from the medium.

IV. REDUCTION OF SYNTHETIC TESTOSTERONE DERIVATIVES

A. RATES

In Table I are listed the relative rates of reduction of a number of

TABLE I

Relative Rates of Reduction of Testosterone Anal ogs

Testosterone (T) = 1

6β–Fluoro T = 6	6β–Methyl T = 0.2
6α–Fluoro T = 3	6α–Methyl T = 0.2
4–Chloro T = 5	4–Methyl T = 0.15
2α–Fluoro T = 3	2α–Methyl T = 0.1

testosterone analogs, namely the 6β-fluoro, 6α-fluoro, 4-chloro, 2α-fluoro, 6β-methyl, 6α-methyl, and 4-methyl 2 α-methyl derivatives.

The reductions were carried out with the supernatant 5β fraction utilizing a TPNH-generating system at pH 5.8. The soluble 5β system was selected to facilitate kinetic studies and possibly to correlate reduction rate with biological inactivity since 5β-androstanes are inactive androgens while the 5α-isomers are in general about equivalent in androgenic potency to the parent Δ^4-3-ketone. In each case the extent of reduction was found by extraction of the steroid and spectrophotometric determination of the disappearance of the α,β-unsaturated ketone chromophore in the ultraviolet. It is apparent from the table that all the electronegative-substituted derivatives were reduced more rapidly than testosterone itself, whereas the methyl-substituted derivatives were very slow to reduce. It is appropriate at this point to note that Glenn and his co-workers (1957, 1959) found that 2α-methylcortisol, 6α-methylcortisol, and 6α-methylprogesterone reduced slowly with a soluble rat liver preparation whereas 6β-fluorocortisol was rapidly reduced. From consideration of Glenn's results in conjunction with our own, it is obvious that this is a general pattern: irrespective of side chain, the alkyl derivatives are slow to reduce whereas the halo derivatives reduce at an accelerated rate. It is further apparent that there is no general relationship between the rate of reduction of the substituted testosterone derivatives with this rat liver system and their androgenic activity in the rat. For example, none of the alkyl-substituted testosterone derivatives are more androgenic than testosterone despite the slower reduction rate and, in fact, the 2α-methyl and 4-methyl derivatives are considerably less active.

B. REDUCTION PATH

1. 6β-Fluorotestosterone

Since the sole measure of reduction rate of these unnatural steroids had been obtained by disappearance of the unsaturated ketone chromophore, it was desirable to investigate the nature of the reduction products. Although Ungar *et al.* had not found Δ^4-3-ol formation in the case of testosterone, reduction in this manner would lead to loss of the ultraviolet chromophore just as would reduction of the double

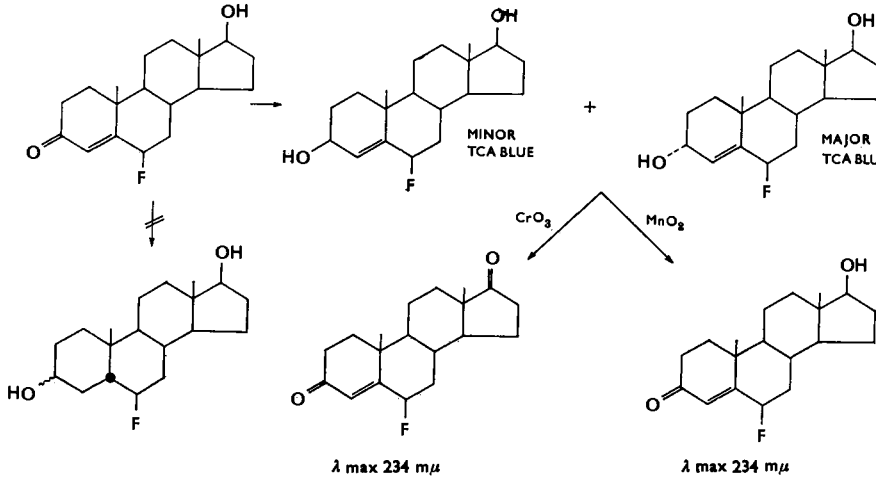

FIG. 4. Reduction of 6β-fluorotestosterone by supernatant fraction.

bond. With this in mind the reduction products of 6β-fluorotestosterone were separated by paper chromatography and their nature was investigated (Fig. 4). It was found that the sole products formed were 6β-fluoro-Δ^4-androstene-3β,17β-diol and 6β-fluoro-Δ^4-androstene-3α,17β-diol, the latter isomer predominating. These products were detected by an intense blue coloration with trichloroacetic acid; their structure was readily proved by oxidation with manganese dioxide and with chromic acid-acetone, which led to 6β-fluorotestosterone and 6β-fluoro-Δ^4-androstene-3,17-dione, respectively. Thus, in contrast to testosterone itself, no reduction of the 4(5)-double bond had occurred. By ultraviolet spectral determination after oxidation it was possible to account for about 75% of the reduced material as 6β-fluoro-Δ^4-3-ol. Reductions carried out at pH 7.2 with DPNH instead of TPNH gave results which were qualitatively similar.

2. 6α-Fluorotestosterone

The reduction products of 6α-fluorotestosterone were similarly separated by paper chromatography and found to consist of a mixture of 6α-fluoro-Δ^4-androstene-3,17β-diol (blue color with trichloroacetic acid) and 6α-fluoro-5β-androstane-3,17β-diol (Fig. 5), the latter product predominating. Quantitative ultraviolet determination before and after oxidation with chromic acid demonstrated the presence of 22% of Δ^4-3,17-diol whereas oxidation of the 4(5)-dihydro zone followed

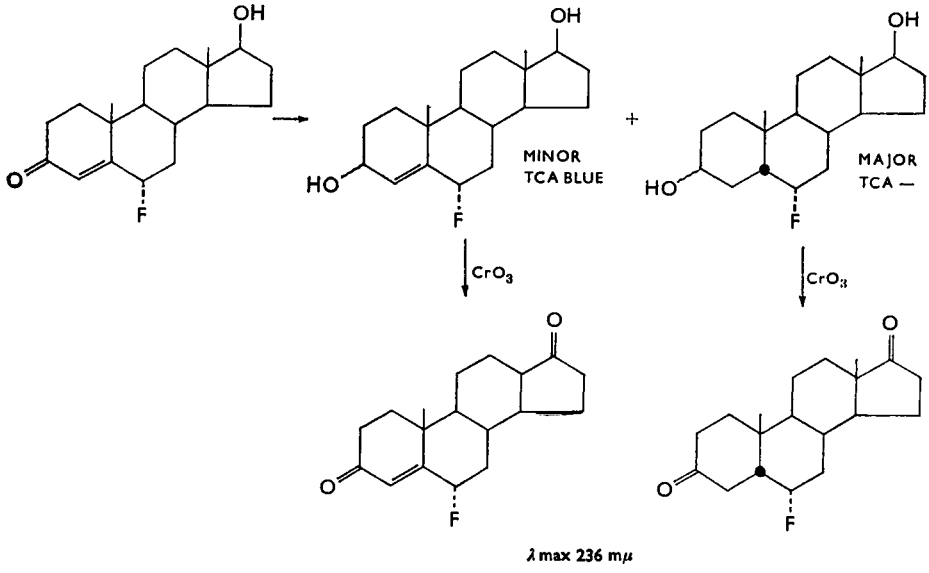

FIG. 5. Reduction of 6α-fluorotestosterone by supernatant fraction.

by quantitative Zimmerman determination accounted for 71% of the reduced material. Thus, in common with the 6β-fluoro case, abnormal Δ^4-3-ol product formation had occurred, although the principal path was the normal reduction of the 4(5)-double bond.

3. 2α-Fluorotestosterone

2α-Fluorotestosterone, which had reduced at three times the rate of testosterone, followed a path similar to that of the 6β-fluoro compound and gave only a mixture of the isomeric 2α-fluoro-Δ^4-androstene-3,17β-diols, no 4(5)-dihydro product being detectable. The 2-fluoro-Δ^4-3-ols, which also gave an intense blue color with trichloroacetic acid, were readily converted to the known 2α-fluoro-Δ^4-androstene-3,17-dione and characterized as such.

Figure 6 tabulates the reduction products of a number of other substrates investigated in our laboratories. 4-Chlorotestosterone with the supernatant fraction underwent disappearance of the ultraviolet chro-

mophore at five times the rate of testosterone. The major product was found to be 4-chloro-Δ^4-androstene-3,17β-diol, and only a trace of 4(5) reduced material could be detected.

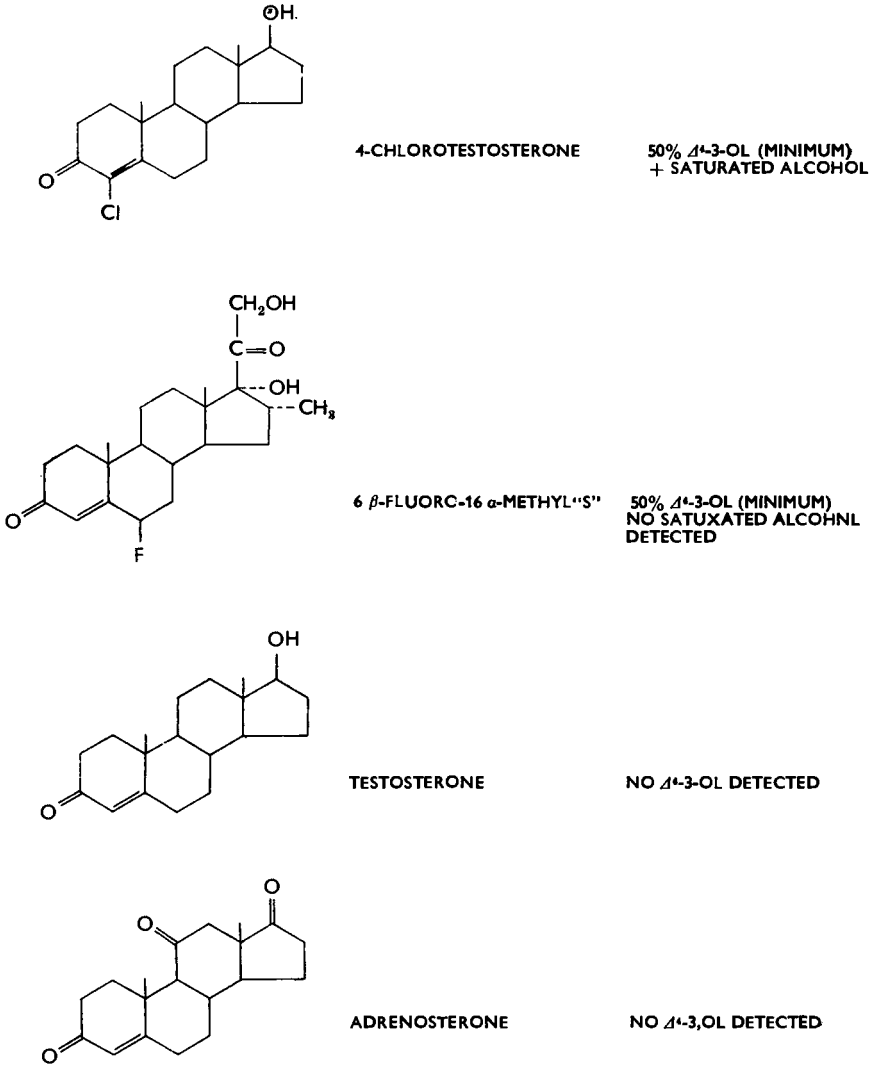

FIG. 6. Other substrates investigated for Δ^4-3-ol formation.

4. *Other Testosterone Derivatives and Pregnenes*

Although we had established up to this point that the 6β-fluoro, 6α-fluoro, 2α-fluoro, and 4-chloro derivatives of testosterone underwent abnormal reduction, the possibility existed that this reduction

was specific only for testosterone derivatives or other C_{19} steroids. Therefore, the reduction rates of 6α- and 6β-fluoro-16α-methyl-Δ^4-pregnene-17α,21-diol-3,20-dione (6-fluoro-16α-methyl "S") were determined versus 16α-methyl "S" as standard. Although both fluoro derivatives reduced faster than the standard, the 6β-fluoro isomer was the fastest; investigation of its reduction products demonstrated that, just as with 6β-fluorotestosterone, the sole reduction products were 6β-fluoro-Δ^4-3-ols, which were readily detected by their blue color with trichloroacetic acid. Structure proof followed from chromic acid-acetone oxidation with scission of the side chain and characterization of 6β-fluoro-16α-methyl-Δ^4-androstene-3,17-dione. Thus Δ^4-3-ol formation is not limited to the androstane series but appears to be a general pattern for androstanes and pregnanes provided an electronegative substituent is present at C-2, C-4, or C-6.

Careful reinvestigation of testosterone with the same enzymatic reduction system failed to reveal the presence of Δ^4-3-ol, nor could such metabolites be found after the reduction of Δ^4-androstene-3,11,17-trione (adrenosterone).

C. EXPLANATION OF ABNORMAL REDUCTIONS

1. *Halogen-Substituted Derivatives*

The question now arises why the fluoro and chloro derivatives undergo this abnormal reduction path. We believe that the answer may be found in a consideration of the relative stability of a saturated versus an unsaturated ketone (Fig. 7). The accepted resonance form of a saturated ketone involves polarization of the carbonyl group with oxygen

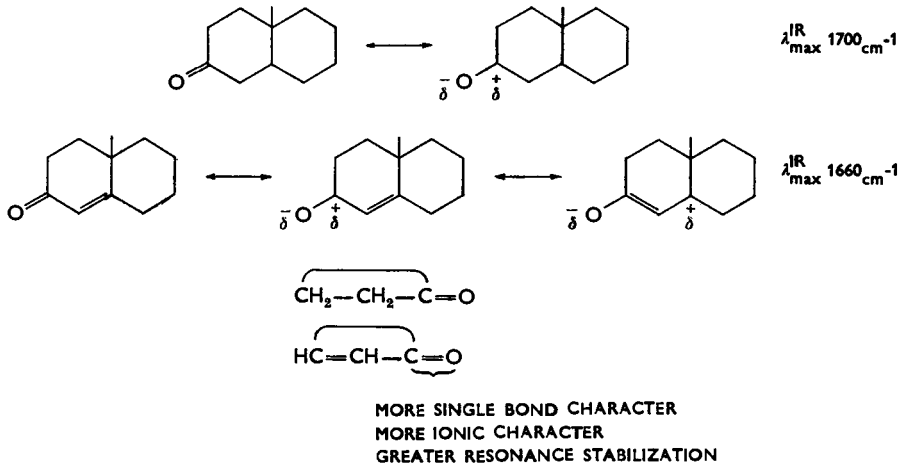

FIG. 7. Resonance contributions of ketones.

assuming a partial negative charge and carbon a partial positive charge. When a double bond is present adjacent to the ketone, the charged forms become more important owing to double bond participation and mobilization. An α,β-unsaturated ketone, therefore, is more resonance stabilized than a saturated ketone and its carbonyl function possesses more ionic character and more single-bond character. Some of these changes are reflected in the carbon-oxygen stretching frequency of the carbonyl in the infrared, which approximates 1660 cm^{-1} for an unsaturated and 1700 cm^{-1} for a saturated 3-ketone.

Owing to its greater resonance stabilization, the carbonyl of an α,β-unsaturated ketone should be more difficult to reduce than a saturated ketone by chemical or enzymatic means. This point is readily demonstrated, for example, by the fact that Δ^4-cholesten-3-one is reduced by sodium borohydride at one-tenth the rate of 5α-cholestan-3-one. Further, it has been established (Norymberski and Woods, 1955) that Δ^4-androstene-3,17-dione undergoes selective reduction at C-17 with a limited quantity of sodium borohydride although 5α-androstane-3,17-dione is preferentially reduced at C-3.

When an electronegative substituent such as fluorine or chlorine is introduced into an α,β-unsaturated ketone on the double bond, or adjacent to the ketone or vinylogous to the ketone, there is an interference with the usual α,β-unsaturated ketone resonance and a net destabilization of the system (Fig. 8). This is due to the powerful induc-

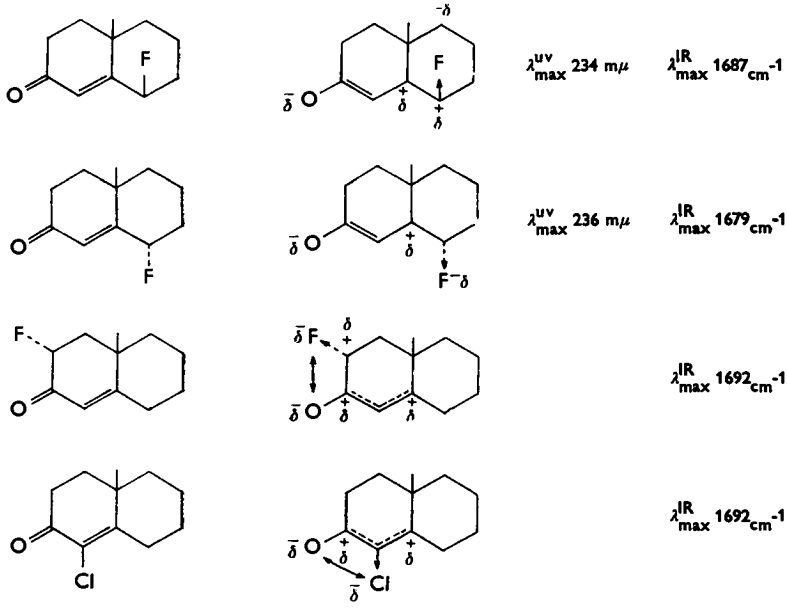

FIG. 8. Destabilization of α,β-unsaturated ketones by electronegative substituents.

tive effect of halogen which places a negative charge on halogen and a positive charge on the carbon atom which bears the halogen atom. In the case of the 6β-fluoro compound, fluorine inhibits polarization of the carbonyl group by transmission of its inductive effect through the double bond while double bond polarization is also inhibited. The same effect is shown by the 6α-fluoro compound, but with reduced intensity due to the equatorial rather than axial nature of the halogen atom. In the cases of the 2α-fluoro and 4-chloro derivatives, the inductive effect is operative and inhibits carbonyl polarization, but of greater importance is the electrostatic field effect of a negative halogen repelling a negative oxygen, the net effect being inhibition of carbonyl polarization and hence resonance. The change is reflected in each case by a shift in the infrared carbonyl frequency, which in the examples of the 6β-fluoro, 4-chloro, and 2α-fluoro compounds approaches the stretching frequency of a saturated 3-ketone whereas 6α-fluorotestosterone is intermediate in carbonyl position.

We believe that the enzyme or group of enzymes in the supernatant liver fraction which catalyze the reduction of saturated 3-keto steroids cannot reduce an ordinary α,β-unsaturated ketone because of the higher energy requirement. Substitution of halogen into the molecule destabilizes the α,β-unsaturated ketone system or in effect converts it into a carbonyl group which electronically resembles a saturated 3-ketone.

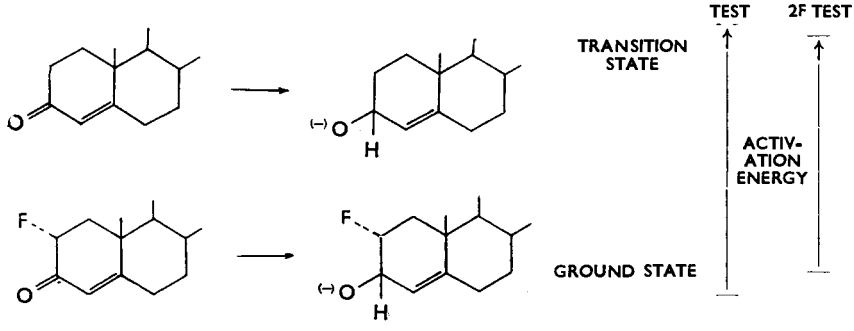

FIG. 9. Decrease of reduction activation energy by 2α-fluoro substituent. Assumptions: (1) enzyme saturation; (2) hydride transfer is rate determining; (3) transition state resembles anion.

Reduction, probably catalyzed by the same saturated 3-keto reductase, can then proceed and lead to Δ^4-3-ols.

Several other mechanistic explanations are possible. One may adopt the very simple argument that the carbonyl group of the halo-substituted compound is relatively electron denuded, hence more positive, and therefore, more susceptible to hydride attack and thus reduction.

More satisfying is a simple transition-state picture illustrated in Fig. 9 for 2α-fluorotestosterone with the reasonable assumptions of enzyme

saturation, a rate-determining transfer of hydride ion, and a transition state that resembles the anion. Since the ground state energy level for the 2α-fluoro compound will be higher than that of testosterone owing to dipole and electrostatic field effects and since the transition state energy level will be lower owing to fluorine delocalization of negative charge, the activation energy of reduction will be less for 2α-fluorotestosterone and Δ^4-3-ol formation can occur more readily.

2. *Methyl-Substituted Derivatives*

The methyl-substituted derivatives will not be discussed at length, but it should be noted that the slow reduction rate of these substances may be readily explained on rather basic organic chemical grounds. Just as electron-withdrawing groups at the C-6 position destabilize the α,β-unsaturated ketone, promoting rapid reduction either of the ketone or double bond, electron-releasing groups such as methyl should stabilize the unsaturated ketone group and hinder reductive processes. A 4-methyl-Δ^4-3-ketone should be relatively inert to double bond reduction because of the greater resonance stabilization of a tetra-substituted double bond. Finally, a 2α-methyl-Δ^4-3-ketone should reduce very slowly, if at all, with this 5β-reductase system because of the + I inductive effect of methyl and, of greater importance, because the equatorial 2α-methyl group would become axial and in severe steric interaction with the 9α- and 11α-hydrogen atoms if reduction to the 5β-dihydro compound occurred.

V. SUMMARY AND PROBABLE BIOLOGICAL ROLE OF Δ^4-3-OLS

In conclusion, we have not established a general relationship between the rate of reduction by liver supernatant fractions *in vitro* and the biological activity of this series of compounds. We have established that electronic changes in the substrate can change the energy requirements of an enzymatic reaction and permit apparently forbidden reactions to proceed. We believe that this accounts for the unusual formation of Δ^4-3-ols and further that we can predict that any chemical alteration of the steroid which destabilizes the resonance of the α,β-unsaturated ketone system will favor Δ^4-3-ol formation.

One question resurrected by this work and by some of the other papers presented at this congress is the possible biological significance of Δ^4-3-ols. It has been noted that many Δ^4-3-ols are as active or more active than the parent Δ^4-3-keto steroids, and the possibility has been suggested from time to time that Δ^4-3-ols may in fact be the active hormonal form. One such potent androgen, 17α-methyl-Δ^4-androstene-3β,17β-diol has been assayed in our laboratories in the castrate rat by

the subcutaneous route over a dose range from 0.2 to 3.2 mg versus the corresponding 3α-deutero compound and Δ^4-3-ketone (Table II).

TABLE II
Androgenic Activity of Δ^4-3β-ols[a]

Compound	Androgenic activity[b]	
	Low dose	High dose
17α–Methyltestosterone	1	1
17α–Methyl–Δ^4–androstene–3β,17–diol	2	0.8
3α–Deutero–17α–methyl–Δ^4-androstene–3β, 17β–diol	1.5	0.2

[a]Assays by Dr. R.I. Dorfman of these laboratories.

[b]Based upon seminal vesicle and ventral prostate response in castrate rats, subcutaneous route of administration.

If the Δ^4-3-ols undergo oxidation *in vivo* to the Δ^4-3-ketone with loss of the C-3 hydrogen atom as a rate-determining step and if, in fact, the Δ^4-3-ketone is the active androgenic agent, then the 3-deutero compound should be less active than the 3-hydrogen compound in accord with the primary deuterium isotope effect (Wiberg, 1955). Our results, in particular the markedly decreased activity of the 3α-deutero compound at high dose levels when the compound is only about one-fourth as active as the 3α-hydrogen compound, lead us to believe that the Δ^4-3-ols, at least in the androgenic series, are not highly active per se, but owe most if not all of their activity to conversion *in vivo* to the corresponding Δ^4-3-ketone.

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DISCUSSION

Adrenal Steroids and Adipose Tissue

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As Dr. Ringold has pointed out, current knowledge does not enable us to state whether hormones acting on their target organs are effective as such or, in order to become active, must undergo modifications of their structure or physicochemical state, through the occurrence of accessory bonds. Moreover, it seems difficult to establish whether hormone action occurs as a systemic process on ubiquitously distributed enzymes or whether it implies a specific site of action at the target organ level, possibly on the basis of an enzymes' spatial arrangement, characteristic for every given species of cell.

Our attention has been drawn to interactions of adrenal steroids and adipose tissue for several reasons, which I shall briefly summarize:

1. The clinical finding of a "*peculiar*" obesity of adrenocortical origin, or at least, with typical adrenocortical features, is well known since the early clinical observations by Pende, the forefather of clinical endocrinology. This finding was later elucidated in its nosographic characters as *hypercortisolism* by the workers of the school I belong to (Antognetti and co-workers). Obesity of this sort is the most constant and among the most typical and early symptoms of hypercortisolism, together with alterations of the metabolism of subcutaneous lipids.

2. After the isolation or the synthesis of several compounds exhibiting adrenocortical activity, the occurrence of iatrogenic obesity, following cortisol or cortical treatment, has been observed.

3. The most recent studies on adipose tissue dynamics enable us to state that an intrinsic homeostasis is at the basis of lipid balance and therefore, at the basis of the various energy sources for cell life.

It has been already accepted that, during starvation or at least at a few hours after a mixed meal, glucose mobilization from glycogen storage (mainly from the liver) is a much less efficient and active process than the more important release of nonesterified fatty acids (NEFA) from adipose tissue.

NEFA release from adipose cells is regulated by a complex mechanism of intrinsic homeostasis. Its principal parameters are (1) the intracellular NEFA pool, deriving from neutral fat hydrolysis; (2) the amount of active glycerol available as acceptor of the free radical to be esterified. It must be pointed out that this acceptor has possibly an energy level comparable to that of glycerophosphate.

The mechanism involved, in brief, is as follows: Intracellular penetration of a greater amount of glucose will lead to an increase in available active glycerol, to a greater re-esterification of NEFA, and consequently to their reduced release from the cell. On the contrary, a decreased amount of intracellular glucose or glucose utilization through pathways which do not readily yield active glycerol will lead to the increase of intracellular NEFA pool and to a higher rate of release from the adipose cell. The latter process

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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needs a plasmatic albumin acceptor in order to make soluble and to circulate NEFA through a water compartment.

Obviously, whichever clinical or experimental cause induces obesity, it should imply positivization of lipid balance at the adipose tissue level. It should, therefore, entail reduction of NEFA release from the cell, related to total intracellular lipid synthesis.

It seems appropriate to suggest that even in hypercortisol obesity, a mechanism of this kind may possibly be involved.

In order to demonstrate that this mechanism is really operative under such conditions, we set up a group of experiments. We have looked for a reproducible, clear correlation between circulating glucose disappearance rate, as evaluated by means of Adezati and Polleri's method, and blood NEFA levels found at various stages of the experiment. It has thus been possible to observe that in normal subjects blood NEFA decrease after glucose intravenous infusion more readily and more intensely than in hypercortisolic patients. Our data indicate, therefore, a slower rate of intrinsic homeostatic reactions between glucose and NEFA.

In order to elucidate the possible mechanisms of action of cortisol at this level, we further investigated the occurrence of the process directly in adipose tissue *in vitro*. Rat epididymal fat pads, incubated in bicarbonate Krebs medium containing human albumin, release NEFA at a certain rate. Upon addition of glucose to the medium, it is possible to evaluate the intrinsic homeostatic regulation by determining the decrease in NEFA released from the tissue.

We were able to see that, whereas cortisol pretreatment of the animal remarkably reduces the homeostatic efficiency of glucose, addition of cortisol *in vitro* does not. The process does not seem to involve any mechanism connected with glucose uptake by the cell, since the latter is not clearly modified by cortisol in adipose tissue.

We have also demonstrated directly the homeostatic efficiency of glycerophosphate added *in vitro*. This substance, however, is ineffective when added to tissue preparations obtained from cortisol-treated animals.

These effects, therefore, are not consistent with the concept of an action dependent on a single enzyme, but indicate an action on homeostatic interaction regulating constants.

The mechanism described, in order to become operative, requires that cortisol remain for a rather long time in contact with cell structures, since cortisol *in vitro* is less effective.

The data on the influence of some nucleoproteins and purine constituents of cell on lipid homeostasis at the adipose tissue level suggest a possible nucleoprotein intermediation of the cortisol effect *in vivo* and recall the well-known cortisol action on such cellular components.

Experiments now in progress will possibly enable us to verify these assumptions, which we were able to make on the basis of the data now available.

Steroids, Nonsteroids, Intermediary Metabolism, Inflammation and Their Probable Interrelationships

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I. Introduction

Adrenocortical steroids exert profound changes on carbohydrate, protein, and fat metabolism. Adrenalectomy reduces glycosuria, increases amino acid metabolism, deranges fat metabolism, and forms ketone bodies in diabetic animals. Classical experiments of Long and others (Evans, 1936; Long *et al.*, 1940, 1960; Long and Lukens, 1936; Thorn *et al.*, 1940) pointed to conclusions that adrenocortical steroids accelerate metabolism of protein and induce gluconeogenesis, presumably from increased availability of amino acids to liver. Other workers (Glenn *et al.*, 1963; Ingle, 1941; Russell, 1939) have shown that adrenocortical steroids influence metabolism of glucose in addition to, as consequence of, or as prelude to, changes in amino acid and protein metabolism. Injection of appropriate steroids to adrenalectomized rats brings about remarkable increases in carbohydrate stores despite increased *requirements* for carbohydrate (Long *et al.*, 1960).

Two principal opposing theories are in vogue regarding primary effects of adrenocortical steroids. They either accelerate protein metabolism or inhibit tissue metabolism of carbohydrate. Initially it was believed that primary metabolic changes occurred in hepatic tissue. However, all presently available evidence suggests that hepatic changes after steroid injection to experimental animals could be due to secondary alterations in substrate availability or other peripheral alterations, not necessarily a reflection of ability of adrenocortical steroids to act primarily on liver (Cahill *et al.*, 1959). Direct effects of adrenocortical steroids on isolated systems of hepatic cells are fragmentary and disappointing indeed. Direct effects on extrahepatic cells are becoming increasingly apparent and readily obtainable (Blecher and White, 1958; Cardoso, 1962; Munck, 1962; Rauch *et al.*, 1961).

Adrenocortical steroids exert profound inhibitory effects when locally applied or directly injected into peripheral sites of inflammation. The original question whether adrenal steroids *have* a peripheral action

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(Long *et al.*, 1960) is no longer of major concern. They do. However, intimate relationships between peripheral anti-inflammatory effects and systemic and local metabolic effects are currently among unanswered questions in this area. Anti-inflammatory effects of adrenocortical steroids are well known, and this *practical* aspect of their action has stimulated widespread interest in their physiologic importance.

The following general approaches to problems of steroid action have been adopted:

1. Attempts have been made to learn details of steroid action in adrenalectomized and intact rats after systemic injection of steroids.
2. Specificities of steroid action have been investigated *in vivo* by examining effects after systemic and direct injection to sites where these substances are generally presumed to act—*inflammation.*
3. It has been shown, using nonsteroidal agents as models, that compounds affecting certain enzyme systems in a general and similar direction to adrenocortical steroids behave similarly on a particular therapeutic end point—*inflammation.* Conversely, agents inhibiting steroid action *in vitro* also inhibit it *in vivo* provided the steroid and inhibitor are allowed to interact at a site where they are generally *presumed* to act.

The guiding principle behind present investigations is that metabolic and anti-inflammatory effects of adrenocortical steroids are *directly* interrelated. Numerous data and ancillary studies are available in this area, but limitations of space prevent discussion: detailed results will be presented in a subsequent publication (Glenn *et al.*, 1963).

II. EXPERIMENTAL

A. METABOLIC EFFECTS OF HYDROCORTISONE IN INTACT AND ADRENALECTOMIZED RATS

Figure 1 shows data obtained after subcutaneous (SC) injection of 2.0 mg hydrocortisone to fasted adrenalectomized rats. The first detectable change is increase in plasma glucose followed in 2–3 hours by glycogenesis in liver. Plasma amino acid nitrogen and protein levels are not significantly altered during equivalent time intervals. A small but significant increase occurs in plasma lactate. It is concluded that liver glycogen accumulation is a function of blood glucose levels. Ashmore (1960) has drawn similar conclusions.

Injection of increasing amounts of hydrocortisone is followed by a typical S-shaped glycogenic curve (Fig. 2). Simultaneous injection of glucose results in a more linear relationship. A 2–3-hour delay occurs in response to steroid injection; this delay is preventable by simultaneous injection of carbohydrate (Fig. 3). Amino acid, protein, and fat injections, during equivalent time intervals, fail to induce the above-mentioned changes. Hydrocortisone appears to be acting on metabolism or disposition of glucose per se.

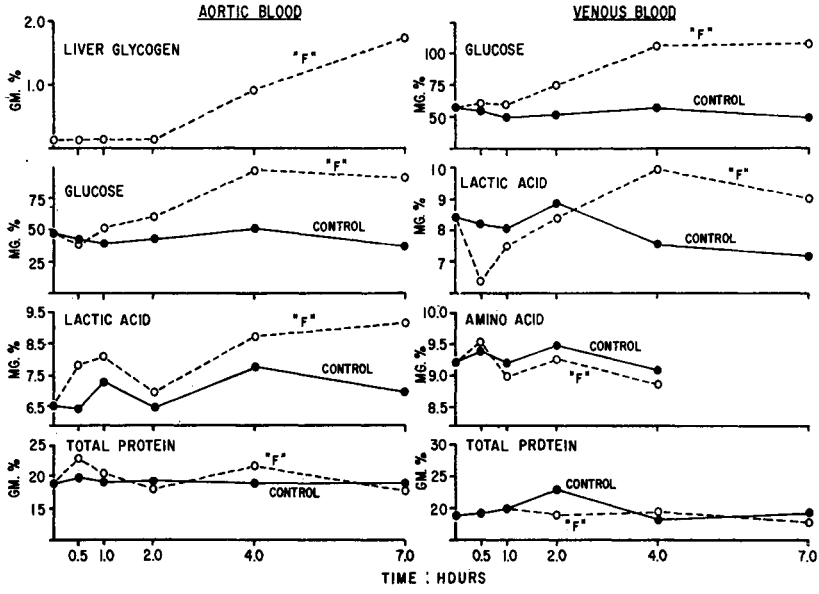


FIG. 1. Time response: effect of hydrocortisone on various blood constituents and liver glycogen (Glenn *et al.*, 1961b). Conditions: hydrocortisone (SC), 2 mg; 5 rats per point.

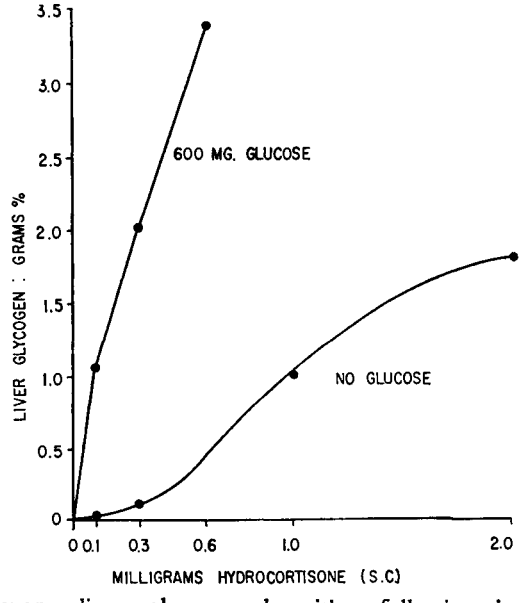


FIG. 2. Dose response liver glycogen deposition following intraperitoneal administration of glucose (Glenn *et al.*, 1961b). Conditions: Livers removed 7 hours after injection. Glucose controls subtracted from each point; 5 rats per group.

Accumulation of muscle glycogen does not occur *unless* glucose is injected along with steroid (Fig. 4). Liver and muscle glycogenic time relationships are identical (Glenn *et al.*, 1961b). Injection of insulin and carbohydrate causes increased glycogen accumulation in muscle; hydrocortisone further *promotes* it (Fig. 5).

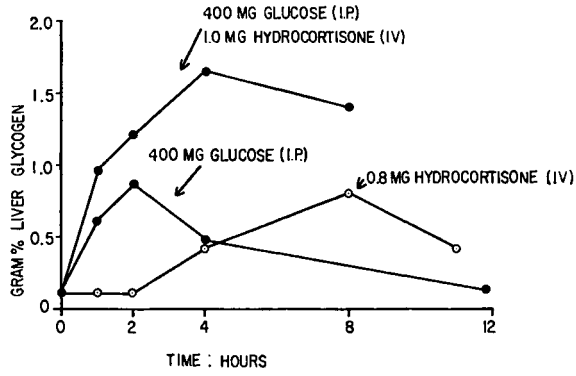


FIG. 3. Liver glycogen time-response curve following the intraperitoneal administration of glucose (Glenn *et al.*, 1961b). Each point is the mean of data for five rats.

Hydrocortisone injection induces decrease in conversion of glucose-1-C^{14} and glycerol-1,3-C^{14} to respiratory $C^{14}O_2$ when substrates are injected in large amount (Fig. 6). Results are to be contrasted to those showing *increased* conversion of palmitate-1-C^{14} to respiratory $C^{14}O_2$ after steroid injection (Fig. 7). Increases or decreases are not observed when glutamate-1-C^{14}, pyruvate-2-C^{14}, pyruvate-1-C^{14}, DL-alanine-2-C^{14}, and DL-proline-1-C^{14} are used as substrates. β-Hydroxybutyrate-1-

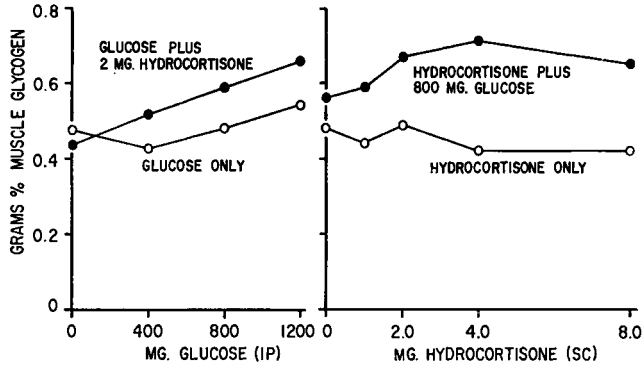


FIG. 4. Dose response of muscle glycogen to glucose and hydrocortisone (Glenn *et al.*, 1961b). Conditions: Gastrocnemius muscles removed 7 hours after injections. Five rats per point.

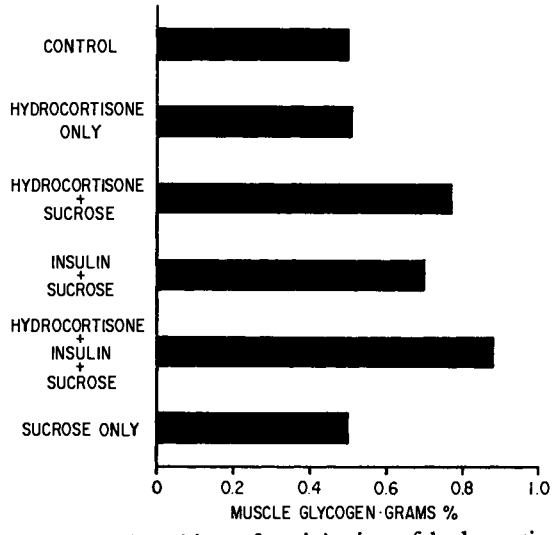


FIG. 5. Muscle glycogen deposition after injection of hydrocortisone and insulin (Glenn *et al.*, 1961a). Conditions: Five rats per point. Insulin, 0.75 units per rat (SC); sucrose, 2.0 gm per rat (oral); hydrocortisone 4.0 mg per rat (SC). Gastrocnemius removed 7 hours after injections.

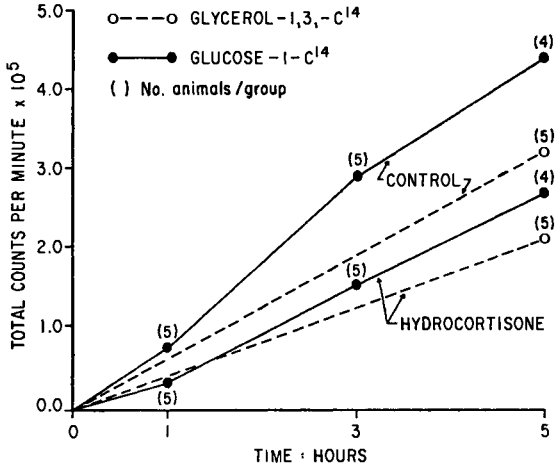


FIG. 6. Effect of hydrocortisone on rate of glucose and glycerol oxidation in fasted adrenalectomized rats (Glenn *et al.*, 1961b). Conditions: Each rat received 500 mg glucose + 2.0 μc glucose-1-C^{14} (IP) or 300 mg glycerol + 2 μc glycerol-1,3-C^{14} (IP), hydrocortisone 2.0 mg per 0.5 ml (SC). Each point is the mean of duplicate determinations. Total barium carbonate at 5 hours: (A) Glucose-treated group: hydrocortisone, 12.875 gm; controls, 12.175 gm. (B) Glycerol-treated group: hydrocortisone, 9.710 gm; controls, 9.600 gm.

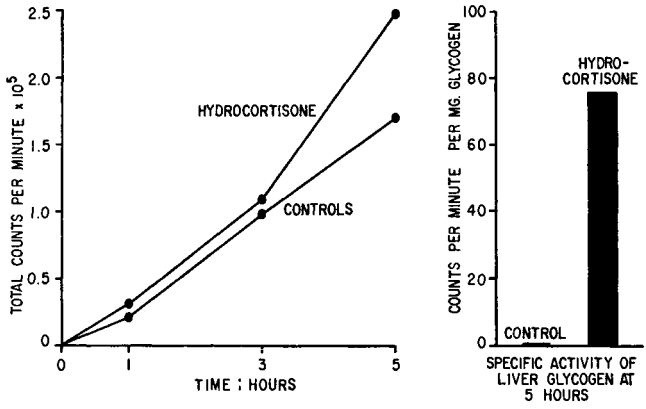


FIG. 7. Effect of hydrocortisone on rate of fat oxidation and its incorporation into liver glycogen (Glenn *et al.*, 1961b). Conditions: Lipomul (5.0 ml) via tail vein (1,986,400 dpm per 5 ml). Five rats per point. Total barium carbonate collected at 5 hours: controls, 11.47 gm, hydrocortisone, 16.17 gm.

C^{14} conversion to expiratory $C^{14}O_2$ is *increased* after steroid injection (unpublished results). Increases in fat oxidation (tripalmitin) parallel changes in *total* CO_2 production after steroid injection (Table I). Conversely, steroid-induced increases in CO_2 production and palmitate oxidation are completely prevented by oral administration of glucose (Glenn *et al.*, 1961b).

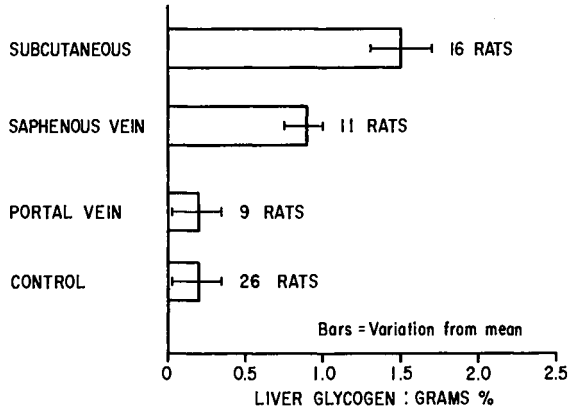


FIG. 8. Liver glycogen response to 1.0 mg hydrocortisone by various routes of administration (Glenn *et al.*, 1961b). Conditions: Livers removed 7 hours after injection for glycogen determinations.

TABLE I

Effect of Hydrocortisone on Total CO_2 Production of Adrenalectomized Fasted Rats[a, b]

Treatment	Number of animals	$BaCO_3$ (mg)	Per cent increase
None	45	11,081	—
Hydrocortisone 2 mg (SC)	45	13,381	20.8

[a]Data from Glenn *et al.* (1961).

[b]CO_2 was collected over 5-hour period after injection of steroid. Increases shown varied from 15 to 41% above normal; they were never the same or below normal.

Respiratory quotients of steroid-injected rats are not different from those of controls (Table II). Slight and insignificant changes occur in

TABLE II

Effect of Hydrocortisone on Respiratory Quotients of Adrenalectomized Rats[a, b]

Treatment	Number of animals	Oxygen uptake (gm/rat)	CO_2 output (gm/rat)	RQ per rat
A. None	4	1.264	1.317	0.75
Hydrocortisone	4	1.724	1.740	0.74
B. None	4	1.363	1.713	0.98
Hydrocortisone	4	1.293	1.639	0.93

[a]Data from Glenn *et al.* (1961b).

[b]Conditions: Group A. A total carbohydrate diet (dextrin : sucrose 2 : 1) was fed for 2 days prior to experiment. Controls ate 118 gm; hydrocortisone treated, 153 gm. Food was removed at 8 : 00 a.m., animals received 2.0 mg hydrocortisone; they had also received 4.0 mg 12 hours earlier. Animals were placed in plastic metabolism cages at 10 : 00 a.m. the above data were obtained during the next 4 hours. Group B. Same as Group A except animals also received 1 gm of sucrose orally at 8 : 00 a.m.

plasma free fatty acids (FFA) of fasted adrenalectomized rats treated with steroid. Results contrast markedly to those to be shown in intact rats. Apparently, fasted adrenalectomized rats utilize free fatty acids at accelerated rates. Steroid has no further effect.

Hepatic glycogenesis is greatest when steroid is injected subcutaneously; less via saphenous vein, and nil via portal vein (Fig. 8). Amounts necessary to induce glycogenesis after portal-venous injection were determined (Fig. 9). Recovery studies were subsequently done on hepatic-venous blood (Glenn *et al.*, 1961b). Extracts of hepatic vein blood were made after portal-venous injection of 16 mg hydrocortisone hemisuccinate (Fig. 10) and reinjected via portal and saphenous veins. Accumulation of glycogen was negligible when extracts traversed portal vein and of expected magnitude when they were intravenously injected.

Data suggest that effects of steroid are due to amounts *escaping* liver, not to steroids *entering* it, i.e., *extrahepatic* (Glenn *et al.*, 1961b).

Some workers (Long *et al.*, 1960) report failure to obtain steroid effects on utilization or plasma levels of glucose in totally eviscerated

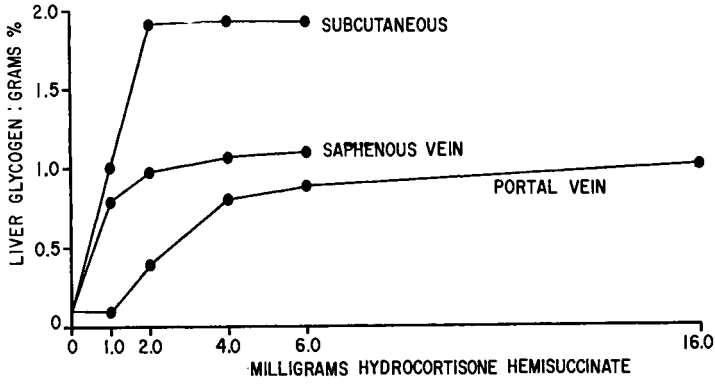


FIG. 9. Dose response curve to hydrocortisone via different routes of administration (Glenn *et al.*, 1961b).

rats. Examination under carefully controlled conditions reveals that hydrocortisone impedes the rate of removal of glucose from plasma of eviscerated rats (Table III). Rats were maintained at surrounding temperatures of 26°C; maintenance at outside temperatures of 37°C

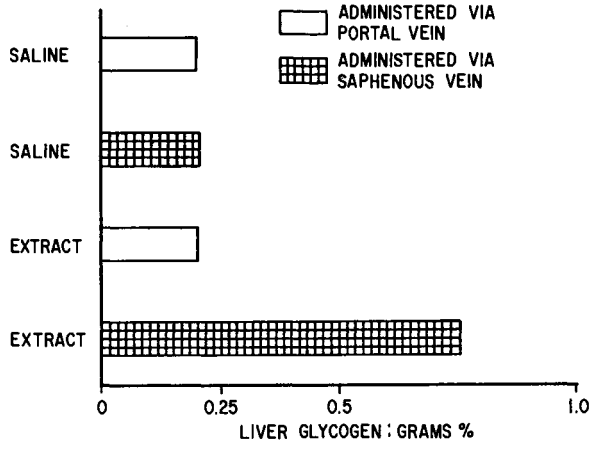


FIG. 10. Glycogenic activity of steroid extracts from the hepatic vein following portal-venous administration of 16 mg hydrocortisone hemisuccinate (Glenn *et al.*, 1961b).

TABLE III

Effect of Subcutaneous Injection of Hydrocortisone on Blood Glucose and Lactate Levels of Eviscerated Rats[a]

Treatment	Dose (mg/rat SC)	Blood glucose (mg%)	Blood lactate (mg%)	Number of rats
Vehicle	0.2 ml	330.0	33.6	6
Hydrocortisone	1.0	508.0	48.1	4
Hydrocortisone	2.0	553.0	46.8	6
Hydrocortisone	4.0	640.0	21.5	3

[a]Inferior vena cavas of intact female rats were ligated 2 weeks before evisceration. Immediately before removal of viscera, animals were injected subcutaneously with the vehicle or hydrocortisone. Animals had been previously anesthetized with sodium cyclopal. Six hours after injection of steroid and removal of viscera, animals were exsanguinated from the dorsal aorta. High blood glucose levels may probably be accounted for by the lack of insulin supply, since exogenous insulin was not injected in these animals. All animals received 200 mg glucose SC 1 hour before evisceration and every 2 hours thereafter in 0.8 ml distilled water for a total of three injections.

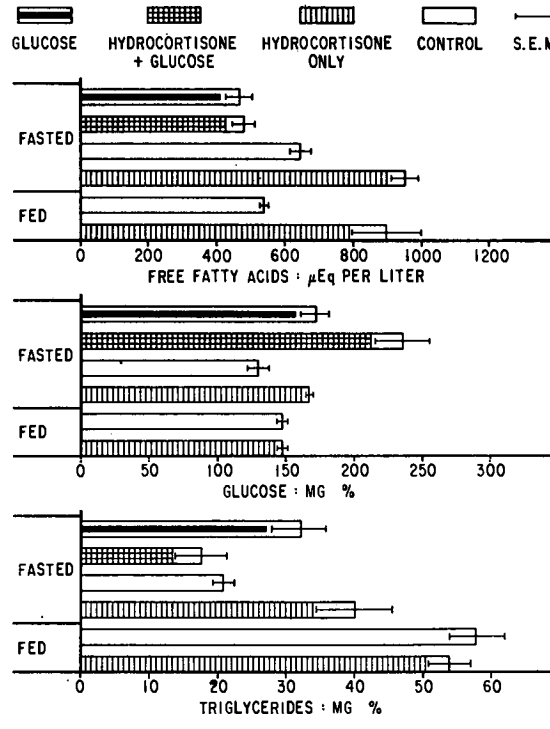


FIG. 11. Effect of fasting, glucose feeding, and hydrocortisone on blood plasma glucose, free fatty acids, and triglycerides. Conditions: Female rats were fasted at 5 P.M. on day 1. Glucose was presented to rats at 8 A.M. on day 2 (ad libitum). Rats receiving hydrocortisone had first injection at 5 P.M. on day 2 and again at 8 A.M. on day 3 (4.0 mg hydrocortisone per rat per injection). Blood was removed 5 hours after last injection. Seven rats per group.

eliminates steroid effect. It is concluded that hydrocortisone inhibits either oxidation or the rate of removal of glucose in fasted adrenalectomized rats, promotes secondary increases in long-chain fatty acid and β-hydroxybutyrate oxidation (since effects occur later), and brings about a *primary* effect on glucose metabolism in tissues other than liver.

Briefly, experiments in fed and fasted intact rats will be summarized. Hydrocortisone injection is followed by increases in plasma glucose, free fatty acids, and triglycerides (Fig. 11). Simultaneous feeding of carbohydrate to fasted rats *prevents* changes in FFA and triglycerides in response to steroid. Again, increased availability of glucose overcomes other effects of steroid. Engel (1950) has already demonstrated that glucose administration prevents increased amino acid metabolism in response to steroid. Recently it has been shown that glucose injection *prevents* increases in blood urea nitrogen after steroid injection to rats (unpublished results). These data would be expected if steroid were *primarily* involved with inhibition of glucose metabolism per se.

B. METABOLICALLY ACTIVE STEROIDS AND EFFECTS ON TUMOR GROWTH AND INFLAMMATION

Increased attention to metabolic effects of adrenocortical steroids is related to the following gross observations. Generally, only metabolically active steroids (glycogenic) are significantly effective C-3-H tumor inhibitors (50–100% inhibition considered significant) in mice (Fig. 12). Only metabolically active steroids are anti-inflammatory agents in rats (Table IV, showing a few representative steroids). For several years

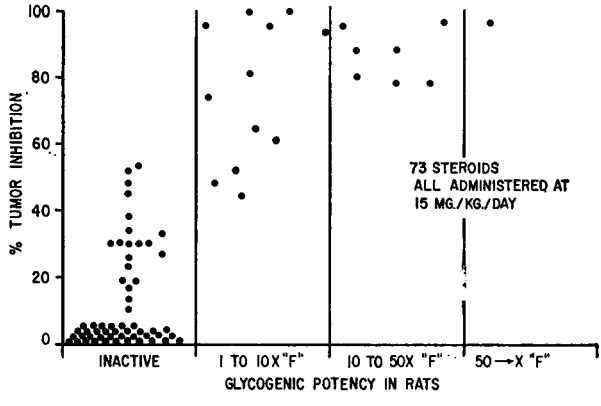


FIG. 12. Relationship between glycogenic activity of C-21 steroids and antitumor activity in C-3-H mice (Glenn *et al.*, 1960). Conditions: Glycogenic potencies were obtained in male adrenalectomized rats; tumor potencies, in C-3-H male mice (10 mice per point, 3–5 dose levels).

TABLE IV

Glycogenic Potencies of Various C-21 Steroids in Rats and Mice[a,b]

Compound	Glycogenic potency		C-3-H Tumor potency	Anti-inflammatory potency, rat
	Rat	Mouse		
Hydrocortisone	1.0	1.0	1.0	1.0
Prednisolone	3.0	5.8	2.5	3.0
9α-Fluoroprednisolone	42.0	15.0	9–10.0	14.0
6α-Methyl-9α-fluoroprednisolone	126.0	3.0	4.2	24.0
6α-Methyl-21-decxy-9α-fluoroprednisolone	25.0	1.0	3.6	135.0
9α-Fluoro-16α-hydroxyprednisolone	33.5	19.0	3.4	6.6
6α,9α-Difluoroprednisolone acetate	443.0	24.0	3.1	66.0
6α-Methylprednisolone	9.6	—[c]	2.0	6.0

[a]Data from Glenn *et al* (1960).

[b]Conditions: Adrenalectomized fasted male rats and intact fasted male mice were used for glycogen assays in the two species; 6–10 animals per group for glycogen assays; 4 dose levels of each steroid were analyzed simultaneously with 4 dose levels of hydrocortisone. At least 4 dose levels (20 mice per point) were used in comparing antitumor potencies in C-3-H mice.

[c]Equal to prednisolone; results were obtained from two separate assays in both C-3-H and Rockland male mice (20–25 gm).

hope has been expressed, and we have been deluded into believing, that separation of metabolic (represented by glycogenic activity) and anti-inflammatory effects could be achieved. The idea was based upon exemplary observation that certain steroids, and only *one* example will be used (oxylone acetate), had marked anti-inflammatory and low glycogenic potency (a ratio of 274 : 10). Glycogen assays were done in male rats; anti-inflammatory, in females. Examination in appropriate sexes revealed that oxylone acetate is 10–11 times as potent as hydrocortisone in male rats; more than 100 times as potent in females as both anti-inflammatory and glycogenic agent (Table V). Therefore, no separation of glycogenic and anti-inflammatory effects exists for this steroid. Castration of female rats fails to alter steroid potency detailed above (unpublished results). Many steroids show similar potency behavior in male and female rats. Explanations for the sex difference are not readily apparent. Liver inactivation may occur at different rates in male and female rats.

TABLE V

Glycogenic and Anti-inflammatory Potencies of 9-α-Fluoro-11β,17α-dihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione 17-acetate (Oxylone Acetate) in Male and Female Rats

Glycogenic potency: hydrocortisone = 1.0		Anti-inflammatory potency: hydrocortisone = 1.0	
Male rats [b]	Female rats [b]	Male rats	Female rats
10.7	127.0	11.1	274.0

[a]These are results of multidose assays which employed at least six levels of compound in each assay. Dose response assays for hydrocortisone in male and female rats for anti-inflammatory and glycogenic activities were essentially identical.

[b]Seven-hour assay. When animals in female anti-inflammatory assay were fasted overnight and their livers were removed for glycogen analysis on following morning, it was shown that the above steroid also had a prolonged *duration* of action and its glycogenic potency was 500–600 times that of hydrocortisone. Thus, potency differences of steroids which employ different assay time intervals, different sexes, and different conditions for comparative purposes are meaningless.

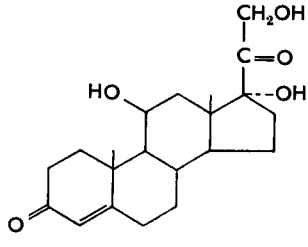
C. STEROID SPECIFICITY IN VIVO AND IN VITRO

Representative steroids, deoxycorticosterone, 11-deoxycortisone, progesterone, pregnanediol, tetrahydrocortisone, dihydrocortisone, and testosterone are systemically inactive with regard to glycogenesis and inflammation when injected subcutaneously (Table VI). Hydro-

TABLE VI

Glycogenic and Anti-inflammatory Effects of Various Steroids Injected Subcutaneously in Female Rats [a]

Compound	Dose (mg/rat, SC)	Liver glycogen (gm %)	Anti-inflammatory (% inhibition)
Vehicle	0.2 ml	0.37	0
Hydrocortisone	2.0	1.89	92.5



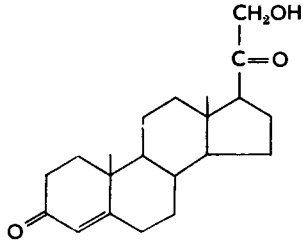
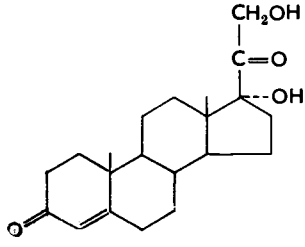
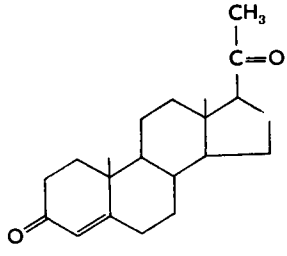
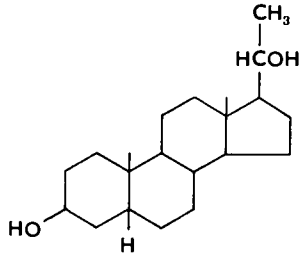
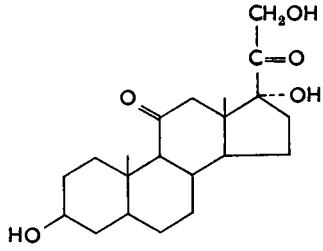
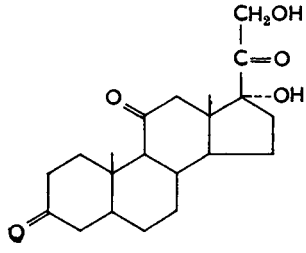
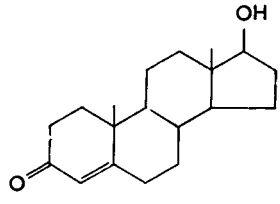
Compound	Dose (mg/rat, SC)	Liver glycogen (gm %)	Anti-inflammatory (% inhibition)
Deoxycorticosterone	1.0, 2.0, 4.0	Inactive	Inactive
			
11–Deoxycortisone	2.0, 4.0, 8.0	Inactive	Inactive
			
Progesterone	1.0, 2.0, 4.0	Inactive	Inactive
			
Pregnanediol	1.0, 2.0, 8.0	Inactive	Inactive
			

Table VI continued

Compound	Dose (mg/rat, SC)	Liver glycogen (gm %)	Anti-inflammatory (% inhibition)
Tetrahydrocortisone	1.0, 2.0, 8.0	Inactive	Inactive
			
Dihydrocortisone	1.0, 2.0, 10.0	Inactive	Inactive
			
Testosterone	1.0, 2.0, 4.0	Inactive	Inactive
			

[a]These assays employed 24-hour fasted adrenalectomized female rats for the glycogen assays (7-hour) and intact female rats for the anti-inflammatory assays, which were conducted over a 7-day period. At least five rats per group were employed in all assays. These are cumulative data; most of the above compounds have been assayed on many occasions, including those shown above.

cortisone is active on both parameters via this route. However, local injection directly into sites of inflammation reveals that hydrocortisone, progesterone, deoxycorticosterone, and 11-deoxycortisone are *all* active anti-inflammatory agents (Table VII). Androgens and reduced

TABLE VII

Anti-inflammatory Effects of Various Steroids When Locally Injected in Croton Oil-Induced Granuloma Pouches [a]

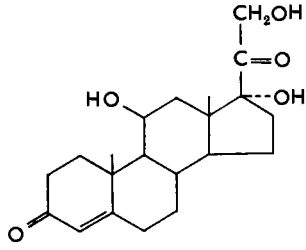
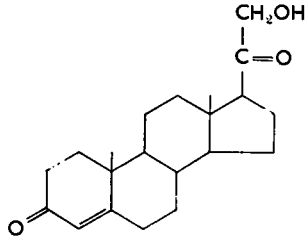
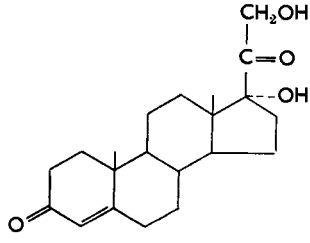
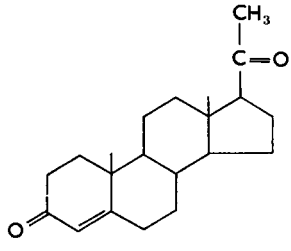
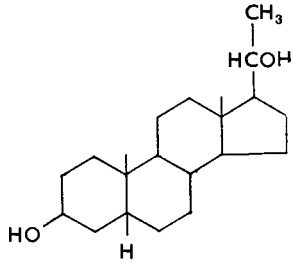
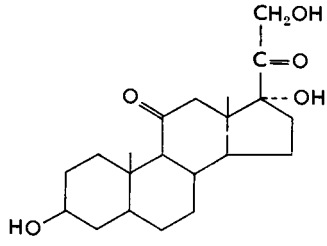
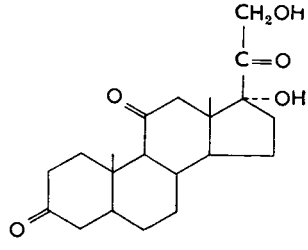
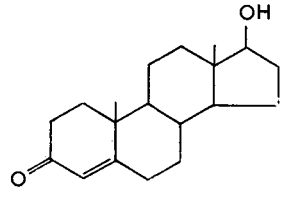
Compound	Dose, local (μg/pouch/day)	Per cent inhibition of inflammation (exudate formation)	Summary
Vehicle	0.2 ml/day	—	—
Hydrocortisone	25.0, 50.0, 100.0	60.0, 74.0, 94.5	Active
			
Deoxycorticosterone	200.0	95.0	Active
			
11-Deoxycortisone	200.0, 400.0	67.5, 85.0	Active
			
Progesterone	200.0	82.0	Active
			

Table VII continued

Compound	Dose, local (μg/pouch/day)	Per cent inhibition of inflammation (exudate formation)	Summary
Pregnanediol	400.0	4.5	Inactive
			
Tetrahydrocortisone	400.0	2.9	Inactive
			
Dihydrocortisone	400.0	0	Inactive
			
Testosterone	100.0, 400.0	10.0, 0	Inactive
			

[a]These results were selected at random from research notebooks. Compounds have been assayed at varying dose levels many times, and results are always in essential agreement with those indicated above. Quantitative relationships have been carefully analyzed, but for present purpose these are unnecessary.

[b]Related derivatives, i.e., 9α-fluoro-11β-hydroxy-17-methyltestosterone, 2α-methyl-9α-fluoro-11β-hydroxy-17-methyltestosterone, and 17-methyltestosterone, are also inactive.

A-ring derivatives of the pregnenes are inactive. Explanations are probably related to the effects set forth below.

D. IN VITRO METABOLISM OF PROGESTERONE AND 6-α-METHYL ANALOGS

When compared to 6-α-methyl analogs, the rate of reduction and subsequent biologic inactivation of the A-ring of progesterone is extremely rapid (Fig. 13). Introduction of 6-α-methyl group on 11-deoxycortisone and progesterone now renders "inactive" compounds capable of causing glycogenesis when subcutaneously injected (Fig. 14). Related studies with 6-α-methyl-17-acetoxyprogesterone show it to have characteristic biologic features of 11-oxygenated steroids (Glenn *et al.*, 1959). Results suggest that 11,21 hydroxylation is not mandatory for "adrenocortical steroidlike" activity. Results lead to the conclusion that discrepancies between *in vitro* and *in vivo* effects of steroids may be related to phenomena just outlined.

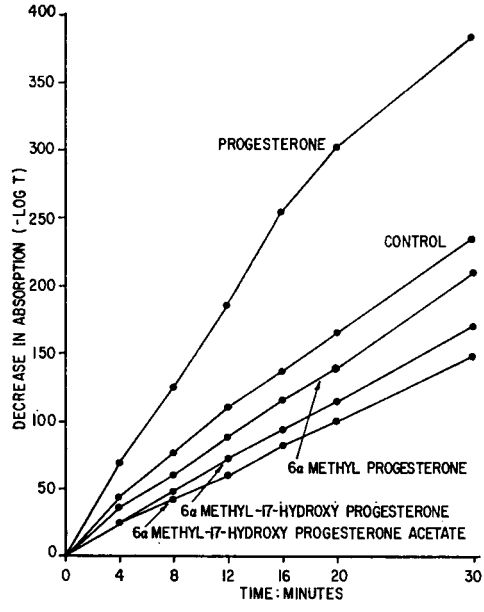


FIG. 13. Effect of progesterone, 6α-methylprogesterone, 6α-methyl-17-hydroxy progesterone, and 6α-methyl-17-hydroxyprogesterone acetate on Oxidation of TPNH (Glenn *et al.*, 1959). Conditions: 3.0 ml reaction mixture contained 9.6×10^{-4} *M* $MgCl_2$; 4.8×10^{-2} *M* phosphate buffer (pH 7.4); 0.25 *M* sucrose; 0.04 *M* nicotinamide; $3.0 \times \times 10^{-4}$ *M* TPNH; 3.0×10^{-4} *M* steroid in 0.01 ml dimethyl-acetamide, 60 meg enzyme per cuvette. Reaction was followed at 340 mμ in cuvette of 1 cm light path. Duplicate determinations from duplicate samples.

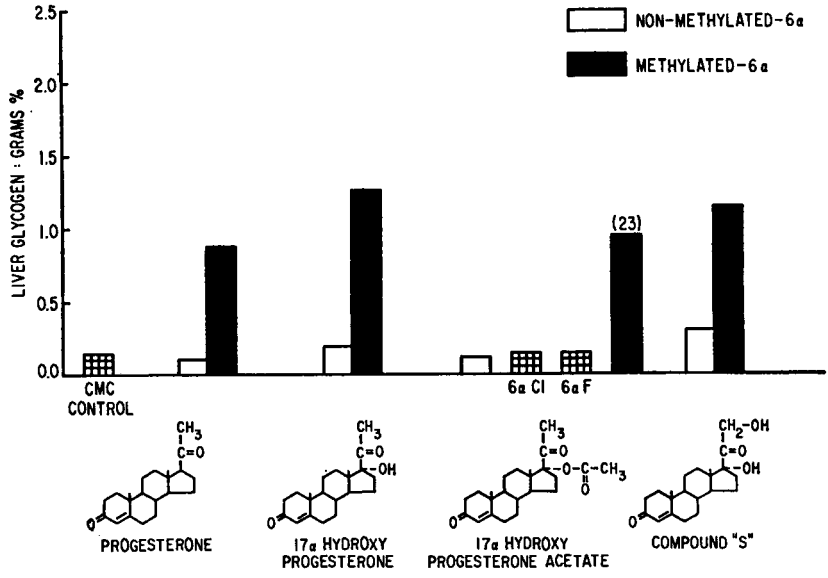


FIG. 14. Glycogenic activity of various 6α-methyl steroids (Glenn *et al.*, 1959). Conditions: All compounds were administered subcutaneously, 2.0 mg at beginning of fast and again 24 hours later. Livers were removed 7 hours after last injection. Each bar represents 6 rats, unless indicated otherwise.

E. STEROIDAL METABOLIC AND ANTI-INFLAMMATORY EFFECTS

Steroids can be locally injected with resultant *direct* anti-inflammatory effects. Attention was directed toward this phenomenon in attempts to relate metabolic to anti-inflammatory activity. It has already been demonstrated that hydrocortisone somehow alters the metabolism of glucose.

Inflammatory granuloma pouches formed in rats by injection of croton oil into pneumodermas are affected as summarized in Table VIII by glucagon, ACTH, growth hormone, L-epinephrine, insulin, and gonadotropin. Daily local injections of thyroxine or 3,3,5-triiodothyronine have no effect on the inflammatory response (unpublished results). L-Epinephrine, under certain conditions, is a locally potent anti-inflammatory agent. The others are inactive in the absence of the adrenal cortex. Neither phenylbutazone nor aspirin are locally effective on the croton oil inflammatory process; since they are active when orally administered, they must exert effects via some entirely different mechanism from hydrocortisone. Adrenalectomy and hypophysectomy have little definite effect, whereas pancreatectomy, but not combined pancreatectomy–adrenalectomy, inhibits an inflammatory response to

TABLE VIII

Summary of Anti-inflammatory and Pro-inflammatory Effects of Various Nonsteroidal Hormones on the Inflammatory Reaction Induced by Croton Oil

Compound[a]	Route of injection	Summary of cumulative assay data concerning activity[b]
Glucagon	Subcutaneous (intact rats)	1.2–1.5 × Hydrocortisone
Glucagon	Local	Inactive
Glucagon	Subcutaneous (Adrenalectomized rats)	Inactive except at toxic dose levels
ACTH	Subcutaneous	Active in intact rats
ACTH	Local	Active at high dose levels in intact, but not in adrenalectomized, rats
L-Epinephrine	Local	Active; 1.0–1.5 × hydrocortisone
Growth hormone	Intramuscular	Inactive
Growth hormone	Local	Inactive
Gonadogen (PMS)[c]	Subcutaneous	Active at dose levels that cause marked ovarian stimulation
Gonadogen (PMS)[c]	Local	Inactive (dose levels employed included thoses which began to stimulate ovaries)
Insulin	Local	Inactive (includes higher dose level that were barely compatible with life)

[a]All the above compounds were injected as indicated at varying dose levels in carboxymethylcellulose vehicle (CMC) for 7 days.

[b]Compounds were injected at 4–6 different dose levels, higher levels included those eliciting characteristic systemic effects.

[c]Pregnant mare serum gonadotropin.

croton oil. More extensive investigations regarding the above phenomena will be presented elsewhere (Glenn *et al.*, 1963).

If inhibition of glucose utilization is somehow involved with steroid action, it should be possible to show nonsteroidal glycolysis inhibitors to be active on the inflammatory process. Lardy *et al.* (1950) has demonstrated that L-glyceraldehyde inhibits the *hexokinase* reaction of brain homogenates. Table IX shows results of addition of D-glyceraldehyde, L-glyceraldehyde, DL-glyceraldehyde, dihydroxyacetone, and glycerol to rat brain homogenates under anaerobic conditions. Three glyceraldehydes alone inhibit glucose conversion to lactate and inflammation when locally injected (Fig. 15). *p*-Chloromercuribenzoate, mercuric iodide, iodoacetic acid, tetramethylthiuram disulfide and many other brain homogenate glycolysis inhibitors markedly reduce the inflammatory reaction when locally injected (Glenn *et al.*, 1963). Glucose and related carbohydrates, in contrast to 2-deoxyglucose and phlorizin, do not inhibit inflammation even when injected in extremely large amounts (unpublished results).

TABLE IX

Effect of Glyceraldehydes and Related Compounds on Anaerobic Glycolysis by Rat Brain Homogenates[a]

Compound	Final molarity	Glucose utilized in 120 min. (mg/gm tissue)	Lactate produced in 120 min. (mg/gm tissue)
Control	—	9.65	7.95
Glycerol	10^{-2}	9.22	7.25
Dihydroxyacetone	10^{-2}	7.72	8.30
D-Glyceraldehyde	10^{-3}	7.49	7.70
	10^{-2}	2.25	2.75
DL-Glyceraldehyde	10^{-4}	4.25	4.50
	10^{-3}	0.80	1.75
	10^{-2}	0.21	0.88
L-Glyceraldehyde	10^{-4}	0	4.50
	10^{-3}	0	2.10
	10^{-2}	0	1.22
Control	—	8.35	7.98

[a]Brain tissue removed from female rats was homogenized in Krebs-phosphate-Ca^{++}-free buffer (pH 7.4) so that bufler contained 1 gm brain / 10 ml. Glucose was added to a final concentration of 100 mg/100 ml. Incubations were carried out at 37°C under anaerobic conditions. All samples were analyzed in duplicate.

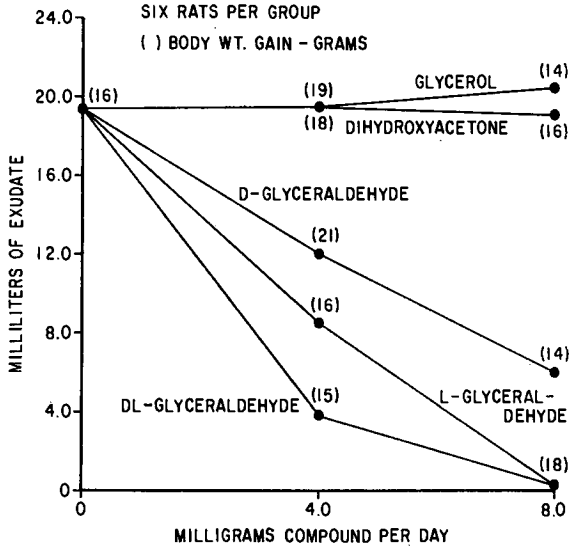


FIG. 15. Comparative local anti-inflammatory activity of glyceraldehyde and related compounds. Six rats per group.

On the basis of the example summarized above, the possibility can be deduced of leaping from observations of gross metabolic effects of certain hormones and showing that nonhormonal agents behave in similar manner on specific biologic end points. DL-Glyceraldehyde actually inhibits glucose metabolism by tissue when it is added *in vitro* to isolated granuloma pouches made with D-α-tocopherol (Glenn *et al.*, 1963).

F. POSSIBLE RELATIONSHIPS BETWEEN IN VIVO AND IN VITRO EFFECTS OF STEROIDS

While the work described above was in progress, Yielding and others (1960) demonstrated DPNH oxidase inhibition *in vitro* with various steroids. Figure 16 shows some of steroids which have been investigated in these laboratories using rat kidney microsomes. Progesterone is the most active *in vitro* inhibitor. Disappointingly, hydrocortisone was inactive, and we have been unable to show inhibition of DPNH oxidase under a variety of experimental conditions. Of ac-

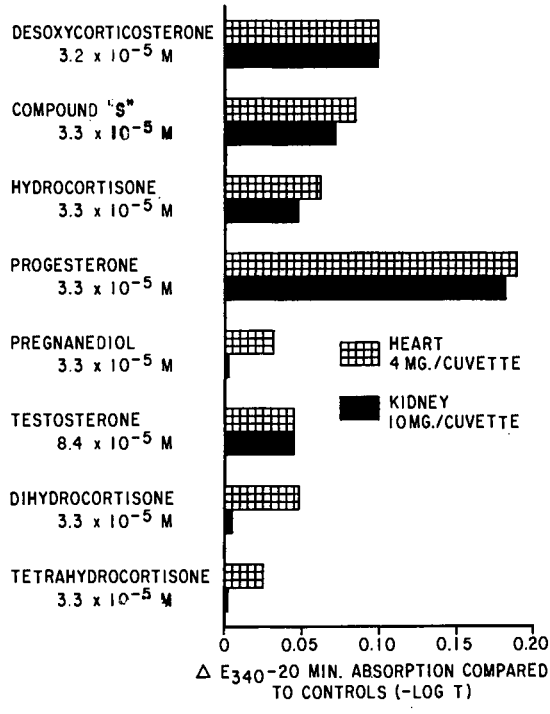


FIG. 16. Effect of various steroids on the oxidation of DPNH by nuclei-free kidney and heart preparations.

tive agents studied, however, all are locally active anti-inflammatory agents; others are inactive on both parameters. Estradiol is extremely effective as local anti-inflammatory agent and DPNH oxidase inhibitor in adrenalectomized rats and *in vitro*, respectively (Glenn *et al.*, 1963). KCN, antimycin A, and *p*-chloromercuriphenyl sulfonic acid, also effective nonsteroidal electron transport inhibitors, are extremely active,

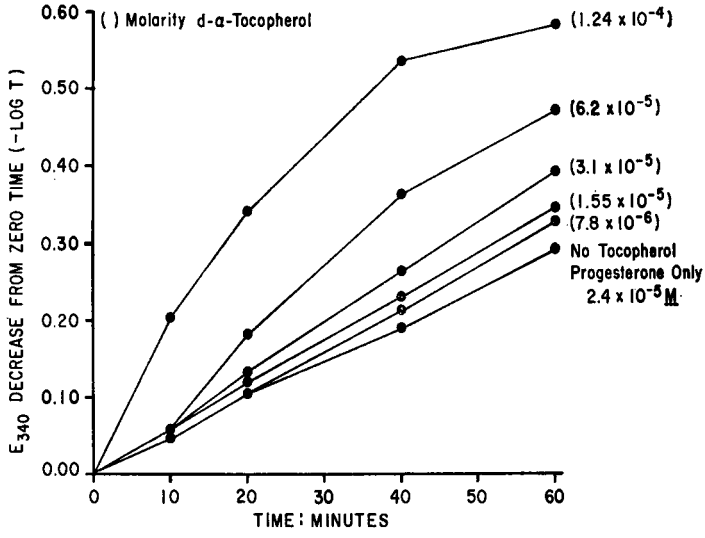


FIG. 17. Effect of D-α-tocopherol on progesterone inhibition of DPNH oxidation.

locally effective, anti-inflammatory compounds (Glenn *et al.*, 1963). Phenazine methasulfate and methylene blue, which *promote* DPNH oxidation, in presence of KCN and electron transport particles *in vitro*, also act as direct anti-inflammatory agents when locally injected (Glenn *et al.*, 1963). Latter two compounds also inhibit the normal course of electron flow from DPNH to oxygen. Presumably, they inhibit synthesis of ATP and other high energy compounds for metabolic work, as do all other compounds discussed.

In confirmation of Yielding and others (1960), D-α-tocopherol addition inhibits effects of progesterone on DPNH oxidase (Fig. 17). Simultaneous injection of D-α-tocopherol directly into inflammatory pouches caused by croton oil also overcomes inhibitory effects of progesterone *in vivo* (Table X).

D-α-Tocopherol injection alone into 25-ml pneumodermas of rats causes a remarkable inflammatory reaction (Table XI). Hydrocortisone overcomes the inflammatory effect when injections are started at initiation or after progression of the reaction (Table XII). Cessation of

TABLE X

Effect of α-Tocopherol on Local Anti-inflammatory Response to Progesterone[a]

Compound[b]	Mg/pouch/rat	Exudate (ml)	Body weight/rat 7 days (gm)
Vehicle	0.4 ml	18.0	14
A. Progesterone	0.1	7.9	22
B. α-Tocopherol	8.0	18.2	20
C. α-Tocopherol	16.0	18.0	17
D. α-Tocopherol	32.0	17.8	21
A + B	—	14.2	20
A + C	—	14.0	22
A + D	—	14.5	24

[a]Fivel rats per group

[b]Vehicle: 0.2 ml CMC + 0.2 ml cottonseed oil per day per pouch per rat; 7 days. Group A also received 0.2 ml cottonseed oil locally.

TABLE XI

Specificity of the Inflammatory Reaction Induced in Pneumodermas of Rats by D-α-*Tocopherol*[a]

Substance injected	Amount injected; day 1 (ml)	Exudate at 7 days (ml)
D-α-Tocopherol	0.5	26.0±1.5
Corn oil	0.5	0
	1.0	0
	1.5	0
Cottonseed oil	0.5	0
	1.0	0
	1.5	0
Linseed oil	0.5	0
	1.0	0
	1.5	0
Mineral oil	0.5	1.4[b]

[a]Five per group; compounds were injected on day 1 only into 25-ml pneumodermas; exudates were removed 7 days later.

[b]Foamy clear exudates which were not characteristic of inflammatory exudates produced by D-α-tocopherol.

steroid treatment allows the response to resume a characteristic growth pattern. Effects of steroid are reversible and its presence required.

In contrast to croton oil-induced reactions, D-α-tocopherol-induced inflammatory reactions persist for many months after one injection of D-α-tocopherol (Fig. 18, showing 5-month-old pouches) and pouches are easily removed from animals (Fig. 19). D-α-Tocopherol pouch exudates are plasmalike in appearance; croton oil pouches, bloody and viscous. D-α-Tocopherol exudates per se do not metabolize glucose;

TABLE XII

Inhibitory Action of Hydrocortisone on D-α-*Tocopherol-Induced Granuloma Pouches and Effect of Subsequent Withdrawal (Local Injections)*

Compound and treament	Mg/day local	Exudate (ml)	Body weight change (gm)	Per cent inhibition
Vehicle (days 1–7)	0.2 ml	34.4±3.0	+23	—
Hydrocortisone (days 1–7)	0.8	0	— 1	100
Vehicle (days 8–22)	0.2 ml	46.6	+60	—
Hydrocortisone (days 1–7 withdrawn days 8–22)	0.8	38.0	+49	18
Vehicle (days 1–15)	0.2 ml	37.4±6.1	+43	—
Hydrocortisone (days 3–15)	0.8	11.0±4.0	+ 5	71

[a]Five to ten rats per group. D-α-Tocopherol was injected on day 1 only (25 ml air+0.5 ml). Hydrocortisone and vehicle were injected once daily directly into pouch lumen for indicated times.

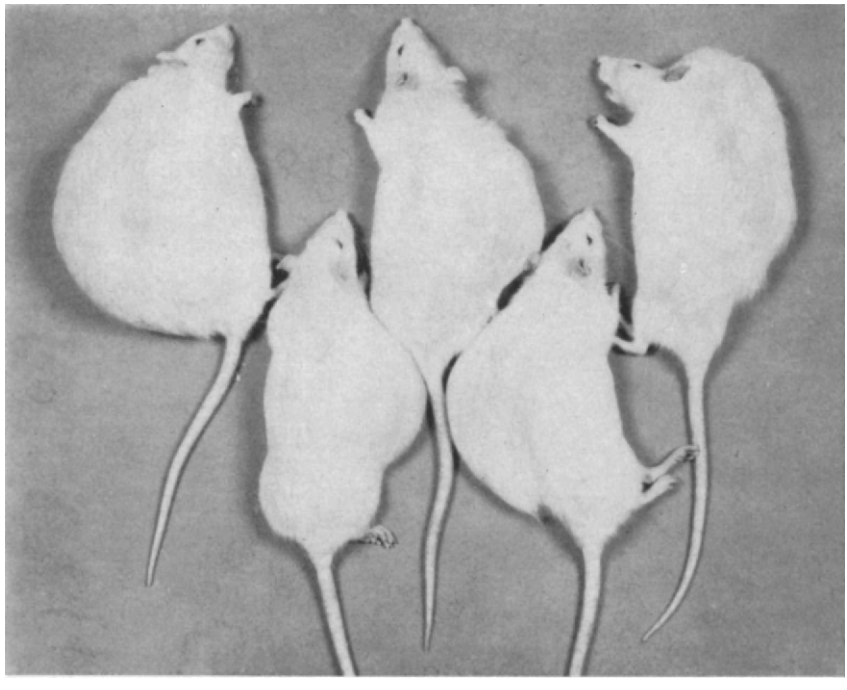


FIG. 18. Five-month D-α-tocopherol-induced pouches.

croton oil-induced exudates do. In Table XIII are results of studies on the chemical composition of D-α-tocopherol-induced pouches contrasted to plasma removed from same animals. Most marked difference is high concentrations of lactate (2 times the plasma concentrations). Numerous studies have been successfully accomplished with D-α-tocopherol pouches and their fluids, but only one or two will be described.

FIG. 19. Removal of 7-day D-α-tocopherol pouches from female rats.

TABLE XIII

Substrate Composition of Plasma and Exudates in Rats with D-α-*Tocopherol Pouches: 7-Day-Old Pouches*[a, b]

Exudate (ml)	Gluc. (mg%)		Lact (mg%)		AAN. (mg%)		Protein(mg%)		FFA (neq/l)		Trig. (mg%)	
	Plasma	Exud.	Plasma	Exud.	Plasma	Exud.	Plasma	Exud.	Plasma	Exud.	Plasma	Exud.
20.4	110 ±3.4[c]	112 ±2.4	20.3 ±0.5	44.3 ±1.1	5.70 ±0.1	6.70 ±0.1	5.49 ±0.1	4.01 ±0.1	389 ±10.5	574 +26.1	45.8 ±3.3	64.7 ±6.8

[a]Ten rats per point. pH of all exudates 7.4–7.5. Urea nitrogen concentrations are same in both plasma and exudates (19–22 mg/100 ml)

	Serum	Exudate
Sodium (meq/l)	137	135
Patassium (meq/l)	4.2	5.6

[b]Abbreviations: Gluc., glucose; lact., lactate; AAN, amino acid nitrogen; FFA, free fatty acids; Trig; triglycerides.
Standard error of the mean.

TABLE XIV

Effect of Prolonged Injections of Hydrocortisone on Plasma and Exudate Levels of Amino Acids, Protein, Lactate, and Glucose in D-α-*Tocopherol Pouches*

Treatment	Exudate (ml)	Plasma (mg%)				Exudate (mg%)			
		Amino acid N	Protein	Glucose	Lactate	Amino acid N	Protein	Glucose	Lactate
None	38.0	5.7	6000	136	13.2	7.9	4600	87	45.0
Hydrocortisone[a]	33.3	5.9	6300	133	19.5	7.4	5000	110	31.9
Per cent change	—12.0	0	+5.0	0	+50.0	0	+8.0	+26.0	—30.0

[a]Hydrocortisone (1.0 mg/day for 5 days, SC, prior to removal of pouches and plasma). Pouches were 7 days old before initiation of steroid injections. Please note rise in exudate glucose and fall in exudate lactate. Of interest is the lack of effect of these dose levels of hydrocortisone on the plasma glucose levels and the highly significant increase in plasma lactate after treatment with hormone. Of considerable importance is that the percentage rise in glucose levels in exudates almost approximates fall in lactate levels in same fluid.

For example, studies done on exudates at weekly intervals reveal that glucose levels steadily decrease; lactate levels increase proportionately. Glucose levels at the end of 1 week in croton oil pouches and D-α-tocopherol pouches are 30–50 mg% and 105–120 mg%, respectively.

Local injection of hydrocortisone into D-α-tocopherol-induced pouches is followed by little biochemical change with the exception of lactate, which decreases, and glucose, which increases (Tables XIV and XV). Plasma glucose levels increase or remain the same, depending upon the amounts of steroid injected. Lactate levels in plasma are *always* increased after steroid injection to intact rats. Increased lactate levels can be induced merely by raising plasma glucose with oral administration of carbohydrate; increases in lactate levels after steroid may merely reflect increasing blood glucose concentrations. Similar results on glucose and lactate levels of exudates following prolonged local injections of hydrocortisone into D-α-tocopherol pouches are shown again in Fig. 20.

TABLE XV

Local Effect of Hydrocortisone on Exudate Levels of Lactate in the D-α-Tocopherol-Induced Granuloma Pouch[a]

Treatment	Exudate (ml)	Lactate (mg%)	Lactate (mg/pouch)
None	27.0	38.6	10.4
Hydrocortisone (200 μg)	25.0	28.5	7.1
Hydrocortisone (400 μg)	23.0	23.1	5.4
Hydrocortisone (800 μg)	22.0	29.7	6.4

[a]Hydrocortisone at the indicated doses was injected subcutaneously into the *wall* of pouches at P.M. and 8 A.M, Pouches were removed at 1 P.M. or 5 hours after last injection of hydrocortisone. Five pouches per group.

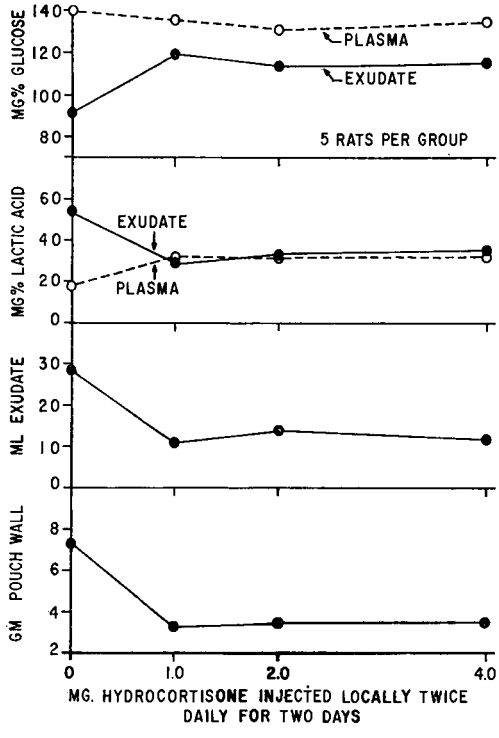


FIG. 20. Effect of prolonged injections of hydrocortisone on blood plasma and D-α-tocopherol exudate levels of glucose and lactic acid.

Prior local injection of hydrocortisone, removal of pouches, and incubation in their own fluid *in vitro* show that steroid prevents subsequent utilization of glucose and production of lactic acid (Table XVI).

TABLE XVI

Effect of Prior Local Injection of Hydrocortisone on Subsequent Utilization of Glucose and Production of Lactate by Isolated D-α-*Tocopherol-Induced Granuloma Pouches*[a]

Prior treatment	Exudate (ml)	Glucose (mg%)		Lactate (mg%)	
		Zero time	120 Min.	Zero time	120 Min.
Vehicle	29.0	112	34	55.7	128.1
Hydrocortisone	27.6	131	73	48.9	111.2

[a]Five pouches per group; removed from D-α-tocopherol pouches that had been made week previously. Five hours before removal of pouches, hydrocortisone (4.0 mg) or the vehicle (CMC) were injected into the SC tissue of *wall* of granuloma pouch. Pouches were removed by quickly excising them from back of animal and clipping skin flap over incision of isolated pouch. Pouches were placed in 100-ml beakers and incubated at 37°C for the indicated time interval in a high humidity atmosphere. Value represent those obtained on exudates alone.

Pouch	Glucose utilized (mg%)	Lactate produced (mg%)
Control pouches	78.0	72.4
Hydrocortisone pouches	58.0	63.3

Inadequate time is available to present or review all the numerous data accumulated as result of studies with D-α-tocopherol-induced granuloma pouches. Hyperglycemic effects of glucagon and hydrocortisone and hypoglycemic effects of insulin can be followed *in situ* by removal of pouch fluid at $^1/_2$-hour intervals for long periods (16 hours) after systemic injections of compounds. Large amounts of substrate (glucose, lactic acid, glycogenic amino acids, and other compounds) can be injected in large amount into pouches of adrenalectomized and intact rats and systemic effects, i.e., glycogen accumulation, followed. These are doses of substrate which kill adrenalectomized rats when injected orally and intraperitoneally. Significantly, D-α-tocopherol pouch fluid has been successfully employed for incubation of adipose and other rat tissues *in vitro*. Since it is a sterile medium, effects of ACTH, glucagon, L-epinephrine, hydrocortisone, and other steroids on glucose and fatty acid metabolism have been successfully demonstrated. Systemic and local anti-inflammatory effects of various selected steroids on D-α-tocopherol- and croton oil-induced pouches closely parallel effects on glucose and FFA metabolism of isolated adipose tissue (Glenn *et al.*, 1962).

III. CONCLUSIONS

1. In rats, hydrocortisone inhibits glucose metabolism *in vivo* and *in vitro* [inflamed tissue of granuloma pouches and adipose tissue (Glenn *et al.*, 1963)].

2. Nonsteroidal compounds, effective inhibitors of glucose metabolism, also inhibit inflammation, lending further support to *initial* observations with steroid in intact, adrenalectomized, and eviscerated rats.

3. Biologically active steroids, i.e., progesterone and estradiol, and nonsteroids which inhibit DPNH oxidase of kidney microsomes also inhibit inflammation. Conversely compounds, i.e., D-α-tocopherol, inhibit steroid effects *in vitro* and *in vivo*, as well as behave on the inflammatory process in entirely opposite manner. However, these statements do not imply that electron transport stimulation or inhibition, as reflected by DPNH oxidase activity, is the primary *modus operandi* of either *pro*-inflammatory or *anti*-inflammatory stimuli.

4. From theoretical considerations and observations, a tool has been devised for study of intermediary metabolism related to a specific therapeutic problem—*inflammation*. Completed studies (Glenn *et al.*, 1963) reveal many interrelated practical uses for this phenomenon.

5. Of furthe importance in understanding problems of steroid action are studies revealing that apparent "specificities of steroids derived from systemic injections alone may be misleading."

6. Inflammation is primarily a metabolic problem with all its inherent subtleties. Knowledge of exquisite biochemical control of process is in embryonic stages. Nevertheless, a firm foundation has been laid.

7. Application of actual data to theoretical considerations leads to the conclusion that inflammation, *whatever* its cause, elicits increased demands upon energy-generating machinery of adjacent cells. Ability to generate increased energy in response to extracellular demands causes the whole spectrum of inflammation to become apparent. Direct interference with the energy-generating process prevents cells from responding to noxious pro-inflammatory stimuli.

From the foregoing account, it appears that both the inciting inducer and terminal inhibitor of inflammation are relatively nonspecific. Increased cellular demands for energy production or its inhibition can be accomplished at either many well-known or undisclosed enzymatic and cellular sites of metabolic control.

ACKNOWLEDGMENT

The author gratefully acknowledges competent technical assistance of Miss Barbara J. Bowman, Mr. Raymond B. Bayer, Mr. M. L. Myers, and Miss Elizabeth Willke.

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DISCUSSION

Aspects of the Anti-inflammatory Action of Prednisolone

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The hitherto unpublished investigations reported here were carried out at our laboratories in collaboration with L. Szporny and E. Ezer.

These investigations are concerned with the migration of colloids and crystalloids under inflammatory conditions. The experiments were carried out according to the following method. The substances under study were labeled with radioactive isotopes: I^{131}-labeled albumin or phosphate ions containing P_{32} were introduced subcutaneously into the paw of male rats. In the case of iodoalbumin the total activity introduced was 0.2–0.3 μc, and in the case of phosphate it was 0.5 to 1 μc, the volume of the introduced isotope being 0.1 ml. Immediately after introduction, activity was registered by means of an end-window GM tube and a rate meter adjusted to the surface of the sole of the paw. Activity was measured for a period of 300 minutes in the case of iodoalbumin, for 15 minutes in the case of phosphate. The inflammatory substance, in the present experiment 10 μg of serotonin, was injected (0.1-ml volume) into the paw simultaneously with the isotope.

11β,17,21-trihydroxypregna-1,4-diene-3,20-dione (prednisolone) was administered to the animals intraperitoneally in doses of 1 mg per 100 gm body weight 24 hours before the experiment and in doses of 0.5 mg/100 gm 4 hours before the experiment. These doses were chosen because in previous experiments they had been found to reduce the volume of edema due to the applied amounts of serotonin, in general, by approximately 50%. Accordingly, changes were likely to occur both in a positive and in a negative direction. The results obtained by experiments with iodoalbumin are presented in Table I; the values reflect the activity observed in the sole area 300 minutes after

TABLE I

Disappearance Rate of I^{131}-labeled iodoalbumin from Hind Paw of Rat

No.	Number of rats	Treatment	Activity as % of starting value	Significance
1	15	Controls	18	1–2, $P > 0.2$
2	20	Serotonin	21.5	1–3, $P < 0.01$
3	15	Prednisolone	50.3	
4	10	Serotonin + prednisolone	37	2–3, $P < 0.01$

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administration of the isotope, calculated as a percentage of initial activity. These values, as compared with values for controls without inflammation, show that the speed of iodoalbumin elimination was not influenced by inflammation provoked by serotonin. When animals were pretreated with prednisolone, iodoalbumin migrated at a much slower rate. Most regrettably, results based on an inflammatory substance plus prednisolone are not yet final. Although the results so far obtained would seem to also indicate that under such conditions the rate of migration is inhibited by prednisolone, no definite statement can be made.

The experiments with phosphate are demonstrated in Table II. The method of evaluation corresponds to that employed before, but this time the activity, as measured 15 minutes after administration, was the basis of calculation of the percentage of the initial value. From Table II it clearly emerges that the disappearance rate of activity was slightly reduced by serotonin as compared with values for untreated controls. Neither in control animals nor in those given pretreatment with serotonin did prednisolone exert any influence whatsoever on the elimination of phosphate, notwithstanding its considerable inhibitive action on the development of edema.

TABLE II

Disappearance Rate of P^{32} Phosphate Ions from Hind Paw of Rat

No.	Number of rats	Treatment	Activity as % of starting value	Significance
1	20	Controls	16	1–2, $P<0.01$
2	20	Serotonin	34	2–3, $P<0.01$
3	20	Prednisolone	15	3–4, $P<0.01$
4	20	Prednisolone + serotonin	38	1–4, $P<0.01$

These results can hardly be reconciled with existing conceptions of the mechanism responsible for inflammation and anti-inflammatory action. It is a generally accepted theory that acute inflammations are accompanied by considerable changes of vascular permeability and of lymphatic drainage which play an important role in the development of edema. The studies described, on the other hand, demonstrate that the action of prednisolone on the absorption of inflammatory edema and its action on that of colloids and crystalloids do not run a parallel course.

The action of prednisolone inhibiting permeability of macromolecules, in this case iodoalbumin, is equivocal. The permeability of crystalloids looks different in the light of quantitative interpretation and in respect to concentration. Judged quantitatively, prednisolone is ineffective; however, the fact that, under the influence of prednisolone, water does not carry with it phosphate, nevertheless permits the inference that an active function must have changed. Only the effect of water migration is perfectly clear, this process being strongly enhanced by prednisolone in the direction of the vascular lumen.

Possible Mode of Action of Adrenocortical Steroids

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The adrenocortical steroids have long been considered to play an important part in the regulation of several major metabolic processes in vertebrates. This concept has been modified in recent years by the findings of Ingle and his colleagues that many of these processes appeared to be regulated by other agents, although they were dependent upon the presence of adrenocortical hormones for their proper functioning (Ingle, 1952). There seems to be good evidence now that all or most of the processes affected by glucocorticoids (cortisone-like steroids) are of this sort; the glucocorticoids are "permissive" rather than "regulatory" in their actions. This is such an important concept that any serious possibility of its being incorrect needs careful examination.

Two aspects seem to need further investigation although they may well be found to confirm, rather than to invalidate Ingle's theory. First, it is now recognized that the constant dose of steroid given in Ingle's "extra control experiments" sets an upper limit on the response to "stress" that is under study; a small dose allows a full response to small "stresses," but only large doses allow the full response to be obtained with a large "stress." Second, much recent work on the association of cortisol with plasma proteins, and on the many factors affecting the rate of elimination of cortisol (or of corticosterone) from the body, suggests that the concentration of freely diffusible cortisol in the tissue may not have been constant during the experiments of Ingle in which rats were kept on constant daily doses of steroids (reviewed, Bush, 1962). On the other hand, it was clear from earlier work that although the adrenocortical hormones were essential for life, and although the glucocorticoids produced profound effects on the metabolism of all the major classes of intermediary metabolites, yet there was no convincing evidence that any of the major pathways of intermediary metabolism contained enzymatic reactions which were crucially dependent upon the glucocorticoids. The adrenalectomized vertebrate maintained on isotonic saline can live, grow, and reproduce if it is protected from severe environmental conditions or lack of food. The exact role of the glucocorticoids in intermediary metabolism remains unsolved although their effects are much better understood now than ten years ago.

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At the chemical level, considerable advances have been made possible by the valuable range of active analogs of cortisol that have been produced by the drug houses, particularly by the work of Fried, Bernstein, Herzog, Hogg, Sarett, and their colleagues. The enhanced biological activities of such analogs are now reasonably explained on the basis of their reduced rate of metabolic elimination from the body, their lower affinity for transcortin, the protection of the 11β-hydroxyl group from oxidation to the 11-ketone group, and a probable intrinsic effect on the increased acidity of the 11β-hydroxyl group in 9α-fluoro-11β-ols (Fried, 1957). The most important result of metabolic studies is that it appears reasonably certain that the intrinsic action of this class of steroids does not depend upon a metabolic reaction of any of their polar groups. There is also good reason to believe that the interaction of such steroids with their receptors takes place with the β-face of the steroid molecule, probably mainly with its "upper half" (carbons 11, 12, 16, 17, 18, 20, and 21). The catalytic action of such steroids thus seems to fall in the class of "physical" rather than "chemical" catalysts. A consideration of the possible chemical events that could be consequent upon such a steroid-receptor combination leads to the tentative conclusion that the number of possibilities is very limited. It seems unlikely, for instance, that such steroids could act as chelating agents, or as diffusible carriers in cell membranes. It is more likely that the interaction is relatively "immobile"; the steroid probably produces its effect by covering up a part of a larger molecule, thus altering the conformation of the latter; or preventing the association of another type of molecule with it. Although there is no decisive evidence either way, it should be emphasized that this type of action could affect any class of large molecule and that the biochemical manifestation could be a direct effect upon the activity of an enzyme, the location of an enzyme, or upon the transport mechanisms in cells.

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DISCUSSION

Glucocorticoids and Alanine α-Ketoglutarate Transaminase Activity

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It is generally believed that the interaction of a hormone with its specific receptor triggers a change in the activity of particular enzymes which mediate the physiologic effect(s) of the hormone. Indeed, the effect may well be on the enzyme-forming system. While the activity of many enzymes has been shown to be influenced by adrenal steroids, no evidence is at hand indicating a direct combination of the glucocorticosteroid with responsive enzymes. However, it is not unlikely that some of the responsive enzymes mediate certain of the biological effects exerted by the corticosteroids.

I want to focus attention on the specificity and possible significance of the increase in alanine-α-ketoglutarate transaminase activity that occurs after treatment with the glucocorticoids. I will consider the following points: (1) the specificity of the response of alanine transaminase to hormones and other treatments (Rosen *et al.*, 1959a); (2) the induction of this transaminase in cortisol-responsive or target tissues; and (3) the correlation between the increase in activity of this enzyme and the glycogenic and thymolytic action exerted by 11 β, 17 α, 21-trihydroxypregn-4-ene-3,20-dione acetate (cortisol).

I. Specificity of Alanine Transaminase Response

We have shown that treatment of rats with various natural or synthetic glucocorticoids results in a marked rise in activity of hepatic alanine transaminase (Rosen *et al.*, 1959b). Examples are shown in Table A. Two steroids, 11-epicortisol and 9-α-methoxycortisol, which are inactive with respect to the glycogen deposition and anti-inflammatory assays, were also ineffective in inducing an increase in the activity of this transaminase.

TABLE A

Effect of Different Treatments on the Alanine-α-ketoglutarate Transaminase Activity in Rat Liver.

Treatment	Dose (mg/rat × 7 days)	Liver alanine transaminase activity
Cortisol	1.0	Increased 5-fold
Prednisolone	0.5	Increased 3-fold
Medrol	0.5	Increased 3-fold
Corticosterone	2.0 (4 days)	Increased 2.5-fold
11-Epicortisol	2.0	Unchanged
9-α-Methoxycortisol	1.0	Unchanged
ACTH	20 *units*	Increased 3-fold
Deoxycorticosterone acetate	3.0	Decreased by 50%
Adrenalectomy of young rats	—	Unchanged
Adrenalectomy of mature rats	—	Decreased by 50%
Hypophysectomy	—	Increased 2-fold

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The level of alanine transaminase in rat liver was lowered to 50% of the normal value by the administration of 21-hydroxypregn-4-ene-3,20-dione acetate (deoxycorticosterone, DOCA). We have attributed this effect of DOCA to interference with the release of adrenocorticotropic hormone (ACTH) from the pituitary (Harding *et al.*, 1961b).

Other studies have shown that adrenalectomy of mature rats, but not of animals less than 6 weeks of age, results in a decrease of as much as 50% in the hepatic activity of alanine transaminase within 36 hours (Harding *et al.*, 1961a). The action of ACTH on this enzyme is mediated by the adrenals, since corticotropin is inactive in the adrenalectomized rat. The endogenous activity of alanine transaminase is twice as high in the liver of hypophysectomized rats as in intact animals; a possible explanation for this finding has been previously reported (Rosen *et al.*, 1959b).

In other studies, it has been found that treatment with testosterone, progesterone, growth hormone, or insulin did not produce an increase in the activity of alanine transaminase. Estrogen and thyroxine caused slight increases in the hepatic level of this transaminase that most likely were due to a stress reaction. (Rosen *et al.*, 1959b).

The activity of aspartate-α-ketoglutarate transaminase, as well as alanine transaminase, was determined in each of the foregoing studies. Neither ablation of the adrenals or pituitary nor treatment with various hormones produced significant changes in the level of aspartate transaminase in rat liver.

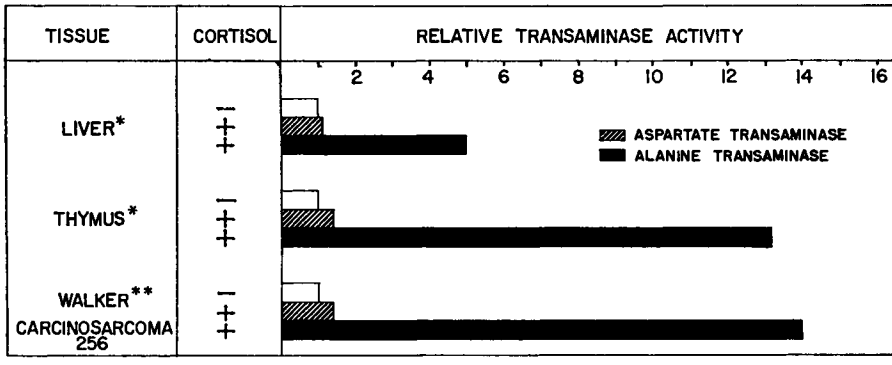
Tyrosine-α-ketoglutarate transaminase (Lin and Knox, 1957) and tryptophan pyrrolase (Knox and Auerbach, 1955) have been found to respond to the administration of cortisol as well as to the substrates of these enzymes. Various differences in the pattern of response of these enzymes in comparison to alanine transaminase have been described previously (Rosen, 1961). Compared to alanine transaminase, it would appear that the endogenous activities of tyrosine transaminase and tryptophan pyrrolase in liver are less dependent on circulating levels of adrenal steroids, since their values are not depressed following treatment with DOCA and only slightly after adrenalectomy.

II. Response of Alanine Transaminase in Several Cortisol-Sensitive Tissues

Is there any correlation between changes in alanine transaminase activity and the response of other tissues sensitive to glucocorticoids? Comparative data for liver, thymus gland, and the cortisol-sensitive tumor Walker carcinoma 256 are shown in Fig. 1. A sixteenfold increase in alanine transaminase activity was observed in thymus tissue following daily injections of cortisol for 1 week. This treatment resulted in a 95% reduction in the size of the thymus gland. The specificity of this response is demonstrated by the slight increase (less than 20%) in activity of aspartate transaminase observed in this tissue. This finding (Rosen *et al.*, 1959b) suggests that the rise in alanine transaminase activity is not due to concentration of enzyme in residual thymus tissue.

The growth of the Walker tumor was markedly impaired (90% inhibition) and its alanine transaminase activity increased fifteenfold following treatment with cortisol for 14 days. In contrast, the activity of aspartate transaminase was not significantly altered. Interestingly, treatment with DOCA stimulated the growth of this tumor slightly in several experiments and at the same time depressed its alanine transaminase activity (Rosen *et al.*, 1961).

In an extension of this investigation, alanine transaminase activity was studied in cortisol-sensitive and -resistant tumors. Suppression of growth was used as an index of sensitivity to cortisol. The inhibitory effect of cortisol on the growth of the cortisol-



* 2.5 mg. cortisol s.c. per rat per day for 7 days
** 3.0 " " " " " " " " 14 "

FIG. 1. Specificity of transaminase response in tissues sensitive to corticosteroids.

sensitive lymphosarcoma P1798 was associated with a marked increase in the alanine transaminase activity of this tumor. In contrast, neither the growth nor the alanine transaminase level of the cortisol-resistant P1798 was significantly affected by treatment with cortisol (Rosen and Nichol, 1961). Although additional studies are needed before this relationship is clearly understood, these results indicate the usefulness of cortisol-sensitive neoplasms for gaining further information concerning responsive tissues.

III. CORRELATION BETWEEN INCREASED ALANINE TRANSAMINASE ACTIVITY AND CERTAIN PHYSIOLOGICAL EFFECTS PRODUCED BY CORTISOL

Because the substrates of alanine transaminase are unique with respect to their high glycogenic potency, it was suggested that an elevation in the activity of the enzyme might be related to the gluconeogenic action of cortisol (Gavosto *et al.*, 1957; Rosen *et al.*, 1958). In support of this hypothesis, Eisenstein (1960) demostrated a diminished glycogenic and alanine transaminase response to cortisol in pyridoxine-deficient rats, in which the pyridoxal phosphate cofactor for the transaminase is limiting. The results presented in Table B confirm the observation of Eisenstein. The increase in activity

TABLE B

Effect of Pyridoxine Deficiency on Certain Physiological Effects Produced by Cortisol

Treatment of pyridoxine-deficient rats[a]	Liver		Thymus	
	Glycogen (%)	Alanine transaminase	Weight (mg)	Alanine transaminase
None	4.5	6.9[b]	0.28	0.28[b]
Cortisol 30 mg/kg × 5 days	4.6	11	0.023	0.40
Cortisol 50 mg/kg × 5 days	5.1	12	0.022	0.85
Cortisol 50 mg/kg + complete diet for 5 days	7.6	38	0.023	3.0

[a]There were 5 rats in each group. The animals were fed the pyridoxine-deficient diet for 3 weeks prior to treatment with cortisol.

[b]Millimoles of product formed per gram of protein per hour.

of alanine transaminase after treatment with cortisol was almost totally blocked in liver and inhibited markedly in the thymus of rats depleted of pyridoxine. However, while the failure of the glycogenic response paralleled the impaired enzyme response in liver, cortisol exerted its usual thymolytic action despite the reduced response of the transaminase obtained in this lymphoid tissue. These results suggest that more than one mechanism may underlie the diverse physiological effects exerted by the glucocorticoids.

Further progress in this area awaits the answers to several questions. What is the primary site of action of the glucocorticoids which results in an increase in enzyme activity? What determines the inducibility of only certain enzymes by the glucocorticoids? Conditions which stimulate gluconeogenesis may initially influence amino acid transport, thus altering the intracellular concentration of amino acids involved in substrateinduced control of enzyme synthesis. Various investigators have considered the possibility that the hormone facilitates the induction of an enzyme by acting directly at some step involved in protein synthesis. However, no direct evidence in support of either of these mechanisms is yet available. With the use of radioisotope-labeled hormones and amino acids, specific cell fractions, and the techniques recently applied to the study of protein synthesis *in vitro*, it should soon be possible to obtain answers to these and other questions concerning hormone–enzyme relationships.

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The Mode of Action of Cortisol upon Lymphocytes[1]

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After the discovery that corticoids accelerate the dissolution of lymphocytes (Dougherty and White, 1945), many workers hoped that because relatively pure suspensions of lymphocytes could be isolated from lymphatic tissues and pure hormonal steroids were becoming available, study of lymphocyte interactions with steroids *in vitro* would lead to elucidation of corticoid action upon this cell. It was felt such a "breakthrough" might open the way to an understanding of the effects of corticoids on other target cells. As you know this has not happened; we do not know how cortisol acts on lymphocytes or for that matter on any cell. Indeed, we do not know how any hormone acts at a molecular level, whether the hormone be steroid, protein, or peptide in nature.

Despite our inability to answer fundamental questions of mechanism relating to cortisol action, much has been learned during the past fifteen years about corticosteroid interactions with lymphocytes. In this presentation we shall not review all the pertinent studies, but shall raise certain questions which, in our view, point up the problems to be solved if the fundamental action of cortisol is to be understood.

I. Does Cortisol Act Directly upon Lymphocytes to Produce Changes in Morphology and Cell Death?

Although *in vitro* corticoid effects were obtained in 1945 by Heilman using hanging drop cultures of lymph nodes, the question then arose whether cortisol acts directly upon lymphocytes or requires the participation of other factors (e.g., lymphoid tissue components). Schrek (1951a,b) showed that trace doses of cortisol increased the destruction of rabbit thymic lymphocytes, surviving 1 or 2 days' incubation at 37° as determined by counting viable cells. Schrek's criteria were that viable cells would resist staining with dyes (such as eosin or safranine), whereas "dead" cells, though grossly "intact", would be stained. Schrek found that doses of the order of 0.01 μg of cortisol produced a cytotoxic effect on lymphocytes. The effects of hormone on cell viability were

[1]This work was supported by grants received from the United States Public Health Service and The American Cancer Society.

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observed first after a lag period of about 7 hours, and the changes were most clearly evident after 22 or 44 hours of incubation. When large doses of steroid, of the order of 200 μg or so, were tested upon lymphocytes, rapid cytotoxic effects were obtained, but Feldman (1950, 1952) showed that such steroid treatment also affected cells other than lymphocytes. Although Schrek reported an excellent correlation between the delayed thymocyte response *in vitro* and the *in vivo* glycogenic activity of a variety of corticosteroids (in contrast to the absence of correlation when high steroid doses were used to produce rapid cytotoxic effects), there appeared to be a major discrepancy in the time course of cortisol action on thymocytes *in vitro* and *in vivo*. Dougherty and White (1944, 1945) had clearly shown *in vivo* that corticosteroids produce an absolute lymphopenia in 3–6 hours associated with marked histological changes in thymus, interpreted to indicate lymphocyte breakdown. Thus the *in vitro* cortisol response described by Schrek seemed slow or less marked than the response *in vivo*. Trowell (1953) also felt that cortisone killed lymphocytes in his *in vitro* culture experiments very much more slowly than *in vivo*. Something seemed to be missing *in vitro*.

The discrepancies between *in vitro* and *in vivo* effects appeared to be resolved in the time-lapse cinematographic studies of Dougherty and his associates at Utah (cf. Dougherty *et al.*, 1960). They were able to show *in vitro* that cortisol and related glucocorticoids rather specifically and rapidly (within minutes, not hours) induce cytoplasmic shedding and disintegration of mouse thymocytes or leukemic lymphocytes. More significantly, the new method clearly showed that different morphological effects were produced by cortisol, depending on the state of maturation of the lymphocytes. Medium-sized lymphocytes responded to cortisol with an unusual cytoplasmic reaction reminiscent in some ways of pinocytosis, where cytoplasmic buds push out at the cell surface and then are pinched off. These cells shed cytoplasm and nuclear shrinkage occurs, but the cells are not killed by cortisol. The lethal effect of cortisol is restricted to the small, mature lymphocyte; the cytoplasm is denuded, the nucleus becomes pycnotic, and finally the pieces break up. This latter process Dougherty designates as *rhexis* rather than *lysis*, and uses the term lymphocytokaryrrhexis instead of lymphocytolysis. Cortisol, even in large doses, has little or no effect on the immature (large) lymphocyte, perhaps because these cells have a large capacity to oxidize cortisol to cortisone (Dougherty *et al.*, 1960). Dougherty's studies, provide strong evidence that cortisol acts directly on lymphocytes to accelerate maturation, but do not explain the lag period observed by others.

II. DOES THE PRIMARY MECHANISM OF CORTISOL ACTION INVOLVE SOME "KEY" ENZYMATIC STEP IN LYMPHOCYTE METABOLISM?

Several attempts have been made to define a biochemical basis for cortisol action in terms of an inhibitory action on some key step of lymphocyte metabolism deemed necessary for maintenance of cell integrity, be it protein synthesis or an energy-producing reaction. Many steroid effects have been found. White and his associates (1961), for example, have shown that cortisol *in vitro* affects many parameters of the metabolism of the thymic lymphocytes, using cellular and broken-cell preparations. These effects range from inhibitions of nucleic acid and protein biosynthesis as well as decreased glucose utilization to effects on the "swelling" and latent ATPase activity of mitochondria. The problem which develops in this type of work is one of interpreting the physiological significance of the diverse *in vitro* effects obtained. The difficulty is that White et al. (1961) find DOC, which has minimal thymolytic activity *in vivo*, to be uniformly more active than cortisol, and 17α-ethyl-19-nortestosterone generally more active than DOC in these *in vitro* systems. In addition to the lack of correlation between *in vitro* metabolic studies and biological activities *in vivo*, the *in vitro* concentrations of corticoid needed were of the order of 10^{-4} to 10^{-5} M, while *in vivo* the concentrations are 10^{-7} M or less.

III. DOES THE MECHANISM OF CORTISOL ACTION INVOLVE CHANGES IN CELLULAR PERMEABILITY?

A. BACKGROUND

For several years our group has been interested in the view that the primary mechanism of hormone action might involve modification of cytostructural barriers, whether at the cell surface or intracellular, influencing the penetration of ions or substrates into (or within) the cell and thereby secondarily altering cell function. Various hormones of diverse chemical structure influence one or more cellular permeability processes in the cells of their respective target organs (Hechter and Lester, 1960), and it is possible that cortisol action involves changes in the permeability characteristics of lymphocytes. Noall *et al.* (1957) showed that, in liver, cortisol increased the transport of AIB (α-amino-isobutyrate), but no information concerning lymphocytes was reported.

Accordingly, studies were undertaken to determine whether cortisol modified the distribution of monovalent cations, D-xylose or AIB in thymic cells. Effects of steroids on permeability were determined under *in vivo* conditions to obviate uncertainties regarding possible differences in the "state" of the cells which might arise because of the particular *in vitro* conditions employed.

B. EXPERIMENTAL

Male rats of the Sprague-Dawley strain (140–180 gm in weight), adrenalectomized for 7 days (maintained on 0.9% NaCl as drinking water), were used for these experiments. In some animals microcrystalline suspensions of cortisol or deoxycorticosterone (DOC), suspended in Special Formula No. 17874 (Upjohn), were administered subcutaneously (0.25 ml per injection), according to certain schedules to be described. In most of the experiments three groups of animals were studied concurrently; a control group received the vehicle only, a second group received cortisol, and a third group received an equivalent dose of DOC. These animals were functionally nephrectomized on the 7th day and injected with either radioactive inulin, sucrose, D-xylose, or AIB. The distribution of the tracer between plasma and thymus or muscle tissue was measured 90 minutes after subcutaneous administration. The total water content of the tissues was also determined. The inulin "space" was assumed to measure the volume of extracellular water; the apparent distribution of tracer in cell water may then be calculated on the further assumption that the concentration of tracer in plasma and extracellular space are identical.

The experimental procedure employed was as follows: under Avertin anesthesia, the renal pedicles were ligated and thereafter one of the following tracers was injected subcutaneously in 0.1–0.2 ml water at the following dose levels (unless otherwise stated): 0.95 mg inulin-C^{14} (1.3 μc), 0.17 mg sucrose-U-C^{14} (1.3 μc), 0.48 mg D-xylose-1-C^{14} (1.09 μc) and 90 μg AIB (1.5 μc). After 90–100 minutes the animals were Nembutalized and a blood sample was drawn from the aorta into a heparinized syringe and immediately processed for plasma. At the same time, the thymus and samples of diaphragm and a gastrocnemius muscle were excised, blotted on filter paper, and weighed. The content of radioactivity in the tissue extracts as well as in plasma was determined by flow-gas counting, using procedures described previously (Eichhorn *et al.*, 1961a,b). In addition the potassium and sodium contents of tissue extracts were determined in suitable aliquots by flame photometry, using lithium as internal standard.

C. RESULTS

Table I shows a comparison of "normal" and "involuted" thymus tissue, the former from untreated adrenalectomized rats, the latter from rats which had received daily cortisol treatment (0.8 mg) for 6 days (so that the thymus tissue : body weight ratio is decreased from 3.6 to 0.8). The former tissue contains as the predominant cell type, small (mature) lymphocytes, whereas in the involuted tissue, the cells remaining are the immature reticular lymphocytes, reticular cells, and RES cells (Dougherty *et al.*, 1960). Permeability characteristics of the cell types in the two kinds of thymus tissue are essentially similar. (Table I).

TABLE I

Distribution of Various Nonmetabolizable Molecules and Ions in Involuted and Control Thymus Tissue of Adrenalectomized Rats

		No treatment	Cortisol (0.8 mg × 6 days)
Inulin	TD[a]	8.1±1.0 (10)	12.2±1.6 (5)
Sucrose	TD	11.6±0.6 (15)	13.3±1.6 (5)
	ID[b]	4	2
D-Xylose	TD	42.2±1.6 (10)	56±2.3 (5)
	ID	48	65
AIB	TD	371±26 (23)	603±68 (10)
	ID	514	885
Potassium	μMole/gm	100±1 (18)	110±4 (7)
	ID	2400	3640
Sodium	μMole/gm	25±2 (35)	36±4 (7)
	ID	15	16
Thymus: body weight	mg/gm	3.6±0.24 (10)	0.8±0.05 (10)
Tissue water	%	79±0.4 (10)	78±0.6 (5)

[a]Per cent distribution in tissue = 100 × cts. per min. per gram tissue/cts. per min. per milliliter plasma.

[b]Calculated per cent intracellular distribution = 100 × apparent concentration per milliliter cell water/concentration per milliliter plasma.

[c]Mean, standard error, and number of observations.

The inulin "space," assumed to be a measure of extracellular volume, is about 10% of the tissue wet weight in both tissues; sucrose and sodium are excluded from most of the cell water; D-xylose penetrates into the cell and has an apparent distribution in about 50–65% of the total cell water; potassium and AIB are accumulated against an apparent concentration gradient. Potassium and AIB appear to be accumulated to a somewhat greater extent by the immature lymphocytes and reticular cells present in involuted thymus tissue relative to the mature lymphocytes present in thymus tissue of untreated adrenalectomized rats.

Table II shows the effect of 4.8 mg cortisol or DOC administered 1 day before sacrifice (in three equal injections at 4-hour intervals given on 6th day after adrenalectomy) upon the distribution of inulin, sucrose, D-xylose, and AIB in thymus; the sodium and potassium content of these tissues is also shown. It will be seen from Table II that 4.8 mg cortisol administered 1 day previously (a) initiates thymic involution as evidenced by decreased ratio of thymus : body weight and (b) produces marked alterations in permeability characteristics of this tissue. Since 4.8 mg DOC, administered under similar conditions, has little or no effect in inducing involution, the cortisol permeability effects which are not reproduced by DOC would appear to be directly

TABLE II

Distribution of Various Substances in Thymus Tissue of Adrenalectomized Rats Treated for 1 Day with Either Cortisol or DOC

		Control	Cortisol[d]	DOC[d]
Inulin	TD[b]	8.1±1.0 (10)[a]	23.5±1.6 (13)	13.7±1.1 (16)
Sucrose	TD	11.6±0.6 (15)	46.5±2.7 (11)	14.0±0.8 (16)
	ID[c]	4	41	1
D-Xylose	TD	42.2±1.6 (10)	71.3±5.9 (10)	54±1.3 (18)
	ID	48	85	62
AIB	TD	371±26 (23)	285±35 (6)	380±27 (7)
	ID	514	466	556
Potassium	μMole/gm	99.7±1.3 (18)	59.2±5.9 (8)	99.2±1.3 (50)
	ID	2400	2300	2880
Sodium	μMole/gm	25.2±1.9 (35)	87.4±3.9 (29)	25.2±1.4 (39)
	ID	15	59	4
Thymus: body weight	mg/gm	3.6±0.24 (10)	2.3±0.16 (8)	3.3±0.15 (10)
Tissue water	%	79.0±0.4 (10)	79.5±0.3 (8)	79.4±0.8 (5)

[a]Mean, standard error, and number of observations.
[b]TD is per cent distribution in tissue.
[c]ID is per cent intracellular distribution.
[d]Dose : 1.6 mg steroid subcutaneously at 16, 20, and 24 hours before sacrifice.

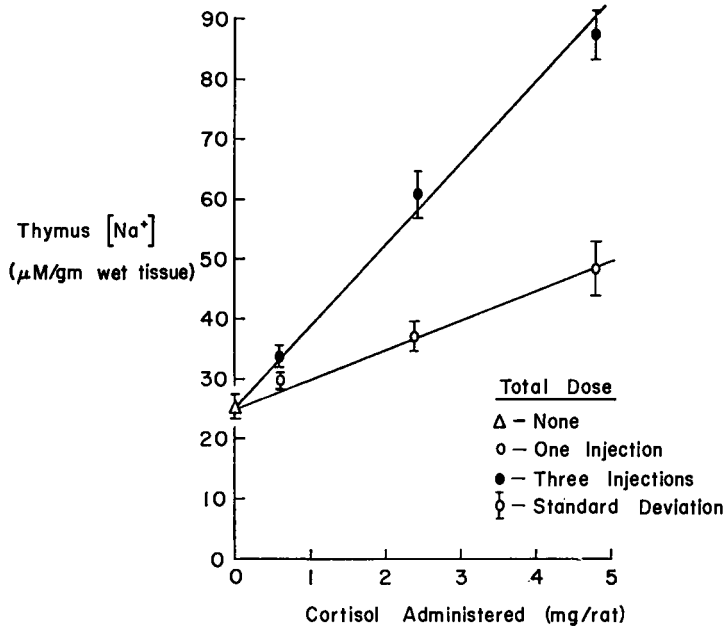

FIG. 1. Relationship between dose of cortisol and sodium content of thymus.

related to the involution process. Table II shows that 24 hours after cortisol treatment, thymus inulin space is increased almost twofold; sucrose penetrates and appears to distribute in about 40% of the total cell water, and D-xylose distribution is increased so that most of the cell water is available for this pentose. DOC produces small but significant increases in the tissue distribution of inulin, sucrose, and D-xylose

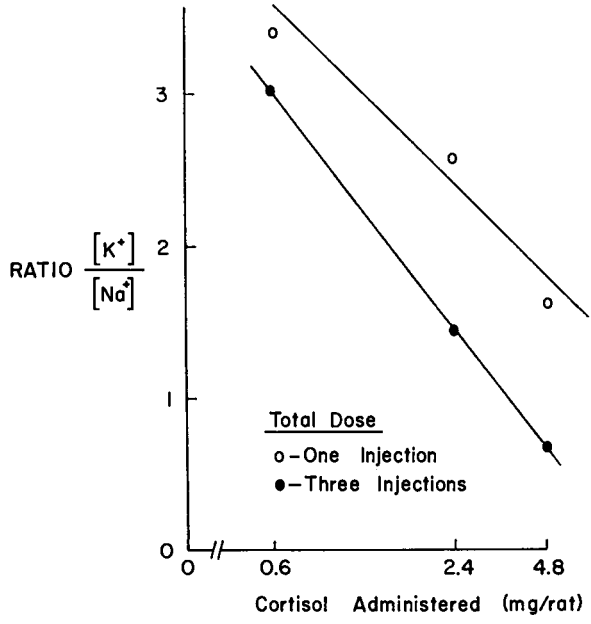

FIG. 2. Relationship between dose of cortisol and potassium : sodium ratio in thymus.

relative to the control. In contrast to the results obtained with cortisol, the increase in sucrose distribution produced by DOC is of the same order as that of inulin so that sucrose may be considered not to enter the cell; the increased tissue distribution of D-xylose is in excess of the inulin space and indicates that DOC, like cortisol, increases the apparent distribution of pentose in the cell water. Neither cortisol or DOC significantly influence the uptake of AIB. Perhaps the clearest difference between cortisol and DOC relates to changes in monovalent cations. Cortisol treatment produces a marked loss of intracellular potassium and a gain of sodium, whereas DOC does not change the tissue sodium or potassium.

Quantitative aspects of the cortisol effect upon thymus sodium and potassium are shown in Table III. Figures 1 and 2 illustrate the relationship between dose of hormone upon sodium content and upon the potassium : sodium ratio, respectively, in thymus tissue. In these experiments a total of 4.8, 2.4, or 0.6 mg of cortisol were administered

TABLE III

Effect of 24-Hour Treatment with Graded Doses of Cortisol on the Potassium and Sodium Content of Thymus Tissue

Dose of cortisol (mg)	Concentration in thymus		K/Na
	Potassium (μMole/gm)	Sodium (μMole/gm)	
None	99.7±1.3 (18)[a]	25.2±1.9 (35)	35.9
1×0.6	101.0±1.9 (8)	29.6±0.9 (8)	3.41
1×2.4	96.1±6.7 (6)	37.3±2.6 (6)	2.58
1×4.8	78.0±3.6 (6)	48.3±4.5 (6)	1.62
3×0.2	101.2±4.2 (8)	33.5±2.0 (8)	3.05
3×0.8	87.5±5.8 (9)	60.5±4.0 (9)	1.45
3×6.6	59.2±5.9 (8)	87.4±3.9 (9)	0.68

[a]Mean, standard deviation, and number of observations.

either as a single injection or in three divided injections at 4-hour intervals, 1 day before experiment. It will be seen that the cortisol effect on cations is much greater when a particular dosage is given in divided injections rather than in a single injection. The observations on the dose dependence of changes in sodium and potassium in thymus tissue, seen 24 hours after cortisol treatment, suggest the possibility of an assay procedure based on thymus sodium content or the potassium : sodium ratio. Such an assay would have definite advantages over methods that depend on weight changes, the usual method of evaluating thymolytic activity of steroids. For example, the cation response is marked 24 hours after hormone administration, whereas several days are required for maximal weight involution. Furthermore, the determination of tissue sodium and potassium presents little in the way of experimental difficulties and obviates the need for careful dissection of the entire thymus gland. Finally, preliminary examination suggests that the index of precision of the dose-response lines compares very well with previously used methods.

Table IV shows the effects of cortisol and DOC upon the diaphragm and gastrocnemius muscles of the animals used to obtain the thymus data in Table II. It will be seen that the level of cortisol treatment which markedly lowered the potassium : sodium ratio in thymus had no significant effect upon the potassium : sodium ratio in either diaphragm or gastrocnemius muscle. On the other hand, DOC decreases the potassium : sodium ratio in both muscles, although it had no effect on potassium or sodium in thymus (Table II). In similar fashion cortisol which increased the inulin space and sucrose penetration in thymus (Table II) is without this effect in muscle, whereas DOC increases the

TABLE IV

Distribution of Various Substances in Diaphragm and Gastrocnemius Muscles of Adrenalectomized Rats Treated with Cortisol or DOC 1 Day Previously

		Type muscle[b]	Treatment		
			None	Cortisol[d]	DOC[d]
Inulin	TD[a]	D	17.6±0.8 (10)[c]	16.5±0.9 (14)	22.9±1.5 (16)
		G	9.5±1.0 (8)	9.1±0.4 (13)	13.9±0.4 (16)
Sucrose	TD	D	23.0±0.8 (15)	19.6±0.8 (11)	21.9±0.7 (16)
		G	11.6±0.4 (13)	11.3±0.6 (11)	14.5±1.0 (16)
D–Xylose	TD	D	42.8±4.1 (10)	55.9±5.5 (5)	59.0±1.7 (18)
		G	18.9±1.3 (8)	21.8±1.0 (10)	27.3±1.2 (18)
AIB	TD	D	104±10 (20)	389±52 (6)	136±15 (7)
		G	44±5 (20)	107±13 (6)	48±2 (7)
K	μMole/gm	D	82.1±1.0 (18)	82.1±1.3 (8)	78.5±0.6 (50)
		G	101.4±2.9 (18)	99.3±1.1 (8)	91.0±0.6 (50)
Na	μMole/gm	D	33.4±1.2 (35)	34.2±2.0 (28)	42.6±0.9 (28)
		G	26.1±2.0 (33)	28.7±2.2 (28)	35.2±1.2 (50)
K/Na		D	2.48	2.39	1.84
		G	3.86	3.45	2.58
Tissue water	%	D	76.0±2.0 (6)	—	—
		G	76.6±1.8 (6)	—	—

[a]TD, distribution in tissue.

[b]D diaphragm muscle; G, gastrocnemius muscle.

[c]Mean, standard error, and number of observations.

[d]Received total 4.8 mg of steroid in three injections on previous day.

inulin space in both muscles. However, DOC does not cause sucrose entry into the muscle cell. Cortisol and DOC both increase the apparent intracellular distribution of D-xylose in muscle as they did in thymus. DOC appears to be more active than cortisol in this regard in muscle whereas in thymus, the reverse was true. Table IV also shows that cortisol, but not DOC, facilitates the uptake of AIB in muscle whereas in thymus, an effect in the opposite direction was observed.

In an attempt to determine whether the permeability changes in thymus observed 1 day following cortisol treatment could be detected at an earlier time interval, 4.8 mg of cortisol or DOC was administered in 3 doses beginning 6 hours before sacrifice. Table V shows that 6-hour treatment with cortisol (which did not influence thymus : body weight ratio) produced small effects relative to those obtained when cortisol was given for 24 hours. Thus, 6-hour cortisol treatment reduced the potassium : sodium ratio from a control value of 4.0 to 3.2, but this is a small decrease relative to the potassium : sodium value of 0.68 observed when cortisol was given 1 day previously; 6-hour treatment with DOC did not lower potassium sodium : ratio. This cortisol treatment

TABLE V

Effect of 6-Hour Treatment with Cortisol and DOC upon Thymus Tissue

		No treatment	Cortisol[a]	DOC[a]
Inulin	TD[b]	8.1±0.4 (10)[c]	12.6±1.0 (9)	—
Sucrose	TD	11.6±0.6 (15)	15.3±0.6 (17)	—
D-Xylose	TD	42.2±1.6 (10)	51.2±1.9 (7)	—
AIB	TD	371±26 (23)	158±10 (10)	346±19 (6)
Potassium	μMole/gm	99.7±1.3 (18)	82.9±2.5 (25)	114.9±3.6 (6)
Sodim	μMole/gm	25.2±1.9 (35)	26.1±1.0 (16)	28.0±2.8 (6)
Thymus: body weight	mg/gm	3.6±0.24 (10)	3.4±0.13 (13)	3.3±0.23 (6)
Tissue water	%	79.0±0.4 (10)	77.0±0.27 (8)	79.5±0.23 (6)

[a]Administered total of 4.8 mg of steroid in three doses.

[b]Per cent distribution in tissue.

[c]Mean, standard error, and number of observations.

also produces in thymus tissue significant increases in the distribution of inulin, sucrose, and D-xylose which are small relative to the effects observed 24 hours after cortisol. The major effect produced by 6-hour treatment with cortisol was a significant reduction of AIB uptake which was not reproduced by DOC. Figure 3 shows the relationship between AIB distribution in thymus tissue and the plasma concentration of AIB in control, DOC-, and cortisol-treated rats. It will be seen that in the control and DOC group the distribution of AIB decreases from about 4.5 to 1.0 as the plasma AIB increases from 0.1 to 0.5 μg/ml. The 6-hour cortisol effect to decrease AIB uptake is seen as an alteration of the slope of the curve so that a significant divergence is observed

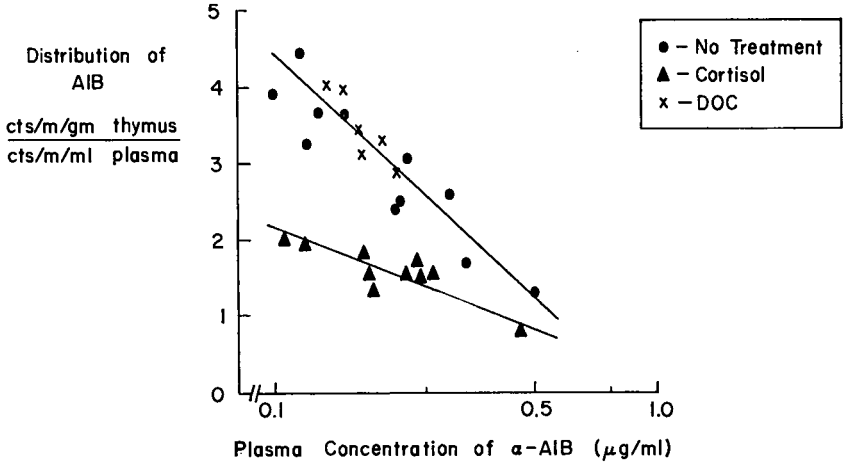

FIG. 3. Effect of 6-hour cortisol treatment on AIB uptake in thymus.

at lower levels of plasma AIB. The significance of these curves relating AIB distribution to AIB plasma level when tracer AIB is given for 90 minutes is not explicable on any simple basis postulating a "saturation" phenomenon. Figure 4 shows the results obtained upon AIB distribution in two experiments where cortisol or DOC were administered for 90–100 minutes. In these experiments either 90 μg (as in the previous experiment) or 300 μg of AIB-1-C^{14} were administered

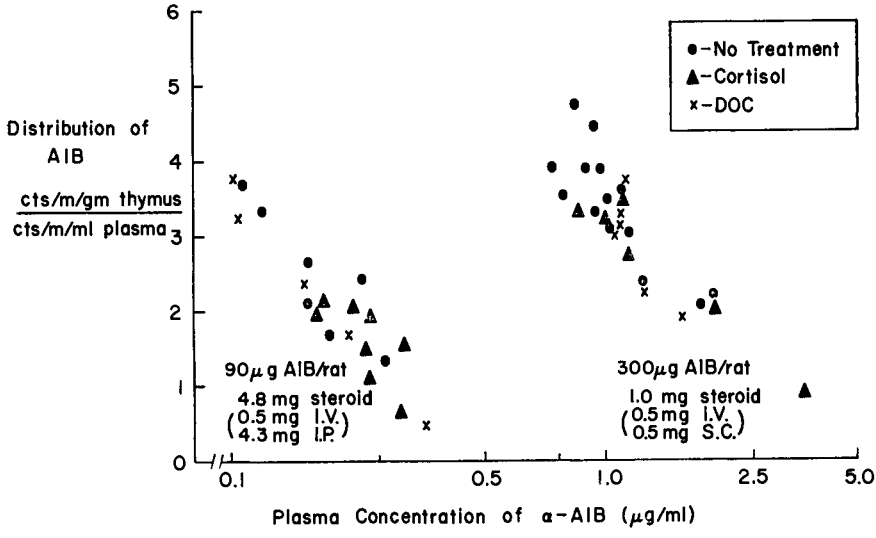

FIG. 4. AIB uptake in thymus 90 minutes after steroid treatment (two levels of AIB administration).

subcutaneously to each rat; bothgroups received 0.5 mg cortisol or DOC intravenously (in saline-10% alcohol) and either 0.5 mg subcutaneously or 4.3 mg intraperitoneally. It will be seen that in these experiments, neither cortisol nor DOC influenced AIB uptake when hormone was given 90–100 minutes earlier. However, it will be noted that in both experiments there is a similar linear relationship between AIB distribution and plasma AIB level, the slopes being almost identical, except that the line representing higher dose of AIB-C^{14} is displaced to the right, a result suggesting that AIB distribution in thymus has not reached a steady state in the 90-minute interval studied. It should be mentioned that neither cortisol nor DOC produced changes in thymus sodium or potassium in these short-term experiments.

D. DISCUSSION

1. Time Course of Cortisol Action to Produce Death of Thymic lymphocytes in vivo, as Evaluated by Permeability

One might think that it should be possible to evaluate the effects of cortisol to produce "death" of thymic lymphocytes *in vivo* by measuring changes in cell permeability. If cells do not disintegrate after death but remain intact with respect to gross morphology (like Schrek's lymphocytes stainable by dyes), it would then be expected that sucrose, sodium, and potassium should equilibrate in these structures, so that the concentrations inside and out are equal. Depending upon the nature of the surface barrier remaining, inulin might or might not be excluded from the interior of the "dead" structure.

Using these criteria, our studies could be interpreted to indicate that 1 day after cortisol treatment the thymic cells are in two states, some viable and the others nonviable but retaining a barrier which excludes inulin. On the basis that sucrose and sodium should equilibrate in all of the water of the nonviable cells, it could then be estimated that such cells represent about 40% of the total number of "cells" present in thymus 1 day after cortisol treatment. Applying these considerations to the effects of cortisol given for only 6 hours, we would conclude that cortisol does not kill lymphocytes in this time interval, because cortisol produces only a small potassium loss without a gain in sodium and does not increase the calculated distribution of sucrose in cell water (which remains close to zero) even though it does induce a small increase in inulin space. Since there is not a significant weight decrease in the 6-hour cortisol treatment, it is most unlikely that cortisol killed a large number of cells which were then subsequently removed from the thymus. Accordingly, it might be concluded that the "lethal" effect of cortisol, evident at 24 hours, is not detected at 6 hours, and in this sense cortisol has a long lag period as reported in the studies of Schrek and others who found delayed responses *in vitro*. The conclusion that cortisol action has a long lag period is not open to us because we carried out our studies *in vivo*. It is well established *in vivo*, in a variety of animals, that 3–6 hours after cortisol administration, histological examination of thymus tissue reveals a classical picture of lymphocyte disintegration (Dougherty and White, 1945). We must, therefore, conclude that the cell fragments that remain in the tissue 6 hours after cortisol, whether cytoplasmic buds or pyknotic nuclei, retain permeability characteristics similar to those initially ascribed to viable cells. We may ascribe the decreases in potassium and AIB uptake, observed at this time interval, to the presence of these cell fragments. It would appear that 18 hours later, when the thymus weight is decreased, indicating the removal of cell fragments, and cells, many fragments which are still present in the thymus tissue, have lost the ability to exclude sucrose and sodium.

2. Does the Mechanism of Cortisol Action Involve an Early Permeability Change in Lymphocytes?

The only early change in cell permeability produced by cortisol, but not by DOC, is an effect at 6 hours, but not at 1.5 hours, to inhibit the transport of AIB. The 6-hour effect could be taken to represent an important aspect of the action of cortisol, since it implies that cortisol inhibits amino acid transport and thus might reduce protein biosynthesis. If protein synthesis is necessary for maintenance of cellular integrity, the cortisol effect on AIB transport might then be related to lymphocyte breakdown. Reasoning from the same type of conceptual framework, Kit and Barron (1953) and later White and his associates (1961) studied inhibitory influences of cortisol on lymphocyte metabolism as the basis of the effect of hormone to induce lymphocyte breakdown.

While it is possible to consider that the effect on AIB tranport is a *primary* action of cortisol, which leads secondarily to lymphocyte breakdown, cortisol action upon lymphocytes would appear to involve "something more" than cellular disruption. It concerns the mechanisms involved in maturation, where cells are transformed from one state to another. The processes whereby intermediate-sized lymphocytes become smaller and smaller by mechanisms involving cytoplasmic shedding and nuclear shrinkage cannot readily be rationalized in terms of cortisol inhibition of protein synthesis, nor does cortisol inhibition of a "key" energy-yielding reaction appear to help. Cytoplasmic budding, with subsequent pinching off and shedding of the buds would seem, mechanistically, to be a process more directly related to molecular mechanisms discussed in connection with pinocytosis or muscle contraction; period. Reduction of nuclear volume without loss of nuclear material to produce the condensed structure we label as pycnotic seems best described as a "change in state" of an ordered polymeric system of deoxyribonucleoprotein. Such a condensed system might exhibit poor protein synthetic activity, but in this case the inhibition of protein synthesis would represent a *result* of cortisol action, not the *cause* of nuclear pycnosis. Accordingly our finding that cortisol inhibits AIB transport might represent a secondary consequence of a change in the state of the cell, rather than a primary action.

3. A Speculation about Cortisol Action at the Molecular Level

We feel, the mechanism of cortisol action involves a cellular receptor, which, after its interaction with cortisol, affects the nucleus and cytoplasm simultaneously. More specifically, we are looking for an action which, starting at a few specific sites, then spreads throughout the cell. In considering the action of insulin and other hormones, the need to postulate some sort of excitation which affects the cell as a whole was

first postulated by Peters in his cytoskeleton hypothesis (1956) and has recently been put forward again and in modified form by Krahl (1958) as well as by us (Hechter and Lester, 1960). In thinking of a reaction which could encompass the spectrum of morphological changes induced by cortisol in lymphocytes, one is reminded of the fundamental role of calcium ion in muscle contraction, cytoplasmic streaming, and influencing maturation of eggs of marine invertebrates (Heilbrunn, 1956) and of the studies of Csapó (1961) which indicate that the action of estrogens and progesterone in uterine muscle contraction somehow influences the "state" or binding of a calcium fraction in the myometrium. By virtue of their polyvalence, calcium and magnesium may be regarded as structure-determining factors, since they may cross-link charged negative sites within a single polymer, or between polymer units when appropriate spatial and steric relationships exist. Thymic nuclei contain calcium and magnesium, the magnesium being associated with the DNA; the calcium with histone and other proteins of the nucleus. In addition to large numbers of arginine and lysine residues, histones also have a considerable number of glutamic and aspartic residues, and it is likely that calcium is held electrostatically at these negative sites. According to Mirsky and Osawa (1961) there is about one magnesium for every 20 phosphate groups of DNA, the remaining phosphate groups being combined mainly with histone.

The question now arises whether cortisol might not act in the lymphocyte by altering the conformation of a specific receptor protein, containing calcium ion so positioned sterically that the divalent ion is released from its binding sites when protein conformation is altered; the calcium thus released is available for interaction with a second component. This reaction with calcium in turn alters conformation and releases another polyvalent ion (for example, ATP, ADP, various adenosine monophosphates, pyrophosphate, histamine) capable of modifying the conformation of a third system, and so on. Alternatively, we may assume that ion jumping modifies water structures (cf. Hechter and Lester, 1960) or initiates a chain reaction thioldisulfide interchange (Jensen, 1959) to succesively alter conformation of the cellular polymers thereby providing a mechanism for the propagation of an "excitation" throughout the cell. In the cytoplasm of the lymphocyte we might visualize calcium acting to produce contraction and streaming of the cytoplasm, so that at certain weak points of the cell surface budding develops and then these buds are pinched off. In the nucleus, the conformation of histone might be altered, releasing calcium and modifying the system so that DNA and protein become crosslinked in a dehydrated nuclear system.

This hypothesis does not define the cellular locus of the specific receptor postulated. It could be either in the nucleus or cytoplasm; indeed there might be receptors in both nucleus and cytoplasm. The receptor

could be an enzyme, but it does not have to be. Neither does the theory demand that calcium ion per se be released from its binding sites in the initial reaction of cortisol with the receptor; this might occur at a second stage. The important point in the idea is that at some very early point, calcium must be released to initiate the sequence of molecular rearrangements we finally recognize as response. As the state of the system is being altered, we would expect changes in the multienzyme sequences involved in biosynthesis and the generation of metabolic energy, and there would be concurrent changes in the permeation and transport of substrates and ions within the cell (Hechter and Lester, 1960). We should emphasize that cortisol action may not release calcium from binding sites in *all* target cells. In fibroblasts where cortisol alters the cell so that it appears to be resistant to the products of inflammation (Dougherty *et al.*, 1961), the cells appear to be morphologically sluggish, almost anesthetized. Perhaps in fibroblasts cortisol alters the conformation of a protein receptor which releases magnesium ions, and then would initiate a characteristic sequence of alterations of polymer conformation. In hepatic parenchymal cells, for example, magnesium release might make this cation available for certain enzymatic systems and thus accelerate biosynthesis of glycogen from glucose or amino acids, etc., in addition to other changes at a nuclear locus. The merit of this speculation should be evaluated by the experimental results it provokes; though many details may have to be modified, it seems to us that the conceptual base of the hypothesis presented, involving a chain sequence of alterations of conformation of polymers, is sufficiently broad to encompass all of the biological actions of corticoids.

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ADDENDUM

Added in proof, August 6, 1963.

Recognition of the role of nucleic acids in regulation of protein synthesis has directed attention to the possibility that diverse effects produced by a hormone in responsive cell types might all result from a single primary effect of a hormone at the genetic locus. This view, which may be designated as the hormone-gene thesis, first advanced in the case of sex hormones (Karlson, *Deutsche Med. Wshschr.* **86**:663 (1961); Zalokar, in "Control Mechanism in Cellular Processes" (ed. D. M. Bonner, Ronald Press Co., New York, 1961) has been suggested for cortisol as well (Segal et al. *J. Biol. Chem.* **237**:PC 3303 (1962); Greengard et al., *J. Biol. Chem.* **238**: 1548 (1963);*Science* **141**:160 (1963), since characteristic tissue responses to this steroid can be abolished by actinomicin D, known to inhibit DNA-dependent RNA synthesis (Reich et al., *Science* **134**:556 (1961); Hurwitz et al. *Proc. Nad. Acad. Sci. (U.S.)* **48**: 1222 (1962) and or by puromycin which block protein synthesis (Yarmolinsky and de la Haba, *Proc. Nat. Acad. Sci. (U.S.)* **45**:1721 (1959); Zamecnik, *Biochem. J.* **85**:257 (1962). Based upon studies with these inhibitors, cortisol action to induce enzyme formation in liver, or increase glycogen, appears to involve stimulation of protein synthesis, presumably by way of synthesis of messenger RNA. These findings, however, do not establish that the primary reaction of cortisol is with a receptor in the nucleus, nor do they exclude the possibility that cortisol action involves release of divalent cations as suggested above.

On Mechanism of Steroid Action at the Molecular Level

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There is a considerable body of evidence that the mechanism of action of most, if not all, steroid hormones involves a combination of steroid with protein, perhaps as the first step. Some steroids may interact with a protein plus another substance, such as a pyridine nucleotide, to form a ternary complex. The differences between tissues—those that are differentially stimulated or inhibited by a specific steroid and those that are not—may be attributed to the presence or absence of specific proteins. Such tissue differences are in turn part of the phenomenon of cellular differentiation, about which very little is known. Each kind of steroid may interact with many different proteins; some of these steroid-protein interactions exhibit a high degree of specificity whereas others do not.

To regulate the rate of metabolic reactions hormones must affect the rate of enzyme systems, either directly by interacting with the enzyme molecule to increase or decrease its activity, or by combining with some protein in the cell membrane or an intracellular membrane. This would alter the rate of transfer of materials across it, increasing or decreasing the supply of substrate or cofactors for certain reactions. The experiments described by Dr. Hechter suggest that cortisol may interfere with the uptake of α-aminoisobutyrate and other materials by thymus cells yet permits sucrose, which is normally excluded, to enter the cell. Cortisol and other steroids have strong surface-active effects at high concentrations and have been shown to bring about the lysis of lymphocytes and of sea urchin eggs.

There is ample experimental evidence that effects of a steroid at 10^{-4} or 10^{-5} M may be qualitatively different, not simply quantitatively different, from its effects in the same system at 10^{-8} M or lower. Under conditions in which DPN in the rate-limiting reagent the addition of 10^{-8} M corticosterone to a system containing beef liver glutamic dehydrogenase leads to a 10% increase in the rate of DPN reduction

[1]Aided by grants from the Charles and Marjorie King Fund and from the Association for the Aid of Crippled Children and by Grant C 2400 from the National Institutes of Health.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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(Engel and Scott, 1960). Yielding *et al.* (1960) could find no stimulation of the glutamic dehydrogenase system by corticoids but reported an inhibition of the enzyme at 10^{-5} *M* steroid. They subsequently found (Yielding and Tomkins, 1960) that high concentrations of diethylstilbestrol, 5×10^{-4} *M*, disaggregate the tetramer form of glutamic dehydrogenase to the inactive dimer or monomer, and that this disaggregation could be reversed by the addition of ADP or DPN. We reinvestigated this and found that, when we duplicated the conditions of Engel and Scott, we could confirm their results (Table I). Progesterone

TABLE I

Rate of Pyridine Nucleotide Reduction by Crystalline Glutamic Dehydrogenase

Pyridine nucleotide	Steroid added		
	None	Corticosterone	Progesterone
DPN	4.54±0.25	5.68[b] ±0.42	7.90[c] ±0.58
TPN	1.87±0.14	1.94±0.16	1.91±0.14
3–Pyridine aldehyde DPN	12.44±0.72	12.45±0.82	12.52±0.82
Deamino DPN	5.18±0.08	4.96±0.66	5.18±0.27
3–Acetylpyridine DPN	10.60±0.73	11.16±0.57	12.14[b] ±0.59
Thionicotinamide DPN	1.4.±0.12	2.01[b] ±0.24	3.38[c] ±0.42

[a]Each value is mean ± S. E. of 20 experiments; rate expressed as moles, of reduced pyridine nucleotide $\times 1^{-1} \times \text{min}^{-1} \times 10^{-7}$.
Complete system: 33 m*M* Tris buffer, pH 7.4, 10 m*M* glutamate, 0.033 m*M* pyridine nucleotide, 4.4 μg enzyme protein per milliliter 10^{-6}M steroid.
[b]$P < 0.05$.
[c]$P < 0.01$.

at 10^{-6} to 10^{-8} *M* increased the rate of glutamic dehydrogenase even more than corticosterone. The effect appears to be specific for glutamic dehydrogenase, for no such effect could be demonstrated in comparable experiments with malic, lactic, or alcohol dehydrogenases, which transfer hydrogen to side one of the pyridine nucleotide, or with α-glycerophosphate or glyceraldehyde-3-phosphate dehydrogenases, which transfer hydrogen to side two of the pyridine nucleotide. Only thionicotinamide DPN, of several analogs tested, could be substituted for DPN and yield a glutamic dehydrogenase system that retained the property of responding to corticosterone and progesterone. Steroid, nucleotide, and enzyme may form a ternary complex with the steroid bound by its β-face to the enzyme and by its α-face to the adenine portion of the pyridine nucleotide. Under these conditions, the steroid may in some way facilitate the transfer of hydrogen from substrate to nucleotide.

Talalay and Williams-Ashman (1958) postulated that all steroid hormones produce their effects by undergoing reversible oxidation and reduction at a ketone-alcohol function and serving as transhydrogenat-

ing systems between TPNH and DPN. A large body of evidence has accumulated which leads to the rejection of the theory that substrate-mediated transhydrogenation plays any physiologic role. Bush and Mahesh (1959) showed that the action of corticoids does not involve ketone-alcohol interconversion at carbon 11 of the steroid. Wiest (1961) demonstrated that the action of progesterone on tissues does not involve ketone-alcohol interconversion at carbon 20; neither 20α-hydroxy nor 20β-hydroxydehydrogenase is involved. The claim by Hurlock and Talalay (1958) that androsterone stimulates transhydrogenation in the liver via 3α-hydroxydehydrogenase could not be confirmed by Stein and Kaplan (1959) or by Bloom (1960). The guinea pig liver and kidney have two separate 17β-hydroxysteroid (testosterone) dehydrogenases, a TPN-specific one in the soluble fraction and a DPN-specific enzyme in the particulate fraction (Endahl *et al.*, 1958). When these enzymes were purified and mixed together in high concentrations, with high concentrations of pyridine nucleotides and testosterone, a very slow rate of substrate-mediated transhydrogenation could be demonstrated (Villee and Spencer, 1960). At lower concentrations of steroids no substrate-mediated transhydrogenation could be demonstrated. Substrate-mediated transhydrogenation with crystalline glutamic dehydrogenase and crystalline lactic dehydrogenase was demonstrated by Holzer and Schneider (1958), but these systems also required high concentrations of substrate, enzymes, and pyridine nucleotides and gave only slow rates of transhydrogenation.

The possible role of substrate-mediated transhydrogenation in the mechanism of estrogen action may be summarized as follows:

1. An estrogen-dependent transhydrogenase (EDTH) has been separated from the estradiol dehydrogenases (EDH) by starch block electrophoresis and paper curtain electrophoresis (Hagerman and Villee, 1959), by density gradient ultracentrifugation in sucrose gradients (Fig 1), and by adsorption to and elution from hydroxyapatite columns (Velle and Engel, 1962). Jarabak *et al.* (1962) described one unsuccessful attempt to separate the enzymes by starch block electrophoresis, but since the initial steps in their enzyme preparations were quite different it is likely that the transhydrogenase was destroyed during the long delays in the initial steps of their procedure.

2. There are a number of differences between the distribution and properties of the dehydrogenases and the transhydrogenase. EDTH is present in the liver and other tissues which do not show estrogen-dependent transhydrogenation. The placenta of the hamster has EDTH but little if any EDH (Kellogg and Villee, 1961). The enzymes differ in their rates of thermal inactivation (Hagerman and Villee, 1959) EDTH, but not EDH, is inhibited by thyroxine, and EDH, but not EDTH, is inhibited by 2′-adenosine monophosphate and by 2′,5′-adenosine diphosphate. The two enzymes respond differently to differ-

ent steroids (Villee *et al.*, 1960). The two dehydrogenases are located in different cells: the DPN estradiol dehydrogenase is located in the cells of the decidua and trophoblast whereas the TPN estradiol dehydrogenase is located in the perivascular stroma of the placenta (Kellogg and Glenner, 1960).

4. When estradiol of very high specific activity, labeled in either position 7 or 17 with tritium, is injected into the rat, it is taken up by the

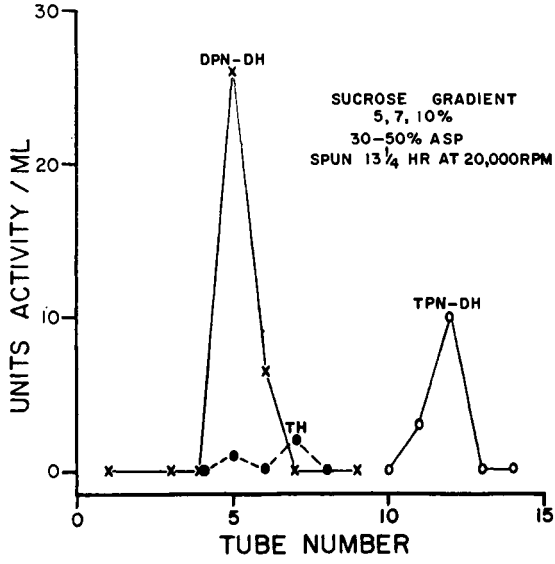

FIG. 1. Distribution of enzyme activities after ultracentrifugation. The material precipitating between 30 and 50% saturated $(NH_4)_2SO_4$ from the nonparticulate fraction of human placenta, obtained by centrifuging 60 minutes at 105,000 *g*, was dissolved in 0.033 *M* Tris, pH 7.4, 0.001 *M* EDTA and dialyzed against 500 volumes of the same solution at 0°C for 4 hours. It was centrifuged for 20 minutes to sediment undissolved protein. Then 0.5 ml of the resulting enzyme preparation, containing 222 units of DPN specific estradiol dehydrogenase, 70 units of TPN estradiol dehydrogenase, and 23 units of estrogen-dependent transhydrogenase, was layered onto 3.5 ml of sucrose solution with a concentration gradient from 5 to 10%. This was centrifuged in a swinging-bucket ultracentrifuge for 13.25 hours at 20,000 rpm. Fractions were collected by piercing the lower end of the tube and collecting 6-drop fractions. Each fraction was mixed with 1 ml of 0.033 *M* Tris buffer and assayed for each of the three types of activity. Recovered: 64.4 units DPN estradiol dehydrogenase (29% of initial), 17.5 units of TPN estradiol dehydrogenase (18% recovery), and 14.4 units of estrogen-dependent transhydrogenase (63% of initial).

uterus and is physiologically effective, but is recovered from the uterus entirely as estradiol (Jensen and Jacobson, 1960). It is not converted to estrone, and it does not lose tritium from position 17. In contrast, estradiol taken up by the liver is rapidly converted to estrone and is

conjugated to water-soluble materials. This is very strong evidence that the physiologic action of estradiol does not involve its oxidation and reduction.

4. Steroid dehydrogenases can carry out substrate-mediated transhydrogenation, as our experiments with the testosterone dehydrogenases showed quite clearly (Villee and Spencer, 1960), but only at high concentrations of all reactants. Dr. Hagerman has applied the general kinetic theory of Hearon (1949) for linked enzymatic reaction systems to the question of substrate-mediated transhydrogenation and has demonstrated that with a single enzyme the rate of transhydrogenation in the presence of excess mediator equals the rate of the slower of the two reactions. With two enzymes the rate of substrate-mediated transhydrogenation depends on the relative amounts of the enzymes present and on their relative rate constants, but other conditions being equal, the rate will be less than the rate for a single enzyme system. With concentrations of estradiol as high as 5×10^{-6} *M*, the concentration employed by Jarabak *et al.* (1962), the transhydrogenation observed could be due to EDTH, to substrate-mediated EDH, or to a mixture of the two. From the published kinetic constants for EDH one can calculate that transhydrogenation, observed at concentrations less than 2×10^{-7} *M*, cannot be due to the substrate-mediated enzyme system, for the rate at this concentration is below that detectable by the methods employed. The experiments of Pearlman, Jensen, and others give an estimate that the concentration of estradiol in tissues is less than 10^{-8} *M*, which again leads to the conclusion that, with estrogens as with other steroids, substrate-mediated transhydrogenation is not of importance in the physiologic role of the hormone. The nature of the estrogen-dependent system in the human placenta must be reinvestigated using assay methods that comply with the requirements imposed by the kinetics of the reactions.

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Nonhormonal Inhibitors of Adrenocortical Steroid Biosynthesis

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Chemotherapeutic control of steroid synthesis and secretion is a fertile field for the study of normal function and dysfunction of endocrine tissues. The discovery of nonhormonal compounds which selectively damaged the cells of the adrenal cortex of the dog (Nelson and Woodard, 1949) or altered the function of the adrenal cortex (Hertz *et al.*, 1950) opened new areas of endocrine research. We shall consider the effects of certain compounds in each of these categories, namely: (1) diaminodiphenyl compounds similar to amphenone, and (2) several analogs and isomers of the insecticide DDD.

I. AMPHENONE, DIAMINODIARYLALKANES, AND DIAMINOCHALCONE

A. ENDOCRINE EFFECTS IN THE RAT AND DOG

Hertz and colleagues (1950) described a marked hypertrophy of the adrenals, thyroid, and uterus of castrated female rats following treatment with amphenone [3,3-bis(*p*-aminophenyl)-2-butanone]. This adrenal effect of amphenone could be dissociated from its goitrogenic action inasmuch as the former was reversed by cortisone and the latter by thyroxine (Hertz *et al.*, 1951). Adrenal hypertrophy did not occur in the absence of the anterior pituitary. Histological evidence revealed a lipid-laden adrenal cortex with lipid concentration in the zona fasciculata. Later, Hume and Nelson (in Hertz *et al.*, 1955) demonstrated that amphenone inhibited the secretion of 17,21-dihydroxy-20-ketosteroids (Porter-Silber chromogens) by the dog adrenal. In clinical investigations, amphenone was observed to suppress biosynthesis of both cortisol and corticosterone in normal subjects and patients with adrenocortical carcinoma (Peterson *et al.*, 1957). Further clinical observations indicated that aldosterone secretion was also reduced (Renold *et al.*, 1956). In perfusion experiments, using the calf adrenal, Rosenfeld and Bascom (1956) demonstrated that amphenone inhibited 11β-, 17α- and 21-hydroxylation and conversion of the Δ^5-3β-hydroxyl to the Δ^4-3 ketone group.

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More recently, endocrine actions of a number of compounds related to amphenone have been studied. The structural formulas of two such types of compounds are seen in Fig. 1. With the exception of the diaminodiphenyl groups common to each, there exists considerable structural modification.

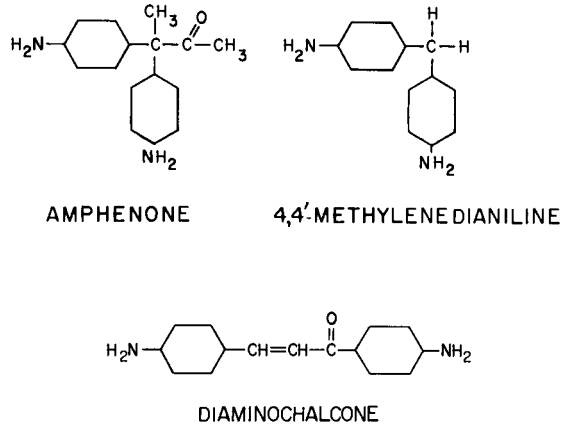

FIG. 1. Structure of amphenone and related diaminodiphenyl compounds.

Data from a representative series of castrated female rats treated with *p,p'*-diaminodiphenylmethane (methylenedianiline) are presented in Table I. The adrenal, thyroid, and uterine weights of the treated animals

TABLE I

Endocrine Action of Methylenedianiline in Castrate Female Rat

Treatment	Number of rats	Final body weight (gm)	Uterine weight (mg)	Adrenal weight (mg)	Thyroid weight (mg)
Methylenedianiline[a]	9	186±26	104±14	102±17	25±4
None	5	225±10	61±15	65±5	13±2

[a]Given as the dihydrochloride at 150 mg/kg in 0.5 ml H_2O daily orally for 14 days

are significantly greater than the controls (Tullner, 1960). In Table II Series A, one may note similar effects following short-term treatment with several diaminodiarylalkanes. The adrenal response to *p,p'*-diaminochalcone (Table II, Series B) is of a lesser magnitude (Hertz and Tullner, unpublished 1961).[1]

[1]We wish to thank Dr. E. Van Heyningen and Mr. D. Cassady of the Eli Lilly Co., Indianapolis, Indiana, for diaminochalcone and diaminodiarylalkanes used in these studies.

TABLE II

Endocrine Effects of Diaminodiarylalkanes and Diaminochalcone in the Castrated Female Rat[a]

Compound	Body weight (mg)	Adrenal weight (mg)	Thyroid weight (mg)	Uterus weight (mg)
	Series A			
1,1–Bis(p–aminophenyl)ethane	220±18	110±17	18±2	148±83
2,2–Bis(p–aminophenyl)propane	224±16	177±19	19±3	125±22
2,2–Bis(p–aminophenyl)butane	217±13	200±42	20±1	232±6
No treatment	252±7	58±6	12±1	62±8
	Series B			
4,4′–Diaminochalcone	281±16	85±9	15±3	70±9
No treatment	278±17	61±4	11±2	68±4

[a]Compounds were administered daily for 5 days. Total oral dose 250 mg 3–5 rats per group.

Certain diaminodiarylalkanes inhibit the secretion of 17-hydroxycorticoids (Porter–Silber chromogens) in the dog. Shortly after the acute intravenous administration of methylenedianiline, there was a substantial suppression of 17-hydroxycorticoid output in the intact dog (Fig. 2).

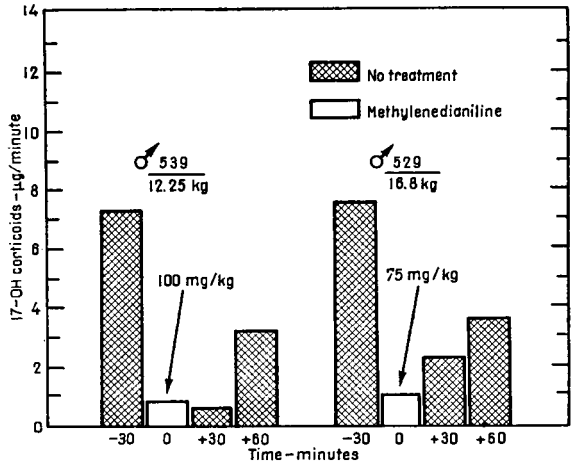

FIG. 2. Effect of methylenedianiline on 17-hydroxycorticoid secretion in the intact dog.

Within 2 hours after drug administration, 17-hydroxycorticoid levels had returned to normal with preliminary chromatographic identification of cortisol as the major secretory product. There was no evidence of increased secretion of 17-hydroxydeoxycorticosterone as seen after treat-

ment with the pyridyl analog of amphenone, metopirone (Liddle *et al.*, 1958; Jenkins *et al.*, 1958).

Although there was only moderate adrenal hypertrophy in the rat after treatment with *p,p'*-diaminochalcone, this agent effectively depressed 17-hydroxycorticoid output in the dog in acute intravenous doses of 50 mg/kg.

Thus we have demonstrated the pharmacologic inhibition of adrenal steroid secretion in the dog by a member of the diaminodiarylalkane series, as well as a representative of a distinctly different class of compounds, the diaminochalcones.

B. ADRENAL-MEDIATED PROGESTATIONAL EFFECTS IN THE RABBIT

One of the most striking effects produced by amphenone and certain diaminodiarylalkanes is the progestational proliferation of the rabbit endometrium (Hertz *et al.*, 1955). The classic experiments of Bouin and Ancel (1910) firmly established the relationship between the corpus luteum of the rabbit ovary and this type of endometrial transformation. Following the isolation of progesterone and in subsequent investigations, the endometrial response in the rabbit was considered to be associated with steroid hormones only.

Results obtained in several experimental series of Clauberg tests are summarized in Table III. It can be seen that the progestational potency

TABLE III

Progestational Response to Amphenone and Diaminodiphenyl Congeners in the Rabbit

Series	Compound	Total dose (mg)	Progestational proliferation[a]
A	Amphenone	3000.	+4, +3, +4, +4
	Progesterone	0.5	+4, +4, +4, +4, +4
	None	—	0, 0, 0, 0, 0,
B	Methylenedianiline	1000.0	+4, +4, +4, +4
	Progesterone	0.5	+4, +3, +4, +4
C	1,1-Bis(*p*-aminophenyl)ethane	1000.0	+3, +3, +2
	1,2-Bis(*p*-aminophenyl)ethane	1000.0	0, 0, 0,
	2,2-Bis(*p*-aminophenyl)prone	1000.0	+2, +3, +3, +2, +2
	2,2-Bis(*p*-aminophenyl)butane	1000.0	+3, +2, +1
	4,4'-Diaminochalcone	1000.0	0, 0, 0
	Progesterone	0.5	+4, +2, 4, +4

[a]Each number represents one animal. Dosage; 10 μg estradiol-17β daily for 6 days followed by 5 days on test compounds. All compounds were injected subcutaneously.

of methylenedianiline is approximately three times that of amphenone and 0.05% that of progesterone. Diaminochalcone is completely inactive. Further, although 1,1-bis(*p*-aminophenyl)ethane is progestationally active, negative results were obtained when the phenyl groups were

on different carbon atoms (1,2-bis(*p*-aminophenyl)ethane.) The activity of the diaminodiarylalkanes demonstrate that the methyl ketone structure of amphenone is not essential for progestational proliferation. This unique action of nonsteroid compounds gave rise to questions as to their mechanism of action. Do these compounds exert their effects directly on the endometrium, as in the case of progesterone (Corner and Allen, 1929), or is the effect a result of altered biosynthetic pathways in other endocrine glands leading to secretion of progestogens? The mode of action of these agents may be better understood by examining results from a series of experiments in which the progestational action of amphenone and methylenedianiline was compared with that of progesterone under a variety of experimental conditions (Table IV).

TABLE IV

Progestational Response to Amphenone and Methylenedianiline Following Alteration of Endocrine Status

Series	Compound	Total dose (mg)	Operative procedure	Progestational proliferation[a]
A	Amphenone	3000.0	Ovariectomy	+4, +4, +3, +1, +3, +3
	Progesterone	0.5	Adrenalectomy + ovariectomy	+4, +4, +4, +3
	Amphenone	3000.0	Adrenalectomy + ovariectomy	0, 0, 0, 0, 0, 0
B	Cortisone	27.5	None	0, 0, 0
	Cortisone + amphenone	27.5 3000.0	None	0, 0, 0, 0
	Cortisone + progesterone	27.5 0.5	None	+3, +4, +4, +4
C	Methylenedianiline	1000.0	Ovariectomy	+4, +4, +4
	Progesterone	0.5	Ovariectomy	+4, +4, +4
	Methylenedianiline	1000.0	Adrenalectomy + ovariectomy	0, 0, 0
	Progesterone	0.5	Adrenalectomy + ovariectomy	+4, +4

[a]Each number represents one animal.

In Series A (Table IV) the progestational effect of amphenone was compared with that of progesterone following ovariectomy or a combination of ovariectomy and adrenalectomy (Tullner, 1958). Ovariectomy had no effect on the uterine response to amphenone. In the absence of the adrenal glands, the progestational response to amphenone was abolished. Progesterone evoked its characteristic endometrial effects under the same experimental conditions. Similarly, Series C illustrates that the progestational effects of methylenedianiline are adrenal dependent (Tullner, 1960).

Subsequent studies by Verne *et al.* (1959) have confirmed the finding that amphenone does not act directly on the rabbit endometrium. These workers have also reported that amphenone is incapable of supporting gestation in the ovariectomized-adrenalectomized rat, although pregnancy was maintained by means of amphenone following ovariectomy alone. The latter findings further establish the fact that the progestational action of amphenone is mediated by the adrenals.

Further, data from Series B (Table IV) demonstrate that cortisone treatment completely suppressed the progestational response to amphenone, although the same dose of cortisone did not alter the progestational response to progesterone. The role of cortisone in inhibiting the secretion of adrenocorticotropin (ACTH) suggested that a decreased rate of ACTH secretion might account for the lack of amphenone activity. One might postulate that elevated ACTH levels in response to amphenone treatment could account for an increased adrenocortical synthesis of progestational steroids. Indeed, Lyons *et al.* (1953) have produced deciduomata in the uterus of hypophysectomized, ovariectomized rats treated with highly purified ACTH.

In our experiments, when immature, estrogen-primed rabbits were given total doses of 500 USP units of ACTH, there was no alteration of the endometrium even though adrenal weight increases in the treated animals exceeded that of controls two- to threefold.

On the basis of more recent experimental evidence, it is apparent that amphenone and its analogs produce alterations in the adrenocortical biosynthetic pathway of the rabbit with consequent formation of a progestationally active metabolite. We have collected adrenal vein blood from methylenedianiline-treated rabbits and prepared the plasma according to the procedure of Zander and Simmer (1954). When the extract was separated on paper in the Bush A system, we observed an unidentified ultraviolet fluorescent spot which was more polar than progesterone and less polar than deoxycorticosterone used for reference standards. No progesterone was found in the extract. Preliminary information in which eluates of the unknown produced submaximal responses in the Clauberg test suggest that the progestational action of this group of diaminodiphenyl compounds is a consequence of altered adrenal cortical metabolism resulting in the increased secretion of a progestational compound.

Biological evidence for progestational activity in extracts from the adrenal cortex was presented in 1930 (Englehart). Later crystalline progesterone was isolated from adrenocortical extracts by Beall and Reichstein (1938). Recently, Balfour *et al.* (1959) have demonstrated the presence of Δ^4-3-ketopregnen-20α-ol in the adrenal venous effluent of young calves. This reduced metabolite of progesterone has been reported to have one-third to one-half the activity of progesterone in the Clauberg test (Zander *et al.*, 1958).

II. ANALOGS AND ISOMERS OF DDD (2,2-BIS(*p*-CHLOROPHENYL)-1,1-DICHLOROETHANE)

A selective cytotoxic effect was first produced in the dog adrenal cortex after chronic oral treatment with the insecticide DDD (Nelson and Woodard, 1949). Later, the active isomer was identified as 1,1-dichloro-2-*o*-chlorophenyl-2-*p*-chlorophenylethane (*o,p'*-DDD) (Cueto and Brown, 1958), although 1,1-dichloro-2-*m*-chlorophenyl-2-*p*-chlorophenylethane (*m,p'*-DDD), another active isomer, has since been described (Nichols, 1961). An unusual characteristic of the DDD compounds is the remarkable species specificity of the adrenocorticolytic effect.

Reported adrenal effects in the rat and mouse have not been confirmed. We have administered *o,p'*-DDD orally to rhesus monkeys for several weeks at doses of 50 mg/kg/day with no depression of adrenal corticosteroid secretion nor apparent histological effects. Acute intravenous infusion of the drug has also yielded negative results in this species. However, clinical findings in cases of adrenal carcinoma (Bergenstal *et al.*, 1959) leave no doubt as to the effectiveness of *o,p'*-DDD in the suppression of adrenal steroid production in man.

We shall describe several aspects of our studies on the DDD-type of compound.

A. HISTOLOGICAL AND FUNCTIONAL CHANGES AFTER *o,p'*-DDD TREATMENT IN THE DOG

Histological demonstration of the progressive cytotoxic effects of *o,p'*-DDD in the dog adrenal is shown in Figs. 3–5. The normal configuration of the cortex is presented in Fig. 3; the effects of 6 weeks' drug treatment at 50 mg/kg daily, showing atrophy of the zona reti-

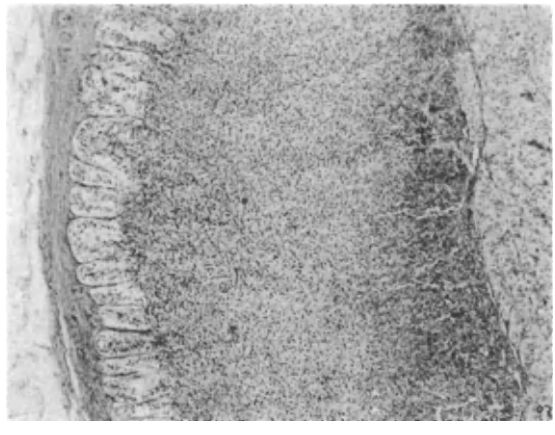

FIG. 3. Histological appearance of the adrenal cortex of normal male dog.

cularis and zona glomerulosa, in Fig. 4. Figure 5 illustrates chemical adrenalectomy in a dog on a similar dosage regimen for 3 months. Note the absence of glandular tissue and the condensation of the stroma in all zones of the cortex. It is noteworthy that there were no histological changes in the gonads or thyroid glands of these animals.

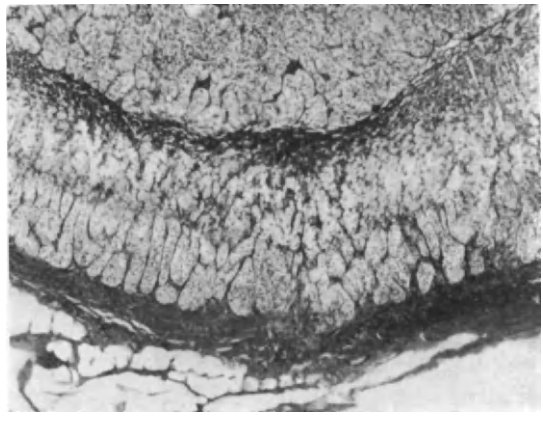

FIG. 4. Histological appearance of the adrenal cortex of male dog after 6 weeks' oral treatment with *o,p'*-DDD.

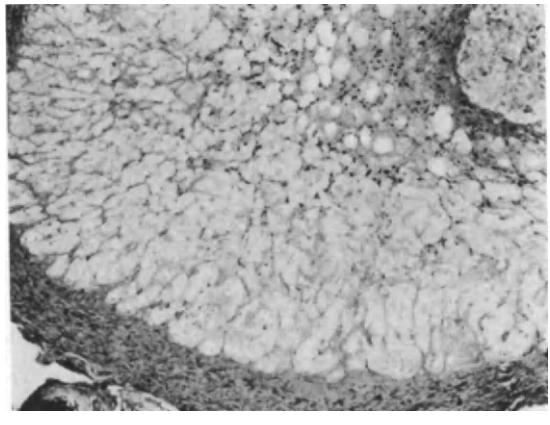

FIG. 5. Histological effects of 90 days' treatment with *o,p'*-DDD on adrenal cortex of male dog.

In short-term observations of the effects of *o,p'*-DDD in the dog (Vilar and Tullner, 1959), histological changes of the adrenal cortex were correlated with 17-hydroxycorticoid output. Normal rates of 17-hydroxycorticoid secretion (Table V) are compared with those after 2–4 days (Table VI) and 6 days (Table VII) of treatment at doses indicated.

TABLE V

Adrenal Vein 17-OH Corticoid Secretion in Normal Dogs

Animal no.	17-OH Corticoids (μg/min)[a]
1	9.4
2	10.5
3	7.4
4	7.0
5	5.0
6	5.5
7	6.4

[a]Mean of three samples.

TABLE VI

Effects of Oral o,p'-DDD on 17-OH Corticoid Secretion in the Dog

Dog no.	Days R_x	Daily dose (mg/kg)	17-OH corticoids (μg/min)
8	2	100.0	0.4
9	2	100.0	0.6
10	2	100.0	1.9
11	4	50.0	6.8
12	4	50.0	0.4

TABLE VII

Adrenal Vein 17-OH Corticoids after 6 Days' Oral Treatment with o, p'-DDD

Dog no.	Daily dose (mg/kg)	17-OH corticoids (μg/min)
13	5.0	6.5
14	5.0	5.0
15	25.0	0.8
16	25.0	0.5
17	40.0	0.6
18	40.0	0.6
19	50.0	0.5
20	50.0	0.3

When cortical histological changes are compared with rate of 17-hydroxycorticoid secretion, after 2 days' treatment, we find that steroid synthesis is reduced to the low levels seen after hypophysectomy even though cortical lesions are minimal with foci of necrosis and leucocytic infiltration confined mainly to the zona reticularis.

After 6 days' treatment, anatomical lesions were diffuse and abundant

throughout the zona reticularis and zona fasciculata. There was little change in the zona glomerulosa.

It was apparent from these acute experiments that inhibition of steroidogenesis preceded the severe adrenocorticolytic effects shown in Figs. 4–5.

B. MORPHOLOGICAL AND FUNCTIONAL REGENERATION OF ADRENAL CORTEX AFTER DDD TREATMENT IN THE DOG

In earlier investigations with technical DDD, regeneration of the dog adrenal cortex was observed on cessation of treatment. When the drug dosage and time of administration are regulated, it is possible to control the degree of adrenocortical destruction. Data from a series of dogs treated with technical DDD (200 mg/kg) daily for 3 weeks, are summarized in Table VIII (Tullner, 1961). Adrenal vein blood samples were collected and biopsies of the adrenal cortex were taken at intervals beginning 1 day after the last treatment day and subsequently at 9–30 days. The secretion rate of 17-hydroxycorticoids increased gradually from the initial low level. Thirty days after termination of drug treatment, corticosteroid output was in the normal range. Concomitant morphological regeneration was demonstrated. Adrenocortical atrophy after 3 weeks' treatment is presented in Fig. 6. This is followed by progressive repair of the zona fasciculata at the 9th (Fig. 7), 14th (Fig. 8), and 30th (Fig. 9) day.

TABLE VIII

Progressive Functional Recovery of the Dog Adrenal Cortex after DDD Treatment

Dog no. and set	17-OH Corticoids (Porter-Silber) (μg/min)[a]		Regeneration (days)
	Initial	Terminal	
1 M	0.66	3.36	9
2 F	0.81	3.63	14
3 M	0.21	6.36	30

[a]For 7 untreated dogs, range = 4.5–10.5 μg/min.

The progressive regeneration of the adrenal cortex accompanied by return of corticosteroid secretion to normal levels indicates that functional cells of the zona fasciculata may be derived from surviving cells of the zona glomerulosa. This is in accord with the hypothesis of Greep and Deane (1949) that mineralocorticoid-secreting cells (zona glomerulosa) may regenerate cells which produce glucocorticoid hormones (zona fasciculata).

FIGS. 6–9. Adrenocortical atrophy and regeneration in the male dog after treatment with technical DDD. Arrows indicate boundaries of cortex.

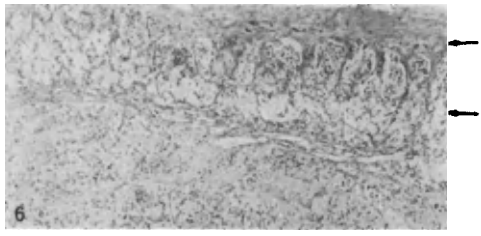

FIG. 6. Adrenocortical atrophy in male dog after 3 weeks' treatment with technical DDD.

FIG. 7. Adrenocortical regeneration in male dog 9 days after cessation of treatment with technical DDD.

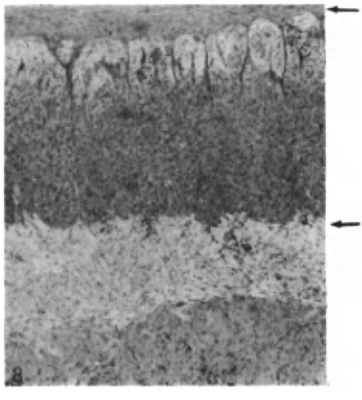

FIG. 8. Adrenocortical regeneration in male dog 14 days after cessation of treatment with technical DDD.

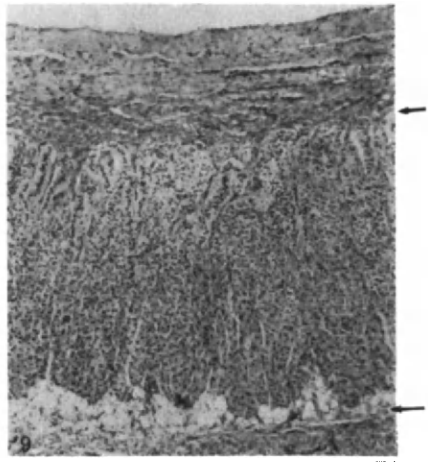

FIG. 9. Adrenocortical regeneration in male dog 30 days after cessation of treatment with technical DDD.

C. EFFECTS OF OTHER ISOMERS OF DDD

Preliminary studies on crude mixtures of the *p,p'* and *o,p'* isomers of difluorodiphenyldichloroethane indicate effective inhibition of 17-hydroxycorticoid secretion in the dog after 6 days' treatment when daily oral doses equivalent to 50 mg per kilogram body weight of the *o,p'* isomer are given. Cytotoxic effects were also noted in the zona reticularis and zona fasciculata. The *p,p'* isomer of difluorodiphenyldichloroethane is inactive under comparable conditions (Landau *et al.*, unpublished 1962).

III. CONCLUDING REMARKS

The mechanism of action of these two groups of adrenocortical steroid inhibitors obviously differs, but neither is adequately understood.

Amphenone and the related compounds described above induce hypertrophy of the adrenal cortex in the rat, rabbit, and dog. This hypertrophy, resulting from lipid accumulation in the inner zones, is similar to that seen after treatment with high doses of ACTH. As previously noted, amphenone-induced adrenal hypertrophy is absent in hypophysectomized rats.

The ACTH level in the peripheral plasma of amphenone-treated dogs is elevated (6–8 mu/100 ml) in contrast to undetectable levels in normal dog plasma (less than 1 mu/100 ml) (Tullner, 1961). Increased ACTH levels are a physiological consequence of decreased biosynthesis of cortisol. The experimental results of Rosenfeld and Bascom (1956) indicate that amphenone exerts its effects directly on the steroid biosynthetic mechanisms of the adrenal cortex. Inhibition of steroidogenesis occurred even in the absence of ACTH.

The toxic necrosis induced by the pharmacologic action of DDD and its analogs remains to be understood. Cazorla and Moncloa (1962) have noted a decrease in adrenocortical glucose-6-phosphate dehydrogenase following *o,p'*-DDD treatment in dogs. These workers suggest that the action of this compound may be related to a biosynthetic block resulting from decreased glucose-6-phosphate dehydrogenase with consequent reduction in levels of reduced triphosphopyridine nucleotide (TPNH) required in the early phases of steroidogenesis.

With prototypes of adrenocortical inhibitors such as amphenone, metopirone, DDD, and their congeners available as model compounds, one may anticipate the synthesis of newer less toxic nonhormonal agents with restricted action on specific loci of steroidogenesis.

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DISCUSSION

Nonhormonal Inhibitors of Adrenocortical Steroid Biosynthesis: Clinical Applications

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This discussion will be restricted to the clinical application of some of the compounds which selectively inhibit certain steps in the biosynthesis of adrenocortical hormones.

A test for pituitary ACTH function has long been needed. The ideal approach for this would certainly be the direct measurement of circulating ACTH in the blood. The available bioassay methods are not, however, suitable for routine clinical studies. Therefore, we are dependent on indirect assessment of this function.

Metopirone (Su-4885) in suitable doses selectively suppresses the enzyme 11β-hydroxylase and thus inhibits the synthesis of cortisol. When the level of circulating cortisol falls, the normal inhibitory influence on pituitary ACTH secretion is withdrawn and there is compensatory increase in ACTH output. The adrenal cortex now responds to the rising blood level of ACTH by augmenting the production of 11-deoxycortisol (compound S) which is a very poor inhibitor of pituitary ACTH release. The secondary rise of compound S in blood or of its metabolites in the urine reflects the pituitary reserve power of ACTH secretion.

When the test is performed on a normal individual by intravenous administration of metopirone, the plasma cortisol level falls, compound S rises, total 17-hydroxycorticosteroids (17-OHCS) show an initial fall followed by a rise, and the urinary 17-ketogenic steroid (17-KGS) rises. The rise in compound S, the rebound rise in total 17-OHCS, and the increase in urinary 17-KGS are due to augmented ACTH secretion in response to metopirone administration.

When this test is done on a patient with pituitary tumor, there is a poor response in the rise of compound S and total 17-OHCS in plasma and of 17-KGS in urine, which are indicative of poor ACTH reserve.

When the metopirone test is carried out on a patient with Cushing's syndrome due to bilateral adrenal hyperplasia, there is an exaggerated response of plasma compound S and urinary 17-KGS. This serves to differentiate between Cushing's syndrome due to bilateral adrenal hyperplasia and that due to an autonomous adrenal tumor; in the latter instance metopirone administration does not provoke a response.

Metopirone has also been used for therapeutic purposes. For instance when metopirone is administered and 11β-hydroxylase is inhibited, it blocks not only cortisol synthesis, but also corticosterone and aldosterone formation. It has been used to sup-

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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press aldosterone synthesis in conditions of secondary hyperaldosteronism such as portal cirrhosis, nephrosis, etc. However, the use of metopirone alone does not help as the secretion of deoxycorticosterone, another salt-retaining hormone, is augmented. Using a glucocorticoid to suppress ACTH and thus inhibiting deoxycorticosterone formation, in conjunction with metopirone has been reported to cause diuresis in patients with refractory edema associated with portal cirrhosis.

Mention should be made of another compound which showed much promise. Melby *et al.* (1961) reported that an inhibitor of cholesterol biosynthesis, triparanol (*p*-*β*-diethylaminoethoxyphenyl)-1-(*p*-tolyl)-2-(*p*-chlorophenyl)-ethanol, also known as Mer-29, caused a reduction in adrenal steroid production. Triparanol inhibits the conversion of desmosterol (24-dehydrocholesterol) to cholesterol by reduction of the C-24 double bond. When triparanol was administered to patients with Cushing's syndrome in doses of 1000–2000 mg/day, marked clinical improvement and a fall in cortisol output occurred. This also caused marked reduction in urinary 17-KS and 17-OHCS in patients with adrenocortical carcinoma and in urinary aldosterone and its metabolites in patients with hyperaldosteronism. The difference between the effects of triparanol and metopirone is that there is no rise in plasma or urinary steroids following triparanol administration. Unfortunately, because of the side effects produced by this drug it has been withdrawn from the market. Nevertheless, this type of approach and development in medical therapy of aberrant adrenal function is very promising and worthy of continued study.

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Studies on Adrenocortical Inhibitors

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The study of agents that have the property of inhibiting the function of the adrenal cortex has undergone changes in concentration of effort and purpose throughout the part two decades and can with some logic be divided into three periods.

After the first report by Humphrey and Donaldson (1942) that suramin sodium caused cellular degeneration of the adrenal cortex, until the activity of amphenone was first described by Allen and Corwin (1950) and Allen *et al.* (1950), sporadic reports of various compounds showing adrenocortical inhibitory activity appeared. These reports, on compounds such as thiouracil, streptomycin, were essentially incidental findings or accounts of undesirable side effects; they were not the type of report which would result from purposeful efforts to develop these compounds as adrenal inhibitors.

With the discovery of the activity of dichlorodiphenyldichloroethane (DDD) (Nelson and Woodward, 1949) and amphenone, results of more purposeful research began to appear, but relatively speaking these efforts did not move rapidly because of the toxicity of these compounds.

With the isolation and characterization of aldosterone (Simpson *et al.*, 1953), the endocrinologist at last had in hand the elusive sodium-retaining principle of the adrenal cortex and was not long in describing the physiologic and pharmacologic properties of this compound. It was also soon learned that irregularities in aldosterone secretion were intimately involved in the pathophysiology of several common illnesses (Gaunt *et al.*, 1955). Therefore, since this time there has been a rash of activity in the search for means of inhibiting the action or the secretion of aldosterone and the other secretory products of the adrenal cortex.

Table I gives in outline form several approaches to a solution of this problem, and they can be with some logic divided into the categories listed.

It is not the purpose of this paper to review this field in detail. That was attempted by the authors of this paper (Chart and Sheppard, 1959), by Browne (1960), and by Gaunt *et al.* (1961). Suffice it to say that from this abbreviated list showing only some examples in each category,

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TABLE I

Some of the more Important Pharmacologic Agents That Have Been Reported to Cause Adrenal Inhibition: Categorized According to Locus of Action

A. Inhibitors of ACTH Secretion
   1. Steroids: cortisol and its analogs — feedback mechanism
   2. Central and autonomic depressants, e.g.:
      a. Most anesthetics (barbiturates, ether, etc.)
      b. Morphine
      c. Phenothiazines
      d. Rauwolfia alkaloids

B. Agents that cause atrophy of the adrenal cortex
   1. DDD and its isomers (e.g., *o*, *p'*-DDD)

C. Inhibitors of cholesterol synthesis
   1. Δ^4-Cholestenone
   2. Triparanol (MER-29)

D. Inhibitors of steroidogenesis in adrenal
   1. Amphenone B
   2. Metopirone (Su-4885)
   3. Indenes and tetralones (Su-9055, etc.)
   4. Thiosemicarbazones

E. Competitive antagonists of aldosterone in renal tubule
   1. Spirolactone, etc.

only a few items have survived the test of clinical medicine for practical therapeutic usage. Those which have the dignity of more than just a few clinical papers are *o,p'*-DDD, triparanol, Metopirone, the spirolactones, and for special purposes, the steroids operant in the pituitary feedback mechanism.

In our laboratories we have concentrated our studies in the category of compounds that interfere with corticosteroid synthesis; we have been concerned mainly with Metopirone and more recently with the indenes and tetralones listed in Table I.

Experimental procedures used in testing these compounds have been described elsewhere (Chart *et al.*, 1962); very briefly, they entailed insertion of a polyethylene cannula into the lumboadrenal vein of an anesthetized intact dog so that periodic samples of adrenal vein blood could be collected. A control sample was obtained, the test compound was administered via a cannula in the femoral vein, and adrenal vein blood samples were collected periodically thereafter.

Figure 1 shows what happens to corticosteroid secretion when adrenal vein plasma is chromatographed according to the system of Zaffaroni and Burton (1951) after intravenous administration of some of the compounds to be described in detail.

Figure 1A shows the results of administration of 5 mg/kg of Metopirone (Su-4885). Steroid standards, cortisol (F), cortisone (E), 11-de-

oxycortisol (S), and corticosterone (B) were placed on the right of the chromatogram, a control sample of dog adrenal vein plasma on the left, and several samples of plasma collected after treatment, as designated, were placed between. The chromatograms were developed for 24 hours, sprayed with a solution of blue tetrazolium, and photographed.

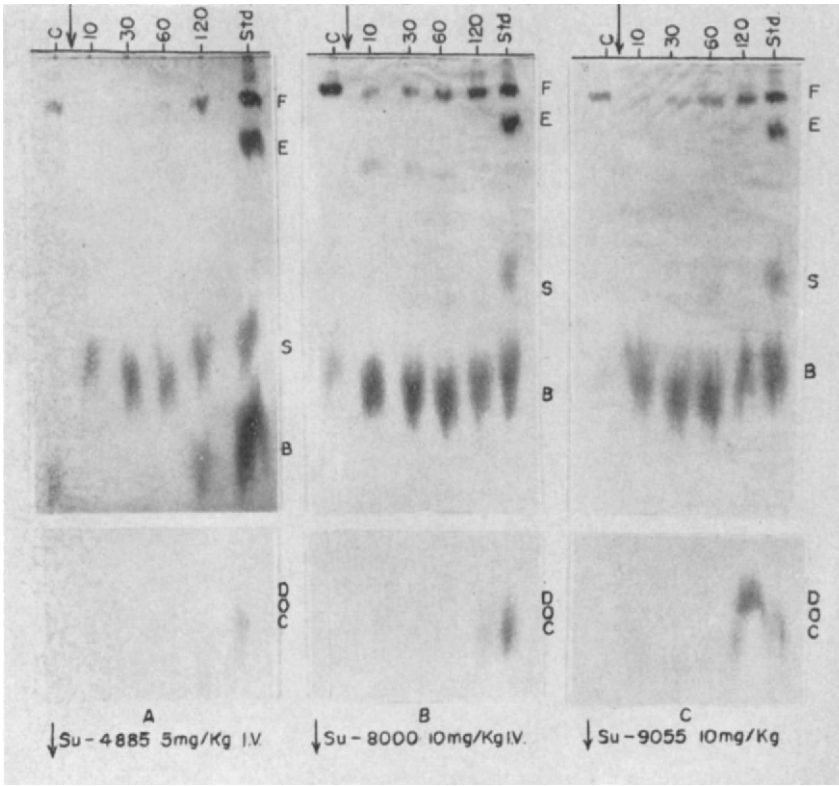


FIG. 1. Chromatographic analyses for adrenal vein plasma corticoids before and after administration in the dog of Su-4885, Su-8000, or Su-9055. *C* = control plasma corticoids; ↓ = drug administration; 10, 30, etc. = time in minutes after injection; *Std.* = standard reference steroids. From J. J. Chart and H. Sheppard, *Endocrinology*, 71, 479, 1962.

Using the reference standards as a guide, it is apparent that the control sample of adrenal vein plasma contains considerable quantities of F and B and only minimal amounts of S. Immediately after the administration of Su-4885 there is a marked drop in the secretion of *F* and *B* and a sharp rise in the secretion of S.

It is evidence of this sort, which followed more direct evidence first supplied by Liddle *et al.* (1958a) and Jenkins *et al.* (1958), which classifies Metopirone as a preferential 11β-hydroxylation inhibitor.

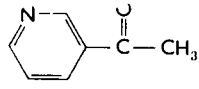
This whole project started with amphenone, and Metopirone was the result of a study of many chemical derivatives of amphenone. Many compounds studied since then appear to have adrenal inhibiting activity qualitatively similar to that of Metopirone. Metopirone was selected for clinical testing because it appeared at that time to be the most potent, and in many respects this is still true.

It soon became evident, however, that this type of activity was not limited to a narrowly defined group of chemical structures, nor did it depend on complicated chemical structures.

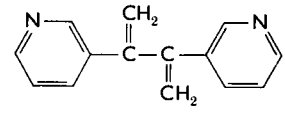
Table II illustrates that many compounds of varied chemical structure have been found to have this activity. The rationale behind the study of these groups is varied. Prototypes of some of these compounds were

TABLE II

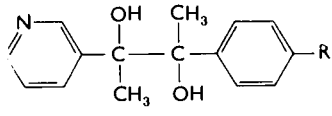
Examples of Various Classes of Compounds that Appear to alter the Secretion of Adrenal Corticoids by Means of 11β-hydroxylation Inhibition.



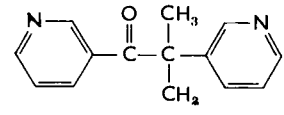
3-Acetylpyridine



(Su 4885)
Metopirone

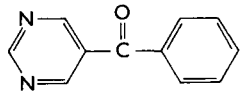


	R_1
Su—8550	H
Su—7648	4-Cl

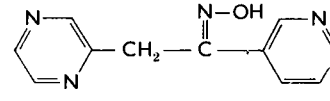


Su-8136

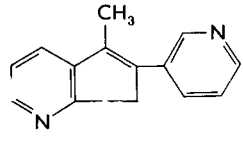
Miscellaneous



Su-10025



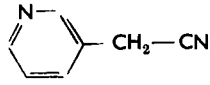
Su-7694

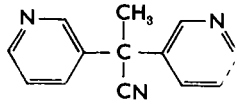


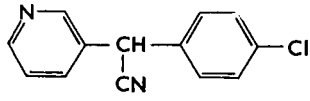
Su-8117

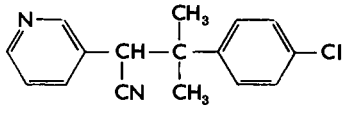
Table II continued

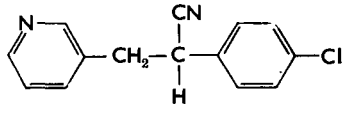
3 Pyridyl and 3–picolyl acetonitriles

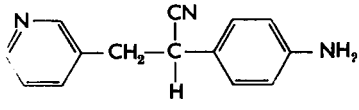
Su–10587 

Su–10195 

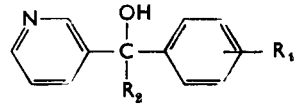
Su–9009 

Su–9076 

Su–8547 

Su–5788 

3–Pyridyl carbinols



	R_1	R_2
Su–9363	3–Cl	CH_3
Su–8605	4–Cl	CH_3
Su–9938	4–OCH_3	CH_3
Su–5729	H	$CH(CH_3)_2$
Su–8676	3–Cl	$CH(CH_3)_2$
Su–8495	4–CH_3	$CH(CH_3)_2$
Su–7550	4–OCH_3	$CH(CH_3)_2$
Su–5730	4–OH_3	$C(CH_3)_3$

obtained as side reactions in the synthesis of Metopirone; the structure and activity of Metopirone suggested others, and reports in the literature suggested investigation of some of these groups.

Table II shows formula for Metopirone and for the 3-acetylpyridine, the rather uncomplicated starting material for this compound. 3-Acetylpyridine shows adrenal inhibition which is qualitatively the same as Metopirone, but it is considerably less potent. Compounds Su-7648, Su-8550, and Su-8136 and ring-closed compound Su-8117 also are derived from 3-acetylpyridine and may be considered as modified intermediates obtained from the synthesis of Metopirone. These compounds are either less active or more toxic than Metopirone.

Table II also shows a group of 3-pyridyl carbinols which have activity qualitatively similar to that of Metopirone. These compounds are of some interest in that their chemical configuration is somewhat similar to that of amphenone. As a matter of fact, this isomeric form of Metopirone is obtained as a side reaction in the synthesis, and it does show

some activity. As a group these carbinols are generally less potent although Su-7550 approaches Metopirone in activity.

Also included in Table II is a group of 3-pyridyl- and 3-picolylacetonitriles. Biologic activity for compounds somewhat similar to these has been described by Rorig (1958) as showing anti-inflammatory activity and by Pittman *et al.* (1960) as having estrogenic activity. This class of compound also shows adrenal inhibiting activity similar to that of Metopirone, and again we find relatively simple structures, as exemplified by 3-pyridylacetonitrile, showing some activity. This compound is the starting material not only for the other compounds shown in this series, but also for another group having activity quite different from that of Metopirone.

The primary purpose of this whole study was to obtain a compound that could cause a generalized inhibition of adrenocortical function. We fell somewhat short of this, but the selective activity of Metopirone was quickly developed and the compound is now well established for use in a test for pituitary insufficiency (Liddle *et al.*, 1958b; Gold *et al.*, 1960).

In an effort to improve on the activity of Metopirone, many new compounds were synthesized; among these were Su-8000 and Su-9055, formulas of which are shown in Table III. Also of interest is the 7-chloro derivative of Su-9055, which bears the designation Su-10603. The activity of these compounds is different from that of Metopirone and appeared to warrant further investigation. Returning to Fig. 1B and C, we see the results obtained with Su-8000 and Su-9055.

As before, the reference standards compounds F, E, S, and B are on the right of the chromatogram. The control sample on the left shows a fairly high concentration of F, some B, and only minimal mounts of S. Immediately after the administration of Su-8000 or Su-9055 there is a marked decrease in the secretion of F and also a decrease in Reichstein's substance S. Concurrently we find a marked increase in the secretion of compound B.

Results obtained with the 7-chloro derivative are qualitatively similar, but this derivative appears to be more potent and is definitely less toxic.

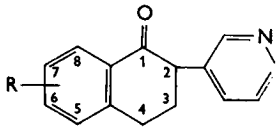
Comparing these new compounds to Metopirone, we find that in common all three decrease the secretion of cortisol, an effect which could be the result of either an 11- or 17-hydroxylase inhibitor. Metopirone, which has been shown to be an 11β-hydroxylase inhibitor, also increases S. The results obtained with Su-8000, Su-9055, and Su-10603, wherein a decrease in S and an increase in B are observed, would strongly suggest that these compounds are 17-hydroxylation inhibitors.

If the hypothesis that Metopirone blocks the 11β-hydroxylating system and the tetralones block the 17α-hydroxylating system is correct, it should be of interest to see what happens when the compounds are given together.

TABLE III

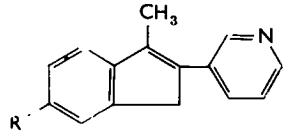
Examples of Compounds that Inhibit 17α-Hydroxylation Reactions in Steroid Biosynthesis[a]

Tetralones



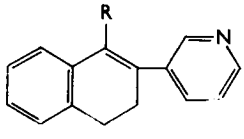
		R
I	Su–9055	H
II	Su–9758	6–Cl
III	Su–10603	7–Cl
IV	Su–10110	8–Cl
V	Su–10196	6–OCH_3
VI	Ba–9479	1–Tetralone Thiosemicarbazone

Indenes



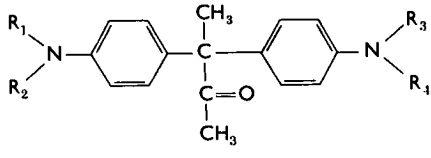
		R
IX	Su–8650	H
X	Su–8000	Cl
XI	Su–9423	NO_2
XII	Su–8954	NH_2

Dihydronaphthalenes



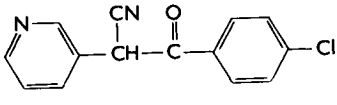
		R
VII	Su–10399	H
VIII	Su–9161	CH_3

N–Acylated amphenones

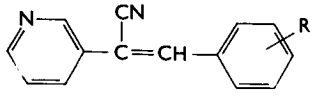


XIII	Amphenone	$R_1 - R_4 = H$
XIV	Su–10744	$R_1, R_3 = CH_3CO$; $R_2, R_4 = H$
XV	Su–8618	$R_1 - R_3 = CH_3CO$; $R_4 = H$
XVI	Su–10266	$R_1 - R_4 = CH_3CO$
XVII	Su–8226	$R_1, R_3 = 3(C_5H_4N)CO$; $R_2, R_4 = H$

3–Pyridyl–substituted nitriles



XVIII Su–9053



		R
XIX	Su–8398	*o*–Cl
XX	Su–9234	*p*–$N(CH_3)_2$

[a]From J. J. Chart, and H. Sheppard (1962), *Endocrinology*, **71**, 479.

FIG. 2. Chromatograms of dog adrenal vein plasma showing the effects of Su-4885 and Su-9055 on corticoid secretion when given simultaneously in the same dog. *C* = control plasma corticoids; ↓ = administration of first test compounds; 10, 30, etc. = time in minutes of postinjection blood samples.

Figure 2 shows the results of an experiment in the dog when 5 mg/kg. of Metopirone and Su-9055 were administered in one injection. Here we see an immediate drop in cortisol secretion, an action which is common to both compounds. However, in Fig. 1C, which shows the results of Su-9055, in addition to a decrease in cortisol there was also an increase in corticosterone secretion. In this experiment the effects of Metopirone, an 11β-hydroxylation inhibitor, prevented this rise in corticosterone. In Fig. 1A, showing the effects of Metopirone, there was an immediate rise in the secretion of compound S. Here this initial rise is prevented by the presence of the 17α-hydroxylation inhibitor, Su-9055. Although we have clearly demonstrated the combined effects of both, it is obvious that we have with Su-9055, a compound that is inherently shorter acting and less potent than Metopirone. Secretion of compound S begins to reappear toward the end of the experiment.

An obvious question concerning the results of these experiments is: "What happens to deoxycorticosterone?" In theory these compounds, which inhibit either 11β-hydroxylations or 17α-hydroxylations, should not interfere with deoxycorticosterone synthesis, which has neither of these hydroxyl groups. One might even expect that it should be secreted in excess. Returning to Fig. 1, it is apparent that such is the case: administration of Su-4885 (Metopirone), Su-8000, or Su-9055, results in an increase in deoxycorticosterone (DOC).

As further confirmation of the activity of Metopirone and Su-9055, and also in order to obtain some preliminary information on mechanisms, *in vitro* studies with guinea pig adrenal slices were performed.

Slices were incubated with ACTH and tritiated progesterone in Krebs–Ringer phosphate buffer at 37°C for 2 hours. Flasks containing tartrates of Su-4885 and Su-9055 at concentrations of 50 mg/liter were incubated along with controls. At the end of the incubation period the medium was extracted and chromatographed according to procedures already described. Ultraviolet photocopies were made, the chromatograms were then cut into 5-mm strips, and radioactivity was determined in a Packard Tri-Carb liquid scintillation counter. The continuous curve of radioactivity drawn to conform to the length of the chromatogram could then be superimposed on the ultraviolet photocopy for identification of peak incorporation of tagged progesterone into corticoids.

Figure 3 shows that when Metopirone and Su-9055 were incubated *in vitro*, results similar to those seen *in vivo* were obtained. Compared with controls, Metopirone shows a marked decrease in the incorporation of tritiated progesterone into F and E and a slight decrease in B. There is a marked increase in the production of tritiated 11-deoxycortisol (S) and also an increase in DOC. In this figure, "E-Zone" does not necessarily mean cortisone. This material has an R_f similar to cortisone but has not been positively identified as such.

With Su-9055 in the medium, the effect on F, the E-Zone, and de-

oxycorticosterone is similar to that observed with Metopirone, but in contrast there is a decrease in the production of radioactive compound S and a marked increase in radioactive corticosterone (B).

Further study with several other series of compounds has disclosed that inhibition of 17α-hydroxylation also is not limited to a narrowly defined group of chemical structures. Table III shows examples of sev-

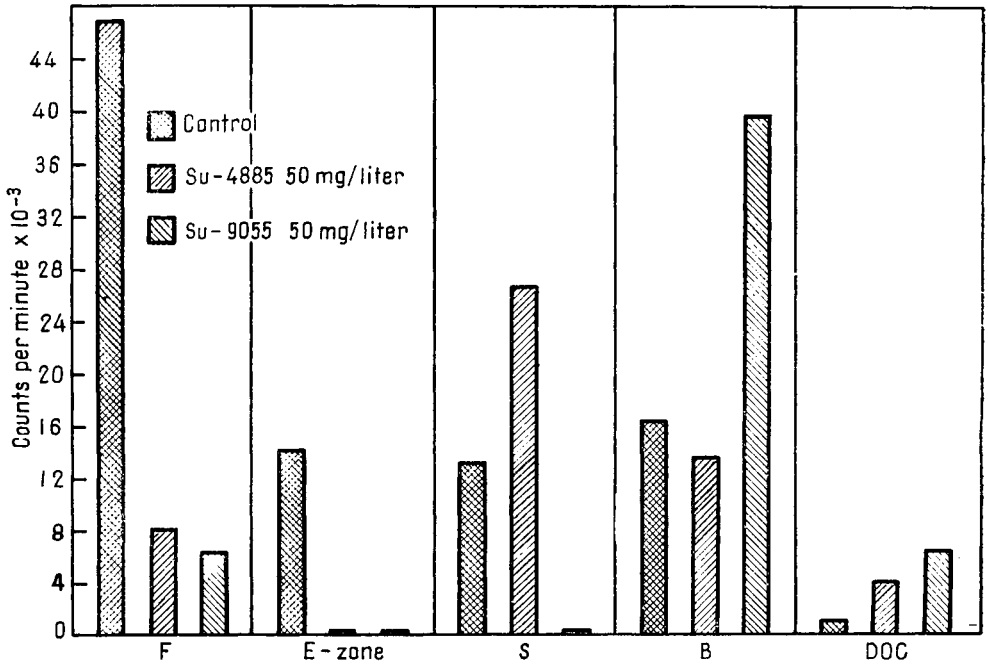


FIG. 3. Comparison of the effects of Su-9055 and Su-4885 on adrenal corticoid production by guinea pig adrenal slices *in vitro*. Individual corticoids are designated on the abscissa; it should be noted that the material designated as "E-Zone" has not been positively identified, although the mobility is similar to that of compound E (cortisone). From J. J. Chart and H. Sheppard, *Endocrinology*, 479, 1962.

eral groups of compounds which appear to have this type of activity.

Examples of the tetralones, Su-9055 and Su-10603, and the indenes, Su-8000, have already been described.

Certain dihydronaphthalenes and the *N*-acylated amphenones also have activity which is characteristic of a steroid 17α-hydroxylation inhibitor. Compound XIII, which is amphenone, is included only for the purpose of comparing chemical structures. Several publications have shown that it inhibits other enzyme systems in addition to 17α-hydroxylation (Rosenfeld and Bascom, 1956).

Compound XIV, diacetyl amphenone, has been described by Korman

(1960) as showing activity like amphenone but as being less toxic. We agree that it is probably less toxic but are of the opinion that the inhibitory activity is more specific for the 17α-hydroxylation reactions than is that of amphenone.

Also shown in this Table are some 3-pyridyl-substituted nitriles. Several acetonitriles were also shown in Table II as having Metopirone-like activity. In fact, 3-pyridylacetonitrile, the starting material for all these compounds, showed 11β-hydroxylation inhibition. Here we have this group which shows 17α-hydroxylation inhibition.

It appears that some form of unsaturation in the linkage between the aromatic rings may be necessary for inhibition of the 17α-hydroxylating system. All the acetonitrile derivatives which had Metopirone-like activity were fully substituted, and we have also found that in several cases reduction of the double bond of the dihydronaphthalenes or the ketone of the tetralones switches the activity from 17α- to 11β-hydroxylation inhibition.

These are only preliminary observations on a limited series of compounds; the structure-activity relationship is one hypothesis on which we are working. It is intriguing that inhibition of these enzyme systems seems relatively nonspecific in that a wide variety of compounds seem to have the property of inhibiting one or the other of these systems. However, in another sense they appear to be highly specific to some relatively small changes in chemical structure.

There is one other aspect of this study which does not deal primarily with adrenal inhibition although it is a natural outgrowth and is related to this problem. We have here a group of compounds that show a certain amount of selectivity for inhibition of 17α-hydroxylation. We, among others (Hall and Eik-Nes, 1962), became interested in what effects these compounds might have on androgen synthesis. It was first shown by these investigators that one of these compounds interfered with the synthesis of testosterone by rabbit testes slices and homogenates *in vitro*. We have tried an indirect approach to answering this question *in vivo*, and so far the preliminary results seem to be encouraging.

Using immature intact rats, an attempt was made by treatment with the 7-chloro derivative, Su-10603, to inhibit endogenous androgen secretion in response to chorionic gonadotropin.

The experimental setup and the results are shown in Table IV. Each group consisting of 12 rats was treated 4 days indicated in the Table; after 4 days of treatment the animals were sacrificed and organ weights indicated in the Table were determined.

There are no significant differences in adrenal and testes weights. We do, however, have differences in the seminal vesicles and the levator ani muscle. Chorionic gonadotropin alone caused a threefold increase in the weight of the seminal vesicles. Su-10603 showed no effect by

TABLE IV

Inhibition of Androgen Secretion by Su-10603 in Rats Treated with Human Chorionic Gonadotropin (HCG)

Group	Daily dose	Organ[a] weight (mg/100 gm of body weight)				
		Adrenal	Testes	S. V.	V. P.	L. ani
Control	0	22.4	630	13.9	45.0	20.2
HCG	2 I. U.	20.9	570	44.3	90.9	29.4
Su-10603	100 mg/kg	25.3	520	14.3	55.3	22.2
HCG + Su 10603	2 I. U. / 100 mg/kg	23.9	550	24.3	87.2	22.0

[a]S.V. = seminal vesicles; V.P. = ventral prostate; L. ani = levator ani muscle

itself, but when it was given in combination with chorionic gonadotropin the seminal vesicle weights are approximately 50% of those treated with gonadotropin alone. With regard to the levator ani muscle, we have some stimulation with gonadotropin alone, but in the animals receiving combination treatment there is no difference from controls. Although this evidence is indirect, it is highly suggestive that we are blocking to some extent androgen secretion in these animals. It is obvious that this blockade is not complete, and this fact may account for the apparent noneffect on the ventral prostate.

These studies on androgen synthesis and secretion afford additional evidence for a preferential inhibition of the 17α-hydroxylation mechanism. Further studies on the interference with this phase of steroid biosynthesis appear to be warranted.

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DISCUSSION

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One of the marks of good research is that it raises as many new questions as it answers. Many of us may have been designing experiments we would like to do with inhibitors of 11β- or 17α-hydroxylation. Dr. Chart has probably thought of, and perhaps done, many such experiments: therapeutic applications the clinician seeks in this type of fundamental physiological work.

Of some concern in gynecological practice is evidence Dr. Chart has shown to suggest that tetralones, like Su-9055, block 17α-hydroxylation not only in C_{21} compounds, but also C_{18} steroids like testosterone. There is a theoretical possibility that they may also block the biogenesis of C_{19} compounds like the estrogens. This possibility arises because the 17α-hydroxylation of progesterone appears to be an essential first step in the biogenesis of estrogens. This raises the possibility that one may be able to block ovarian or placental steroidogenesis while making good the concomitant adrenal deficiency with cortisol. The clinical applications of such a design are of course endless, but I am thinking particularly of ovarian masculinizing conditions such as the Stein–Leventhal syndrome. Here Su-9055 would have two possible sites of action concerned either with preventing the biogenesis of 17α-hydroxyprogesterone, a compound whose alternative metabolism may result in the production of androgenic steroids, or with preventing the formation of testosterone, which may be the androgen concerned.

Dr. Chart has confined his presentation to the inhibition of enzyme systems responsible for hydroxylation at 11β and 11α, but I am sure he has been thinking of other substituents on the steroid nucleus. From the point of view of the adrenal physiologist, presumably the most interesting compounds would be those affecting hydroxylation at C-21 or at C-20, but I have in mind also the possibility of affecting hydroxylation at C-19 which appears to be a crucial step in demethylation at this position in the formation of estrogens. Of course, once we start going round the OH groups which can be attached to various points on the steroid molecule in the anabolism or catabolism of steroid hormones, there is no end to the possibilities.

A further point that is of interest is what happens to the physiology of the intact animal if one blocks with a combination of Su-4885 and Su-9055 his ability to produce compounds F, E, and S. Presumably deoxycorticosterone production rises if all adrenal steroidogenesis is directed along this line. What happens to the pattern of steroid metabolites in urine? It has been known for some years that one of the urinary excretion products from the adrenal is pregnanediol. We have presumed that this arises from the catabolism of progesterone, but there is evidence that DOC can give rise to pregnanediol. It would be very interesting to observe the response of the urinary pregnanediol to medication with Su-4885 plus Su-9055 or Su-10603. Urinary pregnanediol usually responds to ACTH stimulation, albeit not as dramatically as hydroxycorticoids. If, however, the biogenesis of these is blocked, the pregnanediol response to ACTH may be much enhanced. Pregnanediol assay is a simpler, more accurate and specific procedure

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics:
Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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than the assay of most other urinary adrenal metabolites. Herein perhaps lies the germ of another test of adrenal function.

The mechanism by which 11β-hydroxylation is blocked by Su-4885 is not known in any detail. It is an oxidative reaction that requires TPNH and atmospheric oxygen. It may well be that Su-4885 interferes with the enzyme systems involved in hydrogen transfer to and from TPN in this instance. In this context, Dr. Chart mentioned that some form of unsaturation in the linkage between the aromatic rings may be necessary for the inhibition of the 17-hydroxylating system. I wonder whether he has any information on what happens to the unsaturated 3-pyridylacetonitriles when they exert this action. A cell-free preparation of the 17-hydroxylating system from guinea pig adrenals has been made by F. G. Hofman [*Biochim. et Biophys. Acta* **37**, 566 (1960)]. It seems to me that such a system is ideal for investigating the mode of action of these compounds.

The possibilities for physiological investigation by blocking specific enzyme systems in steroid biogenesis are endless. Dr. Chart has shown us some of these possibilities as far as the adrenal is concerned. I hope that in the next few years some of his work will touch upon the gonads and the placenta.

Incretory Modifications Induced by Administration of an Inhibitor of 11ß-Hydroxylase (Su-4885) in Different Functional Conditions of the Diencephalic-Hypophyseal-Adrenal System

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I. Introduction

Research on substances capable of modifying adrenal steroidogenesis have recently led to the synthesis of 2-methyl-1,2-bis(3-pyridyl)-1-propanone (Su-4885, or metopirone), which acts mostly as an inhibitor of the 11β-hydroxylase.

The studies of Liddle *et al.* (1958) and Jenkins *et al.* (1959) demonstrated that administration of small quantities of this compound produces in humans and in animals a partially specific block of 11β-hydroxylase which inhibits the development of some steroidogenetic pathway.

The most important effect caused by the metopirone, in respect to biochemistry, is the blocking of the 11β-hydroxylation of deoxycortisol (Reichstein's compound S) in cortisol production.

The fall of plasma cortisol leads through a feedback mechanism to an increase of ACTH secretion and to the subsequent steroidogenesis of 11-deoxycortisol and to a lesser degree of deoxycorticosterone.

The 11-deoxycorticoids are not able to block ACTH secretion, physiologically obtained only by cortisol action, therefore the compensatory stimulus of ACTH production remains as long as 11β-hydroxylation is blocked. Substance S and its metabolite tetrahydro-S are chromogenic in the Porter-Silber reaction, as is cortisol too.

This chemical behavior enables us to easily evaluate secretory modifications following metopirone administration.

The toxicity of this compound is particularly low in comparison with other adrenostatic compounds, a fact that has contributed to a widespread clinical investigation.

Liddle *et al.* (1958) suggested the use of metopirone in order to evaluate the pituitary reserve of corticotropin. The interest in this field was

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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so stimulating that research was directed to the study of endocrine function rather than to the therapeutic use of this compound.

Besides studies on corticotropin secretion in several endocrine dysfunctions and on the evaluation of the action of hormonal treatments on the "feedback" mechanism, a new interest has arisen to determine the interference caused by nervous system lesions on the adrenocorticotropin response to different stimuli and to clarify the changes induced by various pharmacological compounds in the functioning of the control mechanism for the ACTH secretion.

II. Methods and Clinical Material

The aim of the present paper is to contribute to the study of the secretory modifications found after metopirone administration in different conditions of activity of the hypothalamic-hypophyseal-adrenal system.

The experiments were carried out on patients hospitalized at the Medical Clinic of the University of Milan and on subjects who, although presenting mild dysfunctions, were considered normal in the absence of any clinical and experimental proof of endocrine abnormalities.

The subjects, females, aged between 15 and 50, were classified as follows: 9 normal subjects, 9 cases of Cushing's syndrome (6 of them had bilateral adrenal hyperplasia, 1 had a pituitary adenoma, and 2 had an adrenal adenoma); 3 cases with congenital adrenogenital syndrome; 4 cases suffering from hirsutism of adrenal origin; 2 cases of Stein—Leventhal's syndrome; 2 cases of primary hypothyroidism; and 3 cases of anorexia nervosa.

Cases of primary or secondary hypoadrenalism have been omitted because the usefulness of the metopirone test has already been clearly demonstrated.

In most of these subjects metopirone was administered orally (750 mg every 4 hours for 1 day, ingested with a small amount of milk). In some cases the metopirone test was repeated intravenously (30 mg per kilogram of body weight) as a slow constant infusion over a period of 8 hours. In a few patients metopirone was administered only intravenously.

The Silber and Porter method (1954), was used for the analysis of 17-hydroxycorticosteroids (17-OHCS), the Drekter method (Drekter *et al.*, 1947) for 17-ketosteroids (17-KS), the Klopper method (Klopper *et al.*, 1955) for pregnanediol, the Fotherby and Lowe method (1960) for pregnanetriol, the Henke method (Henke *et al.*, 1960) for 11-deoxycorticoids, and the Brown method (1955) for the estrogen fraction.

The plasma free 17-hydroxycorticoids were determined by the Peterson method (Peterson *et al.*, 1957) as modified by Gold *et al.* (1960c).

ACTH stimulation was induced in all the patients in order to determine the adrenal response and to compare these data to the increase in hypophyseal secretion following the 11β-hydroxylase block. ACTH was administered intravenously at a dosage of 25 units (U) for 1 day and/or of 5, 10, 20 U progressively in 3 subsequent days.

III. RESULTS

A. NORMAL SUBJECTS

Metopirone administered orally induces in normal subjects (Fig. 1) particularly high increases in the absolute value of 17-hydroxycorticosteroids, which include primarily tetrahydro-11-deoxycortisol (THS) of 17-ketosteroids; and, in a smaller amount, of pregnanediol, pregnanetriol, and urinary estrogens.

The increase in amount of THS, of 17-hydroxycorticoids, and of the pregnanetriol appears in an earlier stage and is greater than for pregnanediol, 17-ketosteroids, or estrogens.

Such steroid modifications are clearly found already in the first 24 hours after metopirone administration; they reach a peak on the second day, decreasing to normal or almost normal values the following day.

Intravenous administration of metopirone (Fig. 2) in a single dose of 30 mg/kg produced a smaller increase of steroid excretion than that obtained by oral intake of 4.5 gm of this compound divided in six doses within 24 hours.

The blood determinations of plasma 17-hydroxycorticoids, done in the period after starting the intravenous infusion of the compound, shows a diminution of the initial values, the lowest level being reached at the second hour; there follows a progressive increase that has its highest value around the 6th hour (Fig. 2).

These data, which emphasize the interest of studying this parameter in the evaluation of modifications following metopirone treatment, agree with what has already been published by other authors (e.g., Liddle *et al.*, 1959; Gold *et al.*, 1960a; Brownie and Sprunt, 1962).

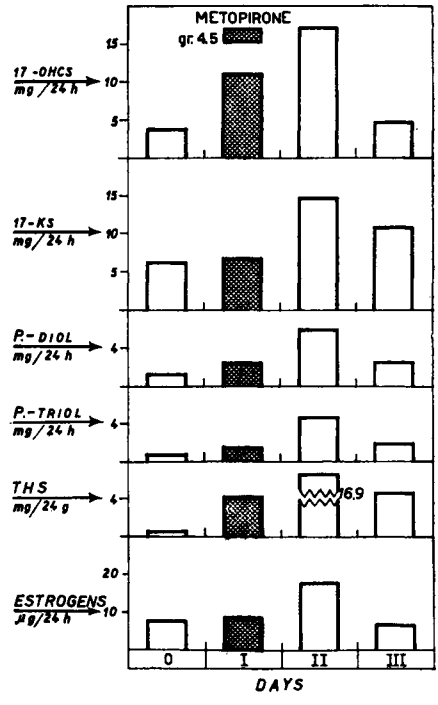

FIG. 1. Response to metopirone in normal subjects.

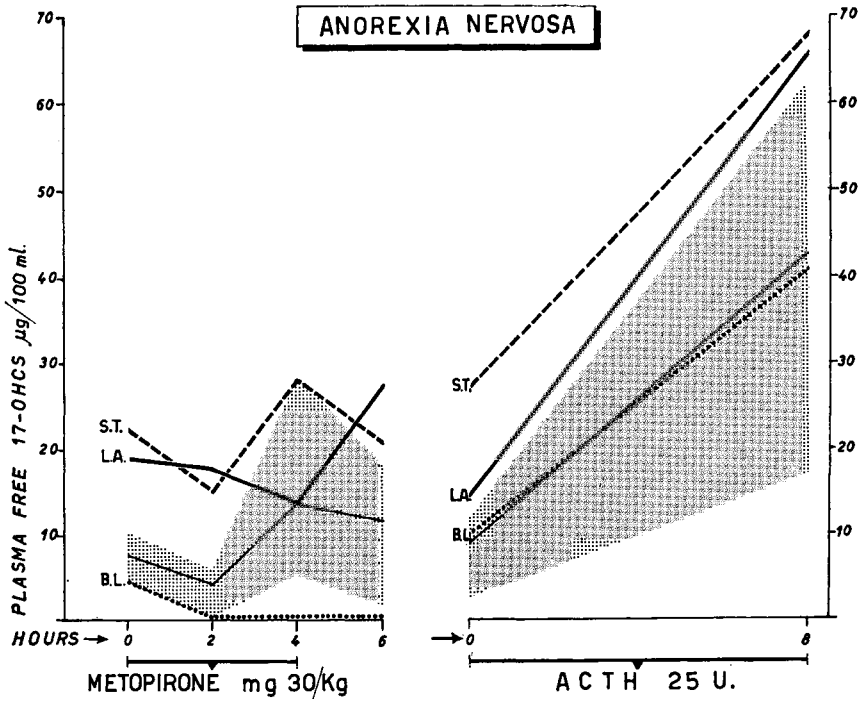

FIG. 2. Response to metopirone and to ACTH in normal subjects and in patients with anorexia nervosa.

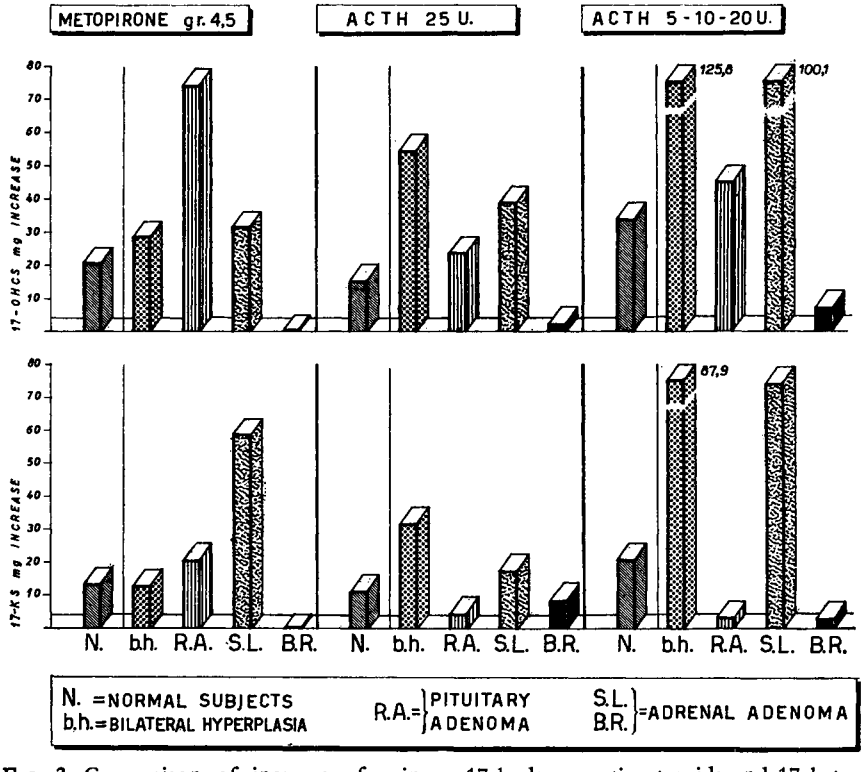

FIG. 3. Comparison of increase of urinary 17-hydroxycorticosteroids and 17-ketosteroids after administration of metopirone and ACTH in normal subjects and in patients with Cushing's syndrome.

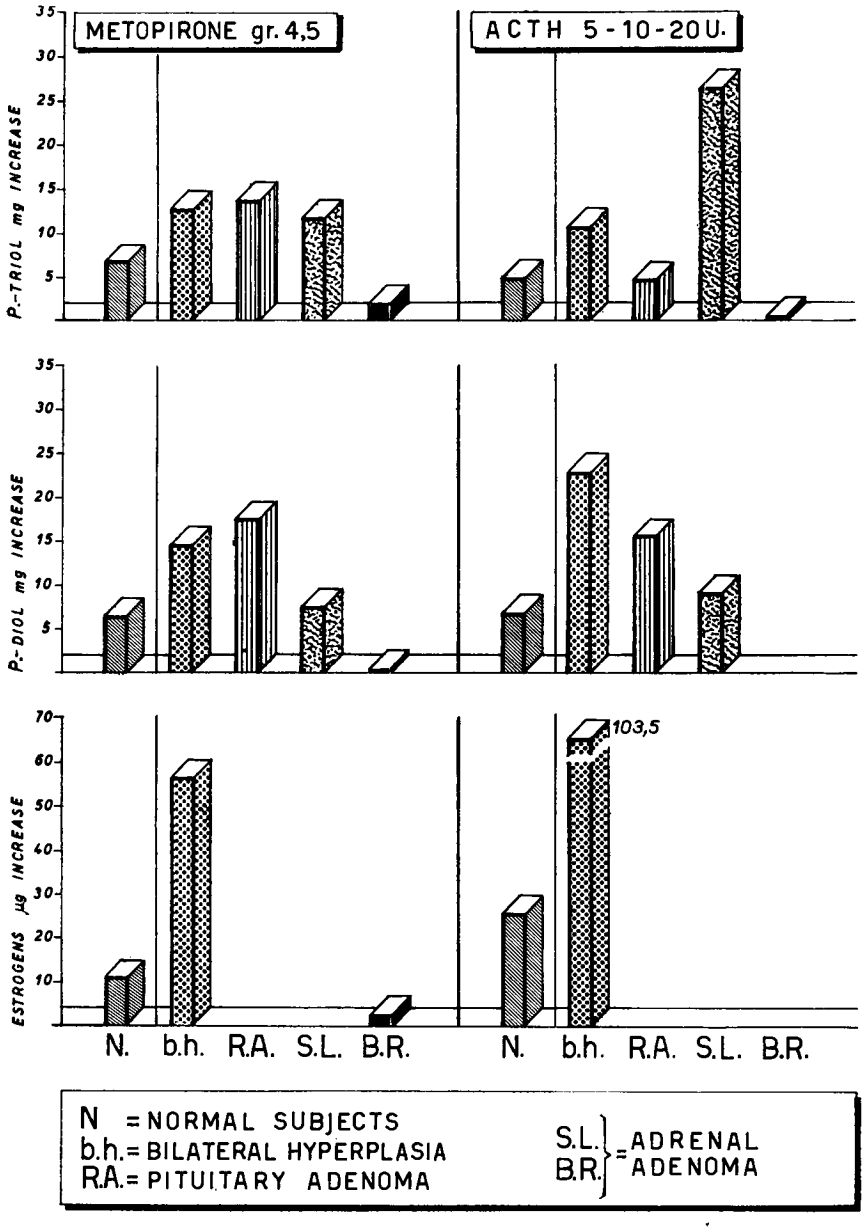

FIG. 4. Comparison of increase of urinary pregnanetriol, pregnanediol, and estrogens after administration of metopirone and ACTH in normal subjects and in patients with Cushing's syndrome.

Comparison between urinary excretion after metopirone administration and-after ACTH infusion (Figs. 3 and 4) shows that the oral administration of 4.5 gm of the adrenostatic compound elicits higher values than those consequent to 25 U of ACTH within 8 hours; the increase in almost all of the steroids analyzed was less than that induced by administration, by similar procedure, of 5, 10, 20 U of ACTH progressively on 3 days.

The plasma increase of the 17-OHCS at the 8th hour over the initial intravenous infusion of ACTH (Fig. 2) is on the contrary much higher than the increase obtained in similar conditions following administration of 30 mg of metopirone per kilogram.

B. CUSHING'S SYNDROME

Administration of metopirone in Cushing's syndrome permits us to analyze the possible pathogenetic mechanism with a compound that creates a physiological pituitary stimulation of ACTH secretion.

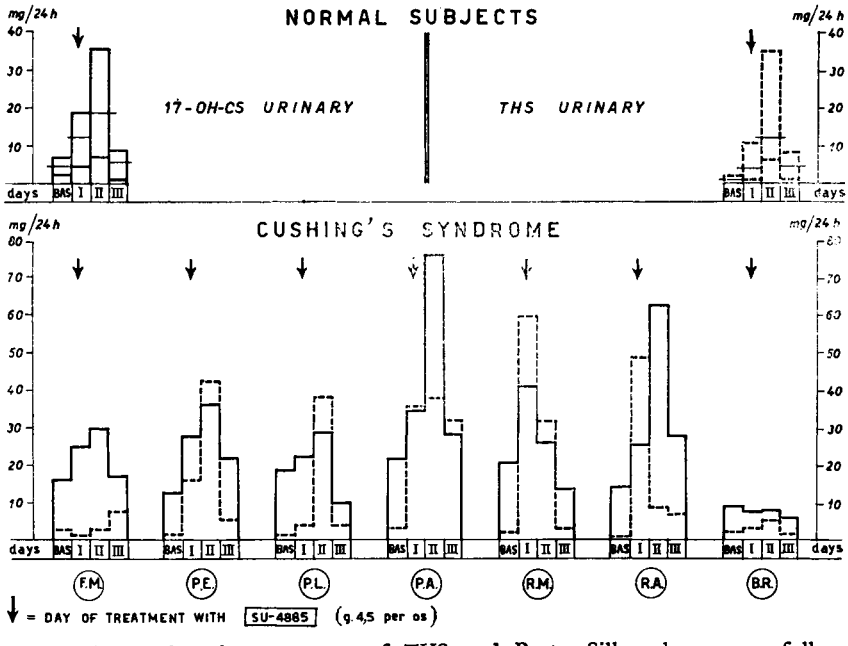

FIG. 5. Changes in urinary pattern of THS and Porter-Silber chromogens following administration of metopirone in normal subjects and in patients with Cushing's syndrome.

Figures 3, 4, and 5 show urinary patterns in 9 patients with Cushing's syndrome in whom histological examination after surgery showed bilateral hyperplasia of the adrenal gland in 6 cases, a pituitary adenoma in 1 case (R. A.), and an adrenal adenoma in 2 other cases (S. L. and B.R.).

In the patients with bilateral hyperplasia the urinary 17-OHCS, and even more the THS, commonly increased to levels higher than those reached in normal subjects. The absolute value found for each steroid in these patients (Figs. 3 and 4) was not uniformly higher than values in normal subjects. The increase and the subsequent decrease of these steroids were chronologically equivalent to the course in normal patients.

Estrogens determined in some patients showed a marked increase. The patient with pituitary adenoma showed, after metopirone administration, particularly high values of 17-OHCS and of THS, values much higher than those found in the normal patients.

In one case (S. L.) of adrenal adenoma the urinary 17-OHCS values were similar to those of the patients with bilateral adrenal hyperplasia, but the second adrenal adenoma patient (B. R.) did not show any modification.

Plasma 17-OHCS increased after intravenous administration of metopirone to a lesser extent than did urinary 17-OHCS following oral administration.

In order to evaluate quantitatively the pituitary response after metopirone administration, the same subjects received an ACTH infusion by means of the technique already explained.

Examination of the results (Figs. 3 and 4), expressed as absolute amount of increase in the two different experiments, shows that in the subjects with bilateral adrenal hyperplasia the urinary excretions of 17-OHCS and 17-KS were much lower than the values obtained after a single ACTH infusion (i.v., 25 U) and markedly lower than values obtained after successive stimulations with 5, 10, 20 U.

In the same patients, urinary increase of pregnanediol and pregnanetriol is similar in amount after either metopirone or ACTH 5, 10, 20 U for 3 days). Urinary estrogens increased in the same patients after metopirone to values higher than those found for normal subjects. This response was definitely lower as compared to the one obtained after ACTH stimulation.

In the patients with pituitary adenoma, the adrenostatic compound produced a response of the adrenal gland much higher than that obtained by means of two types of ACTH infusion.

In one of the patients with adrenal adenoma, modifications produced in the urinary metabolites were similar to those following infusion of 25 U of ACTH, whereas in the other patient, the metabolites were unchanged. The increase in plasma 17-OHCS in the patients with adrenal hyperplasia treated with metopirone, showed a response lower than the modification obtained by intravenous infusion of 25 U of ACTH.

The variety of behavior of the patients with Cushing's syndrome after differential dosage with metopirone gives us the opportunity to consider the dose used and the response obtained.

Liddle *et al.* (1959) in the first period of studies on metopirone found little difference between 6 doses of 550 mg and 6 doses of 750 mg administered orally within 24 hours. Gold *et al.* (1960a) reported that after intravenous administration of the adrenostatic compound, the effect was "all-or-none." Our results show that different doses of metopirone in three cases of Cushing's syndrome (Fig. 6) can lead to quantitatively different results.

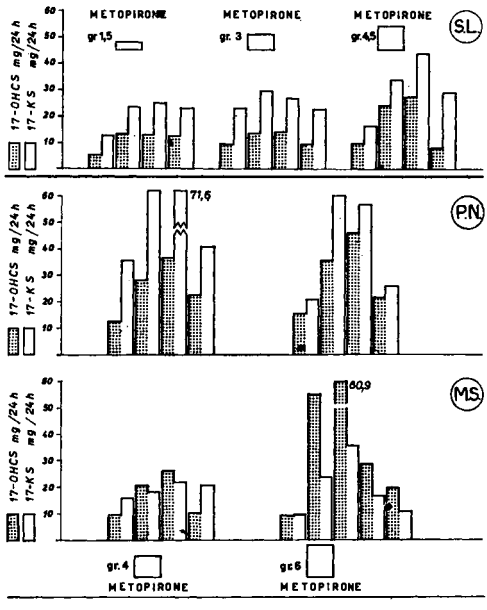

FIG. 6. Urinary steroid excretion in response to different doses of metopirone in three cases of Cushing's syndrome.

Other observations (Fig. 5) show that, although the THS values are higher than the Porter—Silber chromogens because of different extraction procedures, a standard dose of metopirone in patients with Cushing's syndrome does not always cause a similar block of the 11β-hydroxylase resulting in a different pattern of ACTH excretion. We would point out here that in a subject (B. R.) with adrenal adenoma (surgically controlled), the increases in substance S and 17-OHCS were not consistent.

Furthermore, in a patient (R. A.) who was suffering from pituitary adenoma, the block of the 11β-hydroxylation caused a very high and rapid increase of substance S, followed by marked increase of 17-OHCS and rapid decrease of 11-deoxycortisol. This behavior could be explained by adrenal hyperactivity induced by high ACTH levels.

Previous findings show that ACTH hypersecretion and the steroid urinary metabolite modifications are particularly related to quantities of metopirone sufficient to block a large portion of the 11β-hydroxylase. Furthermore, these observations demonstrate that to a certain extent there is an approximate proportionality between the amount of metopirone intake and the increase of residual steroidogenesis. On the other hand, we have to consider that high doses of metopirone can block adrenal steroidogenesis not only in the 11β-position. Liddle *et al.* (1959) hypothesized that the decrease in the total 11-hydroxycorticoids ob-

served in two cases of adrenal adenoma (treated with 750 mg every 6 hours), could be due to a certain degree of interference of that compound in some other steps of corticosteroid synthesis.

C. ADRENOGENITAL SYNDROME

Administration of metopirone to patients with adrenogenital syndrome, with virilism but not hypertension, caused by a deficiency of

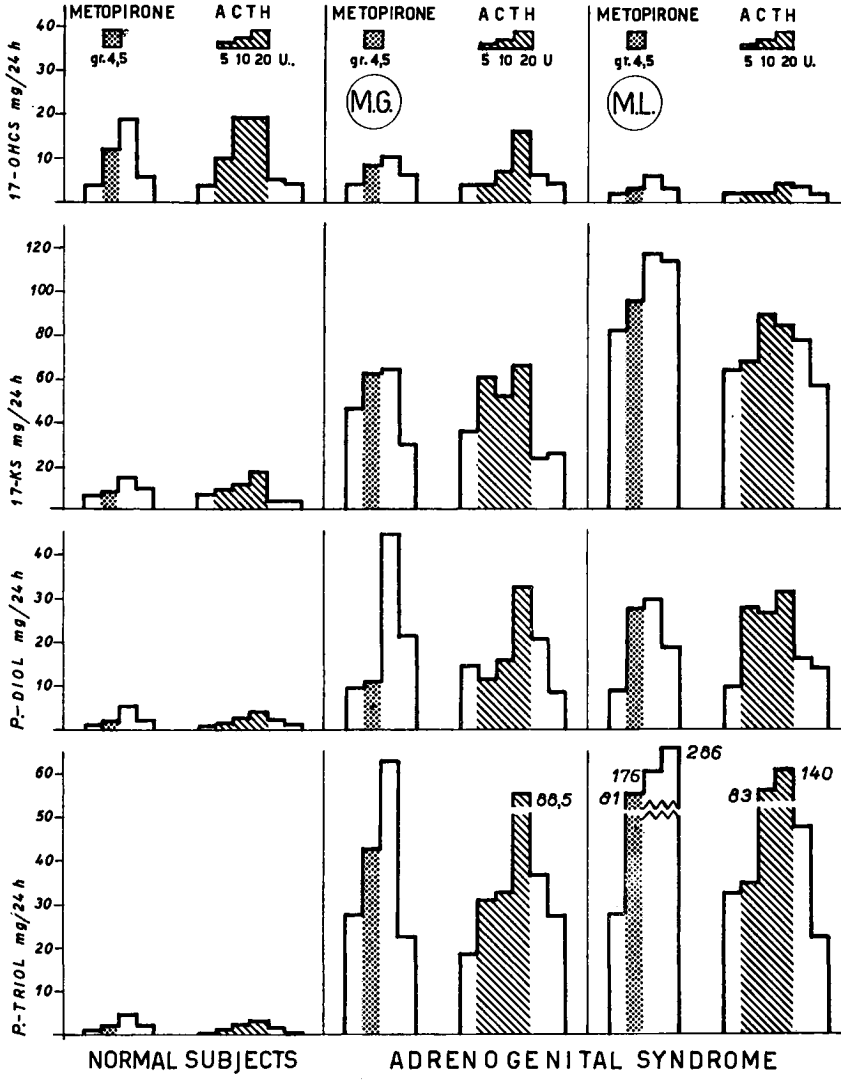

FIG. 7. Changes in excretion of urinary steroids following administration of metodirone in two cases of adrenogenital syndrome.

21-hydroxylation, produced modifications of adrenal steroidogenesis greater than those obtained in normal subjects or in patients suffering from Cushing's syndrome. Such modifications of steroidogenesis are different from what we have found in the subjects previously described.

Urinary excretion of 17-OHCS, 17-KS, pregnanediol, and pregnanetriol was studied after metopirone or ACTH administration in two cases of congenital adrenogenital syndrome (M.G. and M.L.) (Fig. 7), aged 16 and 30, respectively. In each case the 17-OHCS increase was small, much less than in normal subjects. The increase in 17-KS was particularly striking, being much greater than that obtained in normal subjects; nevertheless the percentage increase was similar to that found in normal patients, because the adrenogenital cases have high basal values. Pregnanediol, and particularly pregnanetriol, increased dramatically. These results confirm that in the adrenogenital syndrome ACTH secretion, though already increased by 21-hydroxylase deficiency, can have a still greater increase if the 11β-hydroxylase is blocked. Although in the disease high amounts of pregnanetriolone can be excreted—as described by Cox and Finkelstein (1957), Bongiovanni *et al.* (1959), Cleveland *et al.* (1962)—and though pregnanetriolone synthesis by the metopirone effect on the 11β-hydroxylase can be reduced in favor of pregnanetriol and 17-hydroxypregnanolone, it seems clear that the dramatic increase of pregnanetriol and pregnanediol is the expression of degradation of the precursors such as 17-hydroxyprogesterone and progesterone, accumulated as a consequence of the enzymatic defect.

D. HIRSUTISM

In 4 patients (Table I), aged from 19 to 28 years, suffering from hirsutism and menstrual abnormalities but without other signs of virilization, metopirone administration induced in a similar way, but in a lower degree than in exogenous ACTH infusion, a relatively higher increase of 17-KS than of 17-OHCS. In two of the four patients (G. E. and F. S.), the adrenostatic compound induced, furthermore, an increase in pregnanetriol higher than normal values and similar or greater than the one induced by repeated doses of ACTH.

This behavior, although in some cases variable, demonstrates that the endogenous ACTH, similarly to exogenous ACTH, induces a modification of steroidogenesis from the normal pattern; this modification is an increased amount of 17-KS and sometimes of pregnanetriol.

E. STEIN–LEVENTHAL SYNDROME

In two patients (Table I), aged 20 and 22 and with a clinical Stein—Leventhal syndrome surgically and histologically proved, metopirone administration produced a great increase of 17-KS, similar to the increase found in patients with simple hirsutism.

TABLE I

Metopirone Test in Various Disorders

Diagnosis	Patient	Urinary steroids (mg/24 hr)	Metopirone test				ACTH test					
			Control	Day 1 (4.5 gm oral)	Day 2	Day 3	Control	Day 1 (5 U, i.v.)	Day 2 (10 U, i.v.)	Day 3 (20 U, i.v.)	Day 4	Day 5
Hirsutism	L. A.	17–OHCS	3.7	9.3	8.7	6.	5.0	13.9	34.	33.	8.1	3.6
		17–KS	12.3	6.60	27.2	8.5	12.0	36.8	35.4	41.2	23.8	19.1
		P–3–ol[a]	0.26	1.45	4.7	1.1	0.26	4.20	4.80	11.10	5.0	0.30
	P. A.	17–OHCS	3.6	13.2	30.0	13.1	4.2	11.7	25.6	16.6	4.2	2.4
		17–KS	8.0	15.8	16.0	33.0	7.8	19.9	33.4	39.6	17.0	9.4
		P–3–ol	0.5	0.30	3.0	3.1	1.23	1.80	2.07	2.25	0.5	0.4
	G. E.	17–OHCS	5.5	12.9	22.0	8.0	7.2	7.3	20.0	35.5	7.1	7.3
		17–KS	16.0	18.0	44.0	30.0	1.8	3.0	6.0	8.4	3.0	0.3
		P–3–ol	1.7	3.0	8,4	6.4	6.6	19.5	31.5	35.2	16.9	12.8
	F. S.	17–OHCS	2.95	5.8	26.5	13.8	2.5	4.25	9.9	18.5	3.15	3.8
		17–KS	22.5	24.0	52.5	36.5	20.5	29.0	32.0	39.0	25.0	12.4
		P–3–ol	2.0	32	17.6	10.0	—	—	—	—	—	—
Stein—Leventhal syndrome	M. R.	17–OHCS	6.2	32.9	35.5	9.1	6.0	23.0	39.0	54.0	4.9	4.8
		17–KS	18.4	39.0	51.0	5.8	17.0	33.6	23.2	40.1	16.9	12.7
		P–3–ol	—	—	—	—	—	—	—	—	—	—
	S. I.	17–OHCS	12.0	28.0	31.0	10.8	7.4	10.2	42.5	39.0	9.7	5.4
		17–KS	7.2	28.2	35.0	17.9	11.7	27.5	29.5	41.5	16.0	8.2
		P–3–ol	3.6	0.7	5.1	1.1	2.5	2.5	5.3	9.6	5.7	—

Table I (continued)

Diagnosis	Patient	Urinary steroids (mg/24 hr)	Metopirone test				ACTH test		
			Control	Day 1 (4.5 gm, oral)	Day 2	Day 3	Control	Day 1 (25 U, i. v.)	Day 2
Hypothyroidism	M. A.	17-OHCS	1.44	5.0	15.8	5.2	0.60	11.6	2.30
		17-KS	3.5	2.7	8.5	9.1	6.3	7.2	4.20
	M. M.	17-OHCS	1.3	0.9	7.6	5.1	1.26	5.2	2.85
		17-KS	0.84	0.37	2.4	3.7	1.3	1.5	3.05

[a]P-3-ol, pregnanetriol.

These findings show that at least in some cases of Stein—Leventhal syndrome, endogenous or exogenous ACTH leads to an increased amount of 17-KS.

F. HYPOTHYROIDISM

The conflicting reports in the literature of a reduced metopirone response in patients with hypothyroidism (Gold *et al.*, 1960a), or normal response (Liddle *et al.*, 1959), or both responses (Brownie and Sprunt, 1962) gave rise to our interest in studying two cases of primary hypothyroidism (Table I).

In one of the patients (M. A.) myxedema had been present for a few months. In the other patient hypothyroidism was present at birth; the patient was a 14-year old girl with residual thyroid tissue at tongue level.

The adrenal response (Table I) in both cases was quite small, even smaller in the young girl, although within the lower range of values of normal patients. Similar result was obtained with 25 U of ACTH stimulation.

These data agree with those of Liddle *et al.*, (1960), who emphasized that the loss of corticotropin responsiveness is not peculiar to hypothyroidism. In our cases we had indeed a low degree of ACTH production.

G. ANOREXIA NERVOSA

Metopirone (30 mg/kg) was injected by the usual procedure in three patients suffering from anorexia nervosa (Fig. 2). In one of the patients (B. L.) in almost cachectic condition after a few years of illness, no increase in the plasma 17-OHCS was found after metopirone administration whereas 25 U of exogenous ACTH gave a normal increase. In the other two cases (S. T. and L. D.) normal increase was found in the plasma 17-OHCS after metopirone.

H. NEUROTROPIC DRUGS

Research done in the last few years on animals and humans (De Groot and Harris, 1950; Hume and Wittenstein, 1950; Mason, 1958; Oppenheimer *et al.*, 1961; Egdhal, 1961; Brodish, 1961; Slusher and Hyde, 1961) demonstrated that various zones of the nervous system (cortex, temporal lobe, reticular substance, limbic area, hypothalamus) are capable of regulating corticotropin secretion through a stimulant or inhibiting action. It is well known that some anticonvulsive drugs can interfere with the mechanism of ACTH release (e.g., Bonnycastle and Bradley, 1960; Krieger, 1961). Still controversial is the action of chlorpromazine and reserpine action on Su-4885 responsiveness (Gold *et al.*, 1960b; Feldschue *et al.*, 1961; Krieger, 1961). In view of this previous

TABLE II

Metopirone Test after Psychotropic Drugs

Diagnosis	Subject	Urinary steroids	Before treatment			Treatment	After treatment		
			Control	Day 1 (4.5 gm, oral)	Day 2		Control	Day 1 (4.5 gm oral)	Day 2
Normal	U. M.	17-OHCS	4.9	31.1	15.8	Chlorpromazine 200 mg/day	4.6	9.9	10.2
		17-KS	8.9	14.2	15.0	(7 days)	4.5	8.8	12.3
		P-3-ol.	1.0	1.50	9.54		1.0	2.0	3.9
Cushing's syndrome (adrenal hyperplasia)	P. N.	17-OHCS	12.5	28.1	36.3		11.3	21.3	42.8
		17-KS	35.4	63.2	71.6		37.4	47.6	57.6
		P-3-ol.	1.4	4.0	8.7		1.25	3.6	9.1
Cushing's syndrome (adrenal adenoma)	S. L.	17-OHCS	9.5	13.8	14.0		3.4	18.9	16.2
		17-KS	22.9	29.4	—		17.0	19.4	22.7
		P-3-ol.	1.75	3.1	—		1.3	2.75	3.38
Cushing's syndrome (pituitary adenoma)	R. A.	17-OHCS	14.3	26.0	63.0		12.4	21.0	53.2
		17-KS	9.8	14.9	24.9		6.2	8.1	16.2
		P-3-ol.	0,4	1.6	6.6		1.38	4.3	7.55
Normal	L. M.	17-OHCS	2.8	9.2	8.5	Reserpine, 2 mg/day	3.6	6.1	5.7
		17-KS	4.0	5.3	16.2	(7 days)	4.8	4.6	10.2
Cushing's syndrome (adrenal hyperplasia)	F. M.	17-OHCS	16.0	25.2	29.5		17.3	19.6	36.0
		17-KS	21.8	27.4	23.8		32.8	31.9	33.5
	P. L.	17-OHCS	18.0	22.5	28.8		6.8	3.2	34.7
		17-KS	26.0	27.6	37.7		21.5	14.5	48/5
Cushing's syndrome (adrenal adenoma)	S. L.	17-OHCS	9.5	23.9	27.0		7.2	13.0	15.0
		17-KS	15.8	33.6	43.6		11.6	19.6	22.5

report, the adrenal responses of 3 normal subjects and 5 patients with Cushing's syndrome were studied after metopirone administration and after metopirone plus a pretreatment for 7 days with 200 mg of chlorpromazine (Table II) or with 2 mg of reserpine (Table II) or with 30 mg of α-2-chlor-9-(-3-dimethylaminopropylidene)-thioantene Taractan.[1] In our cases the modifications of adrenal responsiveness to such a drug were inconsistent (for the period and the dosage used).

IV. CONCLUSIONS

From the results obtained, we can draw the following conclusions: The inhibition of the 11β-hydroxylation with subsequent cortisol synthesis induces a major output of corticotropin, with stimulation of the residual pattern of steroidogenesis.

Tetrahyadro-S, 17-OHCS, and pregnanetriol analyses are the most valuable parameters for the evaluation of the adrenostatic action.

We obtained better results from oral administration than by the intravenous route. More valuable data were obtained from urinary samples than from plasma evaluation.

Normal subjects showed, for the majority of the steroids analyzed, a similar pattern after metopirone administration and after intravenous infusion of 25 U of ACTH for 8 hours.

Metopirone administration to patients with bilateral adrenal hyperplasia and Cushing's syndrome induced, frequently but not always, an increased urinary hormonal output, greater than that observed in normal subjects.

Patients with adrenal adenoma showed an indefinite pattern of responsiveness, either increased or subnormal.

In a Cushing patient suffering from pituitary adenoma, we obtained adrenal hyperresponsiveness from the adrenal blocking agent.

Patients with bilateral adrenal hyperplasia showed lesser values after administration of the blocking agent than after ACTH administration; the reverse was true for a patient with pituitary adenoma.

Patients with Cushing's syndrome showed greater excretion of urinary metabolites in relation to the larger amounts of metopirone administered. A certain proportionality on the dose-response effect, at least in these patients, is suggested.

Furthermore, the possibility is emphasized that the blocking action of high metopirone doses could be specific not only to 11β-hydroxylase, but that other pathways might be involved in the same way. In the adrenogenital syndrome the increased amount of pregnanetriol, 17-KS, and pregnanediol are clearly differentiated from the slightly increased 17-OHCS. Metopirone like exogenous ACTH, shows the same type of

[1]Taractan was obtained from Prodotti Roche S. p. A., Milan.

action due to the enzymatic defect peculiar to this disease. Patients with hirsutism of probable adrenal origin and some cases of Stein—Leventhal syndrome showed increased amounts of 17-KS, in quantities higher than for normal patients, and a particularly augmented pregnanetriol excretion, similar to the exogenous ACTH behavior. These findings add evidence of adrenal involvement in some of these patients.

Metopirone administration did not elicit any particular pattern in patients with hypothyroidism or anorexia nervosa.

The administration of psychotropic drugs did not consistently change responsiveness to the metopirone test. For this reason a definite statement about these drugs must await more experimental work.

The discovery of metopirone has added new emphasis to the study of the control of corticotropin release.

The usefulness of this compound is emphasized both in the diagnostic and the therapeutic fields. More studies are required in order to warrant drawing definite conclusions on the metopirone test.

ACKNOWLEDGMENTS

The author is grateful to Dr. Vittorio Ghetti and Dr. Paolo Lucchelli of the Italian Ciba for the generous gift of metopirone (Su-4885) and to Miss Liduina Botalla for her unfailing help.

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The Effects of Treatment with 19-Norsteroids on the Secretion and Metabolism of Cortisol and of Aldosterone in Women[1]

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I. INTRODUCTION

This report is the result of a study that was primarily designed to explore the effects of Enovid on the secretion and metabolism of cortisol and of aldosterone in women. Enovid consists of 98.5% norethynodrel[2] and 1.5% ethynyl estradiol-3-methyl ether (EE3ME). In addition to its action as a progestin, norethynodrel possesses an intrinsic estrogenic activity, whereas EE3ME is a potent oral estrogen (Pincus *et al.*, 1956; Drill and Saunders, 1957). It seemed likely at the start of our study that any effects of Enovid on the metabolism of cortisol and of aldosterone would be the result of a combination of its estrogenic action with an action resembling that of progesterone. Accordingly, we compared the results obtained in groups of subjects who were taking Enovid or norethynodrel with those of subjects receiving EE3ME and progesterone (Layne *et al.*, 1962). Recently we have obtained some data on aldosterone secretion in subjects receiving estradiol benzoate. Our results, therefore, in addition to contributing to our primary aim of investigating and interpreting the effects of treatment with 19-norsteroids, suggest a number of general conclusions on the effects of estrogens and progestins on the synthesis and metabolism of cortisol and of aldosterone.

II. EFFECTS OF TREATMENT WITH 19-NORSTEROIDS ON CORTISOL METABOLISM

Published observations (Layne *et al.*, 1962) have shown that Enovid, EE3ME, and norethynodrel cause a decreased excretion of the tetrahydro derivatives of cortisol and an increased plasma binding of the

[1]Aided by Grant no. 1 from the American Cancer Society (Massachusetts Division) Inc., and by a grant from Mrs. Stanley McCormick.

[2]17α-Ethynyl-17β-hydroxy-5(10)-estren-3-one.

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hormone. These effects are the same as those known to occur as a result of estrogen treatment (Peterson *et al.*, 1960; Mills *et al.*, 1960; Sandberg and Slaunwhite, 1959), and it seems reasonable to ascribe the effects of these 19-norsteroids on cortisol metabolism to their potency as estrogens Our published results have indicated that:

1. Treatment with Enovid, EE3ME, or norethynodrel produces a decreased excretion of each of the three main tetrchydro derivatives of cortisol, namely tetrahydrocortisone, tetrahydrocortisol and allotetrahydrocortisol. No odifference in the relative amounts of these metabolites was detected between the normal and the steroid-treated subjects. This finding suggests that estrogen decreases the excretion of glucosiduronate metabolites of cortisol by limiting the transport of the hormone rather than by an effect on the enzymes responsible for the formation of the tetrahydro derivatives.

2. In subjects treated with Enovid, EE3ME, or norethynodrel the secretion rate of cortisol, as determined from the specific activity of the urinary tetrahydrocortisone following the administration of a tracer amount of 4-C^{14}-cortisol, was slightly but significantly decreased. This result provides strong direct evidence that in addition to reducing the rate of metabolism of cortisol, estrogen treatment can decrease the adrenal secretion of the hormone.

III. EFFECT OF PROGESTERONE TREATMENT ON CORTISOL METABOLISM

Our previous results (Layne *et al.*, 1962) indicated that oral administration of 300 mg/day of progesterone had no major effects on cortisol secretion or metabolism. There was, however, an indication that the plasma cortisol level in the progesterone-treated subjects was lower than that of the controls. We have now studied a total of seven women receiving progesterone. Plasma cortisol and urinary unconjugated cortisol were determined by the Porter–Silber reaction following partition of the extracts on a Celite column in the Bush B-5 system. The values shown in Table I were corrected by a factor calculated from the recovery

TABLE I

Plasma Cortisol and Urinary Unconjugated Cortisol in Control and in Progesterone-Treated Women

Subjects	Number of subjects	Plasma cortisol (μg/100 ml $\pm$ SE[a])	Urinary unconjugated cortisol (μg/24 hours)
Controls	8	13.7±4.3	88±19.6
Progesterone, 300 mg/day	7	6.4±4.8[b]	137±22.4

[a]Standard deviation.

[b]Significantly different from the controls at 2% level of confidence.

of a tracer amount of tritiated cortisol which was added to the samples of blood or urine.

The figures for plasma cortisol in the group treated with progesterone are significantly lower than those of the control group at a level of confidence of 2%. The average urinary excretion of unconjugated cortisol was considerably higher in the progesterone patients than in the controls, although this result did not attain significance because of the very targe spread in the individual values.

It seems possible that progesterone may exert, either directly or indirectly, an effect on the renal clearance of unconjugated cortisol, which leads to an enhanced excretion of the steroid and a decrease of its concentration in plasma. This effect would not be reflected by a significant decrease in the excretion of metabolites of cortisol conjugated as glucosiduronate, since the ratio of the excretion of the free hormone to that of its glucosiduronate-conjugated metabolites is extremely small (Flood *et al.*, 1961).

IV. EFFECTS OF STEROID TREATMENT ON ALDOSTERONE SECRETION AND METABOLISM

In the patients treated with Enovid, the percentage of an injected tracer dose of 7-H^3-aldosterone, which was excreted as the main chromatographic peak of the glucosiduronate fraction of the urine (Flood *et al.*, 1961), was significantly smaller than the corresponding value for the group of control subjects (Fig. 1). In contrast, the percentage of the injected dose which was excreted as the acid labile conjugate of aldosterone was somewhat increased. Similar alterations of the pattern of metabolism of aldosterone were observed in patients treated with EE3ME. The effects are probably due to the estrogenic potency of the orally administered steroids, and the changes in aldosterone metabolism are essentially similar to (though less pronounced than) those observed in late pregnancy by Jones *et al.* (1959).

In agreement with recent results of other workers (Laidlaw *et al.*, 1962), we have found that progesterone treatment causes a significant increase in the secretion rate of aldosterone. Enovid, EE3ME, and norethynodrel also increased the aldosterone secretion rate as shown in Fig. 2. In the progesterone-treated patients, however, no effect was observed in the pattern of metabolism of aldosterone; the percentage of an injected tracer dose of 7-H^3-aldosterone which was excreted as glucosiduronate and that excreted as the acid-labile conjugate of the hormone were similar in control and in progesterone-treated subjects (Fig. 1).

These results suggest that the action of Enovid and of norethynodrel on the secretion and metabolism of aldosterone may be considered as comprised of two components: (1) an estrogenic effect which causes an increased excretion of acid-labile conjugate and a decreased excretion

of metabolites conjugated as glucosiduronate; (2) an effect resembling that of progesterone, which causes an increased secretion of aldosterone.

The increased secretion of aldosterone caused by treatment with EE3ME is difficult to explain if this compound is considered solely

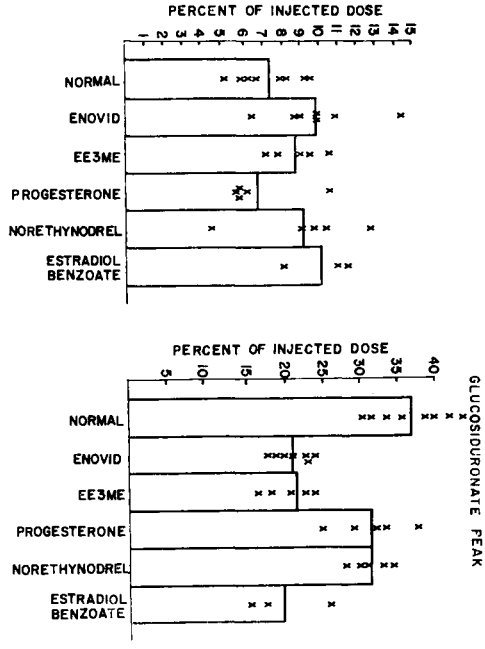

FIG. 1. The effect of oral steroid treatment on the percentage of an injected tracer dose of 7-H^3-aldosterone excreted in the urine as the acid-labile conjugate of the hormone and as the main chromatographic peak of the glucosiduronate fraction.

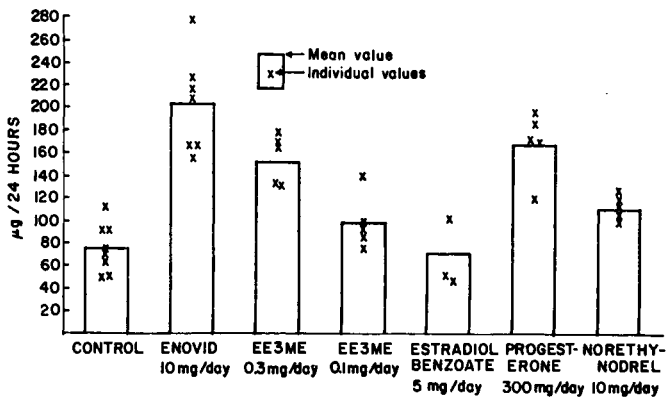

FIG. 2. The effect of oral steroid treatment on the secretion rate of aldosterone in women. Calculated secretion rate of aldosterone (μg/24 hours) based on the specific activity of aldosterone extracted at pH 1.

in the light of its high potency as an estrogen. Preedy and Aitken (1956) found that, in some subjects of a group receiving high doses of estradiol benzoate, a transient decrease in sodium excretion occurred. This suggests that estrogen might depress aldosterone secretion. We have recently determined the secretion rate and examined the urinary metabolites of aldosterone in three women receiving 5 mg/day of estradiol benzoate. The aldosterone secretion rate in these subjects (Fig. 2) was within the normal range, although there were marked effects of the estrogen treatment on the pattern of metabolites of aldosterone (Fig. 1). These results suggest that EE3ME may have, in addition to its action as an estrogen, another effect which resembles that of progesterone in that it increases the secretion rate of aldosterone.

V. THE CLEARANCE RATE OF ALDOSTERONE IN STEROID-TREATED WOMEN

Since the binding of aldosterone by the plasma of women treated with Enovid or with EE3ME was somewhat elevated as compared to that of the plasma of control subjects (Meyer *et al.*, 1961; Layne *et al.*, 1962) it became of interest to compare the clearance rate of aldosterone in treated subjects with that of controls. The clearance rates in 5 subjects receiving 0.3 mg/day of EE3ME were determined in collaboration with Dr. J. F. Tait and his group by the method described by Tait *et al.* (1961), following a single injection of 7-H^3-aldosterone. The results are shown in Table II. No significant difference was observed between the

TABLE II

The Metabolic Clearance Rate and Plasma Protein Binding of Aldosterone in Women Treated with Ethynyl Estradiol-3-Methyl Ether As Compared to Values in Control Subjects

Subjects	Number of subjects	Metabolic clearance rate of aldosterone (liter of plasma/day)	Percentage binding of tracers 7-H^3-aldosterone by 3 ml of plasma[a]
Control subjects	6	1743 mean (1340–2220) range	18 mean 9—23 range
Subjects receiving orally 0.3 mg EE3ME/day	5	1725 mean (629–2195) range	40 mean 30–44 range

[a]Determined after equilibrium dialysis at 37°C against 5% human serum albumin.

clearance rates of the control subjects and those receiving EE3ME, nor was there any apparent correlation between the metabolic clearance rate and the plasma protein-binding capacity in the treated subjects. This suggests that any effect of increased protein binding of aldosterone, which would tend to lower the clearance rate of the hormone, is counterbalanced by other effects in the subjects receiving steroids. Tait *et al.*

(1962), in another communication in this volume, emphasized that the rate of hepatic blood flow and the efficiency of hepatic metabolism, as well as any extrahepatic clearance, are factors that might influence the metabolic clearance rate of steroids. We have no information on the effect of the treatment of our subjects with EE3ME on the hepatic blood flow or on the metabolism of aldosterone in the liver or other tissues.

VI. SUMMARY AND CONCLUSIONS

Enovid, norethynodrel, and EE3ME, when administered orally to women, produced effects on the metabolism of cortisol similar to those known to occur after treatment with estrogen. These effects were a raised plasma level and increased plasma protein binding of cortisol, with a decreased urinary excretion of tetrahydrocortisone, tetrahydrocortisol, and allotetrahydrocortisol. The secretion rate of cortisol was decreased in the subjects receiving each of the synthetic steroids. Treatment with progesterone was found to decrease the plasma level of cortisol and to cause some increase in the urinary excretion of unconjugated cortisol. It is concluded that the effects of Enovid, norethynodrel, and EE3ME on cortisol secretion and metabolism are due to their estrogenic action.

Treatment with Enovid or with EE3ME reduced the percentage of an injected tracer dose of aldosterone excreted as glucosiduronate and tended to increase the amount excreted as the acid-labile conjugate of the hormone. These results are similar to those found in women treated with estradiol benzoate. Enovid, norethynodrel, and higher doses of EE3ME also increased the secretion rate of aldosterone in a manner similar to that found in subjects receiving progesterone. Although the plasma protein binding of aldosterone was significantly increased in women treated with EE3ME, the metabolic clearance rate of aldosterone in these patients was similar to that of controls. The effects of the synthetic 19-norsteroids on aldosterone secretion and metabolism may be interpreted as a combination of their estrogenic action with an effect resembling that of progesterone.

ACKNOWLEDGMENTS

The author is indebted to Dr. J. F. Tait, Mrs. S. A. S. Tait, and Dr. G. Pincus for much discussion and advice; to Dr. E. Wallach and Dr. B. Little for obtaining the plasma samples; and to Mrs. L. E. Cushman for supervision of the patients. Technical assistance was provided by Mr. L. E. Bellaud and Mr. F. DeFeudis.

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DISCUSSION

Interaction of Steroid Hormones

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I. Estrogen effects on cortisol metabolism

The administration of estrogen has two effects on cortisol metabolism: one effect is to increase the protein binding of the steroid in the plasma; the other is to reduce the excretion of glucuronide metabolites in the urine. Part of this reduction is due to the lower secretion of cortisol (Peterson, 1959; Layne *et al.*, 1962), but part is due to an alteration in the metabolism of cortisol so that the percentage of administered C^{14}-labeled cortisol which is excreted as glucuronide is decreased by treatment with estrogen.

The time relations of the two effects of estrogen have not so far been made clear. The rise in protein-bound cortisol is apparent within 3 days (Mills *et al.*, 1960), but change in the excretion of metabolites has not begun to take place at the end of 5 days (Table I). It seems most probable that the effect on protein binding and that on metabolism are separate processes.

Although it is unlikely that the lowered secretion rate of cortisol during estrogen therapy is itself responsible for the altered metabolism, it is of interest to see that the same effects are manifest during continuous replacement therapy. In Fig. 1, the percentage of cortisone which was excreted as 17-ketogenic steroids is plotted for a patient with hypopituitarism maintained on 50 mg per day of oral cortisone. The effect of 0.2 mg of ethynyl estradiol per day was to reduce the percentage excretion progres-

TABLE I

The Effect of Ethynylestradiol (0.5 mg Twice Daily) on Metabolism of Cortisone (100 mg Once Daily) in a Patient with Hipopituitarism

Treatment	Plasma cortisol (μg/100 ml plasma)		Urinary 17-ketogenic steroids
	10 A.M.	Noon	(mg per day)
Control	3.2	37	45
	4.2	37	60
	3.4	34	53
Estrogen	—	—	54
	—	—	51
	9.1	65	56
	11.1	68	57
	10.8	38	51

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sively over 4 months. Toward the end of the estrogen treatment, the free cortisol plus cortisone in the urine rose, as Robertson *et al.* (1959) have shown.

In Fig. 2 similar results are shown for a patient with the adrenogenital syndrome maintained on 37.5 mg of cortisone per day. Ethynylestradiol in a dosage of 1 mg per day reduced the percentage recovery of steroid as 17-ketogenic steroids in the urine

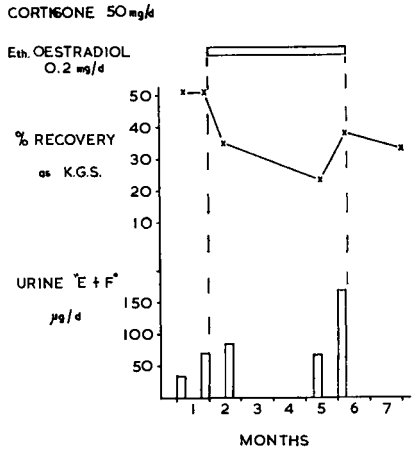

FIG. 1. The effect of ethynylestradiol (0.2 mg/day) on the percentage of administered cortisone excreted as 17-ketogenic steroids (KGS) in the urine and on the urinary free cortisol plus cortisone in a patient with hypopituitarism (female, aged 44) maintained on 50 mg of oral cortisone per day.

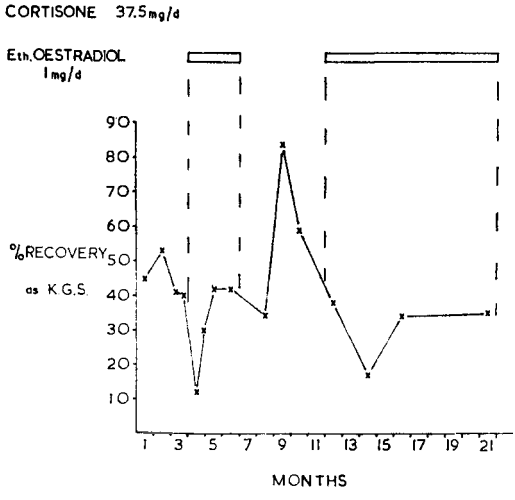

FIG. 2. The effect of ethynylestradiol (1.0 mg/day) on the percentage of administered cortisone excreted as 17-ketogenic steroids (KGS) in the urine in a patient with congenital adrenal hyperplasia (female, aged 15) maintained on 37.5 mg of oral cortisone per day.

to 12%, but over the next 3 months the percentage rose to approximately that of the control period. Two months after the estrogen was stopped there was an overshoot so that 84% of administered cortisone was excreted in the urine as 17-ketogenic steroid. A second course of estrogen was similar in effect to the first.

The effect of estrogen on the metabolism of cortisol appears to have a very long time course, but there is a tendency for the effect to be antagonized with prolonged estrogen treatment.

II. PROGESTERONE EFFECT ON CORTISOL METABOLISM

The data presented by Dr. Layne certainly show that progesterone increases the renal clearance of free cortisol by a factor of about 3.3, but since the urine-free cortisol represents about 0.6% of thedaily secretion, increasing it to 0 9% of the daily secretion could not possibly reduce the plasma level to one-half. This suggests that progesterone alters metabolism either by increasing destruction by the liver or by increasing uptake by the tissues.

III. ALDOSTERONE SECRETION

The effect of the hormones other than estradiol benzoate on aldosterone secretion is very interesting, but it is not possible to conclude that the hormones other than progesterone have their effect by virtue of progesterone-like activity. The factors that control the secretion of aldosterone are very complex. Progesterone probably acts as an aldosterone antagonist, as suggested by the work of Landau and Lugibihl (1958). The other hormones could have their effect by acting directly on the adrenal as angiotensin does (although this seems unlikely) or by altering the sodium balance by interfering with the nonaldosterone hormone mechanism described by de Wardener *et al.* (1961). Until details are available of the effect of these hormones on sodium and potassium balance, it is not possible to say which of these mechanisms is most likely.

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Action of Antialdosterone Compounds in the Laboratory

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I. INTRODUCTION

The important role of aldosterone in the regulation of electrolyte metabolism has been recognized for many years (Gaunt *et al.*, 1955; Ross, 1959). In adrenal insufficiency, absence of the hormone leads to renal loss of sodium and retention of potassium ions, consequent to a disturbance of tubular reabsorptive mechanisms in the kidney. Contrastingly, a condition of hyperaldosteronism creates reverse electrolyte balances, similarly disturbing, and is frequently associated with the production and maintenance of clinical edema (Bartter, 1956; Gaunt *et al.*, 1955; Laragh, 1956; Lieberman, 1958; Luetscher and Lieberman, 1958). As a means of controlling the manifestations of hyperaldosteronism, therefore, a laboratory search was undertaken for pharmacological agents with antialdosterone properties.

First examples of synthetic antagonists with the ability to reverse the renal electrolyte effects of aldosterone in both laboratory studies (Kagawa, 1960a; Kagawa *et al.*, 1957, 1959; Liddle, 1957) and man (Liddle, 1957, 1958; Salassa *et al.*, 1958) were found in steroidal spirolactones (Cella and Kagawa, 1957; Cella and Tweit, 1959) (Fig. 1). Spironolactone or Aldactone[1] has been the most prominent of the antagonists, so far,

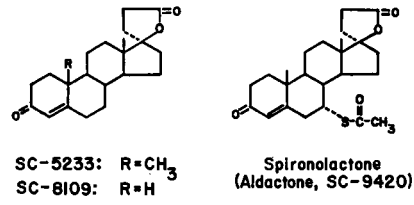

FIG. 1. SC-5233 = 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl)propanoic acid lactone; SC-8109 = 3-(3-oxo-17β-hydroxy-19-nor-4-androsten-17α-yl)propanoic acid lactone; spironolactone = 3-(3-oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl)propanoic acid lactone.

[1]Trademark of G. D. Searle and Company for brand of spironolactone.

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because of its application therapeutically in edema associated with hyperaldosteronism (Gantt, 1961; Streeten, 1961). Both SC-5233 and SC-8109, as prototypes of spironolactone, were relatively more effective, on the basis of dosage, by injection than orally (Kagawa *et al.*, 1959; Liddle, 1958). The present report will discuss interaction of these steroidal spirolactones with mineralocorticoids in the laboratory, primarily with the view of describing pharmacodynamic characteristics of the spirolactones.

II. SPECIFIC AND PHYSIOLOGICAL ANTAGONISM OF MINERALOCORTICOIDS WITH STEROIDAL SPIROLACTONES

Table I demonstrates the action of spironolactone in modifying the

TABLE I

Action of Spironolactone in Reversing Effects of d-Aldosterone-21-acetate and DCA on Urinary Sodium and Potassium in Adrenalectomized Rats

Treatment per animal		Mean urinary response		
Mineralocorticoid	Spironolactone	Na[a]	K[a]	(Na × 10) : K
—	—	1.33	0.90	1.43
0.17 μg Aldosterone	—	0.93	1.08	0.85
0.17 μg Aldosterone	0.25 mg	1.07[b]	0.98	1.10[b]
0.17 μg Aldosterone	1.0 mg	1.29[b]	1.01	1.29[b]
12 μg DCA	—	1.03	1.08	0.96
12 μg DCA	0.25 mg	1.19[b]	1.06	1.13[b]
12 μg DCA	1.0 mg	1.25[b]	1.00	1.25[b]

[a]Log milliequivalents per liter after dilution of sample from 4 rats to 50 ml.

[b]$P < 0.05$ relative to respective mineralocorticoid-treated controls; least change for P of $0.05 = \pm 0.13 \pm 0.11$ and ± 0.12 for Na' K, and (Na × 10): K, respectively; 24 animals per treatment; all treatment was by subcutaneous injection.

urinary effects of mineralocorticoids in 4-hour tests using adrenalectomized rats (Kagawa, 1960a). Injection of *d*-aldosterone-21-acetate alone induced the retention of sodium and loss of potassium ions relative to untreated controls, and accordingly a strong reduction in the ratio of urinary sodium : potassium (Na : K)[2]; all changes were significant compared to the controls ($P < 0.05$). Simultaneous injection of spironolactone inhibited the effects of aldosterone, with the degree of relative change most pronounced for the Na : K index compared to the

[2]Transformation of the responses to logs provided homogeneous variance with different treatments and gave, thereby, statistically useful indexes for evaluation of the data; a change by 0.30 log units involving the comparisons represents a twofold deviation in the basic urinary measurements.

reversal of sodium retention or potassium loss. A similar pattern of electrolyte effects was also evident with deoxycorticosterone acetate (DCA), given at a dose equivalent in mineralocorticoid potency to that of aldosterone, and with the combination of spironolactone and DCA. In general, these data demonstrate a progressive blockade of mineralocorticoid effects with increasing doses of spironolactone, a dose-response relationship also found for other spirolactones (Kagawa, 1960b; Kagawa *et al.*, 1957, 1959).

As the studies on mineralocorticoid antagonism involved the combination of compounds, it was important to question the effects of the spirolactones when given in the absence of mineralocorticoids. Figure 2 demonstrates the action of SC-5233 and spironolactone injected subcutaneously over a wide range of doses (0.0156–16 mg) on urinary electrolytes in a similar 4-hour test using adrenalectomized animals. Significant mineralocorticoid-like changes relative to untreated controls ($P < 0.05$) were found following administration of the 0.25-mg dose of spironolactone (sodium retention) and 0.0156 mg of SC-5233 (potassium loss); however, there was no distinct pattern of response which could be related to dosage (cf. data of 6- and 18-μg dose of DCA). A retention of potassium ions which would oppose mineralocorticoid effects was obtained with the 16-mg dose of spironolactone ($P < 0.05$), but the ratio of Na : K remained unaltered because of a similar shift of the sodium response. The data demonstrated absence of a consistent change of urinary response that would suggest properties opposite to those of mineralocorticoids withSC-5233 and spiron olactone,. Interpretation of these data has suggested that the spirolactones are *specific antagonists* of mineralocorticoids (Clark, 1933, 1937), requiring the presence of mineralocorticoids for electrolyte manifestation (Kagawa, 1960a; Kagawa *et al.*, 1957; Liddle, 1957, 1958; Salassa *et al.*, 1958).

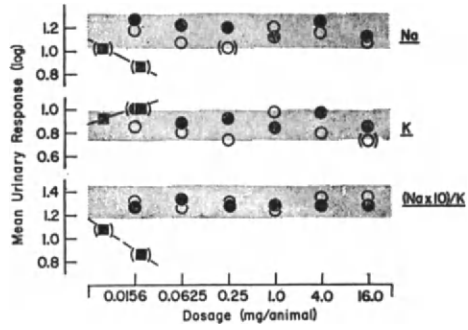

FIG. 2. Renal electrolyte effects following the subcutaneous injection of SC-5233(●) and spironolactone (○) in a drenalectomized rats without mineralocorticoid treatment; shaded areas describe limits for least significant change relative to untreated controls ($P < 0.05$ for responses enclosed in parentheses); 12 animals per treatment; 24 animals for untreated controls; DCA (■) was given at 6- and 18-μg doses in order to obtain reference data.

In the laboratory, the steroidal spirolactones can be differentiated from *physiological antagonists* (Clark, 1933, 1937), which would reverse

action of mineralocorticoids by nonspecific diuretic effects or by mutual antagonism. Investigations demonstrate that the mechanism of renal effects with 2,4,7-triamino-6-phenylpteridine or SKF-8542, a recently described nonsteroid antialdosterone agent (Crosby *et al.*, 1961; Laragh *et al.*, 1961; Wiebelhaus *et al.*, 1961a,b), is by physiological antagonism. In the laboratory, the pteridine is highly effective in reversing both the sodium-retaining and potassium-dissipating actions of mineralocorticoids. However, in the absence of mineralocorticoids, SKF-8542 caused sodium loss and potassium retention relative to untreated adrenalectomized animals (Table II), to show thereby, a unique combination of

TABLE II

Comparative Effeacs of SKF-8542 and Spironolactone Given Subcutaneously on Urinary Excretion of Sodium and Potassium in Adrenalectomized Rats without Mineralocorticoids

Treatment per animal		Mean urinary response		
Compound	Dosage (mg)	Na[a]	K[a]	(Na × 10):K
—	—	1.28	0.93	1.35
SKF-8542	0.03	1.25	0.67[b]	1.58[b]
	0.3	1.47[b]	0.63[b]	1.88[b]
	3	1.67[b]	0.67[b]	2.00[b]
Spironolactone	0.03	1.22	0.87	1.34
	0.3	1.30	0.90	1.40
	3	1.20	0.86	1.34

[a]Log milliequivalents/liter after dilution of sample from 4 rats to 50 ml.

[b]$P < 0.05$ relative to untreated controls; 16 animals for controls and 12 animals per treatment.

intrinsic electrolyte effects which would oppose mineralocorticoid action. The ratio Na : K was increased in accordance with changes in output of the respective ions. These data demonstrate clearly mineralocorticoid-independent effects of the pteridine, to show good agreement with the conclusion of other studies (Herker and Senft, 1961; Liddle, 1961). Contrastingly, spironolactone injected at similar doses produced nonsignificant changes in excretion of the ions ($P > 0.1$). A similar distinction of electrolyte effects has also been noted between the spirolac tones and thiazides, *s*-triazines, aminouracils, acetazolamide, and other diuretic agents which reverse the sodium and/or potassium response to mineralocorticoids nonspecifically by physiological antagonism.

III. Evidence for Competitive Interaction between Spirolactones and Mineralocorticoids

Studies were also undertaken to describe the nature of pharmacodynamic interaction with mineralocorticoids and steroidal spirolactones in adrenalectomized animals. Figure 3 demonstrates data on action of spironolactone in the presence of variable dosage of mineralocorticoids.

Spironolactone given at two different dosage levels with DCA (spironolactone : DCA = 0.48 mg : 12 μg and 7.68 mg : 192 μg) demonstrated a strong blocking response relative to DCA alone. Increasing the dosage of DCA, without change in the dose of spironolactone, progres-

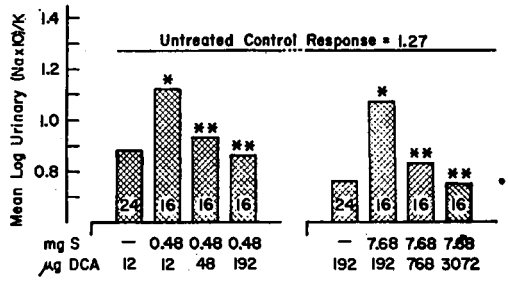

FIG. 3. Reversible inhibition of spironolactone (S) action on urinary Na : K ratio with increasing doses of DCA in adrenalectomized rats; $*P < 0.05$ relative to DCA alone; $**P < 0.05$ relative to DCA plus S; figures in bars indicate number of animals; all treatment was by subcutaneous injection.

sively inhibited the blocking response. Interpolation suggested that approximately ten- and twelvefold increments in dosage of DCA were required for complete neutralization of the blocking response obtained with 0.48 and 7.68 mg of spironolactone, respectively. Essentially similar quantitative data were obtained in other studies with the test of 0.48 mg of spironolactone and *d*-aldosterone-21-acetate, given at doses equivalent to 12, 48, and 192 μg of DCA. These data demonstrate, therefore, ability of mineralocorticoids to inhibit reversibly the blocking response of spironolactone, a fact also made apparent in other studies with SC-5233 (Kagawa *et al.*, 1957, 1959; Liddle, 1958, 1961), and suggest the importance of the dosage rati of spironolactone : mineralocorticoid for renal effects.

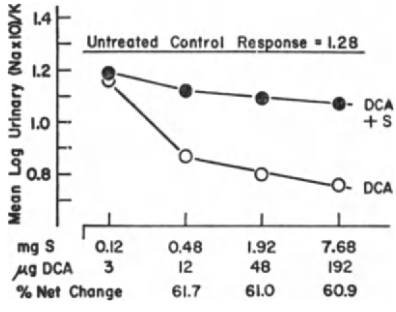

FIG. 4. Urinary Na : K response following treatment with DCA alone or in combination with spironolactone (S) given at a constant dosage ratio (S : DCA = 40 : 1) over a wide range of doses in adrenalectomized rats; 16–24 animals per treatment; least significant change (P of 0.05) between treatments = ± 0.14; the "per cent net change" of DCA response induced by S is shown for each dosage combination, except for the combination with 3 μg of DCA.

In another study, spironolactone was tested in combination with DCA at a constant dosage ratio over a wide range of doses in adrenalectomized rats (Fig. 4), to in-

quire further about their pharmacodynamic interaction. Given alone, DCA produced a strong reduction in the ratio of urinary Na : K at doses of 12, 48, and 192 μg subcutaneously; the simultaneous administration of spironolactone, at a dosage ratio of 40 : 1 for spironolactone : DCA, reversed the response at each dosage level of DCA. In comparison to the action of DCA alone (difference of Na : K response between untreated controls and DCA), spironolactone produced a net change of approximately 60% at each level of dosage combination with DCA, or a relatively constant degree of inhibition which was proportional to the action of DCA.[3] If it is assumed that the responses represent interaction at the responding tissue for electrolyte manifestation, the data provide remarkable support for the idea of mass law effects with spironolactone and mineralocorticoids. No significant changes occurred with either the 3-μg dose of DCA or the combined treatment with 0.12 mg of spironolactone. However, as it is not known to what extent factors related to absorption, transport, excretion, or breakdown of the compounds may have contributed to the observed urinary effects under *in vivo* conditions of the test (Gaddum, 1957), more studies with other dosage combinations will be necessary to describe the quantitative nature of interaction with the antagonist and mineralocorticoid.

IV. MINERALOCORTICOID PROPERTIES OF ANTAGONISTS

Other tests have provided additional information on the biological properties of spirolactones as mineralocorticoid antagonists. One interesting feature was made apparent with the test of SC-8109, the 19-nor

TABLE III

Effects of SC-8109 and Progesterone Given in the Absence of Mineralocorticoids on Urinary Sodium and Potassium Output in Adrenalectomized Rats

Treatment per animal		Number of measurements	Mean urinary response		
Compound	Dose (mg)		Na[a]	K[a]	(Na × 10):K
—	—	6	1.17	0.86	1.31
SC-8109	4	3	1.11	0.91	1.20
SC-8109	16	3	1.03[b]	0.98[b]	1.05[b]
—	—	4	1.26	0.98	1.28
Progesterone	3.2	3	1.23	1.11[b]	1.11[b]
Progesterone	20	3	0.96[b]	1.06	0.90[b]

[a]Log milliequivalents per liter after dilution of sample from 4 animals to 50 ml.

[b]$P < 0.05$ relative to respective untreated controls; 4 animals per measurement; treatment was by subcutaneous injection.

[3] Preference was given to an evaluation based on "per cent net change" to incorporate the progressively decreasing pattern of urinary Na : K response obtained with increasing doses of DCA or the combinations of DCA and spironolactone.

steroidal spirolactone, when given alone at relatively large doses in adrenalectomized animals (Table III). In comparison with untreated controls, SC-8109 at a 16-mg dose subcutaneously produced significant mineralocorticoid-like effects by inducing sodium retention, potassium loss, and a reduction of Na : K in the urine. Comparative tests indicated a potency of 0.5% for SC-8109 (95% confidence limits = 0.02 to 0.13) relative to DCA using the Na : K response as a criterion of mineralocorticoid activity, to confirm other laboratory data following parenteral use of the compound (Kagawa, 1960c; Slater *et al.*, 1959). The data for progesterone emphasize the association of mineralocorticoid properties in still another example of a mineralocorticoid antagonist (Kagawa, 1958; Landau *et al.*, 1955). Thus, in two dissimilar structures, we find examples of compounds which inhibit and, at relatively large doses, mimic the action of mineralocorticoids. This fact may be interpreted as evidence also for ability of the spirolactone and progesterone to occupy the receptor sites of mineralocorticoids.

Table IV summarizes the renal electrolyte properties of steroidal

TABLE IV

Relative Effective Doses of Spirolactones and Various Steroids for a Standard Mineralocorticoid and Antimineralocorticoid Response in Adrenalectomized Rats

Compound	Effective dose[a]		Ratio A:B
	DCA-like (A)	Anti-DCA (B)	
SC-8109	11.3	0.08	141
SC-5233	>19.2	0.22	> 87
Spironolactone	>16.0	0.33	> 49
Progesterone	12.0	1.2	10
Compound S	0.06	(0.0012-0.03)[b]	—
18-OH-DCA	0.12	(0.0003-0.53)[b]	—

[a]Values represent total dosage in milligrams per animal subcutaneously; A=dose equivalent to 6 μg of DCA; B=dose required for 50% block of 12 μg of DCA, using the Na:K response.

[b]Tests revealed no anti-DCA effects over the given range of dosage.

spirolactones, progesterone, and other steroids, in terms of the dose required for a standard mineralocorticoid (A) and antimineralocorticoid (B) response. In the case of SC-8109, the ratio of effective doses (A : B) was 11.3 mg/0.08 mg, or 141; corresponding measurements for progesterone gave a dosage ratio of 12 mg/1.2 mg, or 10, which would suggest a relatively narrow range of effective doses for mineralocorticoid antagonism. Dosage for the standard mineralocorticoid response was indeterminate for SC-5233 and spironolactone, the available data suggesting respective A : B dosage ratios of > 19.2 mg/0.22 mg and > 16 mg/0.33 mg for the two spirolactones. 17,21-Dihydroxypregn-4-ene-3,20-dione (Compound S) and 20-hydroxy-21-acetoxy-

18,20-epoxypregn-4-en-3-one (18-OH-DCA), both relatively weak mineralocorticoids compared to DCA aldosterone, failed to demonstrate anti-DCA properties at dosage levels below those required for mineralocorticoid effects. Thus, there is no *a priori* relationship that a weak mineralocorticoid will antagonize the urinary electrolyte effects of a relatively more potent mineralocorticoid.[4] The data indicate that certain antagonists (i.e., SC-8109 and progesterone) may be "partial antagonists" (Stephenson, 1956) or weak mineralocorticoids with a relatively strong affinity for receptor sites of mineralocorticoids, a circumstance not altogether uncommon in study with nonsteroids in other areas of pharmacology (Ariëns *et al.*, 1955; Stephenson, 1956).

V. NONSELECTIVE MINERALOCORTICOID ANTAGONISM WITH SPIROLACTONES

Early studies confirmed the fact qualitatively that the spirolactones as DCA antagonists were also effective in inhibiting the renal electrolyte properties of aldosterone (Kagawa, 1960a,b; Kagawa *et al.*, 1957, 1959), the presumed major mineralocorticoid secreted by the adrenal glands (Gaunt *et al.*, 1955; Ross, 1959). To obtain quantitative data, therefore, a study was undertaken to describe the comparative efficacies of antagonists, using DCA or aldosterone as the standard mineralocorticoid. Table V lists the median effective dose (MED, the dose for

TABLE V

Relative Effective Doses of Various Antagonists Using DCA or d-Aldosterone-21-Acetate as the Reference Mineralocorticoid in Adrenalectomized Rats

Antagonist	MED (95% limits)	
	DCA	Aldosterone
Spironolactone	0.33	0.33
SC-8109	0.19 (0.07–0.50)	0.23 (0.07–0.75)
SC-10112	0.91 (0.28–2.97)	0.56 (0.21–1.47)
Adrostenedione	5.10 (1.94–13.40)	4.20 (1.60–11.00)

[a]MED = dosage in milligrams per animal subcutaneously for 50% block of the urinary Na:K response to 12 μg of DCA.

50% inhibition of the urinary Na : K response to a standard dose of DCA) for spironolactone, SC-8109, 3-(3β,17β-dihydroxy-4-androsten-17α-yl)propanoic acid lactone (SC-10112) and 4-androsten-3,17-dione (androstenedione), all presumably specific antagonists of mineralocorticoids. The MED for each compound was determined by interpola-

[4]DCA given at doses submaximal for mineralocorticoid effects (0.12–3 μg doses) also failed to inhibit the electrolyte effects of the relatively more potent mineralocorticoid *d*-aldosterone-21-acetate given at a 0.17μg dose.

tion with dose-response data, using the reference response to 0.33 mg of spironolactone. As noted by the comparative rank of MED's, SC-8109 proved to be the most effective antagonist using DCA as the mineralocorticoid, followed in decreasing order by SC-10112 and androstenedione. Against an equivalent dose of *d*-aldosterone-21-acetate, the rank of MED values paralleled those observed with DCA qualitatively; in addition, there was no statistical evidence for quantitative disagreement of the MED values using either of the mineralocorticoids in the evaluation of SC-8109, SC-10112, and androstenedione.[5] Practically, these data would suggest that the search for specific antagonists of aldosterone in the laboratory may be undertaken with DCA as the reference mineralocorticoid, without important loss of accuracy in ranking the antagonists. The data also provide some indirect evidence against the idea of *chemical antagonism* (Clark, 1933, 1937), wherein formation of a pharmacologically inert complex depends on the interaction of specific reactive structures, as inhibition with the antagonists was not dependent on the mineralocorticoid employed for tests.

Table VI provides a list of the various mineralocorticoids which

TABLE VI

List of Mineralocorticoids Inhibited by Steroidal Spirolactones in Adrenalectomized Rats

Mineralocorticoid	Steroidal spirolactone		
	SC-5233	SC-8109	Spironolactone
DCA	+	+	+
Aldosterone	+	+	+
Cortisol	+	+	+
Corticosterone	+	+	+
Compound S	+	+	+
19-Nor-DCA	+	0	0
21-Deoxy-9α-F-cortisone	+	0	0
18-OH-DCA	+	+	+
9α-F-Cortisol acetate	+	+	+

[a] + = Inhibition based on reversal of the Na:K response to the mineralocorticoid; 0 = data nonavailable; all treatment was by subcutaneous injection.

have been inhibited by SC-5233, SC-8109, and spironolactone. Blocking activity was measured by ability of the spirolactones to reverse the Na : K response to mineralocorticoids. The data demonstrate that the spirolactones have been effective against a variety of structures, including cortisol, corticosterone, and Compound S, all of which represent natural corticosteroids and, additionally, synthetic mineralo-

[5] Analysis showed a variance ratio of $F = 0.004$ ($P > 0.05$) in the comparison of the MED differences (2 D. F.) against the pooled estimate of error (48 D. F.), to indicate a close quantitative agreement of MED ratios for the compounds using DCA or aldosterone.

corticoids, 21-acetoxy-19-norpregn-4-ene-3,20-dione (19-nor-DCA), 9α-fluoro-17-hydroxypregn-4-ene-3,11,20-trione (21-deoxy-9α-F-cortisone), 18-OH-DCA, and 21-acetoxy-9α-fluoro-11β,17-dihydroxypregn-4-ene-3,20-dione (9α-F-cortisol acetate). The fact that spirolactones nonselectively inhibit structurally diversified mineralocorticoids negates in part, the view that their interaction involves chemical antagonism. In addition, the likelihood of chemical interaction with spirolactones and mineralocorticoids is believed to be small as judged further by absence of inhibitory interaction *in vitro* (Kumar and Sheth, 1961) and by ability of spirolactones to reverse mineralocorticoid-like, but not other, manifestations of corticosteroids (Kagawa, 1960a; Kagawa *et al.*, 1959).

VI. DISCUSSION

Our laboratory studies indicate that the influence of SC-5233, SC-8109, and spironolactone on renal electrolyte excretion results from specific antagonism of mineralocorticoid effects. The primary evidence for such a concept resides in the fact that the spirolactones require the presence of aldosterone or other mineralocorticoids for electrolyte manifestations. Given alone in the absence of mineralocorticoids, the spirolactones failed to demonstrate properties mutually antagonistic to those of mineralocorticoids, ruling out physiological antagonism as a possible mechanism of their effects. Other studies indicate that the magnitude of electrolyte change depends on the dosage ratio of spirolactones : mineralocorticoids; these data provide strong support for the idea that antagonism with the spirolactones is pharmacodynamically by competitive interaction with mineralocorticoids for receptor sites in target tissues. Chemical antagonism is believed to be unlikely as a possible mechanism of effect, as the spirolactones nonselectively reverse electrolyte effects of both natural and synthetic mineralocorticoids of diverse structures. With appropriate laboratory tests, therefore, the nomenclature of Clark (1933, 1937) has afforded a convenient means to classify antagonists into broad categories according to their pharmacodynamic properties. Its application more generally (Wells, 1958) will make it possible to distinguish between various mineralocorticoid antagonists with known pharmacodynamic differences.

The target tissue for action of the spirolactones is believed to be at the kidney level, the generally accepted site of action for urinary effects of mineralocorticoids (Roemmelt *et al.*, 1949; Murphy *et al.*, 1952; Barger *et al.*, 1958). Experiments with the technique of stop-flow analysis in dogs have localized a distal tubular site of action for the 19-nor steroidal spirolactone SC-8109 (Vander *et al.*, 1960), and for aldosterone (Vander *et al.*, 1958), providing further evidence for action of spirolactones in the kidneys. Whether the spirolactones will operate by specific antagonism of mineralocorticoids extrarenally (Crabbé, 1960;

Ducommun *et al.*, 1960; Henley *et al.*, 1960; Kagawa *et al.*, 1959; Selye, 1960; Sleisenger *et al.*, 1960; Sturtevant, 1958), than at the kidney level alone, remains to be seen.

In general, clinical pharmacology has verified the laboratory evidence that the renal electrolyte properties of steroidal spirolactones result from specific antagonism of mineralocorticoids (Liddle, 1957, 1958, 1961; Salassa *et al.*, 1958). Moreover, there is no clinical evidence that the action of spirolactones is dependent upon changes in secretion, metabolism or excretion of mineralocorticoids (Davidson *et al.*, 1961). As specific mineralocorticoid antagonists, therefore, the spirolactones provide a pharmacological means for the diagnosis and control of clinical conditions associated with hyperaldosteronism (Gantt, 1961; Biglieri *et al.*, 1961; Higgins and O'Sullivan, 1960; North, 1961; Streeten, 1961).

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DISCUSSION

Aldosterone Antagonism and Aldosterone Inhibition

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There is no doubt that the spironolactones provide a very useful and effective pharmacological tool, and in practical medicine they have found wide application in refractory edema. However, although they compete with mineralocorticoids in the target

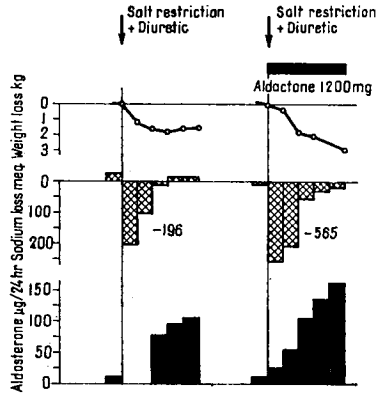

Fig. 1. Comparison of salt restriction with and without Aldactone. Note the increased aldosteronuria.

tissues, such as kidney, salivary gland, and sweat glands, they sometimes increase secondarily the production by the adrenals of the mineralocorticoids, i.e., of aldosterone. Figure 1 shows a balance study in a normal subject, salt restriction and the administration of a diuretic produce weight loss, sodium loss, and hyperaldosteronuria. If we repeat the same experiment in the same subject, giving Aldactone in addition, the weight loss and sodium loss is, as expected, greater, but the aldosteronuria is also greater.

Therefore, the ideal choice in edema therapy is not aldosterone antagonism, but aldosterone inhibition. So far, the most selective inhibitor of aldosterone is the semisynthetic heparinoid Roche, Ro-1-8307. Its action is somewhat slow and to my knowledge its mode of action is still unknown [Scalatmann *et al.*, (1960); Cejka *et al.*, (1960); Veyrat *et al.*, (1962)].

Figure 2 illustrates a study in a normal subject. Both salt restriction and the administration of a diuretic acted as potent aldosterone stimuli, sodium was actively retained in the urine, and the concentration of sweat sodium fell. After an initial drop, the weight

[1]Supported by a research grant from the Swiss National Foundation for research.

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stabilized. Then without change in the diet, the inhibitor was given for 8 days. The urinary aldosterone values came down progressively from 138 to 11 μg/24 hours; the increase in urinary and sweat sodium, as well as the drop in weight, indicate that this decreased aldosteronuria corresponds, in fact, to a diminished aldosterone production by the gland.

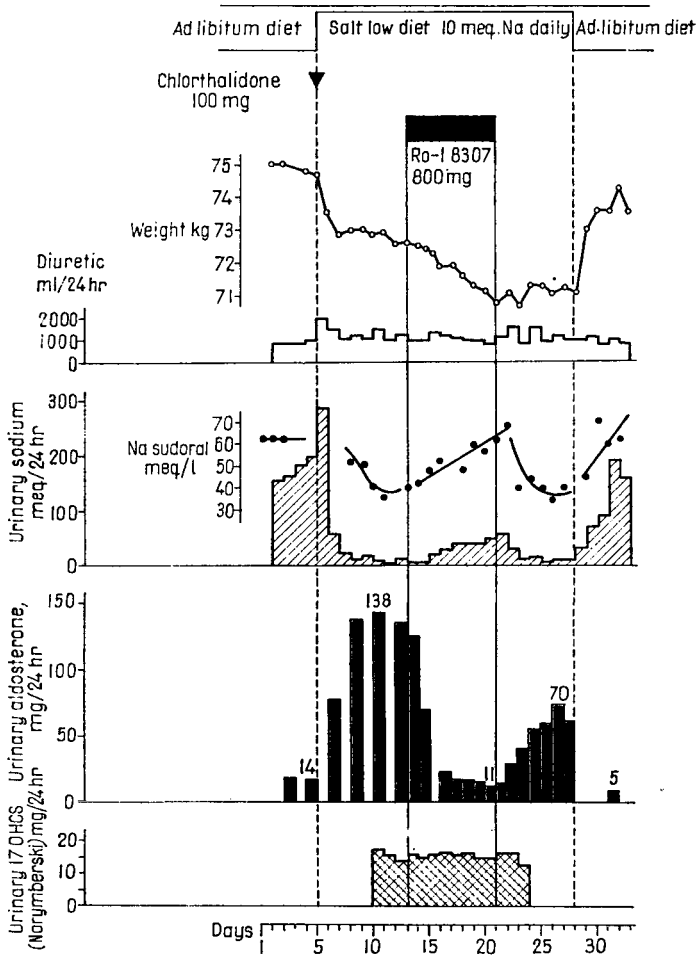

FIG. 2. Aldosterone inhibition in a normal 23-year-old male (5. H.) with a semisynthetic heparinoid Ro-1-8307 (800 mg per day intramuscularly).

As soon as the drug was stopped, aldosteronuria increased. Simultaneously, sodium was again retained in urine and sweat, and the weight also stabilized.

Throughout this study, the 17-ketogenic steroids measured in the urine by the method of Norymberski showed no change.

If in this experiment it is the increased aldosterone production that was inhibited by the heparinoid, the opposite situation can also occur, namely the prevention of the obligatory increased aldosterone production after salt restriction in the diet.

In clinical studies of refractory edema it could be confirmed that the heparinoid Ro-1-8307 is a potent diuretic agent, especially in combination with the usual diuretics.

The next substance, which might turn out to be a potential selective aldosterone inhibitor, has so far been used only *in vitro* and in animal experiments. It is the tetralone Su-9055, which has been considered a 17α-hydroxylase inhibitor (Chart *et al.*,1962).

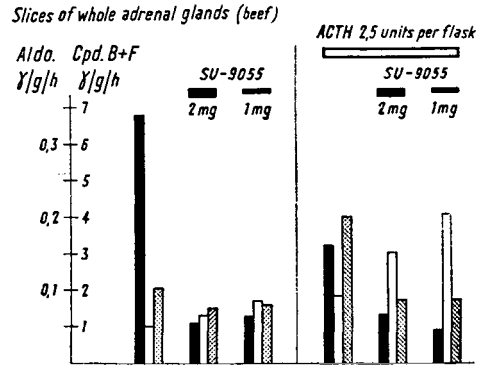

FIG. 3. Effect of high doses of Su-9055 on whole adrenal gland slices with and without ACTH.

We would like to show some preliminary data from *in vitro* studies, illustrating that Su-9055 also blocks, and even more effectively, aldosterone production, perhaps by way of 18α-hydroxylase or dehydrogenase inhibition (R. Neher reported similar findings at the International Congress on Hormonal Steroids).

Figure 3 illustrates the effect of relatively high doses of Su-9055 in slices of whole beef adrenal glands. The black bars indicate aldosterone; the white bars, corticosterone

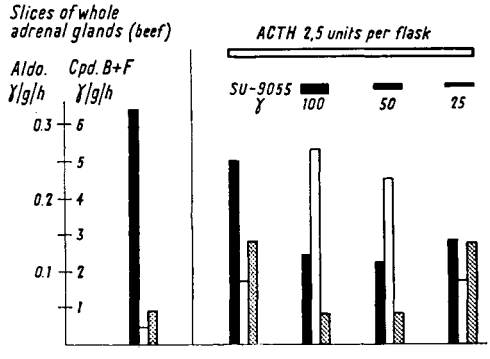

FIG. 4. Effect of decreasing doses of Su-9055 while under ACTH stimulation. As compound F synthesis increases, compound B production decreases.

(compound B); and the crosshatched bars, cortisol (compound F). These high doses of 2 and 1 mg inhibited aldosterone and to a lesser extent, compound F production; compound B increased only very slightly. ACTH in this experiment stimulated compounds B and F and inhibited aldosterone by 50%. If we add Su-9055, while maintaining ACTH,

we see again a very powerful inhibition of aldosterone and of compound F production; however, now, under ACTH stimulation, the increase in compound B becomes very evident.

In the next experiment (Fig. 4), also with slices of whole gland, we used much smaller doses of Su-9055. Again ACTH increased synthesis of compounds B and F, while blocking aldosterone production only slightly. If we compare the effect of decreasing doses of the inhibitor, we see the opposite effect on synthesis of compounds B and F: with Su-9055, compound B production is high and F low; as we decrease the dose of the

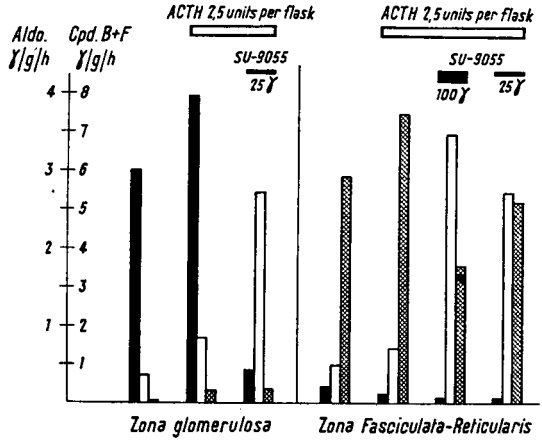

FIG. 5. Comparison of the effect of ACTH plus Su-9055 on the zona glomerulosa and the zona fasciculata. Aldosterone synthesis was inhibited to a much greater degree than compound F synthesis by equal inhibitor doses.

inhibitor, synthesis of compound F rises and B falls; at 25 μg synthesis of compounds B and F is identical to what it was without the inhibitor. However, the production of aldosterone is still suppressed by 50%. This difference in the effect of an identical inhibitor dose on aldosterone and on compound F synthesis is even better brought out in the next experiment (Fig. 5). We compared synthesis of the three steroids in the zona glomerulosa to synthesis in the zona fasciculata-reticularis. In the glomerulosa, ACTH increased aldosterone and compound B production, whereas compound F was practically absent; 25 μg of Su-9055 inhibited aldosterone synthesis considerably and compound B increased correspondingly.

In the fasciculata, where compound B and, mainly, compound F are produced, aldosterone was, for practical purposes, absent. ACTH increased compounds B and F; the little aldosterone that was present decreased even further. At 100 μg Su-9055 inhibited compound F by approximately 50%, whereas compound B increased correspondingly; with 25 μg Su-9055, the percentage inhibition of compound F was very much smaller and therefore, the production of compound B also dropped. It is quite evident that 25 μg Su-9055 plus ACTH will produce about the same amount of compound F as the slices of fasciculata without ACTH However, the production of compound B is far greater. This reflects the ACTH effect on compound B synthesis.

On the other hand, when we compare the effect of 25 μg Su-9055 in the two zones of the gland, we are immediately struck by the relatively small inhibition of compound

F synthesis as opposed to the important blocking effect of the same inhibitor dose on aldosterone production.

In summary then, this new 17α-blocker is also, and it seems even to a much greater extent, an aldosterone inhibitor, probably via 18α-hydroxylase or dehydrogenase inhibition.

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The Actions of Steroid Hormones on Estradiol-17β in Uterine Growth and Enzymorphology

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I. INTRODUCTION

Most studies in basic and human biology have a penchant for uncovering a score of perplexing questions which penetrate ever further into the central thesis of the original work, the aggregate standing as a posture of challenge to accepted theory. The present work is no exception. Witness the newly pointed-up facts emphasizing the metabolic interrelationships at different levels of the endocrine system, as well as among circumscribed areas of the endocrine system and the central nervous system (Benson *et al.*, 1955; Abu-Haydar *et al.*, 1954; Velardo *et al.*, 1956; Szego and Roberts, 1953; Roberts and Szego, 1953) Recent information admits of numerous interactions between estradiol-17β and the estriols (Hisaw *et al.*, 1954; Velardo and Sturgis, 1955a), testosterone (Velardo *et al.*, 1956), corticoids (Velardo and Sturgis, 1955b 1956a,b; Velardo, 1956), and progesterone (Velardo, 1954).

The aforementioned reports indicated that numerous steroids interfered with the action(s) of estradiol-17β-induced uterine growth, but the question yet remains as to the mechanism(s) involved. Therefore, it appeared of interest to reevaluate (1) the steroids involved in uterine growth, (2) the steroids that interfere with estradiol-17β-induced uterine growth, and (3) the role of the adenohypophysis and the adrenal glands in uterine growth as well as during the inhibitory process.

[1]Certain of these studies were conducted during the tenure of a Lederle Medical Faculty Award at the Yale University School of Medicine, 1955–1958. Aided in part by research grants from the United States Public Health Service, National Institutes of Health.

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Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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More specifically, the present report is directly concerned with elucidating: (1) the uterine growth-promoting action of estradiol-17β and its five urinary metabolites (estrone, 16α-hydroxyestrone, 16β-hydroxyestrone, estriol, and 16-epiestriol); (2) the influence of progesterone and several of its C-21 related metabolites on uterine growth; (3) the action of testosterone, glucocorticoids, and mineralocorticoids on uterine growth; (4) interactions of ovarian, testicular, and adrenal hormones on uterine growth; and (5) the mechanism(s) involved in hormonal action and the steroidally induced suppression of estradiol-17β on the uterus.

II. EXPERIMENTAL PROCEDURES

Adult, albino rats, 100 days of age, purchased from the Charles River Breeding Laboratories, each weighing approximately 200 gm, were used in all experiments involving ovariectomized rats. In experiments utilizing ovariectomized-adrenalectomized and ovariectomized-adrenalectomized-hypophysectomized rats, it was found more suitable to use rats 105 days old and weighing 235 gm. These animals survived the triple surgery much better than the younger rats.

All animals were started on experiment 7 days after ovariectomy. The hypophysectomies were performed 1 week prior to the ovariectomies, and the adrenalectomies were started 3–5 minutes after the removal of the ovaries.

The ovariectomized rats were housed six to a 9 × 9 × 18-inch wire-meshed cage; they were fed the standard Purina Lab chow and tap water *ad libitum*. The adrenalectomized-ovariectomized rats were similarly maintained, except for the substitution of 1% NaCl drinking solution in place of tap water. The ovariectomized-adrenalectomized-hypophysectomized rats received the Purina Lab chow, were maintained in similar cages, and received two drinking solutions: 1% NaCl and 1% glucose.

The hormones were dissolved in sesame oil; each dose of hormone was contained in either 0.1 or 0.2 ml of vehicle; administration was by subcutaneous injection daily for 3 days. When two or more hormones were injected, they were administered at different sites. Seventy-two hours after the initial injection(s), necropsies were performed; the uteri were removed and weighed to the nearest 0.2 mg on a Roller—Smith torsion balance. Parallel quantitative data were obtained in each experiment on wet and dry weights of the uterus, percentages of water, total nitrogen, and solids within the uterus. Although only uterine weights are used for the purpose of this paper, their presentation is in essential agreement with the biochemical data. The curves depicted for dry uterine weights follow those of the data for total nitrogen quite precisely. Parallel data on lactic dehydrogenase (LDH) and DPNH oxidase are presented. The methodology has been published in detail elsewhere (Bever *et al.*, 1956).

III. RESULTS

A. UTERINE GROWTH-PROMOTING ACTION OF THE SIX NATURALLY OCCURRING ESTROGENS

A great deal of biochemical research has been in progress on the urinary estrogens and their quantitative determination by Professor Marrian (1955, 1957) and his associates (Marrian and Bauld, 1954, 1955; Marrian *et al.*, 1957a,b; Watson and Marrian, 1955, 1956), Bauld (1954,

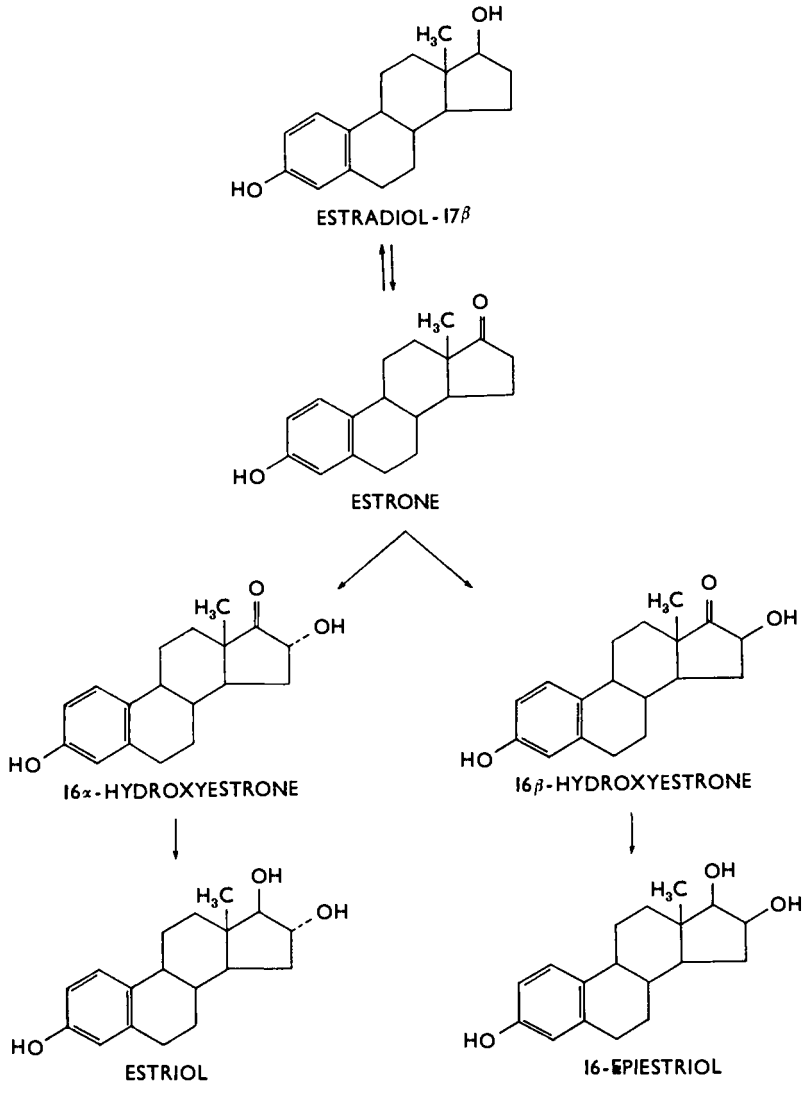

FIG. 1. Hypothetical scheme for the interrelationship of the estrogens in human urine.

1956) and Brown (1952, 1955). Figure 1 depicts a hypothetical scheme for the interrelationship of the estrogens in the urine; it presents a summary of Marrian's research, which has led us to a clearer concept of the biochemical career of estradiol-17β and the $\Delta^{1,3,5}$-estratrienes. Figure 1 indicates that estrone may undergo hydroxylation at the 16α and 16β positions, and that the two hydroxyestrones may then be reduced

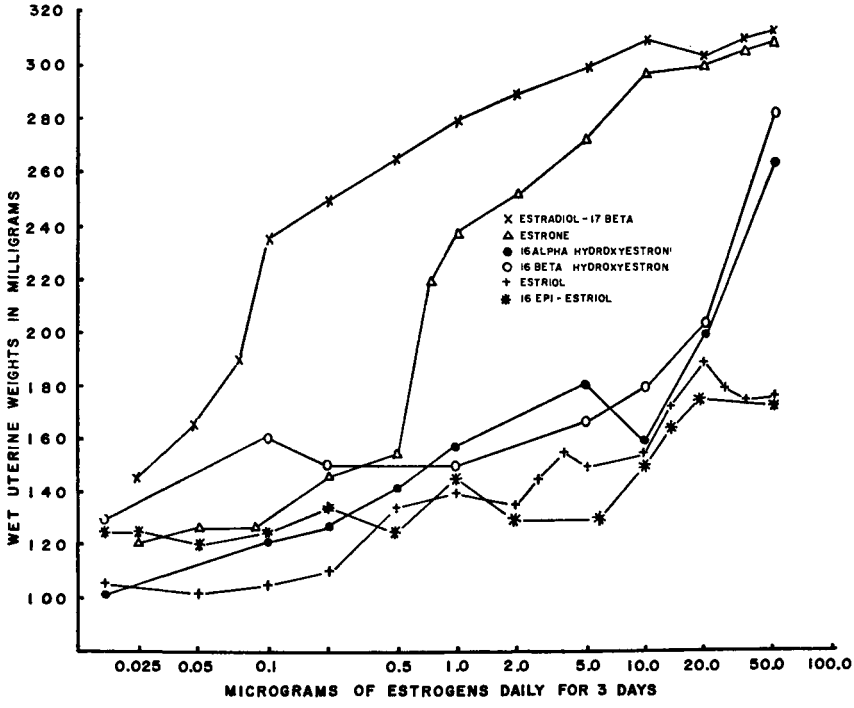

FIG. 2. Uterine growth induced by estrogens. Dosage-response curves showing the comparative effectiveness of estradiol-17β, estrone, 16α- and 16β-hydroxyestrones, estriol, and 16-epiestriol. Rats 100 days of age were ovariectomized and, beginning 7 days later, were given one subcutaneous injection of estrogen daily for 3 days. Necropsies were performed 24 hours after the last injection. Each point on these and subsequent curves represents the arithmetic mean of uterine weight for at least 11–20 animals. The average wet uterine weight for nontreated ovariectomized rats is 118.0 ± 7.0 mg.

at C-17 to give estriol and 16-epiestriol, respectively. Up to 1954, the three different but chemically closely related estrogens isolated from the urine of nonpregnant and pregnant women were estradiol-17β $\rightleftharpoons$ estrone $\rightarrow$ estriol. These estrogens were characterized biologically by numerous investigators, and until 1954 these were the only naturally occurring estrogens that had been biologically characterized and assayed. The recent availability of 16α- and 16β-hydroxyestrones (thanks

to Dr. Thomas Gallagher) and 16-epiestriol (thanks to Dr. Max N. Huffman) gave impetus to the present reevaluation of the biologic activity of estradiol-17β and five of its metabolites, as depicted in Fig. 1.

The potentiality of each estrogen for the promotion of uterine growth was determined by establishing a dose-response curve (Figs. 2 and 3). Maximal uterine growth for the 3-day period was obtained with

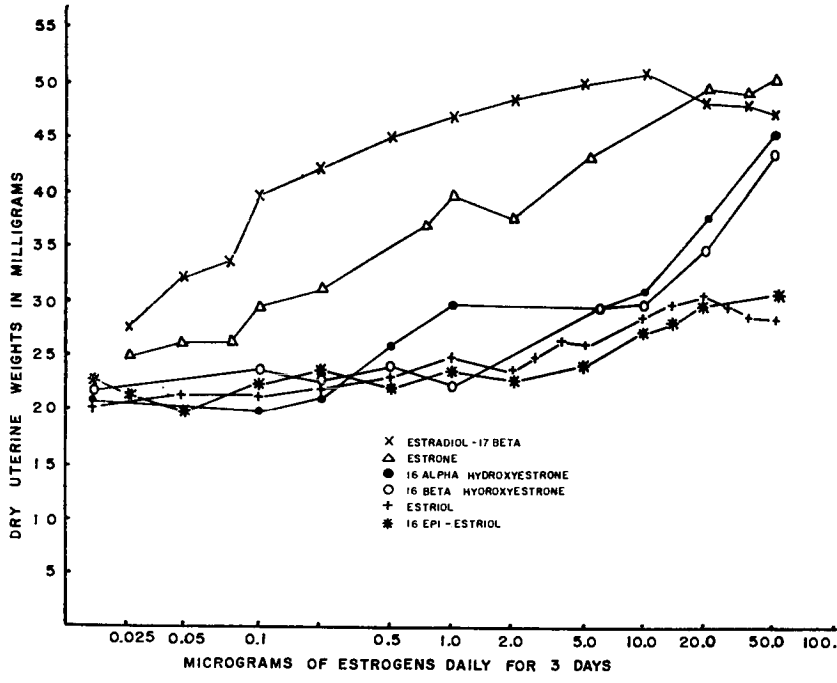

FIG. 3. Uterine dry weights of the corresponding uteri depicted in Fig. 2. The dry weights parallel the growth responses indicated by the uterine weight average for the nontreated ovariectomized rats is 22.0±1.1 mg.

a daily subcutaneous injection of 1.0 μg estradiol-17β, 5.0 μg estrone, 50 μg 16α-hydroxyestrone, 50 μg 16β-hydroxyestrone, 20.0 μg estriol, and 20.0 μg 16-epiestriol. Daily doses as high as 50.0 μg estriol and 16-epiestriol proved less effective than the response elicited with 20.0 μg of the two estriols, respectively (Figs. 2 and 3). Analysis of the biochemical data at hand reveals that the increase in uterine luminal fluid, nitrogen, and solid content of the uteri parallels quite closely the curves depicted for estradiol-17β and its five metabolites. These data show that estradiol-17β > estrone > 16α-hydroxyestrone > 16β-hydroxyestrone > estriol > 16-epiestriol in the promotion of uterine growth.

B. ACTION OF PROGESTERONE, PREGNANEDIOLS, AND PREGNANEDIONES ON UTERINE GROWTH

Considerably more biochemical and physiologic data are available for the estrogens than for progesterone and its C_{21} related metabolites. Recently, Leiberman and Teich (1953) described the C_{21} metabolites related to progesterone (Fig. 4). More recently, Zander has isolated two

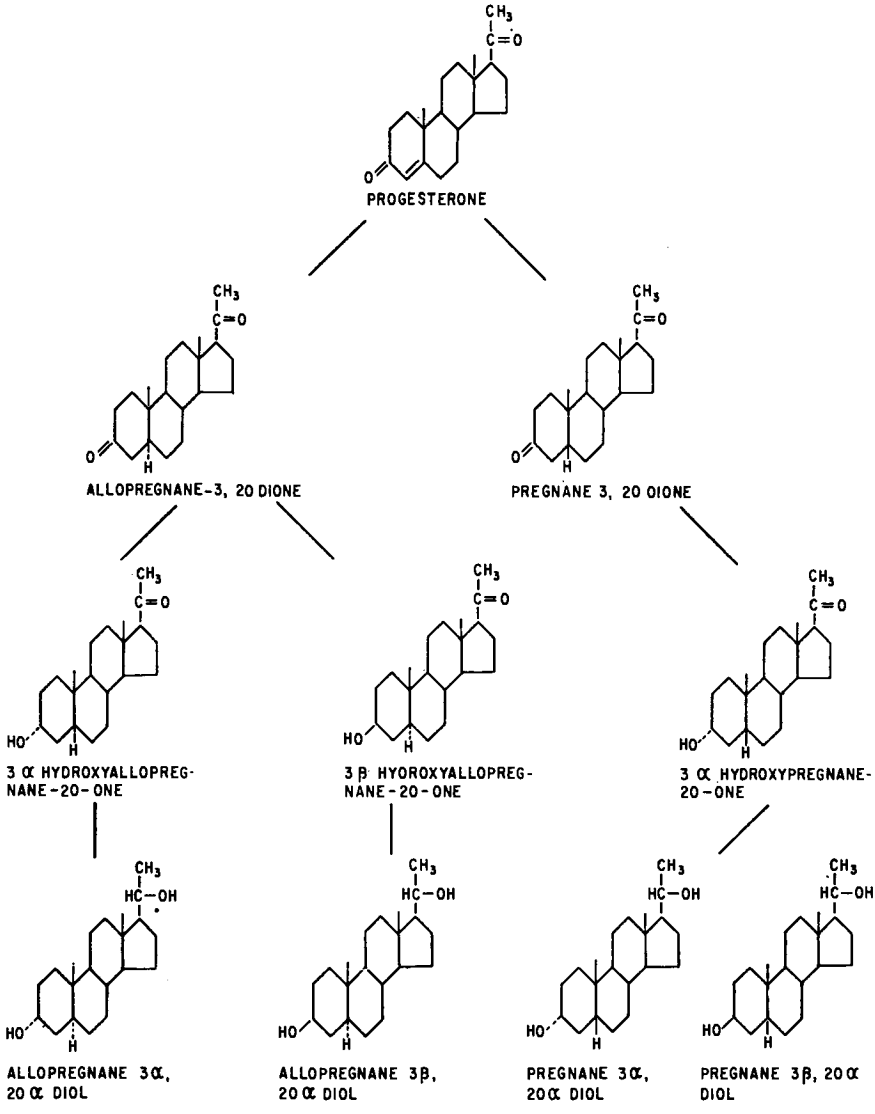

FIG. 4. Schematic representation of progesterone and its C_{21} related metabolites as proposed by Lieberman and Teich (1953).

new progestins from the blood of human females: Δ^4-3-ketopregnan-20α-ol and Δ^4-3-ketopregnan-20β-ol (Zander, 1955). Unfortunately, the newly reported progestins are not yet available for thorough biologic assessment. Therefore, the present report will be concerned with the

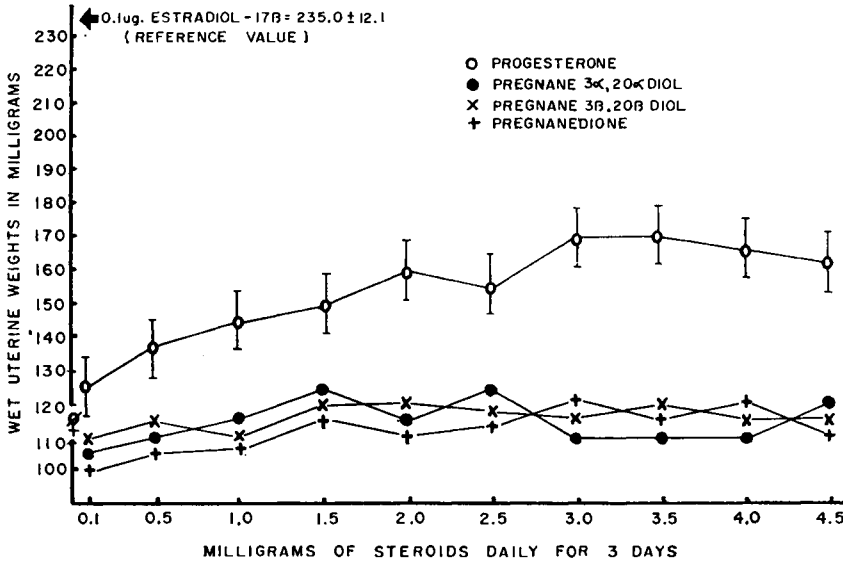

FIG. 5. Influence of progesterone and pregnanes on uterine growth. The vertical bars on the progesterone curve and subsequent curves represent the standard errors of the arithmetic mean.

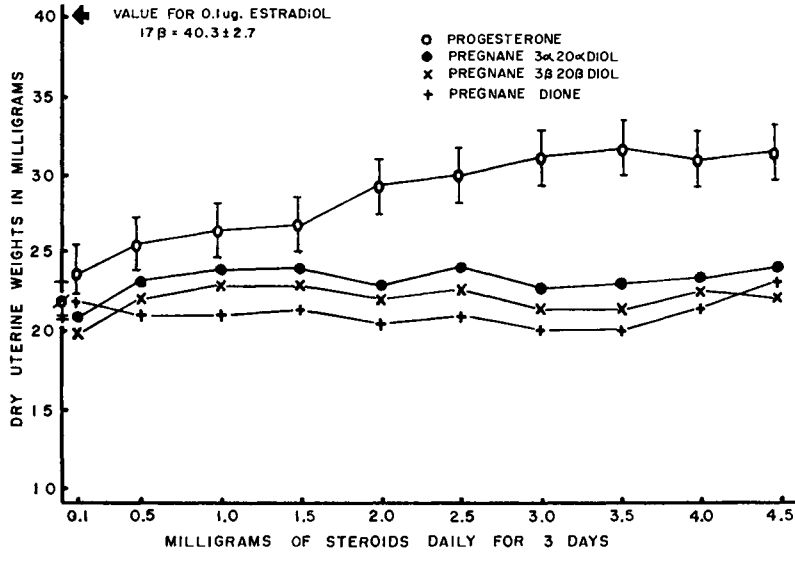

FIG. 6. Uterine dry weights of the corresponding uteri depicted Fig. in 5.

action of progesterone and several of the pregnanediols and pregnanediones on uterine growth. Progesterone displays a slight uterine growth-promoting effect. None of the C_{21} related metabolites of progesterone thus far tested for a uterotropic effect induced uterine growth (Figs. 5 and 6).

C. INFLUENCE OF TESTOSTERONE, GLUCOCORTICOIDS, AND MINERALOCORTICOIDS UPON UTERINE GROWTH

Testosterone in daily doses of 2.0 to 5.0 mg induced uterine growth that was indistinguishable from that produced with three daily subcutaneous injections of 0.1 μg estradiol-17β (Figs. 2, 3, 7, and 8). Of the seven glucocorticoids and mineralocorticoids tested [deoxycorticosterone acetate; cortisone (E); hydrocortisone (F); Δ^1 E; 9α-Fl F; Δ^1 F; and Δ^1, 9α-Fl F], it was observed that deoxycorticosterone was the only corticosteroid which displayed the ability to induce *slight* uterine growth (Figs. 7, 8, 9).

D. INTERACTIONS OF OVARIAN, TESTICULAR AND ADRENAL HORMONES ON UTERINE GROWTH

1. Interactions among the Estrogens

Previously, Hisaw *et al.* (1954) and Velardo and Sturgis (1955a) reported that estriol and 16-epiestriol restricted the uterine growth-promoting action of estradiol-17β. The recent availability of 16α- and 16β-hydroxyestrones provided the impetus for the current extension of these earlier experiments. Combinations of one of the two hydroxyestrones and estradiol-17β were administered to ovariectomized rats. Preliminary data at hand indicate that although the two hydroxyestrones are comparatively weak in inducing uterine growth, as are the estriols, they do not seem to interfere with the action of estradiol-17β. Additional studies involving the action of a wide dose range of the hydroxyestrones on the response of 0.1 μg estradiol-17β are now in progress.

2. Interactions of Estradiol-17β and Progesterone or Pregnanes on Uterine Growth

Progesterone in low doses ($<$ 2.5 mg) interferes with the response of the uterus of the ovariectomized rat to 0.1 μg estradiol-17β, but larger doses (3.5–4.5 mg) do not seem to have an appreciable effect on estradiol (Figs. 10 and 11). None of the pregnanes tested thus far interfered with the uterine growth-promoting action of estradiol.

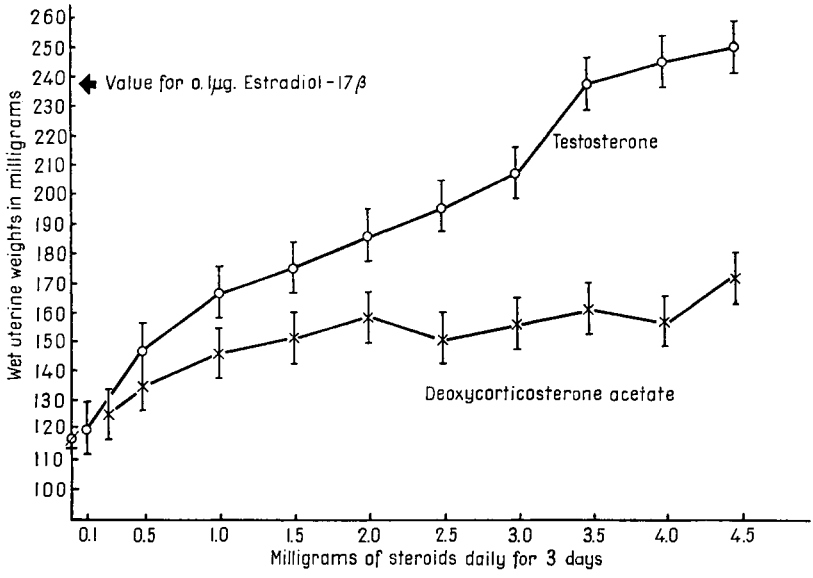

FIG. 7. Curves displaying the uterine growth-promoting action of testosterone and deoxycorticosterone acetate.

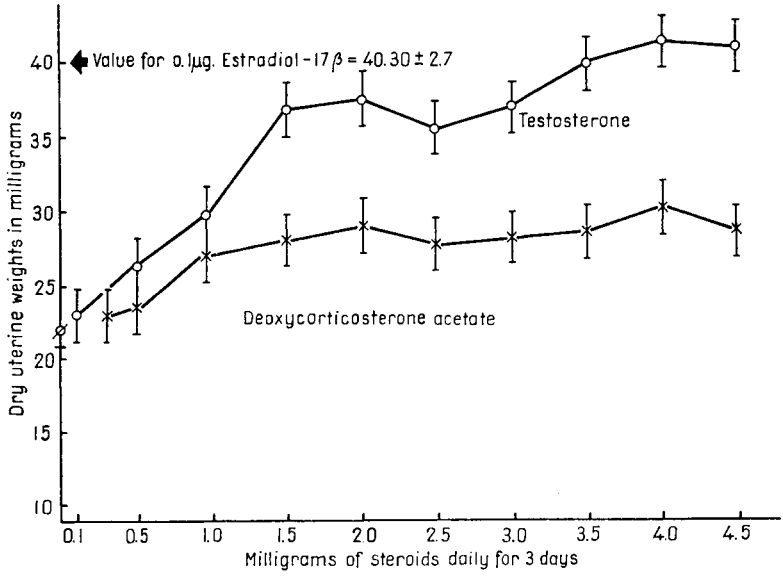

FIG. 8. Uterine dry weights of the corresponding uteri depicted in Fig. 7.

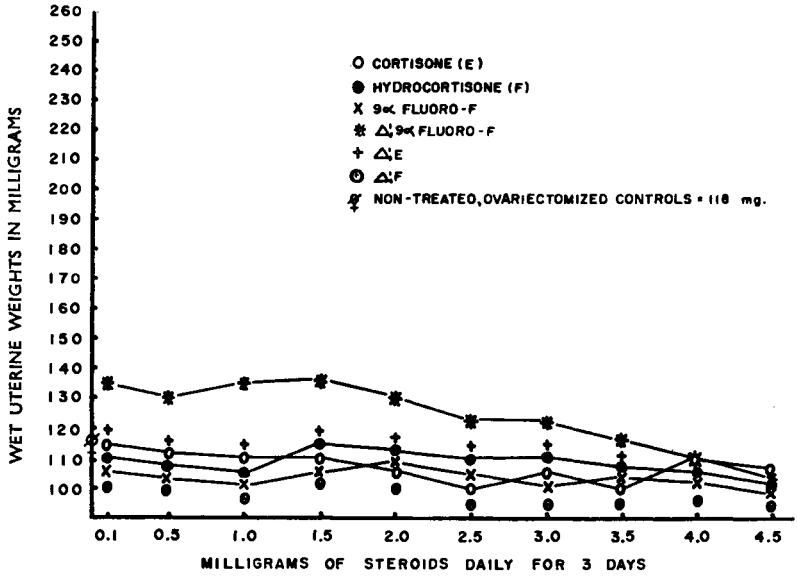

FIG. 9. Curves depicting the inability of cortisone and cortisols to induce uterine growth.

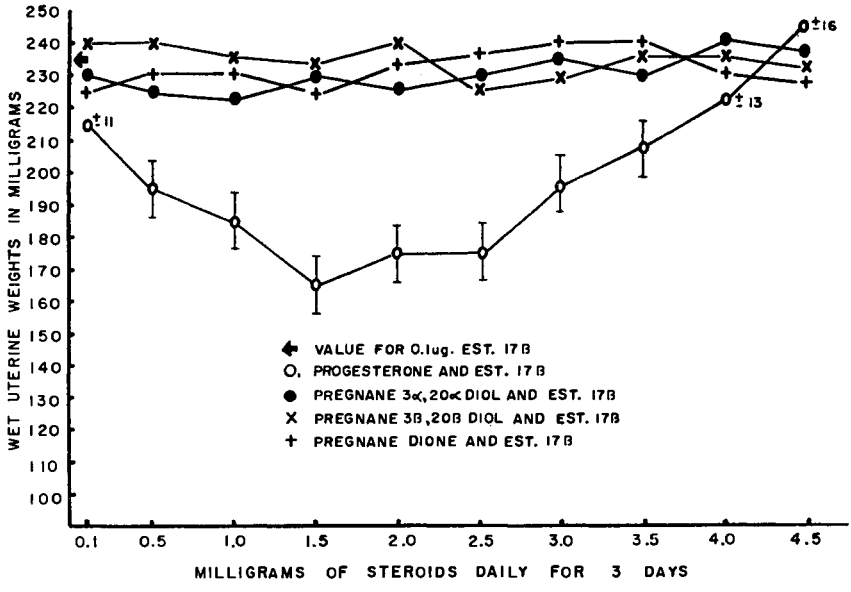

FIG. 10. Curves depicting the restrictive action of progesterone and the nonrestrictive action of the pregnanes on estradiol in uterine growth.

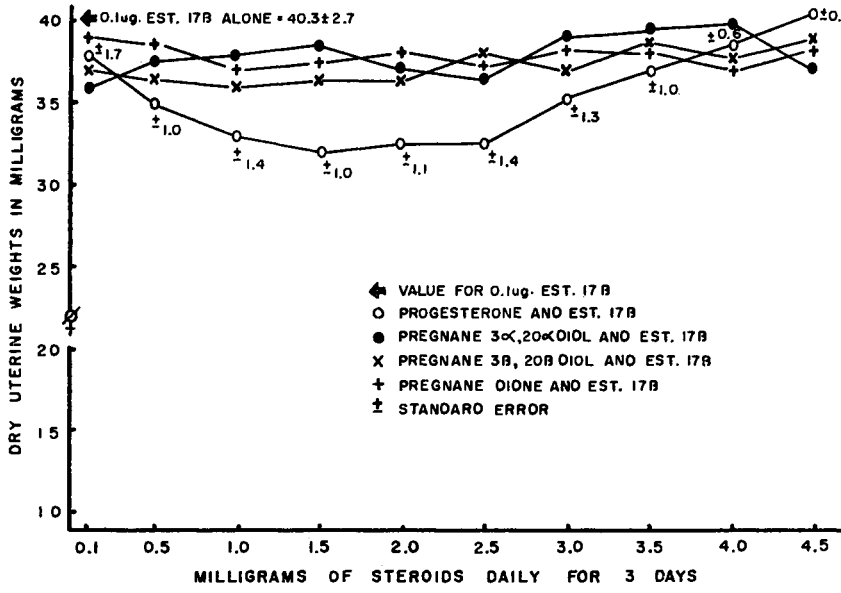

FIG. 11. Uterine dry weights of the corresponding uteri represented in Fig. 10.

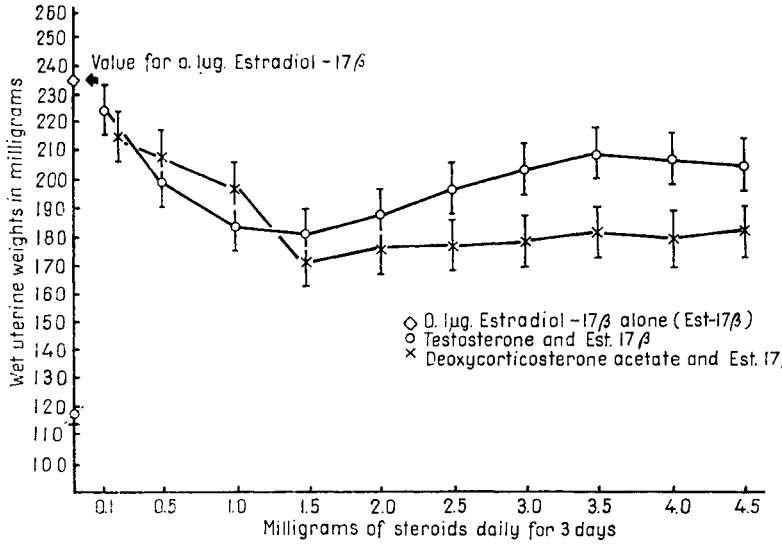

FIG. 12. Curves depicting the inhibitory action of testosterone and deoxycorticosterone acetate on estradiol in uterine growth.

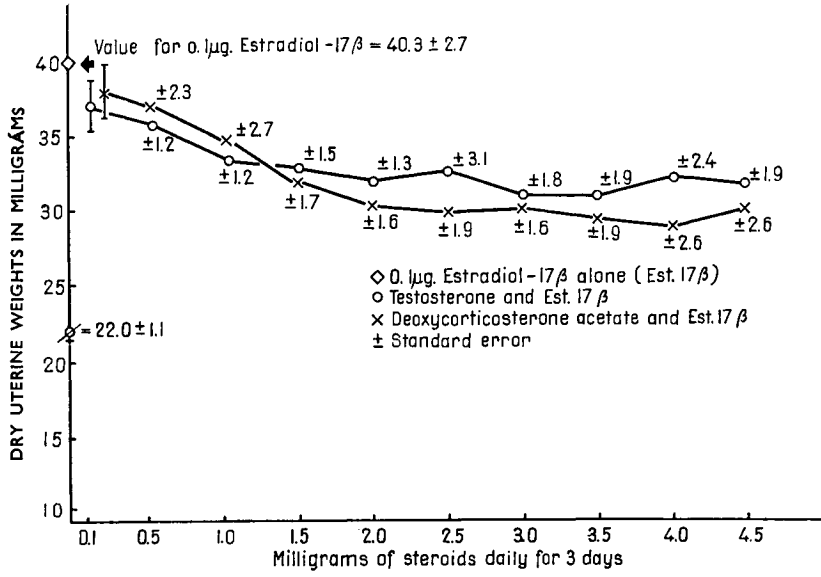

FIG. 13. Uterine dry weights of the corresponding uteri represented in Fig. 12.

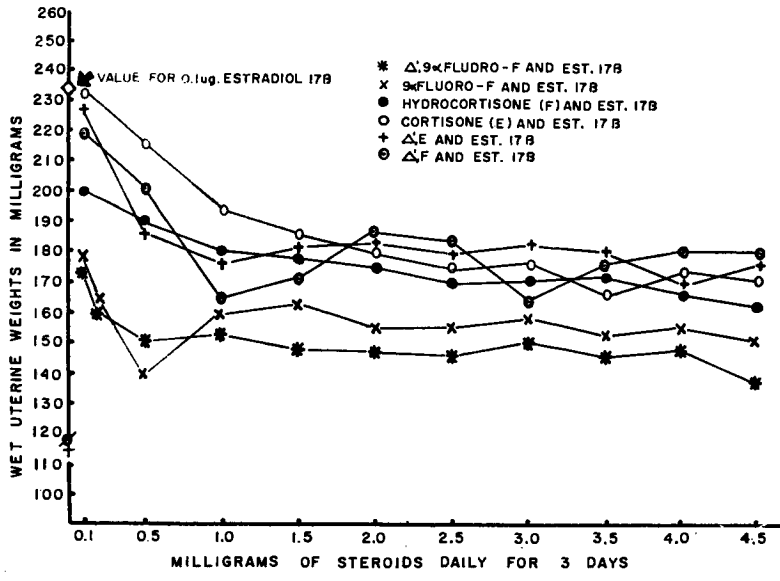

FIG. 14. Suppressive effects of cortisones and cortisols on estradiol-induced uterine growth.

3. Inhibitory Action of Testosterone and Deoxycorticosterone Acetate (DCA) on Estradiol

These data reveal that both testosterone and DCA display similar inhibitory activity on estradiol-17β. Three daily doses of 0.5–1.5 mg of either testosterone or DCA decreased the uterine response from 235.0 mg to 170–180 mg, a significant reduction in uterine growth. Doses above 1.5 mg of either of the two steroids did not induce a greater suppressive effect on estradiol (Figs. 12 and 13).

4. Suppressive Effects of Cortisones and Cortisols on Estradiol-Induced Uterine Growth

All six cortisones and cortisols tested were found to interfere with estradiol. Comparatively, these data reveal that $\Delta^1 9\alpha$FlF > 9αFlF >

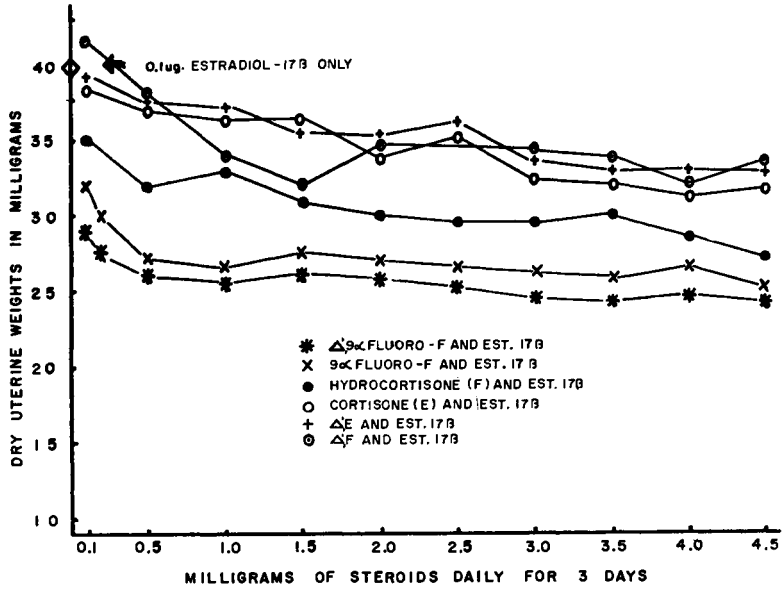

FIG. 15. Uterine dry weights of the corresponding uteri depicted in Fig. 14.

> F > E > Δ^1E and Δ^1F in restricting the uterine growth-promoting action of estradiol. It is of further interest to note that both structural modification of the geometry of ring A of compound F and halogenation at the ninth carbon enhances the suppressive effects of cortisol (Figs. 14 and 15).

E. OBSERVATIONS REGARDING THE MECHANISM OF ACTION OF THE SUPPRESSIVE EFFECTS OF THE DIFFERENT STEROIDS ON ESTRADIOL-17β

The several studies showing the competitive interactions of steroids in reproductive processes have provided a considerable impetus to ascertain the mechanism of action of the suppressive effects of the different steroids on estradiol-17β (Velardo *et al.*, 1956; Szego and Roberts, 1953; Roberts and Szego, 1953; Hisaw *et al.*, 1954; Velardo and Sturgis, 1955a,b, 1956a,b; Velardo, 1954, 1956; Huggins and Jensen, 1954, 1955a,b; Huggins *et al.*, 1954; Szego 1952). Thus far, several approaches to this problem have been initiated. These include studies on (1) the structural specificity of steroids in the growth and inhibition of the uterus by Szego and Roberts (1953, Szego 1959), Huggins and Jensen (1954, 1955a,b) and Velardo (1956); (2) the hormone-enzyme relationships by Bever *et al.* (1953a,b,c, 1956) and Villee and Hagerman (1957); and (3) the endocrine organs involved in the suppression of estradiol-17β-induced uterine growth (Velardo, 1958a, b).

The present results are offered in the nature of a preliminary report on the research in progress in this laboratory on the mechanism(s) involved in the restriction of uterine growth. Previously, it was postulated that estriol might stimulate the release of adrenocorticotropic hormone (ACTH) and consequently adrenocortical steroids. This line of reason-

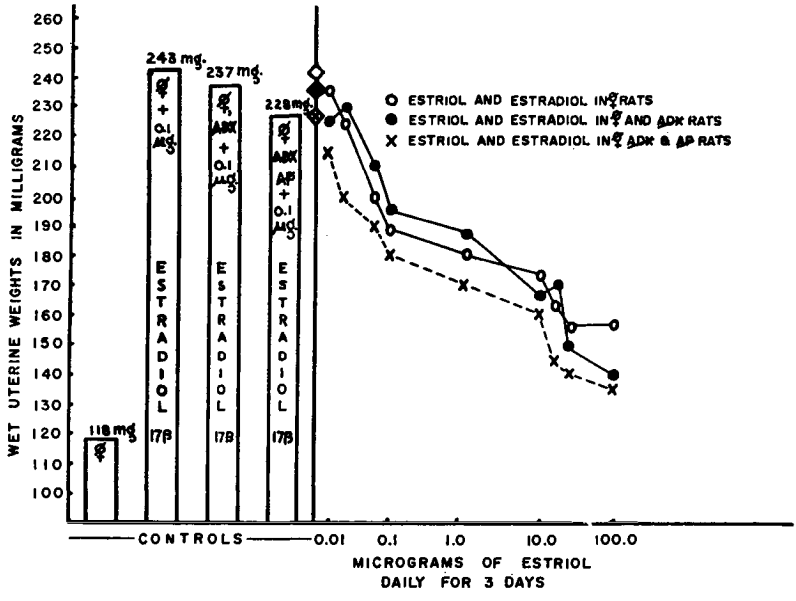

FIG. 16. Demonstration indicating that the restrictive action of estriol on estradiol-17β-induced uterine growth is independent of the adrenal gland and adenohypophysis.

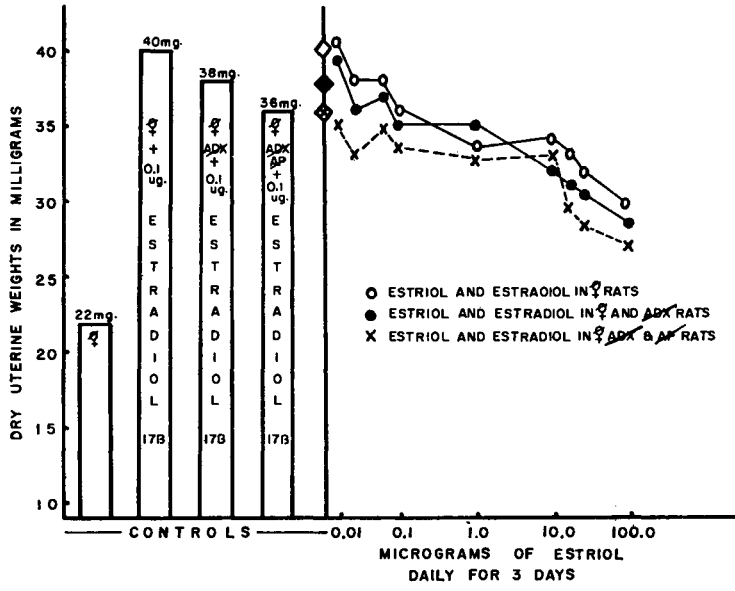

FIG. 17. Dry weight data of corresponding uteri depicted in Fig. 16.

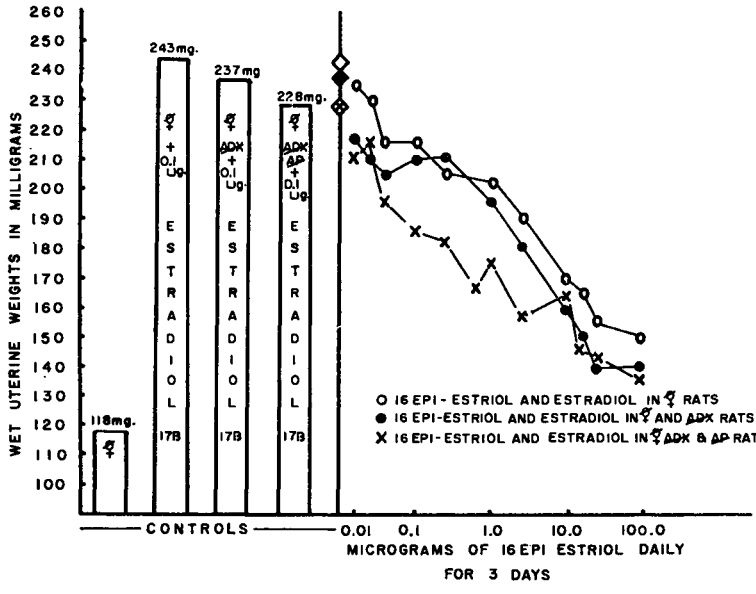

FIG. 18. Exhibit showing that the action of 16-epiestriol on estradiol-17β is not mediated by way of the adrenal glands or the adenohypophysis.

ing seemed to support the thought that the antiuterotropic activity might well be due to the inhibitory effect of the adrenal corticosteroids, an effect known to antagonize estradiol and estrone on uterine growth. Thus, it seemed that a better insight into the factors operative in uterine growth, as well as in the suppressive effects of the different steroids, might be gained if this assumption were tested.

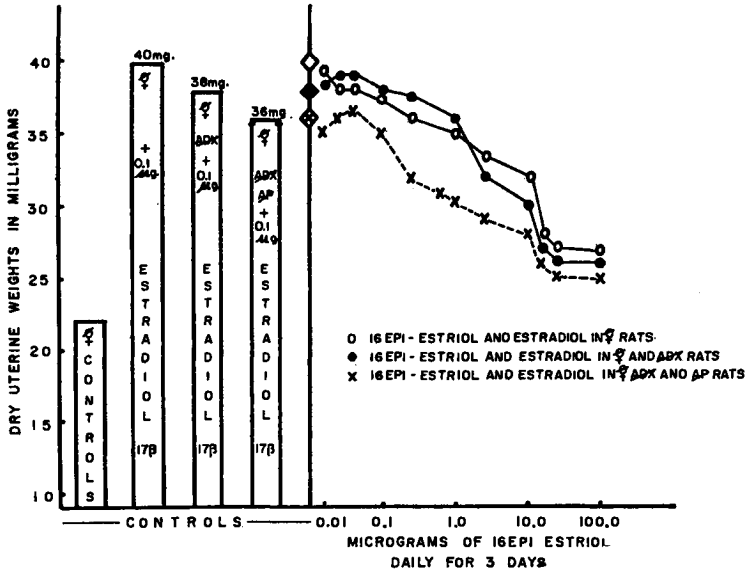

FIG. 19. Dry weight data on corresponding uteri represented in Fig. 18.

Results from the present experiments reveal that: (1) estradiol-17β is an active uterine growth-promoting agent in (1) ovariectomized, (b) ovariectomized and adrenalectomized, (c) ovariectomized-adrenalectomized-hypophysectomized rats; (2) estriol and 16-epiestriol both inhibit estradiol-17β-induced uterine growth in (a) ovariectomized rats—from 0.1 μg estradiol-17β controls of 243 mg down to 156 mg, (b) ovariectomized and adrenalectomized rats—from 237 mg down to 139 mg, and (c) ovariectomized-adrenalectomized-hypophysectomized rats—from 228 mg down to 135 mg; and (3) the suppressive effects of the estriols need not be mediated by way of the adenohypophysis and the adrenal glands (Figs. 16–19).

F. STEROID INFLUENCES ON THE LACTIC DEHYDROGENASE-DPNH OXIDASE SYSTEM OF THE RAT UTERUS

Results from enzymatic determinations indicate that the lactic dehydrogenase (LDH) and specific parts of the enzyme systems concerned with DPNH oxidation may be influenced by estrogens (Figs. 20–22), progesterone (Figs. 23–26), deoxycorticosterone acetate (DCA),

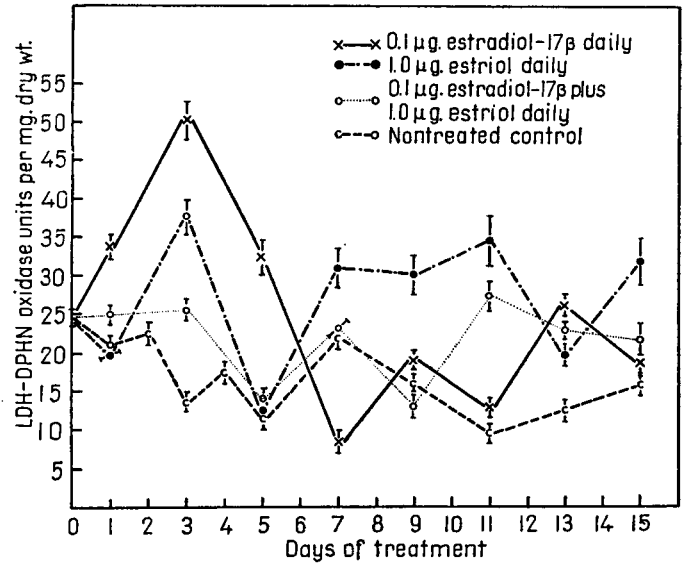

FIG. 20. The effect of estradiol-17β and estriol on ovariectomized rat uterine LDH-DPNH oxidase system. Each point represents the average of 12–18 determinations from 4–6 uteri. After Bever, Velardo and Hisaw, 1956.

cortisone, and testosterone (Figs. 27–30, Table I). Of the four groups of steroids studied, estradiol-17β had the most potent stimulatory effect on DPNH oxidase, while progesterone was the most potent in effecting an increase in LDH activity. DCA had an effectiveness similar to that of progesterone upon LDH. Progesterone, DCA, and estriol all displayed the property of inhibiting the action of estradiol-17β on DPNH oxidase activity. Testosterone and cortisone acetate did not appear to have a strong effect in modifying the action of estradiol in either direction. Estradiol proved quite effective in restricting progesterone and DCA from fully potentiating LDH activity (Bever *et al.*, 1953a,b,c, 1956; Rosa and Velardo, 1954a,b).

Bever further showed that estradiol-17β affects uterine DPNH oxidase, as measured by neotetrazolium reduction, and DPNH-cytochrome c reductase equally. His recent inhibitor studies showed that the two activities respond equally to inhibitors. Furthermore, differential cen-

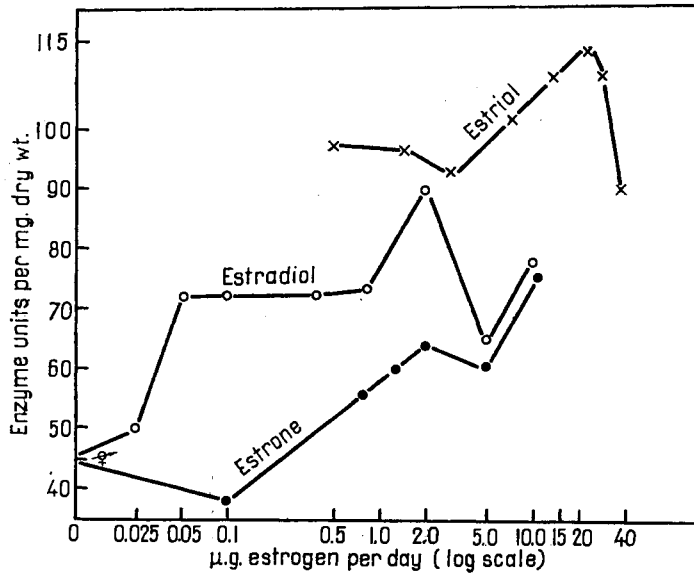

FIG. 21. Effect of the estrogens estradiol-17β, estriol, and estrone on uterine LDH after 3 days of daily administration. Each point represents the average of 4–5 uterine horns distributed in three separately prepared homogenates. After Bever, Velardo and Hisaw, 1956.

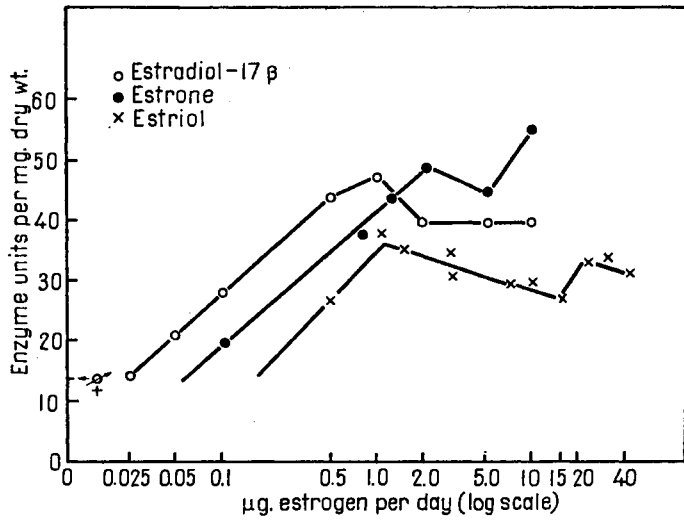

FIG. 22. Effect of estradiol-17β, estriol, and estrone on uterine DPNH oxidase after 3 days of daily administration. Each point represents the average activity of 4–5 uterine horns distributed in three separately prepared homogenates. After Bever, Velardo and Hisaw, 1956.

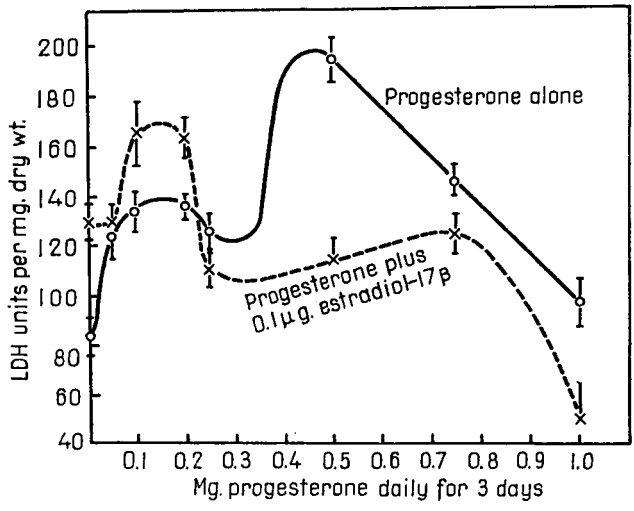

FIG. 23. The effect on LDH of progesterone and of progesterone plus estradiol-17β after 3 days of daily administration. Each point represents the mean value of 8–12 animals in 4–6 determinations using pooled uteri from 2 animals in each homogenate. The vertical lines represent standard error of the mean for each point. After Bever, Velardo and Hisaw, 1953b.

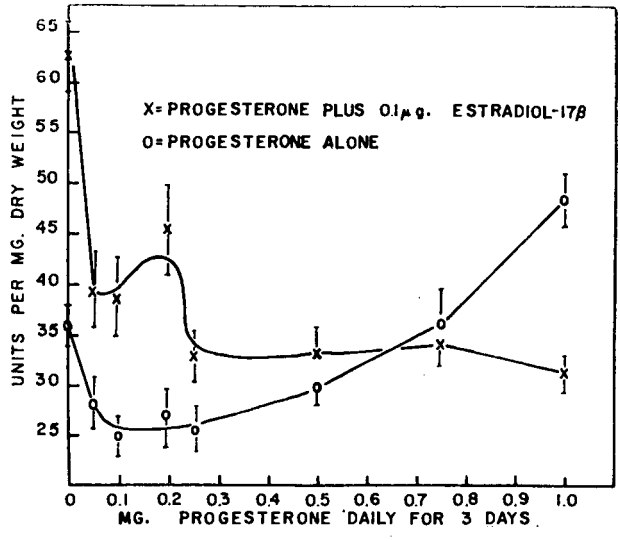

FIG. 24. The effect on uterine DPNH oxidase of progesterone and of progesterone plus estradiol-17β after 3 days of daily administration. Each point represents the mean value of 8–12 animals in 4–6 determinations using pooled uteri from 2 animals in each homogenate. The vertical lines represent the standard error of the mean for each point. After Bever, Velardo and Hisaw, 1953b.

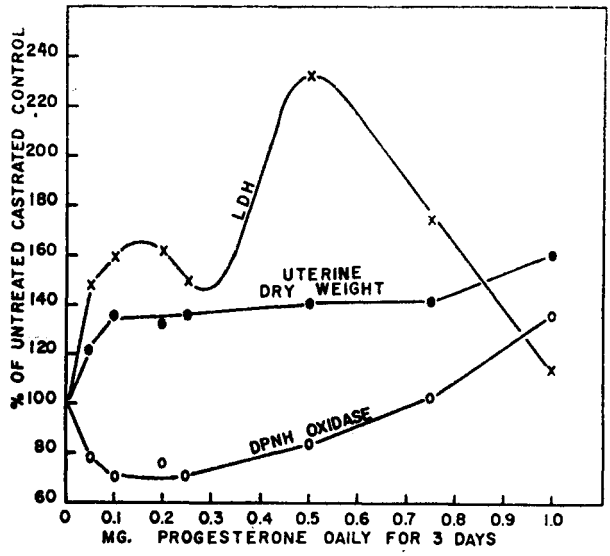

FIG. 25. Influence of progesterone on the rat uterine LDH-DPNH oxidase system. Calculated from data of Figs. 23. and 24. After Bever, Velardo and Hisaw, 1953b.

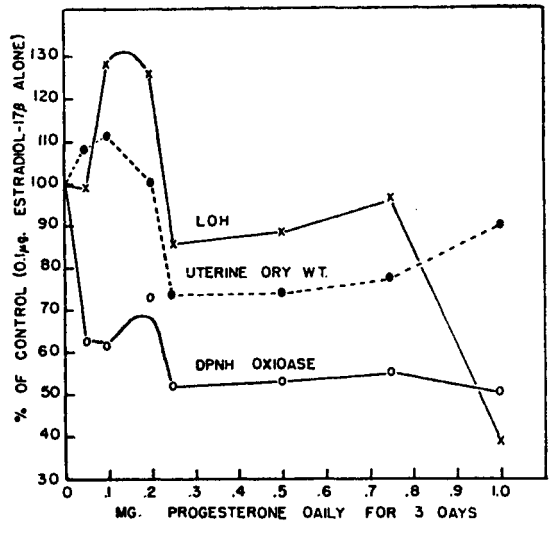

FIG. 26. Influence of progesterone with 0.1 μg estradiol-17β on the rat uterine LDH-DPNH oxidase system. Calculated from data of Figs. 23 and 24. After Bever, Velardo and Hisaw, 1953b.

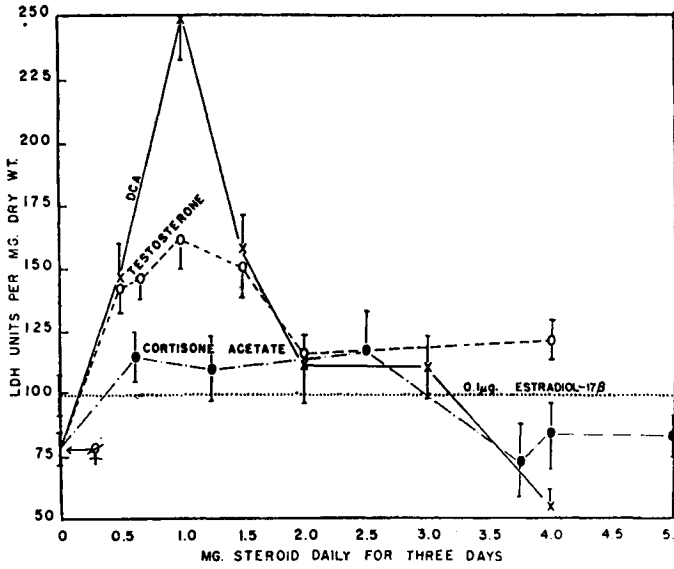

FIG. 27. The effect of deoxycorticosterone acetate (DCA), testosterone, and cortisone acetate on uterine LDH of ovariectomized rats after 3 days of administration. Each point represents the mean value of 8–12 animals in 4–6 determinations. The vertical lines represent the standard error of the mean for each point. After Bever, Velardo and Hisaw, 1953c.

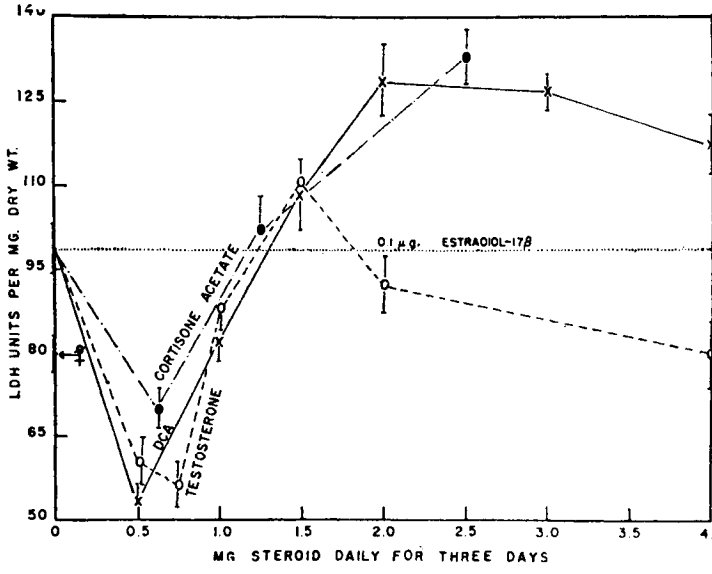

FIG. 28. The effect of deoxycorticosterone acetate (DCA), testosterone, and cortisone acetate when each is administered with 0.1 μg estradiol-17β upon uterine LDH of ovariectomized rats. The steroids were administered daily for 3 days. Each point represents the mean value of 8–12 animals in 4–6 determinations. The vertical lines represent the standard error of the mean for each point. After Bever, Velardo and Hisaw, 1953c.

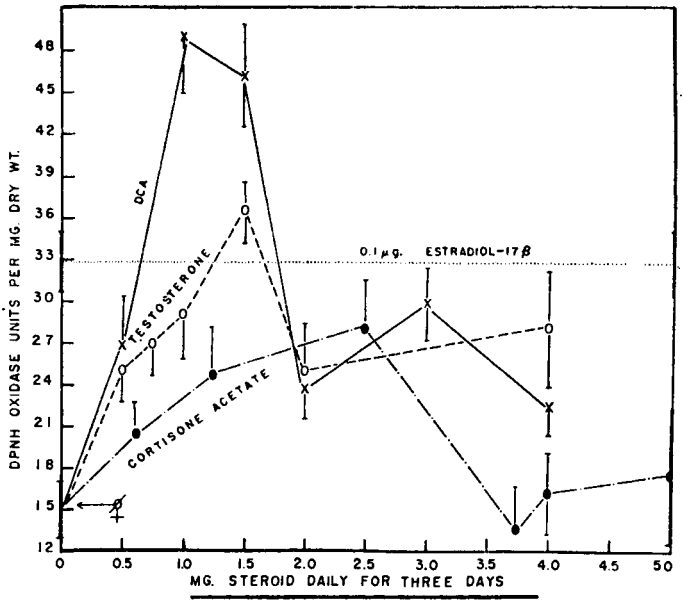

FIG. 29. The effect of deoxycorticosterone acetate (DCA), testosterone, and cortisone acetate on uterine DPNH oxidase of ovariectomized rats after 3 days of daily administration. Each point represents the mean value of 8–12 animals in 4–6 determinations. The vertical lines represent standard error of the mean for each point. After Bever, Velardo and Hisaw, 1953c.

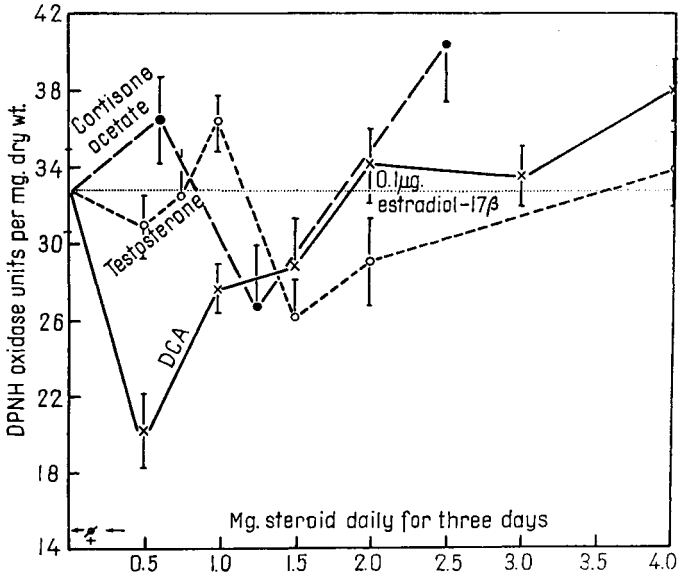

FIG. 30. The effect of deoxycorticosterone acetate (DCA), testosterone, and cortisone acetate when each is administered with 0.1 μg estradiol-17β upon uterine DPNH oxidase of ovariectomized rats. The steroids were administered daily for 3 days. Each point represents the mean value of 8–12 animals in 4–6 determinations. The vertical lines represent standard error of the mean for each point. After Bever, Velardo and Hisaw, 1953c.

trifugal studies indicated an equal distribution of the two activities within the centrifugal fractions (Bever, 1959).

The comparative effects of the steroids upon the two enzymes, alone and with estradiol, are qualitatively summarized in Table I.

TABLE I

Effect of Steroids on Rat Uterine LDH-DPNH Oxidase Activity[a]

Steroid	Relative enzyme activities			
	Effect of steroid alone		Effect of steroid given with estradiol	
	LDH	DPNH oxidase	LDH	DPNH oxidase
Estradiol-17β	+++	++++	—	—
Estrone	++	+++	—	Additive effect
Estriol	++++	++	?	Estriol inhib. estradiol
Progesterone	+++++	—	Estradiol—inhib. prog.	Prog. inhib. estradiol
DOCA	++++	+++	Estradiol—inhib.DOCA	DOCA inhib. estradiol
Testosterone	++	++	Estradiol—inhib. test.	Test. inhib. estradiol
Cortisone acetate	±	+	Estradiol—inhib. cort.	No effect

[a]Reproduced from Bever, A. T. (1959).

IV. DISCUSSION

The results of these experiments reveal the nature of the steroids that are importantly concerned with the growth phase of the uterus, as well as the steroids that are antagonistic to the uterine growth-promoting action of estradiol-17β. The present report also gives a rather comprehensive view of the numerous interactions that occur among hormones of the ovaries and the suprarenal cortex. Superimposed upon these basic studies is a demonstration which indicates that the suppressive effects of estriol and its isomeric form, 16-epiestriol, on estradiol-17β-induced uterine growth are not mediated by way of the adenohypophysis or the suprarenal cortex, but more likely are directly on the endocrine receptor organs.

Courrier (1951) and more recently Velardo (1957, 1958a, b) have summarized the numerous actions and interactions of the luteoids and folliculoids. It is well known that progesterone can restrict in part the estrogens in rodents, and yet in other species these two ovarian hormones may synergize, e.g., the progestational phase of the primate endometrium. Much less is known on a species-wide basis of the actions and interactions of the corticoids, folliculoids, luteoids, and testoids. Information from recent basic studies and clinical observations on the metabolic career of isotopically labeled hormones, coupled with data on the hormonal status of patients with endocrinopathies, offers

important leads to our understanding of the hormonal interrelationships in the human female.

It is well known that the therapeutic problem of dealing with several endocrinopathies is complicated by the recognition that a target organ probably does not respond simply to one hormone alone, but more likely to the total internal hormonal environment of the patient. In both frank myxedema and hyperthyroidism there is consistently observed a marked dysfunction of the female reproductive system which is manifested by either amenorrhea or menorrhagia. In 1956 Velardo and Sturgis, referring to the work of Goldsmith *et al.* (1952), stated that it is not entirely clear whether thyroid disease causes this abnormality by interfering with the release of gonadotropins from the adenohypophysis, by altering ovarian function, or by a direct effect on the uterine mucosa. Since 1956, Engle (Baggett *et al.*, 1956) and Gallagher's (Bradlow *et al.*, 1956) groups have produced a great deal of helpful information which reveals that a thyroid hormone, triiodothyronine, has a marked effect upon the metabolism of the precursor of estradiol-17β, namely testosterone. Gallagher and his associates have shown that triiodothyronine regulates the conversion of testosterone to androsterone and etiocholanolone. It is of direct interest to point out that androsterone is a biologically active androgen. These facts suggest a more complex interrelationship than that hitherto envisaged. Recent evidence indicates that the gonadal hormones may well be influenced by way of hypothalamic-pituitary-thyroidal-adrenal-gonadal axis. This thought is consonant with the thesis that the several metabolic alterations of the female reproductive tract are governed by the sum total of the biologically active hormones *and/or* their metabolites acting *in concert.*

Another clinical problem suggesting the interactions of hormones is the Stein–Leventhal syndrome of polycystic ovaries. During the past few years a considerable amount of evidence has been accumulating revealing that the Stein–Leventhal syndrome is another clinical endocrinopathy that may in a similar fashion involve more than one system of glands of internal secretion. The direct surgical approach on the ovaries as recommended by Stein and Cohen suggested that this disease was limited to the ovaries, but ovarian wedge resection did not invariably reverse the clinical career of the disease. Several subsequent reports have indicated the possibility that in some cases adrenal hyperfunction may be basically involved in the several clinical entities associated with the Stein–Leventhal syndrome: hirsutism, obesity, ovarian lesions, and endometrial activity (Wilkins *et al.*, 1950; Buxton and Van de Wiele, 1954). In this complicated picture it is extremely difficult for the clinician to attack with a high degree of assurance only one of the several possible sites of the endocrine disorder that may contribute to the clinical complaints of obesity, hirsutism, amenorrhea,

and sterility. Therefore it seems logical to approach this problem through a series of hormonal tests, the results of which may be useful as a basis for a rational combination of therapeutic regimens.

Furthermore, the demonstration that cortisone therapy reduces the urinary estrogens in patients with mammary cancer adds further credence to the thought that there are numerous interactions among hormones of the adrenal cortex and the ovary (Smith and Emerson, 1954). Kupperman and his associates (1958) have recently reported that in addition to mechanical barriers, one must consider that thyroid, adrenocortical, and ovarian disturbances may also cause infertility in women. These observations suggest that the several induced metabolic alterations of the female reproductive tract in lower mammals may represent a similarity of expression consistent with and diagnostic of the clinically recognizable symptomatology of reproductive dysfunction.

It is rather difficult to define with clarity the complex hormonal interactions that contribute to the formation of certain recognizable clinical syndromes. Biochemical determinations of hormones and their metabolites offer a chance to separate several of these complicated patterns into their component parts, and thereby call attention to functional and dysfunctional pathways that otherwise may be overlooked. In this connection, it should be mentioned that the splendid contributions by Gallagher and Engel serve as long-awaited breakthroughs in the clarification of certain of the metabolic pathways and precursors of the sex hormones in the human female.

In the final analysis, these data suggest that (1) uterine growth and the attendant metabolic alterations associated therewith are governed by the circulating hormones and their metabolites in a physiological system at a specific time; (2) the estrogens exert their effects *in concert*; and (3) numerous interactions occur (a) among the estrogens, (b) among the estrogens and progesterone, (c) among the estrogens and testosterone, and (d) among the estrogens and adrenocorticosteroids. Thus, it seems that the hormonal action observed is not the result of one hormone alone, but more specifically it appears that the metabolic alterations of the uterus are due to *all* of the hormones and their metabolites acting *in concert*.

V. SUMMARY

1. A detailed evaluation of the steroid hormones involved in (a) uterine growth, (b) suppression of the uterine growth-promoting action of estradiol-17β, and (c) the role of the endocrine organs involved in the restricitive actions of estriol and 16-epiestriol was undertaken in an effort to clarify the endocrines, hormones, and factors operative in the growth and inhibition of uterine proliferation. Therefore, it seemed most germane to start with an assessment of the uterine growth-promoting action of estradiol-17β and its five urinary metabolites, the

luteoids, testoids, a number of glucocorticoids and mineralocorticoids as well as the possible interactions of these compounds with estradiol-17β in uterine growth.

2. The potentiality of each of the naturally occurring estrogens was determined by establishing a dose-response curve. These data reveal that estradiol-17β > estrone > 16α hydroxyestrone > 16β-hydroxyestrone > estriol > 16-epiestriol in the promotion of uterine growth.

3. Progesterone displays a slight uterine growth-promoting effect in ovariectomized rats. None of the C_{21} related metabolites of progesterone [the pregnanedios (3α,20α; 3α,20β; 3β,20β), or the pregnanediones (pregnanedione and allopregnanedione)] induced uterine growth.

4. Testosterone in daily doses of 2.0–5.0 mg for 3 days induced marked uterine growth; daily doses of 3.5–5.0 mg effected uterine weights that were indistinguishable from those produced with 0.10 μg estradiol-17β.

5. Of the seven glucocorticoids and mineralocorticoids tested (deoxycorticosterone, 11-dehydro-17-hydroxycorticosterone acetate (E-AC.) 17-hydroxycorticosterone-21 acetate (F-AC), Δ^1F, Δ^1E, 9αFl F, and $\Delta^1$9αFl F), it was observed that deoxycorticosterone was the only corticosteroid which displayed the ability to induce (slight) uterine growth.

6. Numerous interactions exist among the estrogens: both estriol and 16-epiestriol display modulating effects upon the uterine growth-promoting action of estradiol-17β and estrone. The possible interactions among the 16α- and 16β-hydroxyestrones are currently under investigation.

Progesterone at low doses interferes with estradiol-17β-induced uterine growth but synergizes with estradiol when given in larger doses (< 2.5 mg daily).

Testosterone, which displays androgenic, estrogenic, and progestational activity, exerts a marked suppressive effect upon estradiol.

All corticoids thus far tested inhibit to a large degree the action of estradiol-17β. Comparatively, these data reveal that $\Delta^1$9α-Fl F > 9α-Fl F > F > E > Δ^1E and Δ^1F > DCA in restricting the uterine growth-promoting action of estradiol.

7. Neither the pituitary nor the adrenal glands are required for mediation of the inhibiting action of the estriols, progesterone, testosterone, and the adrenal cortical steroids on estradiol-17β. Secondly, data previously obtained by Bever, Velardo, and Hisaw indicate that the inhibitory effects noted above are associated with differences in effect on the enzyme content of the uterus. They showed that estradiol increases the activity of the lactic dehydrogenase-DPNH oxidase system of the uterus by increasing DPNH, whereas the other hormones tend to increase the LDH component. Thirdly, Rosa and Velardo have histochemically localized the DPN and LDH reactive-areas of the uterus, thus adding credence to the biochemical determinations.

8. These data suggest that (1) uterine growth and the attendant metabolic alterations associated therewith are governed by the circulating hormones and their metabolites in a physiological system at a specific time; (2) the estrogens exert their effects *in concert*; and (3) numerous interactions occur (a) among the estrogens, (b) among the estrogens and progesterone, (c) among the estrogens and testosterone, and (d) among the estrogens and adrenocorticosteroids. Thus, it seems that the hormonal action observed is not the result of one hormone alone; more specifically, it appears that the metabolic alterations of the uterus are due to *all* the hormones and their metabolites acting *in concert*.

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Steroids that Act on the Nervous System

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I. Introduction

The most extensively studied steroids are those possessing hormonal properties. Many hormones affect the nervous system, especially the highest centers, thus causing manifold psychic changes. The effects are partly of an indirect type, e.g., when they are the consequence of an altered sex function or follow the suppression of pain caused by inflammation. They may also be more direct, e.g., when they result from augmented metabolism or from a shift in the sodium : potassium balance. In these instances the nervous effects are obviously linked to the hormonal properties of the substances.

Steroids also affect the nervous system quite apart from the hormonal effects. Many steroids entirely devoid of hormonal activity may act either in a depressant or stimulant capacity. In fact, the steroid nucleus seems to predispose to nervous effects of one kind or another, although only occasional compounds of this type are as active as the most active comparable nonsteroidal drugs. As it is practically impossible to separate the hormonal from the nonhormonal nervous effects in steroids with both properties, this presentation will deal only with *hormonally inactive steroids*. Discussion of effects will be restricted to the action on the brain and spinal cord, although effects on the autonomic nervous system as well as peripheral effects (local anesthesia, muscle paralysis) have also been observed.

Pharmacological classification of drugs acting on the central nervous system is difficult. They may, of course, be categorized broadly as *depressants*, comprising anesthetics, hypnotics, sedatives and tranquilizers, analgesics, and anticonvulsants, or as *stimulants* (or antidepressants), comprising antianesthetics, analeptics, energizers, and convulsants. Within these two groups, however, sharp boundaries are lacking and some drugs may even act as a stimulant in one species and as a depressant in another, or as a depressant shortly after administration and as a stimulant at some later time. As the steroids have been much less widely investigated in these respects than other drugs, it is still more

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difficult to label them correctly. It should be realized that anesthetic steroids may turn out to be much more useful as hypnotics, tranquilizers or anticonvulsants than as general anesthetics. Hence a study using many different pharmacological methods is indicated (see the paper by Bonta and Overbeek in Volume II).

Simple alterations of the molecule not affecting the basic chemical structure, such as esterification of the same alcohol with various acids, may profoundly modify the effect by altering the rate of absorption or excretion and hence the concentration in the blood and/or the liquor. Such esterification may turn an anesthetic into a tranquilizer. For instance we found the hemisuccinate of 3α-hydroxy-5β-pregnane-11,20-dione to be a potent anesthetic, the activity being of the same order of magnitude as that of the hemisuccinate of 21-hydroxy-5β-pregnane-3,20-dione (hydroxydione). The free alcohol also has anesthetic activity, but when esterified with carbaminic acid it is no longer possible to anesthetize experimental animals, although barbiturate anesthesia is prolonged and motility decreased. This is shown in Table I. which

TABLE I

nfluence of Esterification

Parameter	Effective dose (mg/kg)[a]			
	3α-hydroxy-5β-pregnane-11,20-dione			Hydroxydione hemisuccinate
	Unesterified	Hemisuccinate	Carbamate	
Anesthesia				
I. v.	—	36 (40)	—	33 (40)
I. p.	50 (12)	—	>100 (6)	—
Oral	200 (6)	—	>400 (6)	—
Tranquilization				
prolongation of anesthesia i. p.	—	—	50 (6)	—
Exploratory behavior s. c.	—	—	100 (8)	—

[a]Number of mice is in given parentheses.

records the anesthetic and tranquilizing doses as found in mice.

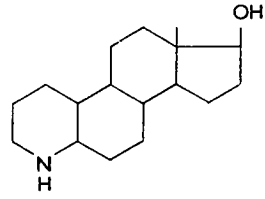
Similar modifications of pharmacological actions could perhaps also be achieved by pharmaceutical means by using a different solvent or by modifying the composition of tablets.

Groups of steroids found to be of particular interest are discussed in the sections that follow. It is perhaps not surprising that a number of these steroids contain nitrogen.

II. STEROIDS CONTAINING NITROGEN

A. NITROGEN IN THE STEROID NUCLEUS

1. 4-Azaestranes (I)



In the 4-azaestranes, carbon atom 4 has been replaced by nitrogen and they are 19-nor compounds. C. C. Bolt (Chemical Research Department, N. V. Organon, Oss, the Netherlands) prepared four of them, all containing a 17β-hydroxy group. Three compounds possessed a keto group at C-3, and at C-17 either H, CH_3 or C_2H_5. The fourth compound was a 17-methyl derivative in which the oxygen atom at C-3 was absent. All four compounds displayed an antidepressant activity in mice. They shortened the duration of anesthesia when they were administered 24 hours before the anesthetic. When given only 1 hour before the anesthetic they had the opposite effect (see the article by Bonta and Overbeek in Volume II). The biphasic effect on the duration of anesthesia is also given by monoamineoxidase inhibitors like iproniazid. However the 4-azaestranes do not inhibit this enzyme. They exert their antidepressant effect by augmenting the elimination of the anesthetics both from the brain and from the whole carcass, as shown by Table II

TABLE II

Effect of 17α-methyl-17β-hydroxy-4-azaestran-3-one on the Elimination of Hexobarbital

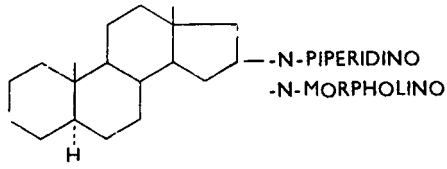
Treatment 24 hours in advance, orally	Time after hexobarbital (minutes)	Hexobarbital (μg/gm ± SE)[a]		Number of mice in anesthesia
		Brain	Carcass	
Control	3.0	63 ± 6.125 (4)	45.5 ± 1.890 (2)	12
	4.5	43 ± 1.225 (4)	42.5 ± 6.930 (2)	9
	6.75	39 ± 4.165 (4)	39.5 ± 10.710 (2)	11
	10.0	35 ± 4.655 (4)	33.0 ± 5.040 (2)	7
Treated 200 mg/kg	3.0	40 ± 3.920 (4)	33.5 ± 5.670 (2)	10
	4.5	36 ± 4.655 (4)	32.0 ± 12.600 (2)	6
	6.75	28 ± 1.225 (4)	28.0 ± 7.560 (2)	2
	10.0	19 ± 1.225 (4)	30.0 ± 2.520 (2)	0

[a]Number of determinations given in parentheses. SE = standard error of the mean.

for hexobarbital. So far we do not know by what mechanism of action this is achieved.

B. NITROGEN OUTSIDE THE STEROID NUCLEUS

1. 16β-N-piperidino- or morpholino-5α-androstanes (II)



The compounds were prepared by C. L. Hewett (Chemical Research Department, Organon Laboratories Ltd., Newhouse, Great Britain). They contained either a keto or a hydroxy group at C-3 or C-17. Among them we found both convulsants and tranquilizers.

16β-*N*-Piperidino-5α-androstane-3,17-dione displayed effects strongly suggesting tranquilizing activity (for the properties of other compounds see the list at the end of this paper). Table III shows the dose-

TABLE III

Effect of 16β-piperidino-5α-androstane-3.17-dione on Exploratory Behavior in Mice

Dose[a] (mg/kg)	Inhibition[b] (%)	95% Confidence limits
10	4	–30/31
25	45	17/75[c]
37	59	37/83[c]
56	76	54/103[c]

[a]Oral administrat on 1 hour before experiment.
[b]Means of 32 mice.
[c]Significant.

dependent inhibition of exploratory behavior in mice (Bonta *et al.*, 1960); Table IV, for the agressiveness of the Siamese fighting fish

TABLE IV

Effect of 16β-piperidino-5α-androstane-3,17-dione on Fighting Behavior in Betta splendens[a]

Dose (μg/ml)	Number of fishes	Inhibition (%)	95% Confidence limits
10	24	46	–12/74
25	28	63	26/81[b]
50	4	86	11/98[b]

[a]Incubation time 45 minutes.
[b]Significant.

Betta Splendens; and Table V, the effect on body temperature. Further-

TABLE V

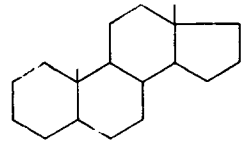
Effect of 16β-piperdino-5α-androstane-3,17-dione on Body temperature in mice[a]

Dose (mg/kg)	Rectal temperature (°C)				
	Control period	Hours after s. c. injection			
		1	3	5	24
0	37.1	36.8	36.7	36.2	35.5
160	36.9	35.8	35.8	36.0	35.3
200	36.9	35.5	34.8	34.8	36.0
250	37.1	34.9	34.4	34.6	36.4

[a]Room temperature 18–22°C. Values are Means of 20 mice.

more, hexobarbital anesthesia was prolonged, amphetamine toxicity in grouped mice was reduced, and after subcutaneous injection a synchronous pattern in the electroencephalogram of the rabbit was induced.

2. 3α-Amino-5α-pregnanes and Pregnene (III)



The 3α-amino-5α-pregnanes containing either a keto group at C-20 (funtumine) or an α-hydroxy group at that site (funtumidine) were found in the leaves of *Funtumia latifolia* by Janot and Goutarel (1958). The pharmacological properties were described by Quevanviller and Blanpin (1958). These appear to be very similar for both substances. They augment the anesthetizing affect of barbiturates, are hypothermic, and were found to have analgesic activity in the test of d'Amour and Smith (1941). (Apart from these effects on the central nervous system, they stimulate the heart and are sympatholytics and hypotensives.) The 3α-aminopregn-5-ene (holamine) also occurs in plant material, i.e., in the leaves of *Holarrhena floribunda*; a pharmacological study was performed by Blanpin (1960). This compound causes convulsions, tremor, and catatonia; it is hypothermic and depresses respiration. The effect of barbiturates is influenced in quite a different manner than by the above-mentioned compounds. The depressant effect on respiration increases the toxicity of the barbiturate but there is no actual reinforcement of the anesthetic effect.

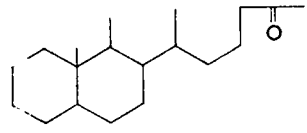
The compounds described above all contained nitrogen. The following examples demonstrate that this is not essential for obtaining effects on the central nervous system.

III. STEROIDS NOT CONTAINING NITROGEN

A. 5α- AND 5β-PREGNANES (IV)

The first marked effects of steroids on the central nervous system were reported by Selye (1941). He could obtain general anesthesia in rats with deoxycorticosterone and progesterone, i.e., two pregnene derivatives. Shortly thereafter he studied a series of 75 steroids in this respect (Selye, 1942). The latter investigation proved that the anesthetic activity was not linked with any hormonal property. Useful information of this kind on a number of other steroids can also be found in the paper by Figdor *et al.* (1957).

The saturated pregnane skeleton (both 5α- and 5β) appears to offer many possibilities for the synthesis of central nervous depressants devoid of hormone properties. Among these pregnanes even general anesthetics, with an activity of the same order as the barbiturates, are found. Various substitutions are possible without losing the activity.

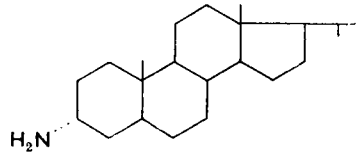


A hydroxyl group may be present at C-3 or C-21, a keto group at C-3 or C-11, and we found a C-nor compound (3β-hydroxy-5α-*C*-norpregnane-11,20-dione) to be a rather potent anesthetic. Selye (1942) described an active D-homo-compound (17aβ-methyl-3β,17aα-dihydroxy-D-homoandrost-5-en-17-one). We have already mentioned (Table I) the anesthetic activity of the two 5β-pregnanes: 3α-hydroxy-5β-pregnane-11,20-dione-3-hemisuccinate and 21-hydroxy-5β-pregnane-3,20-dione-21-hemisuccinate. The latter compound first described by Laubach *et al.* (1955) and P'An *et al.* (1955) has been extensively studied pharmacologically and clinically by many investigators. Although they agree for the most part on the highly satisfactory anesthetic properties of the substance, serious thrombophlebitis has occurred in several instances.

One other interesting pregnane derivative is 6β-hydroxy-3,5-cyclopregnan-20-one, described by Hardwick *et al.* (1957). Animal experiments indicated that this substance might be a new tranquilizer, but clinical results seem not to have substantiated these expectations.

Finally the surprising observations by Glickman *et al.* (1960) and Tilling (1962) that 3α-hydroxy-5β-pregnan-20-one and 3α-hydroxy-5β-pregnane-11,20-dione are pyrogenic should be mentioned. Since this is probably the result of central stimulation it is unexpected that the second substance (an anesthetic) would have this effect.

B. ANDROSTANES (V)



This group contains fewer centrally active substances devoid of hormone properties. The only substance worth mentioning is 3β,16α-dihydroxyandrost-5-ene, first described by Huffman (1956). According to Huffman it would display a powerful tranquilizing effect in acute alcohol withdrawal symptoms in humans, besides having central hypotensive effect.

Several of the 5β-androstanes are pyrogenic (compare the pyrogenic effect of the 5β-pregnanes mentioned at the end of Section III, A.) This has been described, for instance, for 3α-hydroxy-5β-androstan-17-one and for 3α,11β-dihydroxy-5β-androstan-17-one (Glickman *et al.* (1960).

Another 5β-androstane (17β-hydroxy-5β-androstan-3-one) has some anti-LSD 25 activity, although less than several steroids with hormonal activity (Bergen *et al.*, 1960).

IV. CONCLUSION

The above data warrant very few conclusions on possible relations between chemical structure and pharmacological activity. At least it seems that stereochemical differences like 5α or 5β and 3α or 3β have less bearing on effectiveness than is the case when hormone properties are concerned.

As the above description has been classified according to chemical structure, it may be useful to add the following list of the same steroids according to a pharmacological classification (with the restrictions mentioned in the introduction).

1. Anesthetics and hypnotics (?)	21-Hydroxy-5β-pregnane-3,20-dione-21-hemisuccinate
	3α-Hydroxy-5β-pregnane-11,20-dione-3 hemisuccinate
	3β-Hydroxy-5α-*C*-nor-pregnane-11,20-dione
2. Sedatives and tranquilizers	6β-Hydroxy-3:5-cyclopregnan-20-one
	3α-Amino-5α-pregnan-20-one
	3α-Amino-20α-hydroxy-5α-pregnan-20-one
	3β,16α-Dihydroxyandrost-5-ene
	16β-*N*-Piperidino-5α-androstane-3,17-dione

3. Anticonvulsants (antistrychnine)	16β-*N*-Morpholino-3β-hydroxy-5α-androstan-17-one
	3α-Hydroxy-5β-pregnane-11,20-dione
4. Anti-LSD 25	17β-Hydroxy-5β-androstan-3-one
5. Antianesthetics	17β-Hydroxy-4-azaestran-3-one
	17α-Methyl-17β-hydroxy-4-azaestrane
	17α-Ethyl-17β-hydroxy-4-azaestran-3-one
	17α-Methyl-17β-hydroxy-4-azaestran-3-one
6. Convulsants	3α-Aminopregn-4-on-20-one
	17α-Hydroxy-5β-pregnane
	17α-Hydroxy-5α-pregnane
	16β-*N*-Piperidino-17β-hydroxy-5α-androstan-3-one
	16β-*N*-Piperidino-3β,17β-dihydroxy-5α-androstane
7. Pyrogens	3α-Hydroxy-5β-pregnan-20-one
	3α-Hydroxy-5β-pregnane-11,20-dione
	3α,11β-Dihydroxy-5β-androstan-17-one
	3α-Hydroxy-5β-androstan-17-one

The results described in this paper demonstrate that many hormonally inactive steroids exert various actions on the central nervous system. Some of them were found to possess activities quantitatively comparable with activities of nonsteroid drugs. A further exploration of this field seems worthwhile.

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DISCUSSION

Specificity of Steroid Action

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We do not know whether steroids acting as anesthetics are uncoupling agents or on what part of the respiratory chain phosphorylations are uncoupled from oxidations. In this regard 3-α-hydroxy-5β-pregnane-11,20 dione hemisuccinate seems to act in the same manner as hydroxydione.

We must know much more about the specificity of effect of steroids acting on the central nervous system (CNS): Is their effect really specific or is it similar to that of non-steroid drugs acting on the CNS. Do they act by blocking electron transport mechanism, by blocking DPNH dehydrogenase in the case of sedative or hypnotic effects? Do they act by producing DPN analogs or by effecting nervous cell membrane permeability when they are convulsants? Are they able to block any part of the Krebs cycle or to increase the glucose-6-phosphate dehydrogenase activity, i.e., to favor the HMP shunt?

I recall the work of Greig,[1] who showed that barbiturate anesthesia in rats can be reversed by ascorbic acid administration. This was verified by Miss Herold[2] in my laboratory for many other central depressant drugs, such as hydroxydione, and for prolongation of barbiturate anesthesia by androstanolone or progesterone. If we accept the fact that ascorbic acid can act as electron acceptor, this seems to show that blocking the site of electron transport in the respiratory chain is an unspecific effect of all centrally active drugs, including steroids.

On the other hand, it is possible that the sedative effect is a consequence of an increased tryptophan peroxidase (TPO) activity and the antidepressant effect is, on the contrary, due to blocking TPO activity, e.g., with imipramine or Monoaminoxydase inhibitors.

Central excitation can be due also to electron spin resonance of a phenolic ring or to oxidoreduction potential provided by hydroxyl groups of phenolic rings.

We must know whether the pharmacological properties of nonhormonal steroids are due to side chains; in this case the steroid structure could be considered as a carrier.

[1]Greig, M. E., *J. Pharmacol. Exp. Therap.* **87**, 185, 1946.

[2]Herold, M., Georges. G., and Cahn, J. (1958). *In* "Psychotropic Drugs" (S. Garattini and V. Ghetti, eds.) Elsevier, Amsterdam.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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Direct and Indirect Effects of Deoxycorticosterone (DOC) on Skeletal Muscle Electrolyte and Acid-Base Metabolism[1]

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I. GENERAL INTRODUCTION

It is well known that the adrenal mineralocorticoids, deoxycorticosterone (DOC) and aldosterone, enhance the reabsorption of sodium and the excretion of potassium in the renal tubule (see review by Kruhøffer, 1960). If treatment with these steroids is excessive, alterations occur in the normal electrolyte pattern of extracellular fluid. These extracellular changes can cause distortions in cellular electrolyte balance: e.g., hypokalemia promotes a shift of potassium from muscle cells into plasma and of sodium from extracellular fluid into cells (Heppel, 1939; Miller and Darrow, 1940; Muntwyler and Griffin, 1951). Thus, it is possible for an adrenocortical hormone to affect electrolyte concentrations at one body site by exerting its primary effect at another place in the body; this type of change can be referred to as an *indirect* effect of the hormone.

Other effects of the mineralocorticoids upon electrolyte metabolism have been described which are not secondary to their effects upon electrolyte excretion. *In vitro* effects of these agents have been demonstrated on isolated smooth muscle (Bass and Setliff, 1960), isolated frog skin (Taubenhaus *et al.*, 1956), and red blood cells (Wilson, 1957). In addition, effects of these steroids on intestinal electrolyte absorption (Berger *et al.*, 1960) and secretion of electrolytes by exocrine glands, e.g., sweat (Conn, 1949), cannot easily be explained as secondary effects of the drug. Further, gross electrolyte shifts without changes in renal excretion of potassium or sodium have been observed (Barnett and McNamara, 1949; Luft and Sjögren, 1952). Therefore, the mineralocorticoids are capable of producing electrolyte changes in a manner that can be called a *direct* effect of the drug.

The pattern of tissue electrolyte changes produced by the administration of either DOC or aldosterone would be a summation of both

[1] Supported by N. I. H. grants NB-00381 and 2G-153.

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the direct and the indirect effects. Thus, any tissue studies in which the two effects were not clearly separated could give misleading results concerning the basic mechanisms responsible for such a change. Such mechanisms have not been clearly delineated in skeletal muscle. Hence the experiments reported here were designed to study the direct and the indirect effects of the steroid DOC on electrolyte metabolism in this tissue.

II. EXPERIMENTAL INTRODUCTION

A. CHRONIC DOC ADMINISTRATION

Woodbury and Koch (1957) showed that treatment of mice for 4 days with DOC produced an increase in sodium concentration in skeletal muscle cells. Although DOC did not affect sodium content in cerebral cortex in this particular experiment, the authors cited other work from their laboratory demonstrating that DOC was capable of producing a decrease in cellular sodium in brain. In contrast, equi-sodium-retaining doses of aldosterone administered for the same period caused a decrease in intracellular sodium in both brain and muscle. These results were interpreted by the authors as indicating that aldosterone produced a direct effect on transport processes in both tissues. However, they hypothesized that the changes in muscle electrolytes observed after the administration of DOC resulted indirectly from the induced renal potassium loss and not from a direct effect of the steroid on muscle membrane.

Another effect of DOC which has been explained as an effect of the steroid secondary to its promotion of potassium excretion is its distortion of acid-base balance. Many workers have demonstrated that DOC administration causes an increase in blood pH and in plasma bicarbonate concentration (Perera, 1948; Seldin *et al.*, 1954). It is also well known that animals maintained on a potassium-deficient diet exhibit the same acid-base changes (see review by Moore *et al.*, 1955). An observation common to both the DOC and dietary experiments is that tissue and plasma potassium concentrations are below normal. Thus, it has been assumed that DOC alters acid-base parameters because of its marked effect on potassium excretion, which, in turn, lowers total body potassium.

Therefore, it was decided to study cell pH and electrolyte changes by performing experiments in which any effects of DOC mediated through potassium metabolism were minimized. It was felt that this could be accomplished by giving treated animals potassium salts in their drinking water, inasmuch as Ferrebee *et al.* (1941) demonstrated that DOC-induced sodium accumulation in muscle could be prevented by this procedure.

B. EVISCERATION STUDIES

Direct effects of DOC on tissue electrolytes could also be studied by preventing the loss of body potassium usually induced by the drug. This could be accomplished by eliminating the major excretory routes for potassium ion.

In addition to the well-known renal excretion of potassium induced by DOC, Woodbury (1953) demonstrated the quantitative importance of the potassium loss into the gastrointestinal tract during DOC treatment. Therefore it was decided to study the direct effects of DOC upon muscle in an eviscerated-nephrectomized preparation.

III. METHODS

A. CHRONIC DOC ADMINISTRATION

Male Sprague–Dawley rats weighing approximately 150 gm were implanted subcutaneously with twelve pellets (15 mg each) of Percorten (CIBA). The dose of DOC, estimated from the animal's weight and the pellet absorption data of Herxheimer and Woodbury (1960), was approximately 6 mg/kg daily. Potassium chloride was administered by allowing the animals to drink ad libitum a solution containing 150 mEq KCl per liter. The animals were fed ordinary rat chow during the entire experimental period. Thirtyone days later the animals were sacrificed by anaerobic exsanguination via the abdominal aorta. Two microcuries of radioactive (2-C^{14}) 5,5' -dimethyl-2,4-oxazolidinedione (DMO) was given intraperitoneally with a 25 mg/kg unlabeled carrier dose of drug, 4 hours before sacrifice. Blood pH was determined at 37°C and plasma was separated under oil for total carbon dioxide and electrolyte analysis.

Sodium and potassium concentrations in plasma and in 1 *N* HNO_3 extracts of tissue were determined by flame photometry. Chloride analysis of plasma and tissue acid extracts was made by amperometric titration with silver ion (Cotlove *et al.*, 1958). Total carbon dioxide in plasma was measured by the gas chromatographic method. Radioactivity was measured by counting in a thin-window flow counter. Cellular electrolytes were calculated by the use of chloride space, as suggested by Hastings and Eichelberger (1937). Cellular pH calculations based on DMO data were done as suggested by Waddell and Butler (1959).

B. EVISCERATION STUDIES

Male Sprague–Dawley rats weighing approximately 350 gm were functionally eviscerated by the method of Russell (1942). At the same time, the adrenals were removed and a functional nephrectomy was performed by splitting the renal capsule and ligating the renal pedicle. A dose of 50 mg of deoxycorticosterone acetate per kilogram body

weight was given subcutaneously as a suspension in isotonic sodium chloride solution containing Tween 80. Eviscerated animals were injected subcutaneously with 1.5 ml of 20% glucose solution immediately postoperatively and at 2 and 4 hours thereafter. Six hours after surgery the animals were sacrificed under ether anesthesia by exsanguination via the abdominal aorta. Blood and tissue sample analyses and calculation of intracellular electrolyte concentrations were done as described in Section III,A.

IV. RESULTS

A. CHRONIC DOC ADMINISTRATION

The effects of chronic DOC administration on extracellular tissue and plasma electrolyte and acid-base values are given in Table I. The

TABLE I

Effect of DOC and Potassium Chloride, Alone and Together, on Plasma and Tissue Electrolyte and Acid-Base Values[a,b]

Part I: PLASMA

Treatment	H_2O %	pH	Total Co_2 (mmole/l)	Total DMO (cpm/ml)	Na (mEq/liter)	K (mEq/liter)	Cl (mEq/liter)
Control (5)	93.3 ±0.1	7.43 ±0.04	23 8 ±0.9	305 ±17	140.6 ±0.5	4.57 ±0.31	102.3 ±1.1
Control+KCl (4)	93.2 ±0.2	7.43 ±0.01	25.2 ±0.6	327 ±18	140.2 ±0.6	4.11 ±0.13	102.6 ±1.2
DOC (5)	93.1 ±0.1	7.55** ±0.01	34.3** ±2.3	288 ±19	142.1 ±0.7	2.75** ±0.04	90.7*** ±1.1
DOC+KCl (4)	93.1 ±0.1	7.47 ±0.01	28.1* ±2.6	302 ±17	139.4 ±0.5	3.45* ±0.11	98.1** ±1.1

Part II. MUSCLE

Treatment	H_2O (%)	Total DMO (cpm/gm)	Na (mEq/kg wet tissue)	K (mEq/kg wet tissue)	Cl (mEq/kg wet tissue)
Control (5)	74.5 ±0.3	117 ±14	19.82 ±0.81	108.3 ±1.2	15 28 ±0.61
Control+KCl (4)	74.6 ±3.1	101 ±13	20.98 ±0.83	107.7 ±3,2	15.79 ±0.73
DOC (5)	74.8 ±0.5	85 ±11	36.36*** ±1.64	88.5*** ±3.3	15.22 ±1.1
DOC+KCl (4)	75.9 ±0.3	94 ± 4	21.94 ±0.92	103.9 ±2.4	13.90* ±0.39

Part III: BRAIN

Treatment	H_2O (%)	Na	K	Cl
		mEq/kg wet tissue		
Control (5)	78.1	48.60	107.2	31.56
	±0.6	±1.43	±2.7	±1.20
Control+KCl (4)	78.3	46.88	104.6	30.11
	±0.5	±1.69	±2.9	±1.02
DOC (5)	77.7	47 83	108.0	29.69
	±0.6	±1.07	±1.5	±0.88
DOC+KCl (4)	78.2	48.30	107.9	31.59
	±0.9	±2.01	±2.5	±0.99

[a]Values in this and all subsequent tables are means ± standard errors. Numbers in parentheses are the number of animals studied.

[b]Asterisks in this and all subsequent tables denote statistical significance: ***$p = <0.001$; **$p=0.01$–0.001; *$p=0.05$—0.01; none, $p=>0.05$. All statistical comparisons are made to the control group.

steroid caused an increase in plasma pH and total carbon dioxide concentration. Substitution of these data into the Henderson–Hasselbach equation shows that the increase in plasma pH is due to an increase in plasma bicarbonate concentration. DOC treatment caused a significant decrease in plasma potassium and chloride values. Plasma water and plasma DMO and sodium values were not affected by any treatment. Potassium chloride supplementation effectively reversed the increase in plasma pH, but did not entirely correct the DOC-induced distortions in plasma carbon dioxide, potassium, and chloride levels.

Total muscle sodium concentration rose and total muscle potassium concentration fell in response to DOC treatment. These changes were not observed and the values were in the normal range in the DOC-treated animals given extra potassium in their drinking water. Total tissue water and tissue DMO concentrations were not altered in any animal groups.

Brain water and total electrolyte concentrations in brain were unchanged throughout the experiment.

Table II gives the intracellular values derived from data in Table I. Muscle cells in DOC-treated animals show a marked accumulation of sodium and a large depletion of potassium ions. In addition, muscle cells in the same animals became more acid after DOC administration. Potassium treatment did not entirely prevent the cellular distortions in potassium and sodium but did permit the cell pH to remain in the normal range.

Intracellular brain sodium concentration was definitely decreased in the DOC-treated animals. Although the brain cell sodium value in the

TABLE II

Effect of DOC and Potassium Chloride, Alone and Together, on Tissue Intracellular Electrolytes and Acid-Base Values[a,b]

Treatment	Cell pH	Cl space (%)		H_2O (%)		Na		K	
						mEq/kg Cell water			
	Muscle	Muscle	Brain	Muscle	Brain	Muscle	Brain	Muscle	Brain
Control (5)	6.96	13.3	27.4	61.3	50.7	2.77	18.64	175.8	210.1
	±0.08	±0.6	±1.0	±0.9	±1.5	±0.56	±2.11	±0.6	±11.2
Control+KCl (4)	6.87	13.6	26.0	61.2	52.3	2.44	18.71	175.4	198.9
	±0.14	±0.6	±1.6	±1.2	±1.5	±0,47	±1.45	±6.1	±11.8
DOC (5)	6.60*	14.9	29.0	60.0	48.8	24.7***	11.85*	146.9***	220.9
	±0.07	±1.2	±1.0	±1.3	±1.5	±0.9	±2.1	±4.2	± 9.3
DOC+KCl (4)	6.86	12.5	28.5	63.4	49.6	6.49**	15.83	163.2**	216.2
	±0.07	±0.4	±1.0	±0.7	±1.8	±0.84	±1.8	±3.1	±15.9

[a,b]See footnotes, Table I.

potassium chloride-DOC group was lower than normal, the reduction was not statistically significant.

Intracellular water concentration was not altered in any experimental group. Chloride space tended to increase in both brain and muscle, but the increase was not significant.

B. EVISCERATION STUDIES

The results of these studies are presented in Table III. Administra-

TABLE III

Effect of DOC on Total Electrolytes and Water in Plasma, Brain, and Skeletal Muscle of Eviscerated Rats[a,b]

Part I: PLASMA

Treatment	H_2O (%)	Na	K	Cl
		mEq/liter		
Controls (7)	91.67 ±0.11	134.7 ±2.6	5.19 ±0.17	105.2 ±1.54
DOC (7)	92.04 ±0.095	136.2 ±2.1	6.80*** ±0.058	101.1 ±1.8

Part II: BRAIN

Treatment	H_2O (%)	Na	K	Cl
		mEq/kg wet tissue		
Controls (7)	78.36 ±0.21	48.96 ±1.1	107.8 ±1.4	28.87 ±1.2
DOC (7)	78.62 ±0.16	47.59 ±0.88	108.9 ±0.79	32.42 ±1.3

Part III: MUSCLE

Treatment	H_2O (%)	Na	K	Cl
		mEq/kg wet tissue		
Controls (7)	75.12 ±0.26	15.90 ±0.36	103.3 ±1.6	8.95 ±0.45
DOC (7)	75.41 ±0.19	15.31 ±0.56	96.6** ±1.5	11.04* ±0.83

[a,b]See footnotes, Table I.

tion of DOC caused an increase in plasma potassium level with little change in plasma sodium or chloride concentrations. Brain tissue electrolytes exhibited no significant changes following DOC treatment. Decreases in total tissue potassium and chloride concentrations were observed in the skeletal muscle of DOC-treated animals.

Intracellular data from this same experiment are given in Table IV.

TABLE IV

Effect of DOC on Extracellular and Intracellular Distribution of Electrolytes and Water in Brain and Muscle of Eviscerated Rats[a,b]

Treatment	Cl space (%)		H_2O (%)		Na		K	
					mEq/kg Cell water			
	Brain	Muscle	Brain	Muscle	Brain	Muscle	Brain	Muscle
Controls (7)	24.06	7.11	54.38	68.01	29.04	8.84	195.2	151.5
	±1.2	±.33	±1.4	±.46	±1.7	±.86	±6.0	±2.9
DOC (7)	27.67	9.42**	50.51	65.81*	16.82**	3.23**	212.7	145.7
	±1.4	±.72	±1.4	±.83	±3.0	±.25	±6.0	±2.0

[a,b]See footnotes, Table I.

Chloride space increased significantly in muscle after DOC treatment; but the increase in brain chloride space, though definite, was not statistically significant. Brain and muscle intracellular water decreased following DOC administration. Sodium concentration in both brain and muscle cells was lowered in the DOC-treated animals. Cellular potassium changes were variable and not statistically significant.

V. DISCUSSION

Acute treatment of eviscerated animals with DOC caused a decrease in sodium concentration in skeletal muscle cells. Chronic administration of DOC to intact animals, in contrast, caused a sodium ion, as well as a hydrogen ion, accumulation in muscle cells. The chronic effects of DOC in intact animals were essentially prevented by giving treated animals potassium supplements in their drinking water. Brain intracellular sodium levels were decreased by DOC treatment in both the chronic intact and the acute eviscerated preparations.

The effect of DOC on skeletal muscle in the eviscerated preparation is best explained by assuming that DOC enhances sodium transport out of muscle cells. Although definitive experiments have not been done, there is much indirect evidence that DOC increases sodium pump activity in the renal tubule (Kruhøffer, 1960), frog skin (Taubenhaus *et al.*, 1956), and the gastrointestinal tract (Berger *et al.*, 1960). This direct effect of DOC in muscle has also been observed by Vernadakis and Woodbury (unpublished observations) who showed that the steroid decreased muscle cell sodium concentration in neonatal rats in which renal, and probably gastrointestinal, excretion of potassium is limited.

The decrease in brain cell sodium produced by DOC in the eviscerated animals is also due to the increase in sodium efflux produced by the compound. Brain sodium was also decreased by DOC in chronically treated animals, even though plasma potassium values fell in the experiment. However, in contrast to muscle in the same animals, potassium in brain did not fall despite the marked decrease in extracel-

lular potassium. An explanation for this observation is not available at this time.

The muscle electrolyte changes observed in the chronic DOC experiments are, in several ways, strikingly similar to those observed in several classic potassium-deficient-diet experiments (Miller and Darrow, 1940; Irvine *et al.*, 1961). Muscle cells lost potassium and accumulated sodium and hydrogen ions, and extracellular fluid gained bicarbonate and lost potassium ions. The similarity of the above findings to our own results and the fact that, in our experiments, potassium supplements largely prevented these distortions lead to the conclusion that the observed effects are due to potassium losses produced by DOC. Therefore, it seems reasonable to discuss the chronic DOC effect on muscle as it relates to potassium metabolism without, for the moment at least, considering any direct tissue effect of the steroid.

Cooke *et al.* (1952) explained muscle electrolyte shifts secondary to hypokalemia by proposing that extracellular hydrogen and sodium ions exchange with intracellular potassium ions whenever extracellular potassium levels decrease. However, no detailed mechanism for this exchange involving known physiological processes was presented. In another report (Cooke and Segar, 1952), it was suggested that there is a diminished exchange of intracellular sodium and hydrogen ions for extracellular potassium ions whenever extracellular potassium levels decrease. A hypothetical cation-exchange resin was proposed as the normal mediator of this exchange, but again specific mechanistic details were lacking.

A more likely interpretation of the indirect effects of DOC on skeletal muscle can be made by considering the effects of hypokalemia on active ion transport. It is well-known that sodium is actively extruded from muscle cells (Harris, 1956). Caldwell (1958) has reported that hydrogen ion is not passively distributed across muscle cells because the intracellular hydrogen ion concentration is lower than the value predicted from simultaneous membrane potential and extracellular pH measurements. Although an inward transport of bicarbonate ion could explain this relatively alkaline state of the cell, it is more likely that active hydrogen ion transport out of muscle cells accounts for the hydrogen ion deficit (Woodbury, 1960). In addition, it has been proposed that sodium and hydrogen ion are carried by a common mechanism in frog skin (Huf *et al.*, 1951; Schoffeniels, 1956) and in muscle (see Woodbury, 1960). Furthermore, transport of sodium is dependently coupled to potassium influx such that a decrease in extracellular potassium decreases the rate of sodium efflux from cells (Koefoed-Johnsen and Ussing, 1958; Woodbury, 1960). Therefore, hypokalemia would result in a retention of both sodium and hydrogen ions in cells since both are transported out of cells by a common carrier mechanism whose efficiency decreases when extracellular potassium falls. The decreased rate

of potassium entering cells would account for the lowered potassium concentration in muscle observed during DOC treatment. Replacement of potassium in the extracellular fluid would restore the inhibited transport mechanism to its normal rate of activity and correct the electrolyte distortions in muscle caused by hypokalemia.

From the preceding discussion, it is obvious that DOC does indeed exert two effects upon skeletal muscle. First, DOC enhances sodium pumping by a direct effect which is independent of potassium metabolism. This direct effect has not been previously described and has not been fully explored. For example, simultaneous determination of direct DOC effects on cellular hydrogen and sodium ion concentration would be an ideal way to test further the hypothesis that muscle contains a common sodium-hydrogen ion carrier. At the present time, experiments concerning this problem are being done in our laboratories by measuring simultaneously the cell pH and the sodium ion changes in neonatal and eviscerated rats treated with DOC. Second, DOC produces an indirect effect upon skeletal muscle which is linked to potassium excretion. The sequence of events in the production of the indirect effect is as follows: DOC administration causes a marked increase in the urinary and gastrointestinal loss of potassium. This potassium loss, in turn, causes hypokalemia. As a result of the hypokalemia, active extrusion of sodium and hydrogen ions from muscle cells is slowed, and an intracellular acidosis and sodium accumulation occur.

Woodbury and Koch (1957) reported that aldosterone, in contrast to DOC, caused sodium depletion in skeletal muscle cells. These investigators pointed out that the dose of aldosterone used in the experiment produced much less potassium excretion than did an equi-sodium-retaining dose of DOC. That is, when these results are interpreted in terms of our hypothesis, the indirect effect of aldosterone upon skeletal muscle, mediated through potassium loss, is less than that produced by a comparable dose of DOC. Thus, the direct effect of aldosterone upon muscle predominates even in intact animals, and a decrease in cellular sodium results. The fact that two steroids with qualitatively similar properties can produce directly contrasting effects upon muscle in intact animals shows the importance of separating direct from indirect effects in studies of hormonal mechanisms of action. Further elucidation of our understanding of the physiological alterations produced by adrenal mineralocorticoids depends upon investigations in which this separation is made.

ACKNOWLEDGMENTS

The authors wish to thank Miss Adelia Baird and Mr. Dale Reynolds for their excellent technical assistance.

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DISCUSSION

Effects of Anti-inflammatory Steroids on Electrolyte Metabolism

G. Faludi

The results of the beautiful experiments of Drs. Withrow and Woodbury give at least a little insight into the difficult problem of electrolyte metabolism in the living muscle tissue, a problem which is puzzling so many of us.

My field of steroid research is very different, but I too was very curious to know what happens to the electrolyte metabolism of muscles during administration of large doses of anti-inflammatory steroids. Therefore, during induction of myopathy with large doses of anti-inflammatory steroids (100 mg hydrocortisone or the equivalent, 25 mg prednisolone, 20 mg methylprednisolone, 4 mg dexamethasone, or 20 mg triamcinolone daily) in dogs, we did serial serum electrolyte determinations. There were no changes detectable in the blood samples taken at weekly intervals (as compared with the saline control groups) during the entire period of 5 weeks' therapy, although very marked myopathy developed in all the steroid-treated animals.

Our experience was also similar in mice. We treated 288 mice with various anti-inflammatory steroids for a period of 4 weeks. We injected hydrocortisone, prednisolone, methylprednisolone, dexamethasone, triamcinolone, paramethasone, and betamethasone at three different dose levels intraperitoneally, daily. Blood was drawn from each animal at the completion of 4 weeks' therapy (Table I). Each value represents the mean value of 36 animals. There was no significant change in the sodium or potassium levels in any of the steroid-injected groups except those given triamcinolone, in which there was a marked elevation of serum potassium and sodium. The latter finding could not be explained otherwise, but by the fact that there is a markedly and rapidly increased protein breakdown in the affected muscle tissue to an even greater extent than with any of the other steroids.

Although total body potassium determinations and interstitial fluid studies of muscle have not been done, the above findings of normal serum electrolytes in the circulating blood make it very unlikely that potassium deficiency would be a causative factor in the development of steroid myopathy.

Serum Electrolytes of Steroid–Treated Mice

	K (mEq)	Na (mEq)
Saline	5.6	147
Hydrocortisone	4.9	142
Prednisolone	5.1	146
Methylprednisolone	5.3	140
Dexamethasone	5.0	147
Paramethasone	5.0	148
Betamethasone	5.4	147
Triamcinolnoe	7.8	156

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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The Effect of Estrogens, Androgens, and Corticoids on Skeletal Dynamics in Man

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Since the classic studies of Albright and Reifenstein (1948) it has become well established that estrogens and androgens produce net positive calcium balances in patients with postmenopausal and senile osteoporosis.

These effects have been documented in numerous balance studies, and it has also become apparent that, in the case of the estrogens at least, long-term administration exerts a beneficial effect upon the skeleton and prevents further fractures and loss of height.

The positive calcium balance could be achieved either by increasing the rate of bone formation or by decreasing the rate of bone breakdown. Tracer techniques now make it possible to measure one of these processes, namely the rate at which bone-seeking minerals are deposited in the skeleton. This can be done by the use of radioactive tracers, which have the advantage of permitting external counting directly over bones and of being measurable in blood and urine in very low concentrations for periods of weeks or months. We have preferred the use of nonradioactive strontium as the tracer mineral. This avoids the objections to using Ca^{45} or Sr^{89} in young subjects and permits the performance of multiple determinations in the same subject in order to establish firm baselines, reproducibility of method, and the comparison of several compounds in the same subject.

The method of study has been described in detail by Eisenberg and Gordan (1961). It consists of the intravenous injection of 10 mEq of stable strontium gluconate. Blood samples are taken every 24 hours and twenty-four urine specimens are collected for 4 days. Serum and urine strontium and calcium levels are determined on a flame spectrophotometer by the method of MacIntyre (1957) as adapted by Loken *et al.* 1963).

The model for analysis of calcium movement is based on studies in which body calcium is divided into two pools (Anderson *et al.*, 1956; Copp, 1957; Krane *et al.*, 1956; Talmage and Elliot, 1958). One is a

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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small pool of rapidly exchangeable calcium in the extracellular fluid, soft tissues, and surface crystal of bone. The other is a large pool of less readily available calcium in the deeper layers of bone. Calcium is added to the exchangeable pool by intestinal absorption and bone resorption. It leaves the pool by excretion and bone deposition. Thus, if a tracer is introduced into the exchangeable pool and the rate of loss of tracer from the pool and the rate of excretion are measured, the rate of bone deposition can be calculated as the difference between the two. Such analysis can be made by using the usual formulas for measuring pool size and pool turnover rates (Eisenberg and Gordan, 1961). Excretion occurs through the urine, feces, and sweat; urine, the largest component is measured directly. Losses through feces and sweat are relatively small (Eisenberg and Gordan, 1961), and are ignored in order to make the method clinically practical.

Following the intravenous injection of strontium, its serum level declines in a linear fashion when plotted semilogarithmically. Extension of the serum concentration curve back to zero time gives the theoretical strontium concentration at the time of injection if mixing as complete and instantaneous. This value is divided into the dose of administered strontium to give a figure which represents the number of liters of rapidly exchangeable pool into which the strontium is distributed. The slope of the serum clearance curve indicates the total rate of turnover. Subtracting the urinary excretion rate from the total turnover rate gives the rate at which the tracer is deposited in the skeleton. The rate of bone deposition is overestimated by about 10–15% in all jects because excretion in feces and sweat is ignored.

The results obtained in normal subjects and in patients with various endocrine disorders known to affect the skeleton are shown in Table I. At ordinary activity the exchangeable pool is about 40 liters, which

TABLE I

Strontium Dynamics in Various Conditions

Condition	Number of studies	Number of patients	E[a] (liters)	T (l/24 hr)	U (l/24 hr)	B (l/24 hr)
Healthy; ordinary activity	31	25	42.7±1.1	13.5±0.6	3.9±0.2	9.6±0.4
Healthy; athletes	14	14	56.9±2.3	20.7±1.0	5.8±0.2	15.0±1.0
Hyperthyroidism	7	7	48.9±3.6	23.8±3.5	7.0±2.2	16.7±2.0
Acromegaly	9	8	73.8±6.2	23.7±2.6	5.7±0.5	18.2±2.3
Osteoporosis; postmenopausal	40	26	33.0±0.9	8.7±0.3	2.3±0.2	6.4±0.3

[a]Abbreviations: E = exchangeable pool; T = total turnover; U = urinary excretion; B = bone deposition. Values are given as mean ± standard error.

corresponds to about 200 mEq of calcium, or about 4000 mg. Compared to the total amount of calcium in the skeleton, which is about 1.0–1.5 kg, it is evident that the exchangeable pool is less than 0.5% of total body calcium. The last column demonstrates that the bone normally receives in 1 day the amount of tracer in about 10 liters of pool. This corresponds to about 50 mEq or 1000 mg of calcium.

The effects of stress and strain are shown by the studies in fourteen athletes. Their vigorous exercise enlarged the pool and increased the turnover rate; the rate of deposition in bone was 50% greater than in healthy people at normal activity.

Calcium dynamics in conditions associated with osteopenia differed widely. In postmenopausal osteoporosis the exchangeable pool is significantly smaller than normal. The turnover rate is subnormal, and the rate of bone deposition is significantly decreased.

Both hyperthyroidism and acromegaly cause osteoporosis. Tracer studies show clearly that in these two conditions the osteoporosis cannot result from decreased bone deposition, for this is actually accelerated. It must, therefore, be due to increased bone destruction. Both hyperthyroidism and acromegaly enlarged the exchangeable pool. Hyperthyroidism without hypercalcemia increased bone deposition without increasing urinary excretion. Our data, based on the use of nonradioactive strontium, confirm those of Krane *et al.* (1956) using Ca^{45}. With hypercalcemia and normal renal function, bone deposition was increased and urinary excretion greatly increased. In acromegalics the mineral went rapidly to bone and the urinary excretion rate was only slightly accelerated.

The results of administration of steroid agents known to produce positive calcium and phosphorus balance to patients mostly with osteoporosis are shown in Table II. These compounds do not increase the

TABLE II

Skeletal Dynamics: "Anabolic" Compounds

Compound	Number of studies	E (Liters)		T (l/24 hr)		U (l/24 hr)		B (l/24 hr)	
		Control	Exp't	Control	Exp't	Control	Exp't	Control	Exp't
Fluoxymestrone	10	33.4	34.5	9.3	8.8	2.5	2.0	6.9	6.7
Oxymethalone	9	46.0	46.2	15.2	13.0	5.3	3.5	9.8	8.4
Nandrol phenylpropionate	4	46.9	47.6	19.6	16.6	8.0	6.6	16.3	10.1
Norethandrolone	1	35.6	45.9	11.4	10.3	2.7	2.6	8.7	7.7
Growth hormone	1	41.6	44.9	10.0	15.4	4.5	7.2	5.5	8.2
Premarin	6	37.5	34.7	7.3	5.7	3.2	2.1	7.9	6.5
Vallestril	9	30.5	30.5	8.4	7.3	2.2	1.9	6.2	5.4
Manvene	3	29.0	32.4	7.1	5.1	0.9	0.5	6.2	4.7

rate of bone deposition. In fact, this tends to fall somewhat. With the exception of a single study of growth hormone, the one consistent action of these agents is to decrease the urinary excretion of bone-seeking minerals. Their action should not, therefore, be described as anabolic, since bone deposition is not increased, but can more accurately be described as anticatabolic.

All the androgens exert this effect to a similar degree; fluoxymestrone was given in 10-mg doses; oxymethalone, 7.5 mg; nandrol phenylpropionate, 25 mg; i.m. once weekly; norethandrolone, 10 mg per day; growth hormone, 10 mg i.m. per day; Premarin, 2.5 mg per day; methalenestril, 6 mg per day; and Manvene, 20 mg per day.

Table III shows the effects of short-term administration of various

TABLE III

Skeletal Dynamics: Adrenocortical Steroids

Compound	Number of studies	E (liters)		T (l/24 hr)		U (l/24 hr)		B (l/24 hr)	
		Control	Exp't	Control	Exp't	Control	Exp't	Control	Exp't
Hydrocortisone	2	36.2	39.3	11.4	14.4	4.0	4.8	7.7	9.6
Prednisone	4	53.6	52.5	13.2	16.9	4.5	7.1	8.8	9.8
Triamcinolone	8	38.5	43.2	11.7	12.7	3.5	5.8	6.8	9.2
Dexamethasone 1.5	5	49.8	57.5	18.3	22.1	5.5	7.1	12.8	15.0
Dexamethasone 3.0	7	45.5	45.5	13.3	19.9	3.8	8.5	9.5	11.3

adrenocortical steroids. There has been some discussion in the past on whether they exert their calcium-wasting action by catabolic or antianabolic activity. Albright favored the antianabolic action because bone biopsies show no increase in osteoclasts in patients with Cushing's disease. As already suggested, the tracer technique gives a more direct indication of what is going on in bone than can be obtained by histologic methods.

If the action of the adrenocorticoids were truly antianabolic, the rate of bone deposition would be decreased. Such a decreased rate of osteogenesis was not found after administration of cortisol, 200 mg daily; prednisone, 30 mg daily; triamcinolone, 20 mg daily; or dexamethasone, 1.5 mg or 3.0 mg daily. In fact, the rate of bone deposition actually increased slightly. This was probably an attempt on the part of the bone to compensate for the calcium-wasting effect of the compound, which is shown by the increase in urinary excretion rate. All these compounds caused calcium and strontium wasting via the urine. In most cases the pool also increased slightly; this may also reflect a compensatory osteoblastic process.

These data indicate that corticoids exert their effect on bone not primarily by interfering with bone formation, but by increasing bone

TABLE IV
Strontium Dynamics: Adrenocortical Hyperplasia

Condition	Number of patients	E (liters)	T (l/24 hr)	U (l/24 hr)	B (l/24 hr)
Adrenocortical hyperplasia	1	19.8	8.2	5.8	2.4
Adrenocortical hyperplasia	1	28.2	15.2	8.6	6.6

destruction. Thus, they are truly catabolic. The data in patients with far advanced Cushing's disease indicate that eventually the bone is unable to compensate for these losses by increased osteoblastic activity and that ultimately osteoblastic exhaustion and decreased bone formation result.

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DISCUSSION

Evaluation of the Strontium Tracer Technique in the Study of Skeletal Dynamics

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The use of nonradioactive strontium tracer provides us with a practical technique for the study of skeletal dynamics. Care must be exercised, however, in interpreting data obtained by this method. Since fecal excretion was not measured and Eisenberg and Gordan (1961) state that, because of this, "bone deposition is overestimated by 9 to 16 per cent," small changes may appear to be significant when in reality they are not.

When the standard error of the mean is considered, it is difficult to see that the exchangeable pool of the hyperthyroid patient is enlarged.

It is interesting that, whereas acromegaly greatly enlarged the exchangeable pool, the administration of growth hormone did not, although in both cases the total turnover, urinary excretion, and bone deposition were significantly increased. Also it is of importance to note that the androgenic steroids, generally considered to be anabolic, may actually decrease the rate of bone deposition, whereas corticoids which are generally considered to be catabolic or antianabolic, increased the rate of bone deposition at least for the treatment period of these studies. The anabolic or catabolic effects merely reflect the rate of urinary excretion. In the light of these findings, should we still define androgens as anabolic substances or anticatabolic substances when they merely reduce urinary excretion?

Two of the authors of the preceding presentation, Drs. E. Eisenberg and G. S. Gordan (1962) in a recent report, state that using this tracer technique they find that on prolonged corticoid treatment "the rapidly miscible volume of tracer distribution shrinks and the bone deposition rate falls." Therefore, corticoids can be considered to be catabolic even though the rate of bone deposition is increased or unchanged during early treatment.

In this same report, these two investigators stated that large clinical doses of hydrocortisone, prednisone, triamcinolone, and dexamethasone increased urinary excretion of the stable strontium tracer. The administration of a large dose of 6α-fluorotriamcinolone, however, did not increase the urinary excretion rate to the same extent. This finding is extremely interesting in the light of another report by Spencer *et al.* (1962) that in two patients treated with a related steroid, 6α-fluorotriamcinolone acetonide, there was little or no calcium excretion and a slight nitrogen retention was seen in one of the patients. In our own laboratories at the Squibb Institute for Medical Research, we have seen a possible separation of anti-inflammatory and catabolic effects with 6α-fluorotriamcinolone acetonide. The anti-inflammatory activity of this compound in the adrenalectomized rat antigranuloma cotton pellet assay was slightly greater than that of triamcinolone acetonide. The catabolic activity as possibly reflected in the

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adrenalectomized rat liver glycogen assay, however, showed this fluorinated corticoid to be only two-thirds as active as triamcinolone acetonide.

The development of the strontium tracer technique will facilitate the study of the metabolic effects of these new hormonal steroids.

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Inotropic Actions of Certain Steroids upon Cardiac Tissue[1]

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The extensive chemical investigations carried out by W. A. Jacobs from 1922 to 1934 provided the necessary information which enabled subsequent workers to reveal the structures of numerous cardiac glycosides (Fieser and Fieser, 1959). It has been demonstrated that the cardiac glycosides are comprised of three distinct moieties: a central steroid nucleus (cyclopentanoperhydrophenanthrene) to which is attached a 5- or 6-membered, saturated or unsaturated lactone at position C-17, and one or more sugars attached to an oxygen at position C-3. This information has in turn stimulated various workers to study the structure-activity relationship of many cardiac glycosides; among the studies Chen's contributions have been outstanding (Chen *et al.*, 1951, 1952; Chen and Henderson, 1954). One of the few generally accepted conclusions is that the sugar moiety per se is without cardiotonic activity but enhances potency (Chen *et al.*, 1938; Chen and Elderfield, 1940). Other investigators have attempted to clarify the structure-activity relationship of various lactones upon the heart. The pharmacological role of the lactone moiety has not, however, been completely resolved, although most evidence indicates that the lactones per se possess no stimulatory action on mammalian cardiac tissue. As an example, Vick and co-workers (1957) observed only negative inotropic or toxic responses on isolated guinea pig hearts following the administration of a number of simple lactones. They speculated, therefore, that the positive inotropic action could be separated on a structural basis from the toxic effects of cardiac glycosides. By extending this reasoning a little further they hypothesized that perhaps the steroid moiety of the cardiac glycosides might be responsible for producing the positive inotropic

[1]This work was supported by grants from the National Institutes of Health, United States Public Health Service (H-4883) and was completed while the author held support under the Career Development Award Program of the National Heart Institute, U.S.P.H.S. (SG-5759).

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action of the digitalis glycosides. However, even though certain steroids per se have now been shown to produce a direct positive inotropic action upon the myocardium, one cannot infer that the steroid moiety is solely responsible for the production of the increased contractile force produced by cardiac glycosides. Moreover, to this author's knowledge, no steroid glycoside has been shown to possess typical cardiac glycoside activity.

The possibility that certain steroids might be capable of producing a positive inotropic effect by acting directly upon cardiac tissue is not new. In 1928 Clark and White showed that cholesterol was able to increase the force of contraction of the frog heart. Rubin (1950) reported that certain steroids of the estrogen and pregnane series produced augmentation of isolated frog hearts made hypodynamic by reducing the calcium content of the perfusion fluid. And in Rogoff and Stewart's initial observations on adrenal insufficiency in 1926, they noted that there was a diminution in heart rate and speculated that certain adrenal steroids might possess a direct effect upon the heart.

Prompted by the observations of Waud *et al.* (1949a,b) that the phytosterols from Osage orange caused an increase in contractile force of the perfused frog heart, Loynes and Gowdey (1952) were able to show that a number of steroids had the ability to produce a positive inotropic action when added to the perfusing fluid of the frog heart, and in no case were they able to produce systolic arrest. They interpreted their data as suggesting that the lactone moiety of the cardiac glycoside molecule is responsible for the development of systolic arrest and that, at least in part, the positive inotropic action seems to be a function of the steroid portion. In 1956 Fleischhacker went so far as to propose that cortisone and digitalis possess a similar type of action upon the heart.

Subsequently, investigators reported that DOC and progesterone were able to augment the force of frog heart contractions somewhat similar to the increase in force produced by digitoxin (Hajdú, 1957), and augmentation also occurred when deoxycorticosterone acetate (DOCA) and cortisone were added to the perfused guinea pig and frog heart (Hoffmann, 1954). Employing the cat papillary muscle preparation, Emele and Bonnycastle (1956) noted the effects of a wide variety of adrenocortical steroids, but the results obtained were based on only a few observations.

In an attempt to ascertain whether any structure-activity relationships could be found between steroids and their inotropic responses, we initiated a series of studies employing the cat papillary muscle and whole isolated (Langendorff) preparations as our target tissue (Tanz, 1960, 1962; Tanz and Kerby, 1961).[3]

[3]Permission was kindly granted by the Williams & Wilkins Company to reproduce Figs. 1, 2, and 3.

Figure 1 illustrates the results obtained when four different steroids were added to individual papillary muscle preparations. The concentrations indicated were the minimum amounts which consistently elicited an inotropic action, and each curve represents the mean of between 8 and 11 individual experiments. The addition of cortisone acetate and prednisone was followed by a slight positive inotropic response. When the concentration of these steroids was raised to 50 and 100 μg/ml,

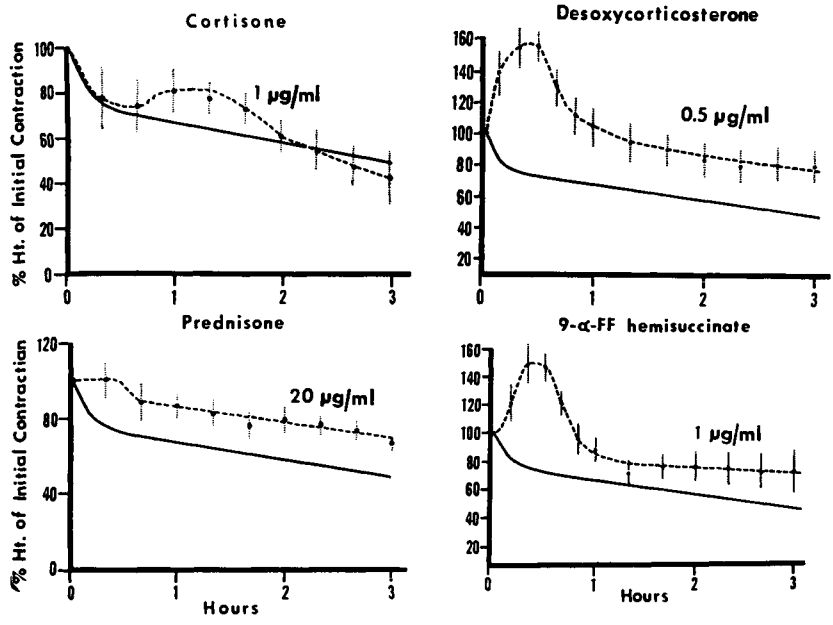

FIG. 1. Mean values obtained on the cat papillary muscle preparation in terms of the percentage height of the initial contraction. Dashed lines = experimental; solid lines = untreated controls; vertical lines = calculated standard error. From Tanz and Kerby (1961).

a negative inotropic action resulted but standstill never occurred. In addition, histological studies were performed comparing freshly excised papillary muscles with those stimulated continuously for 6 hours in the presence and absence of cortisone (1 μg/ml). The untreated control muscles (without cortisone) showed marked tissue degeneration as evidenced by large pycnotic nuclei, a loss of cross-striations, and swelling of the individual cells. In contrast, the fresh and cortisone-treated tissue showed good cellular integrity, lightly stained elongated nuclei, and the typical cross-striations of cardiac tissue. Indeed, the fresh and cortisone-treated muscles looked very similar histologically.

In addition, it was observed that when cortisone acetate (1 μg/ml) was added to the perfusing fluid of hypodynamic cat Langendorff pre-

parations, an increase in contractile amplitude and a return toward control levels of the heart rate and coronary flow occurred. On the basis of these results (Tanz, 1960), we postulated that there must be some substance, perhaps an adrenocortical steroid, which is normally present in the blood stream to permit the heart to function in its normal or physiological manner. This we refer to as a "normalizing" or "protective" action.

Since the addition of both cortisone acetate and prednisone resulted in only a slight positive inotropic action on the cat papillary muscle preparation, we next employed two mineralocorticoids: DOCA and 9α-fluorohydrocortisol hemisuccinate (9α-FF). Figure 1 illustrates the mean results of these experiments and shows that following the administration of these steroids there resulted an immediate and marked positive inotropic action of rather short duration. It was considered unlikely that the presence of the esters contributed to the increased force of contraction in view of the reports by Loynes and Gowdey (1952) that the acetate esters of cholesterol and deoxycorticosterone produced less augmentation on the frog heart preparation than the parent compounds, as well as the previously mentioned observations

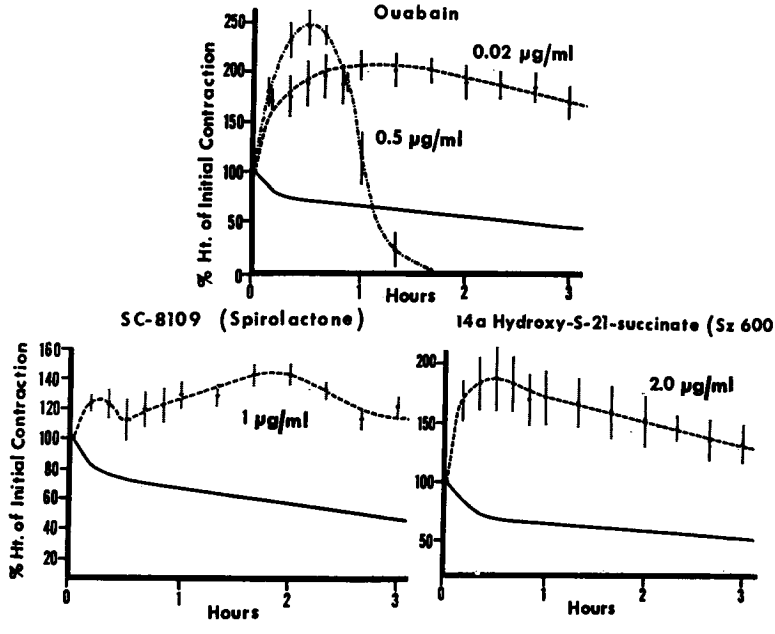

FIG. 2. Mean values obtained on the cat papillary muscle preparation in terms of the percentage height of the initial contraction. Dashed lines = experimental; solid lines = untreated controls; vertical lines = calculated standard error. From Tanz and Kerby. (1961).

by Hajdú (1957) and Hoffmann (1954), who employed DOC and DOCA, respectively.

These results suggested to us that mineralocorticoid structure might produce a more pronounced positive inotropic action than glucocorticoids. However, by comparison to the cardiac glycosides, their duration of action was very short. Figure 2 illustrates the mean results obtained when ouabain in *therapeutic* and *toxic* doses (0.02 and 0.5 μg/ml) was added to the papillary muscle preparation. Following a therapeutic dose of ouabain there was an immediate and marked positive inotropic response and the duration of action was prolonged. When a toxic dose of ouabain was added, a transient and very pronounced inotropic action resulted, but an hour and a half later the muscles no longer contracted to electrical stimulation.

Additional studies were performed employing the spirolactone SC-8109[4] and 14α-hydroxy-*S*-21-succinate (Sz-600).[5] These synthetic steroids were studied because structurally they resemble mineralocorticoids and, in addition, SC-8109 possesses a 5-membered lactone attached to the C-17 position (similar to a cardiac aglycone), whereas Sz-600 possesses an OH group attached to position C-14 (similar to most of the therapeutically useful cardiac glycosides). As can be seen in Fig. 2, the addition of these steroids was followed by a positive inotropic response of prolonged duration.[6] Thus, the inclusion of a C-14 OH and/or a C-17 lactone may contribute to the prolongation of action (Tanz and Kerby, 1961). However, these results should be extended by performing additional comparative experiments with parent compounds before a more definitive statement can be made with respect to steroidal structure-activity relationship.

In 1960, Sayers and Solomon, and Ballard *et al.* reported that the addition of small amounts of aldosterone to the media perfusing the hypodynamic rat heart-lung preparation would produce an increase in the left ventricular work index. These results, however, did not provide evidence for the direct action of aldosterone upon cardiac tissue, since the pulmonary circulation was still present in the circuit.

Figure 3 illustrates the mean results obtained in our laboratory following the addition of tetrahydrocortisol (THF) and *d,l*-aldosterone. In a concentration of 1×10^{-5} μg/ml, aldosterone was found to produce a significant positive inotropic action, whereas the addition of THF (1×10^{-4} μg/ml) did not result in an inotropic response significantly different from that in the untreated controls. Moreover, raising the

[4] SC-8109 was kindly supplied by the G. D. Searle, Co.

[5] Sz-600 was kindly supplied by Organon, Inc.

[6] In a personal communication from Dr. K. W. Thompson of Organon, Inc. (October 7, 1959), confirmation of the positive inotropic action of Sz-600 on the isolated frog heart preparation was reported.

concentration of aldosterone to 1×10^{-2} μg/ml did not result in *standstill.* Thus, the therapeutic index of aldosterone, insofar as it produces a positive inotropic action, is at least 1000, whereas ouabain is about 7.5. On a molecular weight basis d,l-aldosterone was also calculated to be about 1000 times as potent as ouabain on the cat papillary muscle preparation insofar as the minimally effective doses needed to produce a positive inotropic response. However, there is no question but that

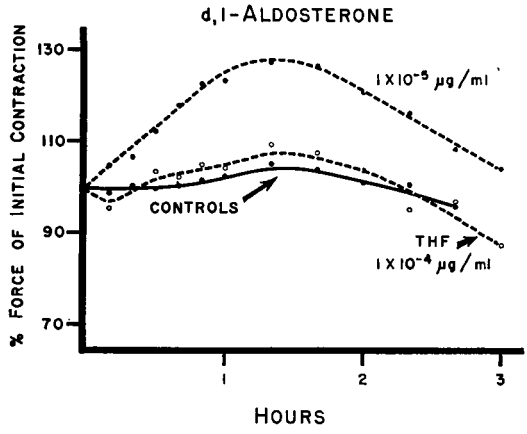

FIG. 3. Mean values obtained on the cat papillary muscle preparation in terms of the precentage force of initial contraction. Dashed lines = *d,l*-aldosterone or THF (tetrahydrocortisol) in the concentrations indicated; solid line = untreated controls. From Tanz (1962).

ouabain produces a greater increase in contractile force than does aldosterone (Tanz, 1962).

Additional studies were performed on the cat Langendorff preparation, and confirmatory observations were obtained showing that small amounts of aldosterone when added to the perfusate of a hypodynamic preparation could increase the force of contraction.

On the basis of our studies and of studies reported by others, evidence is now available which conclusively establishes the fact that certain steroids do possess the ability to produce a positive inotropic action by acting directly upon cardiac tissue. In the case of cat (as opposed to rodent and amphibian hearts), it appears that certain steroid structural considerations may play a role in determining the degree of augmentation and that the duration of action may be related to the possession of a C-14 OH and/or C-17 lactone. However, before these conclusions can be accepted many additional experiments will have to be performed.

In addition, the sensitivity of cardiac tissue to small amounts of aldosterone, which approximate normal plasma levels (Tait *et al.*, 1961), suggests that it may possess a unique role in the etiology of congestive

heart failure. For if aldosterone levels are increased during cardiac failure we might now wish to view this as a homeostatic mechanism by virtue of its apparent ability to exert a direct stimulatory, and therefore beneficial, influence on the failing myocardium.

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DISCUSSION

Actions of Certain Steroids upon Cardiac Tissue

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Dr. Tanz's report led us to consider the problem of the activity of steroid hormones in relations to cardiac function. The subject is undoubtedly suggestive because physicians for centuries have used as cardiotonics digitalis, and its derivatives, which in fact have a chemical structure analogous to that of the steroid hormones. The possibility of modifying cardiac activity with these hormones is also suggested by the fact that they influence, in various degrees and according to their structure, electrolytic, glycosidic, and nitrogenous metabolism even at the cardiac level, as has been recently revealed by Dell'acqua and Gambassi (1961). In spite of these theoretical statements the experimental results are relatively modest.

The experimental results correspond to the impression of the physician, who is never called on to consider cardiac pathology in the strict hormonal sense. The interesting experimental findings by Selye (1958) have not yet a well-defined clinical sig-

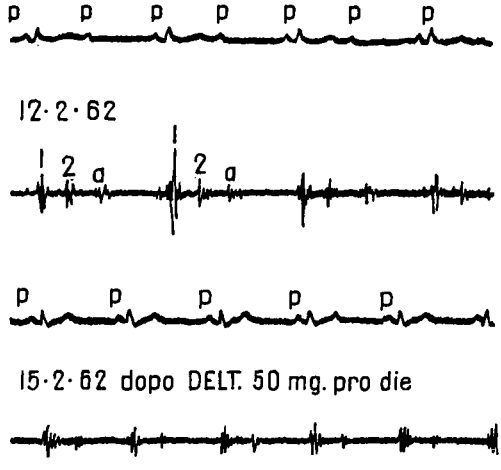

Fig. 1. The atrioventricular block present in the top electrocardiogram (ECG) and phonocardiogram (subject V. M., age 60) disappears (bottom ECG and phonocardiogram) after 3 days of treatment with prednisolone, 50 mg each day.

nificance. On the clinical level cardiovascular pathology in syndromes of deficiency or excess of steroid hormones is to be attributed to a peripheral hemodynamic disorder that secondarily affects cardiac activity, rather than to a primary disorder of the heart. The most typical example in this sense is the cardiac damage that is produced

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by hypertensive syndromes of hypersuprarenalism. In respect to clinical negativity, the electrocardiographic signs are relatively modest. In our experience, we could not identify a pathological electrocardiographic pattern in Cushing's disease, without hypertension.

On the other hand, also in Addison's disease, the electrocardiogram is not fundamentally altered if one excludes an eventual reduction of voltage with flattening of the T wave and lenghtening of the PQ tract, the importance of which is more apparent from a statistical study than in a single case. The lengthening of the PQ tract in Ad-

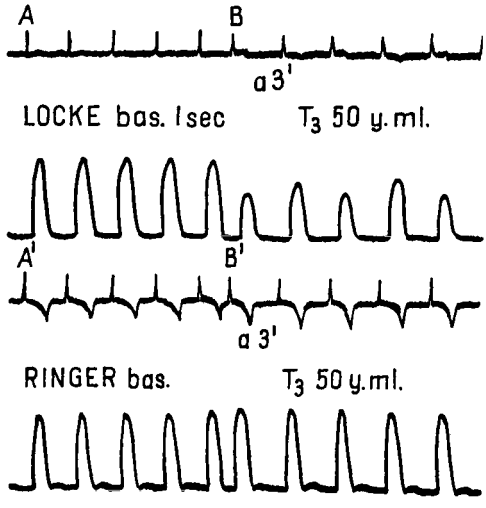

FIG. 2. Simultaneous experiments in two frog hearts perfused with triiodothyronine (T^3): electric and mechanical alternance in the heart perfused with T^3 and Locke's liquid (upper electrocardiogram and pressure curve); in the heart perfused with T^3 and Ringer's liquid (bottom electrocardiogram and pressure curve), mechanical recording increases modestly.

dison's disease is, on the other hand, of particular interest. In fact, recently the possibility has been indicated of correcting an incomplete or complete atrioventricular bloc by means of large doses of cortisones in subjects without evident signs of rheumatic carditis.

I would like to present one of our cases —a 60-year-old patient without valvular lesions, with normal erythrosedimentation rate serum protein and anti-O streptolysin titer, who developed an atrioventricular block with Adams–Stokes crisis. The picture was corrected with prednisolone treatment (Fig. 1). It is interesting to note that with relatively low doses of prednisolone, the block did not disappear and that initially, when the patient did not take the drug during the night, the block was present in the morning and disappeared during the course of the day. Nocturnal administration of the drug caused the block to disappear also in the morning. Therefore, clinical interest is necessarily focused on the relationships between steroid hormones and atrioventricular conduction rather than on the relationships between steroid hormones and inotropism.

It is of some interest to note that the modified sensitivity to digitalis during the treatment with cortisones may be related to an electrolyte imbalance.

The interest in experimental research carried out in isolated organs derives principally from two considerations: (1) The possibility of making an isolated study of cardiac modifications free of every interference by peripheral hemodynamic factors. (2) The possibility of individualizing the direct action of hormones on the heart from that mediated through the neurovegetative system. The latter consideration is of particular importance relative to the thyroid hormones.

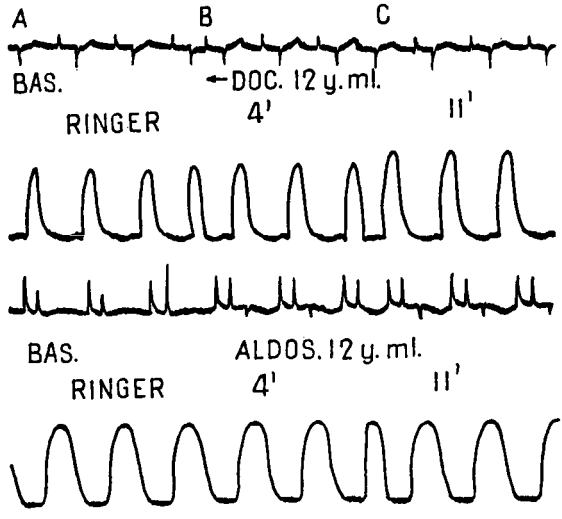

FIG. 3. Experiments in two frog hearts perfused with salt hormones: pressure curve increases in the heart perfused with DOC (upper pressure curve); no modification in the heart perfused with aldosterone.

This is the procedure that Dr. Tänz followed. The discussion of the interesting results seems superfluous. I would like merely to make some general statements on this particular type of experiment. If it is true that in the isolated heart or in fragments of it, the influence from the periphery, either of hemodynamic or neurovegetative nature could be, eliminated, then it is also true that the conditions of this experiment are entirely different from physiological conditions. In fact, not only do those hormone antagonists and potentiators that are efficient in the integral animal fail, but also in general the physiological fluids that are commonly employed are devoid of proteins and therefore the hormone is deprived of those vectors that normally accompany it in the plasma. In these experimental conditions, the most important factor in conditioning the activity of the heart and its response to diverse stimuli is the electrolytic equilibrium of the perfusion liquid.

As the experiment that I will now relate demonstrates, different electrolytic composition of the perfusion liquid has a definite effect in conditioning the response of the heart to thyroid hormone. Figure 2 shows how triiodothyronine diminishes the amplitude of cardiac contraction when the heart is perfused with Locke's liquid and how instead it is not modified and sometimes even increased when the heart is per-

fused with Ringer's solution (Fig. 2). The different electrolytic composition of the two liquids is probably responsible for this opposed behavior (Baschieri and Palagi, 1961; Palagi and Puletti, 1962a).

When one considers that the interference of the thyroid hormone with electrolytic metabolism is modest, and at least as far as we now know, negligible, it is easily understood how this factor can be important in the study of the response of the heart to the corticosuprarenal hormones, whose chief action is to interfer with the electrolyte metabolism.

Our experience concerning the steroid hormones is as yet relatively modest, and the results that we have obtained are not easily interpretable. We have, up to the present time, experimented with hydrocortisone, DOC, and aldosterone. Neither hydrocortisone nor aldosterone in varying concentrations from 0.1 μg/ml to 100 μg/ml modifies cardiac activity. Nor did it appear to us that aldosterone modifies the reaction of the heart to epinephrine. With DOC, however, we observed, with a concentration of the order of 100 μg/ml (Fig. 3), a positive inotropic effect followed subsequently by a negative inotropic effect (Palagi and Puletti, 1962b).

We must therefore conclude that as yet, it remains difficult to draw a definite conclusion from this type of experiment, especially if we refer to the pathlogic conditions of adrenal hyper- or hypofunction.

It is principally from systemic studies performed by physiologists such as Dr. Tanz that physicians await a solution to these problems.

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The Action of Steroids on the Skin

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I. INTRODUCTION

The cutaneous effects of steroids must be considered in relation to the cellular and other elements of which the skin is composed. In the epidermis, for example, cell division in the basal layers and subsequent keratinization and desquamation may be together or independently affected. Similarly, in the melanocytes, hair, and sebaceous glands it may be necessary to differentiate between effects on cell proliferation and effects on subsequent metabolic activity of the cells. In the dermis, as well as the cellular constituents such as fibroblasts, mast cells, and melanocytes, the properties of the extracellular material—the mucopolysaccharide complex—may be influenced.

II. THE SUPERFICIAL EPIDERMIS

A. ANDROGENS

There has been some controversy about the effects of steroid hormones on cell division in the Malpighian layer. Two independent stud-

TABLE I

Effect of Testosterone on Epidermis of Adult Male Rats[a]

Treatment	Thickness of Malpighian layer (μ)	Mitoses per centimeter length
Intact	18.8	47
Castration	20.7	32
Castration + testosterone	19.1	63
Castration + hypophysectomy	24.7	44
Castration + hypophysectomy + testosterone	24.0	46
SE[b]	1.2	8.3

[a]Rats were treated with testosterone implants giving daily uptake of 0.2 mg for 21 days. Eight litters of five rats were used, one rat from each litter being given each treatment. All rats were treated with 0.1 mg of colchicine per 100 gm body weight 5 hours before death (Ebling, 1957a).

[b]Standard error of difference between two means of eight rats.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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ies (Bullough and van Oordt, 1950; Allen, 1957, 1958) showed that testosterone stimulates epidermal mitosis in the mouse. In an experiment on male rats, castration reduced the incidence of mitoses and implantation of testosterone increased it (Table I), though this was not reflected in significant changes in the thickness of the Malpighian layer. In hypophysectomized rats, in which the epidermis was abnormally thick and the incidence of mitoses possibly higher than in castrates, no effect was produced by testosterone. From this experiment it appears that testosterone acts as a stimulant of epidermal mitosis, but that the pituitary is necessary.

B. ESTROGENS AND ADRENAL HORMONES

H. F. Bullough (1943, 1947), W. S. Bullough (1946, 1950a,b), and Allen (1956) have presented evidence that estrogens stimulate mitosis in the epidermis of the mouse. Bullough (1953), from studies on epidermal mitosis *in vitro*, suggested that cell division requires energy derived from carbohydrates and that estrogens affect their utilization; both these conclusions have been challenged by Gelfant (1959a,b, 1960a,b,c). In the adult rat, estrogens do not seem to affect epidermal mitosis (Carter, 1953; Ebling, 1954, 1955), although carbohydrates are necessary for the maintenance of epidermal mitosis *in vitro* (Callard, 1961). But irrespective of any action on cell proliferation, estrogens appear to reduce epidermal thickness. During the estrous cycle, for example, the Malpighian layer is thickest in proestrus (Table II) and is significantly

TABLE II

Changes in Epidermis of Rats during the Estrous Cycle (Ebling, 1954)

Day of cycle	Type of vaginal smear	Thickness of Malpighian layer (μ)		Mitoses per centimeter lenth	
		Mean±SE	*n*	Mean±SE	*n*
1 (proestrus)	Nucleated ephithelial cells	15.2±0.6	10	31.2±4.6	7
2 (estrus)	Cornified cells only	13.2±0,6	24	30.3±2.1	19
3	Many leucocytes with cornified and nucleated cells	11.9±0.6	15	30.4±4.5	10
4	Epithelial cells and few leucocytes	12.7±0.6	20	36.6±4.4	15

reduced at estrus and on the next day, though there appear to be no corresponding changes in the incidence of mitoses that could account for this. Indeed, in this experiment, there was no correlation between epidermal thickness and the incidence of mitoses. In immature female rats, moreover, it was even possible by treatment with estradiol (Table III) to demonstrate a marked reduction in epidermal thickness at the same time as the incidence of mitoses was increased. It must be con-

TABLE III

Effect of Estradiol Benzoate on Epidermis of Immature Female Rats[a]

	Thickness of Malpighian layer (μ)	Mitoses per centimeter length
	Mean±SE	Mean±SE
Normal controls	16.5±0.5	12.9±1.7
Estradiol benzoate	10.9±0.8	43.6±7.6
t	5.9	3.9
p	< 0.01	< 0.01

[a]Eight rats, aged 8 weeks at death, were used in each group. One milligram estradiol benzoate crystals was implanted subcutaneously 3–4 weeks before the rats were killed (Ebling, 1954).

cluded, therefore, that estrogen acts by increasing the rate of keratinization and subsequent desquamation; that is to say the *turnover time* of the epidermis is reduced. This effect of estrogen, unlike the mitotic effect of androgens, is completely independent of the pituitary or adrenals, since it has been shown in hypophysectomized or adrenalectomized rats (Ebling, 1955). The effect cannot, therefore, be due to production of adrenal hormones which are known to inhibit mitosis, for example, cortisone (Ghadially and Green, 1957), hydrocortisone (Bullough, 1952), deoxycorticosterone acetate (Gelfant, 1960b), and adrenaline (Bullough, 1955; Ghadially and Green, 1957; Gelfant, 1960b; Bullough and Laurence, 1961).

III. EPIDERMAL MELANOCYTES

There is conflicting evidence about the effect of androgens on melanocytes. The view that they increase skin pigmentation is supported, for example, by observations on the effect of testosterone in castrate men (Hamilton and Hubert, 1938; Edwards *et al.*, 1941) and in women (Hamilton, 1939).

Androgens have been shown to stimulate pigmentation in certain areas of animal skin, for example, the subcostal region of the male golden hamster (Kuppermann, 1944) and the scrotum of the ground squirrel (Wells, 1945). From quantitative studies on the guinea pig, however, Bischitz and Snell (1959) concluded that in the ear, anterior abdominal wall, and areola, testosterone was incapable of stimulating melanocyte activity, and that in mature guinea pigs melanogenesis in these areas was actually stimulated by castration (Snell and Bischitz, 1959). In the sole of the foot it appeared, curiously, that the amount of epidermal melanin was increased both by testosterone and by orchidectomy.

That estrogens increase skin pigmentation in man and in animals is undisputed. Bischitz and Snell (1960) found that ovariectomy re-

duced the size and melanin content of melanocytes in the guinea pig; conversely estrogen increased the amounts of both free melanin and melanin within the melanocytes. Although a significant increase in the number of melanocytes was shown in one region (anterior abdominal body wall) as a result of large doses of estrogen (Snell and Bischitz, 1960), in general they found that neither ovariectomy nor moderate doses of estrogen had any effect on melanocyte number.

Progesterone appeared to enhance the melanogenic effect of small doses of estrogen. Progesterone by itself increased the amounts of intra- and extracellular melanin, although the melanocytes themselves were unaltered in number or in size; they may even have been made smaller (Bischitz and Snell, 1960; Snell and Bischitz, 1960).

IV. SEBACEOUS GLANDS

A. ANDROGENS AND PROGESTOGENS

In their responses to steroid hormones, the sebaceous glands behave in a similar way to the epidermis. It is well known that androgens enlarge the glands in animals (de Graaf, 1943; Ebling, 1948; Montagna and Kenyon, 1949; Hamilton and Montagna, 1950; Haskin *et al.*, 1953; Lapière, 1953) as well as increasing sebum production in man (Jarrett, 1959; Strauss *et al.*, 1962). In an experiment on male rats (Table IV),

TABLE IV

Effect of Testosterone on Sebaceous Glands of Adult Male Rats

Treatment	Gland volume ($1000\mu^3$)	Cell volume (μ^3)	Cells per gland per day	Turnover time (days)
Intact	502	1990	74	3.8
Castration	368	1772	34	6.7
Castration + testosterone[a]	686	2130	124	2.6
Castration + hypophysectomy	309	1669	41	4.7
Castration + hypophysectomy + testosterone[a]	357	1835	63	3.4
SE[b]	64	78	13	0.9

[a]Testosterone implants giving a daily uptake of 0.2 mg daily were used for 21 days (Ebling, 1957a).

[b]Standard error of the difference between two means of eight rats.

castration reduced the mean volume of the sebaceous glands and implantation of testosterone increased it even above that in intact rats. The effect appeared to be due to increases in both the rate of cell proliferation and cell size; turnover time was reduced. No significant increases in mean volume or in the rate of cell proliferation were, however, produced by implantation of testosterone into hypophysectomized rats;

hence it appears that the action of testosterone requires the permissive role of the pituitary. Lasher *et al.* (1954, 1955) reached similar conclusions; indeed, Lorincz and Lancaster (1957) proposed that the pituitary produces a "sebotropic" factor.

Ebling (1948) reported that progesterone in amounts up to 0.5 mg per day for 30 days had no effect on the sebaceous glands of immature female rats. Haskin *et al.* (1953), however, claimed that doses of 1–10 mg per day caused sebaceous enlargement in spayed adult rats, that some effects could be detected even with a dose of 1 μg per day, and that therefore, progesterone could be compared in its action to testosterone. But though doses of testosterone of the order of 0.2 mg per day for 21 days produce marked effects, similar amounts of progesterone were found to have no effect in either immature or mature, intact or spayed, female rats (Ebling, 1961). Moreover, neither Jarrett (1959) nor Strauss and Kligman (1961a) found any effect on human sebum production from physiological amounts of progesterone.

It was possible to show enlargement of the sebaceous glands by injecting 10 mg of progesterone in oil per day for 21 days into castrated male rats; an increase in cell proliferation was probably involved (Ebling, 1963). One possible explanation is that some metabolic conversion to androgens had occurred; another possibility is that progesterone, by reason of its molecular configuration, is very slightly androgenic. There was no significant increase in seminal vesicle weight, although the preputial glands were significantly enlarged. It is possible that both the preputial and the sebaceous glands are more sensitive to androgens than the seminal vesicles. It is of interest that a synthetic progestin, 17α-ethynyl-19-nortestosterone (Norlutin), having only 3% of the androgenic activity of testosterone when assayed by its effects on seminal vesicles and ventral prostate, has been shown not only to enlarge the sebaceous glands of prepubertal man (Strauss and Kligman, 1961b), but also to cause pseudohermaphrodism of female infants when given to their mothers during pregnancy.

B. ESTROGENS

Estrogens reduce the size of sebaceous glands (Hooker and Pfeiffer, 1943; Ebling, 1948, 1951, 1954). This effect is just as marked in hypophysectomized, as in intact or spayed rats, as shown by an experiment summarized in Table V. Moreover, the reduction does not involve any significant change in the rate of cell proliferation, which was, in fact, above normal in both treated and untreated hypophysectomized rats. Similar results were obtained with adrenalectomized rats (Ebling, 1955).

The conclusion appears to be that estrogens act, not by inhibiting mitosis, either directly or indirectly via the pituitary or adrenal glands, but by causing a more rapid breakdown of cells in the sebaceous glands.

TABLE V.

Effect of Estradiol on sebaceous Glands of Adult Female Rats

Treatment[a]	Total number of sebaceous cells in 15 mm sections	Mitoses per 50 gland sections
Intact controls	301±24	6.7±0.9
Estradiol (2 days)	279±28	9.8±1.9
Estradiol (28 days)	130±14	6.5±0.8
Hypophysectomy	361±45	28.5±6.3
Hypophysectomy+estradiol (28 days)	149±12	23.4±2.4

[a]Implants giving daily uptake of about 3 μg were used. There were 9 or 10 rats in each group, and they were treated with colchicine for 5 hours before death (Ebling, 1955).

It is not difficult to appreciate that, in a steady state, the number of cells making up the gland is equal to the number produced each day multiplied by the number of days each cell remains in the gland (i.e., the turnover time). The size of the gland may be reduced either by a decrease in turnover time or by a decrease in cell proliferation. If the hypothesis that estrogens act by reducing turnover time is true, it should be possible to detect this action even when cell proliferation is simultaneously increased by testosterone. An experiment to test this view is summarized in Table VI. It can be seen that in a group of rats

TABLE VI

Effect of Comcomitant Implantation of Estradiol and Testosterone on Sebaceous Glands[a]

Treatment	Gland volume (1000 μ^3)	Cell volume (μ^3)	Number of of new cells per gland per day	Estimated cell life (days)
Controls	222±20	1955±47	14.9±1.5	8.1±0.8
Testosterone	401±30	2276±108	35.2±2.1	5.1±0.2
Testosterone+estradiol	272±18	2220±80	28.1±4.4	5.4±0.9
Estradiol	118±15	1858±64	11.4±1.2	6.3±1.0

[a]Ten spayed female rats were used in each group (Ebling, 1957b).

treated concomitantly with both estradiol and testosterone, the incidence of mitoses and the mean size of the cells were not significantly less than in rats treated with testosterone alone, even though the glands were smaller and contained fewer cells.

This conclusion that there is no direct antagonism between estrogens and androgens at the target site is in close agreement with that of Strauss *et al.*, (1962), who found that oral ethynylestradiol had no effect on sebum secretion in either prepuberal or adult human males under

simultaneous treatment with methyltestosterone. Nevertheless, as both they and Jarrett (1955) have shown, estrogens do, in fact, suppress sebaceous secretion in normal males, and this finding does not easily fit the hypothesis that they act solely by reducing turnover time, which would leave unaffected the amount of secretion. It is, however, possible to envisage that as the glands are made smaller and their internal surface area becomes reduced, this may ultimately in turn reduce cell proliferation. Moreover, as the life of the cells is reduced, their final contribution to the sebum may be lessened.

Strauss *et al.* (1962) were unable to demonstrate any purely local effect of topically applied estrogens; even at the lowest effective dose sebum secretion was reduced equally on an untreated control site. Thus they argue that estrogens act not locally but systemically, postulating that the secretion of pituitary gonadotropins and hence of testicular androgens is inhibited. At the same time they suggest that estrogens could suppress androgen synthesis by the adrenal, either directly or through the pituitary.

The fact that estradiol was no less effective in reducing the size of sebaceous glands in hypophysectomized or adrenalectomized female rats than in intact or spayed ones, lends scant support to this idea. Equally, it provides no support for the hypotheses that estrogens act by depressing a "sebotropic" or other permissive pituitary factor, or by increasing the output of adrenocortical hormones. This does not, of course, preclude the possibility that both a direct and some such systemic action can occur in man.

C. SYNTHETIC STEROIDS

The effects on the sebaceous glands of the rat of a number of synthetic steroids of miscellaneous hormonal activities are shown in Table VII.

It is of interest that the very weakly estrogenic compounds, estriol-3-methyl ether, 16-epiestriol, and 17-epiestriol were all, in the doses given, as effective in reducing the glands as estradiol-17β. No effect was observed with the 3-methyl ethers of 16-epiestriol and 17-epiestriol, or with 16β-morpholinoestradiol, which is water soluble. Apart from slight effects with 6α-chloro-17α,21-dihydroxy-3,20-diketo-$\Delta^{1,4}$-pregnadiene and 14α,17α-dihydroxy-3,20-diketo-21-chloro Δ^{4}-pregnene, no suppression of the sebaceous glands was produced by corticoids or by any of the other steroids studied. Bullough and Laurence (1960), using the mouse, also have reported that 16-epiestriol, as well as a number of other weak estrogens including estradiol-17α, cause suppression of the sebaceous glands.

No enlargement of the glands was seen with progestogens except possibly 19-nor-17α-methyltestosterone, which was also slightly andro-

TABLE VII

Effects of Miscellaneous Steroids on the Sebaceous Glands

Name of steroid	Hormonal activity	Average number of nuclei per sebaceous gland section	
		Controls	Treated
Estradiol-17β	Strongly estrogenic	9.2 ± 0.3	5.9 ± 0.2
16β-Morpholinoestradiol	Estrogenic	8.8 ± 0.1	10.0 ± 0.6
17-Epiestriol	Weakly estrogenic	10.6 ± 0.5	6.3 ± 0.7
Estriol-3-methyl ether	Weakly estrogenic	8.9 ± 0.5	5.5 ± 0.8
17-Epiestriol-3-methyl ether	Weakly estrogenic	9.0 ± 0.2	8.0 ± 0.7
16-Epiestriol	Weakly estrogenic	9.7 ± 0.3	5.5 ± 0.5
16-Epiestriol-3-methyl ether	Weakly estrogenic	9.8 ± 0.2	9.1 ± 0.4
10β-hydroxy-19-nortestosterone	Weakly estrogenic	8.7 ± 0.7	10.6 ± 0.7
3,6-Diketo-17β-acetoxy-$\Delta^{5(10)}$-estrene	Weakly estrogenic	9.3 ± 0.3	8.4 ± 0.4
6α-Fluoro-16α-hydroxy-Substanz-S-16,17-acetonide	Strongly corticoid	9.4 ± 0.7	8.9 ± 0.7
Prednisone palmitate	Corticoid	8.6 ± 0.4	8.8 ± 0.4
6α-Chloro-17α,21-dihydroxy-3,20-diketo-$\Delta^{1,4}$-pregnadiene	Weak corticoid	9.4 ± 0.7	7.4 ± 0.5
14α, 17α, 21-trihydroxy-Δ^{4}-pregnene-3,20-dione	Very weak corticoid	9.8 ± 0.5	9.8 ± 0.4
14α, 17α, Dihydroxy-3,20-diketo-21-chloro-Δ^{4}-pregnene	Weak antiminera locorticoid	9.9 ± 0.6	7.6 ± 0.4
17β-Hydroxy-2-keto-A-norandrostane	Androgenic	8.3 ± 0.5	9.3 ± 0.4
17β-dihydroxy-17α-methyl-Δ^{5}-estrene-3β	Androgenic	9.2 ± 0.3	8.9 ± 0.5
17β-Hydroxy-2-keto-A-norandrostane caprinate (decanoate)	Androgenic	9.0 ± 0.9	8.5 ± 0.5
Androsterone	Androgenic	8.7 ± 0.3	8.7 ± 0.5
3,17β-Dihydroxy-Δ^{4}-estrene-3,17-diphenyl propionate	Weakly androgenic	10.1 ± 0.4	9.7 ± 0.4
19-Nor-17α-methyltestosterone	Strongly progestational, androgenic	9.8 ± 0.6	11.7 ± 0.1

Name of steroid	Hormonal activity	Average number of nuclei per sebaceous gland section	
		Controls	Treated
Δ^4-17α-Ethynyl-17β-hydroxyestrene	Strongly progestational, weakly estrogenic and androgenic	8.5 ± 0.3	8.6±0.4
Allylestrenol	Strongly progestational	9.3 ± 0.7	8.2 ± 0.7
17α-Ethynyl-6α-methylnortestosterone	Strongly progestational	8.7 ± 0.3	8.1 ± 0.5
6-Chloroestranetriol,3,5,17	Inactive	11.4 ± 0.5	11.5 ± 0.4
1,17β-Dihydroxy-4-methyl-$\Delta^{1,3,5(10)}$-estratriene	Inactive	9.5 ± 0.5	9.1 ± 0.6
1-Hydroxy-4-methyl-$\Delta^{3,1,5(10)}$-estratrien-17-one	Inactive	8.3 ± 0.7	8.5 ± 0.8
3-Keto-17-ethynyl-$\Delta^{4,16}$-androstadiene	Inactive	9.7 ± 0.4	9.6 ± 0.3
6-Bromoestratriol-3,5,17	Inactive	10.1 ± 0.4	9.0 ± 0.7
11α-Hydroxy-19-nortestosterone	Inactive	9.8 ± 0.7	7.7 ± 0.9
Δ^1-17β-Hydroxy-3-ketoethiocholene	Inactive	7.1 ± 0.2	6.5 ± 0.5
4-Phenyl-17α-methyl-3-keto-17β-hydroxy-4-aza-Δ^5-estrene	Inactive	9.8 ± 0.4	8.3 ± 0.5
$\Delta^{4,16(16')}$-16-methyl-17-ketoestradiene	Inactive	9.8 ± 0.8	8.6 ± 0.5
$\Delta^{4,}$-17α-methyl-17β-hydroxyestrenol-17-phenyl propionate	Inactive	9.7 ± 0.5	9.9 ± 0.5
17a-Methyl-3-keto-17β-hydroxy-4-azaestrane	Inactive	8.5 ± 0.6	7.0 ± 0.3
3α, 17α, 20α-Trihydroxy-11-ketopregnane	Inactive	8.2 ± 0.2	8.5 ± 0.2
3-Methyl-19-nor-Δ^4-androstene-3, 17β-diol	Inactive	10.2 ± 0.4	9.7 ± 0.5
3,6-Diketo-17α-methyl-17β-acetoxy-5α-estrane	Inactive	8.9 ± 0.2	8.4 ± 0.8
5β-Methyl-19 norandrostan-17β-ol-3-one	Inactive	8.5 ± 0.3	9.3 ± 0.8
14α, 17α-Dihydroxy-3,20-diketo-Δ^4-pregnene	Inactive	8.1 ± 0.7	8.5 ± 0.7
Δ^4-3, 20-diketo-11β, 21-dihydroxy-17a-pregnene	Inactive	8.0 ± 0.2	9.1 ± 0.4
16β-Morpholinoethiocholan-17β-ol-3-one	Unknown	9.7 ± 0.5	9.1 ± 0.3
16β-Morpholinotestosterone	Unknown	9.8 ± 0.5	9.7 ± 0.3

[a]Each steroid was tested on 6 intact female rats, aged 4 weeks, the littermates forming a control group. For steroids insoluble in water, each rat was given at weekly intervals three subcutaneous injections of $^2/_3$ mg in aqueous suspension and killed 7 days after the last injection. For water-soluble steroids, rats were given 0.2 mg daily for 21 days. The count of the average number of nuclei was based on 25 successive gland sections for each rat. (Ebling, previously unpublished.)

genic, or with four androgens. It is, of course, possible that the dose given, while fully adequate when the compound had reducing activity, was insufficient to produce clear effects with stimulating compounds.

V. THE HAIR FOLLICLE

Inhibition, but not stimulation, of hair growth in animals has been widely observed after administration of steroids, such as estrogens (Williams *et al.*, 1946; Baker and Whitaker, 1949; Whitaker, 1949, 1956; Mohn, 1958) and adrenocortical hormones (Baker and Whitaker, 1948; Whitaker, 1949; Whitaker and Baker 1951). Indeed, Whitaker and Baker (1951) and Fukuyama and Baker (1958) have determined the relative effectiveness of a range of natural corticoids and synthetic analogs. Adrenalectomy accelerates hair growth (Butcher, 1937; Dieke, 1948; Baker, 1951; Houssay *et al.*, 1955; Mohn, 1955), and adrenocorticotropic hormone inhibits it (Baker *et al.*, 1948; Mohn, 1955; Whiteley, 1958). Johnson (1958a,b,c) and Mohn (1958) have analyzed the effects of steroids on hair growth by quantitative studies in the rat. The activity of hair follicles is cyclic, consisting of an active phase during which the hair is formed and a resting period during which the hair is retained as a "club." In animals—though not in man, where the pattern is a mosaic—follicular activity is more or less synchronous, at least in particular regions. In the rat, for example, waves of hair growth and replacement pass over the body from the belly to the back (Butcher, 1934). Thus the action of hormones can be observed at a number of points, namely (1) the time of initiation of follicular activity in any one region, (2) the rate of growth of the hair during the active period, (3) the duration of the active period, and (4) the rate of loss of the club hairs.

In spayed female rats, for example, estradiol delayed the onset of follicular activity in all regions of the body (Table VIII). Conversely, although spaying at 49 days of age did not significantly advance emer-

TABLE VIII

Time of Initiation and Duration of Passage of the Growth Wave in Female Rats[a]

Treatment	Age (days) at emergence of ventral hairs	Time (weeks) from emergence of ventral hairs to spread of the wave over anterior dorsal region
Intact	63.0±0.53	4
Spayed	64.7±0.76	½
Spayed and implanted with estradiol	84.7±2.73	4
Spayed and injected with estradiol (100 μg/day)	98	—

[a]Eight litters, each consisting of 4 rats, were used; each experimental group contained one rat from each litter (Johnson, 1958b).

gence of the ventral hairs due 14 days later, the wave moved much more rapidly, implying that follicular activity in the flank and back was advanced. Similar effects were shown for testosterone (Johnson, 1958b). Adrenalectomy, provided it was carried out about 14 days or more before the expected emergence of hairs, also advanced it, and retardation was produced in intact rats by ACTH. Retardation of the onset of

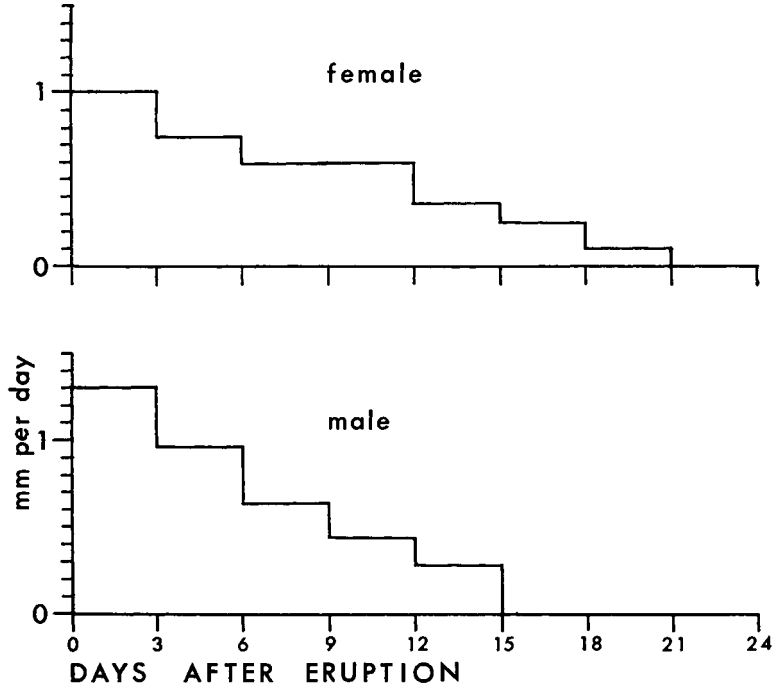

FIG. 1. Daily rates of growth of hairs of the second wave in the mid-side region of the rat. After Johnson (1958a).

follicular activity appears, then, to be an effect common to estrogens, androgens, and corticoids, and there is little doubt that it accounts for the inhibition of hair growth observed by earlier workers.

The rate of growth of hairs was studied by clipping them at skin level at intervals of 3 days. In any particular region, the definitive hair is slightly longer in the male than in the female. It can be seen (Fig. 1) that this is because at each stage the rate of growth is higher, not because the period of activity is longer. By studies of the effects of gonadectomy and of administration of estradiol, it was shown that the difference is entirely due to the activity of estrogens in the female and that the presence of the adrenal is not necessary to produce it. Testosterone had no effect on the rate of growth, nor did it offset the inhibitory effects of estrogen (Johnson, 1958b).

After the new hairs are formed, the club hairs are lost. This process was slightly accelerated by spaying and delayed by treatment with estrogen; testosterone had no such effect (Johnson, 1958b).

VI. THE DERMIS

Steroids have been reported to have many dermal effects, but their precise points of action are unknown. They may, for example, alter the constitution and properties of the collagen-mucopolysaccharide complex. Estrogens are responsible for the swelling of the sex skin of monkeys, which involves a build-up of mucopolysaccharides (Zuckerman *et al.*, 1938; Duran-Reynals *et al.*, 1950). They have also been shown to increase the amount of water in the skin of mice, probably by increasing the amount of ground substance (Cooper and Schmidt, 1957a,b). Corticoids appear to have the opposite effect. According to Mancini *et al.* (1960), in man they cause disappearance of interfibrillar mucopolysaccharides, atrophy of collagen fibers and fibroblasts and dissociation of elastic fibers. Dougherty and Schneebeli (1955), on the other hand, thought that antiphlogistic steroids actually prevent destruction of the fibroblasts in inflammatory states. Wright *et al.* (1960) found a decrease in the ratio of hexosamine to collagen in skin biopsies taken 2 weeks after systemic medication with synthetic corticoids, and Schiller and Dorfman (1957) reported that cortisone or hydrocortisone reduced the uptake of C^{14} and S^{35} by the mucopolysaccharide constituents in the rat.

The cellular constituents of the dermis may also be affected. According to Asboe-Hansen (1957, 1958), corticoids inhibit mast cells and reduce histamine secretion, though others believe that this does not explain their anti-inflammatory action, which is due to the interference with the action of the inflammatory substances (Menkin, 1951a,b; Dougherty and Schneebeli, 1955; Scott and Kalz, 1956).

VII. GENERAL DISCUSSION AND CONCLUSIONS

Steroids affect the epidermis and its derivatives, and it is possible to distinguish between their influence on cell proliferation and their influence on subsequent metabolic activity of the cells. Testosterone, for example, a powerful stimulator of mitosis in the sebaceous glands, has some such effect on the Malpighian layer, but probably no effect on the melanocytes. Estrogens do not stimulate mitosis in the epidermis and sebaceous glands of adult rats, but may do so in some species or age groups. Except for an effect in a single region of large doses, they could not be shown to increase proliferation of melanocytes in the guinea pig. Progesterone has never been shown to affect epidermis, only stimulates the sebaceous glands when given in very large doses, and does not affect melanocyte proliferation. Corticoids have been

shown to inhibit cell division in the epidermis. In view of the very high rate of cell proliferation in the active hair follicle, it is hardly surprising that it does not appear to be affected by estrogens or androgens.

Estrogens appear to affect cell differentiation and activity independently of any possible effects on mitosis. They cause the epidermis to become thinner and the sebaceous glands to become smaller, probably by accelerating cell loss, and they promote melanogenesis. Indeed, the fact that hair growth is slowed down under estrogenic stimulation may be a facet of the same kind of activity; it is possible that the total anabolic capacity of each hair cell is decreased because its speed of keratinization is increased. In the sebaceous glands, at least, turnover time may be reduced also by androgens. Progesterone may have some effect on melanogenesis.

It seems likely that the hormonal effects which have been described will become interpretable in terms of actions on cellular enzymes. Hormones such as androgenic sterois which stimulate anabolic systems will be found to affect mitosis and holocrine synthesis during the early life of epidermal and sebaceous cells. Hormones such as oestrogens and corticoids which have a predominantly catabolic effect when injected may be expected to speed keratinisation and disintegration of the ageing cells. For example, Wilson (1963) has demonstrated that previous treatment of the animals with testosterone or progesterone enhances cholesterol synthesis in slices of rat skin, acting between mevalonic acid and squalene in the Kandutsch-Russell pathway; oestradiol has an inhibitory effect.

Certain hormonal influences on the hair follicle cannot be related to the above effects. A range of steroids, including androgens, estrogens, and corticoids have a common effect in retarding the initiation of activity in resting hair follicles; estrogens, and perhaps to some extent corticoids, delay the shedding of club hairs. The possibility should be borne in mind that these effects are due to changes in the dermal environment, which is known to be sensitive to steroid action.

ACKNOWLEDGMENTS

I should like to thank Dr. G. A. Overbeek and N. V. Organon, Oss, for supplying the steroids and financing the project reported in Table VII.

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DISCUSSION

Influence of Steroids on the Melanocytes of the Skin

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Dr. Ebling has successfully presented a large amount of information in a condensed form. Although I am extremely interested in the effect of steroid hormones on the skin in general, my own work in this field has been devoted to a study of the influence of these hormones on melanin pigmentation. I propose therefore to touch on a number of general points that Dr. Ebling has raised, but will largely confine myself to a detailed examination of the influence of steroids on the melanocytes of the skin.

In his Section II concerned with the superficial epidermis, Dr. Ebling described experiments on castrated rats which showed that testosterone stimulates epidermal mitosis without significantly changing the thickness of the Malpighian layer. Eartly *et al.* (1951) have shown, however, that testosterone increases the thickness of the epidermis in the rat and this effect is antagonized by thyroxine. Burrows (1944) has also reported that testosterone can convert a nonsquamous type of epithelium in the mouse into a keratinized epithelium.

With regard to the effect of estrogens on the epidermis, the majority of workers are agreed that they cause an increased epithelial proliferation, cornification, and desquamation. It would also appear that estrogens have their greatest effect in the perigenital areas and in the nipples (Davis *et al.*, 1945). The observation by Dr. Ebling (1955) that in the adult rat no mitotic effect is produced by estrogens is difficult to explain.

Turning to Section IV on sebaceous glands, it is now generally accepted that sebaceous glands increase in size and activity with the oncoming of puberty in both sexes in the human subject. In the male, experimental evidence shows that testosterone in the presence of the pituitary is responsible for this change. Men castrated before puberty and eunuchoids do not develop acne or seborrhea, but they do so if they are treated with large doses of testosterone (Hamilton, 1941). The observations of Strauss and Kligman (1961) show that there is a definite age factor, for androgenic enlargement of the glands does not consistently occur in very young normal children, although it always occurrs in children of prepuberal age. The same authors believe that the adrenal hormones play an important part in the process in girls, since large amounts of hydrocortisone cause glandular enlargement and both estrogen and progesterone in physiological doses have no effect.

Although Dr. Ebling did not mention this, it is interesting to note that apocrine sweat glands undergo great development during puberty in both sexes (Pincus, 1927). It is believed by some authorities that sebaceous and apocrine glands have a similar reaction to hormones (see Rothman, 1954). However, the application of estrogens or androgens topically to the axilla, or the implantation of pellets of these hormones in the human subject, have been found to have no effect on the apocrine glands (Shelley

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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and Cahn, 1955; Shelley and Hurley, 1953). Montagna (1961) believes that the contradictions which exist between reports on the influence of hormones on apocrine glands is largely due to the fact that most authors have failed to appreciate the enormous variations which occur in the glands even in one normal individual.

In Section V of Dr. Ebling's paper, dealing with the effect of steroids on hair growth, the greatest care must be taken in extrapolating results from one species to another. For example in, man an excess of adrenal cortical hormones may lead to excessive hair growth in specified areas or may have no effect on hair growth (Grant *et al.*, 1950), whereas in rodents the administration of adrenocortical hormones arrests the growth of hair (Whitaker and Baker, 1951). In the human subject it is usual to classify hair into three groups when discussing the influences of hormones on hair growth (Flesch, 1954): (1) Hair that is the same in both sexes and is not primarily under hormonal control. This includes lanugo hair all over the body, eyebrows and eyelashes. (2) Hair under endocrine control. This includes the axillary hair and the major lower part of the pubic hair. (Scalp hair may also be included in this category.) (3) Hair that is a secondary male characteristic, e.g., the beard and moustache.

The influence of hormones on the different types of hair in the human subject has been reviewed by Flesch (1954). Taking testosterone as an example, it has been shown that it is responsible for the growth of the beard and for the complete development of axillary and pubic hair in the male. In eunuchs and prepubertal castrates, the male type of hair fails to develop. In contrast, the male sex hormones create conditions which may lead to early baldness in genetically predisposed individuals (Hamilton, 1942). The influence of ovarian hormones on human hair is not known.

In Section VI on the dermis, Dr. Ebling did not mention the effect of steroids on dermal blood vessels. I wondered whether he had made any observations on this problem. It is just possible that some of the morphological changes noted in the epidermis and its appendages are secondary to circulatory changes in the dermis. Apparently both natural and synthetic estrogens do have a dilator effect on the capillaries of the skin, but not on the arterioles (Reynolds and Foster, 1939). Testosterone is thought to have a vasodilator action and has been used with success in the treatment of vascular disorders (Edwards and Duntley, 1939). The administration of cortisone, on the other hand, brings about a gradual increase of arteriolar tone in the normal skin, as shown by Ebert and Barclay (1952).

Let us return now to Section III concerned with melanin pigmentation of the skin. The literature contains numerous reports of experiments attempting to substantiate a melanocyte–sex hormone relationship. Inconsistent findings are probably due to a number of important factors:

1. Many of the reports have been based on experiments in which intact animals have been used, with an endogenous source of the hormone under test.

2. Many pigmentary phenomena, such as dispersion of melanin granules in the melanocytes, pigment cell proliferation, and melanin synthesis, have been grouped together as a single or related series of effects. This grouping has made the findings of many investigators difficult to assess.

3. The use of different species of animals and different hormone preparations at different dose levels has made comparison of results difficult to interpret.

4. A great number of the observations have been macroscopical only, and most of those that have been microscopical have been confined to vertical skin sections. Vertical sections have the disadvantage that accurate melanocyte counts are impossible and that the appearance of the melanocytes cannot be adequately demonstrated. In my

work, the study of melanocytes has been confined to the superficial epidermis of guinea pig skin; the melanocytes of the hair follicles have been ignored. All the skin specimens were processed under identical histochemical conditions. The specimens that were compared before and after hormone treatment were always removed from adjacent areas in the same skin region in the same animal. Both vertical sections and skin sheets were used.

The effect of androgens on melanogenesis is not clear cut. The results reported by Hamilton (1939) are based solely on macroscopic observations. The results of Edwards and his co-workers (1941) are based upon spectrophotometric analysis of the skin pigments. Spoor (1953) criticized this method on the grounds that there is a variation in standards used for the calibration of the reflectance curves and also that melanin pigments absorb so strongly that their presence masks small variations in pigment content. Bischitz and Snell (1959a&b) found that, in the intact and orchidectomized male guinea pig, testosterone was incapable of producing uniform changes in the melanocytes and melanin except in the anterior abdominal wall of the orchidectomized animals, where a definite reduction in melanogenic activity took place.

Orchidectomy was shown to decrease melanin pigmentation of the skin in men (Edwards *et al.*, 1941) and in the hamster (Kuppermann, 1944). Guldberg (1934), on the other hand, found that melanin pigmentation of the mammary skin in the guinea pig was occasionally increased. Snell and Bischitz (1959) showed that in guinea pigs weighing between 350 and 390 gm orchidectomy had no significant effect on the melanocytes and melanin in the skin of the ear and anterior abdominal wall, but in the areola and sole of foot melanogenesis was stimulated. In heavier, more mature guinea pigs, melanogenesis was stimulated in all the specimens examined.

The female sex hormones have been implicated in pigment formation ever since Lipschütz (1930) showed that transplantation of the ovaries into male guinea pigs produced hyperpigmentation of the areolae. On reviewing the literature, it is surprising to find that the effect of ovariectomy on this process in animals has apparently not been studied. Edwards and Duntley (1949), however, using the spectrophotometer method, reported that in women the skin pigments remained unaltered following ovariectomy. Bischitz and Snell (1960) showed that in the guinea pig ovariectomy inhibited melanogenesis.

The administration of small doses of estrogen to ovariectomized guinea pigs (Bischitz and Snell, 1960) increased the amount of melanin both inside and outside the melanocytes in all the skin regions examined. The greatest effect was produced in the areolar skin, but no significant changes occurred in the melanocyte counts: with large doses of estrogen (Snell and Bischitz, 1960a&b) the effect on melanogenesis was much greater and a significant rise in the melanocyte count occurred in the anterior abdominal wall.

Small doses of progesterone in the ovariectomized guinea pig (Bischitz and Snell, 1960) slightly stimulated melanogenesis. On the other hand, very large doses of progesterone (Snell, 1962) had the opposite effect, causing a reduction in the amount of free melanin. This difference in effect may explain the contradictory results of other workers (Davis *et al.*, 1945; Wheeler *et al.*, 1953; Kuppermann 1944; Tsubota, 1936; Lerner *et al.*, 1953).

It was interesting to find that when small doses of estrogen and progesterone were given together to the ovariectomized guinea pig (Snell and Bischitz, 1960b) melanogenesis was greatly stimulated; the degree of stimulation was greater than that produced by either hormone when given alone in the same dose.

Turning now to other steroid hormones and pigmentation, Shizume *et al.* (1954) have shown that cortisone and hydrocortisone slightly decrease the color of isolated frog skin. Cortisone is, however, capable of inhibiting the release of the melanocyte-stimulating hormone of the pituitary and in this manner causes the skin to become much lighter (Lerner *et al.*, 1954). In patients with Addison's disease the administration of cortisone or hydrocortisone decreases the hyperpigmentation (Hall *et al.*, 1953). In a similar manner, patients who are subjected to a bilateral adrenalectomy can be prevented from becoming hyperpigmented by the administration of cortisone.

To sum up, Dr. Ebling has shown that the many structures which go to make up the skin can be influenced in their activity by steroid hormones. Skin is an ever changing organ, and many of its alterations are revealed by changes in its morphology. But here I must give a word of warning. There are species differences not only in the histology of the skin, but in their response to different hormones at different dose levels. Let us be careful that we do not indulge in dangerous extrapolations from one species to another.

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Hormonal Factors in Aging[1]

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I. INTRODUCTION

This is a report on our observations on the epidermis of aged skin, and on the effects upon the epidermis of topically applied steroids. We have chosen to study the epidermis of the malar region of the face and that of the dorsum of the hand for convenience and because there are here consistent, manifest changes, probably due to the combined effects of aging per se and of years of exposure to sunlight. In other regions of the body, the patterns of aging are more deliberate and perhaps different.

Very little generalization can be made about the epidermis of the skin from the face and of the dorsum of the hand of aged individuals. Not every subject exhibits the same deviations, and focal differences occur in the same biopsy specimen of 7 mm in diameter. Even in those areas where the epidermis shows the greatest mutation, there may be here and there isolated, relatively normal areas. Chronological age is not a valid index of progressive aging changes; the epidermis may be badly damaged in some individuals and practically normal in others of the same age.

In all our preparations, regardless of the magnitude of alterations in the dermis, there is some unaltered papillary body of the dermis immediately underneath the epidermis. Such "normal" dermal tissue may be only a few microns thick and consists of widely spaced delicate collagenous fibers and fibroblasts. The dermis, then, regardless of the amount of degradation in it, attempts to provide a normal bed upon which the epidermis can grow. This point may be significant when one searches for therapeutic agents which might bring about a restoration to a younger condition.

[1]This work was supported, in part, by grants from Revlon, Inc. and the United States Public Health Service RG-6924(C2), administered by Dr. Albert M. Kligman from the University of Pennsylvania.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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II. MATERIALS AND METHODS

These observations were made on four groups of subjects, each group consisting of 10 men and women, 65 years or older. *One side* of the face, and the dorsum of one hand, were anointed daily for at least 6 weeks with an ointment containing a steroid; the opposite side of the face and the other hand were treated with the ointment alone. One group received 1% testosterone propionate, a second group received 1% progesterone, a third group 0.5% ethynylestradiol, and a fourth group 0.5% pregnenolone.

Four biopsy specimens were removed from each experimental subject: one from each side of the face and from each hand before the onset of the treatment, and one from each of these test areas after 6 weeks. The specimens were fixed in Helly's fluid or in 10% formaldehyde, embedded in paraffin, sectioned serially, and stained with a number of histological stains.

III. OBSERVATIONS

A. THE FACE

The epidermis of the face of subjects 21–28 years of age is surprisingly thick. Compared with that elsewhere, the underside is relatively

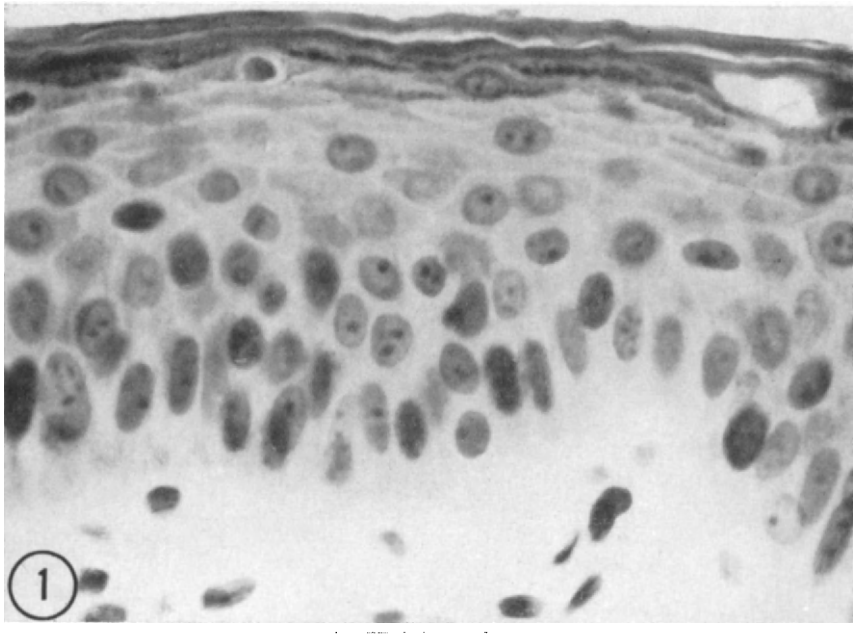

FIG. 1. Epidermis, normal, from the face of a man 27 years old. Observe the even distribution of the cells from the basal layer onto the stratum granulosum. There is a gradual progression of events as the cells flow upward from the basal layer to the higher layers. Stained with hematoxylin and eosin.

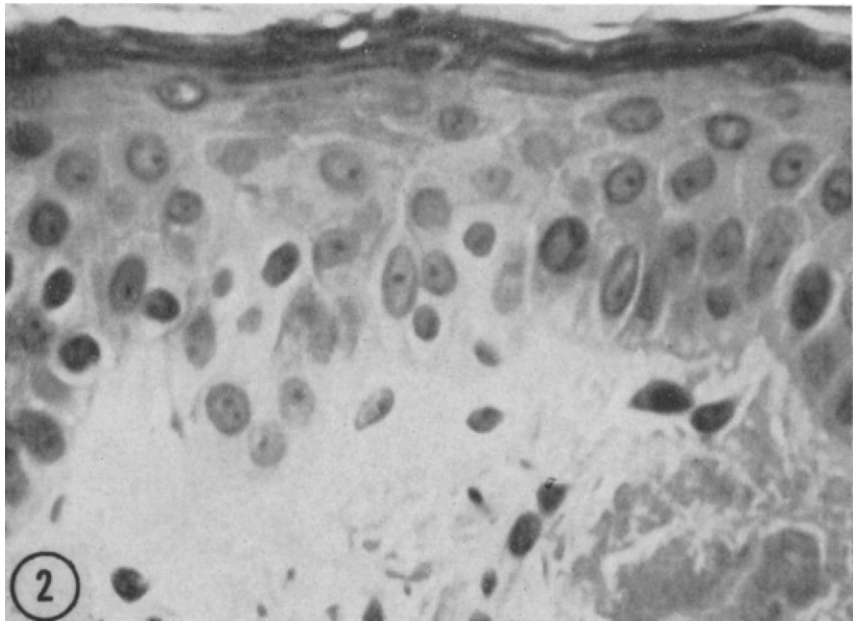

FIG. 2. The epidermis from the face of a man 72 years old; compare with Fig. 1. There is great disparity in the size of the cells and in the size of their nuclei. The orderly arrangement of the cells seen in Fig. 1 is lacking here. A few basal cells on the extreme right look fairly normal when contrasted with those to their left, which are greatly disorganized.

flat, having only an occasional shallow ridge. The basal layer is composed of cells that are cuboidal or columnar. Cells flow upward from the basal layer into the spinous layer, become plump and polyhedral, and gradually flatten out horizontally below the compact, two-cell-thick granular layer (Fig. 1). The moderately thin stratum corneum is compact. Small areas here and there show some disorganization in the arrangement of the cells in lower spinous layers. These focal areas of apparent damage resemble the more generalized changes that occur gradually with aging.

In individuals 60–90 years old, the epidermis may be very thick: often the Malpighian layer is 7 or 8 layers deep, but in the same specimen it may be only three layers thick. A stratum granulosum of flat, long cells, one or two deep, is often discontinuous. The stratum corneum remains thin and compact. Although the underside of the epidermis is flattened, narrow cords of cells may grow for a distance down into the papillary body at the junction of the epidermis with the wall of a pilary canal.

There are many, but subtle, histological deviations of the epidermis. The basal layer and the Malpighian layer are composed of cells that

show great disparity in size and shape and in the size and shape of their nuclei. These cells are tumbled or heaped haphazardly (Fig. 2). Sandwiched among columnar or cuboidal, basal cells may be small round cells or greatly elongated ones with very flat nuclei. Oval cells, oriented parallel to the surface are insinuated between the basal cells and the subjacent dermis. Many basal cells have a bulky, amorphous, achromic cytoplasm; others have a cytoplasm shrunken from the nucleus. A conspicuous, delicately crenated, strongly periodic acid-schiff (PAS)-positive basement membrane follows the contours of the underside of the epidermis, accentuating the denticulation at the underside of the basal cells. There are very few pigmented cells; large tracts of epidermis seem to have no recognizable melanocytes. When visible, melanocytes are gathered in groups or nests. Mitotic activity, which in "normal" epidermis occurs mostly in the basal layer, in aged skin may occur anywhere in the lower cells of the Malpighian layer, and many of the dividing cells appear to be polyploidal.

1. The Effects of Treatment with Steroids

The changes which are induced by the application of these steroids are subtle; the casual histologist might easily overlook them. Yet, in most cases, the tissues from the treated side can be separated unerringly

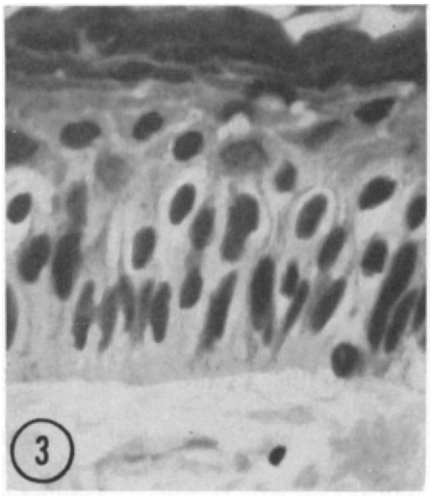

FIG. 3. The epidermis from the untreated skin of a woman 82 years old. Observe the shrunken appearance of all the cells.

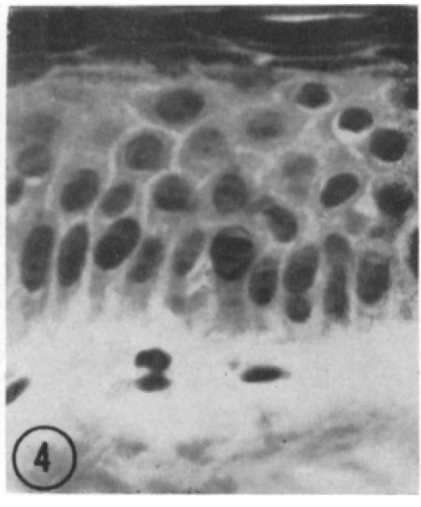

FIG. 4. The epidermis from the skin of the same woman as in Fig. 3, but treated for 6 weeks with testosterone propionate. Compare with Fig. 3. The cells are larger, the nuclei have a more even size, and the cells have an orderly arrangement from the basal layer to the stratum granulosum. Stained with hematoxylin and eosin.

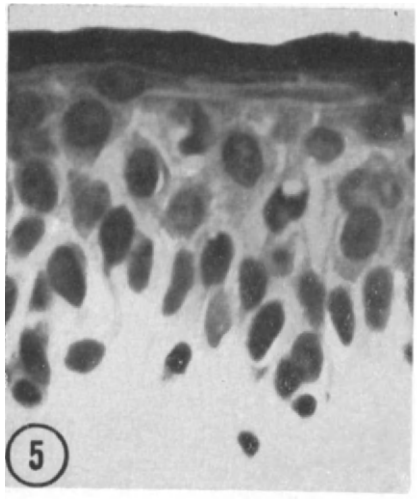

FIG. 5. Epidermis from the face of a woman 71 years old, showing the "typical" senile changes described.

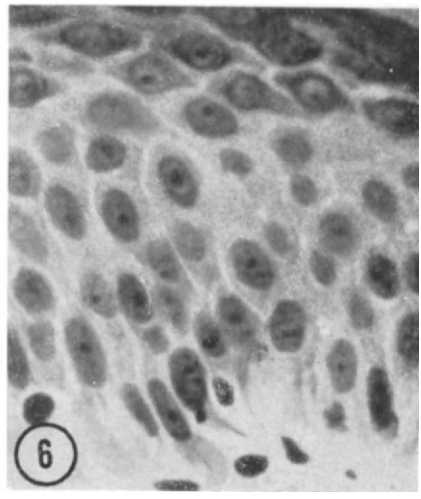

FIG. 6. Epidermis from the face of the subject of Fig. 5, treated with progesterone for 6 weeks. The epidermis has been restored to a condition similar to that found in the skin of young individuals.

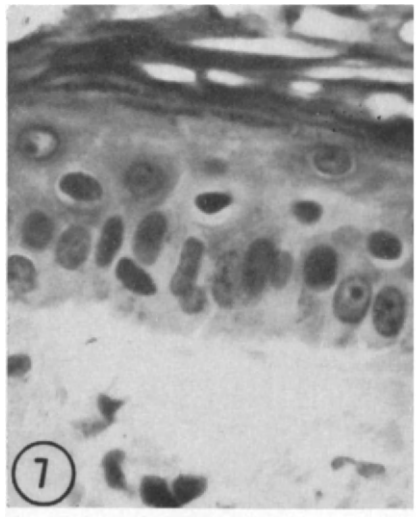

FIG. 7. Epidermis from the face of a woman 88 years old. There is disorganization of the layer and many of the cells are shrunken.

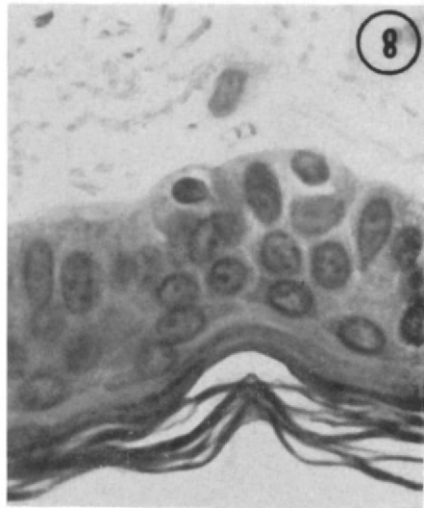

FIG. 8. Epidermis from the face of the subject of Fig. 7, but treated for 6 weeks with pregnenolone. There is nearly complete restoration of the condition found in the epidermis of younger subjects. The cells are all plump; the nuclei are cleanly outlined and they stain well. There is also a gradual progression of the cells from the basal layer to the upper strata.

from those of the untreated side (Figs. 3–8). The modulations which we have noted usually parallel clinical improvements, but *this is not always the case.* Furthermore, even a tissue which has responded well to treatment may have small islands of unchanged cells scattered at random in the epidermis. An appraisal of changes, therefore, can be made only by scanning the entire tissue.

a. Testosterone Propionate, Progesterone, and Pregnenolone

The basal cells become more homogeneously cuboidal and stream gradually upward to the upper Malpighian layers. The cells remain plump and of approximately the same size in any one stratum. The nuclei have a smooth membrane as in younger skin; they are larger, of the same size, and they stain more intensely. There is an appreciable decrease in the number of shrunken cells and "clear" cells, and the stratum granulosum is often thicker. In our best results, the epidermis again looks like that of young subjects (Figs. 3–8).

These three substances have a similar effect upon the epidermis: testosterone and pregnenolone may be slightly more effective than progesterone (Figs. 3–8). Often, the results are more legible in women than in men.

In every case, the epidermis of the contralateral side, which was anointed with the hydrophilic ointment base alone, showed alterations qualitatively similar to those induced by the steroids, but the changes were often minimal.

Grossly visible change was often, but not always, accompanied by the epidermal changes outlined above. A few subjects with splendid clinical improvements revealed modest change in the epidermis. Steroid-induced alterations, therefore, cannot be limited to the epidermis. A fuller report about other changes will be presented in due time.

b. Ethynylestradiol

Those subjects who were treated with this hormone showed some improvement of the epidermis, but the results were equivocal. We could not always distinguish them from those obtained with the ointment alone.

c. Summation

Subjects treated for 6 weeks or longer with ointment bases that contained testosterone propionate, progesterone, or pregnenolone, all showed decided improvement of the epidermis. "Improvement," as used here signifies a restoration to a condition similar to that in the skin of young adult subjects. The ointment base alone, also induced some amelioration, but these were minimal in contrast with those

brought about by the steroids. Estrogen, in contrast with the other three, showed some questionable improvement beyond that caused by the hydrophilic ointment alone.

B. THE DORSUM OF THE HAND

The epidermis on the back of the hand of young subjects combines the features of the glabrous volar surface with those of hairy skin, and it is surprisingly thick. It has a moderate undersculpturing of rete ridges oriented along the axis of the hand. The Malpighian layer may be 6 or 7 cells deep, and its cells have a much more orderly arrange-

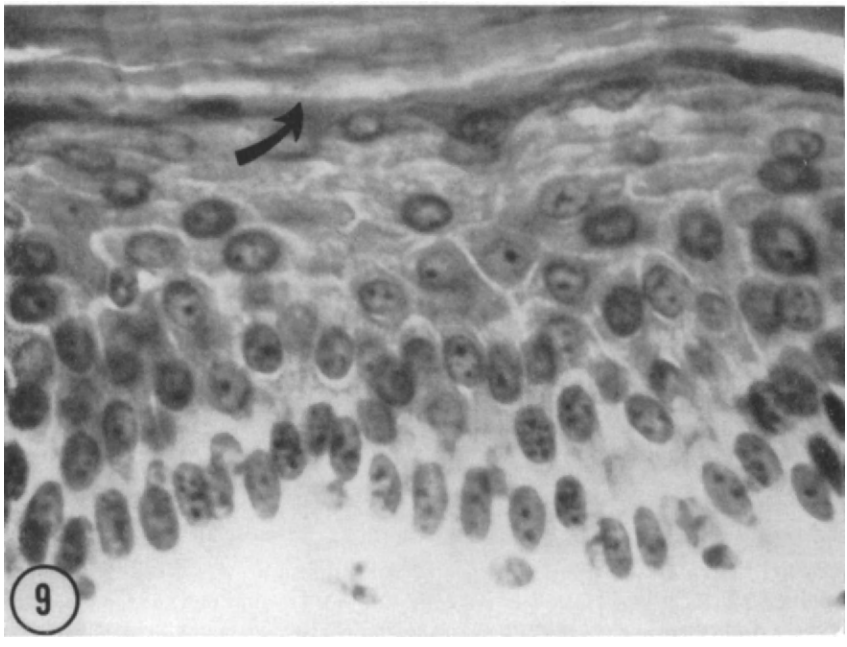

FIG. 9. Normal epidermis from the back of the hand of a man 27 years old. The arrow points to the thin, but clearly delineated, stratum lucidum. Stained with hematoxylin and eosin.

ment than those in the epidermis of the face. The spinous cells below the stratum granulosum, which in the epidermis of the face are flat, in the hand are turgid and almost cuboidal. The continuous stratum granulosum is at least two cells deep and often thicker. The stratum granulosum is separated abruptly from the thick, compact, stratum corneum by a thin stratum lucidum (Fig. 9).

The epidermis of the back of the hand undergoes aging changes similar to those that develop on the face. Even in the oldest subjects studied, the epidermis may be very thin or it may remain relatively

thick; the rete ridges, except at the junction with the ducts of the sweat glands, are largely flattened out. At the site of lentigos, the epidermis is markedly reduced in thickness and has a remarkable growth of digitiform protuberances on its underside. In the basal layer, the palisadelike arrangement seen in the epidermis of young subjects persists only here and there. In aged subjects, the basal layer consists of cells of different sizes with apparently different orientations (Fig. 10).

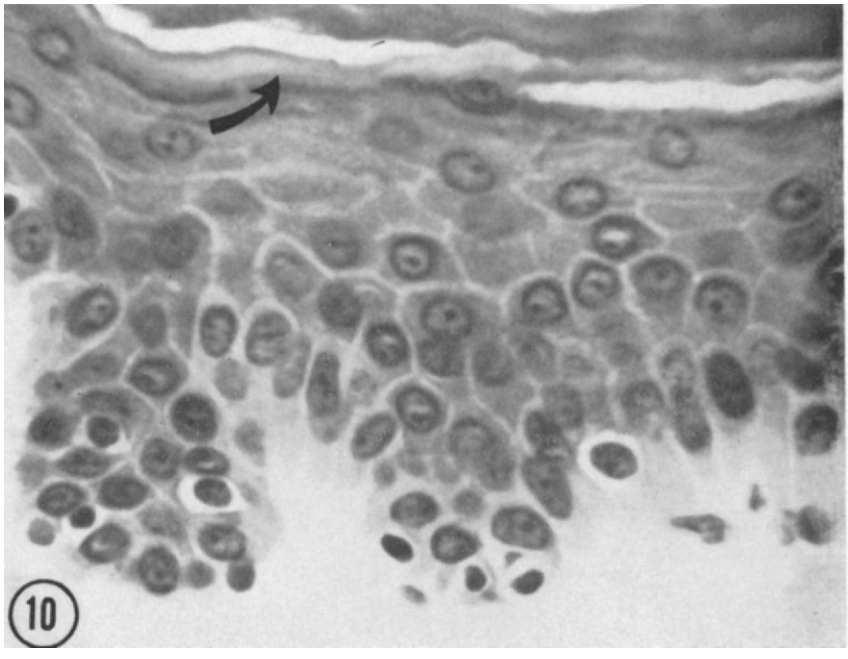

FIG. 10. The epidermis from the back of a hand of a man 72 years old. Compare with Fig. 9. The changes seen here are typical of those seen in most of the older subjects that we have studied. The arrow points to the stratum lucidum.

Except in the areas of lentigos, melanocytes are very few and they usually occur in clusters. Oval cells, which may or may not be clear cells, are often trapped between more typical basal cells and the basement membrane. The cells of the spinous layer show the general disorientation which we have described earlier in the epidermis of the face. The stratum granulosum, the stratum lucidum, and the stratum corneum remain apparently unaltered, regardless of age (Fig. 10).

1. The Effects of Treatment with Steroids

Treatment of the skin with the steroids brings about improvements similar to those seen in the face. As in the face, the effects are spotty, and even in the best cases small islands of epidermis reflected no effects of the treatment.

a. Testosterone Propionate, Progesterone, and Pregnenolone

All three of these substances were effective in bringing about restorative changes. In some cases the entire epidermis appeared surprisingly vigorous and youthful. The major repairs were a restoration of a nearly columnar or cuboidal basal layer and a reestablishment of a spinous layer in which turgid, polyhedral cells flowed upward in an orderly progression. The cells immediately underneath the granular layer were large, with well-stained, round nuclei, as in the epidermis of younger skin. All three steroids produced approximately the same results, testosterone and pregnenolone perhaps being more efficacious than progesterone. The ointment base alone also brought about some minor restorative modulations.

b. Ethynylestradiol

The hormone-treated skin was often difficult to differentiate from that treated with the ointment base alone.

c. Summation

As on the face, pregnenolone, a steroid with apparently no hormone action, testosterone propionate, and progesterone were the most effective in bringing about reparative modulations; ethynylestradiol had considerably less influence upon the epidermis. It is significant that these effects are all local, in spite of prolonged treatment with the steroids.

IV. CONCLUSIONS

Manganotti (1955), after a thorough analysis of aged skin, concludes that there are no characteristic alterations in the epidermis. Our observations largely agree with those of Manganotti, with the exception that we do find the changes outlined herein. There is no reduction in the volume and in the function of its cells, even though other authors speak of "atrophic changes" in aged skin (Hill and Montgomery, 1940; Evans *et al.*, 1943; Cooper, 1942; Knox *et al.*, 1962).

We have seen that, although small islands of epidermis everywhere may be shrunken, most of the epidermis has about the same number of layers as that of young subjects, and in frozen sections, it is almost as thick.

In paraffin sections of aged, untreated skin, the cells of the epidermis are withered, whereas in sections of young subjects, or in those of skin treated with the steroids, the cells are plump. Such shrinkage is not evident in frozen sections. From these observations emerges the thought that the epidermis in aged skin may be heavily hydrated and poorer in its content of protein than that of young skin and that treatment with the effective steroids may bring about a restoration of synthesis of protein to approach that of younger levels. Since a restoration to a "normal," "younger" state can be brought about with steroids, we consider the aging modulations of the epidermis to be reversible rather than permanent.

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DISCUSSION

Action of Estrogens on the Skin

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The results which Professor Montagna has presented suggest most strongly, that the cosmetic application of estrogenic creams to senile skin is not soundly based.

Although enormous doses of steroids were administered in these experiments it is reported that any effects on the epidermis were confined to the site of application even after 6 weeks of treatment. Since large doses of the steroids must have been distributed systemically this result is surpising and I would be pleased to have further comment on its significance.

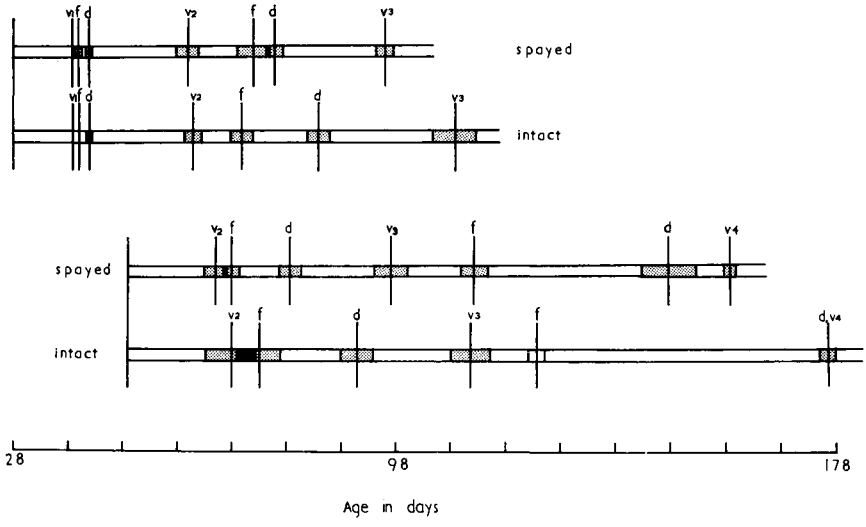

Fig. 1. Mean ages at eruption of hairs of intact female rats and female rats spayed at 4 weeks or 7 weeks of age. The shaded limits represent plus or minus 1.5 times the standard errors. In comparing the different treatments, when the limits do not overlap the differences are significant with $P<0.05$. v = Ventral; f = flank; d = mid-dorsal for wave 1, 2, 3, or 4.

In view of the results presented by Dr. Ebling in the previous paper, on the effects of some similar steroids on the epidermis of the rat, I would be interested to know whether the improvements in the appearance of the epidermis after treatment with the effective steroids were here accompanied by any changes in cellular proliferation.

One might expect that the requirements for the growth of hair, which is an epidermal derivative, would be basically similar to those of the superficial epidermis. I would like to comment briefly on the effect of estradiol on hair growth in the rat during aging.

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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In the rat hair growth occurs in a series of waves of follicular activity which begin ventrally and spread around the body (Johnson, 1958a). In older rats these waves of growth pass around the body more slowly, so that the resting phase of the hair follicles between each period of activity is lengthened.

Spaying of female rats aged 7 weeks accelerates the passage of the second of these waves of hair growth by shortening the length of the follicular resting phase in each region of the body. 17β-estradiol, on the other hand, delays the passage of the wave (Johnson, 1958b).

It was of interest to know what happened to the length of the follicular resting phase in the absence of the gonadal steroids as the rats grew older. After spaying at 4 weeks or 7 weeks of age, observations were therefore continued for several waves of growth.

Spaying at 4 weeks of age had no effect on the first wave of hair growth, which begins ventrally at 39 days of age. This is to be expected since the gonads are not hormonally active at this time.

In the second and subsequent waves of growth, new hairs appeared earlier in each region of the body of the spayed rats as compared with intact controls (Fig. 1).

It is clear, however, from Table I that this earlier eruption of hairs is due only to an

TABLE I

Number of Days between Eruption, in the Ventral Region, of New Hairs of Each Growth Wave

Treatment	Waves 1—2	Waves 2—3	Waves 3—4
Spayed at 4 weeks	21.0 ± 1.31 (NS)[a]	35.8 ± 1.04*	—
Intact	22.8 ± 0,92	48.0 ± 3.40	—
Spayed at 7 weeks	—	31.8 + 1.53**	63.2 ± 2.19 (NS)
Intact	—	41.3 + 0.91	62.5 + 0.70

[a]Comparison with intact controls: NS = not significant; * $P = 0.02$—0.01; ** $P = <0.01$.

initial advancement of follicular activity between the second and third waves of growth. Subsequently, the periodicity of the follicles is the same in spayed and intact rats. The longer time between eruption of new hairs of waves 3 and 4 is due to a lengthening of the resting phase with age, and this was of the same duration in both spayed and intact rats.

It must be concluded therefore, that the aging of hair follicles in female rats, as manifested by this increase in the length of the resting phase, is unrelated to the gonadal steroids.

That the hair follicles in older rats are still capable of being stimulated to earlier activity has been demonstrated by grafting skin to younger rats, whereupon the follicles take up the rhythm of the host (Ebling and Johnson, 1961), but the nature of this stimulus is as yet unknown.

It appears, therefore, that the cells of both the epidermis and the hair follicle are affected by estrogen in young animals, but not in older animals. Obviously the controlling mechanism for cellular activity of these tissues must be looked for elsewhere.

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Influence of Steroids on Enzymatic Activities and Permeability of Cells, Mitochondria, and Lysosomes[1]

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The studies to be described in this presentation stem directly from the detailed description approximately seventeen years ago of the *in vivo* involution of lymphoid tissue caused in a variety of species by the administration of certain adrenal cortical steroids (Dougherty and White, 1945). Within the past five years, our investigations have sought to establish the biochemical basis for the lymphocytolysis induced by certain adrenal cortical steroids, e.g., cortisol (cf., White *et al.*, 1961). Although the mechanism of this steroid-induced lymphocytolysis remains unknown to the present time, the data which have been obtained reveal a wide variety of effects of a number of steroids, both *in vivo* and *in vitro*, on the metabolism of intact normal and malignant rat lymphoid tissue and liver, as well as on subcellular fractions derived from these tissues.

At the outset it should be pointed out, that in our studies the observed relative activities of the various steroids used, as assessed by measurements of oxidative processes, protein and nucleic acid biosynthesis, and energy-yielding pathways, bear no relation to the relative potencies of these steroids as thymolytic substances. For example, although cortisol is an effective thymolytic steroid, whereas deoxycorticosterone (DOC) and progesterone have no or little activity under the usual conditions of bioassay, the last-named compounds are significantly more active than cortisol as *in vitro* inhibitors of a variety of metabolic phenomena in normal and malignant lymphocytes. On the other hand, it may be noted that 17α-ethyl-19-nortestosterone is among the most potent of the steroids in its effects on lymphoid tissue metabolism, and more recently the thymolytic activity of this steroid has been recognized (Aschkenasy, 1959; Llaurado *et al.*, 1959; Saunders, 1960).

[1]The data described in this paper were obtained in investigations aided by grants from the American Cancer Society (P-68), the National Institutes of Health, United States Public Health Service (CY-3154), and the National Science Foundation (G-5711).

Hormonal Steroids, Biochemistry, Pharmacology and Therapeutics: Proceedings of the First International Congress on Hormonal Steroids, Volume 1
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Table I shows data obtained in collaboration with Dr. Melvin Blecher illustrating the relative effects of various steroids upon respiration and glycine-2-C^{14} incorporation by thymic lymphocytes (Blecher and White, 1958, 1959). When added *in vitro* to a suspension of thymic

TABLE I

Relative Effects of Steroids upon Respiration and Glycine-2-C^{14} Incorporation by Thymic Lymphocytes[a]

Steroid	Concentration of steroid ($M \times 10^{-5}$) required to produce a 50% inhibition of		
	Oxygen uptake	Protein synthesis	Nucleic acid synthesis
17α-Ethyl-19-nortestosterone	6.2	3.2	5.2
17α-Ethyltestosterone	9.0	5.3	10
Deoxycorticosterone	17	5.0	8.5
17α-Methyltestosterone	17	15	11
19-Nortestosterone	42	14	27
Corticosterone	36	16	25
Testosterone	70	36	31
Cortisol	143	57	70
Cortisone	192	120	96

[a]Data obtained in collaboration with Dr. Melvin Blecher. (Blecher and White, 1958).

lymphocytes incubated in phosphate buffer, all the steroids studied inhibited glycine-2-C^{14} incorporation into the total proteins and nucleic acids of the cells. The concentration-inhibition data of Table I show: (1) a wide variety of steroids to be effective inhibitors; (2) a rather good correlation between the capacity of a steroid to diminish oxygen uptake and its effectiveness in inhibiting protein and nucleic acid synthesis; and (3) no evident correlation between the inhibitory effectiveness of the steroids in these experiments and their thymolytic activity.

It may be noted that preincubation of lymphocytes with steroids for as long as 2 hours, followed by removal of the steroid from the incubation medium, had no retarding influence on the capacity of the cells subsequently to incorporate glycine-2-C^{14} into proteins and nucleic acids. This might indicate that whatever the basis for the inhibitory effects of a steroid, the latter does not irreversibly alter metabolic processes in lymphocytes, including cell permeability. Data obtained with broken-cell preparations support this point of view and will be discussed subsequently. Koritz and Dorfman (1956) observed that deoxycorticosterone added *in vitro* to reticulocytes, inhibited both oxygen consumption and incorporation of glycine-1-C^{14} into the total proteins of these cells. Evidence was presented that the steroid had no influence on the permeability of reticulocytes to glycine or potassium.

The presence of glucose in our incubation medium, and the dependence of many synthetic processes upon energy-yielding reactions, led

to a study in collaboration with Dr. Lillian Jedeikin of the *in vitro* and *in vivo* effects of various steroids on another aspect of the metabolism of lymphocytes, namely, the oxidation of glucose by these cells (Jedeikin and White, 1958). Studies of the influence of steroids added *in vitro* on the oxidation of uniformly labeled glucose to carbon dioxide by cell suspensions of normal and malignant lymphoid tissue yield data of which those shown in Table II are typical. The inhibitory effects of

TABLE II

Inhibition by Cortisol and Deoxycorticosterone of Endogenous Respiration, Oxygen Consumption, and Glucose Oxidation of Thymus and Lymphosarcoma[a]

Parameter	Cortisol[b]		Deoxycorticosterone[b]	
	Thymus	Lymphosarcoma	Thymus	Lymphosarcoma
Endogenous respiration	16 (1)	44 (1)	46 (1)	65 (2)
Oxygen consumption[c]	32 (8)	55 (2)	86 (2)	66 (2)
Glucose oxidation	31 (8)	69 (2)	93 (2)	88 (2)

[a]Values given are mean per cent inhibition as compared to control without steroid. Values in parentheses indicate number of individual experiments.

[b]In each experiment the concentration of steroid was 180 μg/0.85 ml.

[c]In the presence of 3.5×10^{-3} *M* glucose.

From Jederkin and White, 1958.

cortisol and deoxycorticosterone on the oxidation of glucose by thymic cells were also seen in experiments in which the steroid was injected into the rat prior to removal of the lymphoid tissue for subsequent study *in vitro* (Table III). In these *in vivo* studies with the two steroids,

TABLE III

Effect of in Vivo Injection of Steroids on Glucose-U-C^{14} Oxidation by Thymic Cell Suspensions in Vitro

Type of animal	Steroid injected[a]	μ atoms of glucose carbon oxidized 5×10^8 cells	% Inhibition
Normal	—	3.54	—
Normal	Cortisol	2.30	34
Normal	DOC	2.64	25
Adrenalectomized	—	3.51	—
Adrenalectomized	Cortisol	2.56	27

[a]Single injection of steroid intraperitoneally, 5 mg per 100 gm body weight, given at 4 hours prior to sacrifice of rat. From Jederkin and White, 1958.

only cortisol caused thymic involution, as determined by weight loss and gross appearance. Thus, *biochemical alterations had been induced in lymphoid tissue by administration of a steroid, e.g., deoxycorticosterone, which has been categorized as having no apparent effect on the histology of lymphoid tissue.*

The inhibition of glucose oxidation to carbon dioxide by lymphocytes in the presence of a variety of steroids added *in vitro*, and the possible relationship of this to the inhibitory influence of steroids on protein and nucleic acid synthesis by these cells, led to investigations of the mode of action of these steroids. In collaboration with Dr. Blecher, initial efforts were directed toward development of broken-cell preparations of normal and malignant lymphocytes which might show responses to steroids similar to those seen with intact cells. In addition, attention was focused on certain of the specific reactions in the oxidation of glucose.

Fortified, broken-cell preparations of thymic tissue and of the Murphy–Sturm lymphosarcoma have been obtained which utilize anaerobically either glucose, glucose 6-phosphate, fructose 6-phosphate, or fructose 1,6-diphosphate as substrates (Blecher and White, 1960a). Glycolysis of the first three of these hexoses was inhibited by the addition of DOC *in vitro*. Initial attention was concentrated therefore on glucose as substrate, with the view of ascertaining which aspect of the reaction, glucose to glucose 6-phosphate, might be influenced by the presence of steroid. In view of published reports of an inhibitory influence of cortisol (Bacila and Barron, 1954) and of DOC (Rosencrantz, 1959) on the hexokinase reaction, this enzyme system was studied initially. However, no effect of either steroid on hexokinase activity could be demonstrated (Blecher and White, 1960a). Also, supplementation of our glycolyzing system with yeast hexokinase failed to affect the inhibitory influences of either cortisol or DOC on the anaerobic utilization of glucose by the cell-free preparations of rat lymphosarcoma.

These observations raised the question whether the supply of adenosine triphosphate (ATP), and a possible influence of steroids on this supply, could afford a possible explanation of the data at hand. Studies (Blecher and White, 1960a) with quantities of added ATP varying from a catalytic level (0.33 m*M*) to a stoichiometric amount (3.33 m*M*) revealed that steroid inhibitions of glucose utilization and of 2-phosphoglycerate and lactate formation were evident even at the higher ATP concentrations, although such inhibitions apparently decreased with rising ATP concentrations.

An almost complete negation of the inhibitory influence of DOC on anaerobic glucolysis by broken-cell preparations of lymphosarcoma was achieved by generation of stoichiometric quantities of ATP during the incubation period (Table IV). The apparently greater inhibition of inorganic phosphate (P_i) uptake observed in these experiments, as well as the actual release of P_i to the medium in certain of the studies (Table IV), suggested that DOC might be stimulating hydrolysis of labile organic phosphate esters, e.g., ATP and fructose diphosphate. Unpublished studies revealed that the hydrolysis of fructose diphosphate by fructose diphosphatase was not affected by the presence of steroids, and

TABLE IV

Effects of Source and Quantity of ATP on DOC Inhibition of Glucolysis

ATP source	DOC[a]	Lactate produced (μmoles/hr)	ΔP_i (μmoles/hr)	Glucose utilized (μmoles/hr)
Present initially[b]	—	4.35	3.90	2.20
	+	2.43	0.90	1.00
Generated[c]	—	4.75	3.80	3.30
	+	2.25	1.00	0.70
Present initially[d]	—	7.17	4.38	7.12
	+	3.34	+3.13	2.59
Generated[e]	—	6.68	9.60	5.60
	+	5.58	8.70	4.90

[a]DOC, when added, 0.44 m*M*.

[b]ATP, 0.33 m*M* (catalytic-level).

[c]ADP and P–creatine, each 0.33 m*M*; ADP–P–creatine transphosphorylase, 0.1 μg/ml.

[d]ATP, 3.33 m*M* (stoichiometric level).

[e]ADP and P–creatine, each 3.33 m*M*; ADP–P–creatine transphosphorylase 6.7 μg/ml. (From Blecher and White, 1960a).

that glucose-6-phosphatase is not present in lymphosarcoma homogenates. Therefore, studies were directed toward examining the effects of steroids on the latent ATPase activity of broken-cell fractions of rat lymphosarcoma (Blecher and White, 1960b). For purposes of comparison, selected studies were conducted with rat liver preparations. The data in Table V indicate that a variety of steroids are effective in releasing the latent ATPase of freshly prepared rat lymphosarcoma mitochondria. Progesterone, 17α-ethyl-19-nortestosterone, 17α-alkyltestosterones, and deoxycorticosterone were the most active steroids tested. The 21-acetates of cortisone and cortisol, as well as cholesterol, were essentially inactive in the system under study.

Detailed studies of the effects of deoxycorticosterone on the volume of lymphosarcoma and liver mitochondria have demonstrated (Blecher and White, 1960b) that the addition of 6×10^{-4} *M* steroid *in vitro* initiated a rapid increase in mitochondrial volume. This effect was inhibited by the simultaneous presence of ATP in the medium. The detailed data obtained in experiments in which the effects of added DOC on mitochondrial volume and ATPase activity were assayed simultaneously in the presence of variable initial concentrations of ATP support the conclusion that minimal concentrations of ATP are required to inhibit the steroid-induced alterations of mitochondrial volume. The extent of volume changes was inversely related to *both* the initial and remaining concentrations of ATP. Thus certain steroids may alter the permeability and enzymatic activity of mitochondria, and are added to thy-

TABLE V

Relative Effectiveness of Steroids in Releasing Latent Mitochondrial ATPase Activity[a]

Steroid	ATPase specific activity
Progesterone	1.85
16α-Ethyl-19-nortestosterone	1.43
17α-Methyltestosterone	1.22
17α-Ethyltestosterone	1.11
11-Deoxycorticosterone (DOC)	1.11
21-Deoxycortisol	1.10
Δ^1-Cortisol	0.97
21-Dehydrocortisol	0.94
11-Deoxycorticosterone-21-acetate	0.94
11-Deoxycortisol	0.77
Corticosterone	0.74
Estradiol	0.74
19-Nortestosterone	0.71
Cortisol	0.60
Dihydrotestosterone	0.57
9α-Fluorocortisol	0.43
Δ^1-Cortisone	0.40
11-Deoxycortisone	0.25
Cortisone	0.25
17α-Hydroxyprogesterone	0.25
Testosterone	0.25
Dihydrocortisone-21-acetate	0.17
Cortisone-21-acetate	0.08
Cortisol-21-acetate	0.08
Cholesterol	0.07

[a]Steroid concentration, 0.6 m*M*. All steroids were tested with the same batch of lymphosarcoma mitochondria. Specific activities are corrected for solvent controls. (From Blecher and White, 1960b)

roidal hormones as physiologically important substances which may have, as one of their loci of action, the mitochondrion.

Additional evidence that certain steroids may alter the permeability and enzymatic activity of subcellular particles, as well as of intact thymocytes, has been obtained in recent studies, some of which are currently in progress. In collaboration with Dr. Carl Monder and Mrs. Judith Ben-Ezzer, it has been observed (Monder *et al.*, 1962) that the addition *in vitro* of one of several steroids to a suspension of intact rat thymocytes may alter the glutamic-aspartic transaminase activity of these cells (Table VI). Under the experimental conditions used, progesterone was the most active steroid, producing a relatively large increase in transaminase activity; significant increases were also produced by 17α-ethyl-19-nortestosterone, estradiol, deoxycorticosterone, and tes-

TABLE VI

Effects of Steroids on Glutamic-Aspartic Transaminase Activity of Intact Thymocytes

Steroid ($5 \times 10^{-4} M$)	Oxalacetate[a] (μmoles/2×10^{10} ce s/hr) without steroid	with steroid	t[b]	P[b]
Cortisol	83 ± 17 (11)	67 ± 12	4.35	<0.001
Cortisone	94 ± 21 (10)	73 ± 20	2.57	0.015
Corticosterone	90 ± 21 (10)	82 ± 15	1.91	>0.1
17α-Ethyl-19-nortestosterone	92 ± 20 (12)	105 ± 27	2.60	0.012
Estradiol	87 ± 22 (15)	98 ± 18	3.05	0.005
Deoxycorticosterone	83 ± 17 (11)	100 ± 17	4,16	0.001
Testosterone	89 ± 21 (14)	105 ± 22	5.3	<0.001
Progesterone	92 ± 20 (10)	123 ± 22	5.7	<0.001

[a]Values are means and standard deviations. Number of experiments, with and without steroid, given in parentheses.

[b]Values for t and P calculated from "Student's" formula for differences between paired observations. From Monder, et al, 1962.

tosterone. Cortisol and cortisone produced slight decreases in activity; corticosterone was inactive. None of the steroids influencing glutamic-aspartic transaminase activity of intact thymocytes affected the levels of this enzyme when the steroid was added to disrupted cell preparations of thymocytes. These observations led to studies of the possible influence of steroids on the transfer of glutamic-aspartic transaminase from the cells to the medium during thymocyte incubation. Following incubation of cells with steroid dissolved in propylene glycol or with

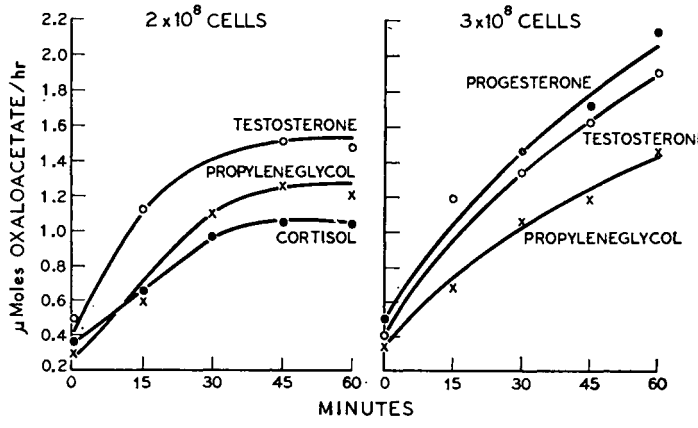

FIG. 1. Effects of steroids (5×10^{-4} *M*) on "leakage" of glutamic-aspartic transaminase from thymocytes (Monder *et al.*, 1962).

this solvent alone, the thymocytes were centrifuged and the glutamic-aspartic transaminase of the supernatant fluid determined. Figure 1 indicates some typical data obtained. The enzymatic activity in the supernatant fluid from cell suspensions incubated with progesterone or testosterone was higher than that of the medium from control suspensions containing no steroid. These results are confirmed and extended in Table VII, in which data are shown for transaminase activity in the

TABLE VII

Effect of Previous Incubation of Thymocytes with Steroids on the Distribution of Glutamic-Aspartic Transaminase Activity between Cells and Incubation Medium[a]

Steroid ($5 \times 10^{-4} M$)	0 Minute of incubation			60 Minutes incubation		
	Broken-cell suspension	Medum	Sum	Broken-cell suspension	Medium	Sum
Propylene glycol alone	3.03	0.32	3.34	2.35	0.84	3.19
Cortisol	2.96	0.32	3.28	2.50	0.88	3.38
Testosterone	2.98	0.32	3.30	2.60	0.95	3.55
Unfractionated control	—	—	3.95, 3.95	—	—	3.55, 3.82

[a]One milliliter Krebs-Ringer phosphate buffer, 0.1 ml propylene glycol or steroid in this solvent, and 0.2 ml thymocytes (1×10^8 cells) were incubated for < 1 minute and for 60 minutes. Cells were centrifuged, and 0.6 ml of the supernatant fluid was analyzed for transaminase activity (Karmen *et al.*, 1955). The cells were washed in 0.5 ml buffer, suspended in 1.3 ml (final volume) buffer, and sonically disrupted for 15 minutes; a 0,2 ml aliquot analyzed for transaminase. Unfractionated controls consisted of suspensions incubated as above, centrifuged, resuspended without separation from incubation medium, and sonically disrupted. Values are expressed as micromoles oxalacetate formed per hour. (From Monder et al., 1962)

medium and that remaining in the thymocytes after incubation with and without steroid. The results indicate clearly that enzymatic activity passed from the cells to the medium during incubation. Other observations lead to the conclusion that the transaminase being measured in studies of cell suspensions is entirely extracellular. This implies that transaminase within the thymocyte could not be measured unless the integrity of the cell was altered, permitting release of the enzyme. The data suggest an approach to studies of selective steroid action on thymocyte permeability.

Finally, reference is made to experiments in progress in collaboration with Dr. George Sachs and Dr. Christian de Duve. Studies of the influence of various steroids *in vitro* and *in vivo* on certain of the lysosomal enzymes of thymic particulate fractions have revealed a specific effect of cortisol, as contrasted with deoxycorticosterone or progesterone. Thus, subcutaneous injection of cortisol in the rat (10 mg/100 gm body weight) at 20 hours and at 4 hours prior to sacrifice has revealed a de-

finite decrease in the latency of certain lysosomal hydrolases and a marked increase in enzymatic specific activity. These increases in specific activity of the three hydrolases studied, namely, β-glucuronidase, aryl sulfatase, and cathepsin, can be attributed entirely to an unchanged *total* enzymatic activity with an accompanying striking decline in the total protein of the thymus. In contrast, under similar conditions, the injection of either progesterone or deoxycorticosterone is without affect on the specific activity of these three hydrolases. Thus, in these studies also, selective steroid effects have been observed on the enzymatic activity and permeability of another subcellular particle, the lysosome. More significantly, there is a correlation between the thymolytic activity of a steroid and the lysosomal properties which have been measured. It remains to be demonstrated whether the data being obtained are pertinent to the problem of the biochemical basis of lymphocytolysis.

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