RECENT PROGRESS IN HORMONE RESEARCH

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COMMITTEE ON ARRANGEMENTS

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PREFACE

This volume contains papers delivered at the Laurentian Hormone Conference held at Franconia, New Hampshire, in September 1948. As in the past, the holding of the Conference was made possible by contributions from the following friends: Armour and Company, Ayerst, McKenna and Harrison, Ltd., Ciba Pharmaceutical Products, Inc., Endo Products, Inc., Charles E. Frosst and Company, Glidden Company, Hoffman-LaRoche, Inc., Eli Lilly and Company, Mallinckrodt Chemical Works, Maltine Company, Nopco Chemical Company, Inc., Parke, Davis and Company, Roche-Organon, Inc., Schering Corporation, G. D. Searle & Co., Sharp and Dohme, Inc., E. R. Squibb and Sons, Upjohn Company, White Laboratories, Winthrop Chemical Company, Inc., and Wyeth, Inc. The Committee on Arrangements acknowledges with thanks the able assistance of the session chairmen: Drs. Konrad Dobriner, Karl Paschkis, Robert L. Noble, Evelyn M. Anderson, Dwight J. Ingle, H. B. Friedgood, Roy G. Hoskins, H. Jensen, and Allan T. Kenyon. The Committee is indebted also to Miss Joanne Sanford and Mrs. Elsie Jackson who acted as secretaries to the Conference.

The new meeting place of the Conference made possible a somewhat larger attendance than in previous years. The full and vigorous discussion of the papers continued undiminished as the printed rescripts attest, and the Committee is grateful to the discussants for illuminating and critical comment. The contributions of the individual authors here speak for themselves. The high standards set by the Committee for the presentation of outstanding original work, of critical general review, of informed, objective commentary have, in our opinion, been met. Our friends will be pleased to know that this series of volumes has proven successful as a publishing venture. We hope to keep it so by the continued publication of timely, balanced papers in the complex and fascinating field covered by the Conference.

GREGORY PINCUS

Shrewsbury, Massachusetts

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I. STEROID HORMONE METABOLISM *IN VIVO* AND *IN VITRO*

Some Aspects of Progesterone Metabolism

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I. General Introduction

1. Historical

It is not my intention to attempt to present a comprehensive review of the whole field of progesterone metabolism: rather I intend to devote the greater part of my time to describing some of the work in this field which my coworkers at the University of Edinburgh—Dr. Nancy Gough, Dr. Ian Sommerville, Miss Elisabeth Sutherland and Mr. Ian Kyle—have been carrying out during the past few years. It would, however, be only fitting to devote a few minutes to recalling some of the important pioneer researches to which all of us who are working in this field today owe so much. Furthermore such a brief historical survey will help us to see the more recent work in its proper perspective.

Pregnane-3(α),20α-diol (pregnanediol) (IV), which is at present believed to be the main metabolic end-product of progesterone metabolism in man, was isolated from human pregnancy urine (24) and its structure in relation to the bile acids fully elucidated (4) several years before anything was known of the chemical nature of the hormone of the corpus luteum. The hormone, later to be named *progesterone*, was isolated as a pure crystalline compound in 1934 almost simultaneously in four different laboratories (1, 5, 15, 32), and the following year its structure was elucidated by Butenandt *et al.* (6) and by Fernholz (12). The close structural relationship between pregnanediol and progesterone which was thus revealed, immediately suggested the possibility that the urinary steroid might be formed in the body by the reduction of the double bond and the two ketonic groups of the hormone. The isolation from human pregnancy urine at about the same time of the C5 stereoisomer of pregnanediol, allopregnane-3(α),20α-diol (V), by Hartmann and Locher (16) provided some support for this attractive possibility, but since no method was then available for the quantitative determination of either pregnanediol or allopregnanediol in urine, there seemed to be no immediate prospect of putting this theory to the test. However, within two years the test was applied and the theory proved to be correct by Venning and Browne at Montreal.

In 1936 Venning and Browne discovered that pregnanediol occurs in human pregnancy urine as a glucuronide and they were able to isolate this glucuronide as a crystalline sodium salt (10). Shortly afterwards Venning

(39) devised a relatively simple method for the isolation of sodium pregnanediol glucuronidate from human urine in nearly quantitative yield and thus, for the first time, provided a procedure by which the pregnanediol content of urine could be approximately determined. Using this method Venning and Browne (40) in 1937 demonstrated that during the menstrual cycle pregnanediol is excreted in significant amounts only during the luteal phase, and they furthermore showed that the administration of progesterone to women "in whom there was a reasonable certainty that no corpus luteum was present" was followed by the appearance of pregnanediol in the urine. The status of pregnanediol as a metabolic reduction product of progesterone was thus securely established.

2. *Origin and Scope of the Edinburgh Research on Progesterone Metabolism*

The results obtained in my department that I want to consider, together with the relevant historical background, can most conveniently be discussed under the three headings of "Urinary Metabolites of Progesterone," "The Quantitative Determination of Urinary Pregnanediol," and "The Conversion of Progesterone into Urinary Pregnanediol." These headings might perhaps suggest that when we started our work on progesterone metabolism we had drawn up beforehand a carefully thought out and comprehensive program of research. It must be confessed, however, that this was not the case.

Our interest in the field was originally aroused in 1944 by a request from the Sub-Committee on Human Fertility of the Medical Research Council for a method of determining urinary pregnanediol which would be simpler to carry out and less liable to error than the Venning method at the low levels of pregnanediol excretion found in nonpregnant women. Eventually, as will be explained later, this *ad hoc* problem was solved to our own satisfaction, and having then a sensitive and accurate method of determining urinary pregnanediol we decided to make use of it in a thorough reinvestigation of some of the factors concerned in the conversion of progesterone to pregnanediol in human subjects. However, at the very outset we accidently stumbled upon an interesting problem in connection with the chemical nature of "sodium pregnanediol glucuronidate," which diverted us for a time from our main objective. This problem was quickly solved, but it led naturally to other chemical problems in connection with urinary metabolites of progesterone which we are still actively investigating at the present time.

II. Urinary Metabolites of Progesterone

1. *Introduction*

Pregnane-3(α),20α-diol is accompanied in human pregnancy urine not only by allopregnane-3(α),20α-diol, but also by the C3 epimer of the latter, allopregnane-3(β),20α-diol (VI), first isolated from that source by Marker *et al.* (20) in 1939. According to Marker *et al.* (21) these three diols are present in human pregnancy urine in the approximate relative proportions of 100:50:12. It is generally assumed that the two allopregnanediols, like pregnane-3(α),20α-diol, are metabolic products of progesterone, but definite proof that this is the case has yet to be obtained. As has been seen, pregnane-3(α),20α-diol is excreted as a glucuronide, but no information is yet available concerning the forms in which the two allopregnanediols are present in human urine.

Human pregnancy urine also contains three stereoisomeric 3-hydroxy-20-ketosteroids: pregnan-3(α)-ol-20-one (I) and allopregnan-3(α)-ol-20-one (II), both of which were first isolated from pregnancy urine by Marker *et al.* (22, 23) in 1937, and allopregnan-3(β)-ol-one (III), first isolated from this source by Pearlman *et al.* (28) in 1942. Although it seems probable that each of these 20-ketosteroids is a metabolic product of progesterone, only in the case of pregnan-3(α)-ol-20-one is there clear proof that this is the case. Pregnan-3(α)-ol-20-one is also the only one of these known to be present in human urine as a glucuronide.

It should be emphasized that the above-mentioned steroids isolated from human urine are not the only ones that have been suspected of being progesterone metabolites.

2. *Sodium Pregnanediol Glucuronidate*

Before considering our own work on the composition of "sodium pregnanediol glucuronidate," brief reference must be made to the elegant work of Heard *et al.* (17) in 1944 which showed that the glucuronic acid in this compound is conjugated with the C3 hydroxyl group of the steroid, and to the synthetic work of Huebner *et al.* (18) which proved that the compound is a β-glucuronide.

Our own interest in "sodium pregnanediol glucuronidate" was stimulated by the failure reported by both Astwood and Jones (2) and Talbot and coworkers (36) in 1941 to find more than about 70% of the theoretical amount of pregnanediol in the products of acid hydrolysis of the glucuronide. This apparent loss of pregnanediol, confirmed by experiments of our own, we believed might be due to the occurrence of side reactions during the hydrolysis, and in an investigation of this possibility we had occasion to

hydrolyze a large amount of supposedly pure "sodium pregnanediol glucuronidate." To our surprise the toluene-soluble products of hydrolysis were found to contain about 20% of ketonic material consisting largely of pregnan-3(α)-ol-20-one. These findings suggested that the so-called "sodium pregnanediol glucuronidate" prepared from human pregnancy urine and purified in the usual way might contain about 20% of a water-soluble derivative of pregnan-3(α)-ol-20-one, and in confirmation of this idea it was found that all samples of "sodium pregnanediol glucuronidate" in our possession gave a definite positive reaction in the Zimmermann test (25). Subsequently, by the use of Girard's reagent T under special conditions, we were able to separate nonketonic and ketonic fractions from ordinary "sodium pregnanediol glucuronidate." The former appeared to be nearly pure sodium pregnane-3(α),20α-diol glucuronidate, while the latter, although not pure, appeared to consist largely of the previously unknown sodium-pregnan-3(α)-ol-20-one glucuronidate (35). More recently Miss Sutherland has been successful in obtaining the latter compound in a state approximating to purity.

Recently Dorfman *et al.* (11) have been able to show the presence of pregnan-3(α)-ol-20-one in the sodium pregnanediol glucuronidate fraction isolated from the urine of a man treated with progesterone. This finding provides the first clear proof that pregnan-3(α)-ol-20-one is a metabolic product of progesterone.

3. *Allopregnane-3(α),20α-diol*

As has already been mentioned it is not yet known whether allopregnane-3(α),20α-diol occurs in pregnancy urine as a glucuronide; nor is there yet proof that it is a metabolic product of progesterone. These problems have been investigated during the past year, and although they are not yet solved, substantial progress towards their eventual solution has been made.

A simple solvent-fractionation procedure has been developed by my co-worker, Mr. Kyle, which permits the isolation of allopregnane-3(α),20α-diol from mixtures containing a large excess of pregnane-3(α),20α-diol. Thus, in an experiment on a mixture of 700 mg. of pregnane-3(α),20α-diol and 13.5 mg. of allopregnane-3(α),20α-diol, 7.0 mg. of the latter were isolated in a pure state. Using this method allopregnane-3(α),20α-diol has been isolated with ease from the crude "pregnanediol" fraction obtained from acid-hydrolyzed human pregnancy urine. It should not, therefore, be a difficult matter to detect it, if it is present, in the corresponding fraction obtained from the urine of men or of post-menopausal women treated with progesterone.

In the meantime the method is being used in an attempt to learn some-

thing of the nature of the allopregnane-3(α),20α-diol conjugate in human pregnancy urine. In one preliminary experiment Mr. Kyle has obtained a small amount of allopregnane-3(α),20α-diol from the hydrolysis products of somewhat crude "sodium pregnanediol glucuronidate," but we are still uncertain whether the allo compound can be obtained from the more carefully purified glucuronidate.

III. The Quantitative Determination of Urinary Pregnanediol

Astwood and Jones (2) in 1941 described a new method for the quantitative determination of urinary pregnanediol which avoided certain of the disadvantages of the original Venning procedure. According to this method the urine is boiled with acid and the pregnanediol thus liberated from the glucuronide is purified by an ingenious precipitation process and weighed. This method was further developed shortly afterwards by Talbot and his coworkers (36), who determined the finally purified pregnanediol by means of the yellow color it yields with concentrated sulfuric acid.

Our own method (34) is essentially a modified version of this "Astwood-Talbot" procedure carried out under rigidly standardized conditions. Except in a few details there are no original features in it and I will not therefore weary you with an account of the work which had to be done before it was finally developed to our own satisfaction.

The method was tested out in a series of recovery experiments in which highly purified sodium pregnanediol glucuronidate was added in varying amounts to different 24-hour samples of men's urine. The results of these recovery experiments, which are shown in Table I, indicate that anything more than about 0.4 mg. of pregnanediol can be estimated in one-fifth of a 24-hour urine specimen with satisfactory accuracy.

This method appears to us to have certain advantages over any others previously described, particularly for use with urines containing less than ca. 10 mg. of pregnanediol per 24 hours. Dr. Sommerville has recently been examining its specificity in a series of experiments with various other urinary steroids. It would seem that allopregnane-3(α),20α-diol as well as pregnane-3(α),20α-diol may be estimated by it, but various other steroids in the amounts likely to be normally present in human urine do not appear to cause any serious error.

IV. The Conversion of Progesterone into Urinary Pregnanediol

1. Introduction

Our present knowledge concerning the conversion of progesterone into urinary pregnanediol in human subjects is in a not altogether satisfactory

TABLE 1

Recovery Experiments

Na Pregnane-3(α), 20α-diol Glucuronidate Added to Men's Urine

Urine specimen	"Men's urine blank" as apparent pregnanediol in one-fifth of 24-hr. specimen (mg.) (av. of duplicates)	Pregnanediol added as glucuronidate to one-fifth of 24-hr. urine specimen (mg.)	Pregnanediol recovered (mg.) Apparent	Corrected for blank	Pregnanediol recovery (corrected) (%)
C4	0.016	0.2	0.017	0.001	0
		0.2	0.012	—	0
A3	0.008	0.2	0.021	0.013	7
		0.2	0.047	0.039	20
B2	0.024	0.2	0.060	0.036	18
		0.2	0.045	0.021	11
D4	0.035	0.4	0.32	0.29	72
		0.4	0.33	0.29	74
B3	0.015	0.4	0.28	0.27	67
		0.4	0.29	0.28	69
A2	0.018	0.4	0.35	0.33	82
		0.4	0.35	0.33	82
A4	0.044	1.0	0.99	0.95	95
		1.0	0.98	0.93	93
C3	0.019	1.0	0.94	0.92	92
		1.0	0.96	0.94	94
D2	0.077	1.0	1.0	0.92	92
		1.0	0.98	0.90	90
B4	0.030	2.0	1.9	1.9	95
		2.0	2.0	2.0	100
D3	0.017	2.0	2.0	2.0	100
		2.0	1.9	1.9	95
C2	0.026	2.0	1.9	1.9	95
		2.0	1.9	1.9	95

state. A number of workers have in the past attempted to study quantitatively the urinary excretion of pregnanediol following the administration of progesterone to subjects of both sexes, but the published data tell us little more than that the proportion of the administered hormone excreted as pregnanediol probably varies over wide limits (0-46%) and is usually rather low (ca. 10%). There is no general agreement on the causes of these variations, and a critical examination of the data leaves one in some doubt whether they are in fact real, or whether if real they are normal.

Two general criticisms may be made about much of the previous work on this problem. Firstly the methods of pregnanediol determination em-

ployed by most workers are ones which are apt to be unreliable at urinary pregnanediol excretion levels below ca. 10 mg. per 24 hours. Since it is doubtful if this level has often been attained in metabolism experiments on human subjects, the possibility cannot be entirely dismissed that the reported variations in the percentage conversion of progesterone into urinary pregnanediol may have been to some extent due to errors in the determination of the latter. Secondly, no published paper dealing with the problem contains data from a long enough series of any single type of human subject to permit conclusions concerning the normal variations that may occur in the conversion. It is in fact difficult to tell from the published data whether the variations are or are not caused by variability in the type of human subject studied.

Just over a year ago Dr. Sommerville and I decided to begin a reinvestigation of the whole problem of the conversion of administered progesterone into urinary pregnanediol since we believed that we had, in our own modification of the "Astwood-Talbot" procedure, a means of studying this problem in a more strictly quantitative manner than had hitherto been possible. We felt that the problem merited a thorough reinvestigation, not only for its own intrinsic biochemical interest, but also because of its possible bearing on the significance of urinary pregnanediol excretion as an index of the output of endogenous progesterone.

Our experiments were carried out on volunteer subjects of both sexes, and in all but a few cases, which will be later described, they were carried out as follows: After a control period of 2-5 days, the subjects were given progesterone dissolved in oil on two successive days. In the majority of cases intramuscular injection was employed, but in a few instances the oily solution was administered in capsules by mouth. Throughout the control period, during the period of progesterone treatment and during the following 5 days, full 24 hour urine specimens were collected. Pregnanediol determinations in duplicate were carried out upon every 24 hour specimen.

2. *Pregnanediol Excretion Following the Administration of Progesterone to Normal Men*

Buxton and Westphal (7) in 1939, Hamblen *et al.* (14) in 1940, and more recently Dorfman *et al.* (11) have demonstrated the excretion of pregnanediol by men following the administration of progesterone. Our work was carried out on three healthy young men, two of whom were experimented upon repeatedly. The results are summarized in Table II and typical pregnanediol excretion curves are shown in Fig. 1.

The constancy of the pregnanediol excretion by the same individual when the same route of administration was used is remarkable, but it is clear

that the extent to which the conversion occurs in different individuals is somewhat variable. There is a suggestion in these figures that a slightly higher proportion of progesterone is converted into pregnanediol when it is given by mouth than when it is given by intramuscular injection. This

TABLE II

Pregnanediol Recovered from Urine of Normal Men After the Administration of Progesterone (2×60 mg.)

Subject	Age	Date of experiment	Route of administration	Per cent recovery of pregnanediol (corrected for control period blank)
D.P.	22	Jan. 12, '48	Intramusc.	10.4*
		Apr. 10, '48	Intramusc.	10.0
		Apr. 17, '48	Intramusc.	10.8
		Apr. 22, '48	Intramusc.	10.0
		May 11, '48	By mouth	12.8*
J.P.	23	Dec. 17, '47	Intramusc.	14.7
		Apr. 17, '48	By mouth	18.6
		Apr. 22, '48	By mouth	17.4
		Apr. 27, '48	By mouth	19.6
A.R.	22	Jan. 12, '48	Intramusc.	9.3
		Aug. 11, '48	By mouth	11.6

*See Fig. 1.

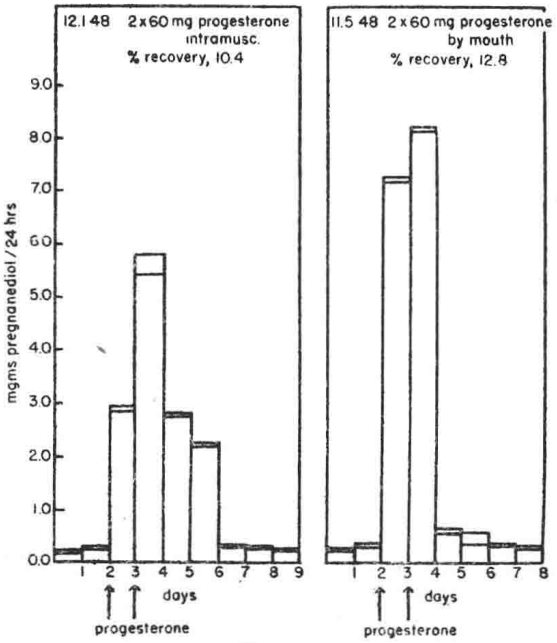

FIG. 1

Normal male, aged 22.

finding would be understandable if, as seems possible, the liver plays a prominent part in the formation of pregnanediol glucuronide from progesterone.

3. *The Effect of Estrogen Administration on the Conversion of Progesterone to Pregnanediol*

Venning and Browne (41) in 1938 obtained results which suggested that pretreatment with estrogen increases the conversion of injected progesterone into pregnanediol glucuronide in women with hypoplastic endometria. Cope (9), on the other hand, in studies on a young secondary amenorrhea case could not confirm these results.

Smith and Smith (33), from studies on toxaemic and diabetic pregnant women treated with progesterone alone and with progesterone *plus* oestradiol benzoate, have concluded, as did Venning and Browne, that estrogen increases the proportion of administered progesterone excreted as pregnanediol glucuronide. Their results are not, however, entirely convincing since the pregnanediol produced from endogenous progesterone must have been a complicating factor in their studies.

Our own experiments on the possible effect of estrogen on the conversion of progesterone to pregnanediol have been carried out on healthy post-menopausal women. Three subjects were treated with progesterone alone and served as controls. Another three were given daily injections of estradiol benzoate for several days prior to, during, and for 2 days following the progesterone injections. The results are summarized in Table III.

TABLE III

Effect of Estrogen Treatment on the Pregnanediol Excretion of Post-Menopausal Women Injected with Progesterone

Subject	Age	Progesterone treatment	Estrogen treatment	Per cent recovery of pregnanediol (corrected for control period blank)
M.E.	65	2×60 mg.	None	15.6
A.	70	2×60 mg.	None	15.6
J.N.	71	2×60 mg.	None	16.0
B.	58	2×60 mg.	30 mg. E.B./day for 10 days[a]	16.1[c]
H.	49	2×60 mg.	30 mg. E.B./day for 10 days[a]	16.1[d]
K.	73	2×120 mg.	30 mg. E.B./day for 20 days[b]	15.1

[a]Progesterone administered on 7th and 8th days.
[b]Progesterone administered on 14th and 15th days.
[c]Biopsy showed thick proliferative endometrium with secretory changes.
[d]Biopsy showed atrophic endometrium.

It seems clear from these results that *under the conditions of our experiments* estrogen treatment has no marked effect on the proportion of the

administered progesterone excreted as pregnanediol. The possibility that under different experimental conditions the conversion of progesterone into urinary pregnanediol may be influenced by estrogen treatment has not, however, been excluded.

4. Pregnanediol Excretion in Hysterectomized Women

The early work of Venning and Browne (41) suggested the interesting possibility that the uterus might be the chief site of pregnanediol glucuronide formation in the body, since these workers were unable to detect the presence of sodium pregnanediol glucuronidate in the urine of two hysterectomized women after the injection of progesterone. Any idea that the uterus might be essential for pregnanediol glucuronide formation was, however, disposed of when Buxton and Westphal (7) demonstrated the excretion of pregnanediol glucuronide by men after the administration of progesterone. Buxton (8), indeed, subsequently showed that pregnanediol glucuronide is excreted in small amounts by hysterectomized women following progesterone treatment. In 1941 the excretion of pregnanediol by hysterectomized women was more extensively studied by Jones and TeLinde (19). In this work three hysterectomized women were injected with progesterone and sodium pregnanediol glucuronidate was subsequently recovered from the urine in each case in an amount similar to that excreted by a normal woman who had been injected with the same amount of the hormone during the follicular phase of the cycle.

Although the findings of Jones and TeLinde appeared to be conclusive we decided that a reinvestigation of the problem with a more accurate method of pregnanediol determination should be undertaken, since we felt that there was still a possibility of reconciling the findings of Venning and Browne with those of later workers by supposing that the uterus is a site of pregnanediol glucuronide formation but not the only site. Our experiments were carried out on three hysterectomized post-menopausal women who were injected in the usual manner with 60 mg. of progesterone on two successive days. The results are summarized in Table IV.

TABLE IV
Pregnanediol Excretion in Hysterectomized Women Injected with Progesterone
(2×60 mg.)

Subject	Age	Weeks after hysterectomy	Per cent recovery of pregnanediol (corrected for control period blank)
H.	49	6	14.1
M.	68	20	13.3
R.*	52	24	14.9

*Hysterectomy and double ovariectomy.