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METABOLISM OF ANDROGENS BY RAT ADRENAL CORTEX —— P.C.M.YOUNG

THE METABOLISM OF 17α-METHYLANDROSTENEDIOL BY THE RAT ADRENAL AND ITS EFFECT ON THE BIOSYNTHESIS OF CORTICOSTERONE BY THE CORTICAL TISSUE IN VITRO

by

Peter C. M. Young

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

Allan Memorial Institute
McGill University
Montreal

Department of Biochemistry
July, 1963.

ACKNOWLEDGMENTS

This work was supported by a grant, MA 1080, from the Medical Research Council.

I wish to express my heartfelt thanks to my research director, Dr. M. Saffran, for his critical guidance, valuable assistance and continued encouragement throughout the course of this study.

I am very grateful to Dr. M. K. Birmingham and the members of her laboratory for their invaluable advice and co-operation, without which, this investigation would not have been possible.

The author is indebted to Dr. S. S. Solomon and Mr. A. Clark for their helpfulness in the preparation and determination of the infrared spectra.

I must also express my sincere appreciation to Miss P. M. Berger who offered me tremendous help during the summer months.

Last of all, but not least in sincerity, I must thank my fellow students and co-workers working in the same laboratory, for they have so nicely put up with me during the past years.

TABLE OF CONTENTS

ABSTRACT

The suppression of the formation of corticosterone by the rat adrenal tissue *in vivo* and *in vitro* by androgens is reported. There is some evidence that androgens can be converted to more polar compounds by the incubated glands. Two such metabolites of 17α-methyl-Δ^5-androstene-3β, 17β-diol (MAD) have been investigated. One is methyltestosterone, probably formed by the oxidation of MAD by the enzyme, 3β-hydroxydehydrogenase. The other may, in addition, contain one or more extra hydroxyl groups. Even more polar compounds have been observed. Whether these originate from the exogenous androgens or arise as a consequence of diversion of corticoid synthesis from corticosterone is not known.

INTRODUCTION

I. Origin of the Problem

In 1959, Saffran and Vogt reported that 17α-methyl-Δ^5-androstene-3β,17β-diol (MAD), added *in vitro* to the rat adrenal glands, did not apparently inhibit the formation of corticoids (1). However, this preliminary experiment did not rule out the possibilities that

a) MAD diverted the production of steroids by the adrenal gland to abnormal metabolite(s), or,

b) the adrenal glands converted the added MAD to substances which interfere with the measurement of adrenocortical steroids, yielding spuriously high values.

The objectives of this study were to determine whether MAD directly suppresses the biosynthesis of corticoids by the rat adrenal cortex, and, if it does so, to ascertain the mechanism whereby it brings about the inhibition.

In the early part of this study, MAD was added to surviving adrenal tissue. After a period of incubation the ultraviolet absorption of the lipid extract of the incubation media was measured. The lipid extract was then chromatographed for the separation of the cortical steroids. Findings of these experiments indicated that added MAD with or without the presence of adrenocorticotrophic hormone (ACTH), was likely metabolized by the incubated adrenal glands because of the

elevated readings of ultraviolet absorption and the appearance of augmented ultraviolet-absorbing areas in chromatograms of lipids from the incubation media. It is, therefore, of interest to take up the study of a neglected aspect of adrenocortical activity---its action on androgens. Further, provided if the possibility under heading (a) exists, such an effect may be due to the action of the androgen itself or some of its metabolites. Knowing the products involved in the metabolic pathways employed by the adrenal tissue for utilization and disposition of the androgen, one can hope to gain some light on the nature of the definitive active substance(s).

II. Biogenesis of Adrenocortical Steroids

In spite of the recent advances and the many discoveries in the field of steroids, the sequence of reactions involved in the biosynthesis of adrenocortical steroids is still not fully known. The current concepts which are most generally accepted today are proposed by Pincus and his colleagues (2, 3). The work of others modified the original scheme but without drastic alteration. The following schemes are based on studies with perfused ox adrenals:-

a) Formation and Utilization of Cholesterol and Cholesteryl Esters

The steps involved in the formation of cholesterol from acetate were well documented. Wright (9) has recently reviewed on this subject. Only the main steps are indicated in Fig. 1.

Fig. 1

Formation of cholesterol and esters from acetate

Acetate --> Mevalonic Acid --> Squalene --> Cholesterol ⇄ Cholesteryl Esters

Fig. 2

Postulated pathway for the formation of progesterone from cholesterol

Cholesterol $\xrightarrow{1}$ 20α-hydroxycholesterol $\xrightarrow{2}$ 20α,22ζ-dihydroxycholesterol $\xrightarrow{3}$ Pregnenolone $\xrightarrow{4}$

Progesterone

1 - 20α-hydroxylase (4)

2 - 22-hydroxylase (5)

3 - 20,22-desmolase (6)

4 - 3β-hydroxysteroid dehydrogenase (7) and isomerase (8)

Cholesteryl esters are included here because of the findings of Griffiths (10), who, in 1960, showed that it is the esters rather than the free corticosterol which disappears from the human adrenal glands on stimulation with ACTH, suggesting that the esters may play some role in the biosynthesis of corticosteroids.

After the formation of cholesterol, progesterone is made from cholesterol and is further hydroxylated in several positions. These reactions are illustrated accordingly in Fig. 2 and Fig. 3.

b) Alternate Biosynthetic Pathways not Involving Cholesterol

Whether cholesterol is an obligatory intermediate in adrenocortical steroid biosynthesis remains a controversial question. Recent work of Werbin and Chaikoff (11) seemed to bring light on this problem. By feeding guinea-pigs with C^{14}-cholesterol and examining cortisol excreted in the urine, they were able to demonstrate that for cortisol biosynthesis cholesterol was an obligatory precursor. In the subsequent year (1962), Caspi (12) established that the biosynthetic origin of nine carbon atoms in C^{14}-cortisol was shown to be the same as those nine carbon atoms in C^{14}-cholesterol formed at the same time from the methyl and carboxyl carbons of 1-C^{14}-acetate perfused through ox adrenals. This also supports the view that most of the cortisol is derived from cholesterol. However, radioactivity was found in certain carbon atoms where none was expected, allowing for the possibility of the existence of an alternative biosynthetic pathway not involving

cholesterol.

III. Steroids Formed by the Rat Adrenal Cortex

According to their physiological activity, steroids formed by the adrenal cortex can be divided into four groups:-

a) Glucocorticoids, which exert an influence on carbohydrate metabolism (13, 14),

b) Mineralcorticoids, which control the electrolyte balance in the body (15),

c) Sex hormones, which includes androgens, estrogens and progesterones, and

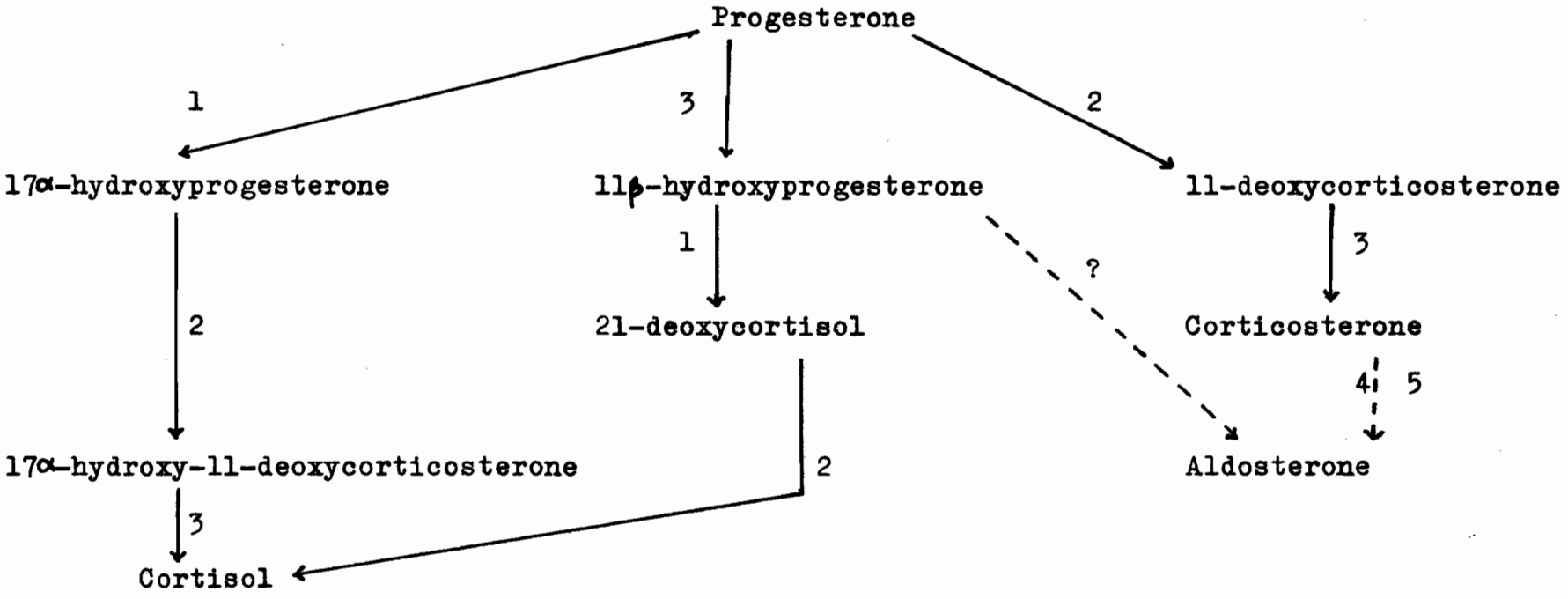
d) Physiologically inactive steroids, which are probably the precursors or metabolites of the active hormones.

Bush (16) was the first to apply methods of chromatography to the study of the secretion of the rat adrenal. He concluded, from the mobility of the products isolated from the adrenal vein blood, that the major secretory product is corticosterone and that the ratio of hydrocortisone to corticosterone is less than 0.05. Similar observations were made on the steroid content of rat adrenal vein blood by Vogt (17), Holtzbauer (18) and Reif and Longwell (19). They further detected an unidentified, non-reducing compound slightly more polar than corticosterone.

The demonstration by Saffran et al (20) that isolated rat adrenals incubated in vitro secrete adrenocorticoids and respond to ACTH provided a convenient, simple method for the study of adrenocortical secretion, which is free of the

Fig. 3

Progesterone hydroxylations in C-21 corticosteroid biosynthesis



1 - 17α-hydroxylase

2 - 21-hydroxylase

3- 11β-hydroxylase

4 - 18-hydroxylase

5 - 18-hydroxysteroid dehydrogenase

technical difficulties of adrenal vein cannulation. Elliott and co-workers (21) developed micromethods for the estimation of adrenocorticoids extracted from the adrenal incubation media and found that measurement by ultraviolet absorption gave higher values than by reduction of tetrazolium, which measures the α-ketol side chain. They concluded that the rat adrenal secretes compounds lacking the characteristic α-ketolic side chain in addition to α-ketolic compounds. Chromatography of the incubation extracts revealed that most of the compounds secreted by the isolated rat adrenal have polarities equal to or greater than corticosterone (22) in agreement with the findings on adrenal vein blood (23). Four major ultraviolet-absorbing regions were obtained by Heard et al (24) on chromatograms of extracts of rat adrenal incubation media developed in the toluene-propylene glycol system of Burton, Zaffaroni and Keutmann (25); these were designated the F, E, X, and B regions in order of decreasing polarity.

On the basis of mobilities of the free and acetylated compound and mixed chromatography with the authentic reference compound, Heard et al (24, 26) suggested that the material in the F zone was hydrocortisone. Reif and Longwell (19) obtained material with the mobility of hydrocortisone from rat adrenal vein blood, and several other workers detected it using in vitro adrenal preparations (22, 27, 28, 29, 30, 31). Mialhe-Voloss et al (32) obtained evidence for the presence of a 20, 21-diol in this region.

Birmingham and Kurlents (33) found that eluates of the F zones possessed hardly any reducing activity and gave a negative Porter-Silber reaction, indicating the absence of a dihydroxyacetone side chain; they concluded that little if any hydrocortisone is present in the F zone.

The material in the E zone was thought to be cortisone by Heard's group (24) who later obtained evidence for its contamination with aldosterone (26). The presence of aldosterone was confirmed by Giroud (29, 34) and Mialhe-Voloss (28). Birmingham and Kurlents (33) could find no evidence for the presence of Porter-Silber positive substances in this zone, and hence no evidence for the presence of cortisone.

The material in the X zone was found to be non-reducing (22, 24, 26, 30, 36). Péron (37) claimed the identification of 6 β-hydroxy-11-desoxycorticosterone as a minor component of this region. The material in this zone resembles in several properties the non-reducing, ultraviolet-absorbing steroid detected earlier in adrenal vein blood by Vogt (17), and is apparently identical with compound RT5 of Giroud (29), steroid 3 of Eisenstein (30) and compound Y of Reif and Longwell (19). That this compound gives a Porter-Silber reaction, presumably specific for the dihydroxyacetone side chain, in spite of its apparent lack of an α-ketolic group, was found for the compound isolated from the adrenal vein blood by Reif and Longwell (19) and independently for the compound produced <u>in vitro</u> by Birmingham and Kurlents (33).

The presence in this region of 17 α-hydroxy-11-desoxycorticosterone claimed by Roberts (35) was ruled out by the significantly greater polarity of the unknown material (19,24). The material in the X zone is a major secretory product; it represents almost all of the Porter-Silber chromogenic material secreted by the rat adrenals in vitro (33), which in turn accounts for one-third to one-half of the total ultraviolet-absorbing material secreted in the absence of ACTH (30, 38).

More recently Ward (40) reported that the major secretory products of the rat adrenal cortex are corticosterone and 20→ 18-hemiketal of 18-hydroxy-11-desoxycorticosterone. The latter was located in Heard's X zone and its identity was first revealed independently by Ward (40) and Péron and Koritz (41) almost at the same time. Aldosterone was found to be the major component of the E zone. Other minor components of the adrenal secretion were identified to be 11-dehydrocorticosterone and 11-desoxycorticosterone. The rat adrenal also secretes material with polarity in toluene-propylene glycol system similar to that of hydrocortisone. This material was found to lack a dihydroxyacetone side chain, a ketone function at C-11 and a hydroxyl group at C-17 and C-6. It was not identical with 20 α - or 20 β -dihydrocorticosterone. It was also pointed out that it might consist of more than one component.

It has been demonstrated by several workers (17, 23, 24, 31, 35) that the B region consists mainly of corticosterone,

on the basis of chromatographic behaviour and reducing properties of the material in that zone.

In addition, other less polar compounds have been detected in smaller amounts. Elliott and Schally (22) found small amounts of material with the mobility of 11-desoxycorticosterone on chromatograms of rat adrenal incubates. Roberts (35) reported the secretion of equal amounts of this compound and corticosterone by the adrenal incubated in the absence of ACTH. An ultraviolet-absorbing steroid of similar mobility was found in rat adrenal vein blood by Reif and Longwell (19).

IV. Adrenal Androgens

That the adrenal gland can synthesize androgens was indicated by the early isolation studies of Reichstein et al who identified adrenosterone (59), 11β-hydroxyepiandrosterone (60) and androstenedione (61) in adrenal gland extracts. These findings are of particular interest in view of the frequent association of virilism in women with hypertrophy or tumour of the adrenal cortex (62). In this connection it is interesting to note that an excess of androgenic hormone has been found to be present in the urine of virilism cases by Simpson (63), and by Simpson, deFremery and Macbeth (64) and Gallagher (65). Callow (66) has reported the isolation of the androgenic substance from the urine of a patient in an adrenal tumour. The androgenic potency of the compounds isolated from the cortex led Reichstein (59, 67) and afterwards Mason, Myers and Kendall (68) to suggest that the

compounds in question had a steroid structure.

The presence in the adrenal of compounds possessing androgenic potency and the theory that the adrenal cortex exercises a direct control over the male accessory sex organs had received support from the work of Davidson and Moon (69). They have shown that treatment of castrated immature male rats with pituitary gonadotropic extract containing the adrenotropic and lactogenic factors, but no growth, thyreotropic or gonadotropic hormones, resulted in not only hypertrophy of the adrenal cortex but also considerable stimulation of the seminal vesicles was observed. It then seemed probable that the adrenals form an androgenic principle.

The fact that excretion of male hormone persists, although on a much lower level, after the cessation of sexual function and after ovariectomy led Callow (70) to the suggestion that the adrenal may be the principal source of excreted male hormone. Callow and co-workers (71) further showed that gonadectomy in either sex did not abolish urinary 17-ketosteroids production, suggesting androgen secretion by the adrenal.

Although adrenosterone was first isolated from the adrenal tissue by Reichstein in 1935, the same compound in pure crystalline state was actually first separated from human urine by Butenandt in 1931 (72, 73). Butenandt's findings were supported by additional data by Butenandt and Tschering (74, 75). A second male-hormone, which was found to be an ester-chloride derivative of dehydroisoandrosterone,

was separated in 1934 by Butenandt and Dannenbaum (76). That the chloride is an artifact obtained from the parent compound dehydroisoandrosterone during extraction procedures was proved by Butenandt and his associates. In the later studies they concluded that man's urine contains practically equal amounts of androsterone and dehydroisoandrosterone (77).

The first androgenic steroid was isolated from adrenal vein blood by Gassner et al (78). In 1953, the presence of 11β-hydroxy- Δ^4-androstene-3,17-dione was demonstrated in human adrenal venous blood (79) and urine (80) after adrenocorticotropin administration. In the subsequent year, Block et al (81) perfused bovine adrenals with homologous blood containing added adrenocorticotropin. They were able to demonstrate the presence of 11β-hydroxy- Δ^4-androstene-3,17-dione as well as adrenosterone and Δ^4-androstene-3,17-dione, in the perfusates.

The production in vitro of the adrenal androgens from acetate by various preparations of human adrenal glands is well established (42). The available evidence suggests that the pathways involved in the formation of androstenedione are similar to those demonstrated for the gonads. The adrenal androgen, dehydroepiandrosterone, was isolated following incubation of H^3-pregnenolone with a homogenate prepared from an adrenal adenoma (43), and 17α-hydroxypregnenolone was shown to be an intermediate by Solomon, Carter and Lieberman (44). Lombardo and Hudson (45) and Cooper and associates (46) isolated 11β-hydroxyandrostenedione from

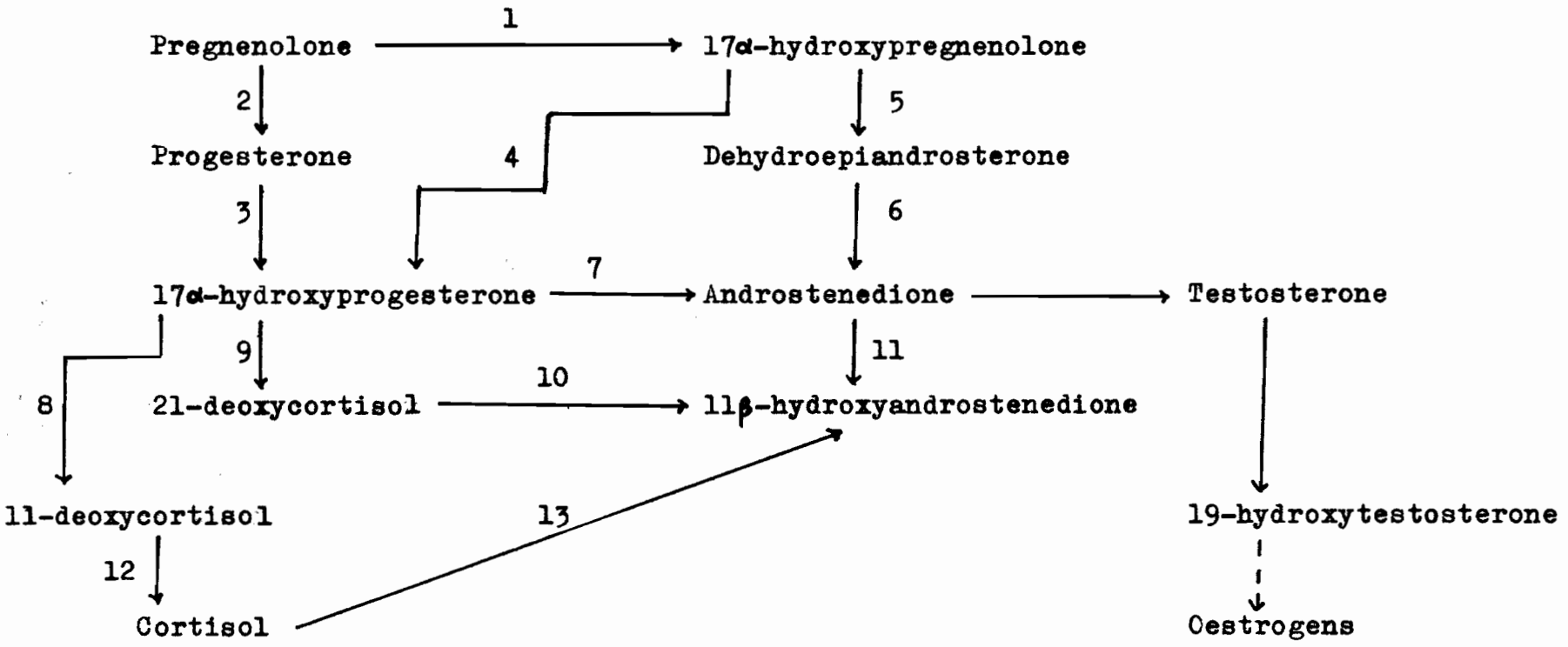
adrenal tissue from patients with metastatic breast carcinoma, prostatic carcinoma, and hypertension. Androstenedione, dehydroepiandrosterone, and 11 β-hydroxyandrostenedione have been demonstrated in human adrenal vein blood (47, 48, 49, 50). The adrenal origin of the aforementioned androgens is well documented by 17-ketosteroid secretory rate studies measured by isotopic dilution (51, 52).

With fresh human adult "normal", atrophic and various "abnormal" adrenal slices, Cohn and Mulrow (53) were able to demonstrate the release of dehydroepiandrosterone, androstenedione and 11 β-hydroxyandrostenedione into the medium after three hours of incubation in Krebs-Ringer bicarbonate buffer, fortified with glucose. They also found that the addition of ACTH to the system increased 11 β-hydroxyandrostenedione release in all kind of tissues studied. They added, however, that ACTH did not stimulate dehydroepiandrosterone and androstenedione release. More recently, Dorfman et al (54) claimed that they were the first ones to demonstrate the potentiality of homogenates of normal and abnormal human adrenals to form testosterone from progesterone. Other biosynthetic products isolated by them in the same study were 17 α-hydroxyprogesterone, androstenedione, and cortisol. The possible pathways of adrenal androgen biosynthesis are summarized in Fig. 4.

Up to 1956, five androgenic steroids had been isolated from adrenal tissue (82). They include a C-21 compound, 17 α-hydroxy-progesterone, and four C-19 compounds,

Fig. 4

Possible pathways of adrenal androgen biosynthesis



Reactions 1, 5, 6, 11 - - - 1957, Block, Dorfman and Pincus (55); later Bloch and Benirschke (56)

1, 5 - -- - - - - Goldstein, Gut, and Dorfman (43); Lipsett and Hokfelt (57)

3, 7 - - - - - - Solomon, Lanman, Lind and Lieberman (58)

Adrenal androgens are converted predominantly from 1 ⟶ 5 ⟶ 6 ⟶ 11 rather than from 2 ⟶ 3 ⟶ 7 ⟶ 11.

adrenosterone, Δ^4-androstene-3,17-dione, androstane-3β, 11β-diol-17-one and 11β-hydroxy- Δ^4-androstane-3,17-dione. And by 1959 three additional androgens had been isolated from this source. An account of the adrenal androgens known to date is given in Table 1 (83).

V. Biosynthesis of Adrenal Oestrogens

The formation of oestrogens from any precursor *in vitro* by normal adrenal tissue has not been demonstrated. The finding by Meyer (84) that bovine adrenal glands are able to introduce a hydroxyl function in the C19 methyl group of Δ^4-androstene-3,17-dione was followed by the suggestion that the biologic removal of the angular methyl group would be preceded by this preliminary hydroxylation. Griffiths (85) obtained similar results with guinea pig adrenals. Hydroxylation at C-19 may, therefore, serve as an intermediate step in the conversion of androgens to estrogens. That this is the case was demonstrated in human placental tissue by Meyer (86), Ryan (87) and Longhampt *et al* (88). Another hypothetical biosynthetic route is shown in Fig. 6. To bring the present state of knowledge up to date, it should be mentioned that Engel and Dimoline (89) have recently reported that surviving slices of human adrenal cortex have only a very limited capacity to carry out the aromatization of testosterone or 19-hydroxyandrost-4-ene-3, 17-dione.

VI. Testicular and Synthetic Androgens

The principal natural occurring androgens are

TABLE 1

C-19 androgenic steroids of adrenal origin (83)

Δ^4-androstene-3,17-dione

Dehydroepiandrosterone

Testosterone

Adrenosterone

11β-hydroxy-Δ^4-androstene-3,17-dione

11β-hydroxyepiandrosterone

6β-hydroxy-Δ^4-androstene-3,17-dione

6α-hydroxy-Δ^4-androstene-3,17-dione

19-hydroxy-Δ^4-androstene-3,17-dione

androsterone, dehydroepiandrosterone and testosterone, the latter being the most active of the three compounds. The isolation and identification of androsterone and dehydroepiandrosterone have previously been referred to. Testosterone was first prepared in crystalline form from bull testis-tissue by Laqueur et al (90). At practically the same time, Butenandt and Hanisch (91) and Ruzicka and Wettstein (92) announced the artificial preparation of testosterone, identical in physical, chemical and biological properties with the natural form.

Much synthetic work has been done on testosterone analogues with 17 α-alkyl substituents and/or 19-nor structures as well as on 9-halogeno-11-hydroxy derivatives. Many androgens are found to have protein-anabolic activity, and much work has recently been done to develop compounds with a high anabolic/androgenic ratio, e.g., 17 α-ethyl-19-nortestosterone.

VII. Influence of Androgens in Metabolism

a) Protein Metabolism

The influence of androgens on protein metabolism has been studied in detail since the demonstration by Kochakian and Murlin (93, 94) and Kochakian (95) that urinary extracts containing androgenic material, and also the pure compounds, Δ^4-androstene-3,17-dione and testosterone, were capable of causing nitrogen retention in the castrated dog. A decrease in the urinary nitrogen of the treated animal was observed, whereas the fecal nitrogen and the nitrogen content

of the blood were unaffected. Similar observations were made by Kenyon et al (96) and by McCullagh and Rossmiller (97) in human beings. Increase in nitrogen excretion exceeding the pretreatment levels followed when androgen treatment was discontinued. With rats, the administration of testosterone propionate produced nitrogen retention in normal, castrate, adrenalectomized, and hypophysectomized animals (98, 99, 100). However, the effect in rat lasts for about one week in spite of continued treatment. In 1950, Kochakian (101) studied the relative nitrogen-retaining activity of a variety of androgens in two strains of rats. He pointed out that there is a strong correlation between the nitrogen-retaining activity and the androgenic potency of these compounds.

The mechanism by which androgens bring about their effect on nitrogen retention has not been fully elucidated. The quantity of nitrogen retained is too great to be explained as an action only on sex accessory tissue. The effect appears to be rather a more general one such as the influence on skeletal muscle. The nitrogen-retaining action of androgens takes place in the absence of the testes, the pituitary, the pancreas or the adrenals. The decreased urinary nitrogen excretion is principally due to a decreased amount of urea. The concentration of ammonia nitrogen is unchanged. Increase in protein synthesis and decrease in the rate of amino acid catabolism had actually been demonstrated by Bartlett (102) in the female dog. The amount of nitrogen

retained under the influence of an androgen varies depending upon the hitrogen intake, age, and differs in certain diseases due to disturbances in endocrine functions.

b) Carbohydrate Metabolism

Early report of Fichera (103) indicated that testosterone propionate caused hyperglycemia and a decrease in liver glycogen in male rabbits. However, the group headed by Gaunt (104) was unable to influence liver or muscle glycogen in rats by treatment with the same androgen. On the other hand, Cahone (105) claimed a decreased concentration of muscle glycogen in guinea pigs treated with testosterone. A decrease in muscle glycogen was also observed in adrenalectomized rats when treated with testosterone propionate.

17α-methyltestosterone was found to be able to decrease sugar tolerance and liver glycogen in the intact rabbit (106). These effects can be nullified by hypophysectomy or thyroidectomy. Testosterone produced no change in sugar tolerance but increased liver glycogen. Decreased sugar tolerance and glycogen stores were also observed in patients with hypogonadism.

VIII. Adrenal Cortex and the Gonads

It has been mentioned previously that the adrenal cortical tissue secretes androgens. The cells of the adrenal cortex arise from the coelomic mesoderm of the posterior abdominal wall near the anterior portion of the mesonephros and condense into a small cluster of acidophilic cells between the root of the mesentery and the genital ridge from

the fourth to the sixth week of embryonal development. This structure eventually becomes the foetal cortex. Inclusion of cells from the genital ridge in the adrenal cortex is responsible in part for the widespread distribution of adrenal rests and for the occurrence of androgenic and oestrogenic tumours in the adrenal cortex in later life.

At the early stage of development, the primitive gonad has the potentiality of developing into either a testis or an ovary. Although embryologists have not finally established the ultimate embryonic origin of each cell type in the mature ovary or testis, the main outlines of gonadal differentiation in each sex are clear,

It is known at one stage of development, the gonad is situated immediately adjacent to the adrenal gland and has a close ontogenic relationship to it. Nests of adrenal cells frequently separate off with the gonad as differentiation proceeds and are found as adrenal rests in the mature ovary or testis. (107)

IX. Effect of Androgens on Adrenal Cortex

That natural and synthetic corticosteroids dimimish or abolish pituitary ACTH production, causing thereby adrenal atrophy and hypofunction, has been known since Ingle and Kendall (108) published their report in 1937. The original work of Cutuly, Cutuly and McCullagh (109), confirmed by Leonard (110), and Zizine, Simpson and Evans (111), showed that androgens and some other steroids (112, 113) would

prevent partially the expected adrenal atrophy after hypophysectomy. Leathem (114) saw the same effect of testosterone propionate but found that it was a time-limited response which faded out after two weeks despite continued treatment. Gaunt, Howell and Antonchak (115) found that MAD and testosterone propionate counteracted markedly the adrenal-inhibiting effects of cortisone. In 1953, Winter, Hollings and Stebbins (117) observed adrenal-maintaining effect of the same two compounds. Gaunt, Tuthill, Antonchak and Leathem (118) reported that cortisone-induced adrenal atrophy in the intact animal could be prevented by testosterone and a large number of other C-19 and C-20, but not C-21, steroids, and that this effect could not be correlated with their androgenic, growth stimulating or other observed biological properties. But it was Reifenstein, Forbes, Albright, Donaldson and Carroll (119) and Brooks and Prunty (120) who pointed out that under conditions when ACTH was available, 17α-ethyl-19-nortestosterone and other androgens suppressed adrenal secretion rather than exerting a beneficial effect on the function of cortical tissues in man. Similar results were obtained by Saffran and Vogt (1) in intact rats treated with MAD.

The findings of Winter et al (117) and Gaunt et al (118) were reproduced by Rinne and Näätänen with nor-androstenolone-phenyl-propionate (121), Llaurado et al with norethandrolone (122), and Kar et al with 19-nortestosterone (123).

Recently (1961), Fekete and Görög (124) found that MAD and norandrosterone propionate had no effect on adrenal hypofunction due to cortisone in spite of their capability of averting adrenal atrophy to a significant degree. They also failed to demonstrate the presence of any close correlation between morphology and function in the same series of experiments.

X. Metabolism of Androgens by Tissues other than Adrenal Cortex

In 1934, Zondek (125) demonstrated that oestrogens were inactivated and destroyed by the liver tissue. This suggested that androgens might undergo similar changes in the body. Earlier in 1933, Bühler (126) had already reported that only a small proportion of the activity of a urinary androgenic extract injected into a human male was recovered in the urine. In 1935, David, Dingemanse, Freud and Laqueur (127) showed that the hormone isolated from the testis was different and more active than any of the androgenic substances which were separated from the urine. Later, Dorfman and co-workers (128, 129, 130, 131, 132, 133) found that when large doses of testosterone were administered to human beings and various animals, androsterone, isoandrosterone, and etiocholanol-3α-17-one were excreted into the urine. Dobriner and associates (134) had since shown that androsterone and etiocholanol-3α-17-one appear in largest quantity in normal urine and in about equal amounts, while isoandrosterone was present in much smaller concentration.

Only very small amounts of etiocholanol-3β-17-one were found.

Three possible routes by which testosterone was metabolized in the body were proposed by Koch (135) to explain the results of excretion studies:-

i) Reduction of ketone group in ring A followed by oxidation of C-17 and subsequent reduction of the double bond;

ii) Oxidation of C-17 followed by a progressive reduction of ring A;

iii) Reduction of the double bond followed by oxidation of C-17 and subsequent reduction of the ketone group on C-3.

The two saturated diones postulated by the third route had since been found in the urine by Lieberman and Dobriner (136). Since Δ^4-androstene-3,17-dione was also found, a fourth probable course would be:-

iv) Oxidation of C-17 followed by saturation of the double bond and then reduction of the ketone group at C-3.

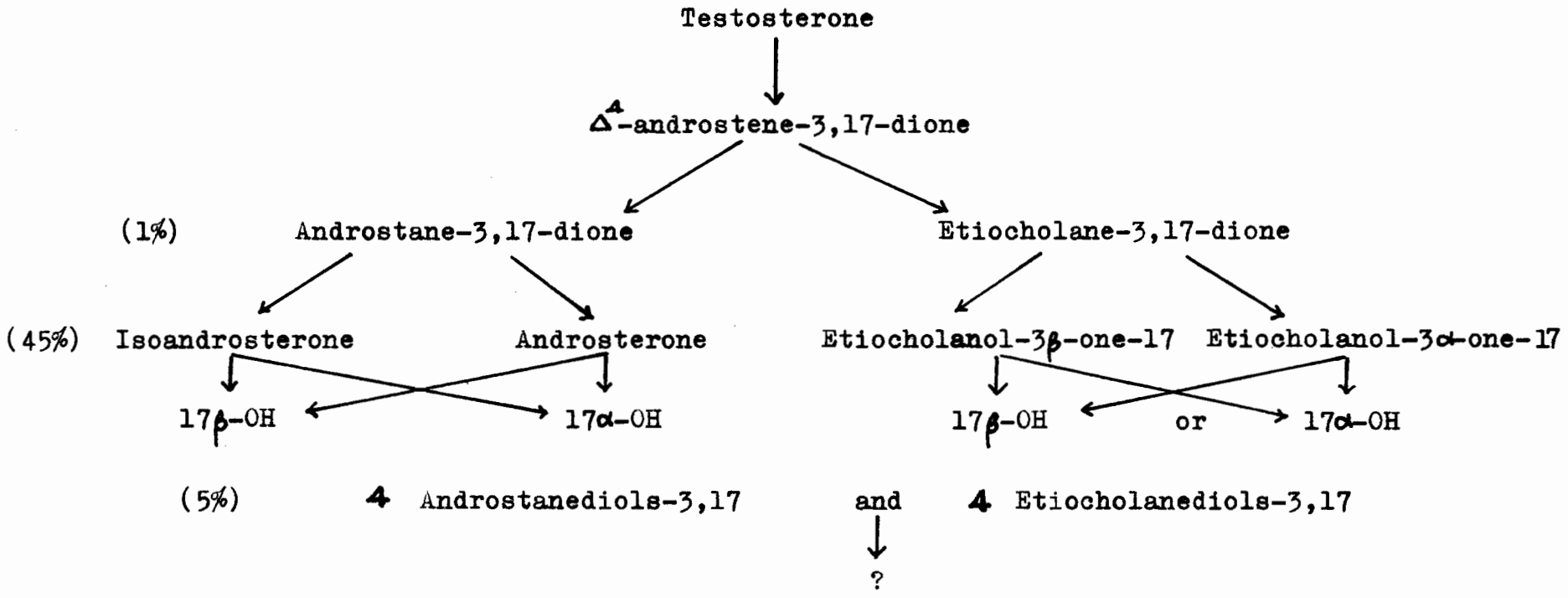
Findings by the same group suggested the following possible metabolic pathway of testosterone as indicated in Fig. 5.

About 50% of the testosterone administered was not accounted for. In addition, the number of organs and enzyme systems involved in these changes were unknown at that time.

That the liver was the major site of androgen inactivation was first confirmed by Biskind and Mark (137). Burrill and Greene (138) showed that the livers of male rats

Fig. 5

The metabolism of androgens by tissues as reviewed by Samuels (143)



( ) Percentage of testosterone administered appeared as the metabolites indicated on the right hand side of the figure.

Possible metabolic pathway of testosterone based on isolation experiments in urine.

All the compounds shown above had been isolated from human urine (with the exception of testosterone).

were also capable of inactivating androgens produced by their own testicular tissue.

Danby in 1940 (139) reported that perfusion of bull testes with blood led to an increase in the androgenic activity of the blood. She also stated that perfusion of liver or kidney of bulls resulted in the disappearance of a large proportion of testosterone introduced into the system. She added that pulped liver or kidney lost the ability to destroy the hormone.

Enzymic destruction of androgens by rat liver tissue

Testosterone, when incubated with minced rat liver tissue in the presence of oxygen, was definitely destroyed (140). The simultaneous disappearance of the 240 mμ absorption band indicated the destruction of α,β-unsaturated ketonic structure. Failure to obtain any colour by the Zimmermann method indicated that 17-ketosteroids were not the final break-down products under such experimental conditions (141, 142). Destruction was shown to be enzymic since boiling deprived the tissue of the ability to destroy the hormone. That oxygen was essential for destruction implied that some oxidative processes were involved.

It is also interesting to know that when DPN was added to the incubation media, the disappearance of testosterone as measured by the decrease in absorption at 240 mμ was accounted for by the formation of 17-ketosteroids. Hence DPN appeared to enhance the destruction of α,β-unsaturated ketonic structure and the oxidation of C-17 of testosterone.

Added citrate only increased the destruction of the α,β-unsaturated ketonic group in ring A. These observations were made with chicken-liver mince. However, with rat-liver mince, only a small proportion of the testosterone molecules in which α,β-unsaturated ketonic structure was destroyed could be accounted for as 17-ketosteroids. When DPN was added, destruction of α,β-unsaturated ketonic structure increased, and a large proportion of these were converted into 17-ketosteroids. Added citrate had the same effect as with chicken-liver mince. It was, therefore, concluded that there were at least three enzyme systems operating:-

i) A system acting slowly on α,β-unsaturated ketonic group in ring A,

ii) DPN-activated enzyme oxidizing C-17 hydroxyl group, and

iii) Another system, affected by citrate, also acts on α,β-unsaturated ketonic structure.

Other androgens such as androsterone and methyl-testosterone are also metabolized by rat-liver mince, the former more rapidly, whereas, the latter more slowly than testosterone (143).

Metabolism of androgens by tissues other than the liver

Few tissues other than the liver metabolize testosterone. The one other tissue which has been found to be significantly active is the kidney, with a rate being approximately equal to one-third of that of the liver (143).

XI. Metabolism of Androgens by Adrenal Gland

Much work has been done on the study of the effect of androgens on the adrenal gland, but until recently little attention has been paid to the action of the adrenal on androgens. Early studies on the metabolism of testosterone by tissues other than the adrenal have been referred to. Only very recently have Chang et al (144) demonstrated that incubation of normal human adrenal homogenate with 4-C^{14}-testosterone yielded, among other compounds, the following metabolites: Δ^4-androstene-3,17-dione, adrenosterone, 11 β -hydroxy- Δ^4-androstene-3,17-dione, and 11 β -hydroxy-testosterone. The latter is found to be the major conversion product. Based on this, the authors concluded that 11 β-hydroxylation seemed to be predominant in adrenal tissue. This should not be surprising in view of the fact that adrenal homogenates have long been known to be capable of introducing a hydroxyl group in the 11 β -position of certain neutral (145, 146, 147, 148) as well as phenolic (149) steroids. It is interesting to note that the usual metabolites of testosterone, such as androsterone and 3α-hydroxy-5β-androstan-17-one were not found in detectable amounts in the incubation extract of Chang et al. When mammalian adrenals of several species were incubated with Δ^4-androstene-3,17-dione as a substrate, metabolites were formed by reduction of ring A (116). This indicates that these mammalian adrenals possess Δ^4-hydrogenase activity.

As Lombardo and Hudson (45) have already demonstrated,

with human adrenal glands, that adrenosterone and 11 β-hydroxy-Δ^4-androstene-3,17-dione do not arise from 3 β-hydroxy-Δ^5-pregnen-20-one, progesterone, 17-hydroxyprogesterone or 17-hydroxydeoxycorticosterone, Chang et al (144) postulated that their 11 β-hydroxy-Δ^4-androstene-3,17-dione may originate from either one of the two sources:

i) Dehydrogenation of 11 β-hydroxytestosterone, or

ii) Direct 11 β-hydroxylation of Δ^4-androstene-3,17-dione.

In addition, they also reported the isolation of a highly polar compound which was thought to be a C-16 or C-6 hydroxylation product formed by the adrenal tissue. Furthermore, there is also evidence supporting the claim that adrenal tissue can convert some androgens to phenolic steroids, presumable oestrogens (144).

The work of Chang et al gained further support from Engel and Dimoline (89), who also demonstrated the conversion by surviving slices of human adrenal cortex of testosterone to 11 β-hydroxytestosterone. They too observed the production of one other polar metabolite of testosterone, whose identity is still unknown. Moreover, a non-polar metabolite having the properties of a testosterone ester has also been mentioned.

XII. Mechanism and Possible Sites of Action of Androgens

It has been known for a long time that treatment with androgens causes a decreased rate of atrophy of the adrenal cortex in hypophysectomized rats. Cutuly and McCullagh (109) thought that "the unexpected high weight of these glands was probably due to variable quantities of lipoidal material

since it has been demonstrated that the medulla does not change greatly in weight after hypophysectomy." Leonard (110), however, attributed this effect to the unchanged state of the cell size. Findings of Zizine and co-workers (111) seemed to agree with the point of view of Cutuly et al. For they succeeded in partially maintaining the adrenal weight and cortical lipid distribution in hypophysectomized rats with testosterone propionate. Since the androgen had the same effect in hypophysectomized male rats which had been simultaneously gonadectomized, they concluded that the effect of the male sex hormone did not seem to be mediated through the action on the pituitary or the gonads.

In spite of the demonstration by Nathanson and Brues (151) that testosterone propionate could increase mitosis in the adrenal cortex, Leonard's experiments (110) indicated that apparently this did not occur in the absence of the hypophysis. It also appeared that an increase in the number of cells could not account for the "heavier" adrenals.

It has equally well been established that adrenal atrophy produced after treatment with large doses of cortisone can similarly be antagonized by androgens (117). Winter and associates suggested that it is possible that the sparing action of androgens on the adrenal cortex of cortisone-treated rats is a consequence of their anabolic action. However, no such action could be demonstrated upon the growth-suppressing action of cortisone in their experiments. In view of the fact that it is not necessary to

assume that steroids have a qualitatively similar action upon metabolic phenomena in all the tissues of the body (152), they proposed that anabolic action of androgens on the adrenals might still be possible.

Attempt has also been made by Rinne and Näätäen (153) and others (117, 118) to study the effect of androgens on the histological picture of the adrenal cortex. Earlier Selye (154-156) reported that androgens exert rather specific effects on the adrenal cortex. In rat they tend to increase cellularity of the glomerulosa and cause capsular fibrosis as well as a pronounced diminution of weight. This usually accompanied by fatty metaplasia of cortical cells. He also pointed out that only if very high doses of testosterone are given does the rat adrenal fail to involute. But Fekete and Görög (124), with reference to Vecsei's Thesis (157), argued that such histological findings offer no basis for drawing inferences concerning the functional condition of the adrenals.

More recently, the effects of steroids hormones are being studied intensively at the molecular level. Some model systems have been worked out for the mechanism of action of oestrogens (150) and some other steroid hormones (150). However, similar systems have not yet been evolved for androgens, although Hurlock and Talalay (158) have found a transhydrogenase in rat liver which is stimulated by 3 α-hydroxysteroids. Since another non-androgenic steroid, 3 α-hydroxy-5 β-androstane-17-one, is found to be as effective

as androsterone in mediating transhydrogenation, the relation between this effect and androgenicity becomes obscured. It was further pointed out by Bloom (159) and Stein and Kaplan (160) that the hydroxysteroid-mediated transhydrogenation had a minor role in liver. Considerable amounts of work have been done on the effects of castration and androgens on the levels of D-amino acid oxidase (161), transaminase and glutamic dehydrogenase (162, 163). Of particular interest is the work of Fishman and Lipkind (164), who demonstrated that levels of β-glucuronidase in the renal tissue of mice was markedly increased by androgens. The specificity of this effect indicates that there is some relation between structure and function.

Enzyme-hormone interaction plays a definite, if not a particularly important, role in the process of metabolism. To elucidate the mechanism of known metabolic antagonism between androgens and cortisone, Kowalewski and Bekesi studied tissue respiration, a function dependent upon enzyme activity, in animals treated with some C-19 and C-21 steroids (165). They found both androgens and cortisone significantly depressed Qo_2 of rat diaphragm and liver slices (166, 167). Kerppola recently reported that cortisone inhibits oxidative phosphorylation in liver mitochondria (168). Earlier Lacroix has provided evidence that enzymatic impairment observed in tissue of cortisone treated animals may be localized at the level of the Krebs Cycle (169). Subsequent work of Kowalewski shows that succinic

dehydrogenase activity in liver mitochondria was depressed by cortisone treatment (170). In spite of the reversible nature of the depression, androgens failed to alter that effect of cortisone. This observation seemed to rule out one of the possible sites of action of androgens, namely at the level of the Krebs Cycle, specifically between succinate and fumarate.

With whole rat adrenal homogenates incubated in the presence of sufficient quantity of triphosphopyridine nucleotide, Tsutsui, Marks and Reich (171) were able to demonstrate that addition of dehydroepiandrosterone decreased the rate of generation of reduced triphosphopyridine nucleotide. When the concentration of TPN is sufficiently low to limit the rate of oxidation of glucose or glucose-6-phosphate to CO_2 through the hexosemonophosphate shunt, addition of dehydroepiandrosterone stimulates the rate of formation of CO_2 , reflecting an increased rate of oxidation of TPNH. On the other hand, the <u>in vivo</u> effect of dehydroepiandrosterone was believed to be on the rate of TPNH oxidation rather than on inhibiting the glucose-6-phosphate dehydrogenase activity, thereby causing a decrease in available TPNH. It is well known that in the adrenal cortex many steps involved in the biosynthesis of steroids require the participation of TPNH (172-176). These steps include the conversion of squalene to cholesterol, the conversion of cholesterol to pregnenolone by side chain cleavage and the conversion of androgens to oestrogens and

various steroid hydroxylations. Decreased rate of formation of TPNH or increased rate of oxidation of TPNH is likely to interfere with steroid biosynthesis.

Another possible site of action of androgens can be localized at the enzymes themselves. Ichii et al (54) showed that virilizing adrenal adenoma possessed a much higher biosynthetic capacity for testosterone and Δ^4-androstene-3, 17-dione from progesterone than normal adrenal tissue. They also observed that when adrenal adenoma homogenate was incubated with progesterone, in spite of the intense increase in the formation of the two androgens, cortisol production per unit weight of tissue is only of the order of 5% of the normal tissue. The authors interpreted this decrease as due to a possible competitive inhibition effect of Δ^4-androstene-3,17-dione on the enzyme, 11β-hydroxylase. This postulate is in good agreement with the recent report from Dorfman and Sharma (177), who found that testosterone, Δ^4-androstene-3,17-dione and dehydroepiandrosterone do not inhibit C-21-hydroxylation of progesterone and 17α-hydroxyprogesterone. This is in contrast to the inhibitory action of adrenal androgens on 11β-hydroxylation step in corticosteroid biosynthesis. Suppression of formation of corticosterone by MAD was also reported.

EXPERIMENTAL

PART I: The effect of MAD on the body weight, adrenal weight and the secretory capacity of the adrenal cortex of rats.

1. Material

   i) Steroid hormones used:

      a) MAD

      b) Cortisol (Compound F)

      c) Testosterone

   ii) Carrier:

      Peanut oil

2. Preparation of solutions

   a) For control:

      A mixture of 10 ml. ethanol (95%) and 11 ml. acetone was added, few drops at a time, to 40 ml. hot peanut oil contained in a 125 ml. erlenmeyer flask. The ethanol and acetone were allowed to evaporate off before the next addition. The flask was stoppered with a cork and the solution left overnight in an oven kept at 70-80° C.

   b) MAD solution:

      Procedures are the same as mentioned above except that 500 mg. of MAD were dissolved in the ethanol-acetone mixture before its addition to the hot peanut oil.

c) Cortisol solution:

As in (a) and (b), except cortisol replaced MAD.

d) Cortisol - MAD:

500 mg. each.

e) Testosterone solution:

500 mg. testosterone.

3. Treatment

Male 180 gm. to 200 gm. rats of the Sprague-Dawley strain, obtained from Canadian Breeding Laboratories, St. Constant were used. They were housed in groups of two per cage. The rats receiving the same treatment were kept in the same cage. No attempt was made to train the animals to handling.

In this series of experiments, rats weighing 180-200 gm. were used. A daily subcutaneous injection of 0.4 ml. oil was given to one group, 5 mg. MAD to a second, 5 mg. cortisol to a third, 5 mg. cortisol combined with same amount of MAD to a fourth, and 5 mg. testosterone to a fifth. All doses of steroids were in 0.4 ml. oil each. Treatment continued for 9 days, and on the tenth day adrenal vein blood was collected and the left adrenal was weighed. The body weight of each animal was recorded every day before the injection was given.

4. Anaesthetic

a) Ether

b) Nembutal (16 mg/ml)

5. Collection of adrenal vein blood

The animal was anaesthetised with 1 ml. of nembutal injected intraperitoneally. 0.6 ml. of heparin was injected via the tail vein. The abdomen was cut open along the mid ventral line. About midway along and perpendicular to the incision, two cuts were made, one to each side, so as to expose the whole abdominal cavity. The viscera were displaced to one side such that the left adrenal gland could be reached without much hindrance. Fatty tissues and connective tissues had to be removed to free the renal vein, which was then bulldogged on the left before it joined the kidney, and on the right before it entered the vena cava. A small slit was made with a pair of fine scissors at the point where the adrenal vein and the renal vein met. Cannulation of the adrenal vein was made with a piece of extremely fine polyethylene tubing (I.D. = 0.023", O.D. = 0.038"). Adrenal vein blood was collected in ice-cooled tared centrifuge tubes for 15-minute periods.

6. Left adrenal gland

When the collection of adrenal vein blood was over, the left adrenal gland was removed and cleaned free of fat in a Petri dish containing filter-paper (Whatman No. 41) moistened with distilled water. The gland was then weighed on an electric automatic balance.

7. Fluorometric measurement of corticosterone content in adrenal vein blood

The blood was weighed and then centrifuged. Transfer of plasma was carried out with micro-pipettes.

Outline of procedures:

a) 0.5 ml. plasma in a 15 ml. pyrex centrifuge tube. Volume brought up to 2.0 ml. with distilled water.

b) Add 4.0 ml. heptane, shake 15 seconds and discard octane layer.

c) Add 2.0 ml. distilled water and 5.0 ml. chloroform. Shake 30 seconds and centrifuge 3 minutes (2,000 rpm). Discard top layer.

d) Add 0.5 ml. 1/10 N sodium hydroxide. Shake 15 seconds. Centrifuge 3 minuts (2,000 rpm).

e) Transfer 4.0 ml. of the chloroform extract to another 15 ml. centrifuge tube containing 1.2 ml. 30 N sulphuric acid. Note the time. Shake vigorously for 30 seconds and centrifuge 3 minuts (2,000 rpm).

f) 1.0 ml. acid extract (bottom layer) transferred to a fluorometer tube. Read 30-40 minutes from the addition of sulphuric acid. Set fluorometer at 40.

g) Set activating wave length at 460 mμ and fluorescence wave length at 510 mμ.

8. Preparation of standard curves

a) Standard stock solution -- A solution of standard corticosterone in 95% ethanol was used. The concentration of the stock solution was made up to 0.1 μg corticosterone per 10 μl solution.

b) Experimental procedures -- 30 μl 95% ethanol, 30, 25, 20,

15, and 10 µl of standard stock solution were placed in 15 ml. centrifuge tubes marked blank, S1, S2, S3, S4 and S5, respectively. Evaporated to dryness. Then add 2.0 ml. distilled water and proceed as outlined under (7).

PART II: The effect of MAD on the secretory activity of the adrenal cortex of rats in vitro.

The methods used in this study were adapted from those of Saffran and Schally (178), and Birmingham and Kurlents (38).

1. Material

i) Steroid hormone (MAD)

ii) Peptide hormone

Adrenocorticotropin (ACTH)

2. Preparation of solutions

a) Incubation medium:

Krebs-Ringer-bicarbonate-glucose solution (178) which was saturated with 95% oxygen-5% carbon dioxide (KRBG).

b) MAD solution:

Weighed out amount of MAD was dissolved in 95% distilled ethanol to give a final concentration of 150 µg MAD per 10 µl solution.

c) ACTH solution:

A few milligrams of USP corticotropin (1.14 U/mg.) were dissolved in an appropriate amount of glass-distilled water such that the final concentration would be 100 mU ACTH per 50 µl solution.

d) Ethanol:

95% distilled.

3. Anaesthetic -- Nembutal: Part I (4) b.

4. Incubation experiments

8 male Sprague-Dawley rats, weighing 200-250 gm. were anaesthetised with nembutal, injected intraperitoneally. The adrenal glands were removed immediately after decapitation. They were cleaned free of fat and quartered in a Petri dish containing filter-paper moistened with KRBG solution. The adrenal quarters were then distributed among eight numbered segments of a second piece of moistened filter-paper in such a manner that each segment contained a quarter of an adrenal from each rat. This filter-paper was kept in a Petri dish, which was placed in a "humidor" to keep the atmosphere moist, and kept cold by sitting the whole set-up on ice.

Adrenal quarters were taken in an orderly way, four at a time, from each segment. The tissue was weighed on a microtorsion balance and placed in a 15 ml. erlenmeyer flask containing 1.5 ml. KRBG solution, and kept on ice. All the flasks were gassed with 95% oxygen-5% carbon dioxide for 20 seconds and stoppered tightly. They were then placed in a Dubnoff Metabolic Shaking Incubator, incubated at 37° C for 60 minutes. At the end of this "pre-incubation" period, the medium in each flask was discarded by suction and replaced with 1.5 ml. of fresh KRBG solution. Ethanol and hormone solutions were added in a manner indicated, as follows:

Flask No.	1	2	3	4	5	6	7	8	9	10	11	12
ETOH (µl)	10		10		10		10		10		10	
MAD (µl)		10		10		10		10		10		10
ACTH (µl)			50	50			50	50			50	50

Flask No.	13	14	15	16
ETOH (µl)	10		10	
MAD (µl)		10		10
ACTH (µl)			50	50

The flasks were then gassed again with 95% oxygen-5% carbon dioxide and stoppered. From the time they left the incubator till the gassing stage, all the flasks were kept on ice.

The flasks were incubated for two hours under the same conditions as set for the pre-incubation. At the end of the incubation, medium from each flask was transferred to a 15 ml. stoppered centrifuge tube and extracted once with an equal volume of methylene chloride. The concentration of Δ^4-3-ketonic steroids was determined by ultraviolet-absorption measurements of the methylene chloride extracts, while the corticosterone content was measured by the fluorometric method.

5. Ultraviolet-absorption measurement

All spectrophotometric measurements in this part were obtained in the Beckman DU spectrophotometer. The samples

were 1 ml. methylene chloride extract of the incubation medium. They were all read against 1 ml. of methylene chloride in the reference cuvette at wave lengths 240 mμ and 255 mμ. Response of the adrenal cortical tissue to the added hormones was estimated by the difference between the optical densities at 240 mμ and 255 mμ per 100 mg fresh weight of tissue.

6. Fluorometric measurement

Fluorometric method was used to measure the corticosterone content of the extract. In this experiment, 0.1 ml. of methylene chloride extract was taken and blown down to dryness under nitrogen. The residue was then treated with 1.2 ml. 30 N sulphuric acid. For fluorometric measurement, 1 ml. of the acid solution was taken and read against the appropriate blank together with a set of standards (Part 1, 8).

PART III: Qualitative studies on the effects of some androgens on the pattern of corticoids produced by the rat adrenals in vitro

1. Material -- Androgens used:

a) MAD (concentrations used were the same as before except where specified)

b) Methyltestosterone

c) Testosterone

2. Pre-treatment

Pre-treatment of the animals with androgens and the method of incubation were exactly the same as mentioned in

the previous sections. The additional physical and chemical means exmployed for these studies are, as follows:-

3. Paper chromatography

a) Preparation of paper:

Whatman No. 42 filter paper (47 or 54 cm. long) was cut along the fibre direction of the sheet to give sheets 18 cm. wide consisting of strips of 1 cm., 2 cm., or 6 cm. wide attached to a common head. These sheets were washed in a Soxhlet apparatus with methanol-benzene (1:1) for 2 days (179), hung in the dark for a few hours to dry, and then stored in the dark until used.

b) Spotting (Application of steroids):

Steroid extracts and the corresponding blanks were taken to dryness with nitrogen in a water-bath kept below 50° C. The residue was taken up with 1 or 2 drops of methylene chloride, and the resulting solution was applied to the paper strip with a micro-pipette. The solution was either confined to a small circle, not more than 0.5 cm. in diameter on narrow strips, or, was applied in the form of a streak on 6 cm. wide strips. A jet of nitrogen was placed close to the spot to help evaporation during spotting. Appropriate reference standard steroids were included on each chromatogram, and so was a dye, either Sudan III or F-5, to facilitate detection of the solvent front in the former case and the position of compound X, 20 → 18-hemiketal-18-hydroxydesoxycorticosterone, in the latter.

c) Solvent system:

The solvent system used was that of toluene-propylene-glycol (25). All solvents used were the highest quality reagent grade available. Toluene and methanol were distilled before use.

i) Solvents: 400 ml. toluene mixed with 100 ml. propylene-glycol.

ii) Wetting of paper: A solution mixture of 60 ml. methanol and 40 ml. propylene-glycol was used. The paper was wetted prior to application of the steroids by passing it through this solution. It was then blotted between two sheets of filter paper.

iii) Temperature: 31 - 34° C.

iv) Equilibration time: Overnight

v) Running time: 8 - 12 or 50 - 72 hours.

vi) Collection of effluent: Small beakers were placed underneath each strip to collect the drips for re-application.

4. Drying of chromatograms

After being removed from the chromatographic tanks, the chromatograms were hung in the dark overnight to dry.

5. Detection of steroids on chromatograms

Paper strips 1 cm. wide were cut from the developed chromatograms and scanned in the Beckman DU spectrophotometer at 240 mμ. The width of paper exposed to the ultraviolet light was confined to 3 mm. by means of a diaphragm placed in the light path in order to avoid any

destruction of the steroid by excessive exposure to ultraviolet light (180). In the later part of the course of this study, an autoscanner (Densicord, Photovolt Inc., N.Y.) was used, which was found to be more efficient and exact in locating the steroid masses. Both machines detect steroids possessing an α, β-unsaturated ketone group.

6. Colour reactions on paper

Colour tests were performed on narrow strips (0.3 cm. wide) cut from 1 cm. wide strips of the developed chromatograms.

a) Porter-Silber reaction:

Compounds possessing the dihydroxyacetone side chain give a positive Porter-Silber reaction (181). Its application on paper was adapted by Birmingham (182). The paper strip is passed through the Porter-Silber reagent (181) at room temperature and the development of the characteristic yellow colour of the phenylhydrazone noted. This colour appears in half to two hours with 2 $\mu g/cm^2$ of steroids containing the dihydroxyacetone side chain and immediately with steroid-21 aldehydes.

b) Reduction of a tetrazolium derivative:

The paper strip was passed through a solution of the tetrazolium derivative M. and B. 1767 (2 mg. of 2:5-diphenyl-3-(4-styrylphenyl)-tetrazolium chloride + 2 ml. of 95% ethanol + 5 ml. of 2.5 N NaOH). It was then passed quickly through distilled water and blotted lightly between two sheets of filter paper. Develop-

ment of a purple colour is characteristic of compounds possessing an α-ketol group, which can be detected at a concentration of 2 $\mu g/cm^2$.

PART IV: Some quantitative studies on the effects of some androgens on the production of corticosteroids by rat adrenals in vitro

Corticosteroids produced by incubated rat adrenals were separated by paper chromatography (Part III, 3). The appropriate ultraviolet-absorbing areas, as located by scanning and comparison with the reference steroid strip, were cut out and eluted by either one or the other of the following two methods:-

1. Method adapted from that used by Birmingham and Ward (40)

The ultraviolet absorbing areas on the chromatogram, which were under investigation, were cut into small pieces in 15 ml. erlenmeyer flasks. Enough distilled methanol was added to cover the pieces of paper. The flasks were left standing overnight in the dark and were shaken in the Dubnoff incubator the next day for 2 hours. The eluates were stored in stoppered test-tubes for later measurement.

2. Method by Saffran and Sharman (183)

The appropriate ultraviolet-absorbing portions of the strips were cut off and eluted with a mixture of ethyl acetate and distilled methanol (2:1) in an all-glass eluter. The solvent was evaporated with a stream of nitrogen and the residue was taken up again with 2 ml. of distilled methanol. Quantitative measurements were performed with a

Beckman DK-2 Spectrophotometer.

3. Tests on eluted steroids

i) Ultraviolet absorption spectra:

Ultraviolet absorption spectra were obtained in the Beckman DK-2 spectrophotometer. A solution of the steroid in methanol was read against a corresponding blank solution. The wavelength range used was 215-280 mμ. The amount of steroid present could be calculated by comparing the difference between the optical densities at 240 mμ and 260 mμ with a similar calculated density of a standard solution of USP reference hydrocortisone. All steroids possessing an α, β-unsaturated ketone group absorb in the ultraviolet region with a maximum at 238-242 mμ.

ii) Colour tests:

a) Porter-Silber reaction

To a steroid-containing solution was added phenylhydrazine reagent (65 mg. of recrystallised phenylhydrazine hydrochloride dissolved in a solution consisting of 62 ml. of concentrated sulphuric acid, 38 ml. of water and 50 ml. of ethanol) to give a concentration of 5-10 μg. steroid per ml. of reagent. Its blank was similarly treated with exactly the same amount of reagent. The solutions were left overnight and read the next day in the Beckman DK-2 spectrophotometer over the wavelength range 310-500 mμ.

b) Reduction of tetrazolium in solution

Ultraviolet absorption spectra were obtained in the Beckman DK-2 spectrophotometer. A solution of the steroid in methanol was read against a corresponding blank solution. The wavelength range used was 215-280 mμ. The amount of steroid present could be calculated by comparing the difference between the optical densities at 240 mμ and 260 mμ with a similar calculated density of a standard solution of USP reference hydrocortisone. All steroids possessing an α, β-unsaturated ketone group absorb in the ultraviolet region with a maximum at 238-242 mμ.

PART V: Identification of some of the isolated steroids formed by the adrenals incubated in the presence of MAD

1. Purification

a) Partition between benzene and water (5:2)

b) Application on alumina or silica gel column and gradient elution with benzene-ethyl ether in the former case, and with methylene chloride-ethanol in the latter.

2. Zimmermann reaction

17-ketosteroids were detected by passing the paper strip containing 5-10 μg. of steroid through a 1% solution of m-dinitrobenzene in ethanolic KOH (184). A characteristic purple colour developed upon drying the paper in the oven.

3. Other colour reactions

a) Porter-Silber reaction (Part IV, 3, iia)

b) Reduction of tetrazolium (Part IV, 3, iib)

4. Mixed chromatography

15 µg. of the eluted steroid and the same amount of reference steroid were mixed and spotted. The chromatogram was developed in Zaffaroni's solvent system (Part III, 3).

5. Sulphuric acid absorption spectra

A solution of 8 µg. steroid in 0.5 ml. concentrated sulphuric acid was allowed to stand at room temperature for two hours. The absorption spectrum was then determined over the wavelength range 220-600 mµ (185).

6. Infrared spectra

240-270 µg. of steroid was dissolved in 0.1 ml. carbon disulphide or compressed in 20 mg. potassium bromide; the infrared spectrum was determined in a Perkin-Elmer double beam spectrophotometer, model 221.

PART VI: Perfusion of rat adrenal gland with Krebs-Ringer-bicarbonate-glucose (KRBG) solution containing MAD

1. Animal

Male Sprague-Dawley rat weighing 380 gm. was used

2. Anaesthetic

1 ml. nembutal solution containing 16 mg./ml.

3. Preparation of perfusate

a) KRBG containing 0.1 ml. distilled 95% ethanol per 100 ml. solution

b) KRBG containing, per 100 ml. solution, 0.1 ml. of MAD solution (50 mg./ml. distilled 95% ethanol).

4. Perfusion of isolated adrenal gland in situ

Technique employed was that developed by Birmingham and Nutik (unpublished).

5. Analysis

The perfusate was extracted with 1 ml. of methylene chloride. The ultraviolet absorption of the extract was measured with the Beckman DK-2 spectrophotometer (Part IV, 3, i).

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Most of the reference steroids used in this investigation were either U.S.P. Steroid Reference Substances or were obtained from Southeastern Biochemicals Inc. 17α-methylandrostenediol was obtained from Organon, Ltd., Newhouse, Scotland, or Charles E. Frosst Co., Montreal. Cortisol was a gift from Merck Co., and testosterone was a generous gift from Dr. Thompson of the Charles E. Frosst Co. All solvents used were the highest quality reagent grade available.

RESULTS

PART I: The effect of MAD on the body weight, adrenal weight and the secretory capacity of the adrenal cortex of rats

1. The ability of MAD to antagonize the catabolic effect and adrenal atrophy produced after treatment with cortisol:

a) Body weight

Table 2 (Groups 1 and 3) showed that MAD, given on its own, did not change the body weight significantly. Treatment with cortisol reduced the body weight to 81.5% of normal. Simultaneous administration of MAD (Group 4) failed to antagonize the catabolic effect of cortisol, but on the contrary, the body weight was further reduced to 77% of normal.

b) Blood flow

The rate of blood flow through the adrenal was measured by the amount of plasma (ml.) collected within 15 minutes. Cortisol and MAD treated rats gave a rate of 0.87 and 0.84 ml. of plasma per 15 minutes, respectively. Control rats or those which received combined treatment of MAD and cortisol had a rate equal to half that of the two previously mentioned groups.

c) Adrenal weight

The weight of the left adrenal was measured in all cases. The mean adrenal weight of rats given the vehicle was 20.6 $\pm$ 0.81 mg. Cortisol reduced the adrenal weight

TABLE 2

Effect of MAD and cortisol on body weight, organ weight, rate of blood flow and secretory capacity of the adrenal cortex of the rats.

Group No. and Treatment	Body Wt. (gm)	Plasma Collected in 15 min. (ml)	Weight of left Adrenal (mg)	% of Normal	Corticosterone Secreted µg/min/gm Adrenal	% of Normal	µg/gland/ hr/kg Body Wt.	% of Normal
1. None (4)	319 ± 11*	0.46 ± 0.01	20.6 ± 0.81	-	27.4± 6	-	112±21	-
2. Cortisol (5)	260 ± 9.9	0.87 ± 0.01	12.2 ± 0.65	59	6.9± 1.1	25	18.9± 3.2	17
3. MAD (6)	289 ± 8.7	0.84 ± 0.01	24.5 ± 1.09	119	8.7± 1.4	32	40.8± 7.2	36
4. MAD & Cortisol (5)	245 ± 10	0.44 ± 0.01	16.4 ± 1.03	80	8.4± 1.1	31	39.7± 7.1	35

( ) No. of animals

* Standard error

MAD 17α- methylandrostenediol

to 59% of the control. The 19% increase above the control caused by MAD was apparent although small. The repair by the combination of MAD with cortisol was not statistically significant. It is interesting to point out that MAD, in the presence or absence of cortisol, increased the adrenal weight by about 4 mg.

d) Adrenal secretion of corticosterone

It was observed that cortisol reduced adrenal secretion of corticosterone to one-fourth of that of the control. MAD had similar effect but to a lesser extent. However, when MAD was administered along with cortisol, it partially antagonized the effect of cortisol on adrenal secretion per gram of tissue.

Owing to the atrophy of the adrenals caused by cortisol, the hourly secretion per gland and per kilogram body weight of this group of animals was also depressed markedly (17% of control). MAD, given alone or with cortisol, gave an hourly secretion almost double that of the cortisol-treated group.

2. The effect of testosterone, as compared to that of MAD, on the body weight, adrenal weight, the weight of testes and the secretory capacity of the adrenal cortex of rats.

Both the artificial (MAD) and the testicular (testosterone) androgens did not cause significant change in the adrenal weight, body weight, rate of blood flow through the adrenals or in the weight of testes. However, treatment with MAD or testosterone decreased the secretory

capacity of the adrenal cortex. In contrast, changes in the secretory capacity were more drastic in animals treated with MAD.

The rate of blood flow through the adrenals as measured by the amount of plasma collected within a period of 15 minutes depends greatly on how successful the cannulation of the adrenal vein was. In the first experiment, MAD-treated animals were found to have a rate of blood flow higher than that of the control group (Table 2, second column). But in the second experiment, the rate of blood flow for the control group was higher than that found in the MAD-treated group (Table 3, second column). This could possibly be explained by the fluctuations in the personal performance of cannulating the adrenal vein in each experiment.

PART II: The effect of MAD on the secretory activity of rats in vitro (Table 4)

The total amount of ultraviolet absorbing steroids produced by the incubated adrenals was estimated by the difference between the optical densities of the methylene chloride extract of the incubation media at 240 mμ and 255 mμ. Response of adrenal cortical tissue to added ethanol or MAD, with and without ACTH, was given as:

(O.D. at 240 mμ - O.D. at 255 mμ)/100 mg. tissue weight

Adrenal tissue incubated with ethanol served as controls. They gave a mean response of 46. Addition of MAD to the incubation media caused an increase in response equal

TABLE 3

Effect of MAD and testosterone on body weight, organ weight, rate of blood flow and secretory capacity of the adrenal cortex of the rats.

Group No. and Treatment	Body Weight (gm)	Plasma Collected in 15 min. (ml)	Weight of left Adrenal (mg)	Weight of Testes (gm)	Corticosterone Secreted			
					µg/min/gm Adrenal	% of Normal	µg/gland hr/kg Body Wt.	% of Normal
1. None (6)	289± 7*	0.75±0.11	22.2±1.4	3.1±0.1	33.2±2.3	-	137±13.1	-
2. MAD (8)	286± 7	0.65±0.19	20.9±0.7	3.1±0.1	17.8±1.8	54	81± 9.3	59
3. Test-osterone (6)	291± 6	0.86±0.24	20.6±0.8	3.2±0.1	23.3±3.9	70	92±17.2	67

( ) No. of animals

* Standard error

TABLE 4

In vitro effect of MAD on production of UV-absorbing steroids by rat adrenals with and without added ACTH.

Addition \ Expt.	1	2	3	4	Mean	Difference Due to MAD
Control	39	54	55	37	46 ± 4.7*	
MAD	117	123	107	95	110 ± 6	64
ACTH	146	155	158	129	147 ± 6.5	
ACTH & MAD	266	168	211	217	215 ± 20	68

* Standard error

Incubation media were extracted with equal volumes of methylene chloride UV-absorption of the extracts were measured in a Beckman DU Spectrophotomer.

Figures represents response as measured by 240-255 mμ/100 mg fresh weight of adrenal tissue.

to 64. Incubation with ACTH alone increased the adrenal response to 147. With added ACTH and MAD, an even higher value was obtained, namely, 215. Thus the net effect of added MAD in the presence of ACTH was an increase of 68. This means that MAD, either in the absence or presence of ACTH, resulted in a two-fold increase in the production of ultraviolet absorbing material by the incubated adrenals of rats.

Table 5 shows the in vitro effect of MAD on the production of "corticosterone", measured by fluorescence (page 42), by rat adrenals with and without ACTH. Production of corticosterone is expressed as µg of corticosterone per 100 mg. fresh weight of adrenal tissue. The control produced a mean of 10.4 µg. In the absence of ACTH, MAD increased the output of corticosterone to 50 µg. above the control level. In the presence of ACTH, MAD caused a slightly higher increment amount, to 59 µg. ACTH, added on its own, stimulated a three-fold increase in corticosterone production as compared to that of the control. This seemed to indicate that MAD was more effective than ACTH in stimulating the production of corticosterone by incubated rat adrenals. However, the addition of concentrated sulphuric acid to the methylene chloride extract of incubation medium containing MAD resulted in a yellow colour suggesting that MAD and the concentrated sulphuric acid also reacted.

To ascertain whether the presence of MAD interferes with the fluorometric measurement of corticosterone, standard

TABLE 5

In vitro effect of MAD on production of "corticosterone" by rat adrenals with and without added ACTH (fluorometric method).

Addition \ Expt.	1	2	3	4	Mean	Difference due to MAD
Control	5.4	12	13	11	10.4 ± 1.8*	
MAD	67	65	56	54	61 ± 3.3	50
ACTH	38	46	48	41	43 ± 2.3	
ACTH & MAD	105	72	130	102	102 ± 12	59

*Standard error

Figures are presented in terms of µg of "corticosterone"/100 mg fresh weight of adrenal tissue.

curves were run in the following manner:-

a) 30 µg. of MAD were added to 0.3, 0.25, 0.2, 0.15, and 0.1 µg. of standard corticosterone (Table 6).

b) 0.5 µg. of MAD was added to a similar set of corticosterone standards (Table 7).

c) Various amounts of MAD were added to a series of fixed concentrations of standard corticosterone (Tables 8 and 9).

A set of standards consisting of 0.3, 0.25, 0.2, 0.15 and 0.1 µg. of corticosterone together with an appropriate blank was included in each of the above determinations.

The absence of parallelism between curve with corticosterone and curve with corticosterone and MAD in either Fig. 6 or Fig. 7 indicated that equal amounts of MAD did not give equal increments in the fluorometric readings.

Figures 8 and 9 showed curves obtained by plotting fluorometric readings vs. total amounts of steroid (corticosterone plus MAD). There was no linear relationship between the quantities of MAD added and the corresponding fluorometric readings. Nevertheless it can be concluded that MAD did invariably give higher fluorometric readings inasmuch as the estimation of corticosterone by this method was concerned.

The inconsistent interference by MAD in the fluorometric method for measurement of corticosterone led to the use of chromatography to purify corticosterone in the investigation of the effect of MAD on isolated rat adrenals.

TABLE 6

Fluorometric measurement of a set of standard corticosterone and another identical set with a series of fixed concentrations of MAD

Tube No.	Steroid Content (µg)		Corrected Fluorometric Reading	Total Amount of Steroid (µg)
	Corticosterone	MAD		
Blank	-	-	0	0
S 1	0.30	-	136	0.30
S 2	0.25	-	87	0.25
S 3	0.20	-	69	0.20
S 4	0.15	-	53	0.15
S 5	0.10	-	35	0.10
M 1	0.30	30	44 (48)	6.04
M 2	0.25	30	54.5 (48)	7.49
M 3	0.20	30	50 (48)	6.86
M 4	0.15	30	49.5 (48)	6.80
M 5	0.10	30	36 (48)	4.95
M 6	-	30	60 (48)	8.23

( ) Dilution factor

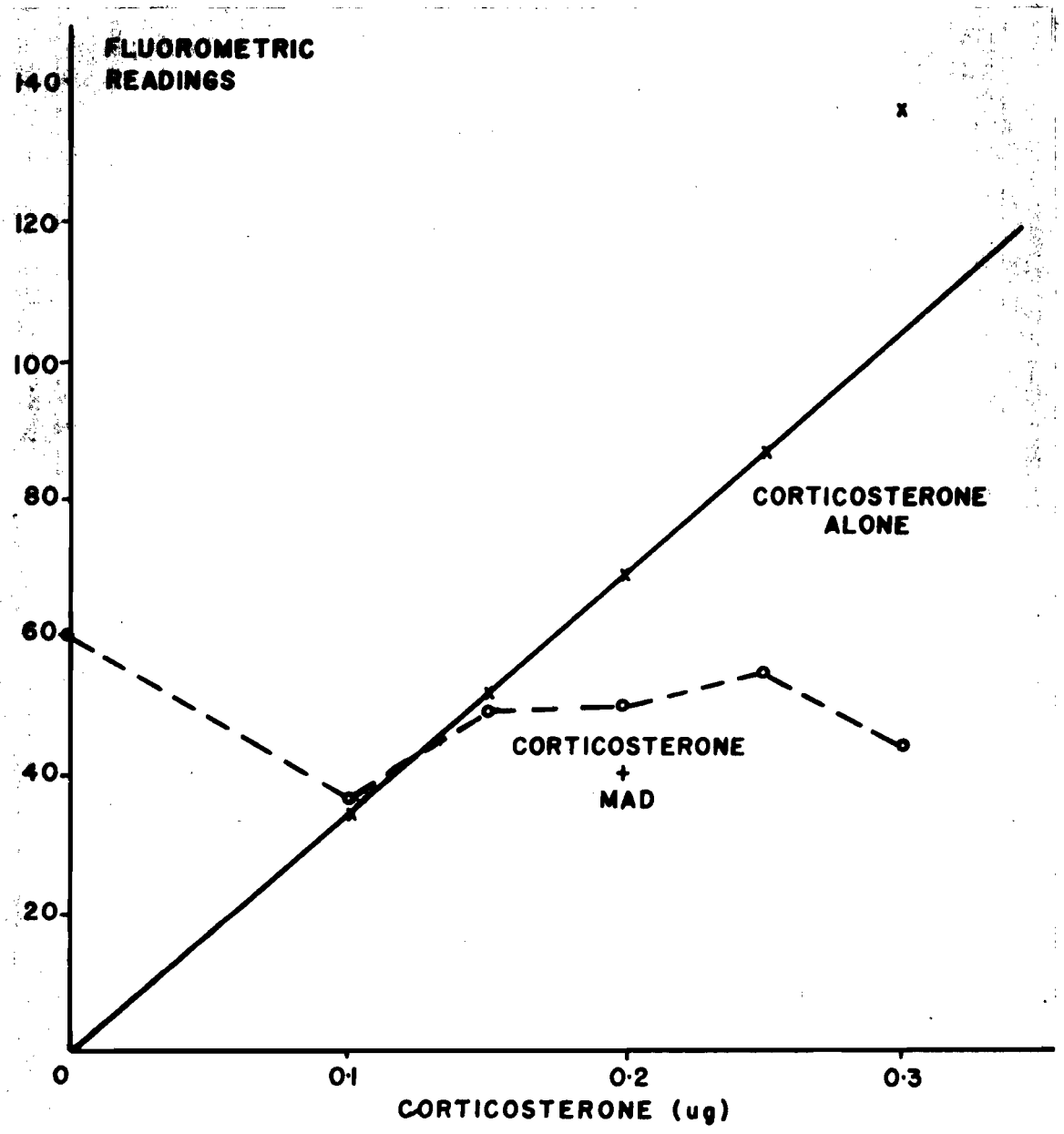


FIG.6 FLUOROMETRIC READINGS vs CORTICOSTERONE

TABLE 7

Fluorometric measurement of a set of standard corticosterone and another identical set with a series of fixed low concentrations of MAD

Tube No.	Steroid Content (µg)		Corrected Fluorometric Reading	Total Amount of Steroid (µg)
	Corticosterone	MAD		
Blank	-	-	0	0
S 1	0.30	-	88.0	0.30
S 2	0.25	-	72.0	0.25
S 3	0.20	-	59.5	0.20
S 4	0.15	-	44.3	0.15
S 5	0.10	-	34.7	0.10
M 1	0.30	0.5	110.4	0.37
M 2	0.25	0.5	92.7	0.31
M 3	0.20	0.5	72.7	0.25
M 4	0.15	0.5	64.7	0.22
M 5	0.10	0.5	44.7	0.15
M 6	-	0.5	8.2	0.03

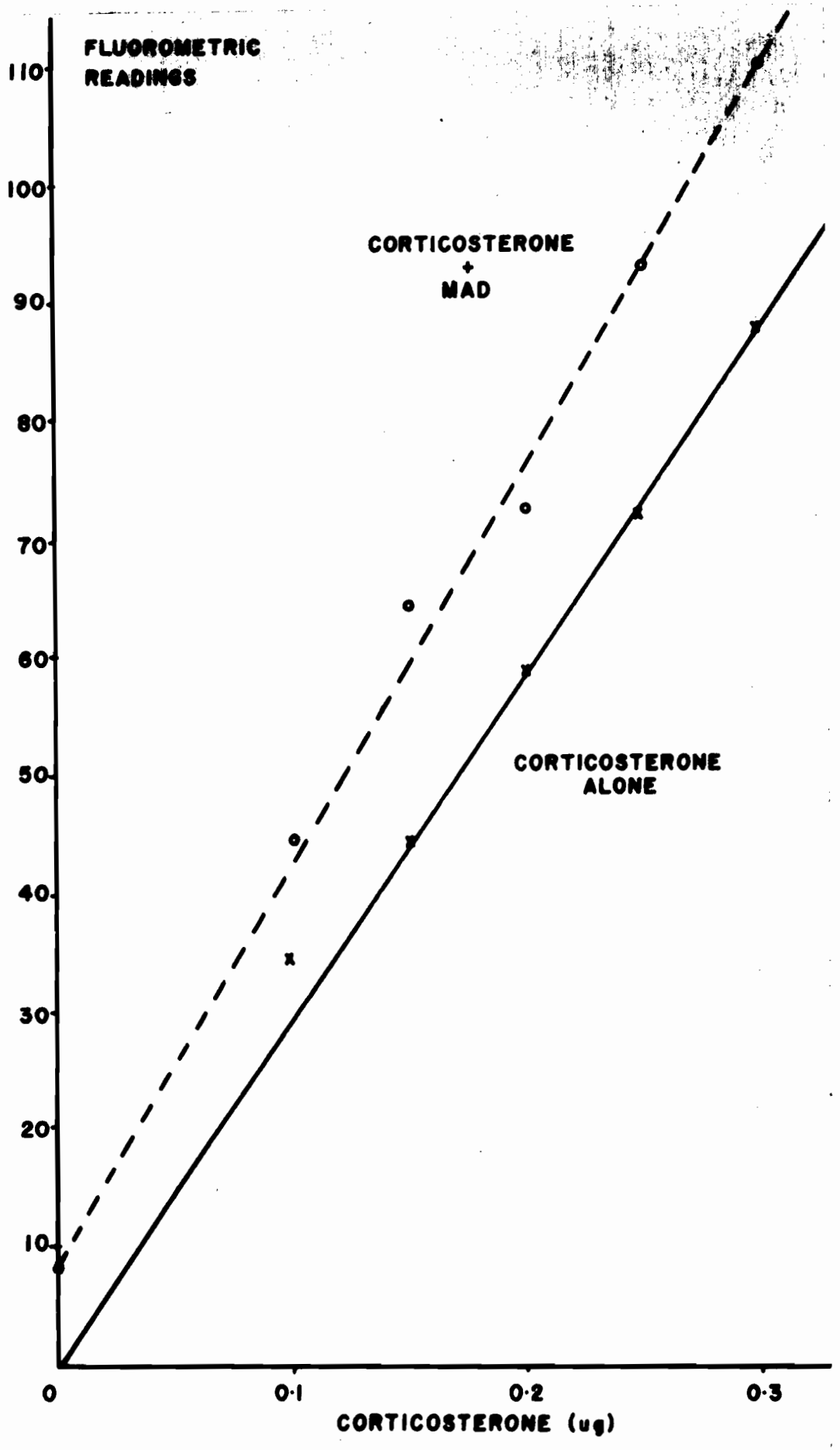


FIG.7 FLUOROMETRIC READINGS vs CORTICOSTERONE

TABLE 8

Fluorometric measurement of a set of standard corticosterone and another set with a series of increasing concentrations of MAD

Tube No.	Steroid Content (µg)		Corrected Fluorometric Reading	Total Amount of Steroid (µg)
	Corticosterone	MAD		
Blank	-	-	0	0
S 1	0.30	-	54.6	0.30
S 2	0.25	-	50.1	0.25
S 3	0.20	-	38.5	0.20
S 4	0.15	-	28.6	0.15
S 5	0.10	-	24.1	0.10
M 0	0.15	-	31.7	0.15
M 1	0.15	0.1	38.1	0.21
M 2	0.15	0.2	38.1	0.21
M 3	0.15	0.5	41.1	0.22
M 4	0.15	0.8	50.6	0.27
M 5	0.15	1.0	47.6	0.26

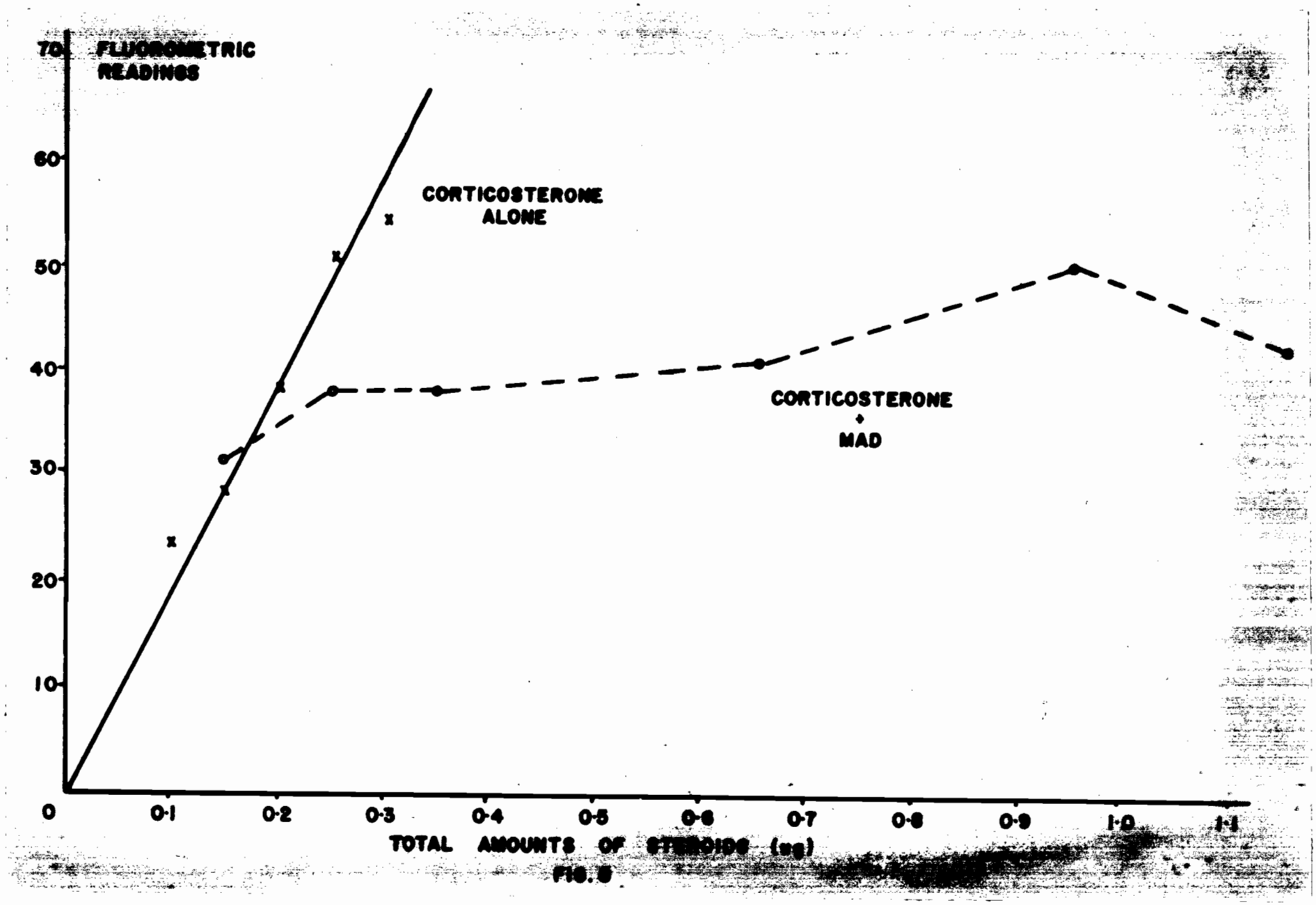

70
FLUOROMETRIC READINGS
60
50
40
30
20
10
0
CORTICOSTERONE ALONE
CORTICOSTERONE + MAD
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
1·1
TOTAL AMOUNTS OF STEROIDS (µg)

FIG. 8

TABLE 9

Fluorometric measurement of a set of standard corticosterone and two other sets with fixed series of increasing concentrations of MAD

Tube No.	Steroid Content (µg)		Corrected Fluorometric Reading	Total Amount of Steroid (µg)
	Corticosterone	MAD		
Blank	-	-	0	0
S 1	0.30	-	81.2	0.30
S 2	0.25	-	65.7	0.25
S 3	0.20	-	56.2	0.20
S 4	0.15	-	40.7	0.15
S 5	0.10	-	25.9	0.10
M 1	0.15	0.1	47.7	0.17
M 2	0.15	0.2	44.2	0.16
M 3	0.15	0.5	50.7	0.18
M 4	0.15	1.0	61.7	0.22
M'1	0.15	0.2	50.7	0.18
M'2	0.15	0.4	60.7	0.22
M'3	0.15	1.0	62.7	0.23
M'4	0.15	2.0	80.2	0.29

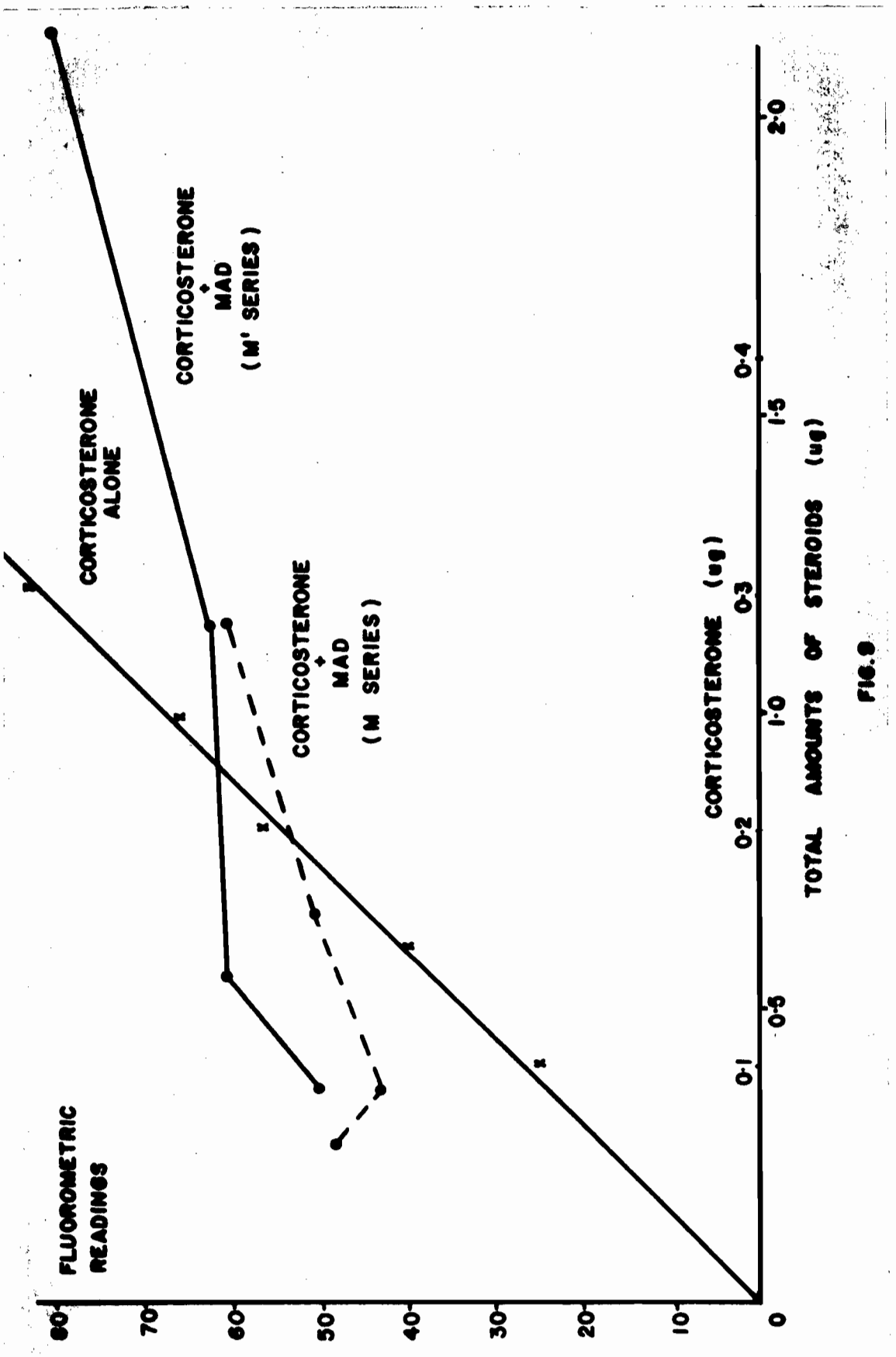

FLUOROMETRIC READINGS
CORTICOSTERONE ALONE
CORTICOSTERONE + MAD (M' SERIES)
CORTICOSTERONE + MAD (M SERIES)
CORTICOSTERONE (µg)
TOTAL AMOUNTS OF STEROIDS (µg)
0
10
20
30
40
50
60
70
80
0·1
0·5
1·0
0·2
0·3
1·5
0·4
2·0

FIG. 9

PART III: Qualitative studies on the effect of some androgens on the pattern of corticoids produced by the rat adrenals in vitro

1. Effect of MAD with and without added ACTH

Table 10 showed the data obtained from an experiment done with eight rats. Ultraviolet absorbing areas detected on the chromatogram were designated as zones F, E, X, B, A and DOC (40). This was done under the guidance of an accompanying paper strip containing the appropriate reference steroids.

Methylene chloride extracts of media from rat adrenals incubated with or without ACTH yielded on chromatography in toluene-propylene glycol system the four major ultraviolet-absorbing zones, F, E, X and B as described by Heard et al (24). The mobilities of these compounds were found to be higher than those observed by Heard et al and Ward (24, 40). A tetrazolium reducing, ultraviolet-absorbing compound with an Rb value (mobility relative to corticosterone) approximated to that of authentic 11-dehydrocorticosterone (compound A) was sometimes detected.

Corticoids produced by the adrenals incubated with MAD exhibited a different pattern. The two most polar compounds, with Rb values of 0.36 and 0.54, had different Rb values than F (Rb = .28) and E (Rb = .40) regions, respectively. The material located in the vicinity of the X zone (Rb = .71) had a slightly higher Rb than 20 → 18-hemiketal-18-hydroxydeoxycorticosterone (Rb = .69). Unlike the

TABLE 10

Effect of MAD on the production of cortical steroids by the rat adrenals in vitro in the presence or absence of ACTH.

ZONE	Reference Standards			Control			MAD			ACTH			ACTH & MAD		
	Rb	r	P	Rb	r	P	Rb	r	P	Rb	r	P	Rb	r	P
F	0.284	?	+	0.27	+		0.36	+		0.28	+		0.38	± +	
E	0.4	?	+	0.425	+		0.54	+		0.44	+				
X	-			0.69		+	0.71		?	0.69		+	0.69		+
B	1	+		1	+		1	+		1	+		1	+	
A	1.93	+		1.76	+		1.85	+		-			-		
	-			-			2.62			-			-		
DOC	3.64	+		-			-			-			-		

Rb = mobility relative to corticosterone; r = tetrazolium reducing; P = Porter-Silber reaction; + sign indicates a positive reaction.

Chromatograms of extracts of incubation media were developed in toluene-propylene glycol system. Zones F, E, B, A, DOC were located with reference standards of cortisol, cortisone, corticosterone, 11-dehydrocorticosterone and 11-desoxycorticosterone. The X zone was assumed to be between the E and B regions, it denotes where 20→18-hemiketal-18-hydroxydeoxycorticosterone should be found.

Reduction of tetrazolium (r) and Porter-Silber reaction (P) were performed on paper strips cut from the chromatograms.

hemiketal, it did not give a positive Porter-Silber reaction. In addition, an augemented ultraviolet-absorbing area, with Rb equal to 2.62, was detected. This material neither reduced tetrazolium nor gave a positive Porter-Silber reaction. It was found to be less polar than compound A (Rb = 1.93).

Incubated adrenals in the presence of ACTH and MAD produced only three ultraviolet absorbing zones. Two of them could be accounted for by compound X and compound B. The remaining one, which had an Rb of 0.38, might be similar to the most polar compound produced by the MAD-treated adrenals.

To ascertain the observation that MAD, with or without added ACTH, affected the pattern of corticoids produced by rat adrenals in vitro, a similar experiment was performed but with doubled amounts of material. Table 11 summarized the results obtained in this experiment. On the whole, figures presented in this table agreed quite well with the previous observations. The only marked difference was found in the adrenals incubated with ACTH and MAD, which behaved as if MAD alone were used.

The newly discovered ultraviolet absorbing material, which ran with an Rb of 2.62, was postulated to be 17α-methyltestosterone. In the next experiment, authentic 17α-methyltestosterone was included in the reference steroids. It was subsequently found that the unknown, postulated to be 17α-methyltestosterone, ran with an Rb

TABLE 11

Effect of MAD on the production of cortical steroids by the rat adrenals in vitro in the presence or absence of ACTH.

ZONE	Reference Standards			Control			MAD			ACTH			ACTH & MAD		
	Rb	r	P	Rb	r	P	Rb	r	P	Rb	r	P	Rb	r	P
	-			-			-			-			0.15		
F	0.24	+	+	0.22	+		0.21	+		0.21	+		0.24	+	
E	0.38	+	+	0.39	+		0.34	+		0.37	+		0.37	+	
X	-			0.64		+	0.79			0.67		+	0.67		+
B	1	+		1	+		1	+		1	+		1	+	
A	1.52	+		1.44			1.48			1.44			1.44		
MT	1.79			-			1.80			-			1.74		
DOC	-			-			-			-					

The MT zone was located with authentic 17α-methyltestosterone.

Also see legends under Table 9.

TABLE 12

Effect of MAD on the production of cortical steroids by the rat adrenals in vitro in the presence or absence of ACTH. (An experiment to demonstrate the reproducibility of data presented in Table 10).

ZONE	Reference Standards			Control			MAD			ACTH			ACTH & MAD		
	Rb	r	P	Rb	r	P	Rb	r	P	Rb	r	P	Rb	r	P
F	0.24	+	+	0.19	+	+	0.21	+		0.17	+	+	0.22	+	
E	0.37	+	+	0.41	+	+	0.34	+		0.34	+		0.37	+	
X	-			0.66		+	0.79		±	0.66		+	0.67		+
B	1	+		1	+		1	+		1	+		1	+	
A	1.52	+		1.48	+		1.45	+		1.45	+		1.44	+	
MT	1.79			-			1.79			-			1.74		
DOC	-			-			-			-			-		

See legens under Table 9.

practically identical with that of the authentic compound. Table 12 also showed the rest of the results obtained in this experiment.

2. Since MAD, 17α-methyltestosterone and testosterone are compounds closely related structurally, they might exert similar or comparable effects on the production of corticosterone and/or on the pattern of corticoids produced by the adrenal cortical tissue. Thus in a series of runs, in which the amount of corticosterone was estimated by absorption of ultraviolet light by the spot on the paper chromatogram, incubation in the presence of MAD and 17α-methyltestosterone resulted in consistently less corticosterone formation than in the absence of added androgen (Table 13). Added testosterone did not cause a consistent decrease. The author is well aware of the fact that the density of the peak on a paper chromatogram is only a semi-quantitative measure of the production of corticosterone. In experiments that followed, the corticosterone was eluted from the chromatogram and estimated more exactly by ultraviolet absorption performed in solution and also by the reduction of tetrazolium.

In chromatograms of lipids from incubation media of rat adrenals in the presence of MAD, 17α-methyltestosterone and testosterone, augmented ultraviolet-absorbing areas, more polar than corticosterone, were detected. These areas appeared consistently with all three androgens, and in amount greater than corticosterone. With MAD and methyl-

TABLE 13

Absorption of ultraviolet light by the corticosterone band from incubation media of rat adrenals, with and without added androgens.

Expt.	Added androgens	Height of Peak
1	None	61
	MAD	44
	Methyltestosterone	46
	Testosterone	49
2	None	52
	MAD	27
	Methyltestosterone	27
	Testosterone	53
3	None	42
	MAD	38
	Methyltestosterone	34
	Testosterone	58

testosterone, the augmented zone had a mobility relative to corticosterone of 0.88, while the Rb of the zone with testosterone was 0.60 (Table 14, under Rb zones 0.55-0.6 and 0.8-0.9). In addition, it was again observed that MAD gave rise to an ultraviolet-absorbing material, which, as had been demonstrated before, had a mobility equal to that of 17α-methyltestosterone, suggesting that MAD was oxidized by the 3β-ol dehydrogenase of the adrenal.

Zones representing compounds more polar than the material in the X region were detected in the extracts of incubation media of rat adrenals in the presence of MAD, 17α-methyltestosterone and testosterone. These materials were present in too small quantities for either qualitative or quantitative analysis.

PART IV: Some quantitative studies on the effect of androgens on the production of corticosteroids by rat adrenals in vitro

Because collection of sufficient amounts of lipids from incubation media of rat adrenals for analytical purposes is rather time-consuming, the latter part of this piece of work was devoted to the study of the effect of MAD on the adrenal cortical tissue of rats. The influence of the androgen on the secretory capacity of the gland was also observed.

Figure 10 showed the typical patterns of corticosteroids produced by rat adrenals incubated with ethanol (control) and MAD. The appearance of augmented ultraviolet-absorbing areas in chromatogram of lipids from incubation media of rat adrenals in the presence of MAD has been mentioned.

TABLE 14

Ultraviolet-absorbing zones in chromatograms of lipid extracts of incubation media of rat adrenals, with and without added androgens.

ADDITION	Rb ZONE							
	0.05-0.1	0.15-0.25	0.3-0.35	0.55-0.6	0.7-0.76	0.8-0.9	1.0	A-Zone
Control	49	50		18	13	13	61	36
MAD	40	14	25	18	26	61	50	21
Methyltestosterone	17		18	21	13	64	34	33
Testosterone	26	36	15	64		35	58	33

A-Zone = material runs with mobility of 11-dehydrocorticosterone.

Figures represent heights of peaks in the corresponding Rb zones.

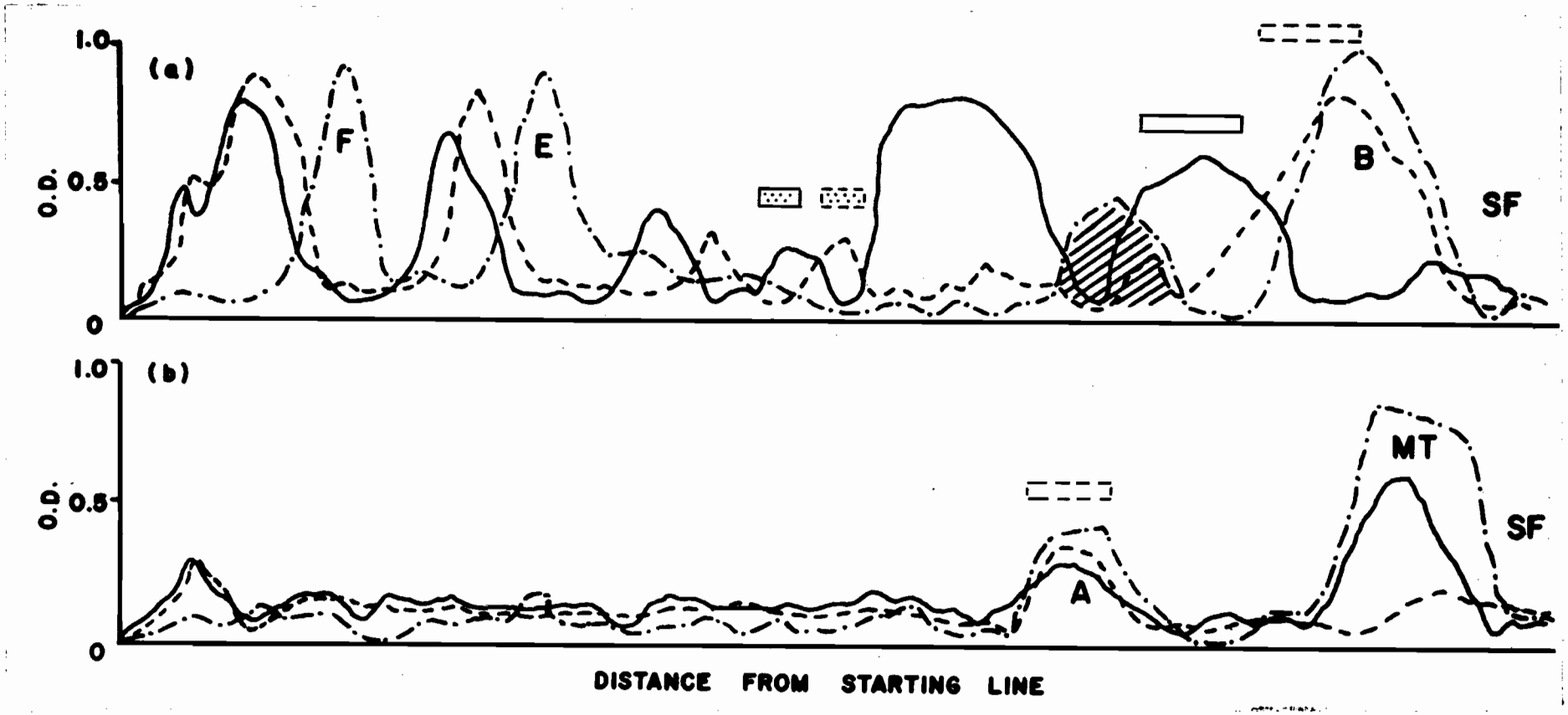


Fig. 10. Ultraviolet-absorbing areas and colour reactions obtained with steroids eluted from paper chromatograms of adrenal lipids developed in toluene-propylene glycol.
(—) Chromatogram of lipids from incubation media of rat adrenals in presence of MAD.
(--) " " " " " " " " " " " " ethanol.
(-.-.) " " reference steroids.
(a) 60-hour run of incubation extracts and reference steroids, cortisol (F), cortisone (E) and corticosterone (B).
(b) 8-hour run of respotted effluents containing incubation extracts and standards dehydrocorticosterone (A) and 17α-methyltestosterone (MT).
open bars - reducing areas
stippled bars - areas giving positive Porter-Silber reaction.
shaded area - impurities from standard dehydrocorticosterone.

No attempts have been made to measure or characterize those which were more polar than 18-hydroxydeoxycorticosterone. The material which ran with an Rb of 0.79 was produced in an amount of 6.9 ± 0.6 µg per 100 mg fresh weight of adrenal tissue. The compound with an Rb between 1.74 and 1.79 was produced in greater quantities, 12 ± 0.7 µg per 100 mg of tissue (Table 15). These measurements were based on ultraviolet absorption.

Table 15 also illustrated that when the production of 18-hydroxydeoxycorticosterone (compound X) and corticosterone (compound B) was measured by ultraviolet absorption, MAD was found to cause a 38% increase in compound X and 22% decrease in compound B. However, when compound X was estimated by the formation of Porter-Silber chromogens and compound B by the reduction of tetrazolium, a decrease of 18% and 45% in dompound X and compound B, respectively, were observed. The percentage increase or decrease in any of the above instances was significant as analysed by the paired t-test.

PART V: Identification of some of the isolated steroids formed by rat adrenals incubated in the presence of 17α-methylandrostenediol (MAD)

Subsequent experiments were performed for the collection of ultraviolet-absorbing lipids located in Rb zones equal to 0.79 or ranging from 1.74 - 1.79 in chromatograms of lipids from incubation media of rat adrenals in the presence of MAD.

TABLE 15

Eluates of certain ultraviolet-absorbing areas in chromatograms of lipid extracts of incubation media of rat adrenals, with and without added MAD.

Expt. No.		1	2	3	4	5	6	Mean ± S.E.
				(µg/100 mg. tissue)				
Total UV Steroids	Control	24.0	25.4	26.0	25.9	20.2	18.7	23.4±1.3
	MAD	53.4	52.6	59.9	57.2	45.1	44.6	52.1±2.5
Rb 0.66 UV	Control	3.9	4.0	3.1	2.9	3.6	2.2	3.3±0.29
	MAD	4.2	4.6	5.4	4.2	4.9	4.0	4.6±0.2
Rb 0.66 P	Control	2.9	3.0	2.6	2.9	3.6	2.9	2.9±0.16
	MAD	2.8	2.7	3.3	1.6	2.2	2.1	2.5±0.24
Rb 0.79	MAD	7.2	6.8	6.8	9.7	5.7	5.6	6.9±0.6
B UV	Control	0	2.4	3.7	3.9	3.8	3.9	3.5±0.27
	MAD	2.5	2.6	2.9	3.4	2.7	2.2	2.8±0.18
B r	Control	0	2.2	3.3	5.1	3.5	4.0	3.6±0.49
	MAD	0.7	2.0	2.0	2.6	1.6	1.8	2.0±0.31
Rb 1.74-1.79 UV	MAD	12.0	12.4	13.4	13.6	10.2	11.1	12.1±0.7

16 rats were used in each experiment. Adrenals incubated with ethanol served as control.

UV - ultraviolet absorption

r - reduction of tetrazolium

P - Porter-Silber reaction

B - Corticosterone

Colour reactions were performed in solution.

1. Zimmermann reaction

Lipids from both zones did not give positive Zimmermann reaction, indicating the absence of a 17-keto group in either of the two compounds.

2. Reduction of tetrazolium

Inability to reduce tetrazolium suggested the absence of an α-ketol group in both structures.

3. Porter-Silber reaction

Both compounds were found to lack a dihydroxyacetone side chain since neither of them gave positive Porter-Silber reaction.

4. Mixed chromatography

The unknown in the less polar region (Rb ranging from 1.74 - 1.79) ran with an Rb identical to that of authentic 17α-methyltestosterone in the toluene-propylene glycol system.

5. Sulphuric acid spectra

The compound in Rb zone 1.74 - 1.79 exhibited a single maximum absorption at 296 - 298 mμ. Authentic 17α-methyltestosterone displayed maximum absorption at 298 mμ. The material in Rb zone 0.79 had absorption maxima at 296, 352, 435 and 495 mμ (Fig. 11).

6. Infrared spectra

The lipid eluted from the less polar region (Rb 1.74 - 1.79) was soluble, and its absorption spectrum was subsequently run, in carbon disulphide. The spectrum contained a band at 3600 cm^{-1} indicating a hydroxyl function, bands at

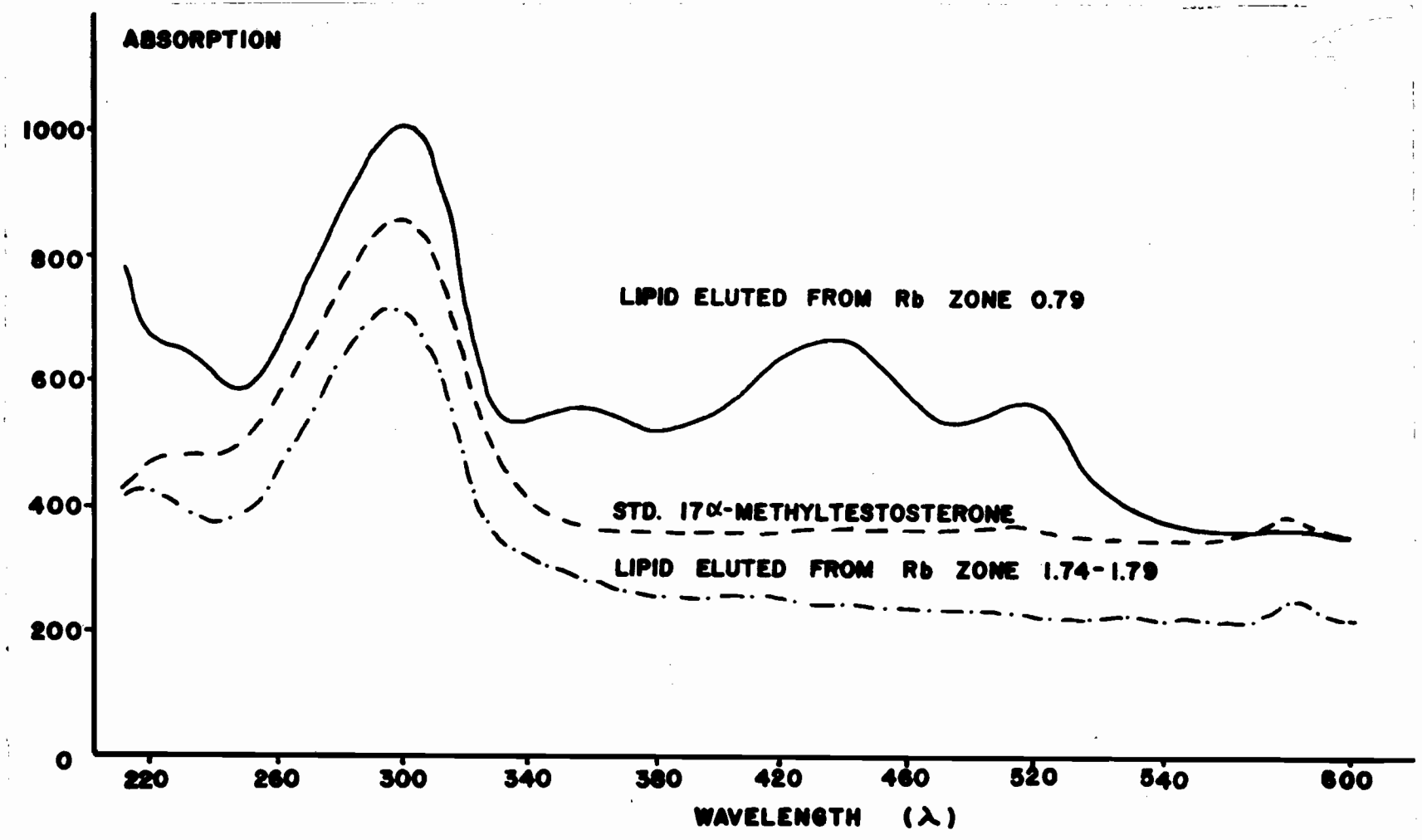


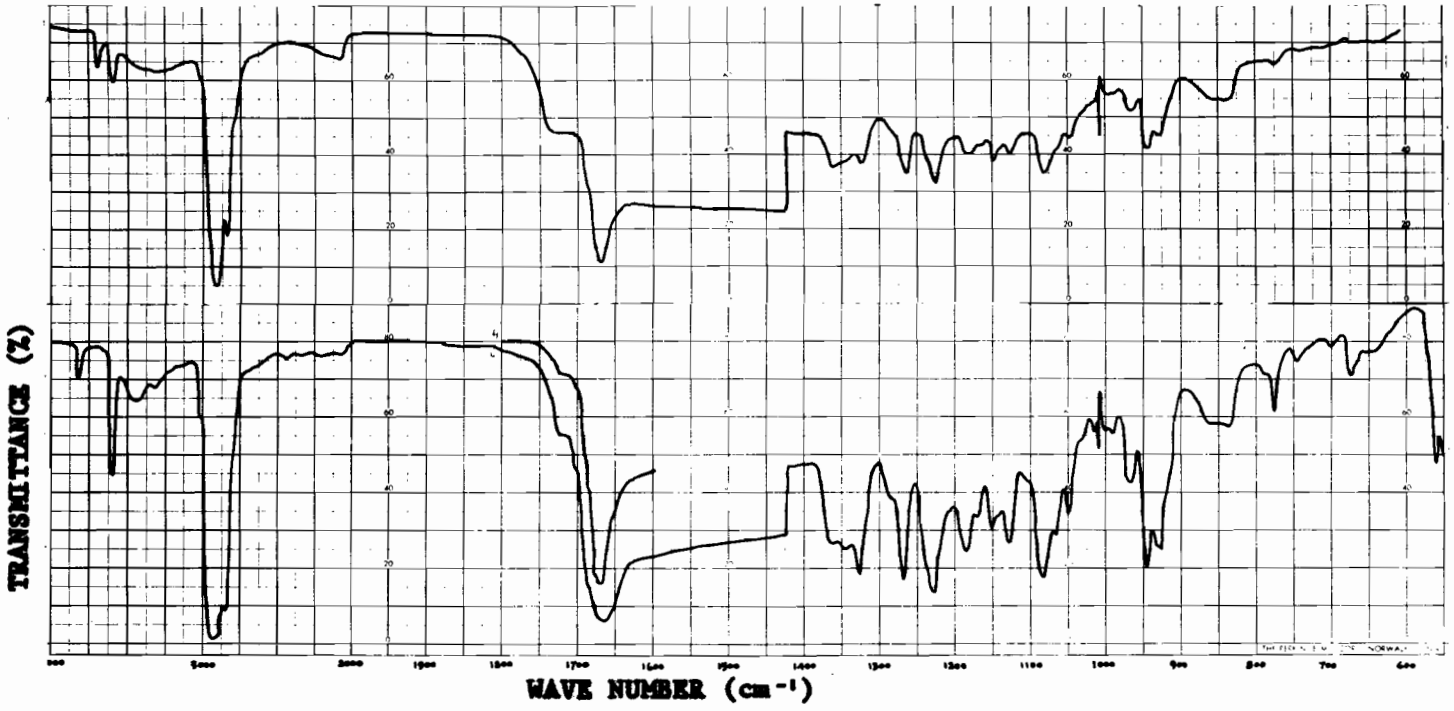
Fig. 11 Sulphuric acid absorption spectra of adrenal lipids eluted from Rb zones 0.79 and 1.74 - 1.79 of toluene-propylene glycol chromatogram and of standard 17α-methyltestosterone.

2925 cm^{-1} (major) and 2850 cm^{-1} (minor) attributable to carbon to hydrogen stretching bands, and a band at 1670 cm^{-1} suggesting the presence of a conjugated ketone group. Bands in the fingerprint region also agreed very well with those observed in the spectrum of authentic 17α-methyltestosterone (Fig. 12).

The infrared spectrum of the lipid isolated from the more polar region (Rb 0.79) was determined in potassium bromide. The absorption bands in this spectrum, especially those of the fingerprint region, were not as sharp and well defined. However, a band at 3400 cm^{-1} , bands at 2920 cm^{-1} (major) and 2850 cm^{-1} (minor), and a band at 1655 cm^{-1} were all noted (Fig. 13).

PART VI: Perfusion study of the isolated adrenal gland

Attempt to perfuse the rat adrenal gland in situ with a suspension of MAD in Krebs-Ringer-bicarbonate-glucose solution was unsuccessful. Probably the suspension was not fine enough to get through the capillaries in the adrenal gland.



Upper figure - eluted lipid

Lower figure - authentic 17α-methyltestosterone

Fig. 12

Infrared spectra of adrenal lipid eluted from Rb zone 1.74 - 1.79 of toluene-propylene glygol chromatogram and of standard 17α-methyltestosterone.

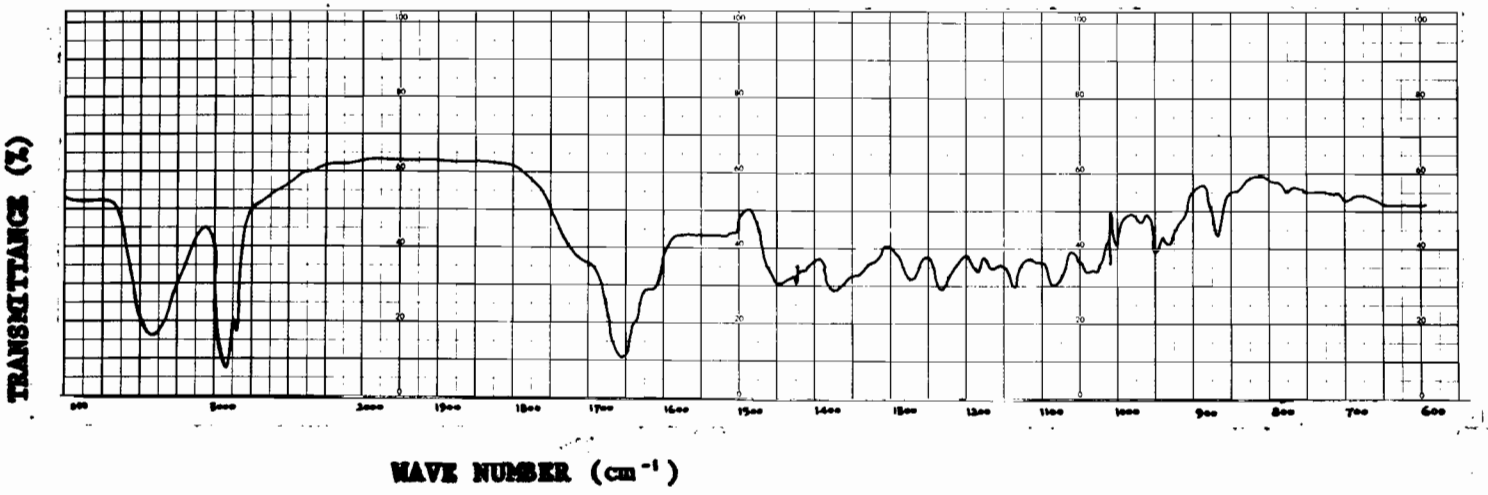


Fig. 13

Infrared spectrum of adrenal lipid eluted from Rb zone 0.79 of toluene-propylene glycol chromatogram.

DISCUSSION

The sparing action of androgens on the adrenal cortex of hypophysectomized or cortisone-treated rats has been well documented. The results of the first part of this study showed general agreement with such previous findings. The original observation, reported by Saffran and Vogt (1), of the suppression of the formation of corticosterone by treatment with 17α-methylandrostenediol (MAD) was also confirmed, using a different strain of rats and a different method for the estimation of corticosterone. In spite of various attempts to avert the action of catabolic glucocorticoids by the use of anabolic steroids supporting metabolism, Winter et al (117), Gaunt et al (118), and others succeeded insofar as to prevent, to a significant degree, adrenal atrophy produced by catabolic glucocorticoids such as cortisone or cortisol. In most of the cases, however, anabolic steroids were found to have no effect on adrenal hypofunction following administration of cortisone. The answer to how androgens brought about their effects on adrenal cortex has long been being searched for. Yet the present state of knowledge could still offer no satisfactory explanations for the mechanism and possible sites of action of those androgens that have been investigated.

That adrenal secretion was repressed by androgens under conditions when ACTH was available to the gland has been

known for some time. The repression of secretion had been observed in man. However, comparatively recent studies of Saffran and Vogt (1), confirmed by the results of this piece of work, demonstrated that similar effect of androgens on adrenal secretion was observed in the rats. The authors postulated that adrenal secretion was probably limited as a consequence of impairment by the androgens of some enzymic process in the cortical tissue. More recent in vitro studies of Dorfman and Sharma showed that Δ^4-androstenedione inhibits 11 β-hydroxylation of 17-hydroxy-11-deoxycorticosterone. It also inhibits the formation of corticosterone by adrenal cortical extract. These findings seemed to give support to the postulation advanced by Saffran and Vogt.

That the suppression by androgens of adrenal secretion could be explained by an inhibition of the release of ACTH is rendered unlikely by Saffran and Vogt. The possibility that MAD could interfere the direct action of ACTH on the adrenal cortex still exists. This was tested by adding MAD in vitro to rat adrenal glands. Incubation was carried out in the presence or absence of ACTH. Results of the second part of this study indicated that MAD neither antagonized nor enhanced the action of ACTH on stimulating the production of corticoids by the adrenal cortex of rats. On the other hand, MAD, added on its own, enhanced the production of Δ^4-3-ketol steroids by the incubated adrenals. The increase was observed to be more than two-fold in one case, and about six-fold in the other.

Saffran and Vogt (1) also pointed out that MAD *in vitro* did not apparently suppress the formation of corticosteroids by the rat adrenals. This was also tested by the same experimental design. The formation of corticosterone was estimated by the fluorometric method of Sweat (186). It was found that whenever concentrated sulphuric acid (3N) was added to an ethanolic solution of steroids containing MAD, invariably an intensive yellow colour developed, suggesting that some chemical reaction had taken place. This yellow colour interfered with the fluorometric readings by yielding high values consistently. No attempt has been made to find out the nature of the reaction or how the colour arises. However, Dr. Just suggested that reactions such as shown in the following would not be unlikely:-

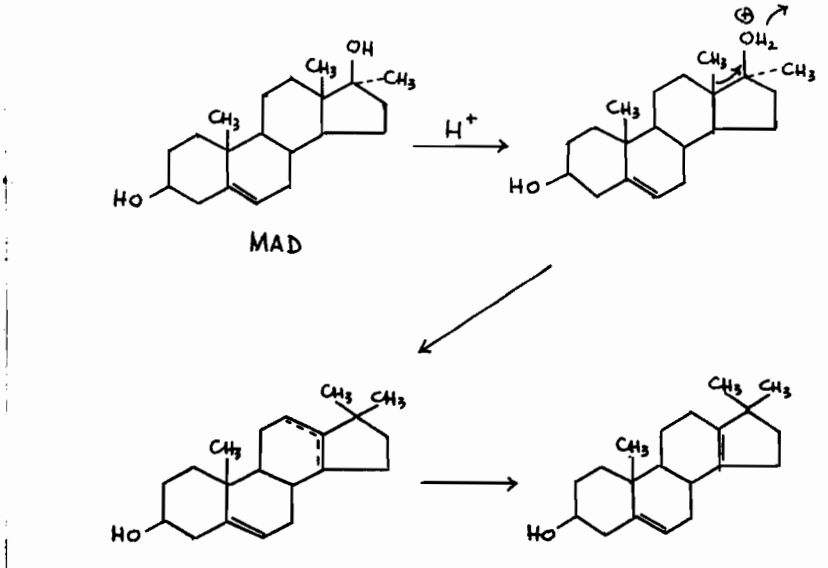


Fig. 14

Whether these reactions are related to the colour change observed is not known.

An attempt to lineate the increments in fluorometric readings with different concentrations of MAD that were present was also unsuccessful. Yet the fluorometric readings might vary with increasing amounts of MAD in a way as predicted in the following diagram:-

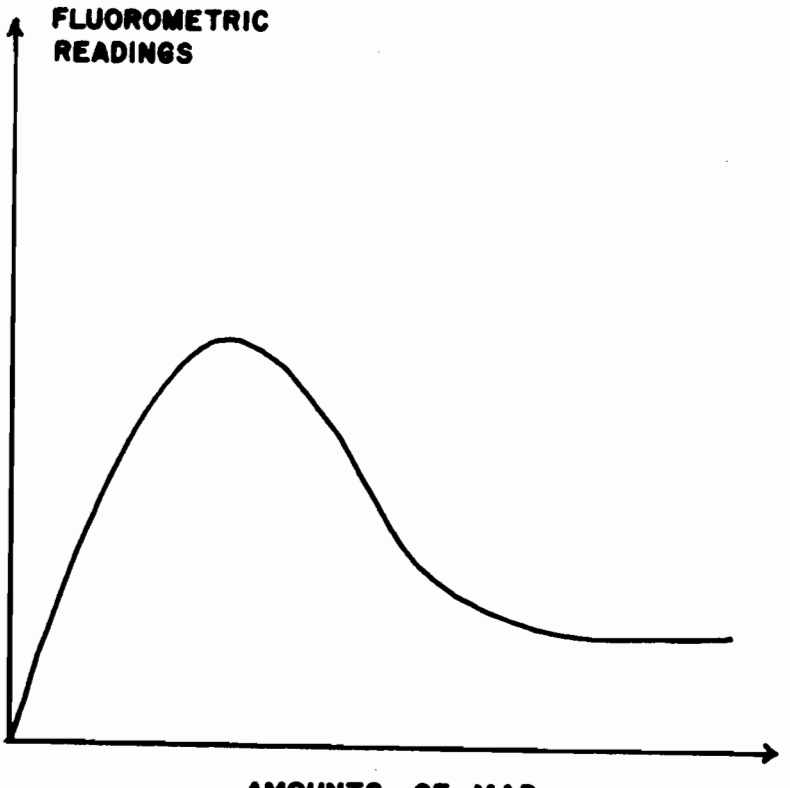


Fig. 15

It is, therefore, concluded that it would be inappropriate to employ the fluorometric method for the estimation of the formation of corticosterone in the presence of MAD.

It has been shown that rat adrenals, when incubated with MAD in Krebs-Ringer-bicarbonate-glucose medium for two hours, gave a two-fold increase in the production of ultra-

violet-absorbing steroids. This could be due to one of the following possibilities:-

1) by exerting an ACTH-like action on the adrenal cortex, MAD brings about its stimulatory effect on the production of corticosteroids; Saffran and Vogt (1), however, demonstrated that MAD does not have an ACTH-like action,
2) MAD itself is metabolized or transformed to Δ^4-3-keto derivatives, thus giving rise to an increase in steroid production by the incubated adrenals.

Subsequent studies on the chromatographic behaviour of various steroids extracted from the incubation media revealed several interesting things. The solvent used was that of Zaffaroni (25). The pattern of adrenocorticoids produced by the rat adrenals treated with ethanol (served as control) or ACTH fell into the classical pattern as first described by Heard et al (24). Incubation with MAD, either in the presence or absence of ACTH, modified the corticoid pattern to a certain extent. The augmented ultraviolet-absorbing zone, more polar than corticosterone, in chromatograms of lipids from incubation media of rat adrenals in the presence of MAD alone, was located near the X region. The steroid represented by this zone, in contrast to that represented by a corresponding ultraviolet-absorbing zone obtained when ACTH was added along with MAD, did not give any positive Porter-Silber reaction. This can be explained by the fact that ACTH stimulates the production of 18-hydroxydeoxycorticosterone by rat adrenals where as MAD acts in the opposite way. The suppression of formation of 18-hydroxydeoxycorticosterone

needs not be complete, since the amount could be reduced to such an extent that it becomes insufficient to give a positive Porter-Silber reaction. Based on this argument, it can be assumed that the so-called "X" region in chromatograms of lipids from incubation media of rat adrenals in the presence of MAD, with or without ACTH, may contain a mixture of at least two components, both of which are ultraviolet absorbing. However, one differs from the other by its ability to give positive Porter-Silber reaction.

Another ultraviolet-absorbing material was detected in chromatograms of steroids produced by rat adrenals in the presence of MAD. The presence of ACTH seems to enhance the production of this material. The density of the peak on the paper chromatograms indicated that this material was produced in amounts greater than corticosterone, suggesting that it could have been derived from the added androgen (MAD). It might be the oxidation product of MAD, namely, 17α-methyl-testosterone. That this oxidation product could be an artefact produced in the course of incubation without tissue, extraction and separation was shown to be unlikely by including an appropriate blank in every experiment. The 17α-methyl-testosterone could be formed from the oxidation of MAD by the 3β-ol dehydrogenase of the adrenal.

It was also found that MAD, in addition to giving rise to two augmented ultraviolet-absorbing areas which were absent in the control, exerted some effect on the metabolism of the more polar adrenocortical steroids. These steroids

were produced in minute quantities. Hence no attempts were made to study the nature of these compounds.

Since 17α-methyltestosterone, testosterone and MAD are structurally related, they may have similar effects on the rat adrenals. In fact, it was observed that incubation in the presence of MAD and 17α-methyltestosterone resulted in consistently less corticosterone formation by the rat adrenals than in the absence of added androgens. Added testosterone did not cause a consistent decrease. The inconsistency in the case of testosterone is difficult to explain in view of the report put out recently by Dorfman and Sharma (177). They found that Δ^4-androstenedione and testosterone can competitively inhibit 11β-hydroxylation, thus bringing about a decrease in corticosterone formation. Testosterone can also inhibit the enzyme 11β-hydroxylase. Since MAD and methyltestosterone have also been shown to cause a decreased production of corticosterone, it is not unlikely that these two androgens bring about their effect by the same or similar mechanism.

It was observed that augmented ultraviolet-absorbing areas, more polar and in amount greater than corticosterone, appeared consistently in chromatograms of incubation extracts in the presence of androgens. Those given rise by MAD have been accounted for. Those that arise due to the addition of 17α-methyltestosterone or testosterone might also be their metabolites as well. It does not, however, rule out the possibility that these three androgens could divert adreno-

cortical steroid synthesis into abnormal pathways whereby resulting in an increase in ultraviolet-absorbing material.

Recently, quantitative studies were carried out in incubation experiments with added MAD. MAD caused a 38% increase in 18-hydroxydeoxycorticosterone as measured by ultraviolet absorption. When 18-hydroxydeoxycorticosterone was estimated by the formation of Porter-Silber chromogens, MAD was shown to give an 18% decrease. One possible explanation for this discrepancy is that MAD *in vitro* might cause the production by the adrenals of a small amount of Δ^4-3-keto steroid which did not form Porter-Silber chromogens but had the same mobility as 18-hydroxydeoxycorticosterone.

The formation of corticosterone by incubated adrenals was definitely suppressed due to the presence of MAD. Estimation based on ultraviolet absorption gave a decrease of 22%. When reduction of tetrazolium was employed for measurement, it revealed a 45% decrease. This could either be due to the presence of some impurities or the production of some abnormal metabolite(s), which absorbed ultraviolet light at 240 mμ but did not reduce tetrazolium.

The inhibition of corticosterone biosynthesis by MAD should not be surprising in view of the findings of Dorfman and Sharma (177). The possible mechanism by which androgens bring about the inhibition has been discussed. The demonstrations of Chang *et al* (144) and Engel *et al* (89) that testosterone can be coverted to 11β-hydroxytestosterone

by human adrenal tissue provide further supporting evidence. It is well known that one step in the biosynthesis of corticosterone involves 11β-hydroxylation of 11—deoxycorticosterone. If androgens can competitively inhibit the enzyme, 11β-hydroxylase, then theoretically one should be able to get some accumulation of 11—deoxycorticosterone. Yet this change might not be detectable owing to the possibility that 11—deoxycorticosterone can be metabolized via other pathways.

The mechanism whereby MAD suppresses the production of 18-hydroxydeoxycorticosterone by the adrenal cortex of the rat is not as obvious. It would not be without reason to speculate that MAD can act in either or both of the two ways:-

1) by blocking the conversion of 11-deoxycorticosterone to 18-hydroxydeoxycorticosterone;
2) by diverting away from preformed 18-hydroxydeoxycorticosterone, giving rise to some more polar steroids.

The first assumption could be a consequence of inhibition of the conversion enzyme by the androgen, whereas the second is based on the detection of a few very polar ultraviolet absorbing areas in chromatograms of incubation extracts in the presence of MAD. Further, there seems to be no concrete evidence for the proof of the current concept that 18-hydroxylation is the terminal step in processes of steroid hormone hydroxylation, especially under such circumstances that C-16 and C-6 hydroxylation might also be possible.

Attempts had been made to characterize and identify

two metabolites of MAD. The material with Rb 1.74 to 1.79 was concluded to be 17α-methyltestosterone, an oxidation product of MAD, on the basis of its practically identical infrared spectrum with the authentic steroid. Other supporting evidence includes ultraviolet absorption at 240 mμ, tetrazolium reduction, Porter-Silber chromogen formation, reactivity toward reagents for the Zimmermann test, mobility in mixed chromatogram and finally, the similarity in sulphuric acid spectra between Rb 1.74 - 1.79 and 17α-methyltestosterone. As to the other metabolite, no conclusion can be drawn upon its identity. It has an Rb equal to 0.79, indicating that it is more polar than corticosterone. This material absorbed ultraviolet light maximally at 240 mμ, suggesting the presence of a Δ^4-3-keto group. If the compound did originate from MAD, then it could possibly be a C-20 steroid. It is also believed to be fairly heavily hydroxylated on basis of its sulphuric acid and infrared spectra.

SUMMARY

Previous findings, reported by Saffran and Vogt, of the effect of 17α-methylandrostenediol (MAD) on the adrenal weight, body weight and the secretory capacity of the adrenal cortex of the rats have been confirmed. Comparable results of its antagonistic effect on cortisol or cortisone have also been demonstrated.

The fluorometric method essentially that given by Guillemin et al (187) for the measurement of corticosterone has been found to be inapplicable for the estimation of corticosterone in the presence of MAD.

MAD does not inhibit or enhance the action of adrenocorticotrophin (ACTH) on the adrenal cortex of rats as measured by the production of ultraviolet-absorbing material by the incubated adrenal tissue.

Evidence has been provided to show that the apparent increase in the production of ultraviolet-absorbing material by the adrenal tissue incubated in the presence of MAD is largely due to the formation of metabolites from the added androgen. Two of these metabolites have been investigated. One of which has been shown to be 17α-methyltestosterone. Supporting evidence was obtained from infrared spectra analysis, study of sulphuric acid spectra, mixed chromatography, tetrazolium reduction, Porter-Silber reaction and Zimmermann test. Infrared and sulphuric acid spectra of the other give

indications that it might be a multi-hydroxylated C-20, Δ^4-3-ketosteroid.

The production of corticosterone and the 18-hydroxydeoxycorticosterone by the rat adrenals is suppressed by MAD added *in vitro*. The inhibition is statistically significant as analysed by the paired t-test.

The effects of MAD, methyltestosterone, and testosterone *in vitro* have been compared. All three androgens inhibited the biosynthesis of corticosterone by the rat adrenals. In the case of testosterone, the suppression was less consistent. It seemed that they all brought about the inhibition by similar, if not the same, mechanism. In addition, augmented ultraviolet-absorbing areas were detected in chromatograms of lipids from incubation media of rat adrenals in the presence of all three androgens. Most of the compounds represented by these areas were found to be more polar than corticosterone.

An attempt has also been made to perfuse the isolated adrenal *in situ* with a suspension of MAD in Krebs-Ringer-bicarbonate-glucose solution. The perfusion was unsuccessful, probably due to the fact that particles of MAD suspended in the perfusate were not fine enough to get through the capillaries of the adrenal gland.

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