THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE REDUCTION OF C_{19} KETO-STEROIDS BY GUINEA PIG TISSUE HOMOGENATES

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degree of

DOCTOR OF PHILOSOPHY

BY

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1957

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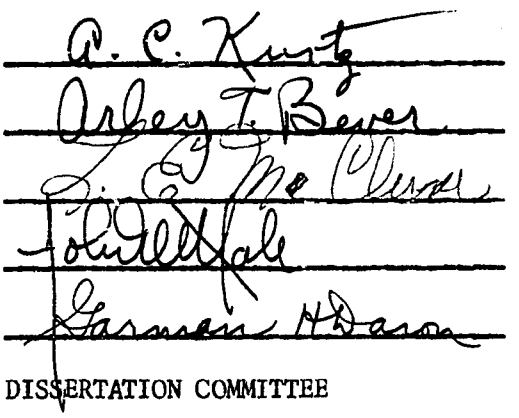

DISSERTATION COMMITTEE

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THE REDUCTION OF C_{19} KETO-STEROIDS BY GUINEA PIG TISSUE HOMOGENATES

CHAPTER I

INTRODUCTION

History

It is just twenty-five years since the existence of several groups of naturally occurring compounds possessing the 1,2-cyclopentenophenanthrene carbon skeleton was first suspected. Chemists, physiologists, and clinicians have shown a great interest in these compounds, and a good deal of research has been done on them in the last twenty-five years.

Prior to 1932 substantial progress had been made toward elucidating the structures of three groups of compounds, the sterols, the bile acids, and the aglycones of the plant heart poisons, which were later to be classified as steroids. The capable researches of Windaus, Wieland and their respective co-workers established the close structural relationship between certain of the sterols and the bile acids.

An x-ray crystallographic examination of ergosterol by Bernal (1) in 1932 stimulated Rosenheim and King (2) to re-examine all the available evidence bearing on the structures of the sterols and bile acids. The

cyclopentenophenanthrene type of formula was the immediate outcome of these researches.

The pioneer work of Allen and Doisy (3) in 1923 on the bioassay of the ovarian estrogenic hormone, and the discovery by Ascheim and Zondek (4), in 1927, of the presence of large amounts of estrogenic material in the urine of pregnant women, encouraged chemists to attempt the isolation of the active principle in a pure state from the latter source. Simultaneously Doisy, et al. (5), Butenandt (6,7), and Dingemanse, et al. (8), in 1929-30, isolated from human pregnancy urine the almost pure crystalline substance, now known as estrone. A few months later, Marrian (9) and Doisy, et al., (10) isolated a second crystalline estrogen, estriol. Butenandt and his co-workers (11) in 1933, proved the estrogens to be phenanthrene derivatives.

Complete proof of the structures of the natural estrogens was achieved by 1934, largely as a result of the brilliant work of Cook and his collaborators (12-14). This work was facilitated by the earlier researches of Girard, et al. (15-18), who had devised simple methods for obtaining pure ketonic estrogens from urine in a high yield by means of their new ketone reagents "T" and "P".

The isolation of estradiol from the liquor folliculi of sow ovaries by Doisy and his co-workers (19,20) in 1935, stands as a landmark to all this early work on the estrogenic substances.

The discovery in 1931, by Kober (21), of a sensitive and highly specific color reaction for the natural estrogens, was a major achievement leading to the development of other color reactions based on similar principles.

The credit for the first total synthesis of a naturally occurring steroid goes to Bachmann and his co-workers (22), who were successful in preparing equilenin in 1940.

Moore, Koch and their collaborators carried out the early work on the androgenic hormone of the testis. McGee (23), a member of this team, was the first to demonstrate clearly the presence of an androgenic principle in extracts of bull testis. However, the first pure crystalline androgen was isolated not from the testis, but from urine. Here again it was Butenandt (24,25) who isolated androsterone from human male urine. Butenandt tentatively advanced a structural formula for androsterone, which was analogous with the probable structure of estrone, which at that time, however, was still unproved. Butenandt's tentative formula was soon proved to be correct in 1934 by Ruzicka and his co-workers (26-28). A second crystalline androgen was isolated from human male urine by Butenandt and Dannenbaum (29) in 1934. It was later proved to be dehydroepiandrosterone. In 1935 the active androgenic principle from testis was isolated in the pure crystalline form in Laqueur's laboratory (30), and named testosterone.

Zimmermann (31,32) facilitated the investigations on the urinary androgens and other related neutral 17-ketosteroids by first suggesting the colorimetric method for their quantitative determination. Many valuable modifications in the Zimmermann method have since been introduced by other workers, and various other methods have also been developed.

Clear cut evidence for the presence in corpus luteum extracts of a principle active in producing progestational changes in the uterus,

was first obtained by Corner and Allen (33) in 1928. Six years later, this active principle was isolated in pure crystalline form simultaneously by four different groups of workers - Butenandt and co-workers (34), Allen and Wintersteiner (35), Slotta and co-workers (36), and Hartmann and Wettstein (37). It is interesting to know that Fraenkel had thirty years previously indicated that the corpus luteum might be an organ of internal secretion. The isolated hormone was named progesterone. Within a few months, its structure was completely elucidated by Butenandt and co-workers, who prepared it from stigmasterol (38), and also from urinary pregnanediol (39). In 1936 Venning and Browne (40) isolated the complex of pregnanediol with glucuronic acid in the form of a crystalline sodium salt from human pregnancy urine, and subsequently a procedure for the quantitative determination of this sodium pregnanediol glucuronidate in human urine was elaborated by Venning (41,42).

The existence of a principle in extracts of the adrenal cortex, active in prolonging the lives of adrenalectomized animals was firmly established during the period 1927-30 by the work of Stewart and Rogoff, of Hartmann and his co-workers and of Swingle and Pfiffner. In 1934 the isolation of an active crystalline preparation from adrenocortical extracts was reported by E. C. Kendall (43), while at the same time Wintersteiner et al (44), reported the isolation of an inactive crystalline substance from the adrenocortical extracts. Kendall and co-workers (45) suggested that the complete physiological activity of adrenocortical extracts might be due to the combined activities of more than one principle, while Wintersteiner and Pfiffner (46) isolated several different crystalline substances from adrenocortical extracts. In 1936, a series of

papers reporting the isolation of a number of different crystalline substances from the adrenal cortex was published by Kendall and his co-workers, Wintersteiner and Pfiffner, and by a newcomer to the field, Reichstein. Soon afterwards Reichstein (47) provided experimental evidence that these substances were steroids.

By 1937 "cortin" activity had been shown to be possessed by four different but closely related compounds, corticosterone, 11-dehydrocorticosterone, 17-hydroxy-11-dehydrocorticosterone, and 17-hydroxycorticosterone. In the next few years, two more compounds, 11-desoxycorticosterone and 17-hydroxy-11-desoxycorticosterone, were added to the list of those possessing "cortin" activity. By 1942 about twenty-eight different steroids had been isolated from adrenal extracts, and the constitutions of most of them had been fully elucidated. Reichstein and his co-workers not only provided us with an extensive knowledge of the adrenocorticoids, but also developed excellent methods for the small-scale separation of pure steroids from mixtures. In 1953-54 Reichstein and his co-workers (48,49) isolated and synthesized aldosterone, the most potent mineralocorticoid in nature.

In the past decade a considerable amount of work has been carried out on the isolation of new urinary steroids, and on the metabolism of the steroid hormones (50-53). Although much has been learned about the metabolism of androgens, estrogens, progesterone, and adrenocorticoids, much more remains to be discovered. It seems likely that many interesting metabolic problems may be solved with the aid of hormones labelled with isotopic carbon.

In the investigations to be described in this dissertation, experiments were designed to observe the influence of pH on the in vitro metabolism of androstanedione and androstenedione by guinea pig tissue homogenates, and also to study the metabolism of androstenedione with different cofactors.

Nomenclature

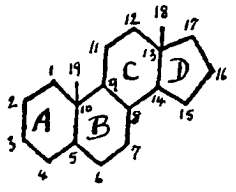
Since the investigations to be described in this dissertation are concerned only with androgens, only such nomenclature as is pertinent is cited (54,55). Androgens are derivatives of cyclopentanoperhydrophenanthrene. The system of designating individual rings and of numbering positions on this nucleus is represented in formula I (Table I). The formulas II and III represent androstane and etiocholane nuclei respectively. Steroid molecules are essentially planar. The symbol α is used to denote bonds which project behind the plane of the steroid rings, and the symbol β to denote those which project in front of the plane of the rings. α configurations are denoted by broken lines; whereas, β configurations are represented by solid lines. The methyl groups at positions 10 and 13 are assumed to have a β configuration. The fusion of rings A and B is trans in androstane series while it is cis in etiocholane series. The fusion of rings B and C is trans in all steroid hydrocarbons. Similarly, the fusion of rings C and D is also trans in all steroid compounds. The name androstane is preferred when carbon 5 is involved in a double bond.

The In Vitro Metabolism of Steroids

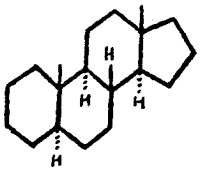
TABLE I

NOMENCLATURE OF ANDROGENS

I

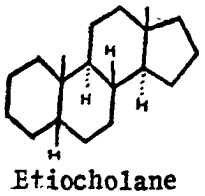


II



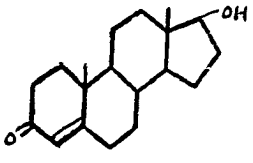
Androstane

III



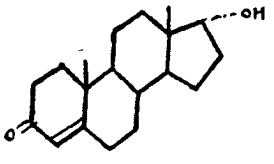
Etiocholane

IV



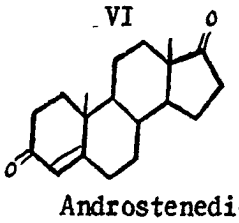
Testosterone
4-Androsten-17 -ol-3-one

V



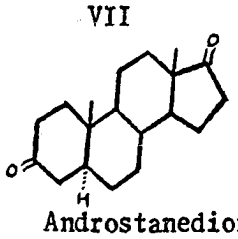
Epitestosterone
4-Androsten-17 -ol-3-one

VI



Androstenedione
4-Androstene-3,17-dione

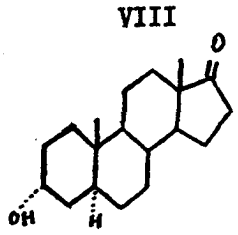
VII



Androstanedione
Androstane-3,17-dione

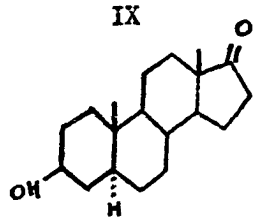
TABLE I - Continued

VIII



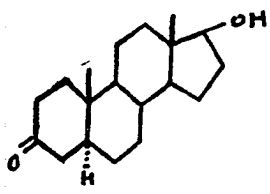
Androsterone
Androstan-3α-ol-17-one

IX



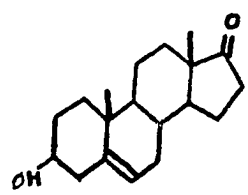
Epiandrosterone
Androstan-3β-ol-17-one

X



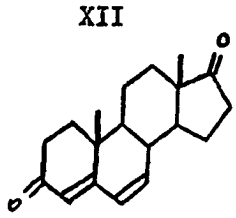
Androstanolone
Androstan-17β-ol-3-one

XI



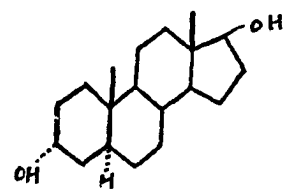
Dehydroepiandrosterone
5-Androsten-3β-ol-17-one

XII



4,6-Androstadiene-3,17-dione

XIII



Androstanediol
Androstane-3α,17β-diol

Since the investigations described in this dissertation involve the reductions of androgens, an attempt is made in this chapter to review the in vitro metabolism of androgens, and the reductions of steroids other than androgens. The in vitro metabolism of steroids has been studied in two ways: perfusion studies developed by Hechter, Pincus, and their co-workers (56-60), and the incubation and assay methods developed by Clark and Kochakian (61,62), and Samuels and his co-workers (63,64).

Perfusion studies, although of use with any steroid, have been used especially in the field of adrenocorticoids. The steroids studied are added to blood which is circulated through intact organs, and the effluent blood is processed to recover the added steroid and its metabolites, which are identified by paper chromatographic techniques. Incubation studies have served as the basis for the elaboration of the metabolic pathways of the androgens. Kochakian and his co-workers (65-71) have studied the in vitro metabolism of many androgenic compounds by these methods, which are discussed in Chapter II.

Androgens. In vitro studies by Clark and Kochakian (62) showed that incubation of testosterone with rabbit liver slices produced 4-androstene-3,17-dione and epitestosterone. The incubation of 4-androstene-3,17-dione with rabbit liver slices under the same conditions led to the formation of testosterone as well as epitestosterone (65). Gongora and Kochakian (72) have demonstrated the conversion of testosterone to 4-androstene-3,17-dione and epitestosterone by rabbit liver homogenates. The incubation of 4-androstene-3,17-dione with guinea pig liver and kidney brei yielded testosterone and androsterone. Testosterone incubated with guinea pig liver homogenate was oxidized to 4-androstene-3,17-dione. The

presence of diphosphopyridine nucleotide increased the yield of product (68).

Incubation of testosterone with the homogenate of liver from female rats resulted in the formation of some 17β-hydroxyandrostan-3-one (73). This steroid had been previously postulated as a possible intermediate in the metabolism of testosterone. C^{14}-labelled estradiol-17 has been isolated by Baggett and co-workers (74) following the incubation of testosterone-3-C^{14} with human ovarian tissue. The formation of 19-hydroxytestosterone as an intermediate product has been suggested. Axelrod and his co-workers (75) perfused testosterone through an isolated male dog liver with heparinized blood as the perfusion fluid. They identified 16α-hydroxytestosterone, 6α-hydroxytestosterone, 2β-hydroxytestosterone, epitestosterone, 4-androstene-3,17-dione, and 6β-hydroxy-4-androstene-3,17-dione as metabolites, using the techniques of paper chromatography. This is the first report of β-hydroxylation at C_2 by a mammalian tissue. Meyer and his co-workers (76) hydroxylated androstenedione by bovine adrenal homogenate preparations. They isolated adrenosterone, 11β-hydroxyandrostenedione, 6β-hydroxyandrostenedione, and 6α, 11β-dihydroxyandrostenedione as metabolites. The conversion of 19 hydroxy-4-androstene-3,17-dione to estrone by human placental tissue slices, cow ovarian follicular fluid, and also by cow adrenals has been reported (77). This report also suggests that the bioconversion of androgens to estrogens proceeds via a C_{19} hydroxylated intermediate.

Schneider and Mason (78) incubated sodium dehydroepiandrosterone hemisuccinate with rabbit liver slices for three to six hours under aerobic conditions and demonstrated the formation of 5-androstene-3β,

17β-diol and a small amount of 5-androstene-3β,16α,17β-triol. The conversion of dehydroepiandrosterone to 5-androstene-3β,17β-diol has been accomplished by liver perfusion and by rabbit liver homogenates (79). Meyer and his co-workers (76) incubated dehydroepiandrosterone with bovine adrenal homogenate preparations, and obtained 4-androstene-3,17-dione, adrenosterone, and 11β-hydroxyandrostenedione as the metabolites.

The incubation of androsterone as the sodium salt of the hemisuccinate with rabbit liver slices for six hours aerobically, resulted in the formation of three steroids, epiandrosterone, androstane-3,17-dione, and androstane-3α,17β-diol (80,81).

The incubation of etiocholan-3α-ol-17-one with rabbit liver slices led to the formation of etiocholane-3,17-dione, etiocholane-3α, 17β-diol, and etiocholane-3α,17α-diol (80,81). Kochakian and his co-workers (71) have recently reported the comparisons of the oxidations of C_{19} hydroxysteroids by guinea pig liver homogenates.

Incubations of androstane-3α,17β-diol with guinea pig liver and kidney from guinea pigs and rabbits have resulted in the formation of androsterone and androstane-3,17-dione (67). Ungar and his co-workers (82) incubated 4-androstene-3β,17β-diol and 4-androstene-3α,17β-diol with rat and chick liver preparations at pH 7.4, using DPN as the co-enzyme. The formation of products followed a sequence consisting of testosterone, 4-androstene-3,17-dione, and epitestosterone. Only trace amounts of ring A reduced ketosteroids were formed. The nature of the reaction indicated that the Δ^4 diols would not be intermediates in the reductive pathway of testosterone catabolism.

Samuels and his co-workers (83) have used citrate as the co-factor for the reduction of C-3 ketone in A ring.

Estrogens. Estrone incubated with mixtures of guinea pig erythrocytes, TPN, and glucose-6-phosphate was converted completely to 17β-estradiol (84). Neither 17α nor any other estradiol appeared.

Progesterone. Taylor reduced progesterone by rabbit liver (85) and rat liver (86) homogenates in the presence of nicotinamide, DPN, and citrate as cofactors. It was suggested that the first stage in the enzymatic reduction of progesterone in liver involves nonstereospecific addition of 4H atoms in ring A to form hydroxyketones, and that the allopregnanedione was formed by subsequent reoxidation mainly of allopregnan-3α-ol-20-one. Slaunwhite and Samuels (87) have reported the in vitro conversion of progesterone to androgens. Incubation of isotopically labelled progesterone with homogenates or slices of testes from hypophysectomized or immature rats injected with chorionic gonadotropin led to the production of 17α-hydroxyprogesterone, 4-androstene-3, 17-dione, and testosterone.

Adrenal corticoids. Glenn and Recknagel (88) have described an enzyme system in rat liver homogenates which catalyses the degradation of the 17,21-dihydroxy-20-ketone side chain of adrenocortical steroids. It requires added nicotinamide, TPNH, anaerobiosis, and isotonic conditions for optimum activity. They also suggested that the C_{21} steroids of the progesterone and adrenocortical steroid groups shared a common pathway so far as the reduction of the C-20 ketone was concerned. The same group of investigators reduced C-20 ketone of adrenocorticoids to C-20 OH by rat liver microsomes (89). Tomkins and Isselbacher (90) have

reported an enzyme system in rat liver which catalyzes the reduction of cortisone to tetrahydrocortisone. TPNH acts as H donor. The reaction can be coupled to the oxidation of d-isocitrate by TPN and isocitric dehydrogenase, or to the oxidation of glucose-6-phosphate by TPN with glucose-6-phosphate dehydrogenase. The reaction proceeds faster under nitrogen. Forchielli and his co-workers (91) incubated 11-deoxycortisol with supernatant fluid from rat liver homogenate. In addition to enzymes capable of reducing the ring A double bond, the C-3 ketone and the C-20 ketone, the presence of enzymes capable of oxidative removal of the α-ketol side chain has been demonstrated. Reaven (92) has reported the reduction of Δ^4 -3 keto group of aldosterone, corticosterone, desoxycorticosterone, and hydrocortisone by human and rat liver slices. The reduction of the C-20 carbonyl group of tetrahydrocortisone and 17-hydroxypregnanolone by a fractionated rat liver homogenate in the presence of DPN, nicotinamide, and Na fumarate, at pH 7.4, has been reported by de Courcy and Schneider (93).

Ganis and his co-workers (94) incubated hydrocortisone with bovine kidney tissue at pH 7.4, and identified 4-pregnene-11β,17α, 20β, 21-tetrol-3-one, 4-pregnene-17α,21-diol-3,11,20-trione, 4-androstene-11β-ol-3,17-dione, and 4-androstene-3,11,17-trione as the metabolites. Grant (95) incubated desoxycorticosterone with a soluble enzyme extracted from acetone-dried ox adrenal mitochondria in the presence of fumarate and TPN, and reported the formation of corticosterone and 6β-hydroxy-11-desoxycorticosterone. The role of fumarate in the 11β-hydroxylation was shown to be related to the production of TPNH. The following reactions were suggested:

Fumarate ⟶ L--Malate

L-Malate + TPN^+ ⟶ Pyruvate + CO_2 + TPNH + H^+

TPNH and molecular oxygen are the only substances required for the 6-β and 11-β-hydroxylation of desoxycorticosterone. Sweat and Lipscomb (96) have also presented evidence that TPNH is a cofactor for 11-β-hydroxylation, and suggest that it is formed by the following sequence of reactions:

Fumarate ⟶ Malate

L-Malate + DPN^+ ⟶ Oxaloacetate + DPNH + H^+

DPNH + TPN^+ ⟶ DPN + TPNH

Dorfman (97) has written an exhaustive review article on adrenocortical steroid metabolism, which covers the ring A reduction of Δ^4-3-ketones, the conversion of 17α-hydroxy C_{21} steroids to 17-desoxy C_{21} steroids, the <u>in vitro</u> conversion of C_{21} to C_{19} steroids and new cortisol metabolites. Tomkins (98) has reported the enzymatic reduction of a variety of α,β-unsaturated 3-ketosteroids to saturated 3-ketosteroids by partially purified rat liver extracts. Reduced pyridine nucleotides function as hydrogen donors.

From the above reports it may be concluded that the enzyme systems in liver and kidney are capable of reducing reversibly the C-3, C-17 and C-20 ketones and also the double bond in the A ring. They also catalyze the oxidative removal of α-ketol side chain of adrenocorticoids. The dog liver has enzyme systems which can hydroxylate C-2, C-6 and C-16. Among the rodents, only rabbit tissues have enzyme systems which can produce epitestosterone. The enzymes of the adrenal tissue can reduce the C-20 ketone and the A ring completely, can β-hydroxylate C_6 and C_{11},

and can hydroxylate C_{17} also. Only adrenal tissue has an enzyme system responsible for 11β-hydroxylation. All these enzyme systems are not only position specific, but also stereoisomerically specific.

It is interesting that the reduction of the double bond in the A ring of C_{19} steroids leads to the formation of androstane derivatives only.

Of significance is the fact that the results of the *in vivo* and *in vitro* experiments complement each other and specifically indicate the reversibility of the steroid metabolic reactions, except for a few differences in the C_{21} series (52,53). The *in vivo* metabolism of C_{21} compounds gives rise to reduced steroids primarily of the pregnane series. The *in vitro* metabolic studies of C_{21} steroids yield mainly allopregnane derivatives. The *in vitro* studies with C_{21} steroids indicate 6β-hydroxylation of these compounds, while 6-hydroxylated steroids isolated from the human urine have 6α-hydroxy configuration. By far, the bulk of *in vivo* reduction studies in man have yielded 20α-hydroxyl groups from the corresponding 20-ketones. The *in vitro* studies with C_{21} steroids have yielded mainly 20β-hydroxy groups from C-20 ketones. All these differences observed between *in vivo* and *in vitro* studies might be due simply to species specific reactions. Most of the *in vitro* studies have been done with bovine or rodent tissues, while the *in vivo* studies have beendone mostly in human subjects.

Because of the comparable results of the *in vivo* and *in vitro* studies with most of the steroids, at least the chemistry involved can be assigned with some certainty. There remain to be studied many more possible steroid metabolic reactions, and the enzyme systems responsible

for these changes. On the latter score, a start has already been made, and many reports have appeared in the last few years.

CHAPTER II

EXPERIMENTAL PROCEDURES

The procedures employed in these studies included preincubation preparation, preparation of tissue homogenates, incubation procedure, separation of tissue components from the steroidal materials, isolation and identification of the steroidal compounds, separation of individual steroids from their mixtures, steroidal assay, and the characterization of metabolites.

Preincubation preparation. The incubation materials consisted of buffer solutions, the steroids to be studied, and the various additives (Table II).

Two-tenths molar disodium phosphate was the buffer solution routinely employed as the incubation medium. Fifty ml. of this buffer solution were used. The buffer solution was ultimately diluted to 0.1 molar strength by the addition of 50 ml. of the aqueous solution of DPNH or TPNH. One hundred ml. of 0.166 molar sodium pyrophosphate solution were used in a few experiments run at pH 9. The buffer solutions were adjusted to the desired pH values by the addition of concentrated hydrochloric acid. A Beckman Glass Electrode pH Meter was used for the determination of pH values.

DPNH or TPNH solution was prepared by the reduction of DPN or

TABLE II

Additives used	Abbreviations
Diphosphopyridine nucleotide (99% pure) (Pabst Laboratories. Wisconsin.)	DPN
Reduced form of diphosphopyridine nucleotide (more than 90% pure) (Prepared in the laboratory)	DPNH
Triphosphopyridine nucleotide (92% pure) (Pabst Laboratories. Wisconsin.)	TPN
Reduced form of triphosphopyridine nucleotide (90% pure) (Prepared in the laboratory)	TPNH
Nicotinamide (U.S.P.) (General Biochemical Inc. Ohio.)	NA
Trisodium salt of isocitric acid (C.P.) (H. M. Chemical Co. Ltd. California.)	Na_3isocitrate
Disodium salt of adenosine triphosphate (Sigma Chemical Co. Missouri.)	$Na_2ATP.3H_2O$
Disodium salt of adenosine triphosphate (Pabst Laboratories. Wisconsin.)	$Na_2ATP.4H_2O$
Disodium salt of fumaric acid (C.P.) (E. H. Sargent & Co.)	Na_2fumarate
Magnesium chloride (C.P.) (J. T. Baker & Co.)	$MgCl_2.6H_2O$
Glucose (Anhydrous, C. P.) (Merck & Co. N. J.)	-

TPN by the modified method of Green and Dewan (99). The modification is discussed by Hamm (100). Nitrogen was flushed through an aqueous solution containing DPN or TPN (0.13%) and sodium bicarbonate (1.0%) for fifteen minutes. A freshly prepared solution of 0.4% sodium hydrosulfite was then added and the passage of nitrogen was continued for about an hour more. The whole reaction was carried out at 38° C. Finally the excess hydrosulfite was removed by aeration. The reduced coenzyme concentrations were determined spectrophotometrically using a Beckman DU Spectrophotometer; this instrument was employed for all the spectrophotometric assays. The extinction coefficient of the reduced coenzyme is 6.22×10^6 (101).

The steroids used in the experiments were purified prior to incubation by alumina adsorption chromatography. Thereafter, the identity of each steroid was confirmed by means of paper chromatography and infrared spectrophotometry. Then, the compounds were crystallized repeatedly from appropriate solvent systems until their melting points agreed with those reported in the literature.

Preparation of tissue homogenates. Adult male guinea pigs were employed in all the experiments. Upon arrival, the weight of the animals was usually between 500-600 grams. The animals were employed in metabolism studies, when their body weight reached 800-1300 grams. The animals were housed in a constant temperature room ($72^{\circ} \pm 1^{\circ}$ F.), in which the lighting was automatically regulated. The animals were fed ad libitum commercial rabbit pellets (Superior Feed Company). In addition, 30-40 grams of carrot and a 25 mg. ascorbic acid tablet were given twice a week. The animals were sacrificed by a blow at the base of the skull,

and then were bled by cutting the throat and severing the jugular vein. The animals were shaved in the abdominal areas in which incisions were to be made, and these areas were thoroughly rinsed with 70% ethyl alcohol in order to avoid bacterial contamination. The desired tissues were removed, weighed, and stored in ice-cold beakers. Usually more than one animal was required, and hence the tissues were chopped into very small pieces with scissors and mixed intimately to minimize the effects of individual animal variation. Twelve grams of tissue were weighed and then transferred to the ice-cold jars of a Waring blendor. Four hundred mg. of nicotinamide (800 mg. in the earlier experiments) and 40 ml. of cold buffer solution were added to the tissue in the jar, and the mixture was homogenized for twenty seconds. The homogenate was transferred to a Fernbach flask of 3 liter capacity by rinsing with the remaining buffer solution. Steroid and additives were then introduced into the flask and the incubation was started.

Incubation procedure. A specially designed water bath was employed for the incubations; this apparatus and the procedure employed have been described by Nall (102). The incubation was carried out at 37-8° C. for ninety minutes. In anaerobic experiments, the tissue was homogenized in an atmosphere of nitrogen, the incubation flasks were flushed with nitrogen for thirty minutes prior to the introduction of the homogenates, and then throughout the incubation, maintaining the flow rate at 3 liters nitrogen per minute per flask. At the conclusion of the incubation the final pH values of the mixtures were determined, and the reactions were terminated by the addition of 700 ml. of redistilled ethyl alcohol which precipitated proteins.

Separation of tissue components. Separation of tissue components was carried out by employing the method described by Nall (102). The alcoholic solutions of the incubation mixtures were heated to boiling and filtered while hot on suction using celite powder as a filter aid. The protein residues were washed several times with hot ethyl alcohol to free them completely of the steroid. The alcoholic filtrates were distilled in vacuo to get rid of alcohol, and the residual aqueous extracts were extracted four times with 200 ml. portions of peroxide-free ethyl ether. The ether extracts were washed with distilled water and then combined and the ether evaporated. The aqueous residues were then transferred to centrifuge bottles and dried overnight by aeration.

The phospholipids were precipitated by using the method developed by Hamm (100). The dried material in the centrifuge bottle was dissolved in a mixture of 1 ml. distilled water and 10 ml. ethyl ether. The phospholipids were precipitated by adding 180 ml. acetone to the mixture with constant stirring. The mixture was centrifuged at 1800 r.p.m. and the supernatant acetone fraction was separated by decantation. The residue was redissolved in the mixture of 1 ml. water and 10 ml. ether and the phospholipids were reprecipitated by the addition of acetone. The whole procedure was repeated twice. The acetone fractions were then combined and evaporated to dryness.

Isolation of steroidal materials. The procedure of Hamm (100) was employed to isolate the steroid substrate and the different metabolites from the dried acetone fractions. Alumina chromatography was employed in the manner developed by Nall (102). The alumina (Harshaw, activated catalyst grade, Al-0109P) was activated prior to its use in the

columns. It was washed with distilled water, adjusted to pH 7.3-7.4 with sulfuric acid, rinsed with distilled water until free of sulfate ion, dried, rinsed with methyl alcohol, redried, and finally heated at 110° C. for three hours. Fifteen to thirty grams of alumina were used for each column, the appropriate amount being determined by the amount of steroid used in the experiment and the weight of the residue which was to be chromatographed. Glass columns with internal diameters of 20 mm. were usually used. The residues were dissolved in minimal volumes of redistilled carbon tetrachloride and introduced into the columns. Steroids were then eluted using a solvent sequence of redistilled carbon tetrachloride, redistilled benzene, benzene mixed with ethyl ether, ethyl ether, ethyl ether mixed with absolute ethyl alcohol, and methyl alcohol (Table III).

Identification of steroidal materials. The fractions from the alumina column were identified by using the paper chromatographic method developed by Kochakian and Stidworthy (103). A 6 inch by 22 inch strip of Whatman number 1 filter paper was dipped in a 1:1 mixture of methanol and propylene glycol, the excess of the mixture on the strip was removed by blotting between paper towels, and then the even distribution of the solvent on the paper strip was insured by passing it through a wringer. The fractions from the alumina column were weighed and dissolved in a suitable solvent, usually dichloromethane, and aliquots containing 0.5 mg. of the solid dry material from each fraction were put on the paper in the form of spots an inch apart. These spots were placed on a straight line 3 inches from the top edge of the paper. A mixture of known steroid compounds (125 µg. of each steroid) was placed on the middle spot. The

TABLE III

Solvent	Grade	Source
Benzene	Thiophene free 99 Mol% minimum	Phillips Petroleum Co. Oklahoma.
Cyclohexane	Pure grade 99 Mol% minimum	Phillips Petroleum Co. Oklahoma.
Carbon tetra-chloride	Analyzed Reagent	J. T. Baker Chemical Co. New Jersey.
Ether	Purified Anhydrous	J. T. Baker Chemical Co. New Jersey.
Ether	Solvent	J. T. Baker Chemical Co. New Jersey.
Methanol	Analyzed Reagent	J. T. Baker Chemical Co. New Jersey.
Acetone	Analyzed Reagent	J. T. Baker Chemical Co. New Jersey.
Chloroform	Analyzed Reagent	J. T. Baker Chemical Co. New Jersey.
Dichloromethane	Analyzed Reagent	J. T. Baker Chemical Co. New Jersey.
Absolute alcohol	100% pure ethyl alcohol, U.S.P.	U. S. Industrial Chemical Co. New York.
Propylene glycol	For laboratory use	The Matheson Co., Inc. New Jersey.

papers were equilibrated in a glass tank containing 1:1 mixture of benzene and cyclohexane for 3 hours, and then the chromatograms were developed by running the same mixture on the papers for 5 hours. The chromatograms were then dried in an oven at 110° C., for 1 hour. Testosterone and androstenedione were detected both by the ultraviolet scanner (104) and by the modified Zimmermann color reaction (31,32,105) which utilizes the spray reagent consisting of a mixture of equal volumes of 15% alcoholic potassium hydroxide and 2% alcoholic m-dinitrobenzene. The color development was facilitated by heating at 110° C. for 3-5 minutes after the spray. Color development using the spray reagent consisting of a solution of 2,4-dinitrophenylhydrazine in acidulated alcohol was also used. Androstanedione, androsterone, epiandrosterone, and androstanolone were detected by the modified Zimmermann color reaction (31,32,105) and also by the 2,4-dinitrophenylhydrazine reaction.

The distance moved by the steroidal compounds on the paper chromatogram is also a valuable tool for the identification of these compounds. Each steroid has a characteristic Rt value which has been defined (103) as the ratio of the movement of steroid and the movement of testosterone in the same period of time.

Separation of individual steroids from their mixtures. Each fraction from the alumina column did not necessarily consist of only one steroid. At times there was a fraction which had more than one steroid. The individual steroids from this mixture were separated by paper strip chromatography (69). The mixture was dissolved in the minimum amount of a suitable solvent, usually dichloromethane, and the solution was quantitatively transferred to the paper processed previously as described in

the above section. The steroidal solution was put on the paper in the form of a strip 3 inches from the top edge of the paper. The chromatogram was developed as described above. Then it was heated in the oven at 110° C. for about an hour. The steroid zones were then detected by means of the ultraviolet scanner and the color reactions which were described in the previous section. The color reactions were applied to the narrow strips cut from the edges of the paper. The steroid zones were cut out from the paper chromatograms and were extracted three-four times with hot ethyl alcohol.

Assay of steroidal compounds. Compounds containing an α, β-unsaturated ketone, namely androstenedione, and testosterone, were assayed spectrophotometrically at 241 mu (106). Androstanedione, androsterone, epiandrosterone, and androstan-17β-01-3-one were assayed by a modification (107) of the Zimmermann reaction.

Characterization of metabolites. The metabolites formed were characterized by paper chromatography, infrared spectrophotometry (108-115) and the determination of melting points (116,117) of the pure compounds and of their oxime or acetate derivatives.

Control experiments. Control experiments with heat inactivated tissue were frequently performed, in a manner similar to the regular experiments.

Pilot columns. Pilot chromatography columns were run along with the regular alumina columns during the experiments in order to ascertain the purity of the steroid used in the experiments. The same amount of steroid as was used in each experiment, was put on an alumina column which was built and run in a similar way to the regular alumina

column run for each experiment. The sequence of solvents put on the pilot column was the same as that put on the regular columns.

CHAPTER III

THE INFLUENCE OF pH ON THE METABOLISM OF ANDROSTANEDIONE BY GUINEA PIG LIVER HOMOGENATES

It has been postulated that the urinary androsterone and epiandrosterone in man are the metabolites of androstanedione (118). The conversion of androstanedione to androsterone has been confirmed by Gallagher and his co-workers (119), by administering deuterium labelled androstanedione and recovering 24% of the deuterium in urinary androsterone. Androsterone is the principal urinary androstane derivative in man (120), whereas epiandrosterone is the main one in guinea pig (121).

Experiments were designed to study the influence of pH on the *in vitro* metabolism of androstanedione. Guinea pig liver homogenates were used as the source of enzyme system(s). The experiments were run in the pH range 5.5-7.5, with 0.2 M disodium phosphate as the buffer medium. The use of DPN and TPN as the hydrogen acceptors, and of DPNH and TPNH as the hydrogen donors, has been well established. Since reductions were involved in our experiments, DPNH or TPNH in the form of aqueous solution was used as the coenzyme. The presence of nucleotidases, the enzymes causing the inactivation of pyridine nucleotides in animal tissues, has been described by several investigators (122,123). It is also demonstrated that NA inhibits (122-124) these nucleotidases, and is involved in the

exchange reaction with the DPN molecule (124). In the experiments undertaken, DPNH or TPNH was used to donate hydrogen to the androstanedione molecule, and be oxidized to DPN or TPN. Nicotinamide was used in order to protect DPN or TPN at this stage, so that it could couple with the appropriate enzyme systems in the liver homogenate, be reduced, and thus be available again for hydrogen donation.

Androsterone, epiandrosterone, and androstan-17β-ol-3-one were identified as the metabolites. In all the experiments, the formation of androstanolone was extremely low. In experiments 1-6 (Table IV), run with DPNH, the quantities of epiandrosterone formed were a little more than those of androsterone, both giving a peak at pH 6.5 (Figure 1). In these experiments, the quantities of androstanolone formed were so low that it was not worth while to plot a curve with them. The change in the gaseous phase at pH 6 (Experiments 2 & 3) did not alter the formation of androsterone or androstanolone, but cut down the production of epiandrosterone to half.

In experiments 7-12 (Table IV), run with TPNH, the general metabolism of androstanedione appeared to be at a little lower level than in those run with DPNH. The amounts of epiandrosterone isolated were more than those of both androsterone and androstanolone, and gave a peak at pH 6.5 (Figure 2). The quantities of androsterone and androstanolone isolated, were so low that no attempt was made to plot curves with them. In these experiments, the change in gaseous phase at pH 6 did not appreciably influence the results.

It is possible that these three metabolites are formed by the action of three different enzyme systems acting on androstanedione, or of

TABLE IV

THE INFLUENCE OF pH ON THE METABOLISM OF ANDROSTANEDIONE BY GUINEA PIG LIVER HOMOGENATES

Expt. no.	Gaseous phase	Steroid mg.	DPNH mg.	TPNH mg.	pH	Androstanedione recovered % by wt.	Epiandrosterone formed % by wt.	Androsterone formed % by wt.	Androstanolone formed % by wt.
1	Air	50	50	-	5.5	81.4	3.2	1.8	0.2
2	Air	50	50	-	6.0	81.2	6.8	4.8	0.6
3	Nitrogen	50	50	-	6.0	78.6	3.4	5.0	0.8
4	Air	50	50	-	6.5	78.4	10.6	5.6	0.4
5	Air	50	50	-	7.0	79.4	7.8	4.4	0.8
6	Air	50	50	-	7.5	80.6	4.0	2.8	0.2
7	Air	50	-	50	5.5	78.6	4.4	1.6	0.4
8	Air	50	-	50	6.0	81.8	7.8	1.6	0.4
9	Nitrogen	50	-	50	6.0	76.4	6.4	1.2	0.2
10	Air	50	-	50	6.5	80.2	8.4	1.8	0.2
11	Air	50	-	50	7.0	78.2	7.0	1.2	0.2
12	Air	50	-	50	7.5	78.8	5.4	1.2	0.2

FIGURE 1

INFLUENCE OF pH ON THE METABOLISM OF ANDROSTANEDIONE BY GUINEA PIG LIVER HOMOGENATES IN THE PRESENCE OF DPNH

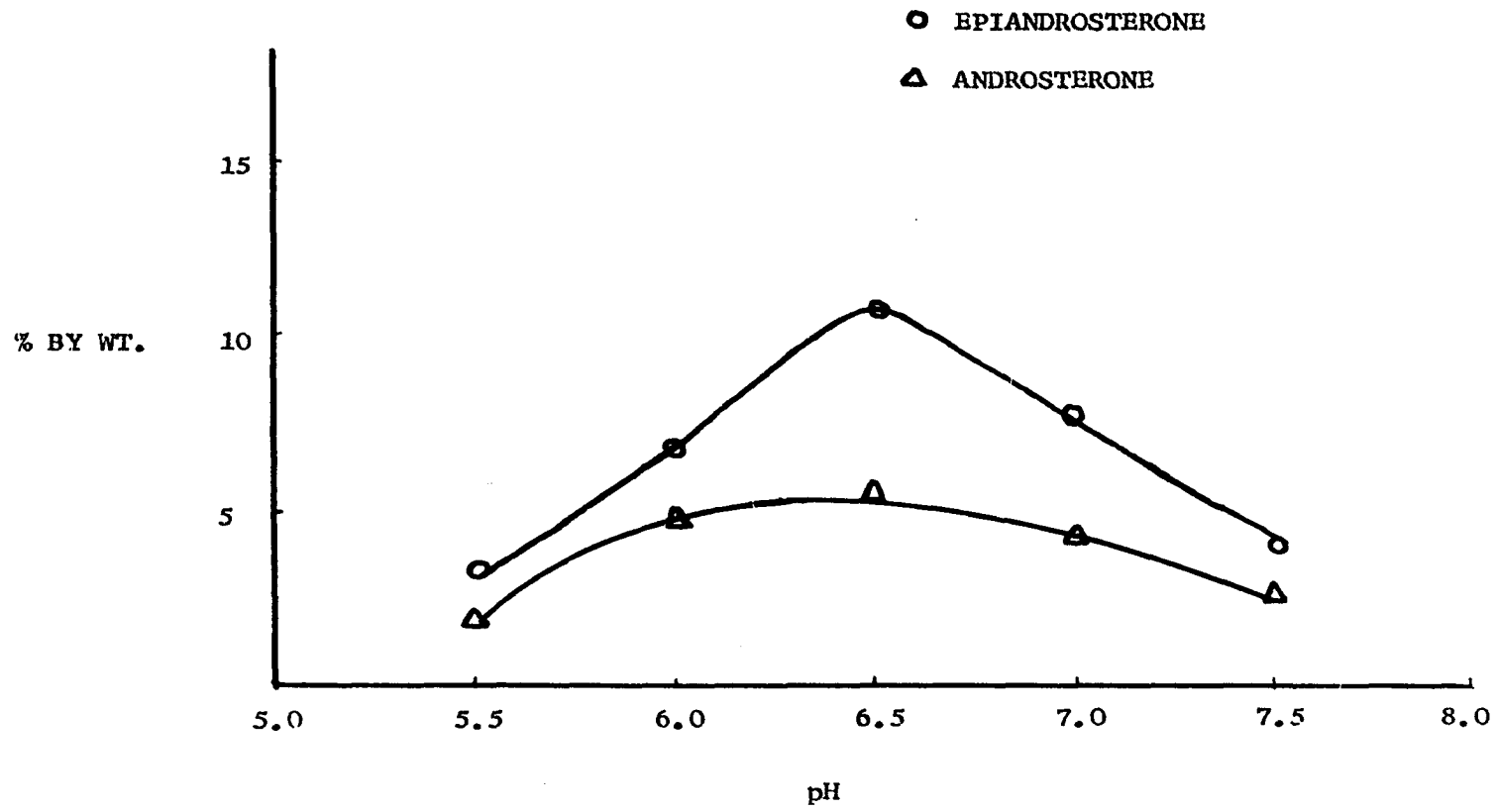
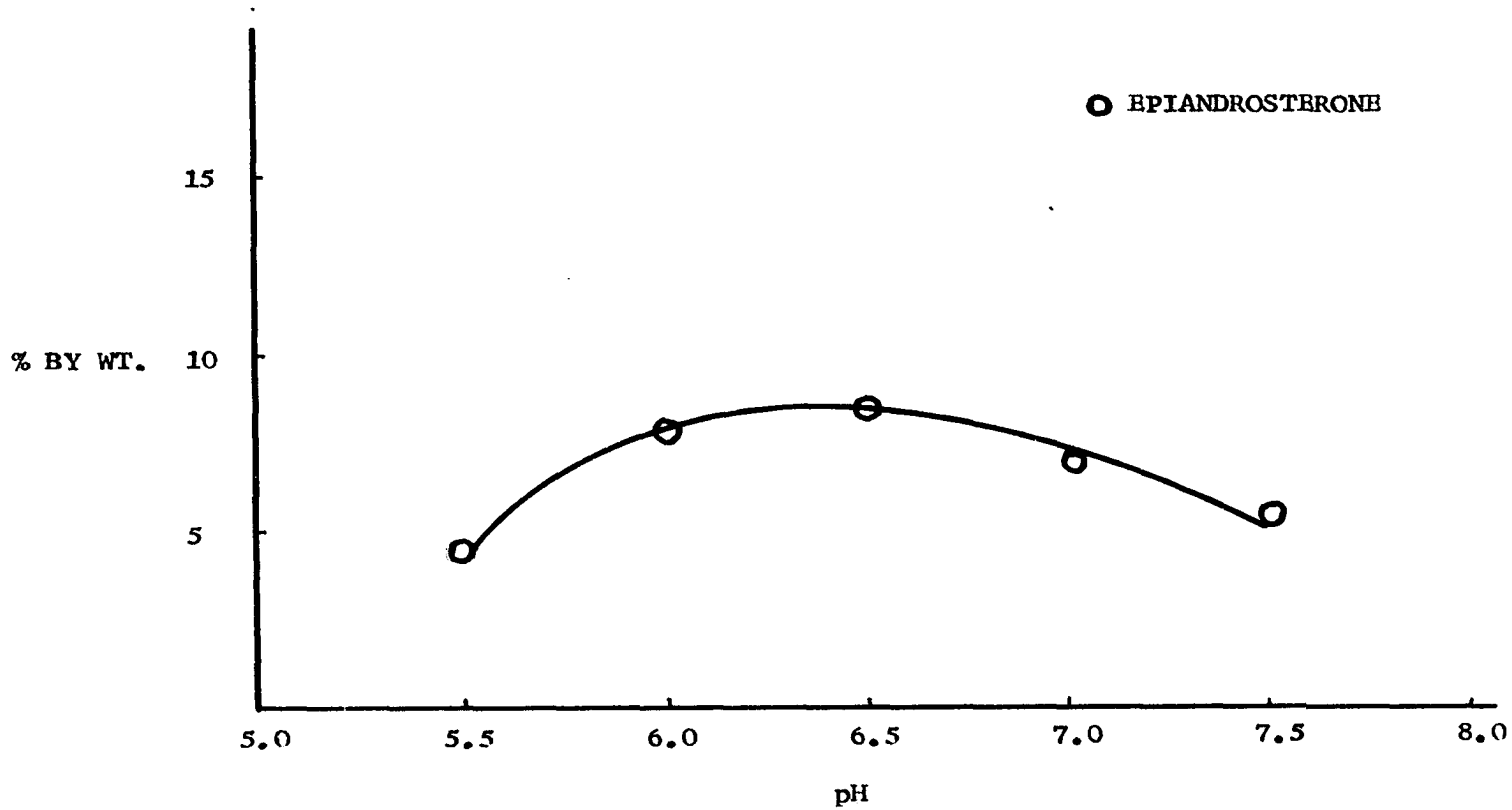


FIGURE 2

INFLUENCE OF pH ON THE METABOLISM OF ANDROSTANEDIONE BY GUINEA PIG LIVER HOMOGENATES IN THE PRESENCE OF TPNH



two enzyme systems, one leading to the formation of two metabolites and the other forming the third metabolite, or of only one enzyme system leading to the formation of all the three metabolites. Talalay and his co-workers (125-129) have reported the isolation of two DPN linked steroid dehydrogenases from cell-free extracts of Pseudomonas. β-hydroxysteroid dehydrogenase catalyzes the reversible DPN linked oxidation of 3β, 16β, and 17β hydroxysteroids, whereas α-dehydrogenase catalyzes the reversible oxidation of 3α-hydroxysteroids of the C_{19}, C_{21}, and C_{24} series. It is also possible that only one enzyme system forms the three metabolites of androstanedione in the presence of different cofactors, and hence the production of all the three metabolites is not the same under fixed conditions.

The formation of epiandrosterone is higher than that of either androsterone or androstanolone, in the presence of both DPNH and TPNH. Thus it appears that pyridine nucleotides favor 3β-hydroxylation rather than 3α or 17β-hydroxylation which might occur optimally with other cofactors. Both epiandrosterone and androsterone give pH curves with a peak at pH 6.5 with DPNH. This suggests that one enzyme system, utilizing DPNH, is responsible for the formation of both these metabolites, but that there are some endogenous factors which inhibit 3α-hydroxylation, and keep the production of androsterone at a lower level.

In TPNH experiments, a pH curve can be plotted with epiandrosterone, but not with androsterone or androstanolone. This suggests that the requirement of DPNH is so specific for the formation of androsterone that its formation is practically nil without DPNH. The production of epiandrosterone is at a lower level with TPNH than with DPNH.

This indicates that the third phosphate group of TPNH inhibits the metabolism of androstanedione, lowering slightly the formation of epiandrosterone and cutting down considerably the formation of androsterone. The third phosphate group might be inhibiting directly the enzyme systems involved, or it might be stimulating some endogenous factors in the tissue, which have an inhibitory effect upon the enzyme systems responsible for the metabolism of androstanedione.

No pH curves could be plotted for androstanolone, either with DPNH or TPNH. This might indicate that the optimal conditions, and perhaps the enzyme system as well, are absolutely different for the production of this metabolite, from those responsible for the production of androsterone and epiandrosterone; or the production of androstanolone may be a minor metabolic pathway.

It also appears that the formation of epiandrosterone is generally favored in the guinea pig. These observations are supported by <u>in vivo</u> experiments (118).

It appears that anaerobic conditions keep the metabolism of androstanedione at a lower level than that under aerobic conditions. This might be due to the specific requirements of the hydrogen transferring reactions and the enzyme systems involved.

The preferential formation of 17-ketone metabolites rather than the 3-ketone compounds observed in the metabolism of androstanedione, is in accord with the preponderance of the former compounds in excretory products (120).

CHAPTER IV

METABOLISM OF ANDROSTENEDIONE BY GUINEA PIG TISSUE HOMOGENATES

It has been postulated (118,130) that androstenedione is formed in the human body by the oxidation of testosterone and dehydroepiandrosterone, then it is reduced to saturated diketones which are further reduced to hydroxyketones. Androstenedione has been shown to be metabolized by rabbit liver slices to testosterone and epitestosterone (65). Formation of testosterone and androsterone from androstenedione by guinea pig tissue homogenates, has been reported by Kochakian and Stidworthy (69).

Experiments were conducted to study the metabolism of androstenedione by guinea pig liver and kidney homogenates, under different conditions of pH and with different additives, using disodium phosphate and sodium pyrophosphate as the buffering media.

Metabolism by Guinea Pig Liver Homogenates

Androstenedione was incubated with guinea pig liver homogenates in sodium pyrophosphate buffer at pH 9. The metabolism to testosterone observed, was only about 1% (experiments 1-3 in Table V). The addition of DPNH to the incubation mixture, and running the incubation at pH 6.8, raised the level of metabolism appreciably (experiment 4). Further

TABLE V

METABOLISM OF ANDROSTENEDIONE BY GUINEA PIG LIVER HOMOGENATES

Expt. no.	Gaseous phase	Steroid mg.	DPNH mg.	TPNH mg.	NA mg.	Other Additives	pH	Androstenedione recovered % by wt.	Testosterone formed % by wt.
1	Air	100	-	-	-	-	9.0	80.9	1.0
2	Air	100	-	-	-	-	9.0	86.9	0.8
3*	Air	100	-	-	-	-	9.0	90.0	-
4	Air	100	87	-	-	-	6.8	76.7	7.4
5	Air	100	87	-	800	-	6.8	75.7	7.4
6	Air	50	-	65	800	-	7.0	73.0	14.2
7	Nitrogen	50	-	65	800	-	7.0	76.0	12.0
8	Nitrogen	50	50	-	800	-	7.0	76.4	5.2
9	Air	50	50	-	400	-	6.0	77.2	6.6
10	Air	50	50	-	400	-	6.5	78.6	4.0
11	Air	50	50	-	400	-	7.0	83.0	3.0
12	Nitrogen	50	50	-	400	-	7.0	81.6	5.4
13	Air	50	50	-	400	-	7.5	86.0	2.4
14	Air	50	50	-	400	-	8.0	84.6	3.4
15	Air	50	-	50	400	-	5.5	81.4	10.2
16	Air	50	-	50	400	-	6.0	80.0	11.0
17	Nitrogen	50	-	50	400	-	6.0	81.6	16.8
18	Air	50	-	50	400	-	6.5	86.6	10.6
19	Air	50	-	50	400	-	7.0	69.9	9.6
20	Air	50	-	50	400	-	7.5	71.6	7.8
21	Nitrogen	50	-	50	-	-	6.5	81.8	11.2
22	Nitrogen	50	-	50	400	-	6.5	80.8	11.0
23	Nitrogen	50	-	50	400	Glucose (36 mg.)	6.5	86.4	11.6

* Boiled tissue used in experiment 3

TABLE V - continued

Expt. no.	Gaseous phase	Steroid mg.	DPNH mg.	TPNH mg.	NA mg.	Other Additives	pH	Androstenedione recovered % by wt.	Testosterone formed % by wt.
24	Nitrogen	50	-	50	400	Na_3.isocitrate (52 mg.)	6.5	72.8	15.8
25	Nitrogen	50	-	50	400	Na_2.ATP.$4H_2O$ (111 mg.)	6.5	72.0	15.8
26	Nitrogen	50	-	50	400	Na_2.ATP.$4H_2O$ (418.1 mg.)	6.5	70.4	16.44
27	Nitrogen	50	-	50	400	Na_2.ATP.$4H_2O$ (418.1 mg.) + $MgCl_2$.$6H_2O$ (136.5 mg.)	6.5	73.4	18.22
28	Nitrogen	50	-	50	400	Na_2.ATP.$4H_2O$ (418.1 mg.) + $MgCl_2$.$6H_2O$ (136.5 mg.) + Na_2.fumarate (107.4 mg.)	6.5	72.8	17.0

addition of NA to the incubating medium did not affect the results at all (experiment 5). Replacement of DPNH by TPNH raised the level of metabolism still further (experiment 6). The change of gaseous phase from air to nitrogen did not seem to affect the results remarkably (experiments 7 and 8).

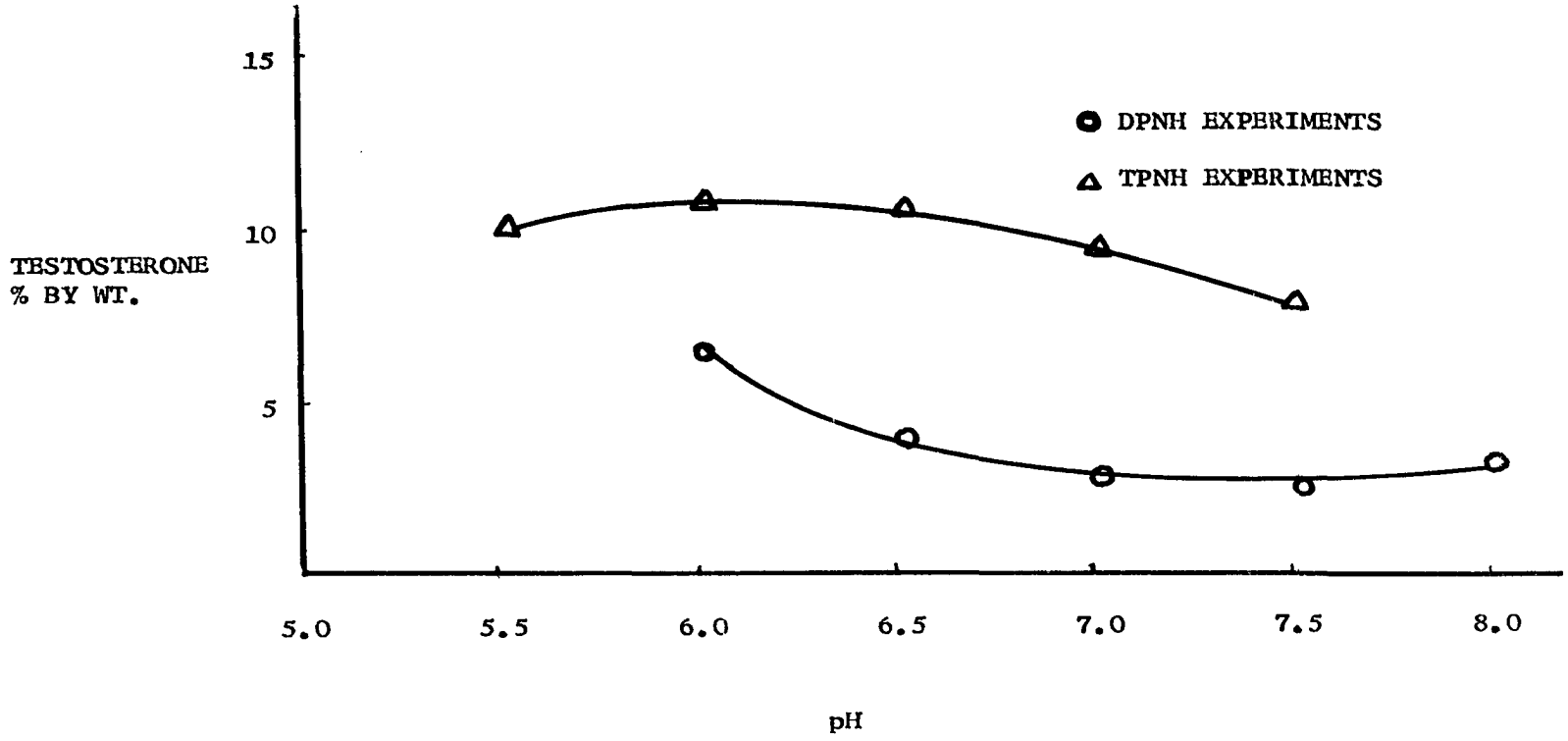
It was then decided to observe the influence of pH on the metabolism of androstenedione by guinea pig liver homogenates, in the presence of the reduced forms of pyridine nucleotides (Figure 3).

Experiments were conducted in the pH range 6-8, with disodium phosphate as the buffering medium, in the presence of DPNH and NA (experiments 9-14). The production of testosterone appeared to be the highest at pH 6. Trace amounts of other metabolites were also detected. They were thought to be epiandrosterone and a substituted unsaturated 3,17-diketone, from the results of the experiments with paper chromatography and infra-red spectrophotometry. The change of gaseous phase from air to nitrogen at pH 7 seemed to favor the formation of testosterone, though it is hard to say positively if this difference was significant, since the metabolism in all these experiments was at a very low level.

Similar experiments were run using TPNH and NA as the cofactors in the pH range 5.5-7.5 (experiments 15-20). The replacement of DPNH by TPNH raised the level of metabolism appreciably. The curve did not show a real peak at any pH, but it had a plateau in the pH range 5.5-6.5. Anaerobic conditions at pH 6 notably favored the formation of testosterone. In these experiments also, a suggestive evidence of the formation of epiandrosterone and a substituted unsaturated 3,17-diketone as the other metabolites was obtained as a result of the experiments with paper

FIGURE 3

INFLUENCE OF pH ON THE METABOLISM OF ANDROSTENEDIONE BY GUINEA PIG LIVER HOMOGENATES IN THE PRESENCE OF DPNH OR TPNH



chromatography and infra-red spectrophotometry.

Androstenedione was incubated with guinea pig liver homogenates, at pH 6.5, under nitrogen, in the presence of different additives (experiments 21-28). It appeared that the addition of NA or glucose to the incubation medium did not affect the results. Isocitrate or ATP raised the production of testosterone. Increase in the amount of ATP used, did not make a remarkable difference in the results. Similarly $MgCl_2$ or fumarate did not affect the results significantly. In all these experiments also, a suggestive evidence of the formation of epiandrosterone and a substituted unsaturated 3,17-diketone in trace amounts was obtained.

Metabolism by Guinea Pig Kidney Homogenates

Metabolism of androstenedione by guinea pig kidney homogenates was studied using different additives (Table VI). Experiments were run with DPNH and NA as the cofactors, in air and under nitrogen (experiments 1 and 2). Amounts of testosterone formed in both the experiments were practically the same. Similar experiments were run with TPNH and NA as the cofactors (experiments 3-7). Anaerobic conditions appeared to favor the formation of testosterone. Further addition of isocitrate to the incubation mixture resulted in a slight increase in the production of testosterone (experiment 8). The addition of ATP (ten moles of ATP per mole of TPNH) to the incubation mixture raised the level of metabolism remarkably (experiment 9). Further addition of $MgCl_2$ did not affect the results (experiments 10 and 11). Addition of ATP and $MgCl_2$ in smaller amounts (only three moles per mole of TPNH) did not make an appreciable difference in the production of testosterone (experiment 12). Further

TABLE VI

METABOLISM OF ANDROSTENEDIONE BY GUINEA PIG KIDNEY HOMOGENATES

Expt. no.	Gaseous phase	Steroid mg.	DPNH mg.	TPNH mg.	NA mg.	Other Additives	pH	Androstenedione recovered % by wt.	Testosterone formed % by wt.
1	Air	50	50	-	800	-	7.0	78.0	7.8
2	Nitrogen	50	50	-	800	-	7.0	77.8	7.4
3	Air	50	-	72.5	800	-	7.0	83.4	6.2
4	Air	50	-	65	800	-	7.0	87.2	5.0
5	Nitrogen	50	-	50	800	-	7.0	75.6	7.6
6	Nitrogen	50	-	50	400	-	6.5	76.4	13.8
7	Nitrogen	50	-	50	400	-	6.5	75.6	13.4
8	Nitrogen	50	-	50	400	Na_3.isocitrate (173.2 mg.)	6.5	78.4	16.8
9	Nitrogen	50	-	50	400	Na_2.ATP.$3H_2O$ (406 mg.)	6.5	69.8	20.88
10	Nitrogen	50	-	50	400	Na_2.ATP.$3H_2O$ (406 mg.) + $MgCl_2$.$6H_2O$ (136.5 mg.)	6.5	70.2	22.28
11	Nitrogen	50	-	50	400	Na_2.ATP.$4H_2O$ (418.1 mg.) + $MgCl_2$.$6H_2O$ (136.5 mg.)	6.5	65.6	19.66
12	Nitrogen	50	-	50	400	Na_2.ATP.$4H_2O$ (125.4 mg.) + $MgCl_2$.$6H_2O$ (41 mg.)	6.5	71.4	15.2
13	Nitrogen	50	-	50	400	Na_2.ATP.$4H_2O$ (125.4 mg.) + $MgCl_2$.$6H_2O$ (41 mg.) + Na_2.fumarate (32.2 mg.)	6.5	73.2	15.5
14	Nitrogen	50	-	50	400	Na_2.ATP.$4H_2O$ (418.1 mg.) + $MgCl_2$.$6H_2O$ (136.5 mg.) + Na_2.fumarate (107.4 mg.)	6.5	72.8	19.2

addition of fumarate to the incubation mixture did not affect the results at all (experiments 11-14). In most of these experiments, trace amounts of epiandrosterone and a substituted unsaturated 3,17-diketone were thought to be formed as the other metabolites.

Discussion

Liver and kidney have many reductases, which are not only position specific, but also stereoisomerically specific. They are also specific in their cofactor requirements (discussed in Chapter I). In the above experiments only testosterone was identified as a metabolite. A suggestive evidence of the formation of epiandrosterone and a substituted unsaturated 3,17-diketone as the other metabolites was obtained.

Thus it appeared that the enzyme systems reducing only C-3 and C-17 ketones to the respective β -hydroxyls, and also reducing the double bond in ring A, were active both in the liver and the kidney of the guinea pig. In the experiments with liver, the addition of NA to the incubating mixture did not affect the results appreciably. This might suggest that the amounts of the reduced pyridine nucleotides were more than sufficient, in spite of the inhibitory action of nucleotidases present in the tissues (122,123), or that the reduced forms of pyridine nucleotides were not acted upon by the nucleotidases. It might also suggest the presence of "NA inhibitor" in the tissues which did not allow NA to take part in the exchange reaction with DPNH or TPNH.

The reduction of androstenedione was favored by acid pH at which the reduced forms of pyridine nucleotides are less stable than they are at the alkaline pH (131). This might lower the availability of the

reduced form of pyridine nucleotides for the reduction of the steroid in acid pH. This might account for fairly low levels of metabolism in our experiments.

TPNH seemed to be more favorable for the formation of testosterone than DPNH. This might be due to the specific characteristics of the enzyme systems involved, or might be due to more stability of TPNH as compared to that of DPNH in acid medium. In a few experiments, anaerobic rather than aerobic conditions seemed to be more suited for the reduction of androstenedione. This is in accordance with the observations of Tomkins and Isselbacher (90).

Isocitrate and ATP were found to be effective additives in our experiments. Isocitrate might be acting as a substrate for the isocitric dehydrogenase present in the tissues. This enzyme should utilize TPN as the cofactor and reduce it to TPNH (132). Thus isocitrate might be favoring the formation of testosterone by keeping a constant supply of TPNH.

$$\text{Androstenedione} + \text{TPNH} \longrightarrow \text{Testosterone} + \text{TPN}$$

$$\text{TPN} + \text{DPNH} \longrightarrow \text{TPNH} + \text{DPN}$$

ATP might be involved in the glycolysis, leading to the formation of DPNH which might be exchanging hydrogen with TPN to produce TPNH as shown above (96). ATP might be responsible for converting TPN to TPNH also, by yielding some energy. H ion required could be picked up from the buffer medium. It might also catalyze directly the reduction of androstenedione to testosterone by TPNH.

It appeared that the ATP requirements in the kidney experiments were about thrice as great as in the liver experiments.

CHAPTER V

SUMMARY

In vitro metabolism of androstanedione and 4-androstene-3,17-dione was studied by using guinea pig tissue homogenates as the source of enzyme systems.

Experiments were designed to show the influence of pH on the metabolism of androstanedione by guinea pig liver homogenates, in the presence of NA and DPNH or TPNH. Androsterone, epiandrosterone, and androstane-17β-ol-3-one were identified as the metabolites. The metabolism of androstanedione was at a higher level in the presence of DPNH than with TPNH. Epiandrosterone was formed in larger amounts than androsterone or androstanolone. In these experiments, pH 6.5 appeared to be the optimal pH for the formation of both androsterone and epiandrosterone in the presence of DPNH, and for the formation of epiandrosterone only, in the presence of TPNH.

Androstenedione was metabolized by guinea pig liver or kidney homogenates. Testosterone was the identified metabolite. Reduced forms of pyridine nucleotides raised the level of metabolism considerably. TPNH was more effective than DPNH. Addition of NA to the incubation mixtures did not affect the results at all. Testosterone formation by liver homogenates was the highest at pH 6.0, in the presence of DPNH,

while it was more or less the same in the pH range 5.5-6.5, in the presence of TPNH. Anaerobic conditions seemed to be more favorable for the production of testosterone than the aerobic conditions.

Isocitrate and ATP appeared to be the effective additives, while $MgCl_2$, glucose, and fumarate were ineffective in raising the level of metabolism both in the liver and kidney experiments. ATP requirements of kidney appeared to be about thrice those of liver. In most of the androstenedione experiments, there was some evidence for the formation of epiandrosterone and a substituted unsaturated 3,17-diketone in trace amounts as additional metabolites.

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