THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE METABOLISM OF ANDROSTANEDIONE AND OTHER ANDROGENS BY GUINEA PIG TISSUE HOMOGENATES AND GUINEA PIG LIVER HOMOGENATE FRACTIONS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

BY

DONALD IVAN HAMM

Norman, Oklahoma

1956

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A DISSERTATION

APPROVED FOR THE DEPARTMENT OF CHEMISTRY

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THE METABOLISM OF ANDROSTANEDIONE AND OTHER ANDROGENS BY GUINEA PIG TISSUE HOMOGENATES AND GUINEA PIG LIVER HOMOGENATE FRACTIONS

CHAPTER I

INTRODUCTION

During the last twenty-five years, compounds containing the perhydro-1,2-cyclopentanophenanthrene[1] nucleus have been demonstrated to be involved intimately in mammalian physiology. Cholesterol,[1,2] the principal sterol[3] of the animal organism, has been known since the eighteenth century as the principal constituent of human gall stones. The chemistry of this material was investigated actively during the nineteenth century. Bile acids, compounds structurally related to the sterols, were isolated in pure form as conjugates by Strecker in 1848. However, the complete

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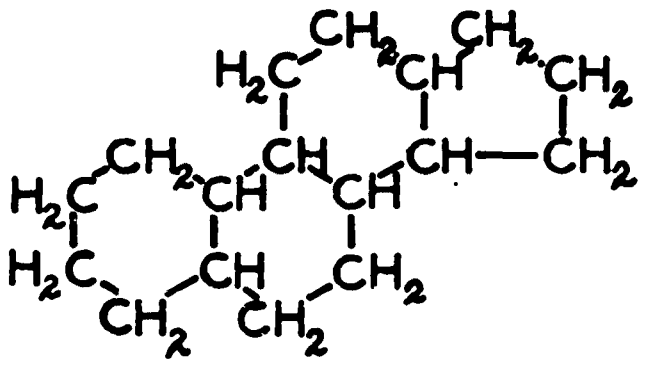
[1]See Table 1.

[2]Greek chole, bile plus stereos, solid.

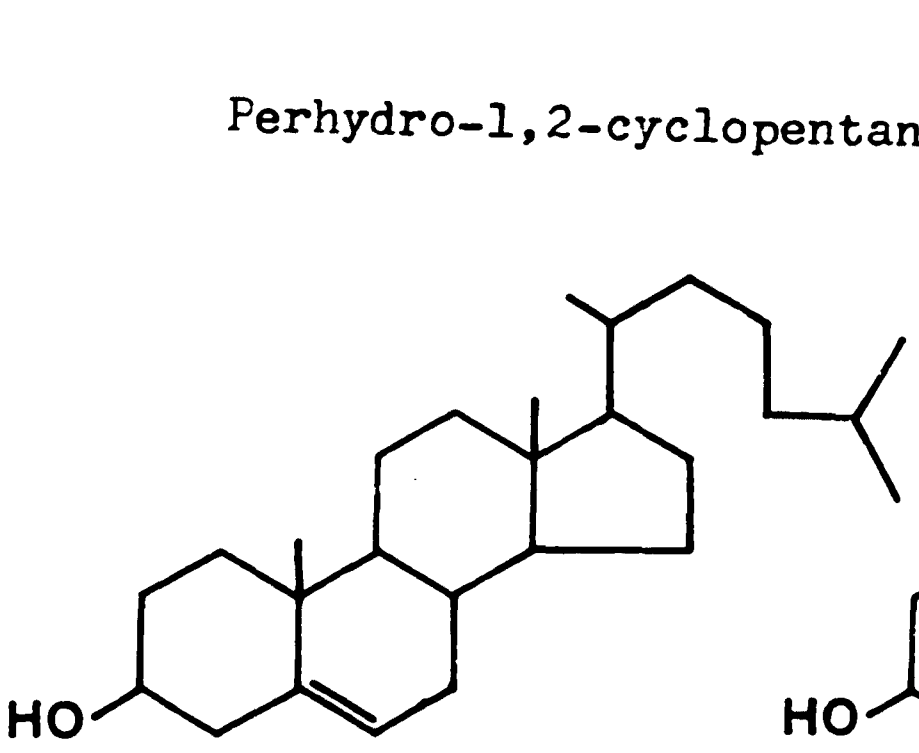
[3]Greek stereos, solid plus the nomenclatural suffix for alcohols, ol; sterols are crystalline alcohols isolated from the unsaponifiable residues of lipids derived from animals and plants.

TABLE 1

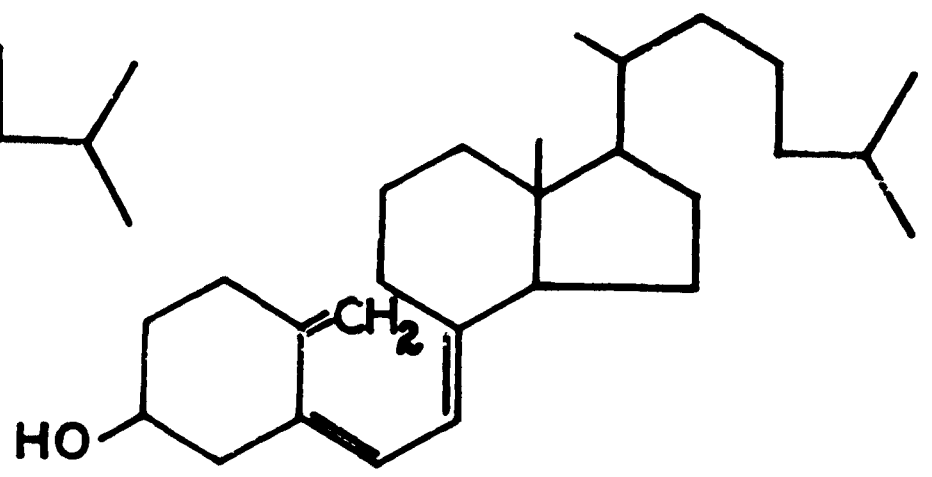
REPRESENTATIVE STEROID COMPOUNDS



Perhydro-1,2-cyclopentanophenanthrene nucleus

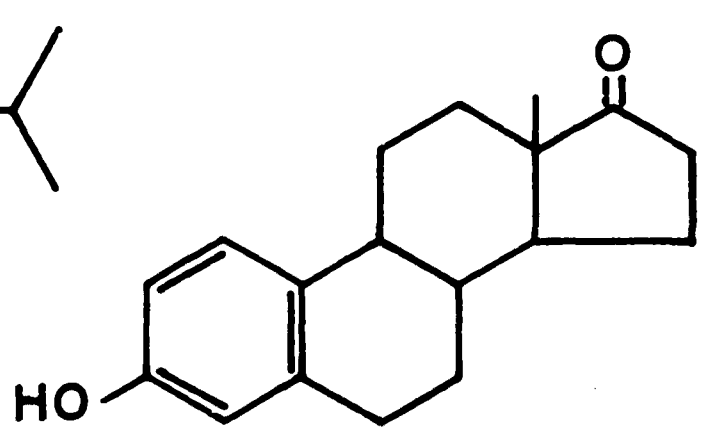


Cholesterol



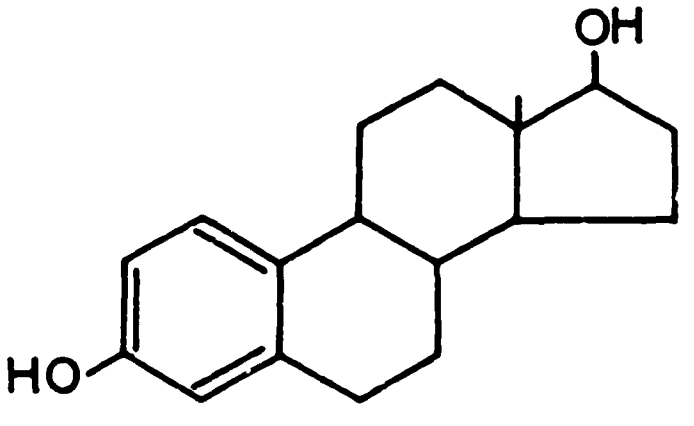
Vitamin D_3

7-Dehydrocholesterol

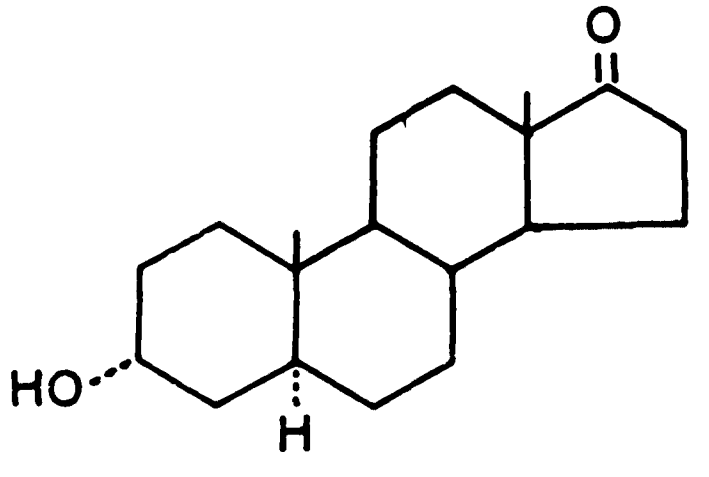


Estrone

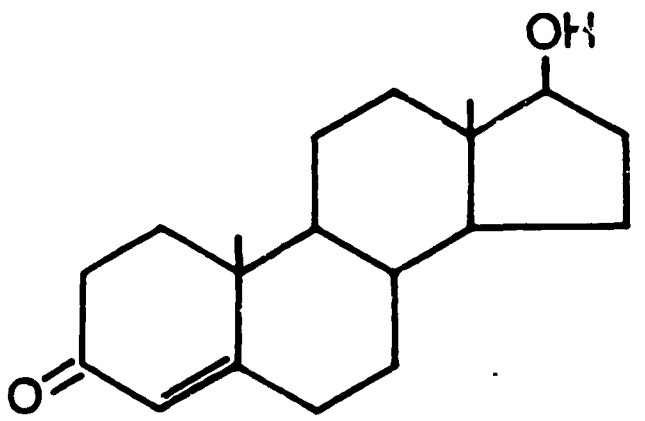
TABLE 1--Continued



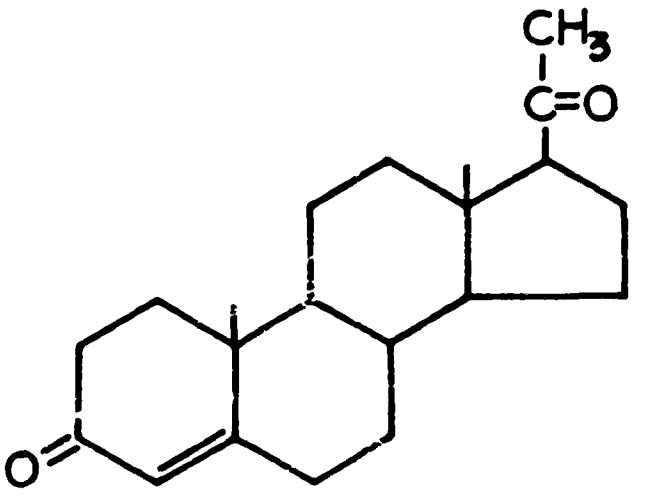
Estradiol



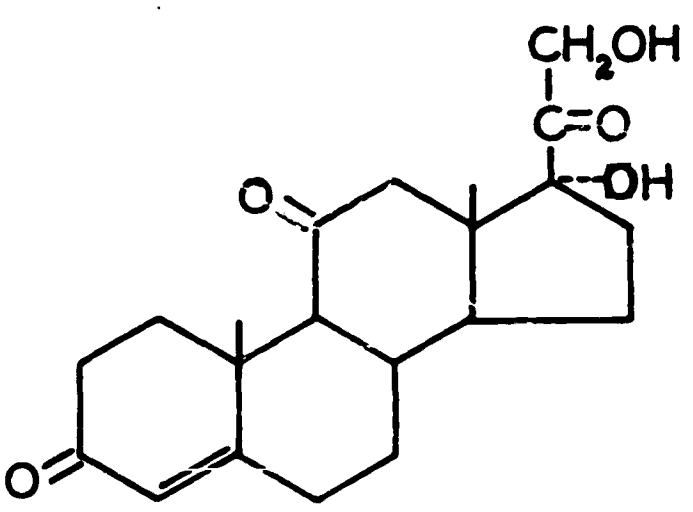
Androsterone



Testosterone



Progesterone



Cortisone

elucidation of the structures of these materials was not accomplished until the last quarter of a century, when the classical work of Wieland, Windaus, Diels, Rosenheim, and King led to the formulation of the structure of cholesterol; and it was during this period that it became apparent that many series of compounds structurally related to the sterols and the bile acids were of the utmost importance. In accordance with the suggestion of Callow and Young (1), compounds chemically related to cholesterol have been designated steroids.

Concentrates of Vitamin D, the antirachitic vitamin, were prepared originally from tuna liver oil and cod liver oil in 1933 and 1935 (2), respectively. Vitamin D_3[1] was isolated in crystalline condition in 1937 (3). The vitamin is prepared both naturally and synthetically by ultraviolet irradiation of the provitamin, 7-dehydrocholesterol[1] (3).

The isolation of estrone from the urine of pregnant women was accomplished independently in 1929 by Doisy (4,5,6) and by Butenandt (7,8). This compound possesses the ability to produce estrus in lower animals; hence it is included among the female sex hormones. The structure now accepted for estrone was postulated originally by Butenandt in 1932 (9). Subsequent investigations revealed the primary female sex hormone, or estrogen, to be estradiol;[1] this compound is 8-10 times more active than estrone in rats (10). Estradiol was synthesized in 1933 (11) and isolated from ovarian tissue

by Doisy in 1935 (12).

The methods employed for the isolation of the estrogens soon led to the isolation of the analogous male sex hormones, or androgens.[4] The first crystalline compound was reported by Butenandt and Tscherning in 1931 (13,14) who named it androsterone;[1] the former worker suggested the correct structural formula in 1932 (15). The primary androgenic hormone, testosterone,[1] was isolated from bull testis by Laqueur in 1935 (16).

The progestational hormone, progesterone,[1] was isolated from corpeus luteum in 1934. Four laboratories reported the isolation within a period of six months; Butenandt published the first report (17,18,19). Slotta deduced the correct structural formula in the same year (20).

Over thirty compounds with the cyclopentanophenanthrene, or steroid, nucleus have been isolated from the adrenal cortex; such compounds are designated adrenocortical steroids or corticoids. Many of these compounds are hormonally inactive, but at least seven exhibit such diverse properties as mineral balance regulatory activity, control of carbohydrate and protein metabolism, and anti-inflammatory activity. The structure of cortisone is given in Table 1.

That the various influences exhibited by the steroid compounds are of extreme importance in mammalian physiology

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[4]Greek andro-, male.

is evident. Twombly (21) has said, "The most profound changes in the animal body, including the development of the secondary sex characters and genesis of certain sorts of cancer, are mediated by steroids." Lieberman and Dobriner (22) introduced a review of the biochemistry of steroids in the following fashion:

> The unique role of the steroid hormones in nearly all phases of life has been established. With the discovery of the sex hormones in the early thirties, it seemed that the physiological action of the steroids was primarily concerned with the phenomena of sex. With the discovery of the steroid hormones of the adrenal gland in the late thirties it was soon found that these compounds had a profound influence on metabolism and that this was also true of the so-called sex hormones.

Various aspects of the influences of steroid compounds continue to be revealed. As is always the case, experimentation designed to give insight into the causes of such influences has lagged far behind the revelation of these influences. Quoting again from Twombly (21), concerning steroid-induced changes, "Although the gross cause and effect relationships are obvious, the mechanisms whereby they are brought about are entirely unknown."

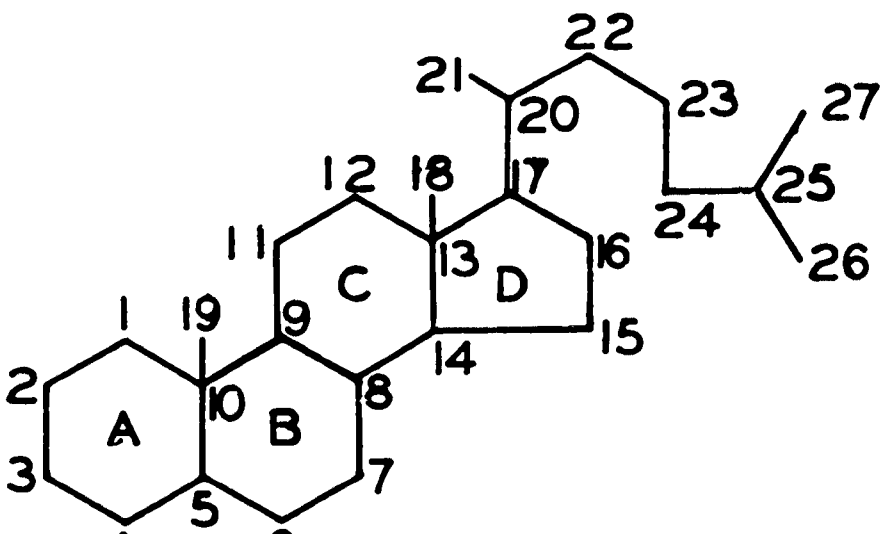
The research reported in this dissertation was undertaken in the hope that it might represent a contribution to the knowledge concerning the metabolism of the androgens.

The Structures of Cholesterol and the Nineteen-Carbon Steroids

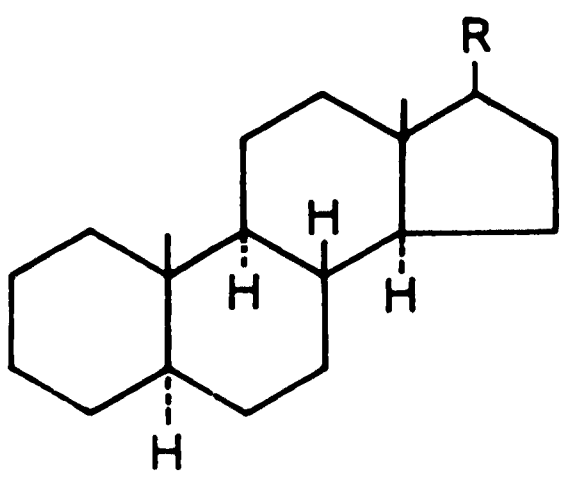
The carbon structure of cholesterol shown in Table 2

TABLE 2

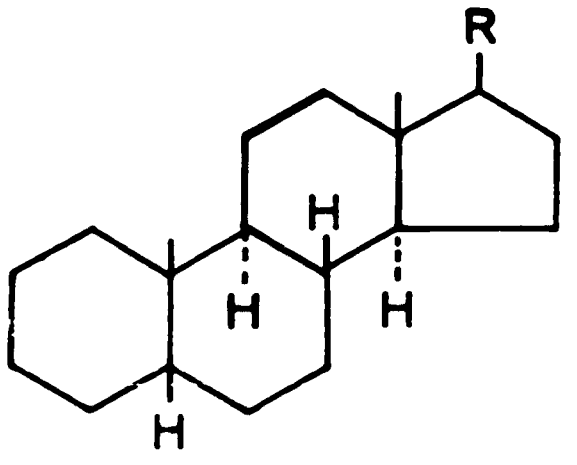
STEROID STRUCTURAL DESIGNATIONS



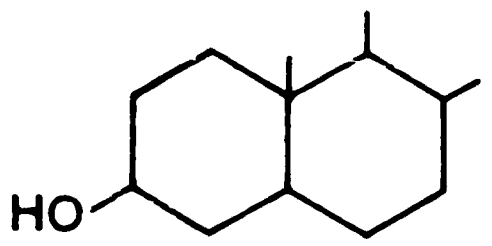
Carbon structure of cholesterol



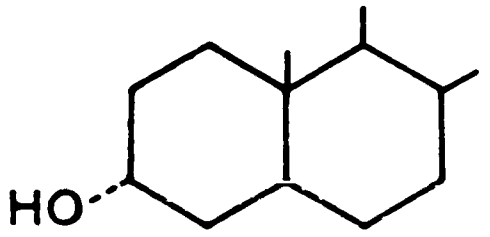
Cholestane R = C_8
Androstane R = H



Coprostane R = C_8
Etiocholane R = H



3-β-Hydroxyl



3-α-Hydroxyl

has been numbered in the fashion usually employed to designate the various carbon atoms numerically. In addition, the common designation of the four carbon rings has been indicated. Those carbon atoms numbered 1-19, inclusive, are common to the androgens,[5] as well as the other series of steroid compounds, with the exception of the estrogens, in which only the carbon atoms numbered 1-18, inclusive, are present.

Cholesterol contains eight asymmetric carbon atoms; they are those numbered 3, 8, 9, 10, 13, 14, 17, and 20. However, cholesterol which has been isolated from natural sources is free from contamination with any of the numerous possible isomers. As has been indicated, the structure of cholesterol has been defined. Those portions of its structure which are pertinent to the structure of the androgens are discussed in the material which follows.

Reduction of the double bond in cholesterol results in the production of an additional asymmetric carbon atom at the position which is numbered 5; the two compounds which result from such reduction are known as cholestanol and coprostanol. The hydrocarbons from which these alcohols are derived are designated cholestane[6] and coprostane,[6]

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[5]The term androgen is frequently applied to any androstane derivative (see Table 2), regardless of the presence or absence of hormonal activity; it is so used in this dissertation.

[6]See Table 2.

respectively. In cholestane the fusion between rings A and B is analogous to that in trans decalin; in coprostane the fusion is analogous to that in cis decalin. Because the possibility of this isomerism is present in all nonphenolic steroids, the two configurations at the carbon atom which is numbered 5 are designated α and β. The hydrogen atoms in compounds in which the fusion is trans (relative to the decalins) are said to have the α-configuration.[7] In essence this configurational designation related the hydrogen atoms at positions 5 to the configuration of the methyl group at position 10; a parallel designation is used for other ring substituents which are capable of giving rise to isomerism. Hence the hydroxyl group at position 3 in cholesterol is designated as β since it is cis to the methyl group at position 10. β-Substituents, including the methyl group at position 10 and the hydrogen atom at position 5, are depicted in planar structural representations using solid lines to join the substituents to the atoms to which they are bonded; α-substituents are depicted using broken lines. This is illustrated in Table 2.

---

[7]The structural designations and the systematic nomenclature employed in this dissertation are those which have been recommended by Fieser and Fieser in their excellent monograph (23). A large portion of the material in this chapter was derived from this source. It is rare that such a pertinent book is available in a narrowly defined research area; the author wishes to express his appreciation for its existence.

The nineteen-carbon hydrocarbons analogous to cholestane and coprostane (in which the alkyl side-chains are absent at position 17) are known as androstane[6] and etiocholane;[6] the hydrogen atoms at position 5 in these compounds are α and β, respectively. The former hydrocarbon is regarded as the parent hydrocarbon of the androgens. This is somewhat arbitrary since testosterone,[1] as well as certain of its derivatives, possesses a double bond at position 4; there is no asymmetry at position 5. Asymmetry is also absent in dehydroepiandrosterone and certain other compounds, which are similar to cholesterol[1] in that they have a double bond between carbon atoms 5 and 6. Furthermore, metabolic reduction of testosterone in vivo gives rise to etiocholane, as well as androstane, derivatives. However, of such reduced derivatives, only those possessing the α-configuration at position 5, the androstane derivatives, are hormonally active. Furthermore, by convention, those compounds lacking asymmetry at position 5, such as testosterone and dehydroepiandrosterone, are arbitrarily named as androstane derivatives when systematic nomenclature is employed.

The fusion of rings B and C is the same in cholestane, coprostane, androstane, and etiocholane, as well as in all other steroid hydrocarbons. The fusion is trans and the hydrogen atoms at positions 8 and 9 are cis, or β, and trans, or α, respectively, to the methyl group at position 10.

The fusion of rings C and D is also trans in all steroid compounds. Compounds isomeric at positions 8, 9, 13, or 14 are unknown. The methyl group and the hydrogen atom at positions 13 and 14 are cis, or β, and trans, or α, respectively, to the methyl group at position 10. Thus the so-called angular methyl groups are cis to each other and are designated β. In androstane (and cholestane), and its saturated derivatives, the ring fusions are all trans. Since the fusions between rings B and C and between rings C and D are common to all steroids, their nature is usually omitted from formulae.

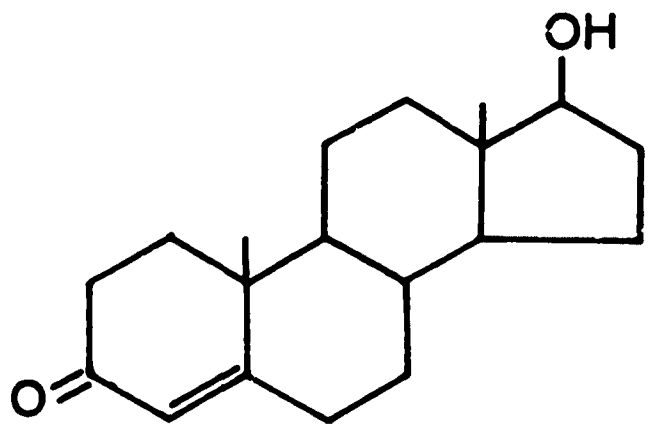
The alkyl side-chain in both cholestane and coprostane is cis, or β, to the angular methyl groups at positions 10 and 13. Although this side-chain is absent in the nineteen-carbon steroids, many compounds containing a hydroxyl group in this position have been isolated from natural sources. In all such compounds the hydroxyl group is also β. The isomeric compounds have been prepared synthetically. Compounds of the progesterone and corticoid series have two-carbon side-chains at position 17; these also have the β-configuration. Certain of the corticoids have both a two-carbon side-chain and a hydroxyl group at position 17; the hydroxyl group necessarily possesses the α-configuration.

The Nomenclature of Androstane Derivatives

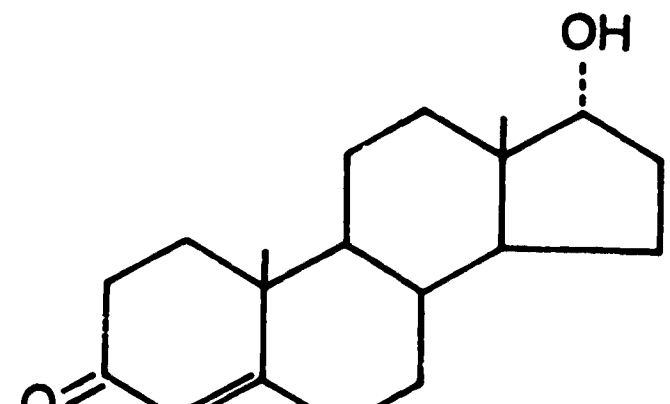
Of necessity the isolation of a new series of compounds requires the formulation of trivial names. Traditionally, such trivial names are displaced only slowly, if at all, by a systematic nomenclature. Although a systematic nomenclature has been devised for the nineteen-carbon steroids (24), it has not yet accomplished such a displacement. Its major influence has been to encourage the spatial designation of functional groups previously included in trivial names. In keeping with the general trend, a mixture of trivial and systematic names is employed in this dissertation. For reference, the trivial names, systematic names, and structural formulae of all the nineteen-carbon steroids discussed are given in Table 3.

TABLE 3

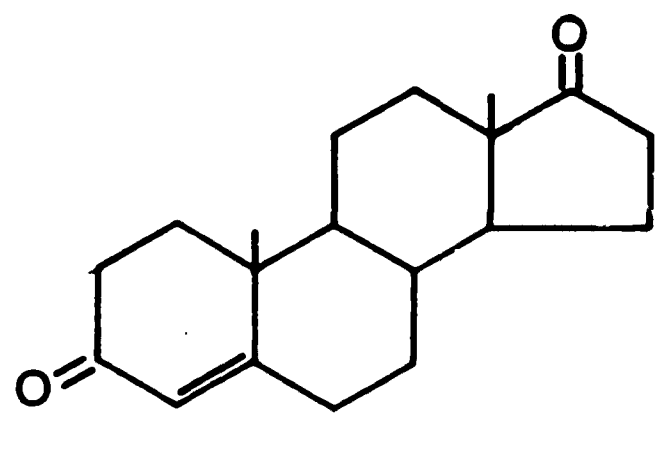
NOMENCLATURE OF ANDROGENS



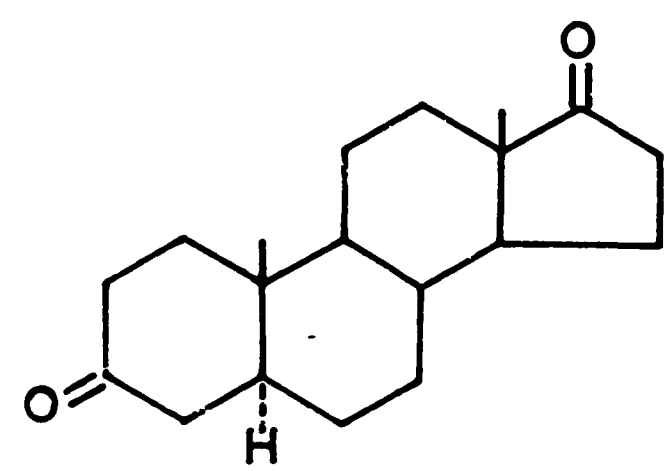
Testosterone
4-Androsten-17β-ol-3-one



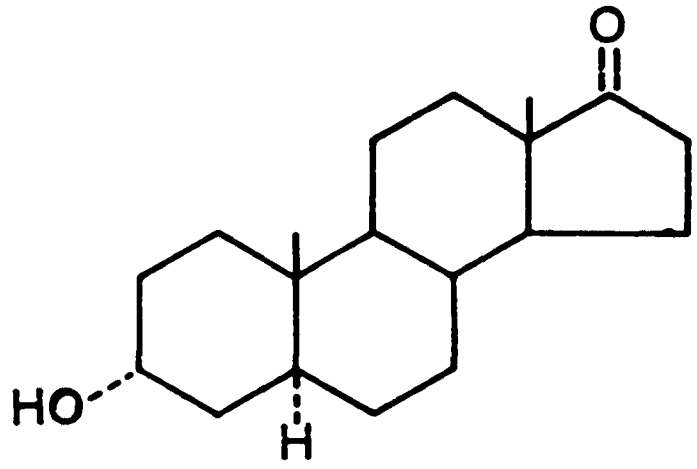
Epitestosterone
4-Androsten-17α-ol-3-one



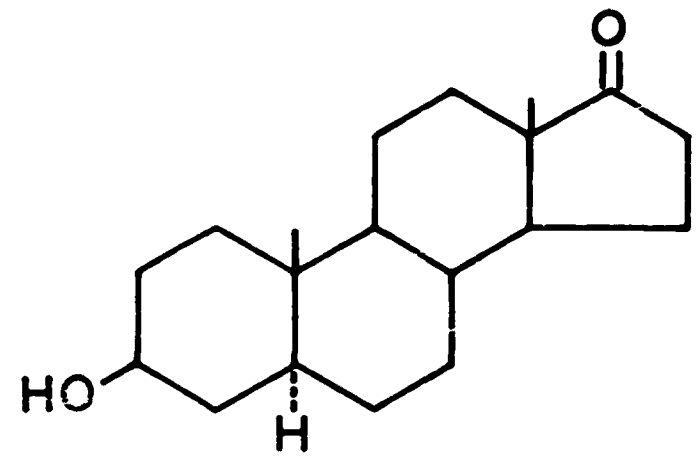
Androstenedione
4-Androstene-3,17-dione



Androstanedione
Androstane-3,17-dione

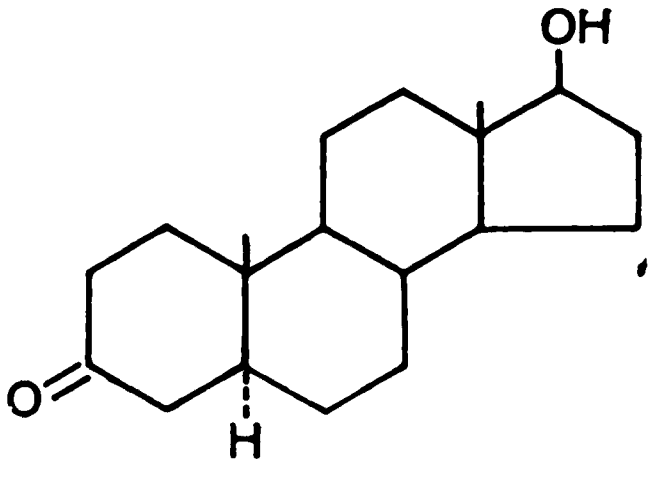


Androsterone
Androstan-3α-ol-17-one

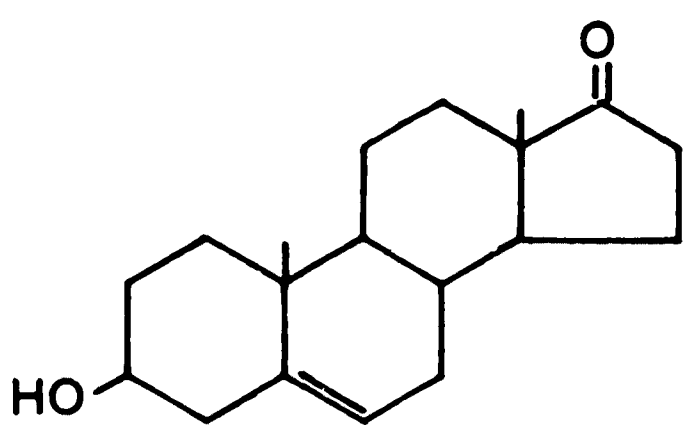


Epiandrosterone
Androstan-3β-ol-17-one

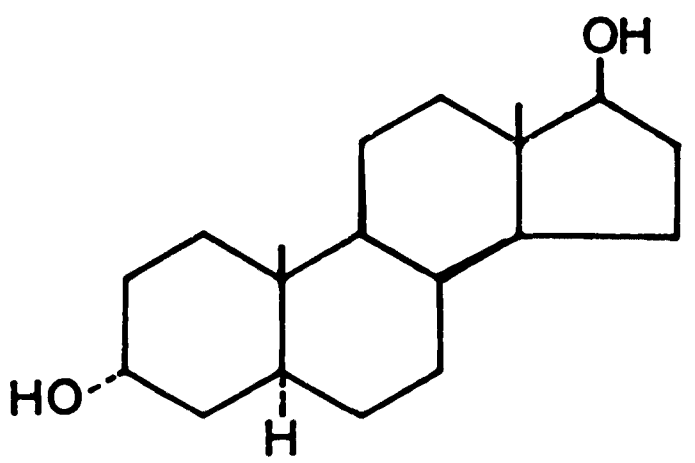
TABLE 3--Continued



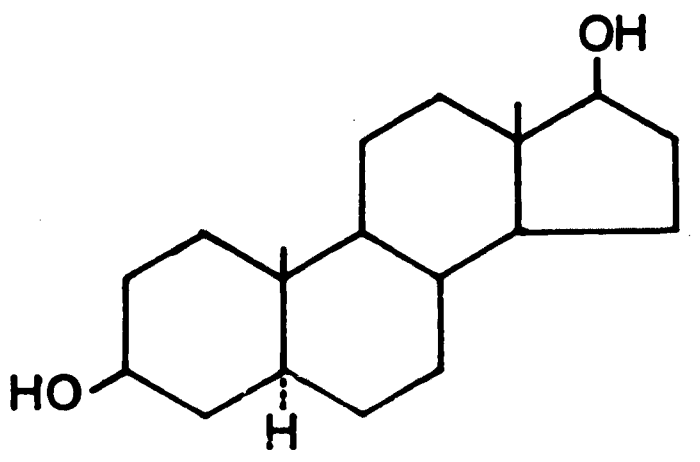
Androstanolone
Androstan-17β-ol-3-one



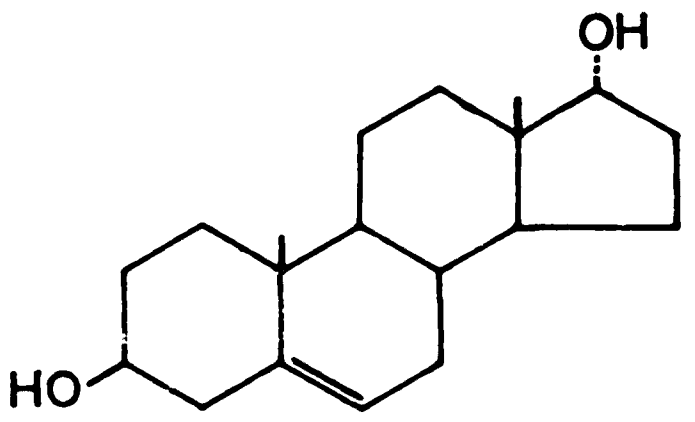
Dehydroepiandrosterone
5-Androsten-3β-ol-17-one



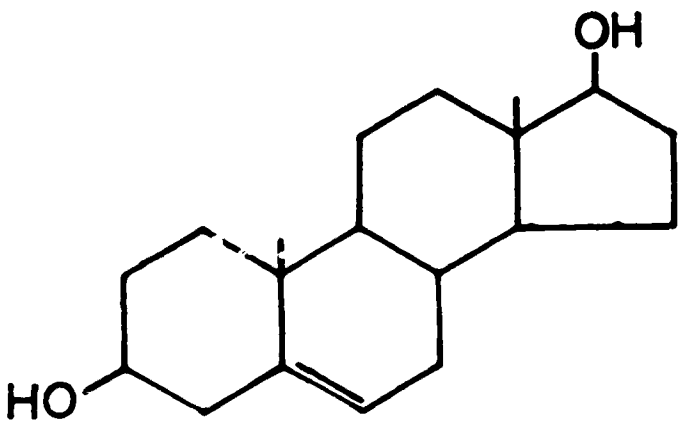
Androstanediol
Androstane-3α,17β-diol



Androstane-3β,17β-diol

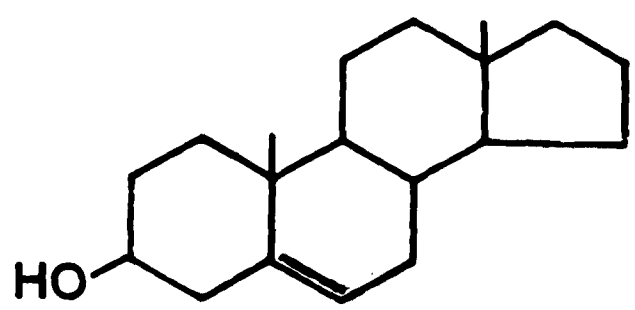


5-Androstene-3β,17α-diol

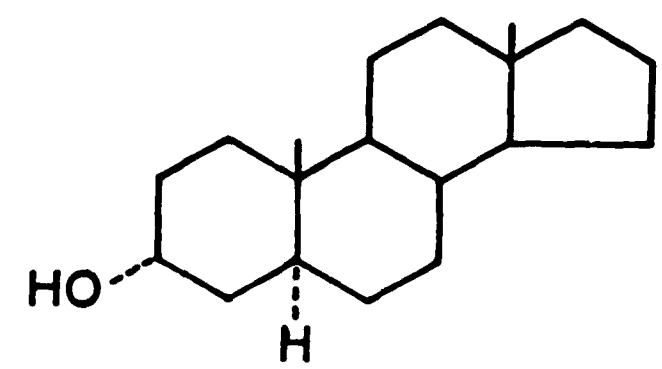


5-Androstene-3β,17β-diol

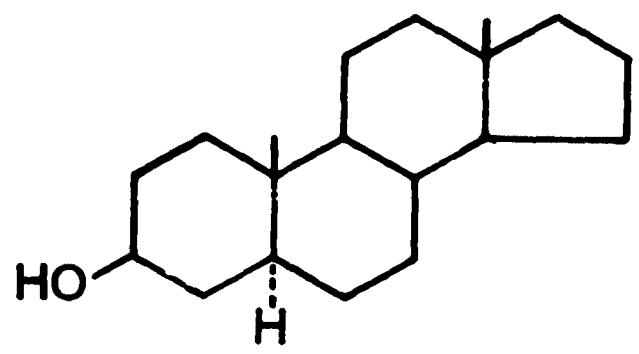
TABLE 3--Continued



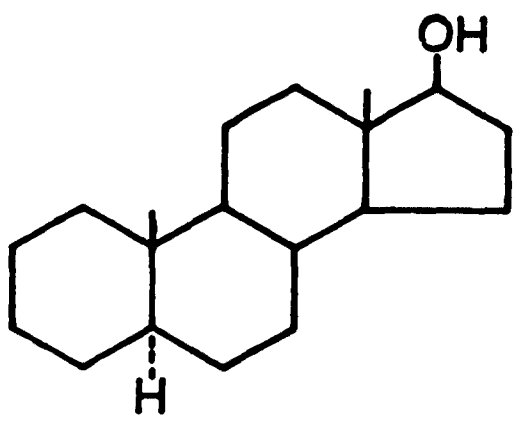
5-Androsten-3β-ol



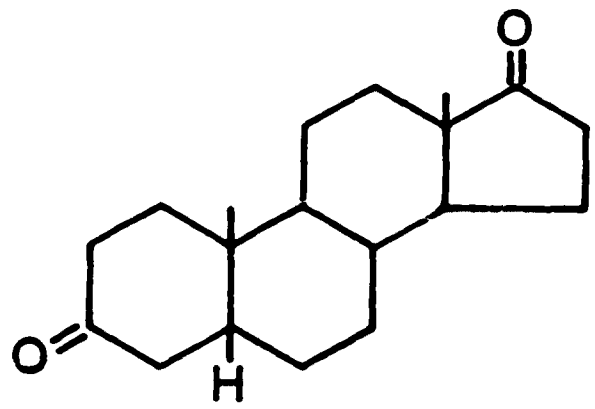
Androstan-3α-ol



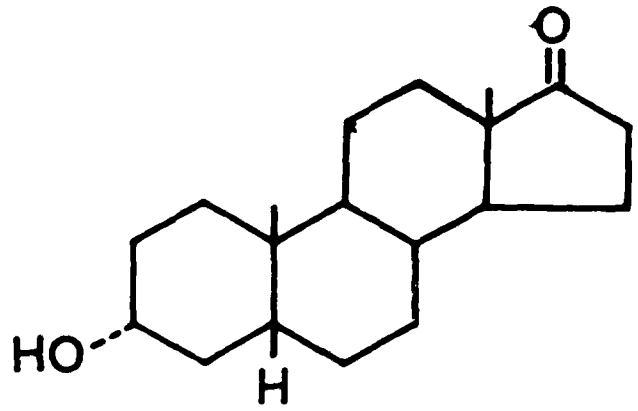
Androstan-3β-ol



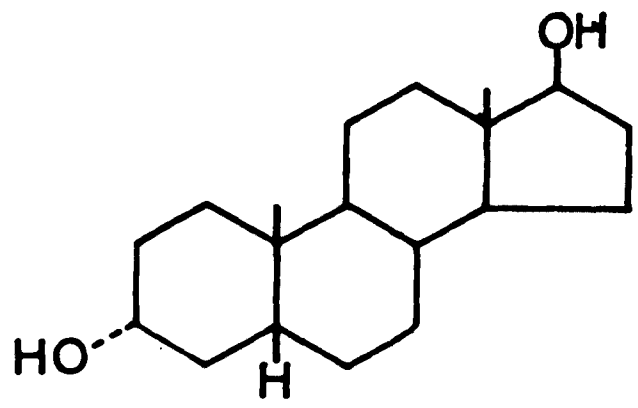
Androstan-17β-ol



Etiocholanedione
Etiocholane-3,17-dione



Etiocholanolone
Etiocholan-3α-ol-17-one



Etiocholane-3α,17β-diol

CHAPTER II

EXPERIMENTAL PROCEDURES EMPLOYED FOR THE STUDY OF THE METABOLISM OF THE ANDROGENS

The various methods which have been employed in an effort to add to the knowledge of the metabolism of the androgens are, for the most part, used similarly for the study of the other steroid compounds. A brief survey of these methods follows.

In vivo studies of the metabolism of the androgens. Probably the first approach used in the study of the metabolism of the androgens involved the administration, primarily to man, of large quantities of the steroids selected for study. After a suitable period of time, the urines of the subjects were subjected to procedures originally employed for the isolation of steroid materials from urine, or to one of various modifications of such procedures. Several reviews of the methods used for the isolation of urinary androgens have been published in recent years (25-29). Such experiments sought not only to demonstrate the principal metabolites of the steroids administered, but also to reveal the presence of trace metabolites which could not be

recognized at the levels in which they normally occurred. The research groups directed by Dorfman and by Dobriner have been the most active in this area.

The following examples are typical of the experimentation in this area: Callow (30) and Dorfman and Hamilton (31) observed increases in the amounts of androsterone[1] and etiocholan-3α-ol-17-one[1] isolable from the urines of hypogonadal males following the administration of testosterone propionate. After the administration of 90 mg. of testosterone daily for forty-five days, Dobriner and Lieberman (32) were able to account for 50% of the injected steroid as an increase in excretion over control levels: 24% as androsterone, 19% as etiocholanolone,[1] 1% as androstanedione[1] and etiocholanedione,[1] and 6% as androstane-3α, 17β-diol[1] and etiocholane-3α,17β-diol.[1] In conjunction with studies of this nature, a systematic investigation of the ketosteroids of normal and pathological urines led to the isolation of forty-two crystalline steroid products, eleven of which had been isolated previously (33,34).

This approach to the study of steroid metabolism is subject to several disadvantages. Principal among these is the possibility that the injection of large excesses of steroid compounds may lead to alterations in the normal routes of metabolism.

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[1]See Table 3.

A variation of the approach just outlined involves the use of steroids which contain isotopic atoms. As Twombly (21) has pointed out, the use of radioactively-labelled steroids is eminently practical for the study of steroid metabolism: "The labelled steroid appears to be chemically identical with the natural one and behaves in the same way; it can be followed in the course of its metabolism without the necessity of complete isolation and identification at every step, and the sensitivity of the methods of detection are such that minute quantities within the range of normal physiological doses are still detectable and their fractions isolatable."

Not only have steroids containing C^{14}, usually at carbon atom number 4, been synthesized and utilized subsequently in metabolic studies, but also many compounds containing non-radioactive deuterium have been used in parallel fashion; the utilization of the two series of compounds varies, in principle, only in instrumentation.

In experiments which typify this approach to steroid metabolism, Gallagher et al (35) obtained deuterium-containing androstanedione, etiocholanedione, androsterone, and etiocholanolone by urinary isolation procedures after the injection of 100 mg. of deuterium-labelled testosterone into a normal male; the excretion of androsterone and etiocholanolone accounted for 26% and 10%, respectively, of the isotopic testosterone. Administration of deuterium-labelled

androstenedione[1] resulted in the recovery of 43% of the deuterium in androsterone and etiocholanolone in equal proportions (35).

In vitro studies of the metabolism of the androgens. In vitro studies of the metabolism of steroids may be divided into three areas on the basis of the approaches used in such studies. Perfusion studies, although not used extensively with androgens, have had wide application in the steroid field, particularly in the realm of the corticoid hormones. Perfusion of adrenal glands has been developed by Pincus, Hechter, and their co-workers (36-40) to such an extent that this method represents one of the most important contributions to adrenal physiology and biochemistry (41). Typically, the steroids being 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. The workers indicated were able to identify fifteen steroid ketols in the perfusates of beef adrenals using paper chromatographic techniques. Danby (42) has performed perfusion experiments with testosterone.

A second approach to the study of the in vitro metabolism of steroid compounds involves the use of spectrophotometric methods of assay. Steroid compounds which contain an α,β-unsaturated ketone in ring A of the steroid nucleus absorb ultraviolet light; maximum absorption occurs at 241 mμ. in ethyl alcohol (43). By working at this

wavelength, it is possible to follow the formation or disappearance of compounds containing this system of conjugation. Samuels and his co-workers have developed such techniques for the study of the androgens, particularly testosterone and androstenedione. Using such techniques, these workers have demonstrated the enzymatic destruction of the α,β-unsaturated ketone system in ring A of testosterone by liver slices and minces (44). This destruction was enhanced by diphosphopyridine nucleotide (DPN) and citrate. Sweat et al. (45) have described the concentration of the enzyme using DPN by extending the techniques developed by Samuels. Talalay (46,47) has extended the knowledge concerning the enzymatic transformations of the androgens; spectrophotometric methods were employed, but the formation or disappearance of DPN, rather than the steroid compounds, was followed (at 340 mμ.) in these studies.

The spectrophotometric studies suffer from their inability to distinguish specific compounds; indeed, changes in transmission of a given wave length of light cannot be attributed absolutely even to steroid compounds, and certainly not to a specific steroid. Furthermore, only three androgens exhibit the structural features necessary for ultraviolet light absorption at 241 mμ; and the studies following the behavior of reaction mixtures at 340 mμ are even more indirect. Nevertheless, this approach has demonstrated a certain utility.

The spectrophotometric methods were preceded by incubation methods developed by Clark and Kochakian (48,49). Testosterone was incubated with liver tissue and the steroid compounds which resulted were isolated by procedures described subsequently. In addition to testosterone, a considerable amount of androstenedione was isolated, as well as a small amount of epitestosterone. This research represented the first demonstration of the action of liver tissue on testosterone in vitro.

Samuels (50) has voiced objections to this approach:

> Although the work of Clark and Kochakian gave specific qualitative information, it depended on actual isolation and identification by the methods of classical organic chemistry. This limited the experiments to grossly unphysiological concentrations of steroid and only gave approximations of quantitative change.

In spite of these objections, this method of approach has laid the foundation for the elaboration of the metabolism of the androgens. Because it applies the methods of classical organic chemistry for isolation and identification, it has been the sole definitive source in the determination of the qualitative metabolic pathways of the androgen compounds. Kochakian and his co-workers (51-56) have extended the investigation of the metabolism of testosterone to well over fifteen androgens.

Experimental Procedures Used in Incubation Studies

Two approaches were employed in order to define the

in vitro metabolism of the compounds studied. In the first of these, 25-100 mg. quantities of steroid were incubated with male guinea pig (and rat) tissue homogenates at 37-8° C. The volume of the incubation mixtures was approximately 100 ml. Metabolites and unconverted substrates were recovered, identified, and assayed. This is the approach developed by Kochakian. The second approach involved a spectrophotometric study of reaction mixtures containing tissue homogenates and quantities of steroid not exceeding 100 μg. These studies were carried out at room temperature. The volume of the reaction mixtures was 3 ml. in most experiments. Because of the small quantities of steroid materials involved, no isolation studies were performed in conjunction with the spectrophotometric studies. These studies resembled those of Samuels and Talalay. The procedures and techniques of the first of these methods are treated in this chapter; those involved in the spectrophotometric studies are discussed in Chapter V.

The procedures employed in the incubation studies included preparation of incubation materials, preparation of tissue homogenates, incubation, separation of tissue components, isolation, identification, and assay of steroidal materials, and characterization of steroidal metabolites formed. These procedures are described in turn.

Preparation of the incubation materials. The materials employed in the incubation reactions included solutions

of buffers, solutions of $DPNH_2$[2] and of FDP, the various steroids, DPN, NA, and NaDPNH.

Three buffer solutions were employed routinely as incubation media: 0.1 molar disodium phosphate, 0.166 molar sodium pyrophosphate, and Krebs-Ringer bicarbonate. The phosphate buffer was usually employed for studies in the pH range 5.0-7.5, the bicarbonate buffer for studies in the pH range 6.8-7.2, and the pyrophosphate buffer for studies in which the pH value was in the vicinity of 9.0. The phosphate and pyrophosphate solutions were adjusted to the desired pH value by the addition of concentrated hydrochloric acid. All pH values were determined with a Beckman Glass Electrode pH Meter, model H2. The Krebs-Ringer bicarbonate buffer solutions were prepared according to the specifications listed by Umbreit, et al. (57); in order to prepare 130 ml. of buffer solution, 21 ml. of sodium bicarbonate solution of appropriate concentration (for the desired pH value) is added to a solution containing 100 ml. of sodium

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[2]The following abbreviations are employed commonly in the literature and are so used in this dissertation:

$DPNH_2$	Reduced diphosphopyridine nucleotide
FDP	Fructose diphosphate
DPN	Diphosphopyridine nucleotide
NA	Nicotinic acid amide
NaDPNH	Sodium salt of $DPNH_2$
TPN	Triphosphopyridine nucleotide
$TPNH_2$	Reduced triphosphopyridine nucleotide
FAD	Flavin adenine dinucleotide

DPN and TPN are frequently designated Coenzyme I and II, respectively. Such terminology is also employed in this dissertation.

chloride solution, 4 ml. of potassium chloride solution, 3 ml. of calcium chloride solution, 1 ml. of primary potassium phosphate solution, and 1 ml. of magnesium sulfate solution. The calcium chloride solution is made up to a concentration of 0.11 molar; the remaining solutions are made up to a concentration of 0.154 molar. A gaseous mixture consisting of 95% oxygen and 5% carbon dioxide is passed through the solution for fifteen minutes prior to the determination of the pH value of the buffer system.

Normally the volume of buffer solution employed was 100 ml. In those experiments in which additives whose nature necessitated their introduction as solutions were employed, 50 ml. of double-strength buffer solution was diluted to normal strength by the addition of the additive-containing solution(s). Reagent grade materials and distilled water were used in all buffer preparations.

Several materials were added to the incubation mixtures which have been demonstrated to be involved in enzymatic transformations. Three such materials were introduced as solids in the state in which they were received. DPN was purchased from Pabst Laboratories. NA was purchased from General Biochemicals, Incorporated. NaDPNH, used in a single experiment, was purchased from Sigma Chemicals. The DPN was guaranteed to have a minimum purity of 95%. The NA was U. S. P. grade.

$DPNH_2$ solutions were prepared by reduction of DPN

using a modification of the method of Green and Dewan (58). Nitrogen is flushed through an aqueous solution containing DPN and sodium bicarbonate for fifteen minutes. A freshly prepared solution of sodium dithionite is added to the DPN solution and the passage of nitrogen is continued for fifteen minutes. The reaction is conducted at 38° C. Upon completion of the reaction, excess dithionite is removed by aeration. The reference cited suggests the use of DPN/bicarbonate and DPN/dithionite ratios of 1.2 and 3.0 (mg.:mg.), respectively. These ratios failed to give satisfactory yields of $DPNH_2$, perhaps due to the age of the dithionite employed. An investigation into the influence of these concentration ratios on the yield of $DPNH_2$ indicated that DPN/bicarbonate and DPN/dithionite ratios of 0.8 and 2.0, respectively, routinely gave yields of $DPNH_2$ of 90%. The concentrations of the bicarbonate and dithionite solutions employed were 1.0 and 0.4%, respectively. $DPNH_2$ concentrations were determined spectrophotometrically using a Beckman Quartz Spectrophotometer, model DU; this instrument was employed for all spectrophotometric assays. The extinction coefficient of $DPNH_2$ given in the literature is 6.22×10^6 (59).

FDP solutions were prepared from the barium salt of FDP by the method of Wald and Hubbard (60). The barium salt is dissolved in dilute hydrochloric acid; barium ion is precipitated as the sulfate by the addition of sodium sulfate; barium sulfate is separated by centrifugation and decantation;

and the resulting FDP solution is neutralized by the addition of dilute sodium hydroxide. These solutions of FDP were prepared within twenty-four hours of their use. The barium salt of FDP was purchased from Nutritional Biochemicals Company.

Steroid compounds were purified prior to incubation by means of adsorption chromatography on alumina. Following chromatography, the state of purity was determined by means of paper chromatography and infrared spectrophotometry. Upon demonstration of purity by these methods, the compounds were crystallized repeatedly from appropriate solvent systems until melting points were obtained which were consistent with those which have been reported in the literature. Diketones were recrystallized from acetone solutions by the addition of water. Monohydroxy monoketones and the dihydroxy compounds were recrystallized by cooling hot benzene solutions of the steroid compounds to which small amounts of absolute ethyl alcohol had been added. Monohydroxy compounds were recrystallized by cooling hot cyclohexane solutions of such compounds. Melting points were obtained using a Fisher-Johns hot stage which had been modified for use in conjunction with a microscope. The melting points[3] of the steroids studied are included in Table 6.

The chromatographic and spectrophotometric techniques

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[3]Literature values.

are discussed in more detail in connection with the isolation procedures. Infrared spectra were obtained with a Perkin-Elmer Infrared Spectrophotometer, model 21.

Preparation of tissue homogenates. Male adult guinea pigs were employed in all but one series of experiments. Upon arrival, the weight of the animals was usually between 500 and 600 grams; when their body weight reached 800-1100 grams, the animals were employed in the metabolism studies. Their diet consisted of commercial rabbit pellets (Superior Feed Company) given ad libitum and 30-40 g. of carrot given twice weekly. One 25 mg. ascorbic acid tablet (Lilly) was given orally twice weekly. The animals were housed in a constant temperature room (72 $\pm$ 1° Fahrenheit) in which the lighting was regulated automatically. The animals were sacrificed by a blow at the base of the skull, and thorough bleeding was induced by severing the jugular vein. Fur was removed by shaving in the areas in which incisions were to be made, and these areas were thoroughly rinsed with 70% aqueous ethyl alcohol in order to minimize bacterial contamination. The desired tissues were removed, weighed, and stored in separate, covered, ice-cold beakers. Usually more than one animal was required, and the tissues were cut into small pieces and mixed intimately to reduce the effects of animal variation. Samples of the desired size, usually twelve grams, were weighed out and transferred to one-quart-capacity, ice-cold Waring blendor jars. In the

original experiments, weighed samples of steroid were introduced into the blendor jars, and the tissue and the steroid were homogenized with approximately 40 ml. of cold buffer solution. Investigation indicated that the metabolism occurring during homogenization was atypical with regard to that occurring during the incubation proper, and only the tissue and buffer solutions were homogenized in subsequent experiments. Originally a two minute homogenization period was employed; this was reduced to twenty seconds in later experiments. Fernbach flasks with a capacity of 2800 ml. were used as incubation vessels. The homogenates were transferred to these flasks by rinsing with the remaining buffer solution. Steroids and additives were then introduced into the flasks and the incubation was initiated. The removal of the steroids from the homogenization step improved recoveries, in that the rather difficult quantitative transfer from the blendor jar to the incubation flask was eliminated.

Rat liver was employed in one series of experiments. One female rat was employed inadvertently. The tissues were handled in the manner just outlined.

One series of experiments was designed to determine the distribution of enzymatic activities following centrifugal separation of homogenates. Homogenates were centrifuged at 1500 times gravity for five minutes at 3-8° C. in an International Refrigerated Centrifuge, model PR-2. The supernatants were decanted into one set of incubation flasks. The

sediments were resuspended in buffer solution, diluted to the volume of the original homogenates, and introduced into a second set of incubation flasks.

Incubation. A specially designed water bath was employed for the incubations; this apparatus and the incubating procedure has been described by Nall (61). Originally the incubations were conducted for two and one-half hours; this period was shortened to one and one-half hours in later experiments. The incubation flasks and their contents were maintained at a temperature of 37-8° C. throughout the incubation period. In those experiments in which the bicarbonate buffer solutions were employed, a gaseous mixture consisting of 95% oxygen and 5% carbon dioxide was introduced into the incubation flasks just above the surface of the mixtures at a rate of 0.4-0.5 liters per minute throughout the incubation period. In anaerobic experiments, the incubation flasks were flushed with nitrogen for fifteen minutes prior to the introduction of the homogenates, for ten minutes prior to the introduction of the steroid and additives, and throughout the incubation using the flow rate indicated above. The incubation flasks were stoppered with cotton plugs to prevent external contamination. Stable pH values were not maintained using bicarbonate buffer solutions in the anaerobic experiments, since there was no provision for the introduction of carbon dioxide. Therefore, only the phosphate and pyrophosphate buffer solutions were employed in

the anaerobic experiments. At the conclusion of an incubation, samples of the incubation mixtures were withdrawn for the determination of the final pH values of the mixtures. After these samples had been returned to their respective incubation vessels, 800 ml. of ethyl alcohol was added to each flask to terminate the reactions by the precipitation of protein. Incubation experiments were usually performed in groups of six, since the water bath was designed to hold six flasks.

In one series of experiments all components of the incubation mixtures were preincubated for fifteen minutes prior to the introduction of steroid. These experiments were performed simultaneously with experiments which were comparable except that only the homogenates were preincubated.

Separation of tissue components. Tissue components were separated using the method described by Nall (61). The alcoholic solutions of the incubation mixtures are heated to boiling and filtered while hot. Filtration is facilitated by suction and by the use of a filter aid. The protein residues are washed several times with 100 ml. portions of hot ethyl alcohol to insure complete removal of steroid. The alcohol present in the filtrates is removed in vacuo, and the residues resulting are extracted five times with 200 ml. portions of peroxide-free ethyl ether. The ether extracts are combined, dried over sodium sulfate, filtered into round-bottom flasks, and the ether evaporated. Moisture is removed

by vacuum desiccation. The dried extracts are transferred to centrifuge bottles and dissolved in 10 ml. of ether. Precipitation of phospholipids is effected by the addition of 180 ml. of dry acetone with stirring. After centrifugation and separation by decantation, the residues are redissolved in ether and the precipitation is repeated twice. The acetone fractions of each sample are combined and evaporated to dryness.

Good phospholipid precipitations were obtained only infrequently by this method. This had been attributed to the presence of traces of water and numerous methods were employed to effect the removal of this component. These included solvent evaporation in the presence of benzene, and prolonged vacuum desiccation in the presence of various drying agents. Special precautions were taken to insure the absence of water in the solvents which were employed. These procedures, used singly or in combination, failed to improve the precipitation of the phospholipid material.

The obviation of this difficulty was accomplished quite by accident. Occasionally a few drops of a solution of magnesium chloride in anhydrous ethyl alcohol were added to the solution of ether and acetone as a last resort to effect precipitation. This procedure was avoided when possible, since the precipitated material could not be dissolved subsequently for reprecipitation purposes. However, in one series of experiments no attempts were made to remove the

last traces of water prior to precipitation; the use of magnesium chloride was intended. All samples gave optimal precipitations upon the addition of acetone. Investigation indicated that a small amount of water (1-2 ml.) was necessary for such optimal precipitations. The ether fractions may be evaporated until the residues are semi-solid, cooled, one ml. of water added, and the acetone added to the mixture. The last stages of the evaporations are facilitated by directing streams of air on the surfaces of the samples.

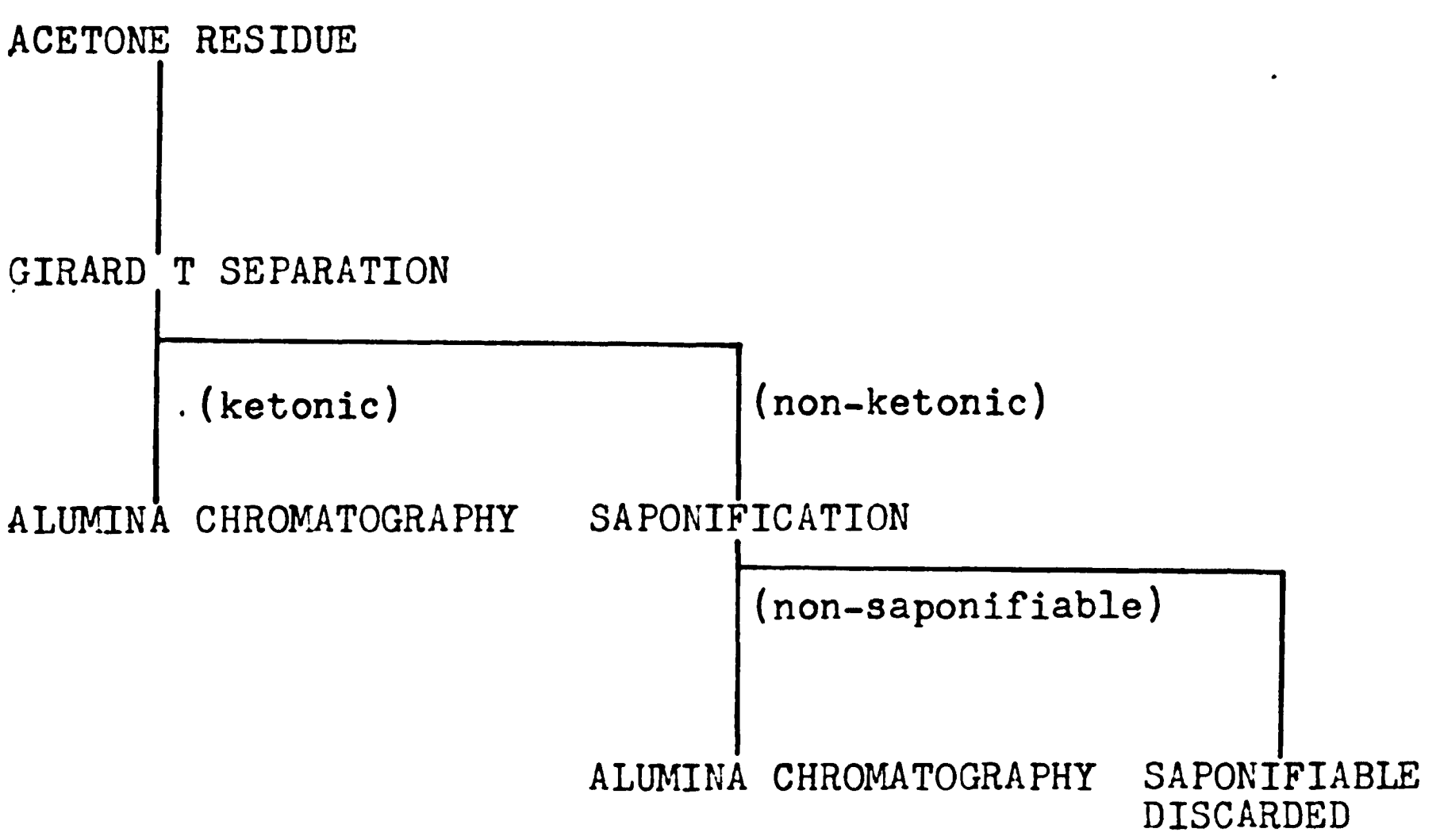
The altered phospholipid precipitation procedure produced several beneficial results in addition to the more effective removal of phospholipids. Any treatment designed to eliminate water produced either a loss of steroidal material, a loss of time, or both. When radioactive testosterone was employed by Nall (61), adsorption occurred on the sodium sulfate used for drying purposes, and on the filter paper used for sodium sulfate removal. Evaporation in the presence of benzene was attended by discoloration of the sample and by loss of steroidal material.

Isolation of steroid materials. In the original experiments, the procedure of Nall (61) was employed to isolate the various steroids resulting from the incubations. The flow sheet shown in Figure 1 is reproduced from the reference cited.

This procedure is laborious, time-consuming, and gives rise to loss of steroidal materials. The latter was

FIGURE 1

ISOLATION OF STEROIDS FROM ACETONE RESIDUES



demonstrated by Nall (61) in studies with radioactive testosterone. It had been demonstrated previously that the Girard separation frequently destroys as much as 15% of the steroidal material subjected to the separation procedure (62). Furthermore, in the majority of the experiments performed, endogenous cholesterol was the only non-ketonic steroidal material isolated. For these reasons it seemed desirable to simplify the isolation procedures.

The first attempt in this direction involved chromatographic separation of the residues obtained by evaporation of the acetone fractions resulting from phospholipid precipitations. Alumina chromatography was employed in the manner developed by Nall (61). The alumina (Harshaw, activated catalyst grade, Al-0109P) is washed with distilled water, adjusted to the desired acidity (usually pH 7.3-7.4) with sulfuric acid, rinsed with distilled water until free of sulfate ion, dried, rinsed with methanol, redried, and heated at 110° C. for two to five hours. Fifteen to thirty-five g. of alumina were used for each column, the appropriate amount being determined by the weight of the residue which was to be chromatographed. Glass columns with internal diameters of ten and twenty millimeters were used in most of the experiments. The residues were dissolved in minimal volumes of solvent and introduced into the columns. The steroids were then eluted using a solvent sequence of carbon tetrachloride, carbon tetrachloride solutions containing

benzene, benzene, benzene solutions containing ethyl ether, ethyl ether, ethyl ether solutions containing absolute ethyl alcohol, and methyl alcohol. Using this system of chromatography, it was observed that the acetone residues could be separated routinely into the fractions indicated in Table 4.

Because no other steroid isolation procedure had been employed, the intermediate fractions rarely consisted of pure steroids or steroid mixtures; usually these fractions contained 50-90% steroidal material. The non-steroidal materials were removed easily, however, by the technique of strip paper chromatography developed by Kochakian and Stidworthy (52). The fractions are dissolved in a small volume of solvent and transferred quantitatively with a micropipet to a 6 inch by 22½ inch strip of Whatman number 1 filter paper which has been impregnated with a 1:1 (by volume) mixture of methyl alcohol and propylene glycol; the steroid solution is placed on a line two and one-half inches from the top of the paper. The chromatograms are developed with a 1:1 (by volume) mixture of benzene and cyclohexane in glass tanks in which the air has been saturated with the vapors of these solvents. Development usually requires four hours for fraction 2,[4] six hours for fractions 3 and 4, and sixteen to twenty hours for fractions 5 and 6. After development, steroid zones on the chromatograms are detected by

[4] See Table 4.

TABLE 4

ELUTION BEHAVIOR OF INCUBATION RESIDUES[a]

Fraction	Eluant	Composition
1	Carbon tetrachloride	Non-steroidal materials; usually highly colored
2	Carbon tetrachloride and benzene	Endogenous cholesterol; monohydroxysteroids
3	Benzene	Diketone steroids
4	Benzene and ethyl ether	Monohydroxy monoketone steroids
5	Ethyl ether and ethyl ether and ethyl alcohol	Dihydroxysteroids
6	Methyl alcohol	Non-steroidal materials; frequently water soluble

[a]See page 32 *et seq.* for chromatographic procedure.

means of color reactions which are discussed in detail in the section pertaining to steroid identification; the color reactions are applied to narrow strips cut from the edges of the chromatograms. The steroid zones are cut from the chromatograms, thoroughly dried to remove propylene glycol, and extracted three to five times with hot ethyl alcohol. Frequently the non-steroidal materials are either immobile or move with the solvent front; this facilitates their separation.

The chromatographic separation into the fractions indicated was enhanced by the modified phospholipid precipitation procedure discussed previously.

After the removal of non-steroidal materials, there remained the separation of the steroids within the various fractions. The compounds studied are grouped according to their elution behavior in Table 5. It would appear that the separation obtained left much to be desired. Actually this was not the case, since the complex mixtures indicated in Table 5 were never encountered in the incubation studies. In the majority of the experiments, the steroid mixture resulting from incubation consisted of endogenous cholesterol, unconverted substrate, and from one to three metabolites. Furthermore, fractions 3, 4, and 5 represent three levels of oxidation, and metabolic conversions always resulted in change of oxidation state; that is, unconverted substrate and metabolic products always occurred in different fractions

TABLE 5

ELUTION BEHAVIOR OF ANDROGENS[a]

Fraction	Steroids Eluted
2	Endogenous cholesterol
	Androstan-3α-ol
	Androstan-3β-ol
	5-Androsten-3β-ol
	Androstan-17β-ol
3	Androstenedione
	Androstanedione
4	Androsterone
	Epiandrosterone
	Dehydroepiandrosterone
	Androstanolone
	Testosterone
	Epitestosterone
5	Androstane-3α,17β-diol
	Androstane-3β,17β-diol
	5-Androstene-3β,17α-diol
	5-Androstene-3β,17β-diol

[a]See page 32 et seq. for chromatographic procedure.

after chromatographic separation. The most complex mixture encountered consisted of four steroids. Various means were employed to effect intra-fraction separations. In this connection it should be noted that the Girard separation would be ineffective in such intra-fraction separations; the mixtures indicated do not result from the omission of this separative procedure since it is employed to separate ketones from non-ketones (63).

In the various experiments, fraction 2 contained only cholesterol or cholesterol and one additional steroid. Cholesterol is conveniently separated from androstan-3α-ol and androstan-17β-ol by precipitation with digitonin (64). Digitonin selectively precipitates those steroids which have a 3-β-hydroxyl group. Separation of cholesterol from the other compounds occurring in fraction 2 is possible by careful alumina chromatography.

Androstanedione and androstenedione may be separated by strip paper chromatography (52). This separation is facilitated by the ultraviolet light absorption behavior of the latter compound; its presence on paper chromatograms can be detected using a chromatogram scanner designed for use with a source of ultraviolet light (65).

The most complex mixtures were encountered in fraction 4. Epiandrosterone occurred in conjunction with androsterone and androstanolone. It can be separated from both of these compounds by the digitonin procedure (64). No losses

are incurred in the digitonin procedure, other than the losses of manipulation, since the steroids which are precipitated by digitonin may be regenerated from their insoluble digitonide complexes by the addition of pyridine (66). However, a mixture of the three compounds indicated is more conveniently separated by means of strip paper chromatography (52). Dehydroepiandrosterone occurred in conjunction with testosterone and epitestosterone. The digitonin procedure (64) is applicable in both instances, but strip paper chromatography (52) is more convenient in these separations also, particularly since both testosterone and epitestosterone exhibit the same ultraviolet light absorption behavior as does androstenedione (43).

Only pairs of steroids were encountered in fraction 5. These pairs were separated by rapid but refined column chromatography.

When the elution pattern of residues consisting of a given mixture of steroids had been defined for a given batch of alumina, only the fractions described were collected. In this fashion, extensive pooling of eluate fractions was avoided and the isolation procedure thereby abbreviated further. Prior to such definition of elution pattern, 100 ml. fractions were collected. The solvent mixtures appropriate for optimal separations were then determined by infrared spectrophotometry. Elimination of extensive pooling had the further advantage of resulting in increased steroid

recoveries.

Identification of steroidal materials. Two techniques were employed to define the steroidal materials present in the fractions obtained from alumina chromatography. The paper partition chromatographic method of Kochakian and Stidworthy has been described (52). For identification purposes (67), aliquots of steroidal samples corresponding to 0.5 mg. of solid are placed on spots one inch apart on the paper strips described previously. An appropriate mixture of known steroid compounds is placed on the middle spot. Elution is effected in the manner previously described. Separate papers are prepared for the detection of ketonic and non-ketonic steroids. After development, the chromatograms are dried in an oven or with a heating fan. Androstenedione, testosterone, and epitestosterone are detected with the ultraviolet scanner mentioned above. Two spray reagents are employed to detect ketonic steroids. The first consists of a mixture of equal volumes of 15% alcoholic potassium hydroxide and a 2% solution of m-dinitrobenzene in ethyl alcohol (68,69). The second consists of a solution containing one gram of 2,4-dinitrophenylhydrazine and ten ml. of concentrated hydrochloric acid per liter of ethyl alcohol. Color development is facilitated by heating when m-dinitrobenzene is employed; and stronger contrast is obtained with the dinitrophenylhydrazine reagent if excess reagent is removed by immersion in a water bath containing a little acid;

phenylhydrazones formed by ketones are insoluble in aqueous acid solutions. A chromatogram may be developed with both reagents if m-dinitrobenzene development is followed by copious spraying with a solution of 5% concentrated hydrochloric acid in ethyl alcohol, drying, and dinitrophenylhydrazine development.

Hydroxysteroids are conveniently detected by spraying the chromatograms with a 2% solution of phosphomolybdic acid in ethyl alcohol (70).

In addition to the characteristic colors exhibited by the various steroid compounds, the distance moved during paper chromatographic separation is also a valuable tool in the identification of these compounds. Each steroid possesses a characteristic R_T value which has been defined (67) as the ratio of the distance moved in a given period of time divided by the distance moved by testosterone in the same period of time.

The colors exhibited toward the various spray reagents are also useful criteria for identification (67). These colors and the R_T values of the various steroids studied are included in the data presented in Table 6.

The second technique employed for steroid identification was that of infrared spectrophotometry. Dobriner, Katzenellenbogen, and Jones have conducted an exhaustive survey of the infrared spectra of the steroid compounds, and an excellent catalog of these spectra has been published (71).

In addition, many analyses of these spectra have been reported (72-78). This method of detection and identification proved to be an extremely valuable tool, particularly in those instances in which only traces of material were available.

Steroid assay. Several methods have been devised for the assay of steroidal materials. Compounds containing an α,β-unsaturated ketone, namely androstenedione, testosterone, and epitestosterone, were assayed spectrophotometrically at 241 mμ. (43). Ketonic steroids other than these three were assayed by a modification (79) of the Zimmerman reaction, which employs m-dinitrobenzene as a color-developing reagent. Dihydroxy steroids were assayed by the Pincus reaction (80), which employs antimony trichloride as a color-developing reagent. No method has been published for the assay of monohydroxy steroid compounds. Since these compounds were studied in only a few experiments, they were assayed by weight.

Characterization of metabolites formed. In addition to the highly characteristic information about the steroid metabolites obtained by paper chromatography and infrared spectrophotometry, melting points of the pure compounds and of their oxime and acetate derivatives were obtained in many instances. In those instances in which sufficient material was available for these procedures, melting points and mixed melting points of the compounds obtained were observed to

agree with the literature values of the compounds which had been indicated by the chromatographic and spectrophotometric procedures. The literature values of the melting points which were obtained are included in Table 6.

Control experiments. Control experiments in which the tissue was inactivated by heating at 100° C. for fifteen minutes were frequently performed in a fashion parallel to that of the regular experiments.

Formation of non-steroidal metabolites. No attempts were made to demonstrate the formation of any metabolites in which the steroid nucleus had been altered or destroyed.

TABLE 6

PROPERTIES USED FOR THE CHARACTERIZATION OF ANDROGENS

Compound	M.p.[a]	R_T[b]	UV[c] 254 mμ.	MDNB[d]	DNPH[e]	PMA[f]
Androstane-3β,17β-diol	164	0.15	-	-	-	blue
5-Androstene-3β,17β-diol	184	0.20	-	-	-	blue
5-Androstene-3β,17α-diol	197-8[g]	0.32	-	-	-	blue
Androstane-3α,17β-diol	223	0.45	-	-	-	blue
Testosterone	155	1.00	+	blue	red-orange	green
Epitestosterone	201	1.35	+	blue	orange	green
Epiandrosterone	175	1.66	-	violet	yellow	green
Dehydroepiandrosterone	153	1.73	-	violet	yellow	green
Androstanolone	181	2.08	-	blue	yellow-or.	green
Androsterone	183	2.72	-	violet	lt. yellow	green
5-Androsten-3β-ol	137[g]	4.60	-	-	-	green
Androstenedione	174	5.80	+	violet	yellow-or.	-
Androstan-3α-ol	147-8[g]	6.85	-	-	-	green
Androstan-3β-ol	148[g]	8.34	-	-	-	green
Androstanedione	133	8.40	-	violet	yellow	-
Androstan-17β-ol	152-3[g]	front	-	-	-	green
Cholesterol	149	front	-	-	-	green
Androsterone oxime	215	-	-	-	-	-
Androsterone acetate	165	-	-	-	-	-
Epiandrosterone oxime	185-6[g]	-	-	-	-	-
Epiandrosterone acetate	96-7[g]	-	-	-	-	-

[a]Unless indicated, melting points are from Fieser (81).
[b]See page 42 for definition of R_T.
[c]Ultraviolet light absorption properties.
[d]Behavior towards m-dinitrobenzene spray reagent.
[e]Behavior towards dinitrophenylhydrazine spray reagent.
[f]Behavior towards phosphomolybdic acid spray reagent.
[g]See reference (82).

CHAPTER III

THE METABOLISM OF ANDROSTANEDIONE BY GUINEA PIG TISSUE HOMOGENATES

Guinea pig liver and kidney homogenates metabolize androstenedione to testosterone, small amounts of androsterone, and trace amounts of epiandrosterone and androstanedione (52). Androstane-3α,17β-diol is metabolized by the liver and the kidney of the rat, guinea pig, and rabbit to androsterone, epiandrosterone, and androstanedione (55). Androsterone, in turn, is oxidatively metabolized to androstanedione by guinea pig liver homogenates and epiandrosterone is converted to both androstanedione and androsterone (53). Similarly, androstanolone is oxidatively metabolized to androstanedione by guinea pig liver homogenates (53). Furthermore, androstanedione has been postulated as the precursor of urinary androsterone and epiandrosterone in man (83). Gallagher (35) has confirmed the androstanedione-androsterone relationship; administration of deutero-androstanedione resulted in the recovery of 24% of the deuterium in urinary androsterone. Androsterone is the principal androstane derivative excreted in the urine of man (83). Epiandrosterone

is the principal androstane derivative occurring in the urine of the guinea pig (84). These inter-relationships are summarized in Figure 2. It would appear that androstanedione occupies a principal role in the intermediary metabolism of the androgenic steroids.

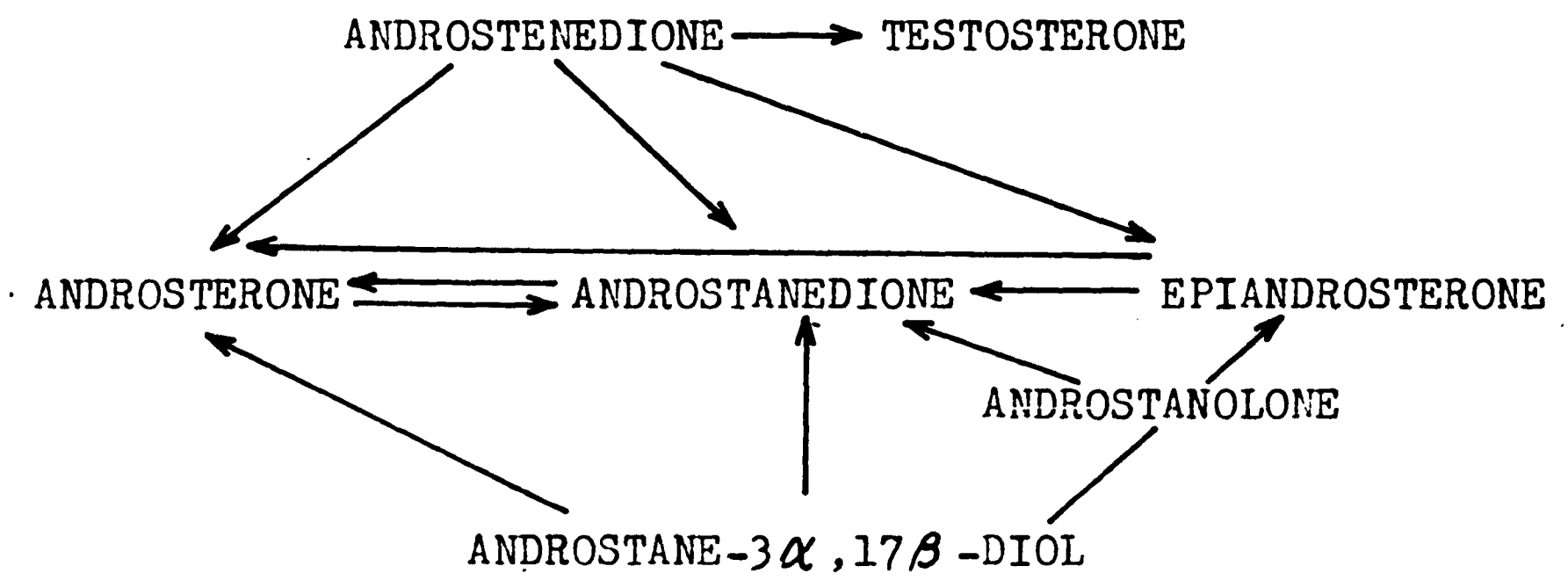
In order to partially define the metabolism of androstanedione, incubation studies were performed using tissue homogenates of male guinea pig liver, kidney, heart, and femoral muscles. Four experiments were performed with fractions obtained by centrifugation of guinea pig liver homogenates. In addition, three experiments were performed with rat liver.

Metabolism by Guinea Pig Liver Homogenates

A total of fifty-nine incubation experiments were performed using guinea pig liver homogenates in order to study the effects of a number of variables which have been demonstrated to influence enzymatic transformations. Androsterone and epiandrosterone were observed as metabolites in each of the experiments. Androstanolone was observed as a third metabolite in those experiments in which the level of metabolism was relatively high. Androstane-3α,17β-diol was detected by paper chromatography in a few experiments; however, the formation of this last compound was never confirmed by infrared spectrophotometry. The extent of metabolic conversion was observed to be dependent upon pH, the

FIGURE 2

METABOLIC PATHWAYS INVOLVING ANDROSTANEDIONE



concentration of the physiological materials DPN, $DPNH_2$, NA, and FDP, and the nature of the gaseous phase employed during incubation.

Table 7 lists the weights of androstanedione, DPN, $DPNH_2$, FDP, and NA used, the buffer solutions employed, and the pH value at the beginning of the incubation period. It also gives the amounts of androstanedione recovered and the amounts of the various metabolites which were formed. The tabular sequence is essentially the order in which the experiments were performed.

Influence of pH. The initial series of experiments was designed to determine the optimum pH for the metabolism of androstanedione. Variation of the pH of the incubation medium between 6.8 and 8.2 (experiments 1-12) failed to produce any significant changes in the conversion of the substrate. This was somewhat surprising since enzymes exhibit pH optima almost without exception. Two possible explanations of the observed behavior may be advanced; either the behavior of one or two enzyme systems (responsible for the metabolism of androstanedione) is masked by the presence of a multitude of enzyme systems in the homogenate; or any differences in the metabolic conversion of androstanedione under the conditions employed are beyond the limits of detection by the methods used.

The experiments did give insight into the nature of the metabolites to be expected. Reduction of androstanedione

TABLE 7

METABOLISM OF ANDROSTANEDIONE BY GUINEA PIG LIVER HOMOGENATES[a]

Expt. no.	Steroid wt. mg.	DPN wt. mg.	$DPNH_2$ wt. mg.	FDP wt. mg.	Buffer and pH	Androstanedione recovered %	Metabolites recovered % Epiandrosterone	Androsterone	Androstanolone
1.	100	-	-	-	KRB[b] 6.9	71.8	0.7	0.3	-
2.	100	-	-	-	" 7.0	70.7	0.6	0.3	-
3.	100	-	-	-	" 7.45	85.6	0.7	0.2	-
4.	100	-	-	-	" 7.95	77.8	0.8	0.4	-
5.	100	-	-	-	" 8.2	71.5	0.5	0.3	-
6.[d]	100	-	-	-	" 8.2[c]	57.0	0.4	0.4	-
7.	100	-	-	-	" 7.45	70.4	-	-	-
8.	100	-	-	-	" 7.45	88.8	0.8	0.4	-
9.	100	-	-	-	" 6.8	84.6	0.5	0.5	-
10.[d]	100	-	-	-	" 8.2[c]	82.8	0.6	0.4	-
11.	100	-	-	-	" 7.1	73.9	-	-	-
12.[e]	100	-	-	-	" 7.4	79.3	1.2	0.4	-
13.[f]	100	-	-	-	" 7.1	69.0	0.6	0.3	-
14.[g]	100	-	-	-	" 7.1	75.2	1.3	0.4	-
15.[h]	100	-	-	-	" 7.1	69.1[i]	1.5	0.3	-
16.[d]	100	-	-	-	" 7.1	45.8	1.3	0.4	-
17.[j]	100	-	-	-	" 7.1	78.8	-	-	-
18.	50	-	-	-	P[k] 7.2	68.8	2.8	1.6	-
19.	50	50	-	170	" 6.5	92.8	1.7	2.1	-
20.	50	-	45[l]	-	" 6.5	92.1	2.6	1.9	-
21.	50	-	-	-	" 5.2	95.4	1.2	0.4	-
SUBSEQUENT EXPERIMENTS WERE PERFORMED WITH 400 MG. OF NA									
22.	50	50	-	-	P 5.2	86.8	1.8	1.0	-
23.	50	50	-	170	" 5.2	78.6	5.8	2.2	-
24.	50	50	-	330	" 5.1	86.8	4.6	1.2	-

TABLE 7--Continued

Expt. no.	Steroid wt. mg.	DPN wt. mg.	$DPNH_2$ wt. mg.	FDP wt. mg.	Buffer and pH	Androstanedione recovered %	Metabolites recovered % Epiandrosterone	Androsterone	Androstanolone
25.[m]	50	100	-	330	P 5.1	83.0	2.0	0.8	-
26.[m]	50	200	-	330	" 5.1	99.2	2.0	0.4	-
27.[n]	50	50	-	330	" 5.1	85.0	5.0	1.0	-
28.[n]	50	100	-	330	" 5.1	83.0	2.5	0.7	-
29.[n]	50	200	-	330	" 5.1	89.8	1.8	0.7	-
30.	50	50	-	250	" 6.7	68.8	6.0	6.4	0.4
31.[o]	50	50	-	250	" 6.7	77.0	6.0	5.0	0.2
32.	50	-	40	-	" 6.7	57.6	21.2	3.8	0.2
33.[o]	50	-	40	-	" 6.7	70.8	11.2	2.0	0.4
34.	50	-	40	-	" 5.6	79.0	4.6	3.4	0.4
35.[o]	50	-	40	-	" 5.6	79.6	2.0	2.0	0.4
36.	50	-	38	-	" 5.5	68.8	7.0	3.5	1.8
37.	50	-	38	-	" 5.9	74.9	12.6	3.6	1.5
38.	50	-	38	-	" 6.5	62.8	16.1	3.0	0.2
39.	50	-	38	-	" 7.0	74.9	12.7	2.1	0.1
40.[d]	50	-	38	-	" 7.5	71.8	9.2	2.1	0.1
41.	50	-	38	-	" 6.5	86.1	-	-	-
42.	50	-	23.5	-	" 6.7	87.8	10.5	3.9	0.4
43.	50	-	47	-	" 6.7	80.8	12.7	3.2	0.4
44.	50	-	47	250	" 6.7	84.0	11.8	5.6	0.3
45.	50	-	47	500	" 6.7	73.2	9.8	5.8	0.3
46.	50	50	-	250	" 6.7	68.8	11.9	5.5	0.3
47.[o]	50	50	-	250	" 6.7	83.6	11.7	6.3	0.4
48.	50	-	19	-	" 6.65	79.6	13.9	5.0	tr.
49.	50	-	19	-	" 6.65	78.6	12.8	5.4	tr.
50.	50	-	38	-	" 6.65	74.4	13.5	5.4	tr.
51.	50	-	38	-	" 6.65	72.4	12.9	5.8	tr.
52.	50	-	63	-	" 6.65	74.6	15.2	5.5	tr.
53.	50	-	63	-	" 6.65	73.6	14.5	5.3	tr.
54.	50	-	37	-	" 6.6	57.0	19.8	4.6	tr.

TABLE 7--Continued

Expt. no.	Steroid wt. mg.	DPN wt. mg.	$DPNH_2$ wt. mg.	FDP wt. mg.	Buffer and pH	Androstanedione recovered %	Metabolites recovered % Epiandrosterone	Androsterone	Androstanolone
55.[p]	50	-	74	-	P 6.6	57.0	26.2	4.6	tr.
56.[p]	50	-	37	-	" 6.6	67.4	15.4	4.8	tr.
57.[p]	50	-	74	-	" 6.6	73.6	15.6	4.4	tr.
58.[q]	50	-	37	-	" 6.6	88.4	2.2	2.4	tr.
59.[q]	50	-	74	-	" 6.6	88.4	7.2	3.0	tr.

[a]Twelve grams of liver and 100 ml. of solution incubated at 37-8° C. with the amounts of steroid and additives indicated.

[b]Krebs-Ringer bicarbonate.

[c]Solution saturated with sodium bicarbonate.

[d]Boiled tissue control.

[e]Incubated 0 minutes.

[f]Incubated 16 minutes.

[g]Incubated 31 minutes.

[h]Incubated 60 minutes.

[i]20-30 mg. spilled in transfer.

[j]Tissue ground.

[k]Disodium phosphate, 0.1 molar.

[l]Sodium salt.

[m]All components but steroid preincubated 15 minutes.

[n]Homogenate and buffer preincubated 15 minutes.

[o]Nitrogen atmosphere.

[p]Supernatant fraction.

[q]Sediment fraction.

could give rise theoretically to four isomeric monohydroxy monoketone compounds: androsterone (3-α-hydroxyl), epiandrosterone (3-β-hydroxyl), androstan-17α-ol-3-one, and androstan-17β-ol-3-one. In addition, four isomeric dihydroxy compounds might result from subsequent reduction of the monohydroxy monoketone compounds. Only androsterone and epiandrosterone were detected. The latter predominated in every experiment but one.

Influence of length of incubation period. Since the level of conversion was extremely low, and since other metabolites were expected, a series of experiments was performed to investigate the stability of androstanedione-reducing enzyme system(s). Various incubation periods were employed (experiments 13-17). The major portion of the metabolism occurred during the time in which the steroid and the tissue were being mixed in the Waring blendor; that is, essentially as much conversion resulted in the incubation mixture which was treated with alcohol immediately (to denature the protein) as in the incubation mixture which was incubated for sixty minutes. This behavior can be interpreted in several ways; the enzyme system(s) responsible for the reduction of the substrate were rapidly inactivated during the homogenization process;[1] the conversions observed were due to the homogenization process, either directly by means of a non-

[1]Such inactivations have been demonstrated; e.g., see (44) and (85).

enzymatic route or indirectly by stimulation of enzymatic processes; or a low concentration of an essential reaction component was rapidly exhausted or destroyed.

A single experiment (number 18) was performed in which the tissue was passed through a meat grinder rather than homogenized. Although there was an increase in conversion, it was not sufficient to indicate destruction by homogenization, particularly since the increase did not greatly exceed the limits of the experimental methods employed. Nall (61) observed no differences in the levels of testosterone oxidation using homogenates produced with Waring blendors and with Erway glass homogenizers. These evidences seemed to eliminate enzymatic destruction as a major consideration. In order to eliminate atypical enzymatic conversion during homogenization, subsequent homogenizations were performed in the absence of steroid. The possibility of homogenization-induced non-enzymatic conversion was ruled out by the use of boiled tissue control experiments; no conversion was observed (experiments 7, 11, 17).

Experiments with Additives

There remained to be demonstrated a dependence upon the concentration of an essential reaction component (other than the enzyme(s) involved). Such co-factors are common among biclogical oxidation-reduction reactions; substances which have been demonstrated to act in this fashion include

DPN, TPN, and FAD. The pyridine nucleotides, of which DPN and TPN are the major representatives, are frequently involved; indeed, they have been referred to as "the workhorses of biological oxidations" (86). Furthermore, DPN has been demonstrated to be obligatory in the enzymatic oxidation of the 17-hydroxyl group of testosterone (46,87). Finally, DPN is rapidly inactivated when tissue cellular structure is destroyed (88-90). Accordingly, this compound was chosen for the initial investigations into this aspect of the metabolic conversion of androstanedione. Since the conversion of this steroid involves reduction, the use of the reduced form of the nucleotide, $DPNH_2$, was indicated.

In the original experiments (numbers 19 and 20), provision for the presence of $DPNH_2$ was made in two ways; the sodium salt of the reduced dinucleotide was introduced as a solid in one instance and solid DPN and a solution of FDP were added in a second experiment. Wald (60) has shown that the latter materials give rise to $DPNH_2$ in the presence of homogenates, probably via glycolysis. Slight increases in the amounts of both metabolites were observed in each instance; total conversion was 4.5 and 3.8%, respectively.

Tissue homogenates have been shown to contain a nucleotidase which exhibits a deleterious effect on DPN (91). NA acts as an inhibitor toward such destruction (92). This material was included in the next series of experiments (numbers 21-23). The experiments were performed at pH 5.2

in consideration of the acidic character of $DPNH_2$. Since $DPNH_2$ is more acidic than DPN, reductions involving this nucleotide should be favored by increases in acidity. More conversion was observed in the presence of DPN and NA than in their absence. In the presence of DPN, NA, and FDP, the total conversion reached 8.0%. The pattern of the metabolite production paralleled that of the early experiments in that androsterone and epiandrosterone were the only metabolites detected; furthermore, the amounts of epiandrosterone formed were two-to-three times those of androsterone.

Influence of preincubation period. These results definitely established the utilization of $DPNH_2$ in the reduction of androstanedione, if not a physiological requirement for this co-factor. In an effort to increase the amount of this compound available to the enzyme system(s) involved, increasing amounts of DPN were employed in the next set of experiments (numbers 24-26). In addition, a preincubation period of fifteen minutes was employed during which all components but steroid were incubated in the usual fashion. These experiments were performed simultaneously with experiments in which only the homogenates were preincubated (experiments 27-29). The preincubation of DPN and FDP did not prove to be beneficial. Increasing amounts of DPN decreased the formation of androsterone and epiandrosterone; total metabolism was 5.8 and 6.0% when 50 mg. of DPN was employed, only 2.4% when 200 mg. of DPN was used.

These data suggested a rather poor formation of $DPNH_2$ from DPN and FDP. Wald (60) suggested that triose phosphate is the immediate hydrogen donor involved in this reduction process. The enzyme triose phosphate dehydrogenase exhibits optimal activity in the pH range 8.5 to 9.0 (93). Activity at pH 5.2 would be of a low order. Furthermore, Wald's experiments indicated that the reaction between FDP (or one of its metabolites) and DPN is facilitated by a nitrogen atmosphere.

Influence of pH and of gaseous phase. These factors as they are related to the reduction of androstanedione were studied in the next group of experiments (numbers 30-35); comparable experiments were performed at two pH values, under aerobic and anaerobic conditions, and in the presence of $DPNH_2$ produced by the homogenate system from DPN and FDP and $DPNH_2$ which had been prepared chemically. The experiments indicated the following items: (1) comparable experiments performed at pH 6.7 (numbers 32 and 33) gave rise to approximately three times as much conversion (25.2 and 13.6%) as experiments performed at pH 5.6 (numbers 34 and 35; 8.4 and 4.4%); (2) comparable experiments performed aerobically with $DPNH_2$ (numbers 32 and 34) gave rise to approximately twice as much conversion (25.2 and 8.4%) as experiments performed aerobically with $DPNH_2$ (numbers 33 and 35; 13.6 and 4.4%); (3) comparable experiments with DPN and FDP gave essentially the same results (12.8 and 11.2%) when carried out

in air (experiment 30) and in nitrogen (experiment 31); (4) an experiment performed with 40 mg. of $DPNH_2$ (number 32) gave about twice as much conversion (25.2%) as a comparable experiment (number 30) performed with 50 mg. of DPN in the presence of FDP (12.8%) when the experiments were performed aerobically, but essentially the same conversion (13.6 and 11.2%) when the experiments were performed anaerobically (numbers 33 and 31); (5) twenty-five-fold increases in the metabolic conversion of androstanedione can be induced by the introduction of the co-factor $DPNH_2$ in sufficient quantity (experiments 1 and 32); (6) dramatic increases in total metabolism are reflected almost exclusively in the amount of epiandrosterone produced (experiment 32); (7) high levels of metabolic conversion are accompanied by the formation of a third metabolite, androstanolone.

The pH effect observed in the first item involves a comparison of experiments in which chemically prepared $DPNH_2$ was employed. A similar but somewhat smaller effect is apparent from a comparison of experiments 30 and 31 with experiment 23; DPN and FDP were employed in these experiments. From the considerations listed previously, the increase in pH should favor the formation of $DPNH_2$ from DPN and FDP due to the value of the pH optimum of the enzyme triose phosphate dehydrogenase; however, the increase in pH should tend to decrease the reduction of androstanedione because of the principle of mass action involved. Since only the latter is

pertinent in the experiments in which chemically prepared $DPNH_2$ was employed, the increase in the reduction of androstanedione which accompanied the increase in pH can only be interpreted as a manifestation of the properties of the enzyme system(s) responsible for the reduction of the steroid. That is, the higher of the two pH values employed in these experiments must be nearer the pH optimum of the steroid-reducing enzyme(s).

Additional insight into the properties of the androstanedione-reducing enzyme system(s) is provided by the observation of the requirement for aerobic conditions. Such requirement, of course, may lie in the reduction step itself, or in reactions coupled with the reduction process, such as hydrogen-transfer reactions. The apparent absence of any aerobic requirement in experiments in which DPN and FDP were used may be attributed to the beneficial effect which nitrogen exerts on the formation of $DPNH_2$ from these compounds; apparently the detrimental effect of nitrogen on androstanedione reduction is compensated for in this fashion.

The increase in conversion produced by the use of chemically prepared $DPNH_2$ verifies the incompleteness of the reaction between DPN and FDP at pH values removed from the optimum. Comparison of aerobic and anaerobic experiments substantiates further the efficacy of nitrogen in the enzymatic formation of $DPNH_2$, since the conversion produced by DPN and FDP is essentially the same as that produced by

$DPNH_2$ when nitrogen atmospheres were employed.

The large increase in reduction of androstanedione induced by the use of $DPNH_2$ is strong evidence of a physiological involvement of this co-factor in the enzymatic transformations operative, due to the pronounced specificity which is exhibited by such transformations. Certain enzyme dehydrogenases which utilize DPN also use TPN with more or less efficiency. Therefore, the results obtained are sufficient to define a requirement for one of the pyridine nucleotides, if not for $DPNH_2$ itself. It has been demonstrated in this laboratory (94) that the enzymatic reduction of androstenedione is enhanced by both $DPNH_2$ and $TPNH_2$; the latter is about one and one-half times as effective as the former. Conceivably a parallel situation might exist in the case of androstanedione.

The preferential increase in the amount of epiandrosterone formed accompanying increased reduction of androstanedione is interesting from a biological viewpoint. The steroid excretion patterns of man and the guinea pig have been alluded to; androsterone is the principal androstane derivative in the former instance (83), epiandrosterone in the latter (84). Such differences could be manifestations of different hormonal relationships. These experiments demonstrate that the independent regulation of the output of these two materials is possible. Conceivably, separate enzyme systems are responsible for the formation of the two compounds

and the epiandrosterone-forming enzyme is more sensitive to the variables studied. Talalay (46,47) has isolated enzymes from Pseudomonas which are specific in their formation of androsterone and epiandrosterone. Again, it is possible that a single enzyme system is involved which exhibits a duality in properties; for example, a preferential requirement for one pyridine nucleotide might be involved in the formation of epiandrosterone, while a different co-factor might be necessary for the formation of androsterone. Such "dual" enzymes are known, e.g., xanthine oxidase.

The formation of androstanolone at higher levels of metabolism is also of interest. Again, several explanations are possible. Separate enzyme systems may be responsible for the formation of these three metabolites. If such is the case, the formation of androstanolone is either a minor metabolic pathway for the reduction of androstanedione, or the optimal operating conditions of the androstanolone-forming enzyme are quite different from those of the enzymes involved in the formation of androsterone and epiandrosterone. Such considerations would also apply to the situation in which one enzyme was responsible for the formation of the three compounds, or to the situation in which two enzymes were operative, one of which was involved in the formation of a pair of the three compounds. The adaptive "β-enzyme" isolated by Talalay (46) from Pseudomonas metabolized both epiandrosterone and androstanolone, but not androsterone.

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 excretion (83).

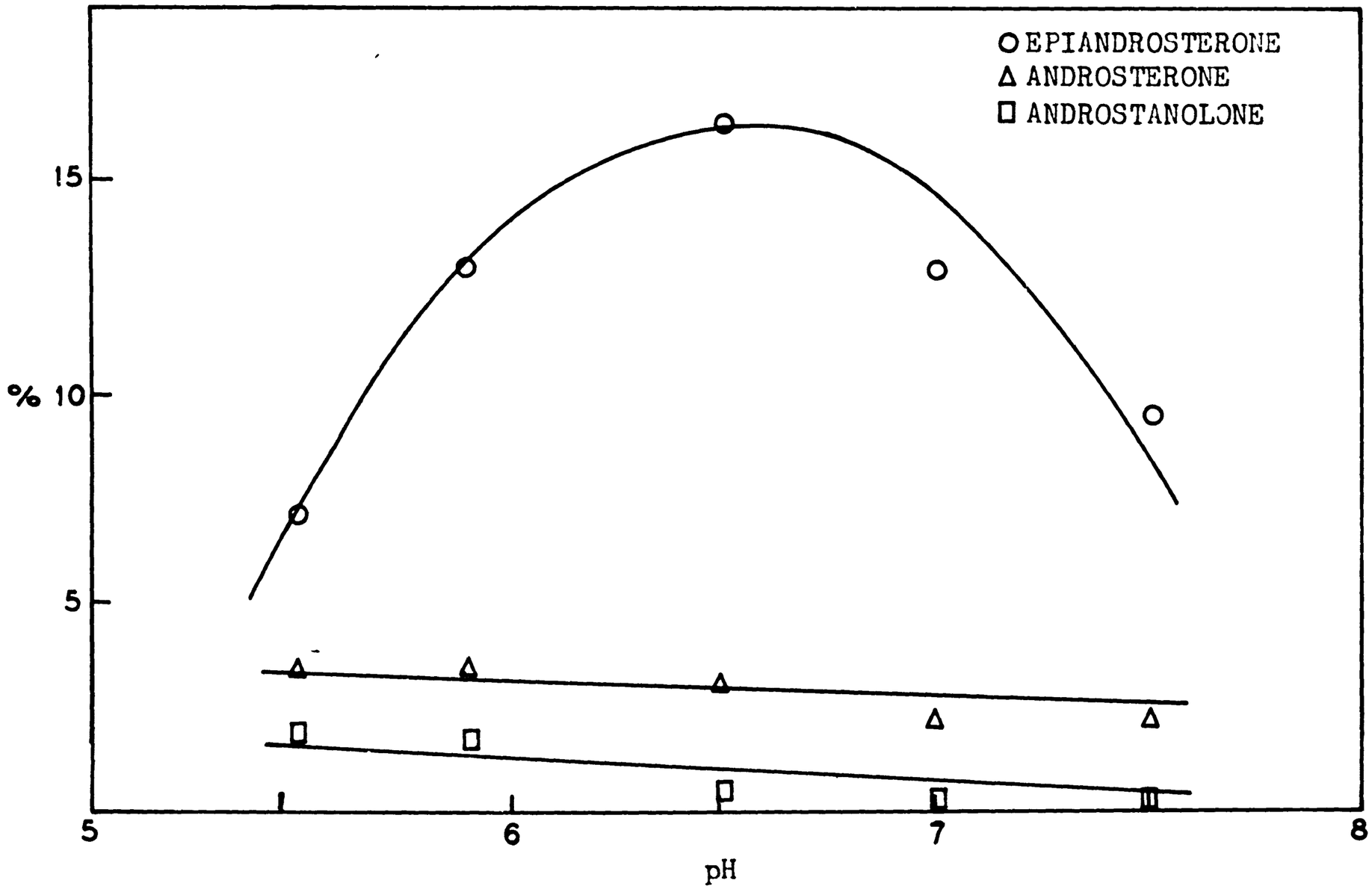
Subsequent experiments were designed to define further the role of the various factors just discussed. The influence of the acidity of the incubation medium was investigated in experiments 36 to 41. The experiments were performed with $DPNH_2$ rather than DPN and FDP since the formation of $DPNH_2$ from these compounds is also pH dependent. Figure 3 indicates the results obtained. The formation of epiandrosterone was markedly dependent on the pH, maximum formation being observed at pH 6.5. The formation of androsterone and androstanolone appeared to decrease with increasing pH, but the observed differences were very small.

Four factors were investigated in the next group of experiments. These included a preliminary investigation into the influence of $DPNH_2$ concentration on metabolic conversion of androstanedione, a further comparison of the influences of DPN and $DPNH_2$, a further comparison of air and nitrogen as the gaseous phase using DPN, and two attempts to increase further the metabolism effected by $DPNH_2$ by the addition of FDP.

Only a slight increase in total metabolism was observed when the concentration of $DPNH_2$ was doubled (experiments 42 and 43). In a subsequent investigation (experiments

FIGURE 3

INFLUENCE OF PH UPON THE METABOLISM OF ANDROSTANEDIONE
BY GUINEA PIG LIVER HOMOGENATES



48-53) no significant variation in metabolism was observed when the amount of $DPNH_2$ was increased from 19 to 63 mg. In contrast to previous experiments, the total metabolism (17.7%) effected by 50 mg. DPN (in the presence of FDP; experiment 46) was essentially equal to that (16.3%) produced by 47 mg. $DPNH_2$ (experiment 43). The total metabolism (17.7%) effected by DPN aerobically (experiment 46) was essentially equal to that (18.4%) produced anaerobically (experiment 47); this was in accord with previous observations. The metabolism effected by $DPNH_2$ was not increased by the addition of FDP (experiments 43-45).

Experiments with fractions prepared by centrifugation. The last series of experiments with homogenates of male guinea pig liver was designed to provide preliminary information concerning the physical nature of the enzyme system(s) involved in the conversion of androstanedione to the metabolites observed. Homogenates were centrifuged at 1500 times gravity, and the metabolism effected by the supernatant and sediment fractions was compared with that effected by the homogenates. Two levels of $DPNH_2$ were employed to reflect any altered co-factor requirements produced by the centrifugal separation.

The sum of the epiandrosterone formed in the separate fractions represented 87.9 $\pm$ 0.9% of that formed in the homogenates. Approximately three-fourths of the epiandrosterone formed by the separate fractions was formed by the

supernatant fractions. The sum of the androsterone formed in the separate fractions represented 159 ± 2% of that formed in the homogenates; approximately five-eighths of the androsterone formed by the two fractions was formed by the supernatant fractions. These results would seem to suggest the possibility of two enzyme systems, since a greater portion of the epiandrosterone-forming activity remained in the supernatant than did the androsterone-forming activity, and since the fractionation gave rise to a pronounced increase in the androsterone formed in the two fractions, but not in the epiandrosterone formed by the two fractions. One would expect the increase to be in androsterone formation if only one increase resulted, since the lower formation of this compound by homogenates probably reflects a poor competition for $DPNH_2$ and/or androstanedione on the part of the androsterone-forming enzyme system.

Metabolism by Other Guinea Pig Tissue Homogenates

Four experiments were performed in which guinea pig tissues other than liver were employed. Androsterone and epiandrosterone were the two metabolites observed. Table 8 lists the tissues, the weights of tissues, weights of androstanedione, the buffers, and the pH values employed. In addition, the amounts of androstanedione and metabolites recovered are listed.

The first three experiments were performed early in

TABLE 8

METABOLISM OF ANDROSTANEDIONE BY OTHER GUINEA PIG TISSUE HOMOGENATES[a]

Expt. no.	Tissue	Tissue wt. g.	Steroid wt. mg.	Buffer	pH	Androstanedione recovered %	Metabolites recovered % Epiandro-sterone	Andro-sterone
1.	heart	9.7	100	KRB[b]	7.45	89.5	0.2	0.4
2.	kidney	12.0	100	KRB	7.45	84.5	0.4	0.5
3.	muscle[c]	12.7	100	KRB	7.45	80.4	0.5	---
4.	kidney[d]	12.0	100	KRB	7.1	84.1	0.3	0.5

[a]Tissue, steroid, and 100 ml. of buffer solution incubated for two and one-half hours at 37-8° C. using a gaseous phase consisting of 95% oxygen and 5% carbon dioxide.

[b]Krebs-Ringer bicarbonate.

[c]Femoral muscles.

[d]Incubation period 0 minutes.

the study to select that tissue most active in the reduction of androstanedione. Since the differences observed were so small, no information was gained for this purpose. Liver was chosen since its role in the metabolism of the androgens has been demonstrated repeatedly (42,48,95). The amount of epiandrosterone formed in tissues other than liver did not exceed the amount of androsterone formed with any of the tissues, except with muscle, from which only epiandrosterone was recovered. These results are contrary to those observed with liver. The small values involved are probably not significant.

The fourth experiment was performed in conjunction with the time study performed with liver homogenates. The results obtained paralleled those obtained with liver.

Metabolism by Rat Liver Homogenates

Three experiments were performed with rat liver homogenates. Androsterone and epiandrosterone were the only metabolites observed. Table 9 lists the weights of androstanedione, DPN, FDP, and NA used. The buffers used, pH values at the beginning of the incubations, and amounts of androstanedione and metabolites recovered are listed also.

The three experiments were performed simultaneously with guinea pig liver homogenate experiments numbers 21, 22, and 23. In addition to determining the influence of the additives used, the experiments were designed to compare the

TABLE 9

METABOLISM OF ANDROSTANEDIONE BY RAT LIVER HOMOGENATES[a]

Expt. no.	Steroid wt. mg.	DPN wt. mg.	FDP wt. mg.	NA wt. mg.	Buffer	pH	Androstanedione recovered %	Metabolites recovered % Epiandrosterone	Androsterone
1.	50	--	--	--	P[b]	5.2	89.6	1.8	0.8
2.	50	50	--	400	P	5.2	85.0	3.8	2.4
3.	50	50	170	400	P	5.2	83.0	3.6	6.0

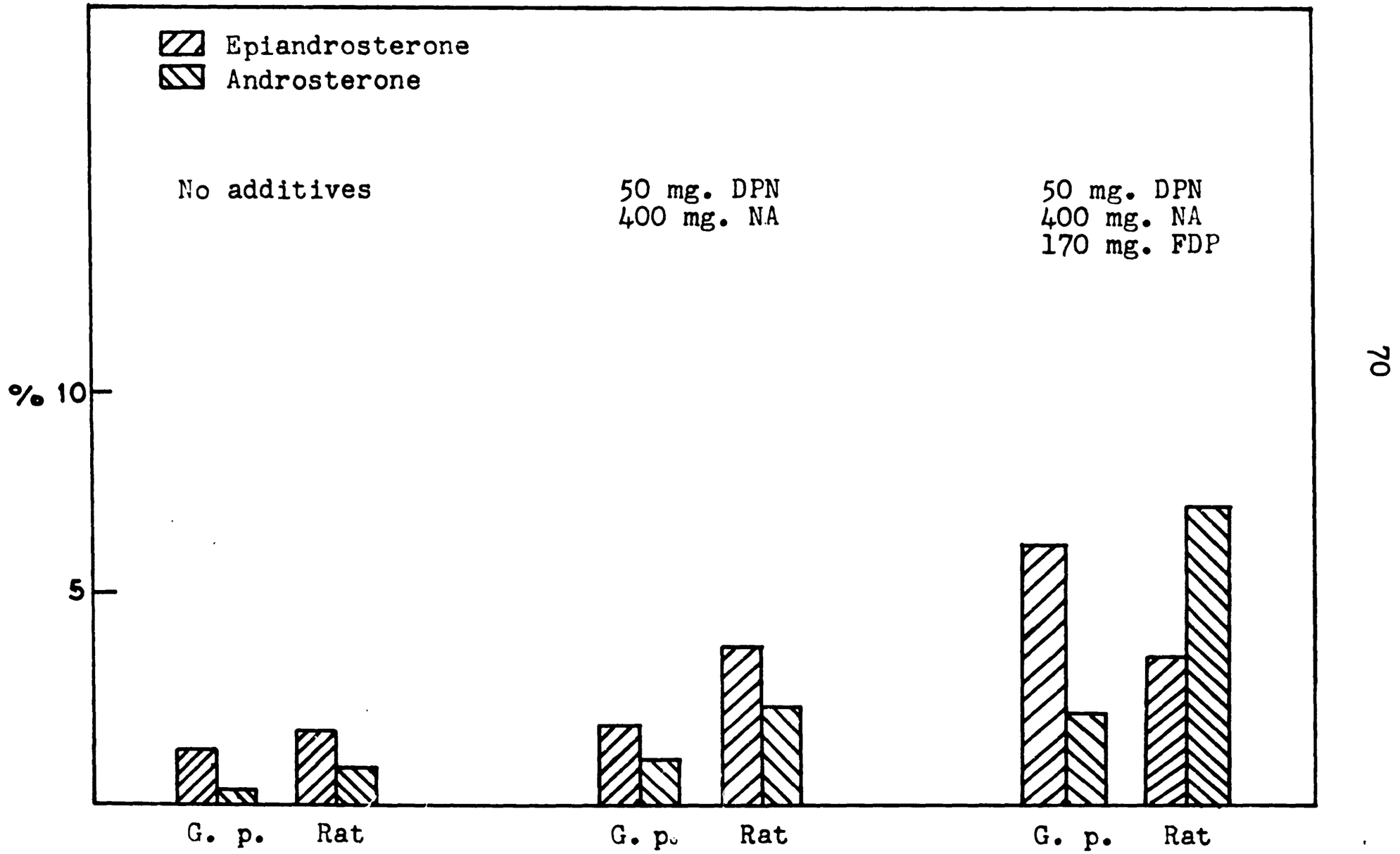
[a]Twelve grams of liver and 100 ml. of buffer solution incubated for one and one-half hours at 37-8° C. with the amounts of steroid and additives indicated.

[b]Disodium phosphate, 0.1 molar.

androstanedione-reducing activities of the two species. A comparison of the two tissues is made in Figure 4.

FIGURE 4

COMPARISON OF THE METABOLISM OF ANDROSTANEDIONE
BY GUINEA PIG AND RAT LIVER HOMOGENATES



CHAPTER IV

THE METABOLISM OF OTHER ANDROGENIC STEROIDS BY GUINEA PIG TISSUE HOMOGENATES

In order to give further insight into the factors regulating the metabolism of androstanedione, twenty-seven additional experiments were performed using other steroids as substrates. Eight experiments were conducted with androstanolone; five of these employed liver homogenates (Table 10), and three were performed with other tissue homogenates (Table 11). Fourteen experiments were carried out using different hydroxysteroids in an effort to determine the influence of chemical structure upon the extent of metabolic conversion (Table 13). Prior to this hydroxyl oxidation comparison study, six experiments were performed with testosterone to determine the appropriate amount of DPN to employ in the comparison study (Table 12). A single experiment with androstanolone is listed in both Table 10 and Table 13.

The Metabolism of Androstanolone by Guinea Pig Liver Homogenates

Since only very small amounts of androstanolone were

recovered from the incubation studies with androstanedione, it seemed desirable to carry out incubation experiments using the former coupound as substrate steroid. In this manner the metabolic interrelationship between the two compounds might be more adequately defined. The incubation of androstanolone was of further interest due to the structural similarity between this compound and testosterone; the former compound is the 4,5-dihydro derivative of the latter.[1] The results of the incubations of androstanolone with liver homogenates are given in Table 10.

Incubation of 100 mg. of the steroid at pH 7.35 resulted in the recovery of 1.2 mg. of androstanedione (experiment 1), thus establishing the reversibility of the reaction in which androstanolone was formed by the enzymatic reduction of the diketone. The enzymatic nature of this oxidative conversion was demonstrated by the failure to recover androstanedione from an experiment in which boiled liver tissue was used (number 2). The reversibility of the reaction involving androstanolone and androstanedione parallels that established by Kochakian and his co-workers (52, 54,96,97) involving testosterone and androstenedione. The latter compounds are the 4,5-dehydro derivatives of the former compounds.[1] Thus the presence (or absence) of a carbon-carbon double bond at position 4 in the steroid nucleus does

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[1]See Table 3.

TABLE 10

METABOLISM OF ANDROSTANOLONE BY GUINEA PIG LIVER HOMOGENATES[a]

Expt. no.	Steroid wt. mg.	DPN wt. mg.	NA wt. mg.	Buffer	pH	Androstanolone recovered %	Androstanedione recovered %
1.	100	---	---	KRB[b]	7.35	91.8	1.2
2.[c]	100	---	---	KRB	7.35	94.0	---
3.	100	100	---	KRB	7.4	55.6	1.8
4.	100	50	400	KRB	7.35	88.9	5.4
5.	50	50	400	PP[d]	9.0	71.6	13.8

[a]Twelve grams of liver and 100 ml. of buffer solution incubated at 37-8° C. with the amounts of steroids and additives indicated.

[b]Krebs-Ringer bicarbonate.

[c]Boiled tissue control.

[d]Sodium pyrophosphate, 0.166 molar.

not qualitatively affect the oxidation-reduction reactions of the carbon-oxygen function at position 17.

Two experiments (numbers 3 and 4) were performed at pH 7.35 in the presence of physiological additives. In the first of these, 100 mg. of DPN was added. No significant increase was observed in the amount of androstanedione formed; however, this may have been due to the extremely low recovery of steroids in this experiment. In the second of these experiments, 50 mg. of DPN and 400 mg. of NA were added to the incubation medium; 5.4% androstanedione resulted. Thus, the requirement of $DPNH_2$ previously observed in the formation of androstanolone from androstanedione, is reflected by a four-fold increase in the oxidation of the former compound to the latter in the presence of added DPN.

A final experiment with liver tissue (which was conducted in conjunction with the hydroxyl oxidation comparison study) was performed at pH 9.0 using 12 grams of liver, 50 mg. of steroid, 50 mg. of DPN, and 400 mg. of NA; 13.8% androstanedione was recovered. From the principles of mass action discussed previously, an increase in oxidation is to be expected from a decrease in acidity when DPN is the oxidant involved. A portion of the increased metabolism may also be due to the two-fold increase in the tissue-to-steroid ratio employed.

Three factors which function in the regulation of the metabolism of androstanedione are apparent. The concentration

of diphosphopyridine nucleotide in the liver will exert a regulatory function; for a given concentration of androstanedione, the extent of metabolic conversion will be partially dependent on the concentration of this co-factor.

The ratio of the oxidized and reduced forms of the nucleotide which is maintained physiologically will also regulate the metabolism of androstanedione. Increases in this ratio will favor the formation of androstanedione from androstanolone (and from androsterone and epiandrosterone); decreases in this ratio will favor the formation of the reduced product(s) from the diketone.

Finally, the acidity will have a regulatory capacity. Not only will the pH partially determine the extent of both the oxidative and the reductive conversions, but also, to some extent, in which direction the reactions will proceed. That is, increases in acidity will favor reduction and decreases will favor oxidation. Tentatively, the hypothesis may be advanced that the acidity exerts some measure of control on the relative amounts of androsterone, epiandrosterone, and androstanolone formed during the reduction of androstanedione (see Chapter III).

The Metabolism of Androstanolone by Guinea Pig Tissue Homogenates Other Than Liver

The incubation of 100 mg. of androstanolone with 10.4 grams of testes, 12.0 grams of kidney, and 9.3 grams of seminal vesicles and prostate resulted in trace, 1.6%, and

no recoveries, respectively, of androstanedione, when the incubations were performed at pH 7.35 in the presence of 50 mg. of DPN and 400 mg. of NA (Table 11).

In two experiments with seminal vesicles and prostate, Nall (61) observed 0.0 and 0.5% conversion of testosterone to androstenedione. Conversions of 0.7 and 1.6% were observed in two experiments with testes. In a single experiment at pH 7.4 using 12 grams of kidney, 100 mg. of testosterone, 50 mg. of DPN, and 333 mg. of NA, 10% androstenedione was formed.

Thus the tissue pattern of metabolism observed with androstanolone is the same as that observed previously with its 4,5-dehydro derivative, testosterone. Metabolism was extremely low or absent in seminal vesicles, prostate, and testes, intermediate in kidney, and most pronounced in liver. Furthermore, the extent to which androstanolone was metabolized by the various tissues investigated appears to be a relatively constant fraction of the extent to which testosterone was metabolized by the same tissues; the latter was metabolized five to six times as extensively as the former. This observation may be stated differently; the ratio of androstanolone oxidation in liver and kidney, approximately three, appears to be the same as that observed with testosterone. This would seem to indicate that a single enzyme system is responsible for the oxidation of these two

TABLE 11

METABOLISM OF ANDROSTANOLONE BY OTHER GUINEA PIG TISSUE HOMOGENATES[a]

Expt. no.	Tissue	Tissue wt. g.	Steroid wt. mg.	DPN wt. mg.	NA wt. mg.	Buffer	pH	Androstanolone recovered %	Androstanedione recovered %
1.	Testes	10.4	100	50	400	KRB[b]	7.35	85.5	tr.
2.	Kidney	12.0	100	50	400	KRB	7.35	95.6	1.6
3.	SVPr[c]	9.3	100	50	400	KRB	7.35	93.3	---

[a]Tissue and 100 ml. of buffer solution incubated for two and one-half hours at 37-8° C. using a gaseous phase consisting of 95% oxygen and 5% carbon dioxide.

[b]Krebs-Ringer bicarbonate.

[c]Seminal vesicles and prostate.

3-ketone-17 -hydroxysteroids. The alternate assumption of the distribution of two enzymes in two tissues such that their concentration ratio is the same in both tissues is certainly less attractive, particularly when the structural similarity of the two steroids is considered. The adaptive "β-enzyme" isolated by Talalay (46) from Pseudomonas is able to metabolize both testosterone and androstanolone, and the latter is oxidized to a lesser extent than is the former.

The Influence of Diphosphopyridine Nucleotide Concentration on the Metabolic Conversion of Testosterone by Guinea Pig Liver Homogenates

Although the metabolism of testosterone by various tissue homogenates has been actively investigated by Kochakian and his co-workers (61,96,97), no definitive experiments have been performed with respect to the optimum concentration of DPN in the incubations performed by this group. A standard set of incubation conditions was desired in order that a comparative study might be instigated using a large number of compounds.

Accordingly, testosterone was incubated with varying amounts of DPN under the conditions indicated in Table 12. The recoveries of metabolite androstenedione are given in this table, and the results are depicted graphically in Figure 5. A marked increase in the recovery of metabolite was observed when the DPN:testosterone ratio (mg.:mg.) was

TABLE 12

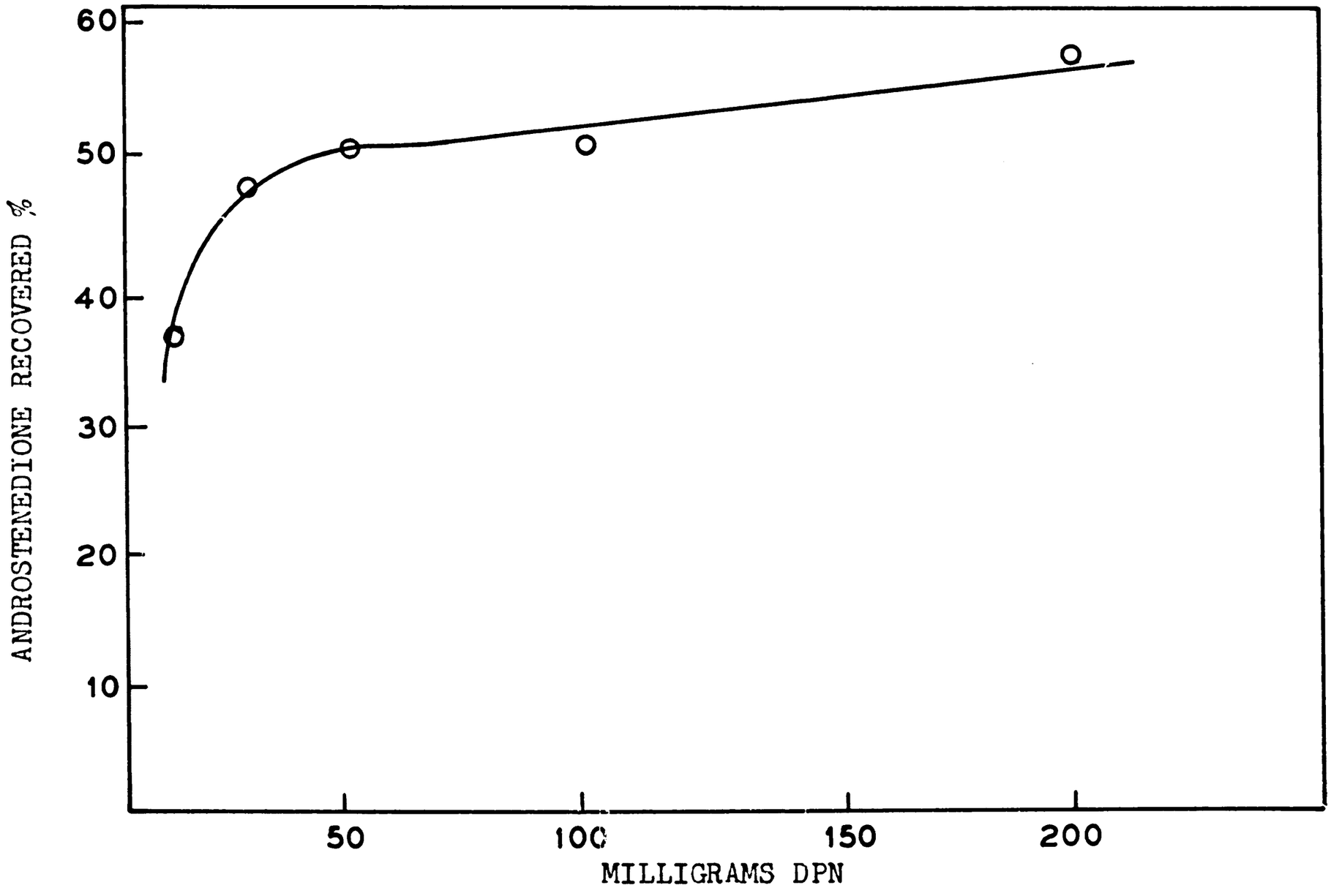
INFLUENCE OF DPN CONCENTRATION ON THE CONVERSION OF TESTOSTERONE BY GUINEA PIG LIVER HOMOGENATES[a]

Expt. no.	Steroid wt. mg.	DPN wt. mg.	DPN:steroid molar ratio	Testosterone recovered %	Androstenedione recovered %
1.	50	10	0.015:0.17	54.6	36.8
2.	50	25	0.037:0.17	42.8	47.2
3.	50	50	0.074:0.17	42.4	50.4
4.	50	100	0.15 :0.17	39.4	50.4
5.	50	200	0.30 :0.17	36.2	56.6
6.	25	100	0.30 :0.17	36.4	63.2

[a]Twelve grams of liver and 100 ml. of sodium pyrophosphate buffer solution, 0.166 molar, pH 9.0, incubated with 400 mg. NA and the weights of steroid and DPN indicated for one and one-half hours at 37-8° C.

FIGURE 5

INFLUENCE OF DPN CONCENTRATION ON METABOLISM OF TESTOSTERONE BY GUINEA PIG LIVER HOMOGENATES



increased from 1:5 to 1:2. This influence diminished rapidly, however, since a further increase in the same ratio from 1:1 to 4:1 produced an increase of only 12% in the amount of androstenedione recovered (experiments 3-5). When the quantities of DPN and testosterone were reduced by one-half, so that the DPN:testosterone ratio of 4:1 was maintained, while the tissue ratio was doubled, an increase of 10% was observed in metabolite formation (experiments 5 and 6).

It is informative to consider the molar ratio of DPN and testosterone involved in these experiments; only in experiments 5 and 6 was there sufficient DPN present to completely oxidize the quantity of testosterone employed, assuming that such oxidation proceeds on a mole-mole basis with no regeneration of DPN by companion reactions. Furthermore, the quantity of androstenedione recovered exceeded the quantity of DPN employed, on a molar basis, in experiments 1, 2, and 3. This would suggest a regeneration of DPN by companion oxidative processes. As might be expected, the extent of re-cycling, or regeneration, of DPN was the greatest in experiment 1; that is, in the experiment in which the least quantity of DPN was employed. Approximately four moles of androstenedione were formed for each mole of DPN present originally.

Fifty mg. of DPN was chosen as the appropriate

quantity to employ in the comparative oxidative study described subsequently.

The Metabolism of Fourteen Nineteen-Carbon Hydroxysteroids by Guinea Pig Liver Homogenates

It is unknown at the present whether the various androgens exert their influences prior to metabolic transformation, during metabolic transformation, or after metabolic transformation to other compounds. Elucidation of this question requires research in at least three areas. The metabolic pathways must be defined. There must be a determination of the role which chemical structure plays in the determination of the biological influences of these compounds. Kochakian and his co-workers have actively investigated the latter area (98-103). Reference has previously been made to the activity of this group in the definition of metabolic pathways. Finally, there should be investigation into the factors of chemical structure which govern the various metabolic pathways.

Of particular interest is the determination of the priority which one metabolic route exhibits over other possible routes. The principal excretion products of testosterone are monohydroxy monoketones and dihydroxy compounds. The former are almost exclusively 3-hydroxy-17-ketone compounds in man, and the hydroxyl group at position 3 is predominantly of the α-configuration, or trans to the angular

methyl group at position 10. The dihydroxy compounds are principally those with the α-configuration at position 3 and the β-configuration at position 17 (83).

Such metabolite distribution is the reflection, ultimately, of the influence of chemical structure upon chemical reactivity.

Androstanedione represents an ideal model compound with which to investigate the various aspects of chemical structure which are pertinent to these considerations. The presence of two ketone groups in a single compound permits a determination of the relative ease of reduction of the two ketones by the reducing system chosen; that is, one can determine whether the formation of 3-hydroxy-17-ketones or 17-hydroxy-3-ketones is favored. Furthermore, the priority of formation of hydroxyl groups of α- and β-configurations at both positions 3 and 17 can be determined, in the event of reduction of the ketones at these positions.

As has been indicated in Chapter III, male guinea pig liver homogenates favor the formation of 3-hydroxy-17-ketones rather than 17-hydroxy-3-ketones when androstanedione is the substrate steroid; that is, the 3-ketone function is more easily reduced than is the 17-ketone function. In addition, the β-configuration is the preferred configuration for the hydroxyl group formed at position 3, since more epiandrosterone was produced than was andro-

sterone, and the β-configuration is also the preferred configuration at position 17, since androstan-17β-ol-3-one, but not androstan-17α-ol-3-one, was isolated from the incubations.

The preponderance of 17-ketones over 3-ketones, and 17-β-hydroxyl compounds over 17-α-hydroxyl compounds, is in accord with the urinary excretion pattern of man cited previously (83). The preponderance of formation of 3-β-hydroxyl compounds over 3-α-hydroxyl compounds, in these in vitro studies, is in contrast to the urinary excretion pattern in man (83), but is in accord with the pattern which has been observed in the guinea pig (84).

Certain theoretical considerations pertinent to these results have been reported. Brown, Brewster, and Schecter (104) have pointed out that five-membered ring ketones are relatively less susceptible to reaction of the ketone than are the analogous six-membered-ring ketones. This would explain the in vitro results observed here as well as the excretion pattern observed in man. The oxidative potentials (105) of comparable compounds lend quantitative support to these considerations; e.g., E^{o}, mv.; cyclohexanone, 162; cyclopentanone, 123.

The relative amounts of the various hydroxyl compounds formed can also be correlated with chemical principles. Epiandrosterone is less strained than is androsterone (106). Chemical reduction of androstanedione with lithium

borohydride gives epiandrosterone as the principal product (106), verifying both the greater ease of reduction of the 3-ketone function and the preferential formation of the 3-β-hydroxyl over the 3-α-hydroxyl. Testosterone (17-β-hydroxyl) is less strained than epitestosterone (17-α-hydroxyl) (107).

However, isomer distribution can also be regulated by factors of an enzymatic nature. Whereas the guinea pig contains reductases which form both 3-hydroxyl isomers and only one 17-hydroxyl isomer (testosterone) from androstenedione, rabbit liver and kidney possess reductases which form only one 3-hydroxyl isomer (androsterone), but both 17-hydroxyl isomers from androstenedione (52,56). Evidently, it is the operation of such enzymatic factors which results in the formation of androsterone as the principle urinary androstane derivative in man (83), but epiandrosterone as the principal urinary androstane derivative in the guinea pig (84).

In order to provide a sounder basis for such considerations, a study was performed in which fourteen compounds containing at least one hydroxyl group, as well as the nineteen-carbon androstane nucleus, were incubated with male guinea pig liver homogenates. Sodium pyrophosphate buffer, pH 8.9, was employed as the incubation medium, and the homogenates and steroids were incubated with 50 mg. of DPN and 400 mg. of NA. The results are given in Table 13.

TABLE 13

METABOLISM OF C_{19} HYDROXYSTEROIDS BY GUINEA PIG LIVER HOMOGENATES[a]

Substrate steroid	Substrate recovered %	Metabolites recovered %
Testosterone	42.4	Androstenedione, 55.4
Androstanolone	71.6	Androstanedione, 13.8
Epitestosterone	77.4	Androstenedione, 5.0
Epiandrosterone	66.0	Androstanedione, 16.8
Androsterone	65.6	Androstanedione, 10.6; Epiandrosterone, 1.4
Dehydroepiandrosterone	80.4	Androstenedione, 8.4
5-Androstene-3β,17α-diol	104.0	Dehydroepiandrosterone, 3.2; Androstenedione, 2.4 Epitestosterone, 1.0
5-Androstene-3β,17β-diol	85.0	Androstenedione, 3.0; Dehydroepiandrosterone, 1.4 Testosterone, 0.2
Androstane-3α,17β-diol	102.6	Androstanedione, 1.1; Androsterone, 0.6 Epiandrosterone, 0.4
Androstane-3β,17β-diol	103.2	Androstanedione, 1.1; Epiandrosterone, 0.9 Androsterone, 0.3
5-Androsten-3β-ol	98.0	----------
Androstan-3α-ol	94.0	----------
Androstan-3β-ol	96.0	----------
Androstan-17β-ol	90.0	----------

[a]Twelve grams of liver and 100 ml. of sodium pyrophosphate buffer solution, 0.166 molar, pH 8.9, incubated with 400 mg. NA and the weights of steroid and DPN indicated for one and one-half hours at 37-8° C.

Monohydroxy monoketone compounds were oxidatively metabolized to the corresponding diketone compounds; androsterone gave rise to epiandrosterone as well as androstanedione. Dihydroxy compounds gave rise to the corresponding diketones via oxidation of both hydroxyl groups, as well as a pair of monohydroxy monoketone compounds, in each instance, corresponding to the oxidation of the two hydroxyl groups singly. Incubation of four compounds containing a single oxygen function, either at position 3 or position 17, did not result in the formation of metabolites in isolable amounts. Metabolism of compounds containing a double bond at position 5, in addition to a hydroxyl group at position 3, resulted in the formation of α,β-unsaturated ketones in the A ring of the steroid nucleus.

In general, the extent of metabolism observed corresponded to the solubility of the compound.[2] This was not rigidly true, since epiandrosterone and dehydroepiandrosterone are both more soluble than testosterone; testosterone was metabolized about three times the extent to which the former compound was metabolized, and about six times the extent to which the latter was metabolized. However, the

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[2]Accurate solubility measurements have been reported for only a few of the androgens (108). In lieu of such measurements, the steroid absorption data which has been determined in various species by Kochakian (98,99,101), has been used as an index of the relative solubility of the androgens. In general, steroid absorption data correlates well with the solubility data which has been compiled.

solubility influence is particularly noticeable when the conversions of the monohydroxy monoketones (5.0-55.4%), the dihydroxy compounds (2.1-6.6%), and the monohydroxy compounds (0.0%) are compared; these values stand in agreement with the relative solubilities of these groups of compounds.[2]

Comparison of the metabolic conversions of compounds containing identical hydroxyl functions provides the best correlations. Six of the compounds studied containing the 17-β-hydroxyl group; testosterone, androstanolone, 5-androstene-3β,17β-diol, androstane-3α,17β-diol, androstane-3β,17β-diol, and androstan-17β-ol. These compounds were metabolized in decreasing amounts in the order listed, and their solubilities decrease in the same order. Essentially the same correlation was observed with the 17-α-hydroxyl compounds, epitestosterone and 5-androstene-3β,17α-diol; the 3-β-hydroxyl compounds, epiandrosterone, dehydroepiandrosterone, 5-androstene-3β,17α-diol, 5-androstene-3β, 17β-diol, 5-androsten-3β-ol, and androstan-3β-ol; and the 3-α-hydroxyl compounds, androsterone, androstane-3α,17β-diol, and androstan-3α-ol.

These results may be due as much to experimental design as to any other factor. The quantity of steroid employed (50 mg.) is far in excess of the solubility of any of the steroids studied. On the other hand, the extent of metabolic conversion of some of the steroids studied, notably testosterone, is also far in excess of the solubility. The

solubility of testosterone in 100 ml. of water is approximately 2.5-3.0 mg. (108); this value is increased slightly in buffer solution. The metabolic conversion of testosterone under the conditions employed approached 28 mg.

If nothing more, the data obtained would appear to give insight into the influence of chemical structure upon the resultant of two unsegregated variables, namely, the solubility of the steroids and/or extent of metabolic conversion under the conditions employed. If of no other value, such information should be useful in therapeutic considerations, since current therapy involves the use of quantities of steroids which approximate the conditions of these experiments.

This research has been extended by other workers in this laboratory (53).

CHAPTER V

EXPERIMENTAL PROCEDURES EMPLOYED FOR THE PREPARATION AND ASSAY OF FRACTIONS OF GUINEA PIG LIVER HOMOGENATES

Certain preliminary investigations into the purification and properties of the enzyme systems responsible for androgen metabolism were performed subsequent to the incubation studies reported in Chapters II, III, and IV. In order to facilitate such studies it seemed desirable to employ methods which would permit a more rapid determination of the results of the various purification procedures utilized. Spectrophotometric methods were used for this purpose.

Sweat et al. (87) have described the concentration of an enzyme from steer liver tissue which promotes the conversion of testosterone to androstenedione; DPN was a necessary cofactor. Talalay and Dobson (46) have reported the partial purification of adaptive steroid dehydrogenase enzymes which were produced in Pseudomonas by the incorporation of testosterone into the growth medium. The latter workers followed the formation and disappearance of $DPNH_2$ spectrophotometrically, and used the rate of change at the appropriate wave

length, 340 mμ., as a measure of enzyme activity in the reaction mixtures studied. The spectrophotometric studies described subsequently employed methods of enzyme activity assay which were patterned after those described by Talalay and Dobson.

These assay methods recognize the role diphosphopyridine nucleotide plays in the metabolic conversion of the androgen compounds. They assume that the principal reaction occurring in reaction mixtures consisting of tissue homogenates, or tissue homogenate fractions, and large excesses of steroid and DPN, will be given by

$$\text{steroid-}H_2 + \text{DPN} = \text{steroid} + \text{DPN}H_2$$

and that any companion reactions involving the conversion of DPN to $DPNH_2$ will be inconsequential when compared to this principal reaction. If such assumptions are valid, the formation of $DPNH_2$ can be taken as a direct measure of the extent of steroid conversion. Furthermore, the rate of formation of $DPNH_2$, under appropriate conditions, can be used as a measure of enzyme activity.

Reductive metabolic conversions can be followed in identical fashion using reaction mixtures consisting of tissue homogenates, or tissue homogenate fractions, and large excesses of steroid and $DPNH_2$. The principal reaction is assumed to be

$$\text{steroid} + \text{DPN}H_2 = \text{steroid-}H_2 + \text{DPN}$$

and the disappearance of $DPNH_2$, rather than its formation, is

followed spectrophotometrically.

In order for the rate of formation or disappearance of $DPNH_2$, and thereby steroid, to be a valid measure of enzyme activity, the quantity of enzyme present in the reaction mixture must be rate-limiting. That such a condition prevails is most easily demonstrated by the continued dilution of the reaction mixtures with respect to tissue concentration; when a given dilution produces a parallel decrease in enzyme activity, the concentration of enzyme present may be considered to be rate-limiting.

In order to follow purification procedures more easily, Talalay and Dobson (46) arbitrarily defined a unit of enzyme activity as the quantity of enzyme which is necessary to produce a change of optical density of 0.001 per minute.

Since this unit has the features of rate of chemical reaction, the number of units present in a reaction mixture will be dependent upon the pH of the medium. For purposes of comparison, all oxidative conversions in this study were performed at pH 9.0, and all reductive conversions were performed at pH 6.75. Sodium pyrophosphate and sodium phosphate buffer solutions, respectively, were employed to obtain these pH values. The preparation of these buffer solutions has been described in Chapter II.

Since chemical reactions are also temperature dependent, the number of units of enzyme activity (as defined

above) present in a reaction mixture will be dependent upon the temperature at which the reaction is conducted. No facilities were available for conducting the reactions at a constant temperature; all reactions in this study were performed at room temperature. Comparisons of enzyme activities were made only when the assays were performed at the same temperature.

Preparation of homogenates. Two types of homogenizers were employed to prepare the tissue homogenates used in these studies. One group of homogenates was prepared with a Waring blendor in the manner described in Chapter II. Homogenates which were employed in cell component fractionation studies were prepared using a motor-driven Erway glass homogenizer, which is a modification of the type developed by Potter and Elvejhem (109). When the latter homogenizer was used, the tissue was homogenized for three minutes in batches of approximately three and one-half g.; ten ml. of suspension medium was used for each batch. Cold disodium phosphate buffer solution, 0.1 molar, pH 6.75, was employed as the homogenization medium when buffer was used, since enzyme activity endured for longer periods of time at this pH value than at other pH values. Cold sucrose solution, 0.25 molar, was employed as the homogenization medium in the cell component fractionation studies. A few studies were performed on homogenates which were prepared in homogenization media containing varying amounts of cold absolute ethyl

alcohol. After preparation, the homogenates were diluted to appropriate volumes, usually ten g. of tissue to one hundred ml. of solution, with the medium employed for homogenization. After dilution, the homogenate solutions were stored in a refrigerator which was maintained at 0-3.5° C.

Fractionation procedures. Tissue homogenate fractionations were performed by protein precipitation with a cold saturated solution of ammonium sulfate, by protein precipitation with cold absolute ethyl alcohol, and by centrifugation. A number of centrifugation programs were employed, including centrifugal fractionation of cell components. Several reviews have been published concerning both the techniques and the results of such fractionations (110-113). The separation of a number of components is described in these reviews, but the isolation of nuclei, mitochondria, microsomes, and a so-called "soluble fraction" is most frequently performed; this pattern was employed in these studies.

Centrifugal fractionation of cell components. The techniques utilized were essentially those described by Umbreit et al. (114). Cold sucrose solution was used to suspend the sediments obtained by centrifugation, since it was also employed as the homogenization medium. Three centrifuges were employed; an International Centrifuge, model V, an International Refrigerated Centrifuge, model PR-2, and a Spinco Preparative Ultracentrifuge, model L.

The first-named centrifuge is unrefrigerated; however, experimentation indicated that the centrifugation periods used were insufficient for heat denaturation.

Nuclear fractions were obtained by centrifugation in the unrefrigerated centrifuge using a speed of 2000 revolutions per minute for a period of fifteen minutes; this speed corresponds to a centrifugal force of 1000 times gravity. Subsequent procedure varied. In some experiments, the sediment obtained after a single centrifugation was designated as the nuclear fraction, and the supernatant fraction was employed for the subsequent centrifugation. In other experiments, the sediment from the first centrifugation was resuspended and centrifuged as before; the second sediment was designated as the nuclear fraction, and the supernatant fractions were combined prior to subsequent centrifugation. In still other experiments, both the original sediment and the original supernatant were recentrifuged, and then handled in the manner described above.

Mitochondrial fractions were obtained by centrifugation in the International Refrigerated Centrifuge using a speed of 11,500 revolutions per minute[1] for periods of ten and fifteen minutes; this speed corresponds to a centrifugal force approximately 8,500 times gravity. This centrifuge

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[1]Actual motor speed employed was 2500 revolutions per minute; a high-speed attachment with a pulley ratio of 4.6 to 1 permits the use of faster speeds.

was operated at a temperature of 3-8° C. The sediment fractions obtained in this fashion were resuspended in sucrose solution and centrifuged as before. The sediment fractions resulting after the second centrifugation were designated as the mitochondrial fractions, and the supernatant fractions were combined and employed for subsequent fractionation.

In the initial experiments, the sediments obtained by high-speed centrifugation were resuspended with stirring. In later experiments this was supplemented by manual homogenization in an Erway homogenizer.

The initial microsomal fractions were obtained in the same centrifuge using a speed of 16,500 revolutions per minute[2] for a period of one hour; this speed corresponds to a centrifugal force approximately 18,000 times gravity. In later experiments a speed of 17,500 revolutions per minute[3] was used; this speed corresponds to a centrifugal force approximately 20,000 times gravity. Certain of the microsomal fractions were obtained using the Spinco centrifuge; a speed of 15,220 revolutions per minute, corresponding to an average centrifugal force 19,200 times gravity, was employed. The Spinco centrifuge was operated at 35° F. Originally the sediment and supernatant fractions so obtained were desig-

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[2]Actual motor speed employed was 3600 revolutions per minute; see footnote 1.

[3]Actual motor speed employed was 3800 revolutions per minute; see footnote 1.

nated as the microsomal and soluble fractions, respectively. In later experiments, both fractions were recentrifuged prior to such designation.

This fractionation program was altered in the last phase of the investigation; mitochondrial and microsomal material was separated as a single fraction by the elimination of the intermediate centrifugation.

No efforts were made to verify the designations applied to the various fractions. Experimentation indicated that there were no detectable differences in either the nitrogen contents or the testosterone dehydrogenase activities of supernatant fractions obtained at 1500 or 2000 revolutions per minute for periods of ten, fifteen, or twenty minutes. This would seem to indicate the separation of discreet fractions. Mitochondrial and microsomal fractions had the visual appearances described by Umbreit et al. (114).

Enzyme activity assay methods. The tissue homogenates and tissue homogenate fractions obtained by these procedures were assayed for enzyme activity spectrophotometrically on the basis of the considerations listed previously. Two spectrophotometers were employed for this purpose; a Beckman Quartz Spectrophotometer, model DU, and a Cary Automatic Recording Spectrophotometer, model 14. The latter instrument was available only during the last stages of these studies. When the Beckman instrument was employed, four

types of reaction mixtures were used in assays of enzyme activity: the first consisted of two ml. of water, and one ml. of an appropriate tissue dilution;[4] this mixture was used as a blank; the second consisted of one ml. of water, one ml. of DPN, or $DPNH_2$, solution (usually containing 300-350 μg. of nucleotide), and one ml. of the same tissue dilution; the third and fourth reaction mixtures, which were prepared in duplicate, consisted of one ml. of nucleotide solution, one ml. of steroid solution (usually containing approximately 25 μg. of steroid), and one ml. of the tissue dilution which was used in the other reaction mixtures. These mixtures were prepared in test tubes, and transferred to spectrophotometer cells, immediately after the addition of the tissue solutions, which was the last component added. The instrument was adjusted so that the first reaction mixture gave an optical density reading of zero (at 340 mμ.), and optical density readings (at 340 mμ.) were determined immediately for the other reaction mixtures. The reactions were allowed to proceed for varying periods of time, usually ten to twenty minutes, and optical density readings (at 340 mμ.) were determined at the end of each two minute interval.

In order to determine the enzyme activity of the tissue solution employed, the values of the optical density readings for the second reaction mixture were subtracted

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[4]Such dilutions were made with one of the two buffer solutions described on page 92.

from the values of the optical density readings of the reaction mixtures containing steroid, and the differences obtained were plotted against time elapsed. The slope of such a plot has the units of change in optical density per minute; the slopes of such plots were employed to determine the number of units of enzyme activity as defined above.

Enzyme activity assays performed with the Cary instrument were performed in similar fashion. This instrument was designed for use with three spectrophotometer cells, two of which, with their contents, were employed to adjust the instrument to give an optical density reading of zero. Reaction mixtures corresponding to the second reaction mixtures described above were placed in these cells. Steroid-containing reaction mixtures, corresponding to the third and fourth reaction mixtures described above, were placed in the third cell. The Cary instrument automatically subtracts the values of the optical density readings of the "blank reaction mixtures," in this work the mixtures which did not contain steroid, from the values of the optical density readings of the "full complement reaction mixtures," the steroid-containing reaction mixtures, and continuously records the difference value. The graph paper on which the difference value is recorded is time-calibrated; the plot so recorded is identical in nature to those obtained manually when the Beckman instrument was used. Again, the slopes of such plots were used to determine the number of units of enzyme activity.

A plot representative of those obtained manually is shown in Figure 6. A tracing of an automatically-recorded plot is reproduced in Figure 7.

Preparation of steroid solutions. Steroid solutions were prepared by shaking appropriate quantities of steroid with appropriate volumes of distilled water for periods of twenty-four to forty-eight hours. When saturated solutions were employed, as was the case with testosterone, the solid remaining after shaking was removed by filtration. The concentrations of unsaturated solutions were predetermined by accurate measurement of the weights and volumes used. The concentrations of saturated solutions of testosterone and androstenedione were determined spectrophotometrically in the manner described in Chapter II. These steroids exhibit maximum absorption at 249 mμ. in aqueous solution.

A mechanical shaker was employed to prepare these steroid solutions. This apparatus has a horizontal displacement of 2½ inches, and a displacement frequency of 250 cycles per minute.

Determination of enzyme purity. Enzyme activity assays performed in the manner described, gave, in themselves, indications of the extent to which total enzyme activity was preserved during the various operations performed. In order to ascertain the degree of purification obtained by these same operations, it was necessary to determine the extent of removal of inactive material. Since such inactive material

FIGURE 6

MANUALLY RECORDED PLOT OF TESTOSTERONE DEHYDROGENASE ACTIVITY ASSAY

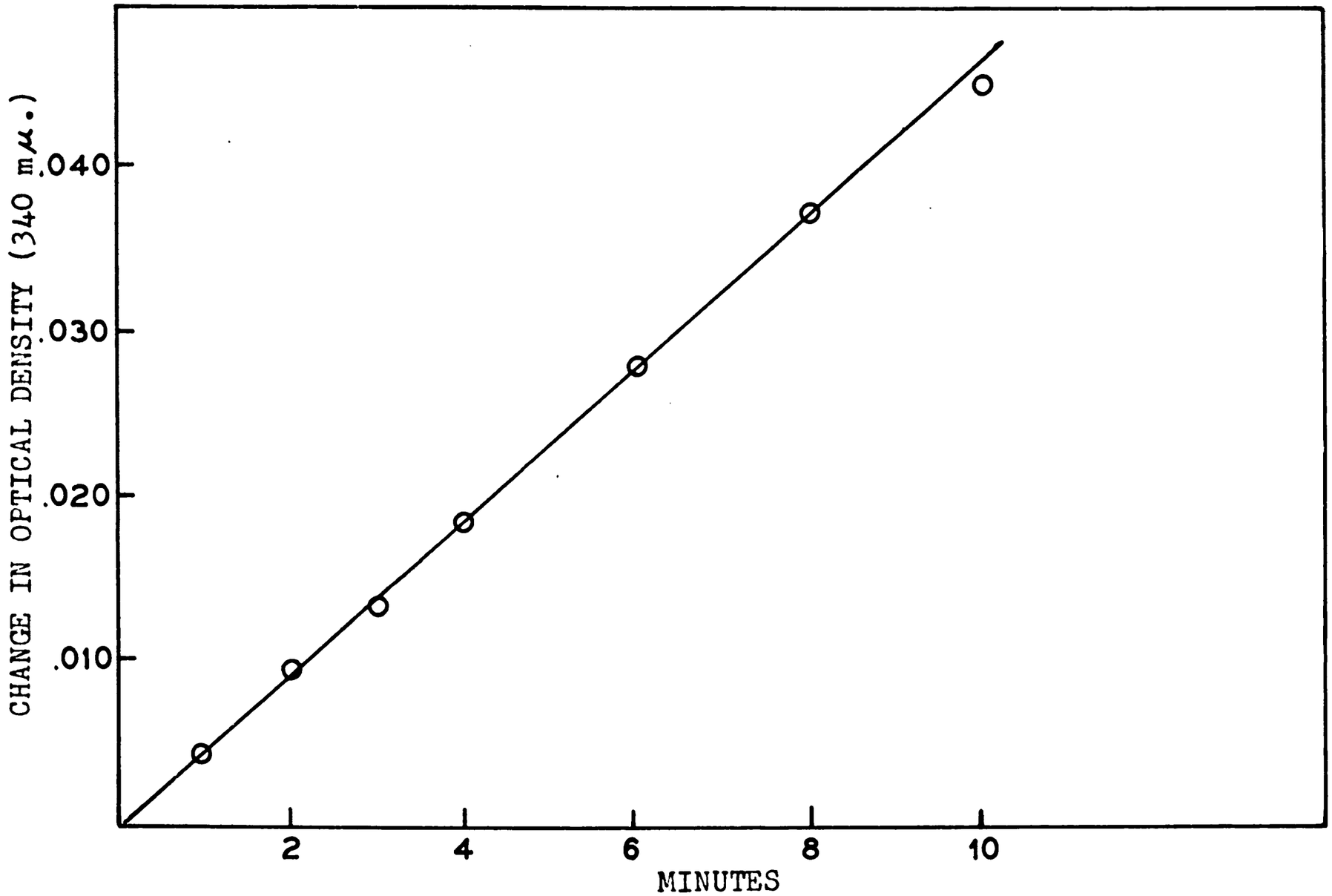
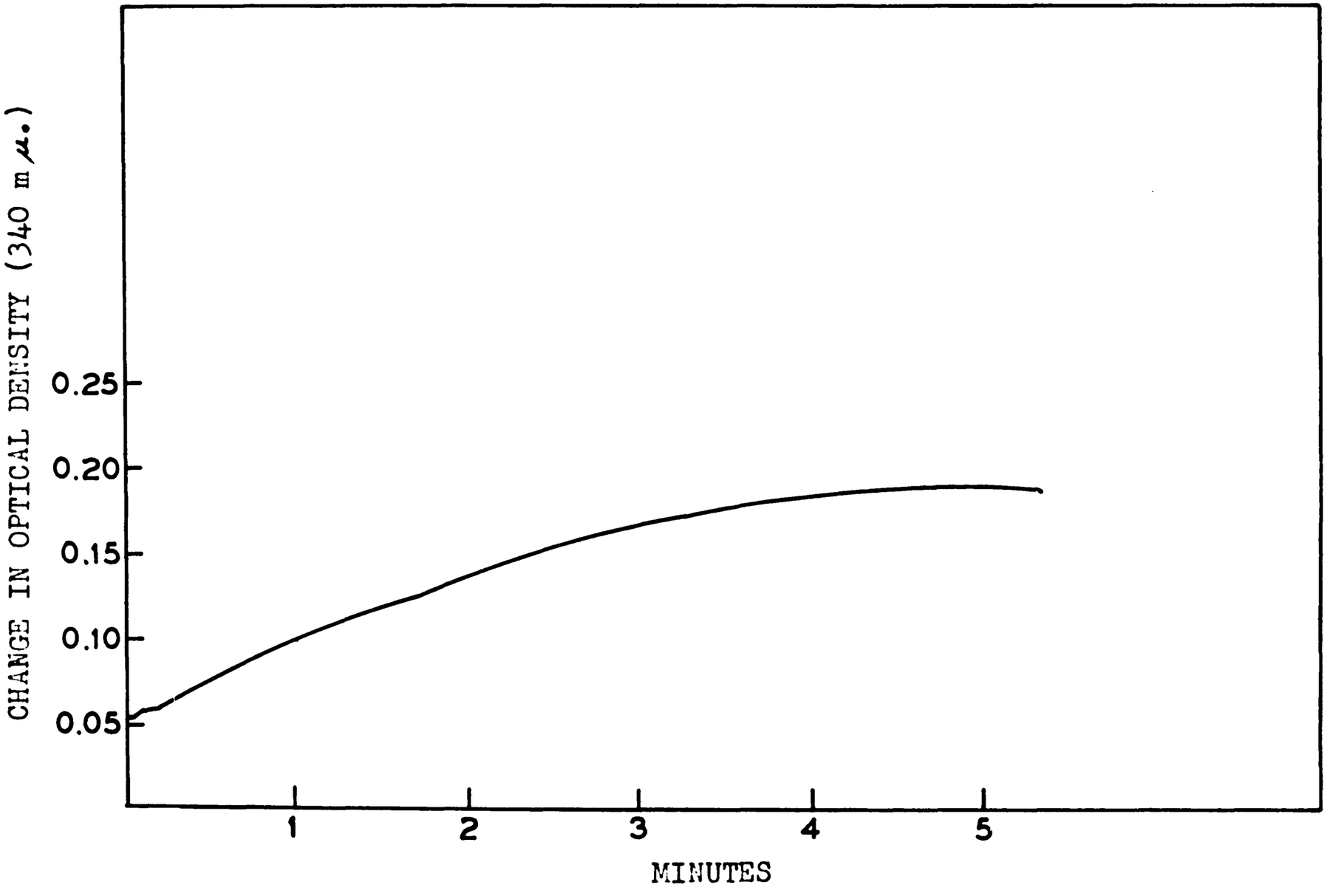


FIGURE 7

AUTOMATICALLY RECORDED PLOT OF TESTOSTERONE DEHYDROGENASE ACTIVITY ASSAY



was primarily protein in nature, the tissue homogenates and tissue homogenate fractions were submitted to the nitrogen analysis division of this laboratory, which performed analyses for that component. Such analyses were made by a micro-Kjeldahl method. The purity of the various tissue homogenates and tissue homogenate fractions, with regard to the steroid dehydrogenase enzymes studied, were based accordingly on the "specific activities" of these fractions, i.e., the units of enzyme activity per mg. of nitrogen.

The majority of the studies carried out in this fashion utilized testosterone as the substrate steroid. This was due to several reasons: testosterone is considered to be the principal androgen (115), and studies of this nature on the enzyme responsible for its oxidative metabolism in the guinea pig have not yet been reported; from the work reported by Talalay (46), as well as the evidence reported in Chapters IV and V of this dissertation, the enzyme responsible for the oxidative metabolism of testosterone may well be responsible for the conversion of other androgens containing either 17-β or 3-β-hydroxyl groups; finally, guinea pig liver homogenates exhibit a more pronounced activity toward testosterone than toward any other androgen.

The results of the investigations outlined in this chapter are reported in Chapter VI.

CHAPTER VI

THE METABOLISM OF TESTOSTERONE AND OTHER ANDROGENS BY GUINEA PIG LIVER HOMOGENATE FRACTIONS

Preliminary centrifugal fractionation. Reference was made in Chapter III to experiments in which guinea pig liver homogenate fractions, obtained by centrifugation, were employed in incubation studies. The results of these incubation studies seemed to indicate a separation of epiandrosterone-forming enzyme activity from androsterone-forming enzyme activity. The initial experiments which were performed using the spectrophotometric methods described in the last chapter were an extension of these experiments. In order to effect partial purification of some of the steroid dehydrogenase enzymes, guinea pig liver homogenates were subjected to various centrifugal forces; enzyme activities and nitrogen contents were determined subsequently in the supernatant and sediment fractions so obtained. Two steroids, androstanedione and testosterone, were employed in the reaction mixtures which were used for spectrophotometric assays. Some of the results obtained in this fashion are shown in Table 14.

TABLE 14

DISTRIBUTION OF STEROID DEHYDROGENASE ACTIVITY AFTER LOW- AND HIGH-SPEED CENTRIFUGATION OF GUINEA PIG LIVER HOMOGENATES[a]

Centrifugal force (x g.)	Fraction	Total activities units[b]		% Total activity	
		Testosterone	Androstanedione	Testosterone	Androstanedione
1,000	Supernatant	38,000	12,500	100	100
80,000	Supernatant	2,500	1,060	7	9
80,000	Sediment	23,350	7,250	60	58

[a]Homogenates were prepared in 0.1 molar disodium phosphate buffer solution, pH 6.75; a Waring blendor was used for homogenization.

[b]See page 92 for definition of units employed.

These data suggested that the separative procedures employed did not effect a separation of the enzyme responsible for the oxidative conversion of testosterone from the enzyme(s) responsible for the reductive conversion of androstanedione, or that a single enzyme was responsible for both conversions. The latter is reasonable since the formation of epiandrosterone is the principal reaction in androstanedione conversion; both testosterone and epiandrosterone can be metabolized by a single enzyme (46).

Since there was a considerable loss in total activity (25-40%) involved in the low-speed (1000 x g.) centrifugal separation, followed by a further loss in total activity (40% of the activity of the 1000 x g. supernatant) when higher speeds were employed, this separative procedure was considered impractical. The procedure was repeated one time, however, in order to determine the specific activity of the high-speed (80,000 x g.) sediment fraction, and to determine the behavior of this fraction toward ammonium sulfate. Since no separation of "testosterone activity" and "androstanedione activity" was realized in the first experiments, assays of enzyme activity using androstanedione were not performed.

The supernatant obtained by low-speed (100 x g.) centrifugation had a specific activity of 188 units per mg. of nitrogen; the sediment obtained by high-speed (80,000 x g.) centrifugation had a specific activity of 357 units per mg. of nitrogen. Thus, less than a two-fold purification of

enzyme activity was obtained, at the expense of approximately 40% of the total activity of the low-speed supernatant. In the initial work, the activities of the various fractions were compared to the activities of the low-speed supernatant fractions from which they were derived, since the latter fractions were much easier to work with than were the homogenates from which they were, in turn, derived.

Ammonium sulfate fractionation. The sediment fraction obtained by high-speed (80,000 x g.) centrifugation was treated with varying amounts of a cold saturated solution of ammonium sulfate, and allowed to stand overnight. Assay of the activity present in the precipitates produced by this treatment, after centrifugation and resuspension, indicated that 90-95% of the enzyme activity present in the sediment fraction was present in the precipitate produced in the presence of a concentration of ammonium sulfate corresponding to 40% saturation. This precipitation work was performed in a cold room which was maintained at 3-8° C. However, the enzyme activity present in the resuspended precipitate decreased on standing. Furthermore, attempts to dialyze this fraction against tap water were unsuccessful; all activity had disappeared after a dialysis period of twenty four hours.

Influence of nicotinamide. An investigation into the influence of NA upon enzyme activities was carried out during the course of these experiments. The NA was dissolved

in the nucleotide solutions in one series of experiments, so that the reaction volumes remained three ml.; in a second series of experiments, the reaction volumes were increased to 3.2 ml. by the addition of 0.2 ml. of NA solution. In most instances 2.4 mg. of NA were employed, corresponding to a NA:nucleotide ratio of approximately 8:1, which was the ratio used in the majority of the incubation studies. NA increased the steroid dehydrogenase enzyme activities in homogenates; in a typical experiment, the enzyme activity was one and two-thirds times greater in the presence of NA than in its absence. This enhancing effect paralleled that observed when NA was employed in the incubation studies, as well as that reported by Sweat et al. (45), and Nall (61). However, an opposite effect was produced in the low-speed (1000 x g.) supernatant fractions studied, since the addition of NA to the reaction mixtures resulted in a decrease in enzyme activity; the magnitude of the decrease was about 20%. A search of the literature revealed that similar observations had been reported previously; Elvejhem and his co-workers (116) observed an increase in malic dehydrogenase activity when NA was added to homogenates, but a decrease in activity when purified preparations of the enzyme were employed. These workers attributed the beneficial effect of NA to its ability to inhibit DPN Nucleotidase, the detrimental effect to a reversible inhibition of the DPN-requiring malic dehydrogenase (116). Apparently the beneficial effect

of NA can only be realized in the presence of the nucleotidase. If this assumption is valid, it follows that low-speed (1000 x g.) centrifugation effects a sedimentation of at least a significant portion of DPN nucleotidase present in guinea pig liver homogenates.

Since NA exhibited these opposite influences, it was not included as one of the reaction components in subsequent assays of enzyme activities.

Fractionations with ethyl alcohol. Varying portions of cold absolute ethyl alcohol were included in the homogenization medium in the next series of experiments.[1] The homogenates resulting were centrifuged as before (1000 x g.), and enzyme activities subsequently determined. Some of the results obtained are shown in Table 15.

Homogenization in a medium containing 20% absolute ethyl alcohol (by volume) gave rise to an altered nitrogen distribution pattern when such homogenates were centrifuged. Normally, the supernatant fraction obtained by low-speed (1000 x g.) centrifugation contained 60-65% of the homogenate nitrogen; when the homogenization medium contained 20% alcohol, only 50% of the homogenate nitrogen remained in the analogous supernatant fraction. Increasing amounts of alcohol decreased the nitrogen content of the supernatant

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[1]All experiments with alcohol were carried out in the cold room.

TABLE 15

DISTRIBUTION OF TESTOSTERONE DEHYDROGENASE ACTIVITY AFTER CENTRIFUGATION OF GUINEA PIG LIVER HOMOGENATES PREPARED IN ALCOHOLIC AND NON-ALCOHOLIC BUFFER SOLUTIONS[a]

Alcohol %	Fraction[b]	Total activity units[c]	Specific activity units per mg. N	Purification
0	Supernatant	5390	82.5	---
0	Sediment	2160	105.	---
20	Supernatant	----	----	---
20	Sediment	7105	195.	2.4

[a]Homogenates were prepared in 0.1 molar disodium phosphate buffer solution, pH 6.75, containing the amounts of cold absolute ethyl alcohol indicated. A Waring blendor was used for homogenization.

[b]Fractions were obtained by centrifugation at a speed corresponding to a force of 1000 x g.

[c]See page 92 for definition of units employed.

still further.

Although a two-fold purification was obtained by the incorporation of alcohol into the homogenization medium, this purification procedure was undesirable, since the residue obtained was difficult to resuspend and was heterogeneous with respect to particle size. Accordingly, attempts were made to use alcohol as a selective protein precipitant. Various quantities of cold absolute ethyl alcohol were added to tissue homogenate solutions after their preparation, and the mixtures were allowed to stand overnight prior to centrifugation. Some of the results obtained in this fashion are shown in Table 16.

These preliminary investigations seemed to offer some promise for the use of alcohol as a purifying reagent. The sediment fraction obtained by centrifugation after addition of alcohol to a concentration of 10% (by volume) contained only 2.7% more of the homogenate nitrogen than did the analogous fraction obtained in the absence of alcohol. Although the sediment produced by 10% alcohol and centrifugation was not assayed for enzyme activity, the corresponding supernatant fraction contained only one-half of the homogenate activity present in the supernatant obtained in the absence of alcohol.

The principal disadvantage involved in the use of organic solvents as protein precipitants lies in the denaturing effect which they often exhibit. The sum of the

TABLE 16

DISTRIBUTION OF TESTOSTERONE DEHYDROGENASE ACTIVITY AND NITROGEN AFTER CENTRIFUGATION OF GUINEA PIG LIVER HOMOGENATES PREPARED IN ALCOHOLIC AND NON-ALCOHOLIC BUFFER SOLUTIONS[a]

Alcohol %	Centrifugal force (x g.)	Fraction	Homogenate nitrogen %	Homogenate activity %	Specific Activity units[b] per mg. N.
--	----	Homogenate	100	100	146
--	1	Supernatant	79.9	---	149
--	1000	Supernatant	64.9	71	164
--	1000	Sediment	36.4	7	45
10	1000	Supernatant	62.2	34	89
10	1000	Sediment	37.2	---	---
20	1000	Supernatant	35.4	0	0
20	1000	Sediment	69.1	26	78

[a]Homogenates were prepared in 0.1 molar disodium phosphate buffer solution, pH 6.75, containing the amounts of cold absolute ethyl alcohol indicated. A Waring blendor was used for homogenization.

[b]See page 92 for definition of units employed.

activities of the two fractions obtained when an alcohol concentration of 20% was employed, 26% of the activity present in the homogenate, suggested that denaturation had occurred in this instance. In an attempt to reduce denaturation, the period of time during which the tissue was in contact with alcohol was abbreviated. Prior to the addition of alcohol, the tissue homogenate solutions used were centrifuged in order to separate the material which could be removed in this fashion, and the supernatants so obtained were diluted with alcohol to a concentration of 20% (by volume). Some of the results are listed in Table 17.

These results indicated that the alcoholic precipitation process was a slow one, since more enzyme activity was sedimented after a five-hour period. In addition, the reagent and/or the concentration and/or the contact periods were rather specific, since the specific activity in the sediment fraction increased with time; material with a rather high specific activity was "precipitated" in the period between four and five hours. Calculation of the apparent specific activity of the material "precipitated" in this interval gave a value ten times larger than the specific activity of the homogenate. However, prior "precipitation" of considerable inactive material had occurred, since the purification obtained after five hours was only slightly greater than two-fold. Attempts to separate the active material from that which was inactive by centrifugations at

TABLE 17

INFLUENCE OF LENGTH OF ALCOHOLIC CONTACT PERIOD ON TESTOSTERONE DEHYDROGENASE ACTIVITY OF A FRACTION OF GUINEA PIG LIVER HOMOGENATES[a]

Alcohol %	Contact period hours	Fraction	Homogenate nitrogen %	Homogenate activity %	Specific activity units[b] per mg. N
--	--	Homogenate	100	100	150
--	--	Supernatant	59	70	178
20	3	Supernatant	52	45	164
20	3	Sediment	16	19	286
--	--	Homogenate	100	100	157
--	--	Supernatant	60	68	178
20	4	Supernatant	46	--	---
20	4	Sediment	17	32	294
20	5	Supernatant	44	--	---
20	5	Sediment	18	42	370

[a]Guinea pig liver homogenates were centrifuged at 1000 x g.; the supernatants were diluted with cold absolute ethyl alcohol to a concentration of 20%, and allowed to stand for the periods indicated prior to a second centrifugation at the same speed.

[b]See page 92 for definition of units employed.

various intervals were unsuccessful, probably due to the minute quantities of enzyme involved.

Longer periods of contact with alcohol did not produce increases in either the extent of purification or total activity in the sediment fraction. Since only 42% of the homogenate activity was present in the sediment fraction in which the purification existed, this procedure did not seem appropriate as an initial step of a purification sequence. Its use in conjunction with other procedures remains a possibility.

Centrifugal fractionation of cell components. A centrifugal fractionation of cell components was undertaken, in the manner described in the preceding chapter, in the next group of experiments. The results are shown in Table 18.

The purification obtained in the microsomal fraction, four-fold, was greater than any which had been obtained previously. Furthermore, the total activity present in this fraction compared favorably with that which had been obtained by other methods. However, the presence of considerable activity in the other fractions seemed to indicate that a rather poor separation of cell components had been effected. Accordingly, some of the refinements listed in the previous chapter, such as resuspension and recentrifugation of sediments, were incorporated into a second centrifugal fractionation. The results are shown in Table 18.

TABLE 18

DISTRIBUTION OF TESTOSTERONE DEHYDROGENASE ACTIVITY IN GUINEA PIG LIVER CELL COMPONENT FRACTIONS

EXPERIMENT 1

Fraction	Homogenate total activity %	Homogenate nitrogen %	Specific activity units[a] per mg. N	Purification
Homogenate	100	100	106	---
Nuclei	15.3	---	---	---
Mitochondria	19.1	8.0	250	2.4
Microsomes	38.3	8.8	454	4.3
Soluble	13.4	40.7	34	---
TOTAL	86.1	---	---	---

EXPERIMENT 2

Homogenate	100	100	67	---
Nuclei	---	36.9	---	---
Mitochondria	32.7	12.2	179	2.7
Microsomes	42.2	9.2	307	4.6
Soluble	12.0	37.0	22	---
TOTAL	86.9	95.3	---	---

[a]See page 92 for definition of units employed.

The refinements utilized effected the shift of enzymatic activity from the nuclear fraction to the mitochondrial and microsomal fractions; the increase in activity was more pronounced in the former. The shift in enzymatic activity was accompanied by an increase in nitrogen content in the two fractions, so that the purification factors exhibited only slight increases; the values observed were 2.7 and 4.6, in the mitochondrial and microsomal fractions, respectively. Both of these factors compared favorably with those obtained by previous methods, and the two fractions possessed 80% of the total activity of the homogenate.

Although the total activity obtained in the microsomal fraction was greater than that obtained in the other fractions in both of the fractionation experiments, the activity present in this fraction was not sufficient to designate the microsomes as the exclusive site of testosterone dehydrogenase activity. The presence of enzyme activity in the mitochondrial and soluble fractions was probably due to microsomal contamination of these fractions. Unfortunately, no data have been reported concerning nitrogen distribution in the particulate fractions of guinea pig liver cells. Such data would permit an assessment of the efficiency of separations in these fractionations. Schneider and Hogeboom (110) have reported the nitrogen distribution in the particulate fractions of rat liver, mouse liver, and rabbit liver, but species variations prohibits any comparisons.

Since this experimentation had, as its primary purpose, the separation of a fraction which contained a large proportion of the enzyme activity originally present in homogenates, in such a fashion that purification was obtained, a third centrifugal fractionation was performed in which the mitochondrial and microsomal materials were separated as a single fraction. The results are shown in Table 19.

The sums of the values observed in the mitochondrial and microsomal fractions for per cent nitrogen and per cent total activity (per cent of homogenate) in the former fractionations are included in Table 19 for purposes of comparison. The results indicate a gradual increase in both values; these increases can be attributed to refinements in techniques and continued experience. Separation of the mitochondrial and microsomal material as a single fraction reduced the manipulation necessary and permitted the isolation of a single fraction having a purification factor of four, which contained 90% of the total activity originally present in the homogenate.

This separation appeared to be satisfactory for use as an initial step in the purification of testosterone dehydrogenase present in guinea pig liver homogenates. It can be performed in less than a day. Ninety per cent of the homogenate activity is preserved during the separation, and a four-fold purification is obtained. Preliminary experiments indicated that the fraction so obtained could be stored

TABLE 19

TESTOSTERONE DEHYDROGENASE ACTIVITY AND NITROGEN CONTENT OF GUINEA PIG LIVER MITOCHONDRIAL AND MICROSOMAL FRACTIONS[a]

Fraction	Expt. no.	Homogenate total activity	Homogenate nitrogen	Specific activity units[b] per mg. N	Purification
Mitochondria+ Microsomes	1	57.4	16.8	357	3.4
Mitochondria+ Microsomes	2	74.9	21.4	234	3.5
Mitochondria+ Microsomes	3	89.5	22.3	283	4.0

[a]The mitochondrial and microsomal fractions were separated as separate fractions in experiments 1 and 2, as a single fraction in experiment 3.

[b]See page 92 for definition of units employed.

in a refrigerator for periods as long as ten days with no loss in enzyme activity.

Partial Characterization of the Activity-Rich Fraction

Attempts at purification were terminated at this point in order to partially characterize the activity-rich fraction which had been obtained by centrifugal fractionation. Such characterization was designed to compare the activity-rich fraction with the homogenate fraction from which it was derived, and to reveal new information concerning the metabolism of the androgens.

Nucleotide utilization by various fractions. The first series of experiments was designed to compare the utilization of DPN and TPN by the homogenate and the activity-rich fraction. The results obtained using the homogenate are shown in Table 20. The activities of several dilutions are indicated, and these activities are reported in units per ml. In addition, the maximum optical densities observed, and the times elapsed when they were observed, are included, since these items reveal pertinent information.

Comparison of the activities at the various dilutions indicates that the concentration of enzyme was not rate-limiting when 1-10 dilutions were employed, since increased dilution produced an increase in activity. However, further dilution did not increase the maximum optical density

TABLE 20

NUCLEOTIDE UTILIZATION BY CELL COMPONENT FRACTIONS OF GUINEA PIG LIVER[a]

Dilution	Cofactor	Activity units[b] per ml.	Maximum optical density[c]	Time elapsed at maximum optical density (minutes)
HOMOGENATE				
1-10	DPN	138	0.145	11
1-25	DPN	240		
1-25	DPN	240	0.147	24
1-50	DPN	230	0.140	55
1-100	DPN	250		
1-10	TPN	107	0.136	14
1-10	TPN	105		
1-25	TPN	140	0.130	45
MITOCHONDRIA + MICROSOMES				
1-10	DPN	106	186	28
1-10	DPN	107		
1-25	DPN	105		
1-10	TPN	0		
1-10	TPN	0		
1-10	TPN	0		
SOLUBLE				
1-10	DPN	17		
1-10	TPN	58		
MITOCHONDRIA + MICROSOMES + SOLUBLE				
1-10	DPN	106		
1-10	TPN	54		

[a]Reaction mixtures contained ca. 25 μg. of testosterone and ca. 300 μg. of nucleotide.

[b]See page 92 for definition of units employed.

[c]At 340 mμ.

values, which can be taken as an index of steroid conversion, but only the time required to attain such values. This would seem to indicate that the maximum optical density value was a function of the concentration of nucleotide and the concentration of steroid, or both, since these concentrations were the same in all reaction mixtures. Comparison of the maximum optical density values, ca. 0.145 and ca. 0.133, observed with DPN and TPN, respectively, indicates a dependence of maximum optical density, and thereby steroid conversion, upon nucleotide concentration; the nucleotides were made up to identical concentrations by weight, but subsequent spectrophotometric assay of the TPN solution indicated a purity of 90%, which was just the ratio of the maximum optical density values observed with TPN and DPN, i.e., 0.133/0.145.

Hence, in the presence of rate-limiting concentrations of enzyme, or tissue solution, the maximum optical density value realized, at constant steroid concentration, was a function of nucleotide concentration, and was independent of the nucleotide employed. In contrast, the rate at which the maximum optical density value was obtained was dependent on the nature of the nucleotide; such values were obtained more slowly in the presence of TPN than in the presence of DPN. This dependence was not too pronounced in the presence of excess enzyme, or tissue solution (1-10 dilution: 14 minutes for TPN, 11 minutes for DPN), but it became quite

evident in the presence of rate-limiting concentrations of enzyme (1-25 dilution: 45 minutes for TPN, 24 minutes for DPN). These differences reveal one area in which spectrophotometric methods are superior to the incubation methods reported in the earlier chapters; the incubation methods are incapable of revealing rate differences.

Calculation of the theoretical optical density change necessary for complete oxidation of the quantity of testosterone present, 25.4 μg., gave a value of 0.183. The observed maximum optical density change corresponds to a conversion of 80%; such calculations assume that there is no destruction of "testosterone-formed" $DPNH_2$.

The results obtained with the fraction which contained mitochondria and microsomes are also shown in Table 20. The activities recorded are not a true indication of activity recovery in this fraction since there is dilution during fractionation. Because of this dilution, less dilution was necessary prior to assay, in order to obtain rate-limiting concentrations of enzyme; 1-10 dilutions of this fraction gave maximum activity in the presence of DPN.

Behavior with regard to nucleotides differed markedly from that observed with the homogenate; there was no evidence of TPN utilization in the mitochondrial and microsomal fraction. Two TPN solutions were employed in these assays; the homogenate exhibited activity with both solutions. Several explanations of this difference are plausible. The

presence of two testosterone dehydrogenase enzymes in the homogenate, one specific for DPN, the other specific for TPN, would explain these results. A prior conversion of TPN to DPN would serve equally well since the presence of such a mechanism in the homogenate, but its absence in the mitochondrial and microsomal fraction, could be assumed.

The duplicates listed in the results with DPN were performed one week apart; the later run was performed eleven days after the sacrifice of the animal and initiation of fractionation. These data indicate the stability of the enzyme in the activity-rich fraction when the fraction is handled in the manner outlined.

Because of the differences between the homogenate and activity-rich fractions, the characterization was extended to include the soluble fraction. The results of this study are shown also in Table 20. Whereas the mitochondria and microsomes fraction had exhibited a preference for DPN, since there was no activity in the presence of TPN, the soluble fraction exhibited a preference for TPN; activity was about three and one-half times as great in the presence of TPN. The DPN activity present in this fraction was undoubtedly due to fractionation technique; 10-20% of the DPN activity was recovered in the soluble fraction after each of the centrifugal fractionations of cell components. However, the activity in the presence of TPN represents 74% of the activity originally present in the homogenate, and cannot be

attributed to technique.

The greater activity in the presence of TPN seemed to eliminate the operation of a mechanism for the conversion of TPN to DPN as a factor responsible for the differences observed between the homogenate and the mitochondria and microsomes fraction. Indeed, it seemed to support the presence of two testosterone dehydrogenase enzymes in the homogenate, only one of which was located in the mitochondria and microsomes.

Equivalent portions of the mitochondria and microsome fraction and the soluble fraction were recombined, and assayed for enzyme activity, in order to complete this aspect of the characterization study. The results obtained are included in Table 20. The activities observed, 106 and 54, with DPN and TPN, respectively, agree favorably with the sums of the activities of the separate fractions, 123 and 58.

Enzymatic assay of aqueous solutions of testosterone. Since the theoretical maximum optical change, for the concentration of testosterone present, was observed with the mitochondria and microsome fraction,[2] it appeared that this fraction could be employed for the enzymatic assay of aqueous solutions of testosterone. Accordingly, two concentrations of testosterone were assayed using 1-2 dilutions of the activity-rich fraction. The results are shown in Table 21;

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[2]See Table 20.

TABLE 21

ENZYMATIC ASSAY OF AQUEOUS SOLUTIONS OF TESTOSTERONE[a]

Testosterone μg.	Maximum optical density change[b] theoretical	observed	Time elapsed at maximum optical density (minutes)
25.4	.183	.192	6
25.4	.183	.188	5
25.4	.183	.188	6
12.7	.092	.104	2

[a]Reaction mixtures contained excess DPN and excess enzyme; guinea pig mitochondria and microsomes used as a source of enzyme.

[b]Assays were performed at a wave length of 340 mμ.

they indicated that such assays were feasible.

Optimum pH for the conversion of testosterone. The optimum pH for the conversion of testosterone in the presence of DPN was determined using the activity-rich fraction. Changes in optical density at six pH values were recorded for a period of ten minutes; values at three representative times, six, eight, and ten minutes, are shown in Table 22. A pH optimum in the vicinity of pH 9.0 was indicated. These results are in accord with those obtained using incubation methods (94).

TABLE 22

INFLUENCE OF PH ON TESTOSTERONE DEHYDROGENASE ACTIVITY OF GUINEA PIG MITOCHONDRIA AND MICROSOMES[a]

pH	Buffer	Change in optical density at 340 mμ		
		6 minutes	8 minutes	10 minutes
6.75	P[b]	0.022	0.027	0.030
7.5	P	0.040	0.051	0.060
8.0	PP[c]	0.055	0.070	0.082
9.0	PP	0.087	0.104	0.122
9.5	PP	0.073	0.092	0.100
10.4	PP	0.067	0.080	0.095

[a]Reaction mixtures contained ca. 25 μg. of testosterone and ca. 300 μg. of nucleotide.

[b]Disodium phosphate, 0.1 molar.

[c]Sodium pyrophosphate, 0.166 molar.

CHAPTER VII

SUMMARY

The incubation of androstanedione with homogenates of male guinea pig liver gave androsterone and epiandrosterone as metabolites. Incubations performed at several pH values and for various periods of time gave essentially the same results. The total yield of metabolites was less than 2% in each instance.

The addition of physiological materials, notably the reduced form of disphosphopyridine nucleotide, markedly increased the recovery of epiandrosterone; the recovery of androsterone was elevated only slightly. A third metabolite, androstanolone, was produced when the level of metabolic conversion of androstanedione was high.

The pH of the medium exerted a definite influence upon metabolite production in the presence of reduced diphosphopyridine nucleotide. This influence was particularly evident in the case of epiandrosterone; maximum formation of this metabolite was observed at pH 6.5. The formation of androsterone and androstanolone appeared to decrease with increasing pH, in the range 5.5 to 7.5, but the observed

differences were very small.

The results of incubations which were performed in the presence of reduced diphosphopyridine nucleotide were dependent upon the nature of the gaseous phase employed. Experiments performed in the presence of a nitrogen atmosphere gave rise to only one-half the quantity of metabolites which was produced in comparable experiments in which air was the gaseous phase.

Incubation of androstanedione with homogenates of guinea pig kidney, heart, and femoral muscle also gave rise to androsterone and epiandrosterone, except in the case of muscle, which gave rise to epiandrosterone only.

Incubation of androstanedione with homogenates of rat liver gave results similar to those observed with guinea pig liver homogenates except that the amounts of metabolites formed were greater.

Androstanolone was used as the substrate steroid in a number of experiments with guinea pig tissue homogenates. Androstanedione was the only metabolite recovered in these experiments. When liver tissue was employed, the conversion of androstanolone was increased by the addition of DPN to the incubation. The conversion of androstanolone was influenced by the pH of the medium; more androstanedione was recovered at pH 9.0 than at pH 7.35.

Incubation of androstanolone with homogenates of guinea pig kidney also gave rise to androstanedione; trace

amounts of the diketone were recovered when testes was the tissue employed, but no metabolism was observed in the presence of seminal vesicles and prostate. The tissue pattern of metabolism observed with androstanolone is the same as that observed previously with testosterone; however, the latter was metabolized five-to-six times as extensively as the former.

Fourteen nineteen-carbon hydroxysteroids were incubated at pH 9.0 in the presence of twelve g. of guinea pig liver, 50 mg. of DPN, and 400 mg. of NA. Monohydroxy monoketone compounds gave rise to diketone compounds; dihydroxysteroids gave rise to the corresponding diketone compounds as well as a pair of monohydroxy monoketone compounds in each instance; no metabolism was observed when monohydroxysteroids were employed as substrates.

Centrifugal fractionation of guinea pig liver homogenates into fractions consisting of nuclei, mitochondria, microsomes, and "soluble" material gave rise to a purification of testosterone dehydrogenase activity in excess of four-fold in the microsomal fraction. When the mitochondrial and microsomal fractions were separated as a single fraction, a four-fold purification was obtained at the expense of only 10% of the activity originally present in the homogenate from which the fraction was derived. This separation could be performed in less than a day, and there was no decrease in activity when the activity-rich fraction was stored in a

refrigerator for ten days.

The activity-rich fraction was employed to assay aqueous solutions of testosterone enzymatically. Assays were performed spectrophotometrically at 340 mμ. by following the formation of $DPNH_2$. Studies at various pH values indicated that testosterone was most rapidly oxidized by the activity-rich fraction at pH 9.0.

Characterization of the cell component fractions and the homogenates from which they were derived with respect to nucleotide utilization seemed to indicate the presence of two testosterone dehydrogenases in homogenates. Whereas the homogenates utilized DPN and TPN, the mitochondrial and microsomal fractions utilized DPN only; TPN activity was present in the "soluble" fraction.

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