PRINCIPLES OF THE ENZYMATIC MEASUREMENT OF STEROIDS*

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Information on steroid concentrations in tissues is important for an understanding of the metabolism of these substances and also for diagnostic purposes in certain clinical conditions. Existing procedures for determining steroids, either singly or in mixtures, are based upon colorimetric methods or tedious bioassays. The merits and limitations of these procedures have been critically appraised (1–3), their principal drawbacks being lack of specificity and sensitivity. This paper concerns the microestimation of steroid hormones and their metabolites by enzymatic methods suitable for the measurement of these compounds in biological systems, and which appear to offer the promise of a high degree of sensitivity and specificity. Enzymatic steroid assays depend upon the quantitative interconversion of certain hydroxy- and ketosteroids by highly purified DPN[1]-linked hydroxysteroid dehydrogenases prepared from *Pseudomonas testosteroni* (4–6). The accompanying changes in DPNH concentration are determined spectrophotometrically at 340 mμ (7) and constitute a stoichiometric measure of the amount of steroid oxidized or reduced. The enzymatic microestimation of steroids was demonstrated in 1953 (4) and its applications extended when more highly purified hydroxysteroid dehydrogenases became available (6). The enzymatic estimation of urinary steroids has been reported briefly (8). The present paper contains a detailed examination of the principles of the enzymatic measurements of steroids and considers their specificity, sensitivity, and accuracy.

At this time, two hydroxysteroid dehydrogenases are suitable for analytical purposes by virtue of their purity and high activity and a knowledge of their substrate specificities. The enzymes are highly active because of their inductive (adaptive) nature, and increases in specific activity of several 100-fold have been easily achieved by adding various steroids to the growth medium (6). The substrate specificities of these hydroxysteroid dehydrogenases have been described in detail (9, 10). In brief,

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[1] The following abbreviations are used: DPN = diphosphopyridine nucleotide; DPN^+ = oxidized diphosphopyridine nucleotide; DPNH = reduced diphosphopyridine nucleotide; DPNase = diphosphopyridine nucleotidase.

3α-hydroxysteroid dehydrogenase (designated as α enzyme) catalyzes the reversible oxidation of 3α-hydroxysteroids of the C_{19}, C_{21}, and C_{24} series, whereas *3β- and 17β-hydroxysteroid dehydrogenase* (designated as β enzyme) reversibly interconverts 3β- and 17β-hydroxysteroids and the corresponding ketones. In order to employ these enzymes for the accurate estimation of steroids in urine, blood, and other tissues, a study of the factors influencing reaction equilibria was undertaken with a view to establishing conditions under which conversions are quantitative in the desired direction and under which interference from reaction products or inhibitors is negligible.

EXPERIMENTAL

Equilibrium Considerations

The reactions catalyzed by these pyridine nucleotide-linked dehydrogenases are of the general type:

$$\diagdown\!\diagup\!\text{CHOH} + \text{DPN}^+ \rightleftharpoons \diagdown\!\diagup\!\text{C=O} + \text{DPNH} + \text{H}^+$$

The oxidations of steroids are freely reversible and the equilibria may be displaced to the right (*forward-reaction*) or to the left (*back-reaction*) by employing mass action principles.

Forward-Reaction—The quantitative conversion of a steroid alcohol to ketone is favored by raising the pH of the reaction mixture, increasing the DPN^+ concentration, and adding a ketone-binding reagent. The influence of pH on the velocity and equilibrium of the conversion of androsterone to androstane-3,17-dione by α enzyme is shown in Fig. 1. A high pH is advantageous in that the velocity of the oxidation is increased and equilibrium is attained more rapidly. Assurance of the displacement of the equilibrium to completion in the forward-reaction has been obtained by incorporating a ketone-binding reagent into the reaction mixture. Hydrazine is both convenient and effective in this respect, and its use is illustrated in Fig. 2, which shows the rate and equilibrium of the conversion of pregnane-3α,17α,21-triol-11,20-dione to its corresponding 3-ketone in the presence of varying concentrations of hydrazine at pH 7.8. Hydrazine is without significant influence on the initial reaction rate, but high concentrations of hydrazine permit the reaction to attain equilibrium more rapidly. By using hydrazine (0.3 M final concentration) in a reaction mixture of pH 9.5, advantage is taken of the greater efficiency of the reaction between hydrazine and ketones in basic solutions. Other ketone-combining reagents such as hydroxylamine (final concentration 1.0 M) and semicarbazide (final concentration 0.5 M) were also effective in shifting the

equilibrium, but these compounds inhibited the reaction rate of α enzyme slightly. Hydroxylamine is also undesirable because of its reaction with DPN^+ (11).

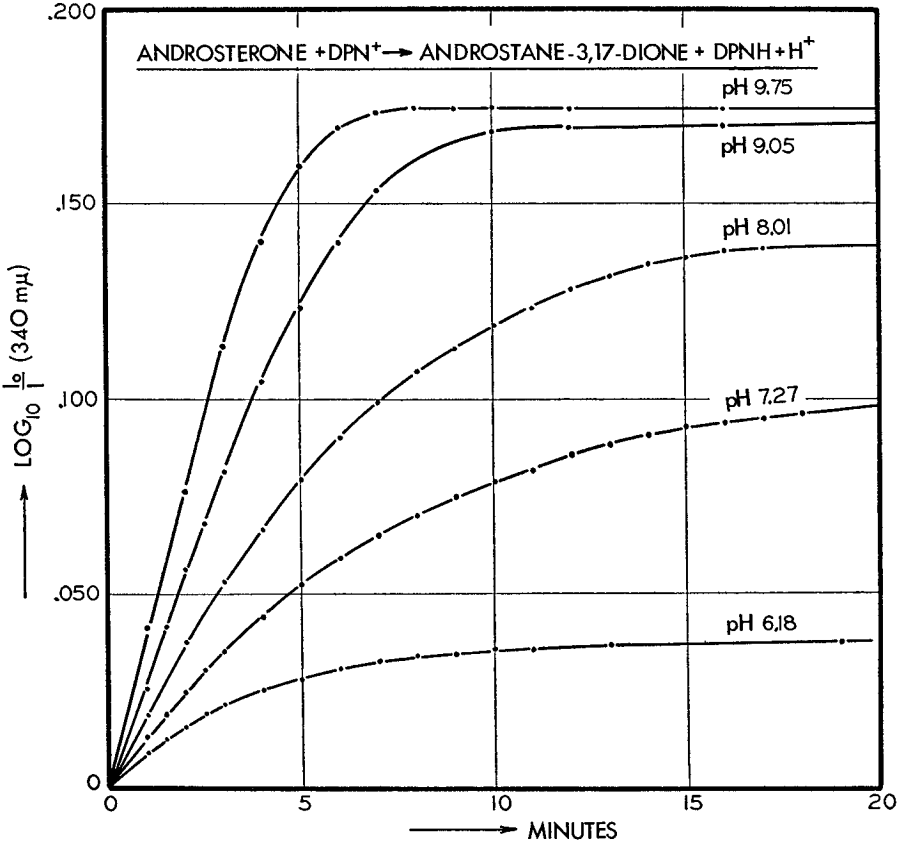

FIG. 1. Time course of the oxidation of androsterone to androstane-3,17-dione by α enzyme at varying pH values. Reactions were carried out in 3.0 ml. volumes in cuvettes containing 100 μmoles of Sorensen's phosphate (pH 6.18 to 8.01) or sodium pyrophosphate (pH 9.05 and 9.75) buffer, 0.5 μmole of DPN^+, 0.085 μmole of androsterone in 0.1 ml. of CH_3OH, and 33 units of α enzyme. Optical densities were measured at 340 mμ at intervals against blank cells containing no steroid. Temperature, 25°.

Back-Reaction—The attainment of complete reductions of ketosteroids to steroid alcohols presents a more complicated problem. The equilibrium can be displaced in the desired direction by increasing the DPNH concentration and by lowering the pH (Table I). In practice, only limited use can be made of these factors, since DPNH is unstable in solutions more

acid than pH 5.5, and the concentration of DPNH must be kept quite low in order to permit accurate measurement of the decreases in optical density. However, for pure steroids the assays are satisfactory, as shown in Fig. 3, which demonstrates the equivalence of assays of epiandrosterone in forward- and back-reactions.

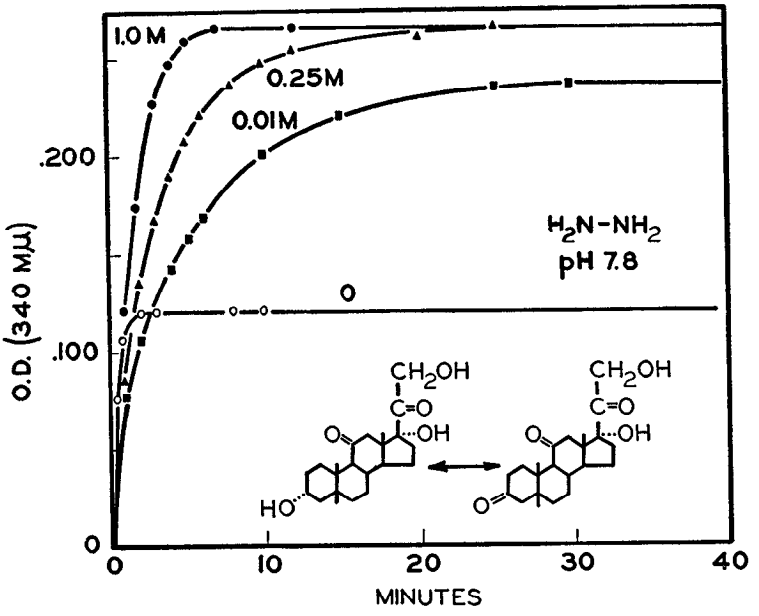

FIG. 2. Time course of the oxidation of pregnane-3α,17α,21-triol-11,20-dione by α enzyme in the presence of varying concentrations of hydrazine (0 to 1.0 M final concentration), as measured by the formation of DPNH at 340 mμ. The reactions were carried out in 3.0 ml. volumes containing 100 μmoles of Sorensen's phosphate buffer, pH 7.8, 0.5 μmole of DPN^+, 0 to 3 mmoles of hydrazine, and 0.130 μmole of steroid in 0.025 ml. of CH_3OH. The reaction was initiated by the addition of 2150 units of α enzyme (specific activity, 47,000 units per mg. of protein). Temperature 25°.

Two methods for obtaining complete reductions in the back-reaction were investigated. By use of relatively large amounts of DPNH, the reduction of steroid was permitted to proceed to completion, the absorption of the excess DPNH at 340 mμ was destroyed by addition of HCl to pH 1, and the DPN^+ was measured by its complex with cyanide in alkaline solution (12). This gave satisfactory values with pure steroids, provided that the DPNH did not contain DPN^+. If the concentration of enzymatically prepared DPNH was raised sufficiently to drive the reaction to completion, there was interference from the α isomer (13) of DPN^+ which is present in enzymatically prepared DPNH, since the α isomer reacted with cyanide

but was not reduced during the enzymatic preparation. In the chemical reduction of DPN^+ with $Na_2S_2O_3$, both isomers react, but, in order to obtain a product suitable for measurement of small amounts of steroids

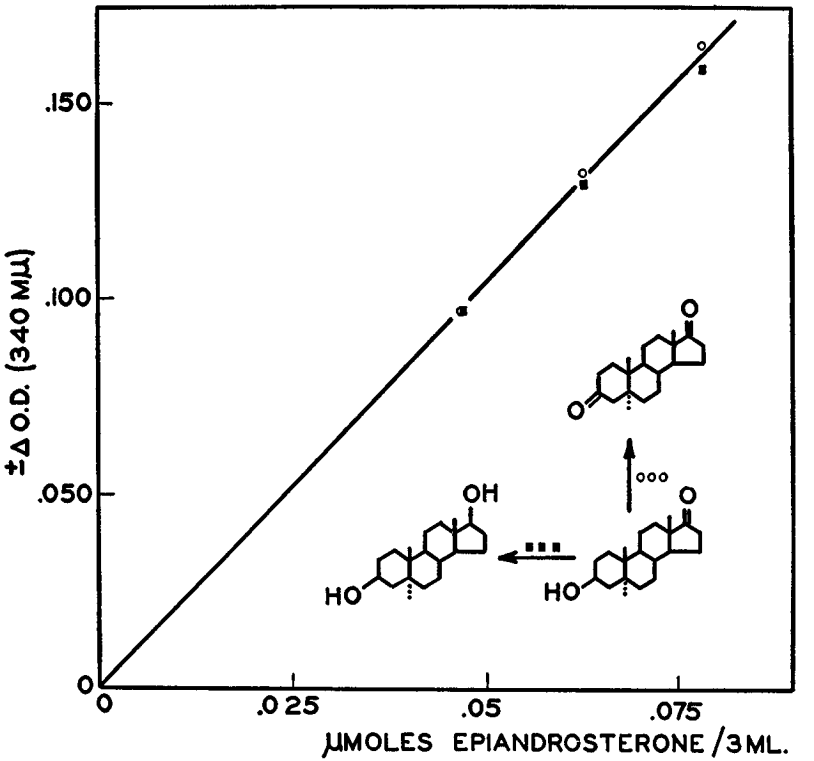

FIG. 3. Enzymatic measurement of epiandrosterone with β enzyme by forward-reaction (○) and by back-reaction (■). Conditions for the forward-reaction were as follows: 100 μmoles of pyrophosphate buffer, pH 9.0, 0.5 μmole of DPN^+, appropriate amounts of epiandrosterone in 0.05 ml. of CH_3OH, all in a total volume of 3.0 ml. Reactions were initiated by addition of 0.02 ml. of β enzyme (118 units of specific activity 15,300 units per mg. of protein). Increases in optical density at equilibrium were determined at 340 mμ. Back-reactions were carried out in 3.0 ml. volumes containing 100 μmoles of Sorensen's phosphate buffer, pH 5.5, 0.5 μmole of chemically reduced DPNH, appropriate amounts of epiandrosterone in CH_3OH, and β enzyme as in the forward-reaction. Decreases in optical density (340 mμ) were measured at equilibrium against a blank containing no steroid and set for an optical density of 0.250. Temperature 25°.

in the back-reaction by the cyanide complex, virtually all traces of DPN^+ must be eliminated, and this presents a goal not easily achieved in routine preparations.

An alternative method for attaining completion in the reduction of

steroid ketones is the removal of the oxidized DPN^+ formed during the reaction by DPNase from zinc-deficient *Neurospora* (14). This enzyme selectively splits the nicotinamide-ribose linkage of the oxidized coenzyme only, and has been recently used to shift the equilibrium in the mannitol phosphate dehydrogenase system of *Escherichia coli* (15). *Neurospora* DPNase has a wide pH optimum, is relatively stable, and easily prepared. These considerations make the addition of DPNase a suitable and convenient method for attaining the desired equilibrium displacement. A comparison of the efficacy of *Neurospora* DPNase and pH changes in shift-

TABLE I

Effect of DPNase and Changes in pH on Equilibrium of Reduction of Androstane-3,17-dione to Androsterone by α Enzyme

pH	Equilibrium decrease in optical density (340 mμ)	Per cent completion of reduction
5.50	0.130	94.9
6.52	0.107	78.1
7.58	0.092	67.1
8.08	0.085	62.1
8.02 + DPNase	0.137	100

Reactions were carried out at 25° in 3.0 ml. reaction volumes in 1 cm. light path cuvettes containing 100 μmoles of Sorensen's phosphate buffer of indicated pH; 0.12 μmole of chemically reduced DPNH (initial optical density approximately 0.250); 0.066 μmole of androstane-3,17-dione in 0.05 ml. of CH_3OH; and 1290 units of α enzyme (specific activity 47,000 units per mg. of protein). Equilibrium was attained in 1 to 5 minutes (depending upon pH). 210 units of DPNase were added in the last instance. Readings were made against a blank containing no DPNH, and compensation was made for the slow spontaneous drop in optical density which the DPNH undergoes during the reaction time. All reactions were run in duplicate and mean values are given.

ing the equilibria is shown in Table I. The reduction of androstane-3,17-dione to androsterone was studied under the conditions indicated; at pH 8.08 the reduction was only 62.1 per cent complete, whereas the addition of DPNase shifted the equilibrium to completion, a condition not even attained by lowering the pH to 5.5. The advantages of using DPNase lie in that contamination of DPNH by DPN^+ is not objectionable, and the concentration of DPNH may be low (about 0.03 μmole per ml.), thereby increasing the accuracy of the differential measurement.

Equilibrium Constants of Steroid Oxidations—The equilibrium constants involved in certain steroid oxidation-reductions have been measured with the aid of α and β enzymes.[2] The values are of some theoretical interest

[2] Talalay, P., and Hurlock, B., unpublished observations.

in providing information on the energy differences between axial and equatorial substituents (16). The equilibrium constants (K_H) for these reactions have been defined as follows (17):

$$K_H = \frac{[\text{>C=O}][\text{DPNH}][\text{H}^+]}{[\text{>CHOH}][\text{DPN}^+]}$$

Values of K_H for the oxidation of various equatorial 3-hydroxyl groups range from 0.9 to 2.1 $\times$ 10^{-9} and for axial 3-hydroxyl groups from 6.7 to 7.5 $\times$ 10^{-9}. The equilibrium constant for the oxidation of the "quasi"-equatorial 17β-hydroxyl group of testosterone was found to be 37.8 $\times$ 10^{-9}. From these value ranges it may be calculated that, in a system containing initially 0.50 μmole of DPN^+, 0.10 μmole of steroid, and with pH 9.5, a 17β-hydroxysteroid will be 100 per cent oxidized. Under these conditions, an *axial* 3-hydroxysteroid will be 99.0 to 99.3 per cent oxidized, whereas an *equatorial* 3-hydroxysteroid will be 93.4 to 96.4 per cent oxidized. In the back-reaction, 0.05 μmole of 17-ketosteroid in a reaction system containing 0.1 μmole of DPNH at pH 6.0 will be 96 per cent reduced at equilibrium. These calculations assume no reaction product to be present initially, and do not include effects of hydrazine (forward-reaction) or DPNase (back-reaction).

Sensitivity, Specificity, and Applications

Sensitivity—The sensitivity of enzymatic assays is largely dictated by the spectrophotometric equipment, the physical dimensions of the cuvettes, and the reaction volumes employed. In routine measurements, which utilize 1.0 cm. light path absorption cells, the volume may conveniently be varied from 0.2 ml. to 3.0 ml. The smaller volumes require the use of microcuvettes (18). By assuming a desired optical density change of at least 0.030, the minimal quantity of steroid accurately measurable is 0.001 μmole, or about 0.3 γ. Possibilities of increasing the sensitivity consist of either working in smaller reaction volumes (18, 19) or of using fluorescence measurements which can detect extremely small amounts of DPN^+ and DPNH (20). Fig. 4 shows the measurement of 0.2 to 1.0 γ of estradiol-3,17β by oxidation to estrone with β enzyme.

Assay of Steroid Mixture—The estimation of single steroids in pure solutions has been amply illustrated in this paper as well as previously (4, 9). In order to examine the suitability of these enzymatic techniques for mixtures of steroids, solutions of four pure steroids in methanol were prepared. All compounds were of high purity, and their concentrations

were determined by α or β enzymes in the forward-reaction in at least two assays for each solution. The steroids used, and their concentrations by enzymatic assay, were as follows: androsterone 2.84 μmoles per ml.; epiandrosterone 0.56 μmole per ml.; dehydroepiandrosterone 0.56 μmole per ml.; and pregnane-3α,17α,21-triol-11,20-dione 0.68 μmole per ml. Accord-

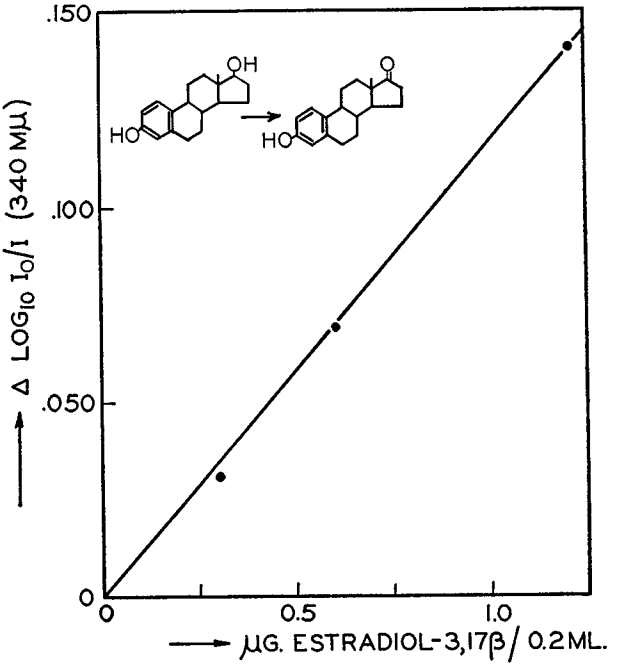

FIG. 4. Estimation of estradiol-3,17β by oxidation to estrone with β enzyme. Reactions were carried out in 1.0 cm. light path microcuvettes in total volumes of 0.2 ml., containing 5 μmoles of sodium pyrophosphate buffer, pH 9.0, 0.05 μmole of DPN^+, and varying amounts of estradiol-3,17β in 0.02 ml. of CH_3OH. The reactions were initiated by the addition of 49 units of β enzyme (specific activity 24,400 units per mg. of protein) in 0.005 ml. Optical density readings were taken at 340 mμ against blank cells containing all ingredients except steroid. Each point represents the mean of two measurements. Temperature 25°.

ing to these assays the steroids varied from 94.0 to 99.5 per cent in purity on the basis of weight. Equal volumes of the four steroid solutions were then mixed and aliquots of the mixture assayed at three concentrations in the forward-reaction, first α enzyme and then β enzyme being used. The 17-ketosteroids were determined separately with β enzyme by the cyanide complex-forming method. The assays are reproduced in Fig. 5, which shows linearity with steroid concentration and reasonable agreement with calculated values. Table II gives the average values of the assays

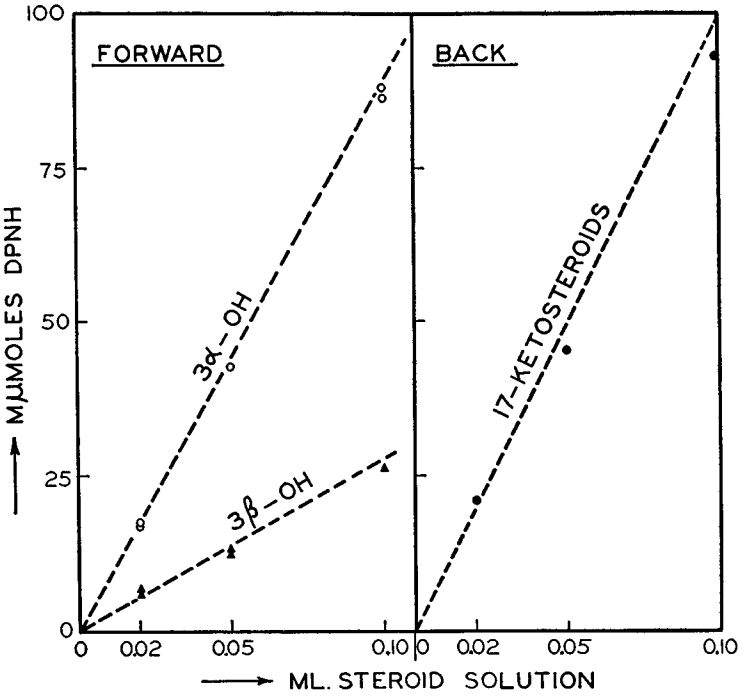

FIG. 5. Analysis of a steroid solution prepared by mixing equal volumes of solutions of androsterone (2.84 μmoles per ml.), epiandrosterone (0.56 μmole per ml.), dehydroepiandrosterone (0.56 μmole per ml.), and pregnane-3α,17α,21-triol-11,20-dione (0.68 μmole per ml.) in CH_3OH. The left graph demonstrates the amount of DPNH formed as a function of the volume of the steroid solution in the forward-reaction with α and β enzymes. The right graph is the amount of DPNH reoxidized in the back-reaction with β enzyme by varying volumes of the steroid solution. The lines are drawn so as to indicate the expected amounts according to the individual concentration of steroids in each solution before mixing. Temperature 25°.

TABLE II

Enzymatic Assay of Steroids in Mixture Containing Androsterone, Epiandrosterone, Dehydroepiandrosterone, and Pregnane-3α,17α,21-triol-11,20-dione in Methanol

Group assayed	Steroid per ml. of mixture, mμmoles		
	Found	Calculated*	Per cent discrepancy
3α-Hydroxyl	860	890	−3.4
3β-Hydroxyl	273	280	−2.5
17-Ketone	963	1000	−3.7

* Calculation is based on enzymatic assay of solutions of individual steroids before mixing. See the text for the design of the experiment.

and the amounts calculated. The assays of functional groups in the mixture were from 2.5 to 3.7 per cent below the calculated values.

Purity of Steroids—During the course of this work, a number of steroids with 3α-, 3β-, and 17β-hydroxyl groups were assayed by the appropriate enzymes, under conditions which assured complete oxidations. The compounds were believed to be of the highest purity, on the basis of preparative history, physical constants, elementary analyses, and infrared and ultraviolet absorption spectra, as well as in some cases by paper chromatography. These compounds were either prepared in our laboratory or obtained from other investigators. By enzymatic assay the purity of these compounds varied from 79.5 to 100.2 per cent according to weight. In many assays, a known amount of a steroid, pure by enzymatic assay, was added at the end of the reaction, and in each case the added steroid was assayed precisely in the presence of steroids of varying purity. Since the molar extinction coefficient of DPNH is known accurately from many independent measurements (21), enzymatic steroid measurements provide a convenient method for determining the purity of certain steroids and contamination by isomers. These determinations of purity are not dependent upon other steroids but merely upon accurate knowledge of the extinction coefficient of DPNH. Contamination of steroids by closely related compounds bearing similar functional groups cannot be detected by enzymatic measurement.

Analysis of Chromatographic Fractions—Enzymatic methods for the analysis of steroids have been useful for the estimation and identification of steroid fractions eluted from paper chromatograms or from chromatographic columns. The latter procedure is illustrated by the chromatographic separation of 400 γ each of 4-androstene-3,17-dione, dehydroepiandrosterone, and testosterone by gradient elution on a column of silicic acid (Mallinckrodt, $SiO_2 . xH_2O$). A column measuring 103 mm. long by 11 mm. in diameter was packed from a slurry of silicic acid in hexane and chloroform (1:1 by volume), and the steroids were applied in a small volume of the same solvent mixture to the top of the column. A reservoir, 300 ml. capacity, provided with a magnetic stirrer was filled with this solvent mixture and connected to the column. The gradient of more polar solvents was established by introducing into the mixing flask methanol and chloroform (1:99 by volume). 5.0 ml. fractions were collected, evaporated to dryness, and redissolved in methanol, and aliquots were used for enzymatic analysis with β enzyme (for dehydroepiandrosterone and testosterone) and by ultraviolet absorption measurements (for 4-androstene-3,17-dione and testosterone) (Fig. 6). The total recoveries of all three steroids were 95 to 100 per cent, and the agreement between ultraviolet absorption and enzymatic analyses (for testosterone) was better than 5 per cent.

Specificity of Measurements—Since α and β enzymes carry out highly selective and stereospecific oxidation-reductions, it is possible to obtain much information on the qualitative and quantitative composition of steroid mixtures by appropriate sequential enzymatic oxidations and reductions. Thus, α enzyme in the forward-reaction measures total 3α-hydroxyl groups of the C_{19}, C_{21}, and C_{24} steroids. In the forward-reaction, β enzyme measures 3β-hydroxyl plus 17β-hydroxyl groups. In the back-

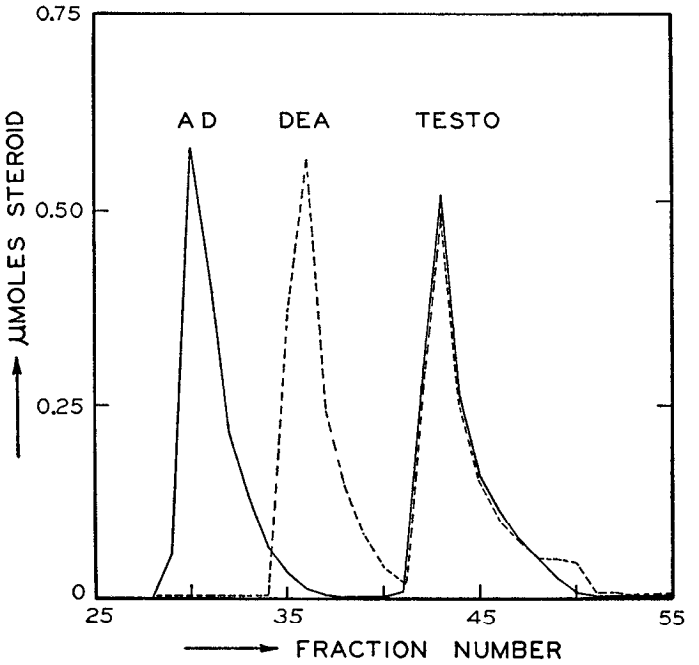

FIG. 6. Chromatography of 4-androstene-3,17-dione (AD), dehydroepiandrosterone (DEA), and testosterone (TESTO) on silicic acid by gradient elution, as described in the text. The fractions (5 ml. each) were analyzed by ultraviolet absorption (—) and enzymatic reaction with β enzyme (- - -).

reaction, α enzyme measures 3-ketosteroids only, whereas β enzyme reduces both 3- and 17-ketosteroids, provided that the ketonic groups are not in conjugation with a double bond. In the back-reaction, if α enzyme is used first and then followed by β enzyme, it becomes possible to obtain individual measurements of 3- and 17-ketosteroids.

In the forward-reaction, 3β- and 17β-hydroxyl groups are measured together; hence a 3β,17β-dihydroxysteroid will cause the formation of 2 equivalents of DPNH for each mole of steroid. Independent measurement of 3β-hydroxyl and 17β-hydroxyl groups may be obtained by measuring the 3-ketone groups with α enzyme in the back-reaction, before and after

oxidation of the steroid mixture with β enzyme; thus the increase in 3-ketosteroids will be equivalent to the 3β-hydroxysteroids originally present. Some of the functional groups which may be measured are listed in Table III. In addition, by the use of the very active steroid isomerase (22) which usually contaminates both α and β enzymes, it is possible to measure the specific increase in absorption at 248 mμ (λ_{max} of α,β-unsaturated ketones in H_2O) and thereby obtain the increase in the α,β-unsaturated ketones following the oxidation of β,γ-unsaturated 3α- or 3β-hydroxysteroids. This measurement should be useful for measuring dehydroepiandrosterone (5-androsten-3β-ol-17-one) and 5-pregnen-3β-ol-20-one.

TABLE III

Groups determined	Reaction
3α-Hydroxyls	α enzyme, forward-reaction
3β- + 17β-hydroxyls	β enzyme, forward-reaction
3-ketones (*not* α,β-unsaturated)	α enzyme, back-reaction
3- + 17-ketones (*not* α,β-unsaturated)	β enzyme, back-reaction
17-Ketones	β enzyme in back-reaction, after α enzyme in back-reaction
3β-Hydroxyls	Measure 3-ketones with α enzyme before and after oxidation with β enzyme (3β-hydroxyls by difference)

DISCUSSION

The estimation of a variety of biochemical substrates by pyridine nucleotide-linked dehydrogenases has become a well recognized procedure. Enzymatic methods have proved especially suitable for steroid determinations for a number of reasons: (1) The substrate specificity of bacterial α- and β-hydroxysteroid dehydrogenases coincides with the most important steroid metabolites found in animal tissues. (2) The reactions catalyzed by these enzymes are readily reversible, and the equilibria can be easily displaced to completion in the desired direction. (3) The enzymes are saturated at very low steroid concentrations, and reasonable reaction velocities during the assays are maintained (10). (4) Enzymatic assay methods are highly specific, and the spectrophotometric methods are very sensitive. These last factors are indispensable in any analytical procedure requiring the measurement of minute quantities of specific metabolites in the presence of large quantities of a variety of other biological constituents.

In contrast to colorimetric methods based on chemical reactions such as the Zimmermann method for 17-ketosteroids (1), the enzymatic methods reported here offer several advantages in addition to those listed above. The measurements depend upon specific changes in light absorption at

340 mμ, under conditions which result in no unpredictable changes at other wave lengths. These changes in light absorption are, unlike those in many chemical methods, due to changes in concentration of DPNH, a well defined compound of known extinction coefficient and absorption maximum. In many chemical methods, the precise nature of the colored complex is unknown and there may be relatively large variations in extinction coefficient and maximal absorption wave length for different steroids. Thus, in the Zimmermann reaction, if 100 be taken as the relative extinction of dehydroepiandrosterone, equal amounts of other steroids give color equivalents from 65 to 130 (23). Moreover, the rate of formation of the colored complex depends upon many factors such as concentration of alcohol, alkali, length of color development, and temperature. Thus, in steroid mixtures, the total amount of color produced depends not only upon the amount of steroids, but also upon the proportion of different steroids and the conditions of color development.

The high degree of specificity of enzymatic reactions offers the advantage in many biological systems that unknown non-steroidal compounds which may be present are not likely to interfere. Thus, in the case of urinary steroid determinations, large non-specific "background" colors are produced when urine is treated with the Zimmermann reagents (KOH and *m*-dinitrobenzene). Many empirical attempts to correct these colors by differential spectrophotometry have not proved entirely satisfactory (1). In contrast, the addition of a few micrograms of enzyme protein to even a pigmented solution has been found not to alter the absorption spectrum appreciably in the region measured.

The sensitivity of these enzymatic methods approaches that required for the measurement of tissue concentrations of steroids. Enzymatic estimations with α and β enzymes permit the measurement not only of ketosteroids but also of hydroxysteroids for which no sensitive and specific methods have thus far been available. We visualize the discovery of further hydroxysteroid dehydrogenases specific for positions other than 3 and 17 on the steroid skeleton. Such enzymes may be of value in extending these methods for the determination of the quantitative and qualitative composition of steroid mixtures.

Methods and Materials

α- and β-hydroxysteroid dehydrogenases were prepared according to improvements of methods previously described (9). The addition of an acetone fractionation at −10 to −20°, after the first ammonium sulfate and protamine steps, has improved the purity of the enzymes so that enzymes of specific activities of 70,000 to 100,000 units per mg. of protein have been obtained routinely before the calcium phosphate gel step.

Neurospora DPNase was prepared according to Kaplan (24) and had an activity of 52,000 units per ml., and 4.2 mg. of protein per ml. The DPN^+ was purchased from the Pabst Laboratories, Milwaukee, Wisconsin, and assayed 85 per cent DPN^+ on a basis of weight (assuming a molecular weight of 663). DPNH was prepared either by reduction of DPN^+ with alcohol dehydrogenase and ethanol (25) or by reduction with sodium hydrosulfite (26). Steroids were all checked as to identity and purity by melting point and optical rotation. Purifications of steroids were carried out whenever necessary. All the solvents were redistilled analytical reagent products. Hexane was purified by treatment with concentrated H_2SO_4 and alkaline $KMnO_4$ and distilled through a fractionating column. Hydrazine sulfate was Baker's analyzed reagent grade.

Assays of hydroxysteroids by the forward-reaction were carried out usually in 3.0 ml. reaction volumes in cuvettes containing 100 μmoles of sodium pyrophosphate buffer (final pH 9.5), 0.5 μmole of DPN^+, 1.0 mmole of hydrazine sulfate (previously adjusted with NaOH to about pH 9.5), 0.01 to 0.1 μmole of steroid in 0.1 ml. of CH_3OH, and sufficient enzyme to achieve equilibrium in less than 15 minutes. Readings were taken against a control cuvette containing all ingredients except steroid. Measurements were based on the increments in optical density at 340 mμ at equilibrium. The molar extinction coefficient of DPNH at 340 mμ was assumed to be 6220 (21).

Assays of ketosteroids by the cyanide complex were carried out in 2.45 ml. reaction volumes containing 100 μmoles of Sorensen's phosphate buffer, pH 5.5, approximately 0.1 μmole of DPNH (chemically reduced), 0.01 to 0.10 ml. of methanol solution of steroid, and sufficient enzyme to achieve equilibrium in 10 to 15 minutes. The initial optical density was about 0.250. Optical density measurements at 340 mμ were taken against a control cuvette containing all ingredients except steroid. When no further decrease in optical density occurred, 0.05 ml. of 6 N HCl was added to control and experimental cells which destroyed all the absorption at 340 mμ. 0.5 ml. of 5 N KCN in 1.0 N KOH was then added to all cells and the optical density again measured at 340 mμ. The assay was based on the increase in optical density upon addition of the cyanide. A molar extinction coefficient of 5150 was assumed for the DPN^+-cyanide complex at 340 mμ (12).

Assays of ketosteroids in the back-reaction with DPNase were carried out in 3.0 ml. reaction volumes containing 100 μmoles of Sorensen's phosphate buffer, pH 5.5, 0.1 μmole of DPNH (chemically or enzymatically reduced), 200 units of *Neurospora* DPNase, 0.1 ml. of methanol solution of steroid, and sufficient enzyme to obtain complete reduction in less than 15 minutes. Optical density readings were taken at 340 mμ against a

control containing no DPNH; an additional control containing all ingredients except steroid was also included. Measurements were based on the decrease in optical density in the experimental cell at equilibrium, compensated for small spontaneous reductions in optical density occurring in the control containing no steroid.

Ultraviolet absorption measurements were made in quartz cuvettes of 1.0 cm. light path in a Beckman DU spectrophotometer. Microcells and a special carriage constructed according to Lowry and Bessey (18) were supplied by W. H. Kessel and Company, Chicago, Illinois.

SUMMARY

The sensitive and specific microestimation of steroids by means of α- and β-hydroxysteroid dehydrogenases has been described. Factors influencing the equilibria of the reactions catalyzed by these enzymes have been examined. Conditions for obtaining complete oxidations of hydroxysteroids or reductions of ketosteroids have been established. Quantitative oxidations of steroids have been obtained by using a high pH and adding a ketone-binding reagent. Complete reductions of ketosteroids have been achieved by the destruction with DPNase of DPN^+ formed in the reaction. The enzymes have been employed for the estimation of 3α-, 3β-, and 17β-hydroxysteroids as well as 3- and 17-ketosteroids, singly as well as in mixtures. The sensitivity of the method is about 1 mμmole of steroid. The methods have been illustrated by the measurement of single steroids, steroid mixtures, and chromatographic fractions. These procedures are also applicable to the determination of steroid purity and contamination by isomers.

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