Enzymatic Estimation of Steroids in Subpicomole Quantities by Hydroxysteroid Dehydrogenases and Nicotinamide Nucleotide Cycling*

(Received for publication, July 20, 1981)

Donna W. Payne, Mikio Shikita§, and Paul Talalay§

From the Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Extremely sensitive methods are described for the measurement of 3α- and 3β-hydroxy-steroids, as well as 3-ketosteroids, based on their reaction with highly purified bacterial hydroxysteroid dehydrogenases and the amplification of the accompanying changes in nicotinamide nucleotides by enzymatic cycling procedures. Conditions have been devised under which the steroid oxidation and reduction reactions lead to the formation of stoichiometric quantities of NADH or NAD′+, respectively, even in the presence of large excesses of reaction products. The scope of these methods is illustrated by application to the analysis of minute volumes of human pregnancy urine, high pressure liquid chromatography fractions derived from such urine samples, and human serum. The steroid contents of milligram quantities of rat prostate have been determined. The methods have been applied also to the measurement of the activities of steroid-transforming enzymes, such as the 3α-hydroxysteroid dehydrogenase of prostate microsomes. At the present time the sensitivity of the described methods allows the accurate determination of 0.2–0.4 pmol of steroids.

This paper describes novel methods for the determination of steroid hormones and their metabolites at subpicomole levels. This degree of sensitivity is achieved by the use of enzymatic cycling to amplify the nicotinamide nucleotide products generated by the activities of specific hydroxysteroid dehydrogenases. The application of the procedures to the measurement of 3α-hydroxy-, 3β-hydroxy-, and 3-ketosteroids in less than milligram quantities of human urine and plasma and in rodent prostatic tissue is illustrated.

The hydroxysteroid dehydrogenases are a family of widely distributed nicotinamide nucleotide-linked alcohol dehydrogenases that promote the reversible interconversion of hydroxyl and carbonyl functions on the steroid skeleton and side chain according to the following stoichiometry (Talalay, 1963):

\[
\text{Hydroxysteroid} + \text{NAD}(P)^+ \rightarrow \text{ketosteroid} + \text{NAD}(P)H + H^+ 
\]

Soon after the first highly purified enzymes of this class became available, it was shown that these enzymes displayed a high degree of positional and steric specificity, and it was suggested that they could be used for the estimation of steroid hormones, bile acids, and their metabolites (Hurlock and Talalay, 1956, 1957, 1958; Talalay, 1960), for carrying out stereospecific oxidations and reductions of steroids, and for the resolution of enantiomeric steroids on a minute scale (Talalay and Levy, 1959). The methods involve the quantitative enzymatic oxidations of hydroxysteroids (forward reaction) or reductions of ketosteroids (reverse reaction) and the measurement of the accompanying changes in nicotinamide nucleotides by ultraviolet or fluorescence spectroscopy. Applications of these methods to the measurement of steroids in urine and chromatographic fractions of steroid mixtures were described (Hurlock and Talalay, 1956, 1957, 1958).

The most readily available highly purified 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50) of Pseudomonas testosteroni has been widely used for the measurement of bile acids and bile salts in plasma and other biological fluids (Iwata and Yamasaki, 1964; Palmer, 1969; Murphy et al., 1970; Fausa, 1975). More recently such measurements have been extended to 3α,7α-dihydroxy-bile acids by the use of partially purified 7α-hydroxysteroid dehydrogenases of Escherichia coli B (Hasselwood et al., 1973; MacDonald et al., 1973, 1974).

The intrinsic advantages of these enzymatic methods are: (a) the high degree of positional and steric specificity of the hydroxysteroid dehydrogenases; (b) availability of highly purified bacterial enzyme preparations which are uncontaminated by each other and have high specific activities; (c) low Michaelis constants for steroids; (d) the ease with which the reaction can be driven to completion in the desired direction even in the presence of large quantities of steroid products; (e) the ease with which the reactions can be reversed under suitable experimental conditions, thus permitting the assay of both hydroxy- and ketosteroids; (f) applicability to the measurement of groups of steroids in crude extracts of tissues or body fluids, thus obviating the need for tedious separation and isolation methods.

The principal drawbacks of these methods have hitherto been their relatively low sensitivity. The limits of precise assay of steroids by direct measurement of changes in fluorescence of NADH are of the order of 5 nmol. To enhance the sensitivity of the enzymatic measurements, a number of investigators (Koritz and Moustafa, 1971; Härkönen et al., 1974, 1979; Nicolas et al., 1980) have proposed the application of the nicotinamide nucleotide cycling methods of Lowry (Lowry et al., 1961; Lowry, 1973). In this way, measurements to the limits of 5–10 pmol of pregnenolone (Koritz and Moustafa, 1971), 0.02–0.01 pmol of 17β-estradiol (Härkönen et al., 1974), 0.12 pmol of vitamin D metabolites (Härkönen et al., 1979), and 0.3 pmol of bile acids or salts (Nicolas et al., 1980) have been reported.

Our own interest in these methods was rekindled by the...
need to measure the levels of various C19 steroids in minute quantities of prostatic tissue, in connection with experiments on the experimental induction of prostatic hyperplasia in dogs (DeKlerk et al., 1979). Information from various sources (Geller et al., 1976, 1978; Gloyne et al., 1970; Krieg et al., 1979; Belis, 1980; Corpechot et al., 1981) indicates that the levels of testosterone¹, dihydrotestosterone, 5α-androstan-3α,17β-diol, and 5α-androstene-3β,17β-diol and related steroids in the prostatic glands of man and dog are in the range of 0.15–10 ng (0.5–30 pmol) per g of wet tissue. Consequently, if analyses are to be carried out on 100-mg samples of tissue, it becomes necessary to measure 0.05–3 pmol of steroid. Plasma concentrations of many steroids in the same species also fall into the range of 1–30 pmol per ml and thus require similar sensitivities of measurement.

This paper describes methods for the measurement of 3α-hydroxy-, 3β-hydroxy-, and 3-ketosteroids. These methods are directly applicable to the measurement of 17β-hydroxysteroids such as testosterone, although at present highly active and purified preparations of 17β-hydroxyysteroid dehydrogenases are available. The practical lower limit of sensitivity of measurement is 0.2–0.4 pmol. The present limit of sensitivity is imposed by the magnitudes of the blank values which arise from the presence of small quantities of contaminating nicotinamide nucleotides in the analytical enzymes. Among the options for improving upon the ultimate limits of sensitivity is the use of smaller reaction volumes and more highly refined enzyme preparations. Our methods have comparable sensitivity to radioimmunoassay procedures (Collins and Hennan, 1976), which are, however, characterized by intrinsic limitations of their own, such as cross-reactivities of antibodies and specificities that are not always fully characterized.

**EXPERIMENTAL PROCEDURES**

**Materials**

Single distilled water was passed through deionizers and redistilled in an all-glass system. MES, Hepes, and CAPS buffers were obtained from Calbiochem-Behring, Chromatographically pure NAD and NADH was obtained from Sigma, which supplied 100 mg of protein), pig heart glutamate-oxaloacetate transaminase (specific activity 1200 units/mg of protein), pig heart glutamate-oxaloacetate transaminase (specific activity 200 units/mg of protein), and oxaloacetic acid were supplied by Boehringer Mannheim. A special grade of crystalline yeast alcohol dehydrogenase (specific activity 300 units/mg of protein) low in nicotinamide nucleotides was obtained from Sigma, which also supplied L-malate, 2-aminopropyl alcohol, and L-glutamic acid. Glusulase, a partially purified mixture of sulfatase (42,000 units/ml), and β-glucuronidase (75,000 units/ml) were obtained from Heliz pomata was obtained from Endo Laboratories, Garden City, NY. Reagent grade methanol was distilled twice. Absolute alcohol was obtained from Pharmco, Linfield, PA. Reagent grade concentrated sulfuric acid and L-ascorbic acid were from J. T. Baker Chemical Co., Phillipsburg, NJ. Triton N-101 was supplied by Rohm and Haas Co., Philadelphia, PA, and crystalline bovine plasma albumin was obtained from Reheis Chemical Co., Phoenix, AZ. Silica gel (Silica Woelm TSC) was from ICN Nutritional Biochemicals, Cleveland, OH. Spectroscopic grade glycerol was obtained from Fisher, and spectroscopic grade hexane and ethyl acetate were from Burdick and Jackson Laboratories, Muskegon, MI. Steroids were purchased from Syntex, Palo Alto, CA, or Steroids, Inc., Wilton, NH. Highly purified 3α- and 3β-hydroxysteroid dehydrogenases were prepared from Pseudomonas sp. BT according to minor modifications of the procedure of Shikita and Talalay (1979), and various preparations of these enzymes had specific activities of 130–240 and 55–90 units/mg of protein, respectively.

Borosilicate tubes (10 × 75 mm) for fluorimetric measurements (e.g. disposable culture tubes, Fisher) are essential to prevent high blank readings. Fluid transfers were usually made with Lang-Levy construction pipettes (H. E. Pedersen, Copenhagen, Denmark) or in a series of multiple additions, with microliter glass syringes fitted with automatic repetitive dispensers (model PB600, Hamilton Co., Reno, NV).

In order to minimize background fluorescence, all glassware was washed in boiling 50% (v/v) nitric acid and rinsed in glass-distilled water. Pipettes were washed by sequential aspiration of 50% nitric acid, 1 N sodium hydroxide, distilled water (twice), and acetone.

**Enzymatic Cycling**

The principles of the procedure are based on the cycling method of Kato et al. (1973) and are illustrated in Fig. 1. The three-part analytical procedure involves the following steps. (1) Quantitative oxidation or reduction of steroid: reaction of the steroids to be measured with highly purified 3α- or 3β-hydroxysteroid dehydrogenases under conditions which result in the formation of stoichiometric quantities of NADH or NAD from the hydroxysteroids and the ketosteroids, respectively. (2) Cycling: amplification of the amount of NADH or NAD formed in Step 1 by the enzymatic cycling methods devised by Lowry et al. (1961, 1972). (3) Indicator reaction: quantitative oxidation of the L-malate formed in the cycling step by malate dehydrogenase and measurement of the accompanying formation of NADH by fluorimetry. Full details of the application of these methods to the measurement of steroids in subpicomole quantities are summarized in the following description. Helpful further information on techniques is to be found in the monograph of Lowry and Passoneau (1972).

1a. Oxidation of Steroids—Samples to be assayed (standard steroid solutions in methanol), HPLC fractions, tissue extracts) are placed in borosilicate tubes (6 × 50 mm) and evaporated to dryness on a vacuum centrifuge (Speed Vac Concentrator, model SVC-100, Savant Instruments, Inc., Hicksville, NY). The quantity of steroid used for each assay should not exceed 2 pmol since after amplification by cycling and completion of the indicator reaction, the final concentration of NADH approaches 15 μM. At higher values, the fluorescence is not proportional to concentration (cf. Kato et al., 1973). To the dry residue in each tube are added 10 μl of a buffer mixture prepared from 9 volumes of 100 mM CAPS, 0.03% (v/v) Triton N-101, 0.001% (w/v) albumin, 6 μM NAD, and 1 volume of methanol. The CAPS-Triton-albumin buffer (adjusted to pH 9.75 with NaOH) is stored in small aliquots at −20 °C. Just before use the requisite amount of 100 mM NAD solution (stored at −20 °C and stable for several months) is added to give a concentration of 6 μM. The contents of the assay tubes

---

¹ The abbreviations and trivial names used are: testosterone, 17β-hydroxyandrost-4-en-3-one; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography; albumin, bovine plasma albumin; CAPS, 3-(cyclo-hexylamino)-2-(N-morpholino)ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; malate, L-malate; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; dehydroandrosterone, 3β-hydroxy-5α-androstan-17-one; androsterone, 3α-hydroxy-5α-androstan-17-one.

---

**Fig. 1. Principles of the enzymatic estimation of steroids.** Schematic presentation of the enzymatic estimation of hydroxy- and ketosteroids with the aid of hydroxysteroid dehydrogenases in conjunction with nicotinamide nucleotide cycling.
are mixed and adjusted to stand for 10 min at ambient temperature. Alternatively, a solution of steroids in methanol may be diluted with buffer mixture to achieve the above final concentrations of all the components. Identical standard curves are obtained by either procedure.

To the 10 ml of reaction mixture is added 1 ml of 3α- or 3β-hydroxy steroids dehydrogenase (suitably diluted in 100 mM phosphate potassium buffer, pH 7.0, 1.1% w/ v) albumin, 20% (v/v) ethanol, and 0.01% w/v of NAD, pH 7.0 (2.4 millimilliions of activity (0.44-4 ng of protein). Two types of assay blanks are prepared by omitting either the hydroxysteroid dehydrogenases or the sample to be assayed (see "Results and Discussion"). After 15 min of incubation at 25 °C, 2 ml of a solution containing 1.5 X HCl and 24 mm ascorbic acid are added to destroy unreacted NAD. Both temperature and time are critical. The tubes are then cooled rapidly in an ice bath until the addition of the cycling reagent.

1b. Reduction of Steroid—A buffer mixture composed of 100 mM MES, 0.05% (v/v) Triton N-101, and 0.001% (w/v) albumin (adjusted to pH 6.0 with NaOH) is stored in small aliquots at -20 °C. NADH (5 mM) stored at -80 °C in sodium carbonate buffer, pH 10.6, is stable for at least 1 month. The NADH solutions are heated at 80 °C for 30 min before each use to destroy NADH formed during storage. Fresh solutions of ascorbic acid (0.5 M) are prepared for each experiment. Samples to be assayed for ketosteroids are prepared as described for the hydroxysteroids above. Each assay tube receives 10 ml of a solution prepared by mixing above the above MES-Triton-albumin buffer with NADH (2.28 μM final concentration), ascorbic acid (8.8 mM final concentration), and methanol (10% by volume). The contents of the tubes are mixed and allowed to stand for 10 min at ambient temperature. This solution is then incubated for 20 min at 25 °C. This solution contains 2 ml of a solution containing 1.5 X HCl and 24 mM ascorbic acid are added to destroy unreacted NADH. The presence of ascorbic acid retards any slight oxidation of NADH to NAD which may occur during the acid destruction of NADH. Following incubation at 25 °C for 10 min, 2 ml of 0.5 N NaOH are added to achieve a basic pH, the tube is then cooled in an ice bath, and the cycling reagent is added as soon as possible.

2. Cycling Reaction—Cycling reagent (50 μl) is added to each assay tube, and the contents are mixed thoroughly. The cycling reagent has the following composition: 250 mm Hepes buffer (adjusted to pH 8.0 with NaOH), 2 mM 2-mercaptoethanol, 300 mm ethanol, 2 mM oxaloacetic acid (stock solution in 0.5 M HCl at -80 °C), cysteamine, and malate dehydrogenase (5 μg/ml). After 20 min of incubation at 25 °C, 2 ml of a solution containing 0.5 X HCl and 24 mM ascorbic acid are added to destroy unreacted NADH. The presence of ascorbic acid retards any slight oxidation of NADH to NAD which may occur during the acid destruction of NADH. Following incubation at 25 °C for 10 min, 2 ml of 0.5 N NaOH are added to achieve a basic pH, the test tube is then cooled in an ice bath, and the cycling reagent is added as soon as possible.

3. Indicator Reaction—With the aid of a 60-μl constricted pipette, the complete contents of each tube are transferred to a borosilicate tube, 10 X 75 mm, containing 1 ml of the indicator reagent of the following composition: 50 mM 2-amino-2-methylpropanoic acid (adjusted to pH 9.9 with HCl), 200 mM NaCl, 10 nm sodium glutamate, 0.3 mM NAD, 0.18 mM NADH, 0.1% (w/v) albumin, 0-70 mM steroid in 0.05 ml of methanol, and 50-200 milliunits of 3α- or 3β-hydroxysteroid dehydrogenase. The concentrations of NADH and NADH were determined from decreases in absorbance of NADH at 340 nm in 3.0-ml assay systems containing 50 mM 2-amino-2-methylpropanoic acid (adjusted to pH 9.9 with HCl), 2.5 mM NAD, 33 mM glutamate, 12 units of yeast alcohol dehydrogenase, and 12 units of malate-oxaloacetate transaminase (2 mg/ml), and malate dehydrogenase (5 μg/ml). Each set of determinations includes duplicates of the two types of blanks described above and also a series of standard amounts (0.5-2.0 pmol) of steroids (e.g., 3a-androstan-3α-17β-diol, dehydroepiandrosterone, or dihydrotestosterone). Standard amounts of L-malate (0 and 10 nmol) are also assayed with 1 ml of indicator reagent to determine the apparent cycling rate. The fluorescence of all tubes is determined after 20 min of incubation at ambient temperature in a Farrand-2 ratio fluorometer with entrance slit at setting 3 and the range switch usually at position 1. The primary excitation filter is Corning 7-37, and the fluorescent radiation is filtered through a combination of Corning filters 4-72 and 3-72. The instrument is calibrated for each set of determinations with a standard of 0.14 μM quinine sulfate in 5 mM H2SO4. This standard is sealed tightly and the mixture was centrifuged after each extraction. Alternatively, portions of the dried powder can be extracted with acetone. The results obtained by the two procedures are similar. The pooled organic solvent extracts were reduced to dryness on the vacuum centrifuge. The oily residue was dissolved in 1 ml of a mixture of methanol and water (7:3, by volume) and extracted with three 1-ml portions of hexane which were discarded. The aqueous methanol layer was again reduced to dryness on the vacuum centrifuge, and the residue finally dissolved in a volume of methanol such that 1 μl represented approximately 0.25 mg of lyophilized prostate powder.

**Spectrophotometric Standardization of Substrates**

Steroids employed for standard curves were recrystallized and sublimed in a vacuum. Stock solutions were prepared in methanol, standardized spectrophotometrically by enzyme assay, and stored at -20 °C. The concentrations of 3α-androstan-3α-17β-diol and dehydroepiandrosterone were determined by measuring the formation of NADH at 340 nm in 3.0-ml assay systems containing 50 mM 2-amino-2-methylpropanoic acid (adjusted to pH 9.9 with HCl), 200 mM NaCl, 10 nm sodium glutamate, 0.3 mM NAD, and 0.18 mM NADH, over a period of 15 min at 25°C. The results obtained by the two procedures are similar. The pooled organic solvent extracts were reduced to dryness on the vacuum centrifuge. The oily residue was dissolved in 1 ml of a mixture of methanol and water (7:3, by volume) and extracted with three 1-ml portions of hexane which were discarded. The aqueous methanol layer was again reduced to dryness on the vacuum centrifuge, and the residue finally dissolved in a volume of methanol such that 1 μl represented approximately 0.25 mg of lyophilized prostate powder.

**RESULTS AND DISCUSSION**

**Blank Values**—The lower limits of sensitivity of the assay equipment consisted of a Waters Associates, Inc. (Milford, MA) P6000A solvent delivery system, USK universal injector, model 660 solvent programmer and R401 differential refractometer. Steroids were detected by refractive index (nanomole quantities) or by enzymatic cycling (picomole quantities).

**Preparation of Biological Samples**

Urine—Portions (0.4 ml) of 24-h collections of human pregnancy urine were adjusted to about pH 5 by additions of 0.1 ml of 2 m sodium acetate buffer (pH S.2). Following dilution to 2 ml with distilled water and the addition of 1 drop of chloroform, the sample was washed for 24 h at 37.5°C with a mixture of β-glucuronidase (880 units) and sulfatase (210 units). The mixture was extracted three times with 2 ml of ethyl acetate, and the extracts were pooled, with a 0.5 g of silica gel, and reduced to dryness on the vacuum centrifuge. The silica gel pellet was extracted three times with 2 ml of 1-ml of the extract was derived from 0.1 ml of the original urine sample. Another portion of the urine was carried through the same procedure except that the β-glucuronidase and sulfatase mixture was omitted from the incubation.

**Prostate Extracts**—Ventral and dorsolateral lobes of prostate were removed from six adult Sprague-Dawley rats (350-400 g body weight) under pentobarbital anesthesia. The pooled weights were 4.62 g for the ventral and 2.29 g for the dorsolateral lobes. Each organ pool was minced and homogenized with 3-5 ml of water by means of a Polytron homogenizer (model P 10; Brinkmann Instruments) and dried in a thin layer under vacuum from the frozen state. The powders (673 mg or 14.6% of wet weight for the ventral and 402 mg or 18.0% of wet weight for the dorsolateral lobes) were stored at -20 °C in a desiccator over P2O5. Portions of the dried powders (50-75 mg) were suspended in 1 ml of H2O with the aid of a conical all-glass homogenizer, extracted three times with 3-ml portions of dichloromethane, and the mixture was centrifuged after each extraction. Alternatively, portions of the dried powder can be extracted with acetone. The results obtained by the two procedures are similar. The pooled organic solvent extracts were reduced to dryness on the vacuum centrifuge. The oily residue was dissolved in 1 ml of a mixture of methanol and water (7:3, by volume) and extracted with three 1-ml portions of hexane which were discarded. The aqueous methanol layer was again reduced to dryness on the vacuum centrifuge, and the residue finally dissolved in a volume of methanol such that 1 μl represented approximately 0.25 mg of lyophilized prostate powder.
system described in this paper are governed by the magnitude of the fluorescence of controls (blanks), since these values must be subtracted from the assay measurements. The principal source of interfering fluorescence is the contamination of the cycling enzymes and hydroxysteroid dehydrogenases by minute quantities of nicotinamide nucleotides which are amplified by the cycling process. Consequently, only enzymes low in nicotinamide nucleotide content can be used. A small contribution to the undesired fluorescence may arise also from incomplete destruction of the excess NAD$^+$ or NADH used as substrates in the steroid oxidation and reduction reactions, respectively. Therefore, it is mandatory that the levels of added nicotinamide nucleotides be kept at the minimum required to attain completion of the steroid oxidation-reductions.

Typical blank values encountered in these assays are shown in Fig. 2, which demonstrates the assay of 1.0 pmol of 5α-androstane-3α,17β-diol by oxidation to dihydrotestosterone with 3α-hydroxysteroid dehydrogenase. The fluorescence observed in the assay system is strictly proportional to the duration of cycling (Fig. 2, top curve). Thus, the fluorescence of sample blanks from which either the steroid (middle curve) or the hydroxysteroid dehydrogenase (lower curve) has been omitted also increases with the cycling time. The hydroxysteroid dehydrogenase makes a much smaller contribution to the blank fluorescence than the cycling enzymes which are added in considerably larger quantities.

An additional contribution to the blanks occurs in the ketosteroid reduction reaction. Excess NADH is efficiently destroyed at pH 2, whereas the NAD$^+$ formed by the hydroxysteroid dehydrogenase reaction is not affected. However, during the acid destruction of NADH, some NAD$^+$ or NAD$^-$ like substances are produced, and these may undergo amplification by the cycling process. The formation of such products is minimized by carrying out both the steroid reduction reaction and the acid destruction of NADH in the presence of ascorbic acid.$^2$ Furthermore, NAD$^+$ formed spontaneously in NADH solutions during storage must be destroyed by heating the NADH solutions at high pH before each set of experiments. Nevertheless, because of higher blank values, the sensitivity currently attainable in the steroid reduction assay is somewhat lower than in the steroid oxidation assay.

Under standard assay conditions and the customary cycling time of 60 min, the maximal blank values (steroid solution omitted) are 8-15 fluorescence units (as defined by the fluorometer settings given under "Experimental Procedures") for the steroid oxidation and 15-25 fluorescence units for the steroid reduction reactions. This contrasts with blank values of less than 2 units for the malate standards. Increased sensitivity of the assay can probably be attained by reducing the volume of the assay system (from the present size of 11 μl) so that the quantities of reagents and consequently the magnitudes of the blank values are decreased. A promising procedure for achieving this goal is the oil drop technique devised by Lowry (1963).

**Measurement of Standard Solutions of Hydroxysteroids and Ketosteroids**—The final fluorescence observed in the assay system is strictly proportional to the quantity of steroid added over the range of 0.2-2.0 pmol. Fig. 3A illustrates the magnitude of fluorescence of varying quantities of 5α-androstane-3α,17β-diol by their oxidation to dihydrotestosterone in the presence of 3α-hydroxysteroid dehydrogenase, whereas in Fig. 3B, 3β-hydroxysteroid dehydrogenase has been used to oxidize dehydroepiandrosterone. Both assays were conducted at pH 9.75 in the presence of excess NAD$.^+$ A linear relation between fluorescence and quantity was also observed when comparable quantities of carefully standardized NADH were subjected to the same cycling procedure (Fig. 3C). Moreover, the slopes (fluorescence/ pmol of steroid or NADH) of the three assays (Fig. 3, A–C) are essentially identical demonstrating not only that complete oxidations of the steroids were attained but also that the stoichiometric quantities of NAD$^+$ formed remained stable under the assay conditions.

The measurement of dihydrotestosterone (0.16-1.6 pmol) by reduction to 5α-androstane-3β,17β-diol catalyzed by 3β-hydroxysteroid dehydrogenase is shown in Fig. 4A. Results (not shown) are similar when 3α-hydroxysteroid dehydrogenase is used to reduce the dihydrotestosterone (to 5α-androstane-3α,17β-diol). The reactions were conducted at pH 6.0 in the presence of excess NADH. When comparable quantities of carefully standardized NAD$^+$ were subjected to the cycling process, strict proportionality between fluorescence and quantity was observed (Fig. 4B). The slopes (fluorescence/ pmol of steroid or NAD$^+$) were almost identical for the ketosteroid and the NAD$^+$ assays, thus supporting the view that the ketosteroid undergoes complete reduction and that the stoichiometric quantities of NAD$^+$ formed in these reactions remain stable during the assay process.

Since the reliability of the enzymatic assays requires complete conversions of hydroxy- to ketosteroids (and vice versa), we present (see "Appendix") the theoretical basis for devising conditions under which these requirements are met. The degree to which steroid oxidations and reductions are complete depends upon a number of factors: (a) the concentration of steroid; (b) the concentration of nicotinamide nucleotide; (c) the equilibrium constant of the reaction which in turn is governed by the conformation of the hydroxyl group; (d) the pH of the medium; and (e) the possible presence of the products of the reaction. The last mentioned factor is relevant where it is necessary to measure hydroxysteroids in the presence of an excess of ketosteroids (or vice versa) in body fluids.

---

2 O.H. Lowry, personal communication.
Enzymatic Estimation of Steroids

Fig. 3. Assay of standard solutions of 3α- and 3β-hydroxysteroids by oxidation with hydroxysteroid dehydrogenases. A, oxidation of 5α-androstane-3α,17β-diol (0.2–2.0 pmol) by 3α-hydroxysteroid dehydrogenase; B, oxidation of dehydroepiandrosterone by 3β-hydroxysteroid dehydrogenase, followed by amplification by enzymatic cycling of the NADH formed (see "Experimental Procedures"). Each point represents the mean (±S.D.) of 5 determinations. A blank (minus substrate) of 9.4 fluorescence units has been subtracted. The slope of the linear regression line is 46.1 fluorescence units per pmol of NADH by the same 3-step procedure, except that the hydroxysteroid dehydrogenases were omitted from Step 1. Each point represents the mean of duplicate measurements from which 9.4 fluorescence units (blank values for A and B) have been subtracted. D, typical standard curve for 2.4–14.7 nmol of L-malate obtained by the indicator reaction (Step 3) only. Each point is the mean of duplicate determinations. The slopes of the linear regression lines are: for A, 99.2; for B, 63.3; and for C, 65.2 fluorescence units per pmol of substrate, and for D, 107 fluorescence units per pmol of L-malate. The apparent cycling rates computed from these values are 5533 per h for A and 5916 per h for B.

Fig. 4. Assay of a standard solution of dihydrotestosterone by reduction with 3β-hydroxysteroid dehydrogenase. A, reduction of 0.16–1.6 pmol of dihydrotestosterone by 3β-hydroxysteroid dehydrogenase followed by amplification by enzymatic cycling of the NAD" formed (see "Experimental Procedures"). Each point is the mean (±S.D.) of 6 determinations. A blank (dihydrotestosterone omitted) of 15.8 fluorescence units has been subtracted from each value. B, assay of 0.16–1.2 pmol of NAD" by enzymatic cycling as described under "Experimental Procedures." Each point is the mean (±S.D.) of triplicate determinations, from which 15.8 fluorescence units have been subtracted. The slope of A is 46.1 fluorescence units per pmol of dihydrotestosterone and that of NAD" is 48.1 fluorescence units per pmol of NAD". The apparent cycling rate is 3988 per h.

or tissue extracts. Under the prescribed conditions, the enzymatic conversions of 2 pmol of various hydroxy- and ketosteroids alone are more than 99.5% complete. But even in the presence of 50 times larger quantities of steroid products, which would tend to displace the equilibrium, the reactions are more than 95% complete. These predictions were confirmed experimentally by assaying 2 pmol of dihydrotestosterone with 3α- or 3β-hydroxysteroid dehydrogenase in the presence of 25 pmol of the respective products of each enzymatic reaction. Similarly, 5α-androstane-3α,17β-diol was measured in the presence of a 13-fold excess of dihydrotestosterone. In none of these measurements was significant interference by products encountered.

The lower limits of sensitivity of measurement of hydroxysteroids (Fig. 3) and ketosteroids (Fig. 4) are at present 0.2 and 0.4 pmol, respectively. This limit is based on the stringent assumption that reliable fluorescence measurements must be at least twice blank values. The blank values have been subtracted from all measurements shown in Figs. 3 and 4. The coefficient of variations [(S.D./mean) × 100] for repetitive measurements (carried through all three steps described under "Experimental Procedures") is ±5–12%, irrespective of whether the measurements are made as part of the same experiment or different experiments.

Standard quantities of malate are assayed by means of the indicator reaction (Step 3, see "Experimental Procedures") in each experiment. The malate standards are strictly linear and highly reproducible in repetitive assays. In seven assays we obtained 10.3 ± 0.98 fluorescence units per pmol of malate when corrected for the blank (indicator reagent only). The ratio of the slope of the steroid standard curve to that of the malate standard is used as a measure of the "apparent" cycling rate. The range of apparent cycling rates observed in our assays is 5000–8000 per h. Unusually low cycling rates may reflect intrinsic difficulties with the cycling system (such as loss of activity of the cycling enzymes) or errors in the concentrations of the standard steroid solutions (e.g. very dilute steroid solutions may be partially adsorbed to storage vessels). Discrepancies are sometimes encountered between the apparent cycling rates observed when hydroxysteroids or ketosteroids are assayed. Such differences are usually caused by the fact that the conditions of the cycling system (e.g. pH) are not the same. A method for measuring the absolute cycling rate has been described (Kato et al., 1973).

Analysis of Chromatographic Fractions—Many studies on purified hydroxysteroid dehydrogenases indicate that these enzymes display a high degree of positional and steric specificity for hydroxyl groups on the steroid skeleton and side-chain but usually can react with closely related steroids bearing such functional groups. These enzymes must, therefore, be regarded as specific reagents for "functional groups." Cur-
rent information indicates that the 3α-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni* and *Pseudomonas* sp. BT (Shikita and Talalay, 1979) has a broad range of specificities for many 3α-hydroxysteroids of the C19, C21, and C24 series in which the A/B ring fusion may be either *cis* or *trans*. In contrast, the 3β-hydroxysteroid dehydrogenase of *Pseudomonas* sp. BT will oxidize 3β-hydroxysteroids of the C19, C21, and C24 series but requires that the A/B ring fusion be *trans* or that a double bond be present at C-5 (6).

One potential advantage of the use of group-specific reagents in the analysis of steroid profiles in tissue samples and body fluids is the possibility of detecting the presence of compounds of unknown structure which may be of biological importance.

When combined with conventional separation techniques, the enzymatic cycling assay can provide measurements (and partial identification) of individual steroids at levels too low to be detected by conventional analytical methods (refractive index or UV absorption). This application is demonstrated in Fig. 5 which shows the analysis of a 6 pmol of each of six steroids (androstrene, 5α-androstan-3α,17β-diol, 5α-androstan-3β,17β-diol, 5α-desmosterolone, 5α-desmosterolone, and testosterone). Each fraction was assayed with both 3α- and 3β-hydroxydehydrogenases, and no activity was detected where substrates were not present. Thus, androstrene and 5α-androstan-3α,17β-diol were detected with 3α-hydroxysteroid dehydrogenase; 5α-androstan-3β,17β-diol and dehydroepiandrosterone were detected with 3β-hydroxysteroid dehydrogenase; dihydrotestosterone and testosterone were not detected. The recovery of steroids subjected to HPLC and detected by enzymatic cycling was linear over the range of 0.2-200 pmol (data not shown), and the recoveries in the chromatographic fractions were 80-100% in various experiments. With the enzymatic cycling assay technique, it is thus possible to utilize conventional HPLC equipment to separate steroids at the picomole level and to recover and detect such quantities satisfactorily. Since the HPLC techniques are relatively rapid, many fractions can be analyzed conveniently by the cycling procedure, and the possibility exists of developing methods for obtaining steroid "fingerprints" of individual small tissue samples.

**Steroid Determinations in Human Pregnancy Urine and Serum**—The cycling methods have been used to determine the total amounts of 3α- and 3β-hydroxysteroids directly in untreated samples of human pregnancy urine. Some appreciation of the sensitivity of the method is given by the fact that unconjugated 3α-hydroxysteroids can be easily and accurately measured in less than 0.05 μl of urine and that after hydrolysis with sulfatase and β-glucuronidase, as little as 0.002 μl of urine is required (Fig. 6). The quantity of 3α-hydroxysteroids excreted per 24 h in late pregnancy urine is about 146 μmol (after hydrolysis of conjugates) and 13 μmol (before hydrolysis). Thus, less than 10% of the 3α-hydroxysteroids is unconjugated. These values are comparable to published values determined by other methods (Breuer et al., 1976). One of the advantages of the high sensitivity of the cycling methods is that 100-fold diluted urine samples can be assayed directly without prior extraction and almost identical results are obtained.

Steroids can also be easily detected in ethyl acetate extracts of only 1–2 μl of human serum in late pregnancy. For example, we measured 0.35 μM (untreated) and 3.2 μM (hydrolyzed with glucuronidase and sulfatase) 3α-hydroxysteroids in the serum of a pregnant woman at 32 weeks of gestation. The corresponding values for 3-ketosteroids were 0.26 μM and 0.78 μM, respectively.

Since the 3α-hydroxysteroid fraction includes bile acids,
some of which may be conjugated, the overall group-specific measurements do not exclusively reflect metabolites of steroid hormones. Further information on the composition of individual steroid components may be obtained on fractionation by HPLC. This is exemplified in Fig. 7 which provides the 3α-hydroxysteroid profile of a sample of human pregnancy urine in which the chromatographic pattern obtained from the equivalent of 0.5 μl of urine treated with sulfatase and β-glucuronidase (Fig. 7A) is compared with that of 5 μl of untreated urine (Fig. 7B). The major component, eluted at 20 min, is probably 5α-pregnane-3α,20α-diol, which increases about 10-fold upon enzymatic hydrolysis of conjugates. Certain other components (e.g. the peak eluted at 11.25 min)

![Graph]

FIG. 7. HPLC analysis of 3α-hydroxysteroid profiles in human pregnancy urine. Extracts of human pregnancy urine (A, hydrolyzed; B, untreated) prepared as described under “Experimental Procedures” were subjected to HPLC on a normal phase column with a nonlinear gradient (model 660 solvent programmer, Water Associates, program 10) starting with a mixture of hexane and ethyl acetate (1:1, by volume) and reaching a limit of 100% ethyl acetate at 18 min. The flow rate was 1 ml/min, and fractions were collected at 0.5-min intervals from 5–22 min. The fractions were evaporated in a vacuum centrifuge and assayed for 3α-hydroxysteroids by oxidation with 3α-hydroxysteroid dehydrogenase and nicotinamide nucleotide cycling. The arrows indicate the expected positions of elution of androsterone, 5α-androstan-3α,17β-diol (3α-diol), and 5α-pregnane-3α,20α-diol (pregnanediol). The urine extracts applied to the column were: A, 5 μl of an ethyl acetate extract of hydrolyzed pregnancy urine representing 0.5 μl of original urine containing 45 pmol of 3α-hydroxysteroids; and B, 50 μl of an ethyl acetate extract of unhydrolyzed pregnancy urine representing 5 μl of original urine and containing 40 pmol of 3α-hydroxysteroids. Of the total 3α-hydroxysteroids applied to the column, 41% (A) and 37% (B) were detected in the fractions assayed. The ordinates are calculated from the fluorescence of each fraction and the slope of a standard curve of 5α-androstan-3α,17β-diol (126.8 fluorescence units/pmol of steroid).

Similarly increased about 10-fold after hydrolysis, whereas other constituents (e.g. the peak at 14.5 min) are present in even greater amounts in the hydrolyzed urine. In routine measurements, about 80% of picomole quantities of pure steroid hormones are recovered in the HPLC fractions. However, in the HPLC separation of urinary steroids, the total amount of 3α-hydroxysteroids eluted in the chromatographic fractions during the time monitored is only 40% of the quantity applied. The calculated contents of 3α-hydroxysteroids are 7.0 and 1.4 pmol per mg dry weight for the ventral and dorsolateral lobes, respectively.

![Graph]

FIG. 8. Determination of 3α-hydroxysteroid content of the ventral and dorsolateral lobes of rat prostates. Methanol solutions of extracts of the ventral (○) and dorsolateral (×) lobes of rat prostates were prepared (see “Experimental Procedures”) such that 1 μl represented 0.25 and 0.24 mg of dry prostate weight, respectively. Portions (0.5, 1.0, and 1.5 μl) of each solution were assayed (in triplicate) for 3α-hydroxysteroid content by oxidation with 3α-hydroxysteroid dehydrogenase followed by enzymatic cycling. The quantities of 3α-hydroxysteroids were calculated on the basis of a standard curve for 5α-androstan-3α,17β-diol (63.2 fluorescence units/pmol). For reasons discussed in the text, the fluorescence in the tissue samples is not strictly proportional to the amount of extract analyzed, especially in the case of the higher levels found in the ventral lobe. The interrupted line has been drawn through the origin and the lowest quantity analyzed, to indicate the deviation from proportionality. The calculated contents of 3α-hydroxysteroids are 7.8 and 1.4 pmol per mg dry weight for the ventral and dorsolateral lobes, respectively.

likewise increased about 10-fold after hydrolysis, whereas other constituents (e.g. the peak at 14.5 min) are present in even greater amounts in the hydrolyzed urine. In routine measurements, about 80% of picomole quantities of pure steroid hormones are recovered in the HPLC fractions. However, in the HPLC separation of urinary steroids, the total amount of 3α-hydroxysteroids eluted in the chromatographic fractions during the time monitored is only 40% of the quantity applied. The calculated contents of 3α-hydroxysteroids are 7.0 and 1.4 pmol per mg dry weight for the ventral and dorsolateral lobes, respectively.

Steroid Determinations in Prostatic Tissue—The quantities of steroids present in tissues such as the prostate are small, and their measurement requires highly sensitive methods such as those described in this paper, especially if only milligram quantities of tissue are available for analysis. This is the case if it is desired to correlate steroid levels with the histological characteristics of different regions of a small organ. Fig. 8 illustrates measurements of 3α-hydroxysteroids in extracts of less than 0.5 mg of dry weight (3.5 mg of wet weight) of rat ventral and dorsolateral prostate. The quantities of hydroxysteroids measured are reasonably proportional to the amount of extract analyzed. Furthermore, the addition of 0.51 pmol of androsterone to varying quantities of ventral and dorsolateral prostate extracts leads to recovery of 83–117% of the added androsterone (Table 1). This demonstrates that steroid standards can be recovered with satisfactory yields in the presence of tissue extracts containing a mixture of various steroids.
While the satisfactory recovery of a standard steroid which is a good substrate for the analytical enzyme is essential for the validation of the method (e.g., Table I) certain additional precautions are required to assure the validity of such analyses. With some extracts, the measurements are not strictly proportional to the amounts of tissue analyzed (e.g., Fig. 8). In such instances, it has been found that longer incubation periods with the hydroxysteroid dehydrogenases or incubation with larger quantities of these enzymes establishes linearity. These deviations from linearity are undoubtedly the result of the presence in the extracts of steroids that are poor substrates for the dehydrogenases and/or the presence of steroidal (and even nonsteroidal) inhibitors of the enzymatic reactions. It is, therefore, mandatory to carry out additional control experiments to ensure that complete oxidations and reductions of steroids by the hydroxysteroid dehydrogenases have been accomplished.

Preliminary studies of the 3α-hydroxysteroid content of prostates indicates variation with age and microscopic appearance of the glands. For example, the 3α-hydroxysteroid content reaches a maximum of 3 pmol/mg dry weight in the ventral prostates of young Sprague-Dawley rats near puberty and declines to very low values in the adult. In young beagles with histologically normal prostate glands, the 3α-hydroxysteroid content is 1-2 pmol/mg dry weight, and this value declines to about 0.3 pmol/mg dry weight in the hyperplastic prostates of old beagles. These estimates of the 3α-hydroxysteroid content fall into the same range as those reported by radioimmunoassay for the content of 5α-androstane-3α,17β-diol (2.5-50 fmol/mg wet weight or 0.02-0.3 pmol/mg dry weight) in rat, dog, and human prostates (Meikle et al., 1978, 1979; Bartsch et al., 1980; Krieg et al., 1979; Corpechot et al., 1981).

Measurement of Hydroxysteroid Dehydrogenase Activity—The above illustrations describe the sensitive determination of steroid hormones and their metabolites. The methods described in this paper are equally applicable to the determination of steroid hormones and their metabolites. The methods of these studies.

| TABLE I |
|---|

**Assay of 3α-hydroxysteroids in extracts of ventral and dorsolateral lobes of rat prostate; recovery of a standard quantity of added androsterone.**

Each assay was carried out in triplicate (coefficient of variation was less than ±5%). The androsterone solution was standardized spectrophotometrically, and 3 different amounts were assayed in the hydroxysteroid dehydrogenase-cycling assay. The final fluorescence was calculated on the basis of the difference between assays of the prostate extract in the presence and absence of 0.51 pmol of androsterone.

| Prostate lobe extract | Quantity of prostate analyzed | Amount of androsterone added | Amount of 3α-hydroxysteroids found | Recovery of androsterone |
|-----------------------|-------------------------------|-----------------------------|-----------------------------------|-------------------------|
| Ventral               | mg dry weight                 | pmol                        | pmol                              | pmol                    |
| 0.250                 | 0.125                         | 0                           | 0.988                             | 0.602                   |
| 0.250                 | 0.125                         | 0.51                        | 1.57                              | 0.602                   |
| Dorsolateral          | 0.125                         | 0                           | 1.84                              | 0.602                   |
| 0.24                  | 0.51                          | 0.601                       | 0.434                             |
| 0.24                  | 0.51                          | 0.372                       | 0.422                             |

**FIG. 9. Measurements of 3α-hydroxysteroid dehydrogenase activity of dog prostate microsomes.** The figure shows a linear relationship between the amount of 5α-androstane-3α,17β-diol formed from dihydrotestosterone and the time of incubation in the presence of 2.95 and 4.19 fmol of micromolar protein. The microsomes were isolated by differential centrifugation from homogenates of dog prostates. The enzyme assays were carried out in final volumes of 20 μl containing 100 mM MES (adjusted to pH 6.0 with NaOH), 0.02% (by volume) Triton N-101, 0.001% (w/v) albumin, 8.5 μM dihydrotestosterone in 0.05 μl of methanol, and 1.5 μl of microsome suspension. The reaction was initiated by addition of 1.5 μl of 10 mM NADH. Incubations were carried out at 25 °C for the indicated times. The reaction was arrested by the addition of 2 μl of 3 N HCl, and the steroids were extracted with three 200-μl portions of cyclohexane. The solvent extracts were reduced to dryness in a vacuum centrifuge and redissolved in 100 μl of methanol. Portions (10 μl) were analyzed by oxidation with 3α-hydroxysteroid dehydrogenase, and amplification of the NADH was formed by nicotinamide nucleotide cycling as described under “Experimental Procedures.”

**Acknowledgments**—We acknowledge with gratitude the invaluable contributions of Donna E. Geoffrion and Marquie E. Renaud to these studies.
Enzymatic Estimation of Steroids

**Table A**

Equilibrium constants ($K_W$) for the oxidation of hydroxysteroids to ketosteroids

Calculation of the degree of completion of oxidations and reductions under standard assay conditions is based on:

$$K_W = \frac{[\text{ketosteroid}] [\text{NADH}] [\text{H}^+]}{[\text{hydroxysteroid}] [\text{NAD}^+]^2}$$

| Reaction | Conformation of hydroxyl group | $K_W$ ($\times 10^2$) M | Oxidation\(\text{Hydroxysteroid alone}\) | Oxidation\(\text{Hydroxysteroid + 10 pmol product}\) | Reduction\(\text{Ketosteroid alone}\) | Reduction\(\text{Ketosteroid + 10 pmol product}\) |
|----------|-------------------------------|-------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Androsterone = 5α-androstan-3α,17β-dione = 5α-androstan-17β-ol-3-one | Axial | 7.42 | 99.91 | 99.45 | 99.92 | 99.56 |
| 5α-Androstan-3α,17β-diol = 5α-androstan-17β-ol-3-one | Axial | 6.68 | 99.90 | 99.39 | 99.93 | 99.60 |
| 5α-Androstan-3β,17α-diol = 5α-androstan-17α-ol-3-one | Equatorial | 2.11 | 99.68 | 97.71 | 99.98 | 99.87 |
| 5β-Androstan-3α,17β-diol = 5β-androstan-3,17-dione | Equatorial | 1.57 | 99.57 | 97.47 | 99.98 | 99.91 |

* Determined at 25 °C. Data are from Talalay (1963).

**APPENDIX**

Equilibria of Oxidations of Hydroxysteroids and Reductions of Ketosteroids

A fundamental requirement for the validity of the enzymatic methods of steroid analysis described in the text is that the oxidations of hydroxysteroids (forward reaction) and reductions of ketosteroids (reverse reaction) be complete in the desired directions even in the presence of excesses of reaction products, so that under all conditions the amounts of NADH or NAD formed are stoichiometrically related to the quantities of steroids to be measured. The magnitudes of the equilibrium constants of the oxidations of various 3-hydroxysteroids have been determined (Talalay, 1963), and experimental conditions for achieving completion of the reactions in either the direction of oxidation or reduction can be easily attained by adjustment of the reaction conditions.

Table A lists the equilibrium constants ($K_W$) at 25 °C for the oxidations of several 3α- and 3β-hydroxysteroids in which the hydroxyl groups are in axial and equatorial conformations, based on the following relationship:

$$K_W = \frac{[\text{ketosteroid}] [\text{NADH}] [\text{H}^+]}{[\text{hydroxysteroid}] [\text{NAD}^+]^2}$$

The degree of completion of oxidation or reduction of 2-pmol quantities of various steroids either alone or in the presence of 10 pmol of the reaction product under the assay conditions described in the text is also shown in Table A. Satisfactory degrees of completion of the reactions, in most cases exceeding 99%, are achieved in both the forward and reverse directions.

Nearly complete oxidations and reductions are also obtained under the specified conditions if the reaction products are present in greater excess. For example, if it is desired to measure 2 pmol of androsterone in the presence of 100 pmol of product, the degree of completion of the oxidation reaction will be 95.52%, rather than the 99.45% achieved in the presence of only 10 pmol of product. Similarly, the degree of completion of oxidation of 1 pmol of androsterone alone is 99.55%. This extent of oxidation of 1 pmol of androsterone drops to 99.51% and 95.63% in the presence of 10 and 100 pmol of product, respectively.

**REFERENCES**

Bartsch, W., Krieg, M., and Voigt, K. D. (1986) *J. Steroid Biochem.* 23, 259-264

Belis, J. A. (1980) Invest. Urol. 17, 332-336

Breuer, H., Helm, D., and Kükskenzer, H. L. (eds) (1976) *Methods of Hormone Analysis*, pp. 279, 286. John Wiley and Sons, New York

Collins, W. P., and Hennan, J. F. (1976) *Mol. Aspects Med.* 1, 3-128

Corts, C., Baulieu, E. E., and Redol, P. (1983) *Acta Endocrinol.* 96, 127-133

DeKlerk, D. P., Coffey, D. S., Ewing, L. I., McDermott, I. R., Reiner, W. G., Robinson, C. H., Scott, W. W., Strandberg, J. D., Talalay, P., Walsh, P. C., Wheaton, L. G., and Zarkin, B. R. (1979) *J. Clin. Invest.* 64, 842-849

Fausa, O. (1975) *Scand. J. Gastroenterol.* 10, 747-752

Geller, J., Albert, J., López, D., Geller, S., and Niwayama, G. (1976) *J. Clin. Endocrinol. Metab.* 42, 586-588

Geller, J., Albert, J., Loza, D., Geller, S., and Niwayama, G. (1976) *J. Clin. Endocrinol. Metab.* 42, 586-588

Gloyna, R. E., Siiteri, G., and Wilson, J. D. (1970) *J. Biol. Chem.* 249, 1746-1753

Harkonen, M., Adlercreutz, H., and Groman, E. V. (1974) *J. Steroid Biochem.* 5, 717-725

Harkonen, M., Adlercreutz, H., Dabeck, J. T., and O’Riordan, J. L. (1979) *J. Steroid Biochem.* 11, 1205-1208

Haslwood, G. A. D., Murphy, G. M., and Richardson, J. M. (1973) *Clin. Sci.* 44, 95-98

Hurlock, B., and Talalay, P. (1979) *Proc. Soc. Exp. Biol. Med.* 163, 560-564

Hurlock, B., and Talalay, P. (1957) *J. Biol. Chem.* 232, 37-52

Hurlock, B., and Talalay, P. (1958) *Endocrinology* 62, 201-215

Iwata, T., and Yamashita, K. (1964) *J. Biochem. (Tokyo)* 56, 424-431

Kato, T., Berger, S. J., Carter, J. A., and Lowry, O. H. (1973) *Clin. Endocrinol.* 4, 334-338

Krieg, M., Bartsch, W., Janssen, W., and Voigt, K. D. (1979) *J. Steroid Biochem.* 11, 615-624

Lowry, O. H. (1963) *Biochem. (Tokyo)* 43, 134-138

Lowry, O. H., and Talalay, P. (1958) *J. Biol. Chem.* 233, 37-52

MacDonald, I. A., Williams, C. N., and Mahoney, D. E. (1973) *Biochim. Biophys. Acta* 280, 243-253

Meikle, A. W., Burton, G. V., Stringham, J. D., and Fang, S. (1979) *J. Steroid Biochem.* 11, 1503-1505

Meikle, A. W., Stringham, J. D., and Olsen, D. C. (1978) *J. Clin. Endocrinol. Metab.* 47, 900-913

Murphy, G. M., Billing, H., and Baron, D. N. (1970) *J. Clin. Pathol.* 23, 594-598

Nicolas, J. C., Chaintreul, J., Descompbs, B., and Crastes de Paulet,
Enzymatic Estimation of Steroids

A. (1980) Anal. Biochem. 103, 170–175
Palmer, R. H. (1969) Methods Enzymol. 15, 280–288
Shikita, M., and Talalay, P. (1979) Anal. Biochem. 95, 286–292
Talalay, P. (1969) Methods Enzymol. Anal. 8, 119–143
Talalay, P. (1963) in The Enzymes (Boyer, P. D., Lardy, H., and Myrbäck, K., eds) 2nd Ed., Vol. 7, pp. 177–202, Academic Press, New York
Talalay, P., and Levy, H. H. (1959) in Steric Course of Microbiological Reactions, Ciba Foundn. Study Group No. 2 (Wolstenholme, G. E. W., and O'Connor, C. M., eds) pp. 53–78, Churchill, London
Talalay, P., Shikita, M., and Renaud, M. E. (1982) J. Steroid Biochem. (in press)
Enzymatic estimation of steroids in subpicomole quantities by hydroxysteroid dehydrogenases and nicotinamide nucleotide cycling.
D W Payne, M Shikita and P Talalay

J. Biol. Chem. 1982, 257:633-642.

Access the most updated version of this article at http://www.jbc.org/content/257/2/633

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/257/2/633.full.html#ref-list-1