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The Mechanism of the Bacterial C-1,2 Dehydrogenation of Steroids. III. Kinetics and Isotope Effects*

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ABSTRACT: The kinetics of the C-1,2 dehydrogenation of 3-keto steroids with cell-free preparations of *Bacillus sphaericus* in the presence of menadione and 2,6-dichlorophenolindophenol as electron acceptors indicate that the enzyme undergoes intermediate reduction–oxidation. Lineweaver–Burk plots of $1/V$ *vs.* 1/acceptor gave parallel slopes for several fixed concentrations of steroid and the replot of intercepts *vs.* 1/steroid gave $1/V_{max} > 0$. Deuterium isotope effects were studied by enrichment procedures, by steroidal product isolation, and by spectrophotometric kinetic assays. In the case of 5α-3-keto steroids, a significant kinetic isotope effect was found for deuterium at the 1α but not the 2β position while for the Δ^4-3-ketone, deuterium at either position affected the rate at V_{max}. The kinetic evidence, which is in accord with the previously proposed enolization–hydride abstraction mechanism, indicates that the rate-determining step in the over-all sequence appears in the steroid dehydrogenation step rather than in the enzyme reoxidation step.

Previous studies from this laboratory (Hayano *et al.*, 1961; Ringold *et al.*, 1962, 1963) demonstrated that the enzymatic introduction of a double bond into the C-1,2 position of 5α- or Δ^4-3-keto steroids by sonically disrupted cell-free preparations of *Bacillus sphaericus* (ATCC 7055) in the presence of added external electron acceptors, preferably menadione, proceeds *via* a *trans*-diaxial elimination of the 2β,1α-hydrogen atoms. Tritium-incorporation studies established that in the absence of an electron acceptor a preferential introduction of heavy isomer into the 2β position can be effected. This, coupled with a number of mechanistic considerations, led to the proposal of a dehydrogenation mechanism consisting of a two-step process of enolization followed by hydride abstraction, a mechanism essentially identical with the nonenzymatic C-1,2 dehydrogenation of steroids by dichlorodicyanoquinone (Ringold and Turner, 1962).

In this paper we report a spectrophotometric assay for the reaction which couples the reduction of 2,6-dichlorophenolindophenol with reduced menadione. Utilizing this assay the kinetics of reaction with a 5α-3-keto steroid, a Δ^4-3-keto steroid, and with their corresponding 1α- and 2β-mono- and -polydeuterio derivatives have been studied. In addition, the isotope effects have been determined by enrichment and by steroidal product isolation procedures, which have in general given good agreement with the spectrophotometric method. The kinetic evidence has been found to be consistent with the general mechanism for two-substrate systems in which the enzyme exists in oxidized and reduced states, the steroid reacting with the oxidized form and the external electron acceptor with the reduced form. The primary deuterium isotope effects,

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which represent a complex kinetic situation, indicate that steroid oxidation rather than enzyme reoxidation is rate limiting. Further, loss of the two hydrogen atoms during steroid dehydrogenation does not appear to be a synchronous process which offers additional support for an enolization–hydride loss mechanism.

Materials and Methods

Cell-Free Enzyme Preparation

A starter culture of 1 ml of *B. sphaericus* (ATCC 7055) was added to a sterile solution of 400 ml of distilled water containing 0.3% yeast extract (Difco) and 0.5% N-Z Case (Peptone (Difco)). After 48 hours' agitation at 30° (aerobic) the bacteria were harvested by centrifugation for 5 minutes at 3000*g*. The cells (*ca.* 2.5 g) were washed with 0.03 M potassium phosphate buffer (pH 7.0) and, after suspension in 25 ml of the same buffer, sonically disrupted for 20 minutes with a Raytheon sonic oscillator (10 kc, 250 w, Model DF 101). The debris was removed by centrifugation at 6000*g* for 15 minutes and the supernatant solution (referred to hereafter as enzyme solution A) which contained the enzyme was kept at 0° until ready for use. Such solutions could be maintained for only 3–4 hours without a marked loss of activity although whole cells, after storage overnight at 0°, gave preparations of satisfactory activity. Enzyme solution A contained, on the average, about 125 μg of protein/0.5 ml. It should be noted that over a period of months the preparation of dehydrogenase by the above procedure gave reasonably constant enzyme activity. Nevertheless, the comparison of a deuterated and nondeuterated steroid substrate was always carried out with the identical enzyme preparation.

Preparation of Deuterated Steroids

2β-Deuterio-5α-androstane-3,17-dione. 2α,3α-Oxido-5α-androstan-17-one (125 mg) (Iriarte *et al.*, 1955), lithium aluminum deuteride (245 mg), and anhydrous ether (20 ml) were heated under reflux for 4.5 days. The reaction was terminated by the dropwise addition of saturated sodium sulfate solution followed by the addition of solid sodium sulfate. The ethereal solution was filtered and the residue was washed several times with ether. Removal of solvent left 134 mg of solid which, in the infrared, showed strong hydroxyl absorption (3350 cm^{-1}), carbon–deuterium stretching frequencies (2150 and 2200 cm^{-1}), and an absence of carbonyl absorption. The crude diol was dissolved in 5 ml of acetone, chilled to 0°, and oxidized with 8 N chromic acid in sulfuric acid (Bowers *et al.*, 1953). The excess reagent was destroyed by the addition of methanol, and water was added yielding 85 mg of 2β-deuterio-5α-androstane-3,-17-dione, mp 124.5–125.5°. Recrystallization from acetone–hexane raised the melting point to 130–132°. In the infrared the carbon–deuterium stretching frequency appeared at 2146 cm^{-1} ($CHCl_3$).

Anal.[1] Found: 0.96 g-atom of deuterium.

A sample of this material which was dehydrogenated by a cell-free preparation of *B. sphaericus* in the presence of menadione by the procedure previously described (Ringold *et al.*, 1963) exhibited an 86% loss of deuterium.

2β-Deuterioandrost-4-ene-3,17-dione. This material, containing 0.64 g-atom of deuterium (75% 2β, 25% 2α), was prepared by zinc–deuterioacetic acid dehalogenation of 2α-iodoandrost-4-ene-3,17-dione as described by Ringold *et al.* (1963).

1α-Deuterio-5α-androstane-3,17-dione. Reduction of 17-hydroxy-5α-androst-1-en-3-one with 10% palladium–carbon in an atmosphere of deuterium gas, followed by chromic acid–acetone–sulfuric acid oxidation and alkaline equilibration, gave material containing 0.856 g-atom of deuterium that was shown by dehydrogenation studies to consist of 96% 1α isomer and 4% 1β isomer.

1β-Deuterioandrost-4-ene-3,17-dione. The preparation of this substance containing 0.65 g-atom of deuterium (78.5% 1β, 21.5% 1α) has been previously described (Ringold *et al.*, 1963).

1α-Deuterioandrost-4-ene-3,17-dione. A sample of 1α-deuterio-5α-androstane-3,17-dione containing 0.80 g-atom of deuterium (85% 1α, 15% 1β) was converted to the desired Δ^4-3-ketone with the same deuterium content and distribution by the procedure previously described (Ringold *et al.*, 1963). This material was used for the enrichment experiments. A second sample of 1α-deuterioandrost-4-ene-3,17-dione containing 0.856 g-atom of D (96% 1α, 4% 1β) was prepared from the saturated 1α-deuterio-5α-dione described above and was used for the menadione–indophenol spectrophotometric runs and for the kinetic determinations with menadione as sole electron acceptor.

2,2,4,4-Tetradeuterio-17β-hydroxy-5α-androstan-3-one. 17β-Hydroxy-5α-androstan-3-one (1.0 g), anhydrous diglyme (20 ml), and 0.72 g of sodium were placed in a 100-ml flask and the flask was immersed in an ice bath while a solution made from 13 ml of deuterium oxide (99.8%) and 13 ml of diglyme was added dropwise. The solution was heated in an oil bath at 70° overnight. After 16 hours the oil bath temperature was gradually increased to 100° and the reaction was finally terminated by cooling and pouring the solution into 250 ml of chilled water containing 2.8 ml of 37% HCl. The precipitate was filtered, washed with water until the washings were neutral, and dried in a vacuum desiccator. The crude product was chromatographed on 35 g of silica gel. All fractions eluted with 2% ether–benzene except the first, which was yellow, were combined to give 0.756 g of product, mp 178.5–181.5°. Recrystallization from acetone–hexane gave white crystals, mp 180–183°, 0.70 g. Deuterium analysis indicated the incorporation of 3.75 g-atoms. In the infrared (CCl_4) the C-2 and C-4 methylene peaks at 1418 and 1427 cm^{-1} (Jones and Cole, 1952) were absent.

2,2,4,6-Tetradeuterioandrost-4-ene-3,17-dione. Testosterone (1.16 g) was exchanged for 4.5 hours at 75° with sodium deuterioxide in deuterium oxide–diglyme as

[1] Deuterium analysis by Mr. Josef Nemeth, Urbana, Ill.

described for the tetradeuterio-5α-androstan-3-one above. The product was chromatographed on a column of 45 g of silica gel and all benzene–ether 7:3 fractions, except the first two and last two, were combined to yield 0.96 g of product which was homogeneous on thin layer chromatography. One recrystallization from acetone–hexane yielded 0.83 g of tetradeuteriotestosterone, m.p. 155–157°. Oxidation of 0.2 g of this material at 0° with 8 N chromic acid–sulfuric acid in acetone gave, after recrystallization of the crude product, 0.12 g of title compound, m.p. 173–174.5°. In the infrared the C-4 proton peak at 870 cm^{-1} was markedly reduced in intensity and the C-2 methylene peak at 1418 cm^{-1} was absent. A moderate peak at 1432 cm^{-1} indicated that exchange at C-6 was not complete.

Anal.[1] Found: 3.6 g-atoms of deuterium.

1α,2,2,4,6,6,16,16-Octadeuterioandrost-4-ene-3,17-dione. 1α-Deuterioandrost-4-ene-3,17-dione (0.856 g-atom of D, 96% 1α, 4% 1β) (47 mg) was exchanged with sodium deuteroxide as described above, and the product was chromatographed on a 1 mm thick silica gel plate (benzene–ethyl acetate, 7:3). Crystallization from acetone–hexane gave 25 mg of product, m.p. 170–172.5°. In the infrared (KBr), the C-2 methylene peak at 1418 cm^{-1} and the C-16 methylene group at 1402 cm^{-1} (Jones and Cole, 1952) were absent. A band at 1038 cm^{-1} attributed (Jones *et al.*, 1955) to CD_2 scissoring at C-16 appeared, while the C-4 proton band at 870 cm^{-1} was markedly reduced in intensity and the C-6 methylene group at 1430 cm^{-1} appeared as only a shoulder.

1α,2,2,4,4,16,16-Heptadeuterio-5α-androstane-3,17-dione. The exchange of 123 mg of 1α-deuterio-5α-androstane-3,17-dione (0.856 g-atom of D, 96% 1α, 4% 1β) was effected as described for 17β-hydroxy-5α-androstan-3-one. The precipitate of crude product was chromatographed on two 1 mm thick silica gel plates with benzene–ethyl acetate (7:3) as developing solvent. Elution of the main zone with acetone gave 107 mg of product which, after crystallization from acetone–pentane, melted at 128–129°.

Anal.[1] Found: 6.78 g-atoms of deuterium.

In the infrared the completeness of deuterium exchange was demonstrated by the absence of the characteristic methylene bands at 1417 and 1426 cm^{-1} (CCl_4).

Procedure for Enrichment Experiments

The 6000*g* supernatant derived, as described above, from 400 ml of *B. sphaericus* culture medium was diluted to 50 ml with 0.03 M phosphate buffer, pH 7.0. The solution, which was held at 25° in a 250-ml erlenmeyer flask, was treated successively with 1 ml of ethanol containing 5.0 mg of menadione and 0.8 ml of a 3:1 ethanol–propylene glycol solution containing 10 mg of deuterated steroid which had been diluted with nondeuterated material to the desired and known deuterium content. The reaction mixture was agitated at 25° for the appropriate length of time (7–10 minutes) to effect approximately 50% reaction and the steroids then were isolated by extraction with ethyl acetate. The extent of formation of 1-dehydro steroid, in the case of the saturated steroid, was determined by gas chromatography of an aliquot of the ethyl acetate extract. In the case of the starting Δ^4-3-ketone, the extent of reaction was determined directly by chromatography of an aliquot on silica gel thin layer plates in a system of benzene–ethyl acetate (7:3). The Δ^4-3-one and the more polar $\Delta^{1,4}$-3-one zone were located by ultraviolet scanning and the requisite spots were cut out, eluted with acetone, and taken to dryness. The residue was dissolved in ethanol and the optical density was read at 240 and 244 mμ, respectively. From the known extinction coefficients of the Δ^4-3-one and the $\Delta^{1,4}$-3-one, the per cent of reaction was readily calculated. For the actual isolation of starting material from the Δ^4- and from the 5α incubations, the residue from ethyl acetate extraction was applied to 1 mm thick silica gel plates and chromatographed in the benzene–ethyl acetate (7:3) system. The unreacted starting material, after separation from the dehydrogenated product and recrystallization from acetone–hexane, was analyzed for deuterium content by mass spectroscopy.

Procedure for Kinetic Runs with Analysis by Steroid Isolation

A. 5α-Androstane-3,17-dione Deuterated and Nondeuterated. Phosphate buffer (70 ml, 0.03 M, pH 7.0), 0.5 ml of ethanol containing 3 mg of menadione, and 5.0 ml of enzyme solution A (see above) was placed in each of eight 250-ml erlenmeyer flasks which were then kept at 25° and gently agitated. The deuterated and nondeuterated steroid (2.0, 1.5, 1.0, or 0.5 mg) contained in 0.2 ml of ethanol–propylene glycol (3:1) was added at 30-second intervals to an erlenmeyer flask and each concentration was incubated for an identical total period of time (5 minutes). The reactions were quenched by the addition of 25 ml of ethyl acetate and the reaction product was isolated by extraction with the same solvent. The washed and dried extracts were evaporated *in vacuo* and the residues were chromatographed on 1-mm silica gel plates (benzene–ethyl acetate, 7:3). The zones corresponding to unreacted starting material and to the 1-dehydro substance were removed together in each case and eluted with acetone. The steroid mixture was analyzed on an F & M Model 720 dual-column gas chromatograph, using helium as the carrier gas and a 4-ft silicone XE-60 on Anachrom ABS 0.25-in. column. The column temperature was 225–230°, the helium flow 100–110 cc/min, port temperature 265–300°, and detector temperature 270–290°. Under these conditions there was a 2 minute difference in retention time of 5α-androstane-3,17-dione and 5α-androst-1-ene-3,17-dione which gave almost base-line separation. The per cent composition of reactant to product was calculated by the trace, cut, and weigh method. Analysis of a known mixture containing 19.47% 1-dehydro compound gave 20.06%.

B. Androst-4-ene-3,17-dione Deuterated and Nondeuterated. Dehydrogenation was carried out with agitation at 25° by addition of the steroid (4, 3, 2, and 1 mg) in 0.2 ml of ethanol–propylene glycol (3:1) to a

TABLE I: Enrichment of Deuterated Derivatives of Androst-4-ene-3,17-dione by Interrupted Dehydrogenation with Cell-Free *B. sphaericus* Preparations in the Presence of Menadione.[a]

	G-atom of Deuterium/ Mole of Starting Steroid[b]	% Compn of Starting Steroid[c]	% Reaction	G-atom of Deuterium/ Mole of Unreacted Steroid[b]	% Compn of Unreacted Steroid[d]	% of Each Species Reacted[e]	Isotope Effect[f]
Ia	0.80	(1αD-1βH) 68	50	0.90	(1αD-1βH) 84.0	38	$V_H/V_{1\alpha D} = 2.0$
		(1αH-1βD) 12			(1αH-1βD) 6.0	75	
		(1αH-1βH) 20			(1αH-1βH) 10.0	75	
b	0.40[g]	(1αD-1βH) 34	57	0.61	(1αD-1βH) 57.1	28	
		(1αH-1βD) 6			(1αH-1βD) 3.9	72	$V_H/V_{1\alpha D} = 2.6$
		(1αH-1βH) 60			(1αH-1βH) 39.0	72	
II	0.64	(2αH-2βD) 48	34	0.74	(2αH-2βD) 62.4	14	
		(2αD-2βH) 16			(2αD-2βH) 11.6	52	$V_H/V_{2\beta D} = 3.7$
		(2αH-2βH) 36			(2αH-2βH) 26.0	52	
III	0.65	(1αD-1βH) 14	80	0.67			
		(1αH-1βD) 51					$V_H/V_{1\beta D} \cong 1$
		(1αH-1βH) 35					

[a] The procedure for enrichment and determination of the per cent of reaction is detailed under Materials and Methods. [b] Determined by mass spectroscopic analysis. [c] Determined by dehydrogenation to completion followed by mass spectroscopic analysis of Δ^1 product (see Ringold *et al.*, 1963). [d] The per cent of (1αH-1βH) or (2αH-2βH), *i.e.*, nondeuterated species, is 100[1 − (g-atom of D/mole of unreacted steroid)]. It is assumed that 1α and 2β stereospecificity are absolute and that there is no isotope effect for deuterium at the 1β or 2α positions. Under these conditions the starting ratio of (1αH-1βD) to (1αH-1βH) remains unchanged in the unreacted steroid; *e.g.*, in Ia, $(1\alpha H\text{-}1\beta D)_{unreact} = 10.0(12/20) = 6.0\%$. The per cent of $(1\alpha D\text{-}1\beta H)_{unreact}$ follows by simple difference from 100%. The same considerations pertain for the C-2 deuterated substances. [e] Per cent of each species reacted = 100[(% species in starting material) − (fraction of total steroid unreacted)(% species in unreacted steroid)]/(% species in starting material); *e.g.*, in Ib, $\%(1\alpha D\text{-}1\beta H)_{reacted} = 100[34 - (0.43)(57.1)]/34 = 28\%$. The fraction of total steroid unreacted = [100 − (% reaction)]/100. [f] Calculated on the basis of zero-order kinetics; V_H/V_D = % H species reacted/% D species reacted. [g] Prepared by dilution of Ia with nondeuterated substrate.

mixture of enzyme solution A (5.0 ml) and phosphate buffer (5.0 ml, pH 7.0, 0.03 M) containing 0.4 mg of menadione (in 0.05 ml of ethanol). Following a 7-minute reaction period, 10 ml of ethyl acetate was added, and the product then was isolated by extraction with the same solvent. The per cent of reaction was found in each case by separation on thin layer plates followed by ultraviolet spectral determination as described in the enrichment experiments.

Procedure for Kinetic Runs with Spectrophotometric Determination

The kinetics were measured by following the disappearance in optical density of the indophenol maximum at 600 mμ by means of a Perkin-Elmer 202 or a Cary Model 11 ultraviolet recording spectrophotometer. Each determination was made at 25° by adding the appropriate volume of enzyme solution A (usually 0.50 ml) and 0.03 M phosphate buffer, pH 7.0 (2.00 ml), to both the reference and sample 3-ml cuvets. Then, 0.05 ml of ethanol solution containing 50 μg of 2,6-dichlorophenolindophenol with or without 50 μg of menadione was added to the sample cell which gave an optical density of about 1.2. The spontaneous decoloration of indophenol (0.1–0.2 OD units) leveled off asymptotically and was negligible after 1.5 minutes, at which point the steroid, in 0.05 ml of ethanol, was added. (It was found that ethanol alone caused no further decoloration.) The zero reading was taken as the first optical density reading obtained after the sample cell which contained the steroid was returned to the instrument, and readings were taken from zero to several minutes. Six concentrations of 5α-3-keto steroids varying from 20 to 70 μg/2.6 ml and of Δ^4-3-keto steroids from 50 to 300 μg/2.6 ml were used. Each run at a particular steroid concentration was followed immediately with a run of the deuterated substance at the same concentration. All runs were made within 3 hours after sonic disruption of the bacillus and velocities are reported for the early portion of the curve where the oxidation obeyed zero-order kinetics. Maximum velocities were determined by double reciprocal plots of concentration and of optical density and are reported as changes in optical density units per given time for the indicated

TABLE II: Enrichment of 1α-Deuterio-5α-androstane-3,17-dione by Interrupted Dehydrogenation with Cell-Free *B. sphaericus* Preparation in the Presence of Menadione.[a]

G-atom of Deuterium/ Mole of Starting Steroid	% Compn of Starting Steroid	% Reaction	G-atom of Deuterium/ Mole of Unreacted Steroid	% Compn of Unreacted Steroid	% of Each Species Reacted	Isotope Effect
0.50	(1αD-1βH) 48	44	0.61	(1αD-1βH) 59.4	31	
	(1αH-1βD) 2			(1αH-1βD) 1.6	57	$V_H/V_{1\alpha D} = 1.9$
	(1αH-1βH) 50			(1αH-1βH) 39.0	57	

[a] See footnotes in Table I.

TABLE III: Comparison of the Rates of Dehydrogenation of Deuterated and of Nondeuterated Androst-4-ene-3,17-dione by Cell-Free *B. sphaericus* Preparations in the Presence of Menadione with Analysis by Isolation of 1-Dehydro Steroid.[a]

	Steroid Concn (mg/10 ml)	Mg of Steroid Dehydrogenated		
		H	D	$[V_H/V_D]^b$
A. Nondeuterated	1.0	0.520	0.294	1.77
vs. 2β-D[c]	2.0	0.712	0.360	1.98
	3.0	0.696	0.365	1.91
	4.0	0.560	0.480	1.17
B. Nondeuterated	1.0	0.154	0.070	2.20
vs. 1α-D[d]	2.0	0.170	0.110	1.55
	3.0	0.216	0.097	2.21
	4.0	0.207	0.122	1.70

[a] The procedure for these experiments is given under Materials and Methods. [b] The velocities of the deuterated compounds are *not* corrected for nondeuterated or for 2α- or 1β-deuterated species. [c] The 2β-deuterio compound contained 0.64 g-atom of deuterium with an isomer distribution of 75% 2β, 25% 2α. [d] The 1α-deuterio compound contained 0.856 g-atom of deuterium (96% 1α, 4% 1β).

concentration of enzyme, menadione, and indophenol.

Results

Isotope Effect by Deuterium Enrichment. Table I demonstrates that the interrupted dehydrogenation of deuterated androst-4-ene-3,17-dione with cell-free preparations of *B. sphaericus* in the presence of menadione led to an enrichment of isotope in the recovered substrate when deuterium was present primarily at either the 1α or 2β position. Since only the 1α- and 2β-deuterium atom undergo loss during dehydrogenation, the kinetic isotope effects are readily calculated. Corrections for the small amount of 1β- or 2α-deuterio Δ^4-3-ketone were made by assuming that these isomers and the nondeuterated substrates reacted at the same rate, as is evident from compound III which was primarily 1β-deuterated and did not show significant enrichment even after 80% dehydrogenation. Isotope effects were calculated on the basis of zero-order kinetics but, since the reactions were carried beyond the very initial stages, a first-order component was also undoubtedly present and the true isotope effect is somewhat greater than that calculated.

The 1α-deuterium isotope effect in the case of the Δ^4-3-ketone was 2.0 and 2.6 in separate experiments while the 2β-deuterium effect was substantially greater, $V_H/V_D = 3.6$. In the case of the saturated 5α-androstane-3,17-dione (Table II), a 1α-deuterium isotope effect of 1.9 was found. The attempted enrichment of 2β-deuterio-5α-androstane-3,17-dione by interrupted dehydrogenation led, in two separate experiments, to recovered material containing less than the initial concentration of deuterium. This loss, which apparently occurred during the work-up and chromatography, precluded establishment of the deuterium isotope effect in this substrate.

Isotope Effects by 1-Dehydro Product Isolation. Table

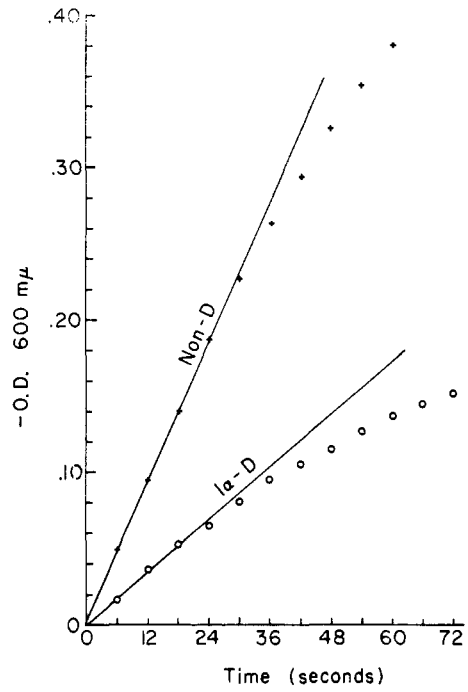

FIGURE 1: Initial rate of dehydrogenation of androst-4-ene-3,17-dione and its 1α-deuterio derivative as measured by the reduction of indophenol. The system contained 150 μg of steroid, 50 μg of 2,6-dichlorophenolindophenol, and 50 μg of menadione in a total volume of 2.6 ml.

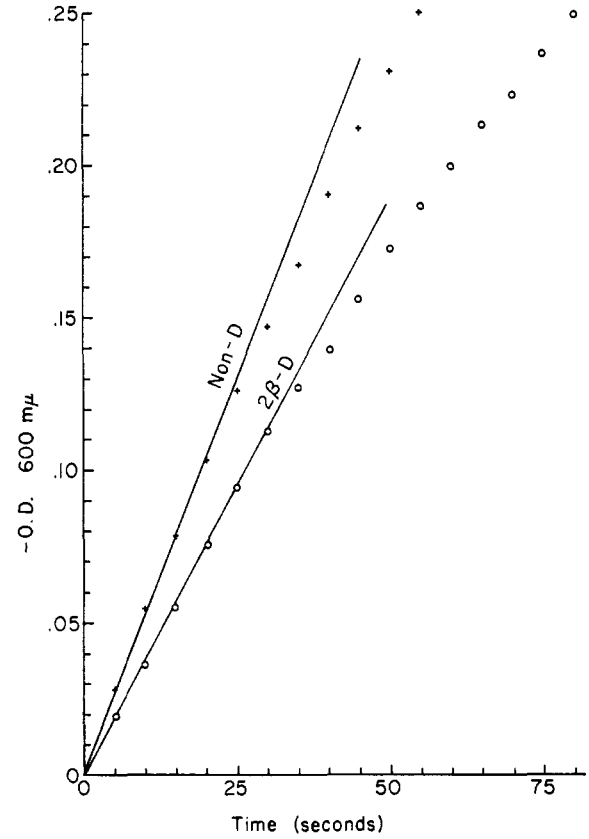

FIGURE 2: Initial rate of dehydrogenation of 5α-androstane-3,17-dione and its 2β-deuterio derivative as measured by the reduction of indophenol. The steroid concentration was 70 μg/2.6 ml in each case.

III illustrates the influence of deuterium substitution on the rate of introduction of the 1,2 double bond into androst-4-ene-3,17-dione as measured by isolation of the dehydrogenated product. Four concentrations of each substrate were incubated, but it will be noted that dehydrogenation was a function of concentration with both substrates at only the 1- and 2-mg/10 ml levels with plateauing occurring at higher levels. It should further be noted that the 2β-deuterio compound contained only 0.64 g-atom of deuterium of which 75% was at the 2β position. The substantial 2β-deuterium isotope effect of 1.77–1.98 is therefore a minimal figure. The 1α-deuterio substance was more completely deuterated (0.856 g-atom, 96% 1α), and the isotope effect of 1.55–2.20 is close to the maximal value. In Table IV the effects of 2β- and of 1α-deuterium substitution on the rate of dehydrogenation of 5α-androstane-3,17-dione are summarized. In contrast to the substrates of Table III, both deuterio derivatives were almost completely deuterated at the requisite position. The V_H/V_D values were close to unity for the 2β-derivative (1.06–1.17) and indicated a minimal isotope effect, while the 1α-deuterio isotope effect was somewhat greater (*ca.* 1.5) but still much less than that for the corresponding Δ^4-steroid.

Isotope Effects by Spectrophotometric Dehydrogenation in Presence of Menadione plus Indophenol. As detailed in Materials and Methods, dehydrogenation carried out in the presence of 50 μg each of menadione and 2,6-dichlorophenolindophenol could be readily followed by the disappearance of the indophenol maximum at 600 mμ.

Figure 1 illustrates the time course of the dehydrogenation of androst-4-ene-3,17-dione and the corresponding 1α-deuterio derivative at a concentration of 150 μg/2.6 ml at pH 7.0. Zero-order kinetics were obeyed for only about 30 seconds and in all runs velocities were measured by extrapolation of this initial portion of the curve. Figure 2 presents the time course of the dehydrogenation of the saturated 5α-androstane-3,17-dione and its 2β-deuterio derivative. Again it may be noted that zero-order kinetics pertained for only the first 25–30 seconds.

Figures 3–6 are representative Lineweaver–Burk double-reciprocal plots of the dehydrogenation of androst-4-ene-3,17-dione, of 5α-androstane-3,17-dione, and of the 2β-deuterio, the 1α-deuterio, and the 1α,2β-dideuterio derivatives. Concentrations are given as μg of steroid/2.6 ml, while the velocity of dehydrogenation is presented in terms of the change in optical density units/2 minutes as based on the initial velocity. At least four individual runs were made for each deuterated

TABLE IV: Comparison of the Rates of Dehydrogenation of Deuterated and of Nondeuterated 5α-Androstane-3,17-dione by Cell-Free *B. sphaericus* Preparations in the Presence of Menadione with Analysis by Isolation of 1-Dehydro Steroid.

	Steroid Concn (mg/10 ml)	Mg of Steroid Dehydrogenated		
		H	D	$[V_H/V_D]^a$
A. Nondeuterated *vs.* 2β-D[b]	0.5	0.240	0.227	1.06
	1.0	0.370	0.322	1.15
	1.5	0.464	0.411	1.13
	2.0	0.550	0.468	1.17
B. Nondeuterated *vs.* 1α-D[c]	0.5	0.135	0.090	1.50
	1.0	0.213	0.146	1.46
	1.5	0.242	0.158	1.53
	2.0	0.284	0.185	1.54
	0.5	0.153	0.131	1.17
	1.0	0.236	0.175	1.35
	1.5	0.312	0.212	1.47
	2.0	0.362	0.186	1.95

[a] Velocities are not corrected for nondeuterated or for 2α- or 1β-deuterated species. [b] The 2β-deuterio compound contained 0.96 g-atom of deuterium (84% 2β, 16% 2α). [c] The 1α-deuterio compound contained 0.856 g-atom of deuterium (96% 1α, 4% 1β).

TABLE V: Kinetics of the Dehydrogenation of Deuterated and Nondeuterated 3-Keto Steroids by Cell-Free *B. sphaericus* Preparations. Spectrophotometric Assay Procedure.[a]

Compd	K_m (M)	$V_{max}(H)/V_{max}(D)$
Androst-4-ene-3,17-dione		
Non-D	1.5×10^{-4}	2.48
1α-D	1.4×10^{-4}	
Non-D	1.4×10^{-4}	2.38
2,2,4,6,6-Penta-D	1.0×10^{-4}	
Non-D	1.7×10^{-4}	4.47
1α,2,2,4,6,6,16,16-Octa-D	9.7×10^{-5}	
5α-Androstane-3,17-dione		
Non-D	6.1×10^{-5}	1.88
1α-D	3.9×10^{-5}	
Non-D	5.2×10^{-5}	1.21
2β-D	4.5×10^{-5}	
Non-D	5.5×10^{-5}	2.12
1α,2,2,4,4,16,16-Hepta-D	4.0×10^{-5}	
17β-Hydroxy-5α-androstan-3-one		
Non-D	3.5×10^{-5}	1.31
2,2,4,4-Tetra-D	2.9×10^{-5}	

[a] The kinetic method is described under Materials and Methods. All runs were made with 50 μg each of menadione and 2,6-dichlorophenolindophenol.

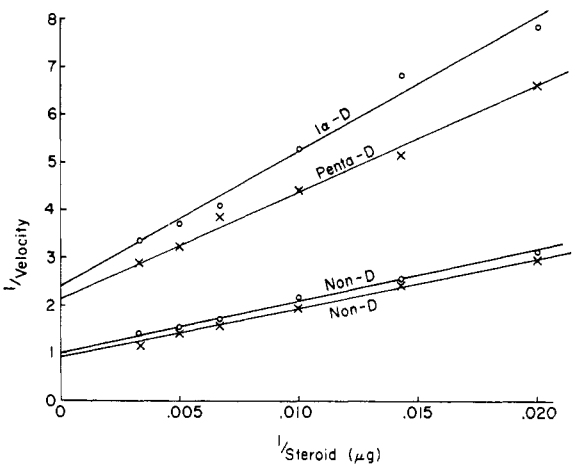

FIGURE 3: Double-reciprocal plots of 1/velocity *vs.* 1/steroid for androst-4-ene-3,17-dione and its 1α-deuterio and 2,2,4,6,6-pentadeuterio derivatives. The concentration of menadione and of indophenol was 50 μg/2.6 ml. Velocity is expressed as optical density units and steroid concentration as μg/2.6 ml. Points for 1α-D and its non-D standard are shown as open circles while points for penta-D and its standard are indicated by (×).

substrate *vs.* the nondeuterated compound. Maximal velocities were determined by extrapolating the Lineweaver–Burk plots to the origin and K_m was calculated in each case in the conventional manner. Each K_m value pertains for the stated concentration of indophenol and menadione although it has been shown (see below) that these values are essentially unchanged at infinite acceptor concentrations.

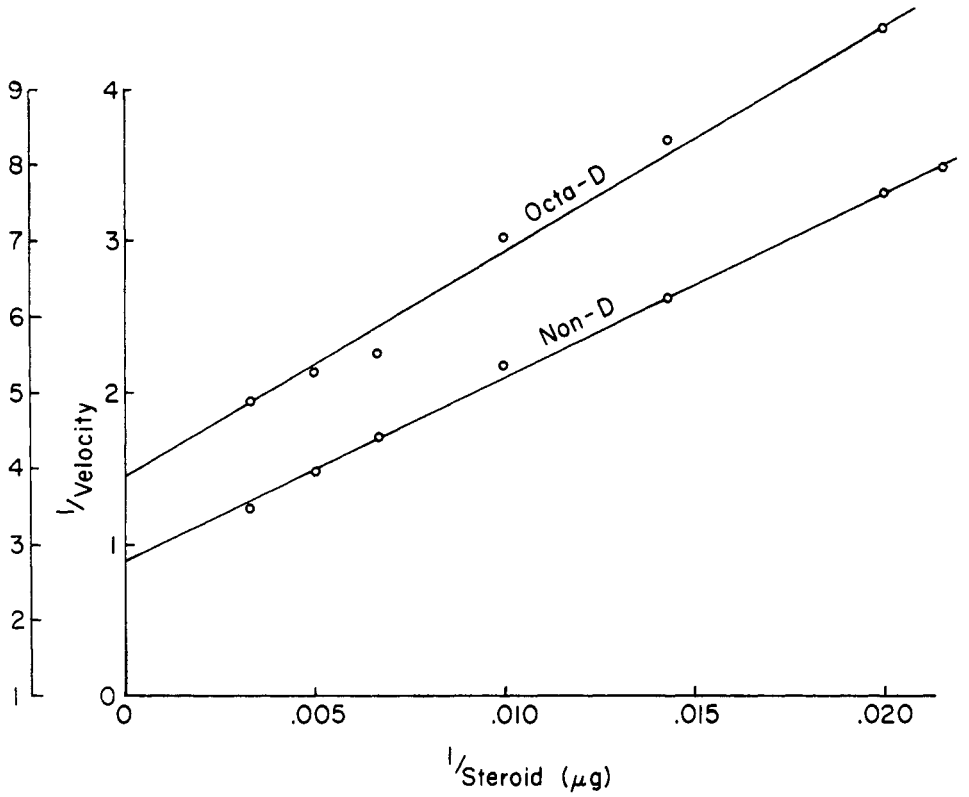

FIGURE 4: Double-reciprocal plots of 1/velocity *vs.* 1/steroid for androst-4-ene-3,17-dione and its 1α,2,2,4,6,6,-16,16-octadeuterio derivative. The outer 1/velocity scale is for the deuterio compound and the inner scale for non-D. Menadione and indophenol concentrations of 50 μg/2.6 ml.

Table V lists the average K_m for each substrate and $V_{max}(H)/V_{max}(D)$ for the 1α-, 2β-, and 1α,2β-deuterio derivatives. In the case of the Δ^4 compound, V_H/V_D for the 1α-deuterium was 2.48, and 2.38 for the 2β-deuterio. The 1α,2β-deuterio compound exhibited V_H/V_D = 4.47. With 5α-androstane-3,17-dione, the 1α isotope effect was 1.88, the 2β effect 1.21, and the combined 1α,2β effect 2.12. Another 5α-saturated 3-ketone, 17β-hydroxy-5α-androstan-3-one, exhibited a 2β-isotope effect of 1.31. In each case, K_m of the deuterated compound was smaller than that of the nondeuterated substance and K_m of the saturated 5α derivatives was approximately one-third of the Δ^4 derivatives.

Table VI demonstrates that the isotope effect of the 2β-deuterated compounds remained essentially constant in the pH range from 6.3 to 7.7. Although the effect was slightly higher at pH 6.3 and 7.7 than at pH 7.0, the difference did not appear to be significant.

Kinetics in the Presence of Varying Concentrations of Indophenol as Added Electron Acceptor in the Absence of Menadione. Figure 7 illustrates the effect of varying the concentration of 2,6-dichlorophenolindophenol on the rate of dehydrogenation of fixed concentrations of androst-4-ene-3,17-dione as measured spectrophotometrically by the disappearance of indophenol. Typical Lineweaver–Burk double-reciprocal concentration curves were obtained but the slopes were essentially similar at each steroid concentration. In Figure 8, secondary plots of the intercepts and of the slopes *vs.* the reciprocal of steroid concentration are shown. Both the slopes and intercepts were calculated by the method of least squares. Since the slopes are essentially parallel, the reaction may be classed as one involving enzyme oxidation–reduction (ϕ_{12} = 0) by the terminology of Dalziel (1957) and, since $1/V_{max}$ extrapolates to a finite value, a limiting velocity is reached and enzyme substrate complexes must be formed. From the secondary plots, K_m for indophenol at infinite steroid concentra-

TABLE VI: Effect of pH on Deuterium Isotope Effects.

Substrate	pH	$V_{max}(H)/V_{max}(D)$
5α-Androstane-3,17-dione	6.3	1.36
vs. 2β-D	7.0	1.21
	7.7	1.30
Androst-4-ene-3,17-dione	6.3	2.65
vs. 2,2,4,6,6-penta-D	7.0	2.38
	7.7	2.68

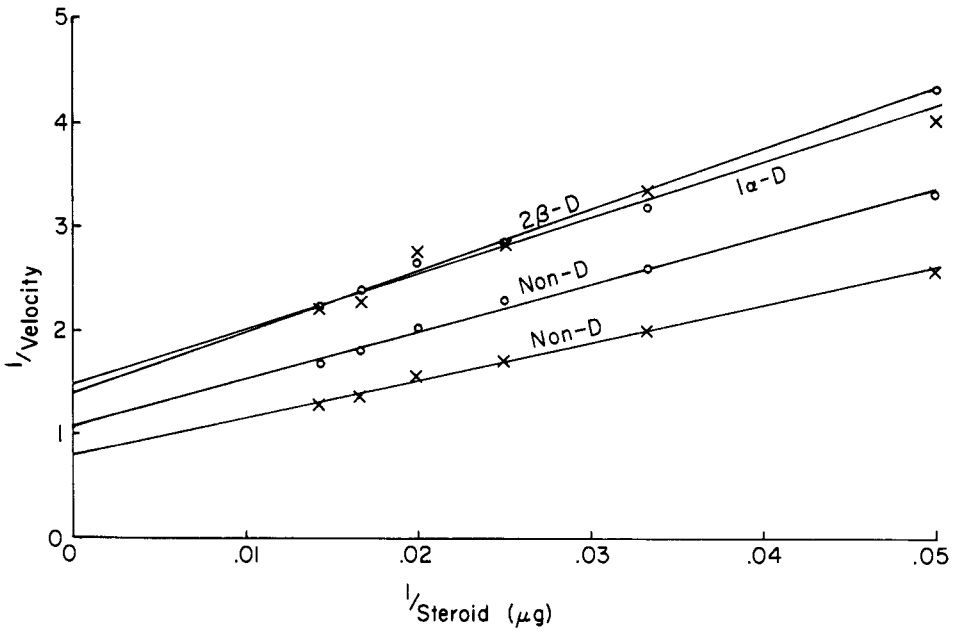

FIGURE 5: Double-reciprocal plots of 1/velocity *vs.* 1/steroid for 5α-androstane-3,17-dione, and its 1α- and 2β-deuterio derivatives. Menadione and indophenol concentrations are 50 μg/2.6 ml. Points for 2β-D and its non-D standard are shown as open circles; points for 1α-D and its standard are indicated by (×).

tion is found to be 7.4×10^{-5} M, while K_m for androst-4-ene-3,17-dione is 1.23×10^{-4} M at infinite indophenol concentration.

Kinetics in the Presence of a Fixed Concentration of Indophenol and Varying Concentrations of Menadione as Electron Acceptors. Figure 9 compares the rate of dehydrogenation of 300 μg of androst-4-ene-3,17-dione in the presence of 50 μg of indophenol alone and in the presence of 50 μg of indophenol plus 50 μg of menadione. Both the initial rate and extent of reaction are seen to be essentially doubled by the inclusion of menadione. Table VII indicates the effect of menadione concentration on the rate of dehydrogenation and shows that a marked increase in rate results from the addition of only 2.5 μg of menadione and maximal velocity is reached at 50 μg, but higher concentrations lead to a slight inhibition.

TABLE VII: The Effect of Menadione Concentration on the Dehydrogenation of Androst-4-ene-3,17-dione as Measured by the Decrease in Absorbancy of 2,6-Dichlorophenolindophenol at 600 mμ.[a]

Menadione (μg/2.6 ml)	ΔOD/ minute
0	0.148
2.5	0.227
50	0.293
125	0.263
250	0.239

[a] All runs with 300 μg of androst-4-ene-3,17-dione and 50 μg of the indophenol contained in a final volume of 2.6 ml. The procedure is detailed under Materials and Methods.

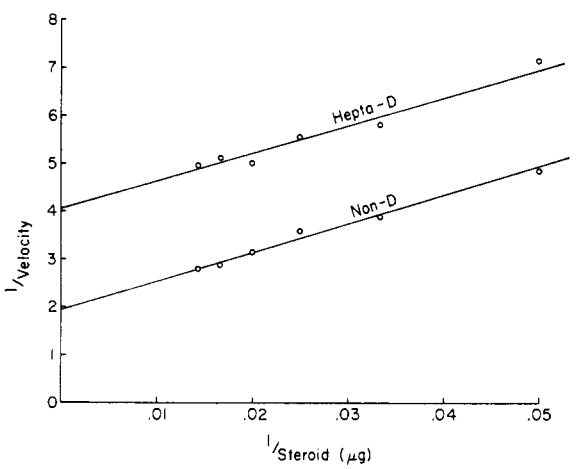

FIGURE 6: Double-reciprocal plots of 1/velocity *vs.* 1/steroid for 5α-androstane-3,17-dione and its 1α,2,2,4,-4,16,16 heptadeuterio derivative. Menadione and indophenol concentrations of 50 μg/2.6 ml.

Figure 10 shows that in the presence of 50 μg of indophenol, concentrations of menadione of 1.5–10 μg gave satisfactory Michaelis–Menten kinetics and double-reciprocal Lineweaver–Burk plots. As with indophenol alone, the slopes were seen to be essentially

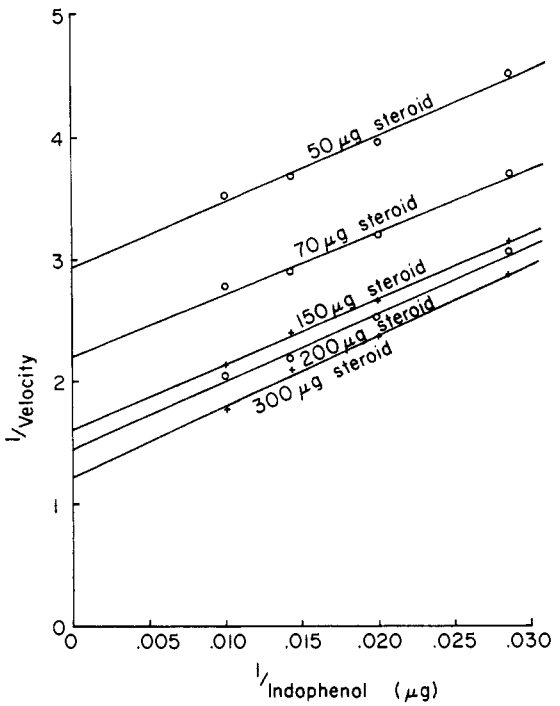

FIGURE 7: Double-reciprocal plots of 1/velocity *vs.* 1/2,6-dichlorophenolindophenol at several fixed concentrations of androst-4-ene-3,17-dione in the absence of menadione. Steroid and indophenol concentrations are expressed as μg/2.6 ml.

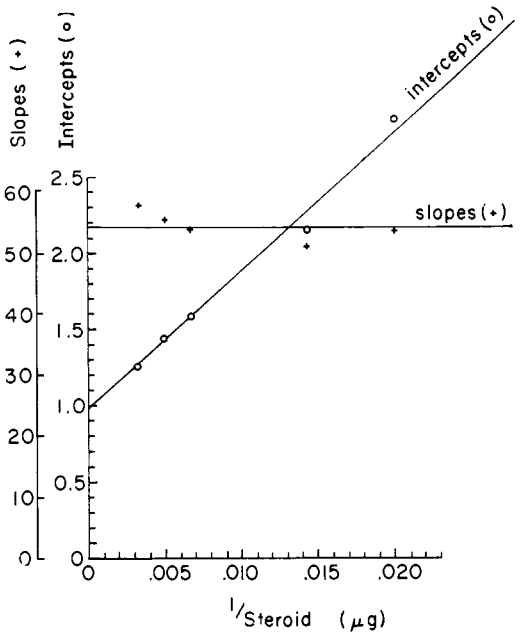

FIGURE 8: Replots of slopes and intercepts from Figure 7 *vs.* reciprocal of androst-4-ene-3,17-dione concentration. The slope line represents the average value.

parallel (Figure 11) and $1/V_{max}$ was >0. The secondary plots of $1/V_{max}$ and slopes from Figure 10, as shown in Figure 11, allow calculation of K_m for the steroid (1.4×10^{-4} M) and K_m for menadione (1.7×10^{-6} M).

Simultaneous Dehydrogenation of 5α-Androstane-3,17-dione and Androst-4-ene-3,17-dione. Table VIII illustrates that the simultaneous dehydrogenation of the two substrates at concentrations considerably higher than their K_m values was not cumulative. This is in accord with a single enzyme effecting the dehydrogenation of both substrates.

Effect of pH on the Rate of Dehydrogenation of Androst-4-ene-3,17-dione. The variation of dehydrogenation rate of two concentrations of androst-4-ene-3,17-dione (50 and 300 μg/2.6 ml) was shown to reach a maximum at about pH 7 (Table IX). Proceeding from pH 5.7 to 7 more than doubled the initial velocity of dehydrogenation at the higher substrate concentration.

Relative Rates of Dehydrogenation of Nondeuterated Substrates and Inhibition Experiments. Utilizing the identical cell-free enzyme preparation it was shown that

TABLE VIII: The Effect of Combined Substrates on the Rate of Reduction of 2,6-Dichlorophenolindophenol by Cell-Free Preparations of *B. sphaericus*.[a]

Substrate (μg)	ΔOD/minute
Androst-4-ene-3,17-dione (A) (300)	0.350
5α-Androstane-3,17-dione (B) (70)	0.245
A (300) + B (70)	0.320

[a] Kinetics by the usual spectrophotometric assay with menadione (50 μg), indophenol (50 μg), and the steroid contained in a total volume of 2.6 ml.

TABLE IX: Effect of pH on the Rate of Dehydrogenation of Androst-4-ene-3,17-dione by Sonic Preparations of *B. sphaericus*.

pH	Concn of Steroid[a]	μg/2.6 ml of Indophenol Reduced/Minute	Concn of Steroid[a]	μg/2.6 ml of Indophenol Reduced/Minute
5.7	50	2.50	300	3.15
6.4	50	2.75	300	4.60
7.0	50	2.85	300	6.95
7.4	50	2.50	300	5.70
7.7	50	2.15	300	4.85

[a] Dehydrogenation carried out with 50 μg each of menadione and indophenol.

in the presence of 50 μg each of menadione and indophenol the rates of dehydrogenation of 5α-androstane-3,17-dione and of androst-4-ene-3,17-dione were identical at V_{max}. It was further found that there was no product inhibition since the addition of 50 μg of androst-1,4-diene-3,17-dione had no influence on the rate of dehydrogenation of 30 μg of androst-4-ene-3,17-dione.

Discussion

Stereochemistry. Previous studies established an absolute preference for 1α-hydrogen loss and a preferential, but not necessarily mandatory, 2β-proton loss. Unfortunately, the present study has not resolved this point since 2-deuterio-5α-androstane-3,17-dione, which should have been the pure 2β isomer by its method of chemical synthesis from the 2α,3α-oxide, underwent only an 86% loss of deuterium on dehydrogenation. This may be the result of only partial specificity in the enzyme process, but we favor the possibility that the deuterated substance was not the pure 2β-isomer. Partial inversion of the 2β-deuterium atom could have occurred during the final chromic acid oxidation step in the chemical synthesis since such loss of steric homogeneity has been noted in related chromic acid oxidations. The possibility also exists that the 2α,3α-oxide, which was in turn derived from the $\Delta^{2(3)}$-olefin, contained some 3α,4α-oxide as a result of contaminating $\Delta^{3(4)}$-olefin in the starting material. The 3,4 isomers may not have separated from the 2,3 compounds on thin layer chromatography and would have led to 4β-deuterio-5α-androstane-3,17-dione which would not lose deuterium on dehydrogenation. In the calculation of isotope effects complete stereospecificity for the enzymic process has been assumed, however.

Kinetic Mechanism. As noted in Results, the parallel slopes of Lineweaver–Burk plots in Figures 7 and 10 and the secondary plots of Figures 8 and 11 showing that $\phi_{12} \cong 0$ and $1/V_{max} > 0$ are consistent with the general mechanism given by Alberty (1953) for a two-substrate system in which the enzyme exists in an oxidized (ox) and reduced (red) state and the substrates combine with a particular form of the enzyme (equations 1 and 2). This kinetic behavior, which appears

$$E_{ox} + \text{steroid} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}\cdot\text{steroid} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_{red} + \text{steroid}_{ox} \quad (1)$$

$$E_{red} + \text{acceptor} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_{red}\cdot\text{acceptor} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E_{ox} + \text{acceptor}_{red} \quad (2)$$

to be characteristic of flavoprotein-mediated dehydrogenation, and the requirement for a quinone in the electron transport chain (menadione, Q_9 or the natural vitamin $K_{2(35)}$) (Stefanovic *et al.*, 1963), strongly indicate classification of the steroid Δ^1-dehydrogenase as a flavoprotein, which has been previously suggested by

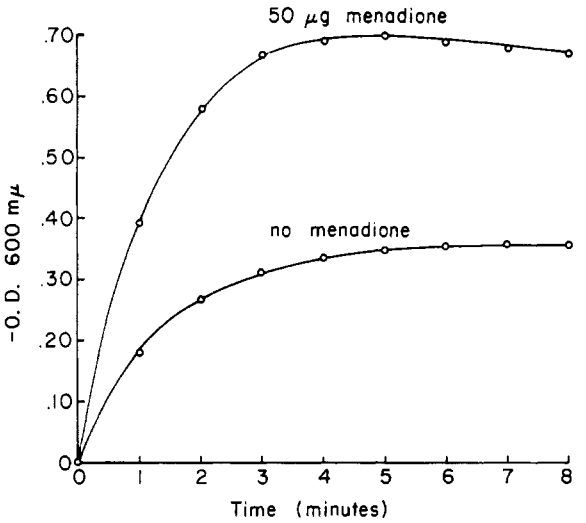

FIGURE 9: Time course of the dehydrogenation of androst-4-ene-3,17-dione in the presence and absence of menadione as measured by the decrease in absorbancy of indophenol at 600 mμ. The steroid and indophenol concentrations were 300 μg/2.6 ml and 50 μg/2.6 ml, respectively.

Levy and Talalay (1959) for other Δ-dehydrogenases.

From steady-state kinetics the following expressions for V_{max} and K_m may be readily derived (Massey and Veeger, 1963). The K_m for menadione (1.7×10^{-6} M)

$$V_{max} = \frac{k_3 k_7 E_0}{k_3 + k_7} \qquad K_m(\text{steroid}) = \frac{k_7(k_2 + k_3)}{k_1(k_3 + k_7)}$$

$$K_m(\text{electron acceptor}) = \frac{k_3(k_6 + k_7)}{k_5(k_3 + k_7)}$$

and for indophenol (7.4×10^{-5} M) clearly indicates that at equivalent concentration of electron acceptors (50 μg of each), as utilized in the spectrophotometric determination, menadione is involved in enzyme reoxidation while the role of indophenol is simply reoxidation of reduced menadione. It should further be noted that, in the case of the Δ^4-3-ketone, K_m for the steroid was found to be essentially the same when indophenol plus menadione were acceptors. Fortuitously, V_{max} for the Δ^4-3-ketone and 5α-saturated 3-ketone are essentially identical. From inspection of the equation for V_{max} this can occur in one of two ways. Bearing in mind that k_7 is the same for both substrates (since the steroid is not involved in the enzyme reoxidation step), if k_3 is very fast compared to k_7 then V_{max} reduces to $k_3 k_7 E_0/k_3 = k_7 E_0$. However, if this were the case and k_7 could be considered as the true rate-determining step in the over-all oxidation, then the deuterium isotope effects in the oxidation of the two different steroid substrates should be identical, which is not the case. Further, the fact that K_m for the Δ^4-3-keto steroid did not significantly differ when indophenol was the sole electron acceptor or when indophenol *plus* menadione served

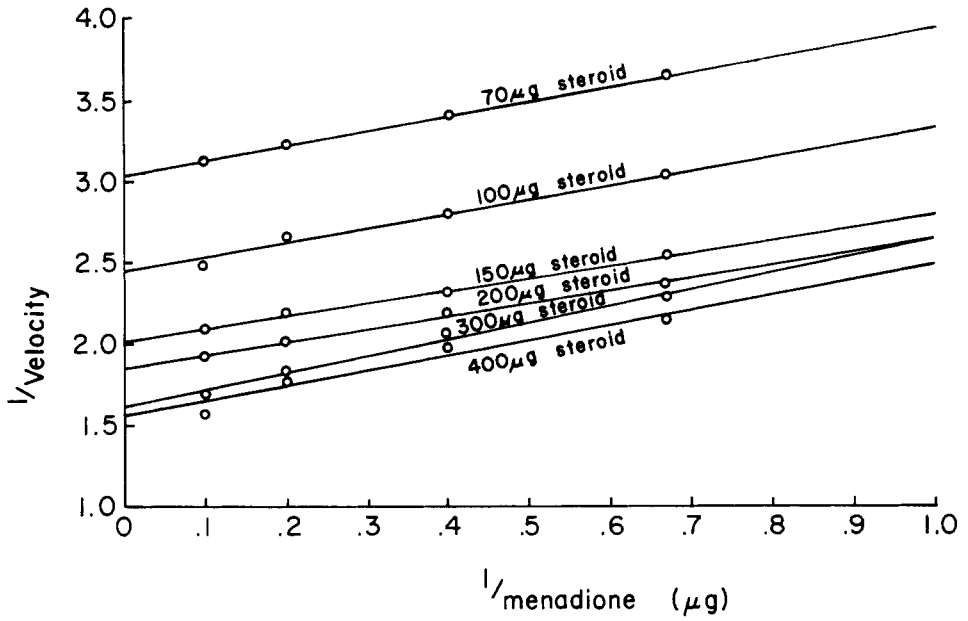

FIGURE 10: Double-reciprocal plots of 1/velocity *vs.* 1/menadione at several fixed concentrations of androst-4-ene-3,17-dione. The concentration of indophenol was fixed at 50 μg/2.6 ml. Steroid and menadione concentrations are expressed as μg/2.6 ml.

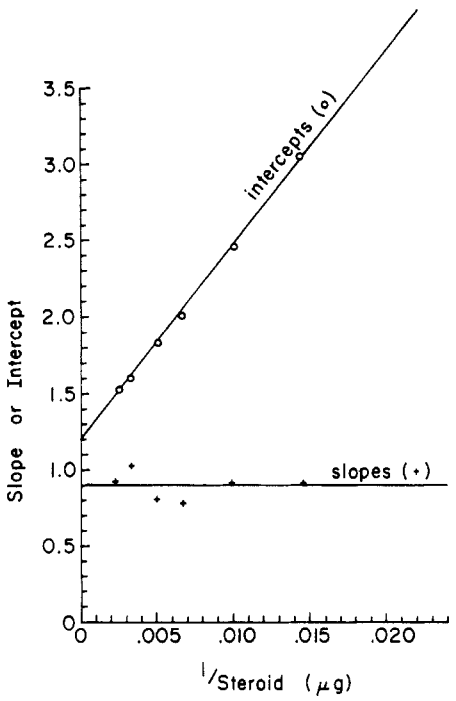

FIGURE 11: Replots of slopes and intercepts from Figure 10 *vs.* reciprocal of androst-4-ene-3,17-dione concentration. The slope line represents the average value.

as acceptor indicates that k_7 must be fast compared to k_3. V_{max} for the two substrates will also be identical if $k_3(5\alpha) \cong k_3(\Delta^4)$. This appears to be the only reasonable explanation for the data although unfortunately it does not allow a decision as to the magnitude of k_3 relative to k_7, except that k_3 cannot be significantly larger than k_7. It could, in fact, be of the same order of magnitude as k_7 or much smaller. In view of the marked 2β-deuterium isotope effect with the Δ^4-3-ketone and the virtual absence of this effect in the 5α-saturated 3-ketone, it appears reasonable to assume that k_3 is considerably smaller than k_7 and therefore $V_{max} \cong k_3E_0$. With this simplifying assumption, the expressions for K_m of the steroid may be reduced to $K_m = k_2 + k_3/k_1$ and, since $K_m(\Delta^4) \cong 1.5 \times 10^{-4}$ and $K_m(5\alpha) \cong 5.5 \times 10^{-5}$ and k_3 is equal for both substrates, then the true enzyme–steroid dissociation constant (k_2/k_1) for the 5α-steroid is smaller than that for the Δ^4-3-steroid. In the absence of information on the relative rates of k_2 and k_3, this expression for K_m cannot be further simplified.

Isotope Effects. In a flavoprotein-mediated dehydrogenation, where the enzyme itself undergoes oxidation–reduction, the introduction of deuterium into the steroid substrate may lead to an isotope effect at either the substrate oxidation step (k_3) or at the enzyme reoxidation step (k_7). This is true because the deuterium originally present in the substrate undergoes transfer to the enzyme. However, from the arguments presented in the previous section, V_{max} of the over-all reaction very likely depends only upon k_3 and therefore the kinetic isotope effects depend only upon the steroid dehydrogenation step.

In conformity with the mechanism previously proposed, the dehydrogenation step k_3 may be formulated as a reversible enolization followed by a hydride-abstraction step (equation 3). The over-all kinetic isotope effect ($k_3(H)/k_3(D)$) will be dependent upon the

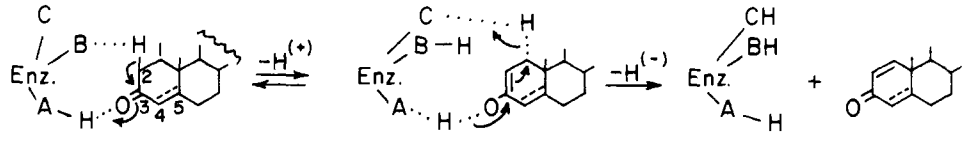

FIGURE 12: Proposed mechanism of enzymatic dehydrogenation in the presence of menadione and 2,6-dichloroindophenol.

$$\text{enzyme·steroid} \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} \text{enzyme } (H^+)\text{·enol} \xrightarrow{k_{11}} \text{enzyme } (H_2) + \Delta^1\text{-steroid} \quad (3)$$

$$k_3 = \frac{k_9 k_{11}}{k_9 + k_{10} + k_{11}}$$

rate of enolization (k_9), the rate of enol reprotonation (k_{10}), the rate of hydride abstraction (k_{11}), and the isotope effect in the individual steps. Deuterium at the 2β position will affect k_9 and k_{10} while deuterium at the 1α position will affect only k_{11}. Although 2β-deuterium will slow the rate of enolization, it will also slow the rate of reprotonation of the enol providing that the deuterium atom is not lost from the enzyme by exchange with the medium. There would not appear to be a substantial basis for estimating the relative magnitude of this isotope effect in the two directions, but it need not be unity and it need not be the same for both substrates.

From Tables I, III, and V it may be seen that the introduction of deuterium at either the 2β or 1α position of the Δ^4-3-ketone led to a substantial deuterium isotope effect (the greatest reliance is placed on the figures in Table V (k_H/k_D = 2.4–2.5) since only the spectrophotometric assay measured the initial velocities). Also, introduction of deuterium at both positions gave an isotope effect (4.5 times) considerably higher than the individual effects, but not equal to their product (5.9 times). In the case of the 5α-3-ketone, the 2β-deuterium isotope effect was minimal (1.2–1.3 times) while the 1α effect was again substantial (1.9 times), although less than in the Δ^4-3-ketone. The appearance of a substantial isotope effect from 1α- or 2β-deuterium in the Δ^4-3-ketone presents, on the surface, a dichotomy of two "rate-determining" steps in a single reaction. Since two such steps are an impossibility, the observed $V_{max}(H)/V_{max}(2\beta D)$ and $V_{max}(H)/V_{max}(1\alpha D)$ for the Δ^4-3-ketone cannot be true isotope effects for single rate constants such as the enolization step (k_9) or the hydride-transfer step (k_{11}), but are, instead, composite results of a complex rate situation. As such, the true $k_9(H)/k_9(D)$ and $k_{11}(H)/k_{11}(D)$ of the Δ^4-3-ketone must be considerably greater than 2.5 and it is also probable that in the case of the 5α-3-ketone the 1α-deuterium effect of 1.9 is less than the true $k_{11}(H)/k_{11}(D)$. Unfortunately there are no examples in the literature of enzymic oxidation reactions which have allowed determination of k_H/k_D for the isolated hydride-transfer step. From strictly chemical reactions, however, hydride-transfer isotope effects varying from three (Swain *et al.*, 1961) to five (Burstein and Ringold, 1964) have been reported. In view of this broad range, no estimate can be made of the possible maximum isotope effect in the enzymatic hydride-transfer step. Further, there is no assurance that the magnitude of isotope effects in the Δ^4-3-ketone and in the 5α series will be parallel. With respect to the magnitude of the isotope effect in the enolization step (k_9), an enzymic enolization with k_H/k_D of 5.35 in the proton-loss step has been observed with Δ^5-3-ketoisomerase (Malhotra and Ringold, 1965). This would indicate that the 2β-deuterium isotope effect is not a pure measure of $k_9(H)/k_9(D)$.

Although certain simplifying assumptions can be made with respect to the individual rate constants and the possible magnitude of the isotope effect in each step of the reaction, no completely consistent picture emerges for both substrates. For example, in the case of the 5α-steroid, if $k_{10} \gg (k_9 + k_{11})$, $k_3 = k_9 k_{11}/k_{10}$. Under these conditions, a 1α-deuterium isotope effect can be anticipated in the hydride-transfer step k_{11} while the isotope effect in k_9 from 2β-deuterium would be balanced by the same effect in k_{10}. With respect to the Δ^4-3-ketone, if k_9 is assumed to be small and k_{10} and k_{11} are of the same magnitude, $k_3 = k_9 k_{11}/k_{10} + k_{11}$. Then deuterium at either the 1α or 2β position would lead to an isotope effect. Considering the great number of variables that are involved, it appears pointless to speculate further on the significance of these effects or even to attempt the selection of a single reaction step as a slow one. These studies do serve to emphasize the complexity which is inherent in the interpretation of isotope effects in an enzymatic transformation.

Expansion of the previously proposed reaction mechanism, to accommodate the intermediate oxidation–reduction of the enzyme, leads to the over-all dehydrogenation sequence shown in Figure 12 with menadione acting as a secondary electron acceptor. The primary enzyme-bound acceptor is very likely a flavin;

however, the crude state of the enzyme has precluded serious characterization efforts.

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The Formation of Estradiol-3-glucuronoside-17α-*N*-acetylglucosaminide by Rabbit Liver Homogenate*

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ABSTRACT: When 17β-estradiol-6,7-^{3}H was incubated with rabbit liver homogenates at pH 7.6 with uridine diphosphate *N*-acetylglucosamine in the presence of uridine diphosphate glucosiduronic acid, estradiol-3-glucuronoside-17α-*N*-acetylglucosaminide was formed in amounts equivalent to about 10% of the total conjugated radioactivity. The double glycoside was identified by countercurrent distribution and then treated with β-glucuronidase. The estradiol-17α-*N*-acetylglucosaminide thus formed was identified with an authentic sample of this material by countercurrent, chromatographic, and isotope techniques. When estradiol-6,7-^{3}H and uridine diphosphate *N*-acetylglucosamine were incubated with liver homogenate in the absence of uridine diphosphate glucosiduronic acid, no transfer of *N*-acetylglucosamine to estradiol took place unless a prior incubation of the steroid and homogenate with uridine diphosphate glucosiduronic acid has been carried out. The results establish the presence in rabbit liver of a mechanism for the transfer of *N*-acetylglucosamine to the 17α-hydroxyl of estradiol and strongly indicate that the receptor is not the free steroid but the 3-glucuronoside. No evidence was found for the transfer of *N*-acetylglucosamine to the 17β-hydroxyl of estradiol.

The transfer of glucosiduronic acid from uridine diphosphate glucosiduronic acid (UDP-glucosiduronic acid[1]) to phenolic steroids by liver microsomal systems was indicated by the preliminary results of Isselbacher (1956). Smith and Breuer (1963) reported that estrone-3-glucuronoside is formed by the incubation of estrone and UDP-glucosiduronic acid with washed rabbit liver microsomes. Recent work (Layne *et al.*, 1964; Layne, 1965) has shown that much of the 17α-estradiol excreted in rabbit urine is in the form of the 3-glucuronoside-17α-*N*-acetylglucosaminide. This indicates that the rabbit possesses an enzyme system capable of transferring *N*-acetylglucosamine to the estrogen molecule. The failure to find evidence of the excretion of estrogen conjugates with glucosiduronic acid at position 17 or with *N*-acetylglucosamine at position 3 suggested that the two conjugating sites on 17α-estradiol might exhibit a high degree of specificity for the sugars. It also

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[1] Abbreviation used in this work: UDP, uridine diphosphate.