Mechanisms of Steroid Oxidation by Microorganisms III

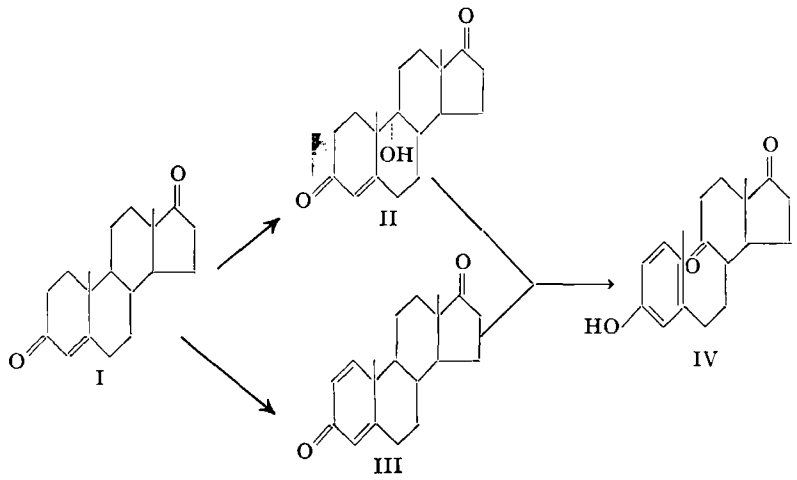
Enzymatic Mechanism of Ring A Aromatization

By CHARLES J. SIH and A. M. RAHIM

Evidence is presented to show that the aromatization of 9α-hydroxyandrost-4-en-3,17-dione and 19-hydroxyandrost-4-en-3,17-dione involves a 1,2-dehydrogenation by the steroid 1-dehydrogenase to afford vinylogs of β-hydroxyketones which may undergo spontaneous nonenzymic rearrangement (reverse aldolization) to give their respective phenols. This mode of aromatization differs from the human placental system in that it requires a typical flavoprotein electron acceptor for activity; oxygen and $NADPH_2$ are not required for aromatization. Also, the relative rates of oxidation follows the order 19-norandrost-4-en-3,17-dione > 19-oxoandrost-4-en-3,17-dione > 19-hydroxyandrost-4-en-3,17-dione.

ONE PATHWAY of steroid degradation by microorganisms involves a 9α-hydroxylation, followed by a 1,2-dehydrogenation (or vice versa) with the formation of a 9,10-seco phenol (1). The degradation of androst-4-en-3,17-dione could thus be visualized as follows: androst-4-en-3,17-dione (I) → 9α-hydroxyandrost-4-en-3,17-dione (II) or androsta-1,4-diene-3,17-dione (III) → 3-hydroxy-9,10-seco-androsta-1,3,5(10)-triene-9,17-dione (IV).

We have previously postulated that the microbial aromatization of 9α-hydroxyandrost-4-en-3,17-dione and 19-hydroxyandrost-4-en-3,17-dione probably involved a 1,2-dehydrogenation followed by reverse aldol condensation, their results were obtained solely from the intact microorganism. A preliminary communication (3) on the enzymatic mechanism of this aromatizing reaction has appeared. In view of the importance of this mechanism we wish to record in detail



Scheme I

This mechanism of Ring A aromatization is of biochemical interest since it bears close similarity to the formation of estrogens from androgens in mammals (2). Although Dodson and Muir (1) our examinations of reactions using partially purified enzyme preparations which are significant in establishing and confirming the proposed mechanism of Dodson and Muir.

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EXPERIMENTAL

Materials and Methods

Cytochrome *c*, nicotinamide-adenine dinucleotide (NAD), nicotinamide-adenine dinucleotide phosphate (NADP), reduced nicotinamide-adenine dinucleotide ($NADH_2$), and reduced nicotinamide-

adenine dinucleotide phosphate ($NADPH_2$) were obtained from Sigma Chemical Co. Phenazine methosulfate was purchased from the Aldrich Chemical Co. Whatman No. 1 paper from H. Reeve Angel and Co. was used for paper chromatography. 2,3 - Dichloro - 5,6-dicyanobenzoquinone (DDQ) was purchased from the Ealing Corp. 9α-Hydroxyandrost-4-en-3,17-dione was prepared according to the method of Sih (4). 9α-Fluorohydrocortisone was synthesized according to the method of Fried and Sabo (5). All melting points were uncorrected and were determined in open soft-glass capillaries. Values of $[\alpha]_D$ have been approximated to the nearest degree. Ultraviolet absorption spectra were determined on a Cary model 11 MS recording spectrophotometer. Infrared spectra were recorded on a Beckman IR 5A double beam infrared recording spectrophotometer. Microanalyses were carried out by Mr. J. Alicino of Metuchen, N. J. Protein was estimated by the method of Gornall, *et al.* (6). Phenolic steroids were detected on paper chromatograms by spraying with diazotized sulfanilic acid (7). Spectrophotometric measurements were made on a Beckman DU spectrophotometer equipped with a Gilford multiple sample absorbance recorder model 2000 (8).

The conditions and the procedure used for the growth of *N. restrictus*, the purification of the steroid 1-dehydrogenase, and the details of the phenazine methosulfate-cytochrome *c* assay have been described previously (9). The solvent system for paper chromatography consisted of toluene-propylene glycol (10) and the R_f values of the steroids used in this work are shown in Table I. Δ^4-3-Oxo and $\Delta^{1,4}$-3-oxo steroids were assayed quantitatively after their elution from the paper chromatogram; their absorbances at 240 mμ were taken as a measure of their concentration (9). Phenolic steroids were assayed spectrophotometrically by measuring the increase in absorbance at 280 mμ (11).

Enzymatic Transformation of 9α-Hydroxyandrost-4-en-3,17-dione (II) into 3-Hydroxy-9,10-seco - androsta - 1,3,5(10) - triene - 9,17 - dione (IV).—A 100-mg. quantity of phenazine methosulfate and 50,000 units of 1-dehydrogenase (specific activity 1000) in a total volume of 400 ml. of 0.03 *M* phosphate buffer, pH 7.0, was added to 200 mg. of II. After a 4-hour incubation, the reaction was terminated by the addition of 6 *N* HCl and the protein precipitate was removed by filtration. The filtrate was extracted with three 150-ml. portions of chloroform, dried over sodium sulfate, and taken down to dryness. The residue was taken up in 75 ml. of benzene-ether (1:1) and extracted with three 35-ml. portions of 5% NaOH. The aqueous layer was acidified and extracted with 40-ml. portions of chloroform three times; the chloroform layer was dried over sodium sulfate and concentrated to dryness to give 168 mg. of residue. Two recrystallizations from acetone-petroleum ether afforded 94 mg. of IV, m.p. 122–124°; $[\alpha]_D^{25}$ +98° in chloroform (c, 0.9); $\lambda_{max.}^{alc.}$ 280 mμ (ε 2700); $\lambda_{max.}^{Nujol}$ 2.95, 5.75, 5.86, 6.23, and 6.66μ, identical to an authentic specimen.

Anal.—Calcd. for $C_{19}H_{24}O_3$: C, 75.97; H, 8.05. Found: C, 76.23; H, 8.53.

Enzymatic Dehydrogenation of 9α-Fluorohydrocortisone.—To 100 mg. of 9α-fluorohydrocortisone was added 50 mg. of phenazine methosulfate and 50,000 units of 1-dehydrogenase (specific activity 700) in a total volume of 500 ml. of 0.03 *M* phosphate buffer, pH 7.0. After incubation for 16 hours, the reaction was terminated by the addition of HCl and the mixture was extracted with 100 ml. of methylisobutyl ketone three times. The combined methylisobutyl ketone extract was dried over sodium sulfate and concentrated to dryness to give 89 mg. of residue. Two recrystallizations from ethanol gave 65 mg. of crystals, m.p. 265–269° dec., identical in all respects (mixed melting point and infrared spectrum) with an authentic sample.

Reaction of 9α-Hydroxyandrost-4-en-3,17-dione with 2,3 - Dichloro - 5,6 - dicyanobenzoquinone.—A 500-mg. quantity of DDQ was added to 400 mg. of 9α - hydroxyandrost - 4 - en - 3,17 - dione in 20 ml. of dioxane. The mixture was refluxed for 16 hours under nitrogen. On cooling, the hydroquinone separated and filtered off. The filtrate was diluted with an equal volume of chloroform and poured onto an acid washed alumina column (20 Gm.). Elution with chloroform afforded 20 mg. of a compound, m.p. 120–122°, $\lambda_{max.}^{alc.}$ 280 mμ (ε 2600), whose infrared spectrum was identical to an authentic sample of IV.

Enzymatic Conversion of 19-Hydroxyandrost-4-en-3,17-dione into Estrone.—To 20 mg. of 19-hydroxyandrost-4-en-3,17-dione was added 10 mg. of phenazine methosulfate and 5000 units of 1-dehydrogenase (specific activity 1000) in a total volume of 100 ml. of 0.03 *M* phosphate buffer, pH 7.0. After a 12-hour incubation, the reaction was terminated by the addition of 6 *N* HCl and extracted with 50-ml. portions of chloroform three times. The combined chloroform extract was dried over sodium sulfate and taken down to dryness. Two recrystallizations from chloroform-methanol afforded 10 mg. of crystals, m.p. 260–262°, identical in all respects (melting point, mixed melting point, and infrared spectrum) with an authentic specimen of estrone.

In a separate experiment 1 mg. of 19-hydroxyandrost-4-en-3,17-dione was mixed with 500 mcg. of phenazine methosulfate and 1000 units of 1-dehydrogenase (specific activity 900) in a total volume of 4 ml. of 0.03 *M* phosphate buffer, pH 7.0. After a 2-hour incubation, the mixture was distilled and the distillate gave a positive reaction for the presence of formaldehyde (12). By omitting the 19-hydroxyandrost-4-en-3,17-dione from the reaction mixture, the distillate gave no purple color.

TABLE I.—*Rf* VALUES OF STEROIDS IN THE TOLUENE–PROPYLENE GLYCOL SYSTEM

Compd.	*Rf*
Androst-4-en-3,17-dione	0.90
9α-Hydroxyandrost-4-en-3,17-dione	0.35
Androsta-1,4-diene-3,17-dione	0.82
Androst-4-en-3-one,17β-ol	0.45
Androsta-1,4-diene-3-one-17β-ol	0.40
19-Hydroxyandrost-4-en-3,17-dione	0.10
19-Oxoandrost-4-en-3,17-dione	0.75
19-Norandrost-4-en-3-one-17β-ol	0.40
3-Hydroxy-9,10-seco-androsta-1,3,5(10)-triene-9,17-dione	0.33
3-Hydroxyestra-1,3,5-triene-17-one	0.37
9,10-Seco-androst-4-en-3,9,17-trione	0.85

RESULTS

In a previous paper we have reported the conversion of 9α-hydroxyandrost-4-en-3,17-dione into the 9,10-seco phenol (IV) by crude cell-free extracts of *N. restrictus* (13). Since phenazine methosulfate was required for this transformation, it was suspected that the steroid 1-dehydrogenase was involved in this conversion. Figure 1 shows that, using partially purified steroid 1-dehydrogenase (tenfold), a similar conversion could be obtained, evidenced by the rapid disappearance of absorbance around 240 mμ, accompanied by an increase in absorbance at 280 mμ. Thus all subsequent experiments were performed using this partially purified enzyme preparation. To substantiate further the participation of the steroid 1-dehydrogenase in this aromatization reaction, several quinones were tested for their ability to serve as electron acceptors because steroid 1-dehydrogenase has been implicated as a flavoprotein. Table II shows that the relative efficiency of these quinones as electron acceptors were the same with either androst-4-en-3,17-dione or 9α-hydroxyandrost-4-en-3,17-dione as the substrate. Figure 2 shows that the pH optimum of this aromatization reaction is around 9.0, identical to that of the oxidation of androst-4-en-3,17-dione. Because of the difficulty in preparing 9α-acetoxyandrost-4-en-3,17-dione and microorganisms contain esterases capable of hydrolyzing acetoxyl groups, 9α-fluorohydrocortisone was incubated and was readily converted into 9α-fluoroprednisolone by the same enzyme system. (See *Experimental.*)

Two possible mechanisms of this Ring A aromatization reaction involving the steroid 1-dehydrogenase were apparent and these can be represented as

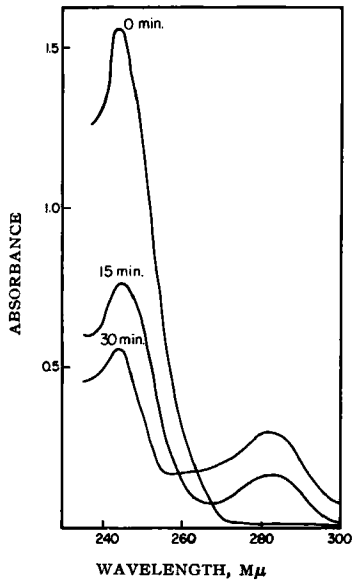
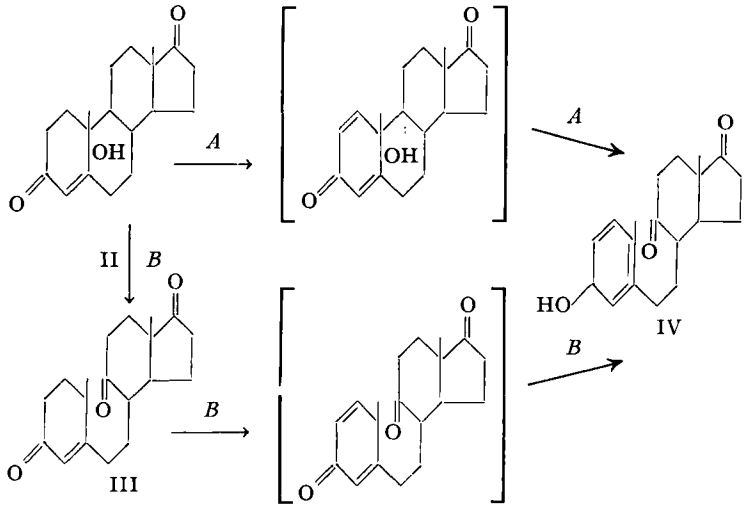


Fig. 1.—Oxidation of 9α-hydroxyandrost-4-en-3,17-dione to 3-hydroxy-9,10-seco-androsta-1,3,5(10)-triene-9,17-dione by purified steroid 1-dehydrogenase. The reaction mixture consisted of 600 mcg. of 9α-hydroxyandrost-4-en-3,17-dione, 500 mcg. of phenazine methosulfate and 25 units of 1-dehydrogenase (specific activity 1000) in a total volume of 4 ml. of 0.03 *M* phosphate buffer, pH 7.0. At the indicated time intervals the reaction was terminated by the addition of HCl and extracted with 1 ml. of chloroform. After separation of the layers by centrifugation, 0.2 ml. of the chloroform layer was diluted with 2.8 ml. of chloroform.



Scheme II

Mechanism *A* involves the introduction of a 1,2-double bond to form a vinylog of a β-hydroxyketone which could undergo facile rearrangement (reverse aldolization) to give a 9,10-seco phenol (IV). Mechanism *B* involves the abstraction of the 9α-hydroxyl proton to yield 9,10-seco-androst-4-en-3,9,17-trione (III); introduction of a 1,2-double bond into the latter compound affords a $\Delta^{1,4}$-dienone system which could also undergo chemical rearrangement to give a 9,10-seco phenol (IV). In order to distinguish these two mechanisms, 9α-hydroxyandrost-4-en-3,17-dione and 9,10-seco-androst-4-en-3,9,17-trione were incubated with the steroid 1-dehydrogenase. Figure 3 shows that the former compound was readily oxidized whereas 9,10-seco-androst-4-en-3,9,17-trione was not. This experi-

ment eliminates the latter compound as a possible intermediate and makes Mechanism *B* an unlikely one.

Table III shows that in the absence of phenazine methosulfate little if any 9α-hydroxyandrost-4-en-3,17-dione was metabolized. The slight decrease is probably due to the contamination of a trace amount of the natural electron acceptor. Addition of NAD and NADP did not stimulate the rate of metabolism of 9α-hydroxyandrost-4-en-3,17-dione, whereas on addition of phenazine methosulfate a rapid disappearance was observed.

On paper chromatograms we have been unable to detect the presence of 9α-hydroxyandrosta-1,4-diene-3,17-dione; the first demonstrable product has been the 9,10-seco phenol even when careful kinetic studies were made within the pH range of 4.8 to 9.0. Although it is well known that vinylogs of β-hydroxyketones undergo rearrangement very easily even by ordinary chemical means, methyl-3,11-dioxo-14,19-dihydroxy-$\Delta^{1,4}$, 14β-etiadienate has been obtained in very small yields as a by-product of a chemical reaction (14). This encouraged us to attempt to synthesize 9α-hydroxyandrosta-1,4-diene-3,17-dione by using mild chemical methods. However, when 9α-hydroxyandrost-4-en-3,17-dione was refluxed with DDQ, only 9,10-seco phenol (IV) was obtained in low yields; we were unable to detect the presence of the desired product on paper chromatograms.

Because this aromatization reaction bears close similarity to the mammalian conversion of androgens into estrogens, several of the intermediates in the mammalian pathway were incubated with the microbial system. 19-Hydroxyandrost-4-en-3,17-dione (V) was converted into estrone (VI). (See *Experimental.*) The distillate of the reaction mixture gave a positive test for the presence of formaldehyde (12), but the ratio of estrone to formaldehyde has been erratic and varied from experiment to experiment. In the absence of an electron acceptor such as phenazine methosulfate, very little 19-hydroxyandrost-4-en-3,17-dione (V) was metabolized. $NADH_2$ and $NADPH_2$ gave no stimulation (Table IV). 19-Oxoandrost-4-en-3,17-dione was also transformed into estrone by the same enzyme preparation; no attempt was made to determine the one-carbon fragment. Figure 4 shows the relative rates of oxidation follows the order: androst-4-en-3,17-dione>19-norandrost-3-en-3,17-dione>19-oxoandrost-4-en-3,17-dione>19-hydroxyandrost-4-en-3,17-dione. These rates were identical when the same experiment was carried out under anaerobic conditions.

TABLE II.—EFFECT OF DIFFERENT ELECTRON ACCEPTORS ON THE METABOLISM OF 9α-HYDROXYANDROST-4-EN-3,17-DIONE AND ANDROST-4-EN-3-ONE-17-OL[a]

Electron Acceptor	Relative Rate: 9α-Hydroxyandrost-4-en-3,17-dione	Relative Rate: Androst-4-en-3-one-17β-ol
1,2-Naphthoquinone	100	100
Phenazine methosulfate	83	86
Menadione	62	65
1,4-Naphthoquinone	47	51
2,6-Dichlorophenol indophenol	50	48
2,3-Dichloro-5,6-dicyanobenzoquinone	30	34

[a] The reaction system contained 1.5 μm. of 9α-hydroxyandrost-4-en-3,17-dione or androst-4-en-3-one-17β-ol, dissolved in 0.1 ml. of dimethylformamide, 1.5 μm. of various electron acceptors, and 20 units of enzyme (specific activity 500) in a total volume of 4.0 ml. of 0.03 *M* phosphate buffer, pH 7.0. After 10 minutes, the reaction products 3-hydroxy-9,10-seco-androsta-1,3,5(10)-triene-9,17-dione and androsta-1,4-diene-3-one-17β-ol were assayed as in Fig. 2.

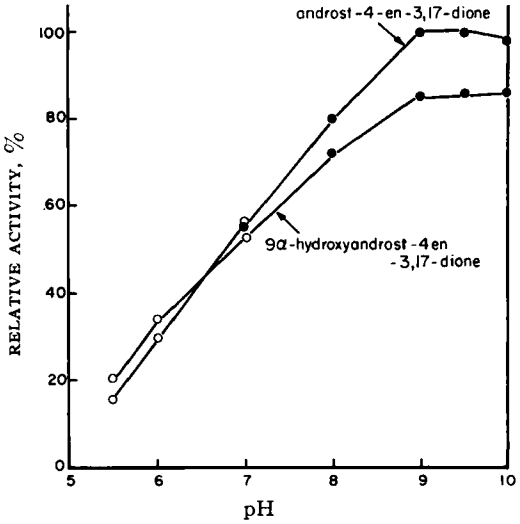
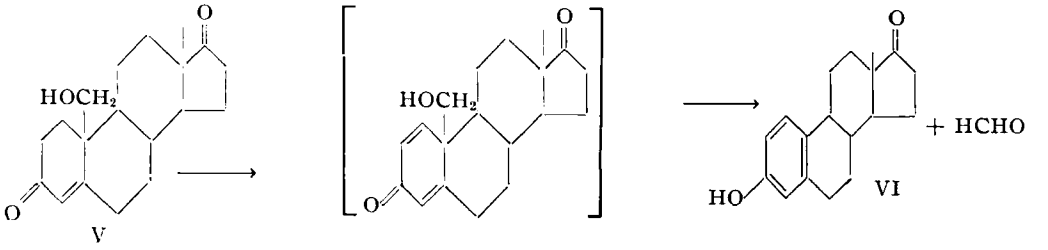


Fig. 2.—Effect of pH on the rate of oxidation of androst-4-en-3,17-dione and 9α-hydroxyandrost-4-en-3,17-dione. Key: O——O, 0.03 *M* phosphate; ●——●, 0.03 *M* tris (hydroxymethyl) aminomethane buffer. The reaction systems contained 1 mg. of androst-4-en-3,17-dione or 9α-hydroxyandrost-4-en-3, 17-dione, 500 mcg. of phenazine methosulfate, and 25 units of 1-dehydrogenase (specific activity 800) in a total of volume of 4 ml. of 0.03 *M* phosphate or Tris buffer. After 10 minutes, the reaction was terminated by acidification; androsta-1,4-diene-3,17-dione was assayed by the paper chromatographic method, and 9,10-seco phenol (IV) was assayed as in Fig. 1.



Scheme III

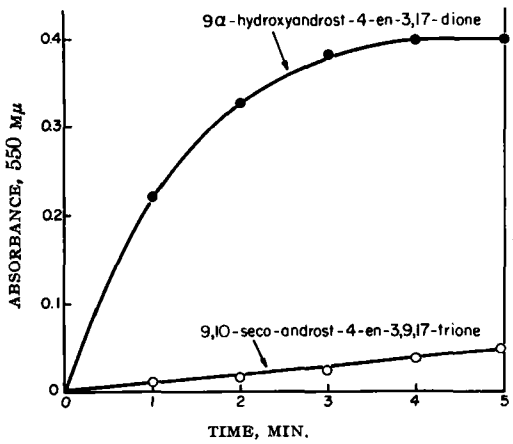


Fig. 3.—Reaction of 9α-hydroxyandrost-4-en-3,-17-dione and 9,10-seco-androst-4-en-3,9,17-trione with purified 1-dehydrogenase. The assay system contained 2 mg. of cytochrome *c*, 100 mcg. of steroid in 0.1 ml. of methanol, and 25 units of enzyme (specific activity 1000) in a total volume of 3 ml. of 0.03 *M* phosphate buffer, pH 7.0. The reaction was initiated by the addition of 0.1 ml. of a 1% phenazine methosulfate solution.

TABLE III.—EFFECT OF PHENAZINE METHOSULFATE ON METABOLISM OF 9α-HYDROXYANDROST-4-EN-3,17-DIONE[a]

Additions	9α-Hydroxyandrost-4-en-3,17-dione, μm. 20 min.	45 min.
None	1.59	1.36
NAD (1.5 μm.)	1.57	1.43
NADP (1.5 μm.)	1.56	1.48
Phenazine methosulfate (1.5 μm.)	0.03	0.00

[a] The reaction mixture contained 1.65 μm. of 9α-hydroxyandrost-4-en-3,17-dione in 0.2 ml. of dimethylformamide and 40 units of enzyme (specific activity 1000) in a total volume of 4 ml. of 0.03 *M* phosphate buffer, pH 7.0. The reaction was stopped at the indicated time intervals by the addition of 1 ml. of chloroform; 9α-hydroxyandrost-4-en-3,17-dione was determined by the paper chromatographic method.

DISCUSSION

The results described show that the conversion of 9α-hydroxyandrost-4-en-3,17-dione into 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione requires the presence of an electron acceptor characteristic of flavoproteins (15); the relative efficiency of these electron acceptors follows the same order regardless of whether androst-4-en-3,17-dione or 9α-hydroxyandrost-4-en-3,17-dione was used as the substrate. It is interesting to note that DDQ is also capable of serving as an electron acceptor for the steroid 1-dehydrogenase which confirms the results of Ringold and Turner (16), who have shown that chemical 1,2-dehydrogenation using DDQ involves the abstraction of trans-diaxial hydrogens similar to that of the microbial system. The same enzyme system was capable of converting 9α-fluorohydrocortisone into 9α-fluoroprednisolone and the aromatization reaction exhibited an alkaline pH optimum around 9.0 which is characteristic for steroid 1-dehydrogenase. All these results support the view that the aromatization of 9α-hydroxyandrost-4-en-3,17-dione involved the introduction of a 1,2-double bond by the steroid 1-dehydrogenase.

TABLE IV.—EFFECT OF PHENAZINE METHOSULFATE ON METABOLISM OF 19-HYDROXYANDROST-4-EN-3,17-DIONE[a]

Additions	19-Hydroxyandrost-4-en-3,17-dione, μm. 20 min.	45 min.
None	1.57	1.44
NAD (1.5 μm.)	1.56	1.49
$NADH_2$ (1.2 μm.)	1.56	1.50
NADP (1.5 μm.)	1.59	1.56
$NADPH_2$ (1.2 μm.)	1.58	1.52
Phenazine methosulfate (1.0 μm.)	1.48	1.16

[a] The reaction mixture contained 1.65 μm. of 19-hydroxyandrost-4-en-3,17-dione in 0.2 ml. of dimethylformamide, and 40 units of enzyme (specific activity 900) in a total volume of 4 ml. of 0.03 *M* phosphate buffer, pH 7.0. The reaction was terminated by the addition of 1.0 ml. of chloroform at the indicated time intervals and the 19-hydroxyandrost-4-en-3,17-dione was estimated by the paper chromatographic method.

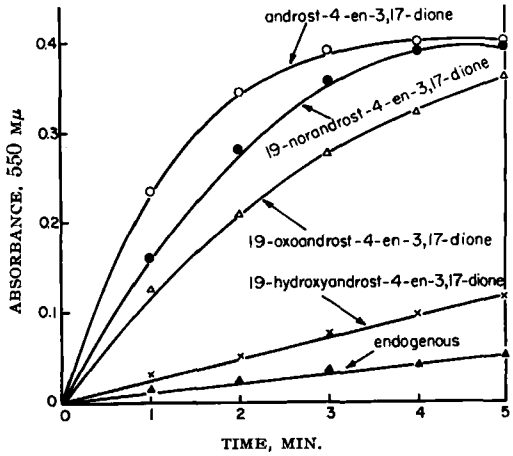


Fig. 4.—The relative rates of oxidation of 19-methyl, 19-nor, 19-oxo, and 19-hydroxy steroids by steroid 1-dehydrogenase. The assay system is the same as that of Fig. 3.

As the enzyme system failed to metabolize 9,10-secoandrost-4-en-3,9,17-trione, this obviously eliminated the alternate pathway B as depicted under Scheme II.

In the absence of an electron acceptor very little 9α-hydroxyandrost-4-en-3,17-dione was metabolized, and NAD and NADP showed no stimulation. Also, the kinetics of 9α-hydroxyandrost-4-en-3,17-dione oxidation (Fig. 3) showed no lag phase. All these results indicate that the first step in the metabolism of 9α-hydroxyandrost-4-en-3,17-dione involves a 1,2-dehydrogenation by the steroid 1-dehydrogenase. Although we have been unable to detect the presence of 9α-hydroxyandrosta-1,4-diene-3,17-dione, it is well known that vinylogs of β-hydroxyketones could undergo rearrangements very easily, and many attempts to prepare this type of compound have failed; *e.g.*, treatment of 19-acetoxyandrosta-1,4-diene-3,17-dione with $NaHCO_3$ or esterase resulted in the formation of estrone (17, 18).

19-Nor, 19-hydroxy, and 19-oxo androstenediones were converted into estrone when incubated with the same enzyme system. However, in the absence of an electron acceptor little 19-hydroxyandrost-4-en-3,17-dione was metabolized. This again eliminated an alternate mechanism involving deformylation prior to 1,2-dehydrogenation.

In view of the foregoing evidence, the aromatization of 9α-hydroxy and 19-hydroxyandrost-4-en-3,17-dione involve a direct 1,2-dehydrogenation to give vinylogs of β-hydroxyketones which undergo spontaneous nonenzymic rearrangements (reverse aldolization) to give their respective phenols. The microbial aromatizing system differs from that of the human placental microsomes in that (*a*) the relative rate of oxidation follows the order 19-nor>19-oxo>19-hydroxy, (*b*) oxygen and $NADPH_2$ are not required in the aromatization reaction, and (*c*) a suitable electron acceptor characteristic of flavoproteins is needed.

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Identification of Sympathomimetic Amines as Tetraphenylborates

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The tetraphenylborate salts of sympathomimetic amines are readily isolated in a highly pure state even from low concentrations in complex mixtures. Therefore, melting points, infrared, and ultraviolet spectral characteristics of these salts were studied as an aid to the identification of the medicinally important sympathomimetic amines.

CHATTEN AND LEVI (1) and Fischer and Plein (2) have prepared derivatives for the identification of sympathomimetic amines and have listed references for the identification of these amines. Characterization through tetraphenylborate (TPB) salts would be a valuable addition to these present methods for identifying sympathomimetic amines.

Isolation of organic bases as their TPB salts and subsequent identification by melting point is well established (3–12). Determination of various alkaloids and chemotherapeutic agents by ultraviolet spectra of their TPB salts has also been reported (13, 14). Chatten, Pernarowski, and Levi (15) have prepared the TPB salts of a series of local anesthetics and have reported their ultraviolet and infrared spectra.

In general, TPB derivatives of organic bases

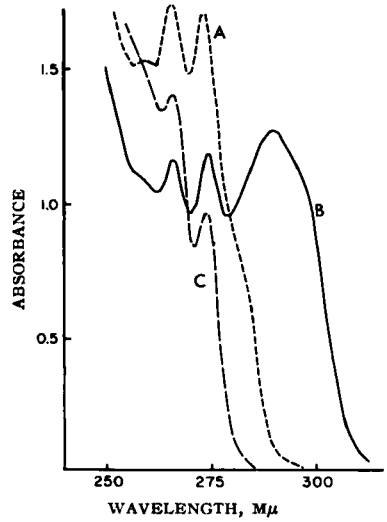

Fig. 1.—Ultraviolet spectra in methanol. Key: A, phenylephrine TPB ($2.53 \times 10^{-4}M$); B, methoxamine TPB ($6.46 \times 10^{-4}M$); C, ephedrine TPB ($4.83 \times 10^{-4}M$).

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