# Mechanisms of Steroid Oxidation by Microorganisms. VII. Properties of the 9α-Hydroxylase*

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An active cell-free preparation from *Nocardia restrictus* capable of introducing a 9α-hydroxyl group into steroids has been obtained. Several precautions toward obtaining this active preparation as well as the properties of the 9α-hydroxylase system are described in detail. Among the steroids tested, androst-4-ene-3,17-dione, pregn-4-ene-3,20-dione, and 21-hydroxypregn-4-ene-3,20-dione were good substrates for the 9α-hydroxylase, whereas 3β-hydroxyandrost-5-en-17-one, cholest-5-en-3βol, cholest-4-en-3-one, and estra-1,3,5(10)-triene-3,17β-diol were inactive. The 9α-hydroxylase preparation converted androsta-4,9(11)-diene-3,17-dione into 9α,11α-oxidoandrost-4-ene-3,17-dione. Androsta-4,9(11)-diene-3,17-dione has been shown to be a competitive inhibitor of the 9α-hydroxylase which establishes that the 9α-hydroxylase system is indeed involved in both hydroxylation and epoxidation. However, the 9α-hydroxylase system appears to be more complex than the 9α,11α-epoxidase system. Cell-free extracts obtained from cells induced with androsta-4,9(11)-diene-3,17-dione possessed good epoxidase activity, but no 9α-hydroxylase activity was observed with such preparations.

The microbiological hydroxylation of steroids is a well-documented fact. Hydroxyl groups can now be introduced into practically every position on the steroid nucleus by exposure of the steroid molecule to a suitable microorganism (Peterson, 1963). However, little information is available concerning the enzymatic mechanism of steroid hydroxylations because little success has attended attempts to demonstrate hydroxylations in cell-free extracts of microorganisms (Talalay, 1957). Although cell-free preparations from mammalian tissues have been obtained (Hayano, 1962) which are capable of catalyzing the introduction of hydroxyl groups onto several positions in the steroid molecule, to our knowledge only one successful attempt has been recorded to date on hydroxylations by cell-free extracts from microorganisms. Zuidweg *et al.* (1962) demonstrated the conversion of 17α,21-dihydroxypregn-4-ene-3,20-dione into 11β,17α,21-trihydroxypregn-4-ene-3,20-dione using a cell-free preparation from *Curvularia lunata*. In view of the paucity of knowledge in this area we should like to report our studies with the 9α-hydroxylase system of *Nocardia restrictus*.

## EXPERIMENTAL

*Materials.*—All solvents and inorganic chemicals were reagent grade. $NADH_2$ and *p*-mercuribenzoate were products of Sigma Chemical Co. $NADPH_2$ was purchased from California Corp. for Biochemical Research. 2-Amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride was a gift from Dr. A. Pohland of Eli Lilly and Co. Phenazine methosulfate was obtained from the Aldrich Chemical Co., Inc. Androsta-1,4-diene-3,17-dione was a product of Steraloids, Inc. Androsta-4,9(11)-diene-3,17-dione, 9α,11α-oxidoandrost-4-ene-3,17-dione, and 9α-hydroxyandrost-4-ene-3,17-dione were prepared according to the method of Sih (1961). 3-Hydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione was prepared by fermentation (Wang and Sih, 1963). 9α,11α-Oxidoandrosta-1,4-diene-3,17-dione was prepared by reaction of 9α,11α-oxidoandrost-4-ene-3,17-dione with 2,3-dichloro-5,6-dicyanobenzoquinone. All the radioactive steroids used in this work were products of the New England Nuclear Corp. These include: androst-4-ene-3,17-[4-$^{14}$C]dione (22 mc/mmole), pregn-4-ene-3,20-[4-$^{14}$C]dione (46 mc/mmole), 3β-hydroxyandrost-5-en-17-[4-$^{14}$C]one (41 mc/mmole), cholest-5-en-3β-[4-$^{14}$C]ol (45 mc/mmole), cholest-4-en-3-[4-$^{14}$C]one (46 mc/mmole), 21-hydroxypregn-4-ene-3,20-[4-$^{14}$C]dione (35 mc/mmole), and estra-1,3,5(10)-triene-3,17β-[4-$^{14}$C]diol (8 mc/mmole).

*Culture Methods.*—*Nocardia restrictus* (Lee and Sih, 1964) was grown in 4.8 liters of Difco nutrient broth (twelve 2-liter flasks) on a rotary shaker (250 rpm, 1 in. stroke) at 25°. After 24 hours, 960 mg of androst-4-ene-3,17-dione in 4 ml of dimethylformamide was distributed equally to the flasks and the fermentation was continued for 4 hours. The cells were then collected by centrifugation and washed with cold 0.03 M potassium phosphate buffer *p*H 7.6, containing 1 mM glutathione and 2 mM of EDTA.

*Preparation of Cell-free Extract.*—The cells were suspended in 10 volumes of the same buffer and placed in a sonic field of a Raytheon magnetostrictive oscillator (10 kc) for 15 minutes. All subsequent steps were carried out in a cold room maintained at 4°. The cell debris was removed by centrifugation for 15 minutes at 860 × *g*. The supernatant was further centrifuged at 14,000 × *g* for 20 minutes. The sediment was dissolved in 4 ml of the same buffer and was homogenized in a Potter-Elvejhem homogenizer; this fraction served as the source of the hydroxylation enzyme for all subsequent experiments.

## RESULTS

*Standard 9α-Hydroxylase Assay.*—Because of the similarity of the chemical structures between the substrate and the product, a radioactive paper-chromatographic assay was chosen for this work. This assay possesses the desired sensitivity and at the same time permits the quantitation of both the substrate and the product. The standard assay system contained androst-4-ene-3,17-[4-$^{14}$C]dione (7500 cpm), 0.06 μmole of $NADPH_2$, and varying amounts of the enzyme preparation in a total volume of 2 ml of 0.03 M phosphate buffer (containing 1 mM of glutathione and 2 mM of EDTA), *p*H 7.4. This mixture was incubated for 5 minutes at 25° with air as the gas phase. The reaction was terminated by 2 N HCl and extraction of the mixture with 3 ml of chloroform. Two ml of the chloroform layer was transferred to another test tube and

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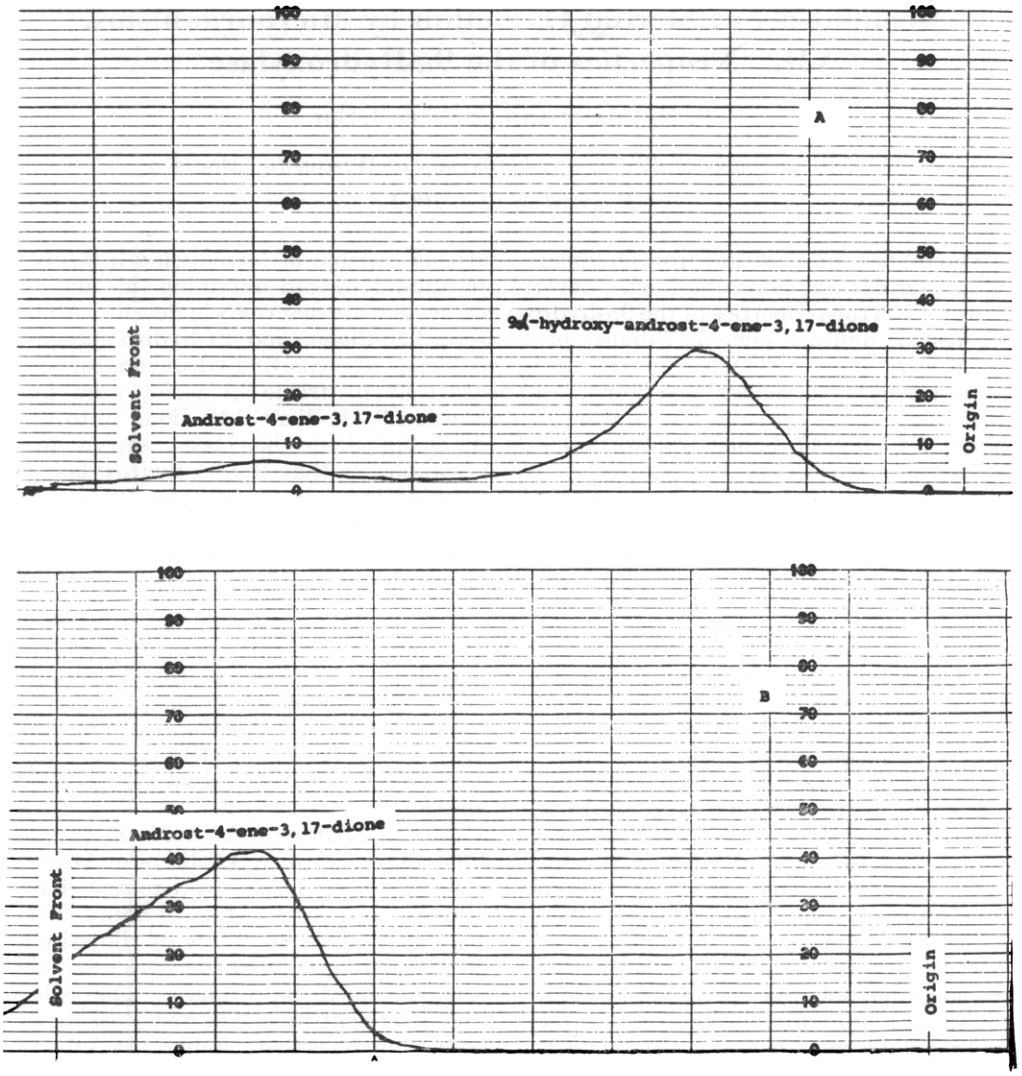


FIG. 1.—Demonstration of 9α-hydroxylase activity. The reaction mixture contained androst-4-ene-3,17-dione-[4-$C^{14}$](7500 cpm), 0.06 μmole of $NADPH_2$, 0.2 μmole of androsta-1,4-diene-3,17-dione, and 4.8 mg of protein in a total volume of 2 ml of 0.03 M phosphate buffer, *p*H 7.4.

TABLE I

EFFECT OF 9α-HYDROXYANDROST-4-ENE-3,17-DIONE ON THE RECOVERY OF RADIOACTIVE STEROIDS[a]

| 9α-Hydroxy-androst-4-ene-3,17-dione (μg) | Hydroxylation (%) | Per Cent of Total Radioactivity Recovered |
|---|---|---|
| None | 27 | 72 |
| 2.5 | 27 | 80 |
| 5.0 | 28 | 88 |
| 10.0 | 43 | 89 |
| 25.0 | 45 | 92 |

[a] The assay system is the same as that of Table II except 9α-hydroxyandrost-4-ene-3,17-dione was varied as shown.

evaporated to dryness. The residue was dissolved in 0.05 ml of chloroform: 0.03 ml was applied on Whatman No. 1 paper (1.5 × 72 cm) and developed for 3 hours in a toluene–propylene glycol system (Zaffaroni *et al.*, 1950). The radioactivity on the paper strips was quantitatively assayed with a Vanguard Autoscanner Model 880 ADS with the automatic data system. The linearity of this assay follows the equation $Y = 0.330X$, where $Y$ is the mμmoles of product formed per minute and $X$ is the mg of protein. The standard deviation of $Y$ for all values of $X$ determined (0.33, 0.66, and 0.99) was less than ±0.012. Most of the subsequent work was performed using this assay system because of the small quantities of enzyme preparation required.

As the product, 9α-hydroxyandrost-4-ene-3,17-dione, was further metabolized by the cell-free extract, a deviation in linearity of the above assay method was observed if the incubation period was longer than 5 minutes or if the protein content of the enzyme preparation exceeded 1 mg. For example, if the above-mentioned assay mixture was left standing for a 2-hour period, no radioactivity could be detected on the paper chromatogram. However, if 0.2 μmole of an inhibitor such as androsta-1,4-diene-3,17-dione was added to this assay mixture, a linear relation between 9α-hydroxylase activity and the enzyme preparation was again observed, even though the protein concentration exceeded 1 mg and the incubation period was extended to as long as 1 hour. The linearity of this assay follows the equation $Y = 0.320X$, where $Y$ is the mμmoles of product formed per minute and $X$ is the mg of protein. The standard deviation of $Y$ for all values of $X$ determined (0.8, 1.6, 2.4, 3.2, and 4.8) was less than ±0.001. Androsta-1,4-diene-3,17-dione was selected as the inhibitor because it has been previously shown that 1-dehydro steroids are powerful inhibitors of the steroid 1-dehydrogenase (Sih and Laval, 1962) which catalyzes the oxidation of the product, 9α-hydroxyandrost-4-

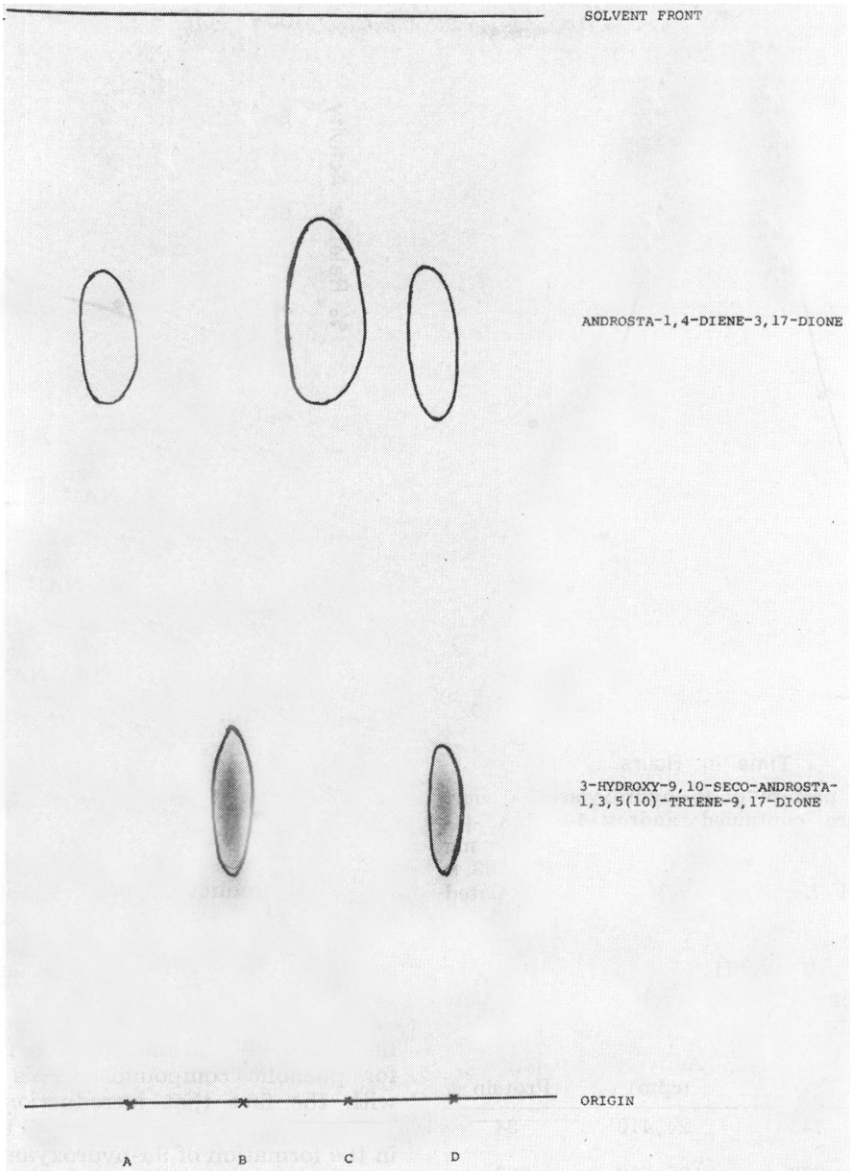


FIG. 2.—The conversion of androsta-1,4-diene-3,17-dione into 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione by 9$\alpha$-hydroxylase. The reaction mixture contained 100 $\mu$g of androsta-1,4-diene-3,17-dione, 3 mg of $NADPH_2$, and 5 mg of enzyme protein in a total volume of 2 ml of 0.03 M phosphate buffer, *p*H 7.4. The mixture was incubated for 2 hours at 25° and extracted with chloroform. An aliquot of the chloroform extract was spotted on Whatman No. 1 paper and developed for 3 hours in the toluene–propylene glycol system. The phenolic steroids were detected by spraying the paper with the diazotized sulfanilic acid reagent. (A) Androsta-1,4-diene-3,17-dione. (B) 3-Hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione. (C) Boiled-enzyme mixture. (D) Reaction mixture.

ene-3,17-dione, into 3-hydroxy-9,10-secoandrosta-1,3,5-(10)-triene-9,17-dione (Sih and Rahim, 1963). The 9,10-seco phenol could then be further oxidized in a series of reactions which eventually leads to $CO_2$ and $H_2O$ (Sih, 1962). Thus, by including androsta-1,4-diene-3,17-dione in the assay mixture, better recovery of the radioactivity in 9$\alpha$-hydroxyandrost-4-ene-3,17-dione could be obtained. Table I further demonstrates that the total amount of radioactivity recovered and the amount resided in the product, 9$\alpha$-hydroxyandrost-4-ene-3,17-dione, could be increased by the addition of an exogenous source of nonradioactive 9$\alpha$-hydroxyandrost-4-ene-3,17-dione. This experiment further confirms the fact that the product, 9$\alpha$-hydroxyandrost-4-ene-3,17-[4-$^{14}$C]dione, was being further metabolized by the cell-free extract.

*Removal of the Endogenous Inhibitor.*—The 9$\alpha$-hydroxylase activity in the sonic extract after removal of the cell debris was consistently low; the specific activity (cpm/mg protein) was around 34. However, by centrifuging the sonic extract at 14,000 $\times$ *g*, we obtained a sediment that showed a considerable increase in 9$\alpha$-hydroxylase activity. The total activity increased 8-fold and the specific activity increased 30-fold after separation from the supernatant. Little 9$\alpha$-hydroxylase activity remained in the supernatant fraction (Table II). This result suggested the presence of an inhibitor in the supernatant fraction. The inhibition of 9$\alpha$-hydroxylase activity was proportional to the amount of supernatant fluid added. The inhibitor appears to be heat stable since the inhibition pattern remained unchanged after the addition of boiled supernatant fluid to the enzyme. On the other hand, if the supernatant fluid was ashed, little if any inhibition of 9$\alpha$-hydroxylase activity was observed with the added ash; this result suggested that the inhibitor was organic in nature.

*Identification of the Product of Reaction.*—Under the

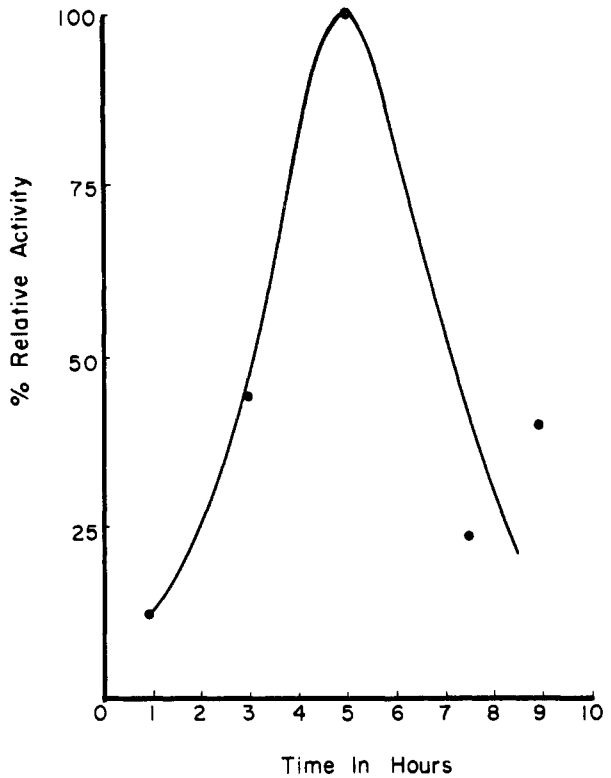


FIG. 3.—Induction pattern of 9α-hydroxylase activity. The reaction mixture contained androst-4-ene-3,17-[4-$^{14}$C]dione (7500 cpm), $NADPH_2$, 0.06 μmole, and 1 mg of enzyme protein in a total volume of 2.0 ml of 0.03 M phosphate buffer, *p*H 7.4. The mixture was incubated for 5 minutes at 25°.

TABLE II

DISTRIBUTION OF THE 9α-HYDROXYLASE ACTIVITY[a]

| Fraction | Protein (mg) | Total Activity (cpm) | Cpm/mg Protein |
|---|---|---|---|
| Initial sonic extract | 743 | 24,410 | 34 |
| Sediment | 200 | 196,500 | 983 |
| Supernatant | 531 | 14,750 | 28 |

[a] The initial extract was centrifuged at 14,000 × *g* for 20 minutes. The residue obtained is designated as the sediment fraction. The conditions used for assay are described in the text.

conditions described with 0.2 μmole of androsta-1,4-diene-3,17-dione as inhibitor, 9α-hydroxylase activity could readily be demonstrated as shown in Figure 1. The radioactive peak corresponding to androst-4-ene-3,17-[4-$^{14}$C]dione has disappeared with a corresponding appearance of a new radioactive peak with a mobility consistent with 9α-hydroxyandrost-4-ene-3,17-[4-$^{14}$C]-dione (Fig. 1A). When the enzyme was boiled for 10 minutes at 100°, the added androst-4-ene-3,17-dione-[4-$^{14}$C] did not disappear and no appearance of this new radioactive peak was observed (Fig. 1B). Since the relative mobilities on paper for 9α-hydroxyandrost-4-ene-3,17-dione and 17β-hydroxyandrosta-1,4-dien-3-one are very similar in the toluene–propylene glycol system and the enzyme preparation used has been known to contain both 17β-hydroxy steroid dehydrogenase and steroid 1-dehydrogenase, the following experiments were conducted to eliminate 17β-hydroxyandrosta-1,4-dien-3-one as a possible reaction product. When the radioactive product was treated with chromic trioxide in acetic acid, its mobility on paper did not

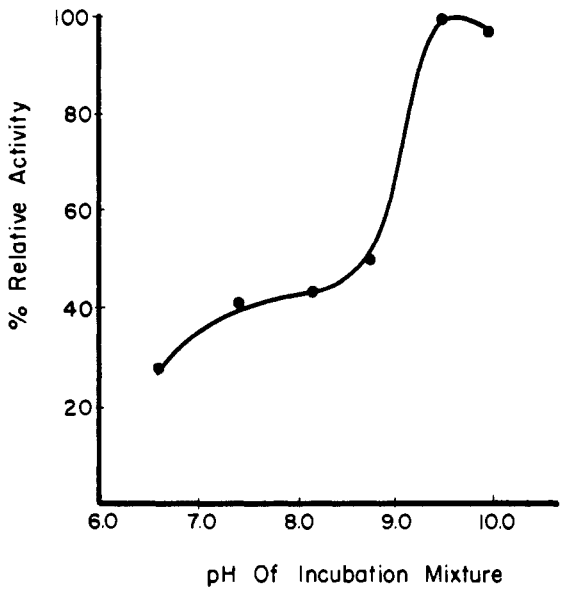


FIG. 4.—Effect of *p*H on 9α-hydroxylase activity. The reaction mixture contained 1 mg of protein, 0.06 μmole of $NADPH_2$, and androst-4-ene-3,17-[4-$^{14}$C]dione (7500 cpm) in a total volume of 2 ml of 0.1 M Tris buffer at the *p*H values indicated. The mixture was incubated for 5 minutes at 25°.

change. This indicates that the hydroxyl group in the product is tertiary in nature. When androsta-1,4-diene-3,17-dione was incubated with the enzyme preparation, a product appeared having a mobility corresponding to 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione. This product gave an orange color after the paper chromatogram had been sprayed with diazotized sulfanilic acid (Stowe and Thimann, 1954) (Fig. 2). Also, this product after elution from paper had an absorption maximum at 280 mμ, characteristic for phenolic compounds. This result is consistent with the fact that introduction of an 9α-hydroxyl group into androsta-1,4-diene-3,17-dione would result in the formation of 9α-hydroxyandrosta-1,4-diene-3,17-dione which could undergo facile reverse aldolization to yield 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,-17-dione (Sih and Rahim, 1963). Furthermore, this product gave a magenta color with the Zimmerman reagent, indicating the presence of a five-membered ring ketone. This phenolic product was absent with a boiled-enzyme preparation. These data conclusively established the fact that the enzyme activity in the sediment fraction was indeed an 9α-hydroxylase. In some enzyme preparations used, the concentration of steroid 1-dehydrogenase was higher than normal. Under these conditions a mixture of 9α-hydroxyandrost-4-ene-3,17-dione and 3-hydroxy-9,10-secoandrosta-1,3,-5(10)-triene-9,17-dione was obtained when androst-4-ene-3,17-dione was incubated with these preparations.

*Induction.*—The 9α-hydroxylase appears to be an adaptive enzyme as no 9α-hydroxylase activity was observed if the cells were grown in the absence of a steroid inducer. Figure 3 indicates the induction pattern. The maximal 9α-hydroxylase activity was obtained in about 5 hours after the addition of the steroid inducer and the enzyme activity declined sharply after that time. If the cells were harvested 12 hours after the addition of androst-4-ene-3,17-dione, no 9α-hydroxylase activity was detected.

*Stability.*—The 9α-hydroxylase preparation was unstable to freezing. Approximately 65% of the activity was lost after freezing the preparation for 36 hours

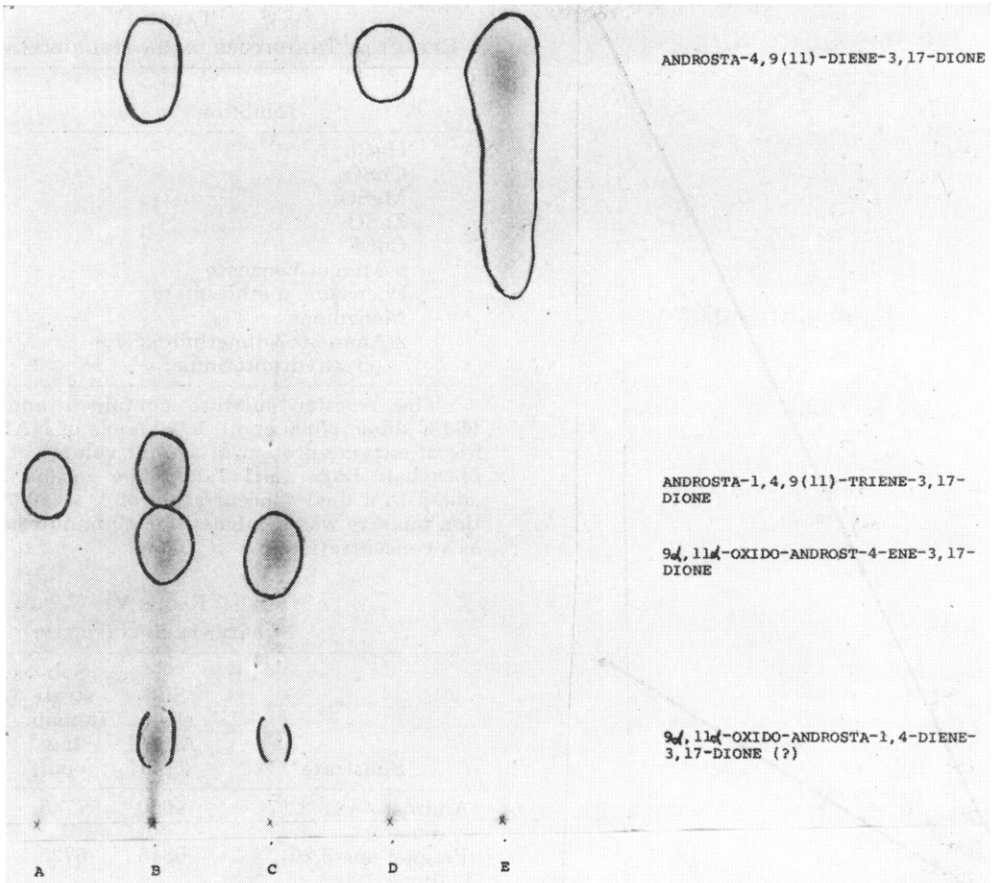


FIG. 5.—Epoxidation of androsta-4,9(11)-diene-3,17-dione by the 9α-hydroxylase. The reaction mixture was the same as those described in Fig. 2 except 100 μg of androsta-4,9(11)-diene-3,17-dione was substituted for androsta-1,4-diene-3,17-dione. The steroids were detected by spraying the paper chromatogram with the Zimmerman reagent. (A) Androsta-1,4,9(11)-triene-3,17-dione. (B) Reaction mixture. (C) 9α,11α-Oxidoandrost-4-ene-3,17-dione. (D) Androsta-4,9(11)-diene-3,17-dione. (E) Boiled-enzyme mixture. The paper chromatogram was developed for 30 hours in an isooctane–propylene glycol system.

Although higher enzyme activity was obtained if the sonic extract was prepared in 0.03 M phosphate buffer containing glutathione and EDTA than if prepared in plain distilled water, both of these preparations were most stable at about *p*H 7.4. When such preparation at *p*H 7.4 was stored at 0°, the half-life of the enzyme activity was about 2.5 days.

*Effect of* pH.—The 9α-hydroxylase activity was maximal at *p*H 9.5. At *p*H 7.4 the activity corresponded to about 40% of that at *p*H 9.5 (Fig. 4).

*Effect of $NADH_2$ and $NADPH_2$.*—A definite stimulation of 9α-hydroxylase activity was observed by the addition of an external source of either $NADH_2$ or $NADPH_2$ (Table III). However, no absolute requirement for these pyridine nucleotides was demonstrable,

TABLE III
EFFECT OF $NADH_2$ AND $NADPH_2$ ON 9α-HYDROXYLASE ACTIVITY[a]

| Concentration (M) | Hydroxylation (%) $NADH_2$ | $NADPH_2$ |
|---|---|---|
| None | 27 | 27 |
| $1.6 \times 10^{-5}$ | 35 | 28 |
| $8.0 \times 10^{-5}$ | 42 | 42 |
| $16.0 \times 10^{-5}$ | 42 | 45 |

[a] The assay system was the same as those described in the text except the reduced pyridine nucleotide concentrations were varied as indicated.

which is not at all surprising in view of the crude preparation used.

*Inhibitors.*—The 9α-hydroxylase activity was found to be sensitive to mercuric ions and *p*-mercuribenzoate, indicating the presence of a reactive sulfhydryl group in the enzyme. Zinc and cobalt salts were less effective. Both phenazine methosulfate and menadione were effective inhibitors of the enzyme activity at a concentration of $10^{-3}$ M. Because tetrahydropteridine has been implicated in steroid hydroxylation by mammalian tissue (Hagerman, 1964), it was decided to determine whether some stimulation could be obtained by the addition of tetrahydropteridine to the 9α-hydroxylase preparation. At a concentration of $10^{-5}$ M, 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine gave a 15% inhibition (Table IV).

*Substrate Specificity.*—Among the steroids tested, androst-4-ene-3,17-dione, pregn-4-ene-3,20-dione, and 21-hydroxypregn-4-ene-3,20-dione were equally active substrates for the 9α-hydroxylase. The following compounds were found to be inactive with the 9α-hydroxylase: 3β-hydroxyandrost-5-en-17-one, cholest-5-en-3β-ol, cholest-4-en-3-one, and estra-1,3,5(10)-triene-3,17β-diol (Table V).

*Relation between Hydroxylation and Epoxidation.*—Bloom and Shull (1955) postulated that "A microorganism capable of introducing an axial hydroxyl function at $C_n$ of a saturated steroid also effected the introduction of an epoxide grouping axial at $C_n$ in the corresponding unsaturated substrate." In accord with this

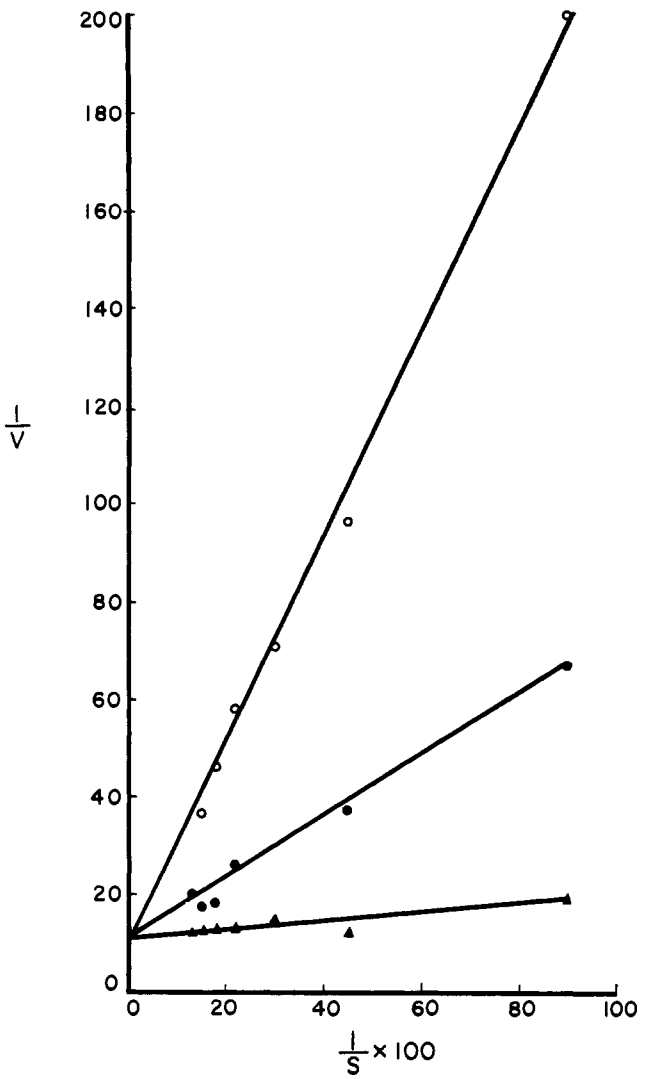


FIG. 6.—Competitive inhibition of 9α-hydroxylase activity by androsta-4,9(11)-diene-3,17-dione. The conditions used for assay are the same as those described in the text. ▲—▲—▲, no androsta-4,9(11)-diene-3,17-dione, ●—●—●, 1.6 mμmoles of androsta-4,9(11)-diene-3,17-dione, O—O—O, 3.2 mμmoles of androsta-4,9(11)-diene-3,17-dione.

theory, the 9α-hydroxylase should be able to introduce an epoxide into androsta-4,9(11)-diene-3,17-dione. Since the validity of this theory has yet to be challenged with a cell-free preparation from microorganisms, androsta-4,9(11)-diene-3,17-dione was incubated with the 9α-hydroxylase. Figure 5 shows that a product appeared having the same mobility on paper as 9α,11α-oxidoandrost-4-ene-3,17-dione; the mobility of this compound remained unchanged after its reaction with *m*-chloroperbenzoic acid or with chromic acid in acetic acid. These results are all consistent with the structure of 9α,11α-oxidoandrost-4-ene-3,17-dione. Because the enzyme preparation also contained steroid 1-dehydrogenase, androsta-1,4,9(11)-triene-3,17-dione and 9α,11α-oxidoandrosta-1,4-diene-3,17-dione were also obtained. In order to establish whether hydroxylation and epoxidation take place at the same site on the enzyme, a mixture of androst-4-ene-3,17-dione and androsta-4,9(11)-diene-3,17-dione was incubated with the 9α-hydroxylase system to determine the inhibition pattern. Figure 6 shows that androsta-4,9(11)-diene-3,17-dione was a competitive inhibitor of the 9α-hydroxylase, which suggests that hydroxylation and epoxidation take place on the same enzyme site. In order to ascertain whether the hydroxylation system is identical to the epoxidation system, cells were induced with androsta-4,9(11)-diene-3,17-dione. Although such extracts possessed good epoxidase activity, no 9α-hydroxylase activity was observed in these preparations.

TABLE IV
EFFECT OF INHIBITORS ON 9α-HYDROXYLASE ACTIVITY[a]

| Inhibitor | Inhibition (%) |
|---|---|
| $HgCl_2$ | 100 |
| $CuSO_4$ | 10 |
| $MgSO_4$ | 0 |
| $ZnSO_4$ | 35 |
| $CoCl_2$ | 43 |
| *p*-Mercuribenzoate | 97 |
| Phenazine methosulfate | 62 |
| Menadione | 92 |
| 2-Amino-6,7-dimethyl-5,6,7,8-tetrahydropteridine[b] | 15 |

[a] The reaction mixture contained androst-4-ene-3,17-[4-$^{14}$C]dione (7500 cpm), 0.06 μmole of $NADPH_2$, and 0.25 mg of enzyme protein in a total volume of 2 ml of 0.03 M phosphate buffer, *p*H 7.65. The various inhibitors were added to a final concentration of $1 \times 10^{-3}$ M. The reaction mixture was incubated for 10 minutes at 25°. [b] Used as a concentration of $1 \times 10^{-5}$ M.

TABLE V
SUBSTRATE SPECIFICITY[a]

| Substrate | Substrate Added (cpm) | Substrate Remaining (cpm) | Product Formed (cpm) | Conversion (%) |
|---|---|---|---|---|
| Androst-4-ene-3,17-dione | 8050 | 7745 | 555 | 7[b] |
| Pregn-4-ene-3,20-dione | 6815 | 5725 | 1057 | 7[b] |
| 21-Hydroxypregn-4-ene-3,20-dione | 6300 | 5705 | 892 | 9[b] |
| Cholest-4-en-3-one | 7800 | 7705 | 0 | 0 |
| Cholest-5-en-3β-ol | 6505 | 6422 | 0 | 0 |
| 3β-Hydroxyandrost-5-en-17-one | 7100 | 6785 | 0 | 0 |
| Estra-1,3,5(10)-triene-3,17β-diol | 6825 | 6785 | 0 | 0 |

[a] The assays were performed in 2 ml of 0.03 M phosphate buffer, *p*H 7.6, with 0.06 μmole of $NADPH_2$, 0.3 mg of protein, and different radioactive substrates as shown. The reaction time was 5 minutes at 25°. [b] The specific activity of androst-4-ene-3,17-dione was 22 mc/mmole, and of pregn-4-ene-3,20-dione was 46 mc/mmole, and 21-hydroxypregn-4-ene-3,20-dione was 35 mc/mmole, which must be taken into consideration.

## DISCUSSION

Because little success has attended attempts in obtaining active cell-free extracts from microorganisms capable of introducing hydroxyl groups into steroids, the properties of the 9α-hydroxylase were extensively studied with a view of providing background information which might be useful in the investigation of other steroid hydroxylases. Furthermore, before any solubilization and fractionation of the 9α-hydroxylase system can be made, its properties must be thoroughly known in order that one may manipulate the enzyme with a minimal loss of hydroxylase activity. The 9α-hydroxylase reported herein provides an active system which now could be examined with respect to its biochemical mechanism.

Two key factors were essential in obtaining the active 9$\alpha$-hydroxylase preparation. These were the time of induction and the removal of the endogenous inhibitor; other factors were not as critical. The addition of $NADH_2$ or $NADPH_2$ at $10^{-5}$ M gave a definite stimulation of 9$\alpha$-hydroxylase activity but substantial activity was noted even in their absence. It is highly probable that the stimulation by reduced pyridine nucleotides may become more pronounced and specific when the enzyme preparation is in a more purified state. Zuidweg *et al.* (1962) observed that with crude preparations from *C. lunata* both $NADH_2$ and $NADPH_2$ gave a slight stimulation of 11$\beta$-hydroxylase activity, whereas in partially purified preparations only $NADPH_2$ stimulated the reaction. The 9$\alpha$-hydroxylase system compares favorably with other hydroxylase systems in terms of its specific activity (m$\mu$moles of product formed per hour per mg of protein). The 11$\beta$-hydroxylase preparation from *C. lunata* (Zuidweg *et al.*, 1962) gave a specific activity of 15 after ammonium sulfate fractionation. The mammalian 11$\beta$-hydroxylate preparation from adrenal mitochondria (Sharma *et al.*, 1962) showed a specific activity of 12. Our 9$\alpha$-hydroxylase preparation after separation from its endogenous inhibitor has a specific activity of 20.

It is interesting to note that the 9$\alpha$-hydroxylase was capable of catalyzing the epoxidation of androsta-4,9-(11)-diene-3,17-dione. This result is in good agreement with the data of Kurosawa *et al.* (1961), who showed that the 11$\beta$-hydroxylase system from adrenal mitochondria could also convert 17$\alpha$,21-dihydroxypregna-4,9(11)-diene-3,20-dione into 9$\beta$,11$\beta$-oxido-17$\alpha$,-21-dihydroxypregn-4-ene-3,20-dione in low yields. Although it always has been assumed that the hydroxylase and epoxidase systems are similar, one could not preclude the possibility that two distinct enzyme systems exist for hydroxylation and epoxidation, especially in view of the crude preparations used. However, since androsta-4,9(11)-diene-3,17-dione has now been shown to be a competitive inhibitor of the 9$\alpha$-hydroxylase, it leaves little doubt that both epoxidation and hydroxylation must take place on the same enzyme site, which further strengthens the theory of Bloom and Shull (1955). Cell-free extracts obtained from cells induced with androsta-4,9(11)-diene-3,17-dione were incapable of introducing an 9$\alpha$-hydroxyl group into androst-4-ene-3,17-dione. This result establishes the fact that the epoxidase system is not identical to the hydroxylase system. From a chemical viewpoint, it is not at all surprising in that a higher activation energy is required for an $OH^{\oplus}$ species to displace the 9$\alpha$ proton as compared to its reaction with the $\pi$ electrons in the olefin. Biochemically speaking, it appears that the 9$\alpha$-hydroxylase system probably consists of all the enzymes and coenzymes required for epoxidation plus one or more enzyme(s) and coenzyme(s) to displace the 9$\alpha$ proton, for we have been able to selectively destroy the 9$\alpha$-hydroxylase activity while retaining the 9$\alpha$,11$\alpha$-epoxidase activity with the hydroxylase preparation.

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