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PURIFICATION AND SOME PROPERTIES OF CHOLESTEROL 20α-HYDROXYLASE FROM HOG ADRENAL MITOCHONDRIA

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SUMMARY

1. A method has been developed for the determination of cholesterol 20α-hydroxylase complex using [4-^{14}C]cholesterol and 20α-hydroxy[7α-^{3}H]cholesterol.

2. An approx. 50-fold purification of the cholesterol 20α-hydroxylase complex from acetone-dried mitochondria of hog adrenal cortex was accomplished by $(NH_4)_2SO_4$ fractionation, Sephadex G-200 and DEAE-cellulose column chromatography.

3. Addition of non-heme iron-protein as well as NADPH to the incubation mixture was essential for both the 20α- and 22ξ-hydroxylation of cholesterol.

4. The activities of cholesterol 22ξ-hydroxylase and cholesterol C-20–C-22-lyase were not separated from the activity of cholesterol 20α-hydroxylase during the purification of the enzyme.

5. The K_m value of the cholesterol 20α-hydroxylase for cholesterol was estimated to be $0.57 \cdot 10^{-5}$ M at pH 7.4 in the presence of optimum amounts of NADPH and non-heme iron-protein.

6. The influence of pH, metal ions and pregnenolone on the activity of cholesterol 20α-hydroxylase was examined.

INTRODUCTION

The hydroxylation of cholesterol at the C-20α position seems to be a particularly important step in the biosynthesis of steroid hormones by endocrine organs, since it has been reported that the regulatory mechanism(s) involving ACTH and gonadotrophic hormones in steroidogenesis is primarily related to this step[1–3]. The requirement of a flavoprotein, non-heme iron-protein and cytochrome P450 for the activity of steroid 11β-hydroxylase (EC 1.14.1.6) has been elucidated by OMURA *et al.*[4]. However, little is known about the enzymatic properties of cholesterol 20α-hydroxylase

* Trivial names used: pregnenolone, 3β-hydroxy-pregn-5-ene-20-one; progesterone, pregn-4-ene-3,20-dione; deoxycorticosterone, 21-hydroxy-pregn-4-ene-3,20-dione; corticosterone, 11β, 21-dihydroxy-pregn-4-ene-3,20-dione.

because no method for the determination of the activity of this enzyme has been available.

In this report, a method for the determination of cholesterol 20α-hydroxylase will be described, together with some properties of this enzyme in a partially purified preparation from acetone-dried hog adrenal mitochondria.

EXPERIMENTAL

[4-^{14}C]Cholesterol (40 μC/μmole) was purchased from New England Nuclear Corp. 20α-Hydroxy[7α-^{3}H]cholesterol (10.9 mC/μmole), 20α-hydroxycholesterol were generously supplied by Dr. K. SHIMIZU, Tottori University, Yonago, by Dr. M. GUT, Worcester Foundation for Experimental Biology, Shrewsbury, Mass. These sterols were purified by paper chromatography before use.

Preparation of acetone-dried mitochondria from hog adrenal cortex

Hog adrenal glands, obtained at slaughter, were placed in crushed ice and transported to the laboratory. The cortex was separated from medulla and the acetone-dried powder of cortical mitochondria was prepared according to the procedure described by HALKERSTONE, EICHHORN AND HECHTER[5].

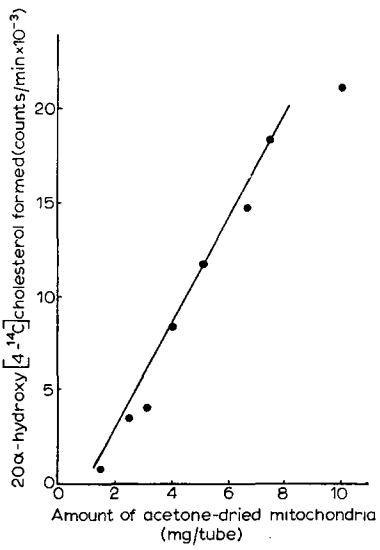
Determination of 20α-hydroxylase activity

Difficulties which are encountered in determining the activity of 20α-hydroxylase lie in the fact that the 20α-hydroxycholesterol derived from cholesterol by the action of 20α-hydroxylase is subsequently transformed to 20α, 22ξ-dihydroxycholesterol[6], pregnenolone[5] and various steroid hormones. The method for determination of 20α-hydroxylase activity explored in the present study is based on measurements of total 20α-hydroxy[^{14}C]cholesterol formed from [^{14}C]cholesterol (substrate) during the incubation. The loss of 20α-hydroxy[^{14}C]cholesterol due to the subsequent biotransformation was corrected for by the recovery of internal standard: a known amount of 20α-hydroxy[^{3}H]cholesterol added to the incubation system. Assuming that the formation of 20α-hydroxy[^{14}C]cholesterol and the disappearance of 20α-hydroxycholesterol in the incubation mixture occur at constant rates and that the 20α-hydroxy[^{14}C]cholesterol formed and the 20α-hydroxy[^{3}H]cholesterol added are mixed immediately and evenly with each other during incubation, the total amount of 20α-hydroxy[^{14}C]cholesterol formed from [^{14}C]cholesterol during the incubation period could be calculated by the following equation:

$$\text{20α-hydroxy[}^{14}\text{C]cholesterol formed} = \frac{\text{20α-hydroxy[}^{3}\text{H]cholesterol added}}{\text{20α-hydroxy[}^{3}\text{H]cholesterol recovered after incubation}} \times {}^{14}\text{C recovered as 20α-hydroxycholesterol}$$

In the standard assay conditions, each incubation tube contained, besides 0.2 ml [4-^{14}C]cholesterol suspension (100 000 counts/min, 10 μg) suspended in water with the aid of Tween 80 (ref. 7) and 0.2 ml of enzyme solution, the following additions; 200 mμmoles of NADPH, 1.5 mg of non-heme iron-protein fraction, 0.5 ml of 50 mM phosphate or Tris–HCl buffer (pH 7.4) in a total volume of 1.8 ml. The amount of enzyme used for the incubation was between 150 and 200 μg protein per tube, depending upon the specific activity of the preparations. Incubations were carried out at 37°

in air with gentle shaking. 1 min after starting the incubation, when the rate of formation of 20α-hydroxy[^{14}C]cholesterol was assumed to have reached a constant rate, 0.2 ml of 20α-hydroxy[^{3}H]cholesterol suspension (10 000 counts/min, 0.5 μg) which had been preheated at 37°, was pipetted into each tube and the incubation was allowed to continue for 30 min. The concentration of 20α-hydroxycholesterol in the incubation medium may have changed during the incubation and the change in the concentration of 20α-hydroxycholesterol may consequently have resulted in changes in the rate of its disappearance. Furthermore, the time of adding 20α-hydroxy[^{3}H]cholesterol could not be determined on a theoretical basis. However, it was necessary to establish experimental conditions which afforded a linear relationship between the rate of reaction and enzyme concentrations (Fig. 1). Addition of 20α-hydroxycholesterol to the incubation system resulted in the "product inhibition" of 20α-hydroxylase (Fig. 2).



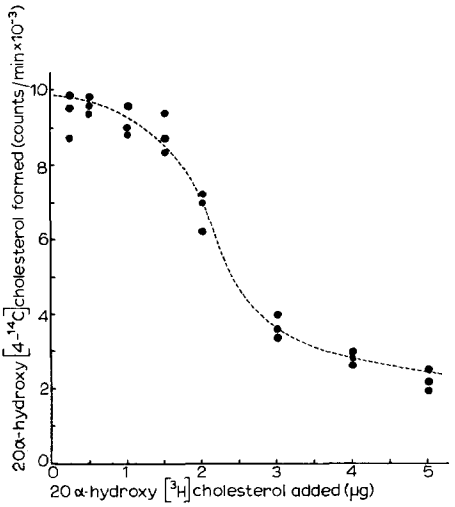


Fig. 1. Amount of acetone-dried mitochondria and the rate of formation of 20α-hydroxy[4-^{14}C]-cholesterol. The mitochondrial acetone-powder was suspended in 50 mM phosphate buffer (pH 7.4) and added to the incubation mixture. Other incubation conditions and the calculation of the rate of 20α-hydroxy[4-^{14}C]cholesterol formation are described in the text.

Fig. 2. Influence of various amounts of 20α-hydroxy[7α-^{3}H]cholesterol added to the incubation mixture on the activity of cholesterol 20α-hydroxylase. Additions and incubation conditions are described in the text. 1 μg of 20α-hydroxy[7α-^{3}H]cholesterol contains radioactivity of 20 000 counts/min.

However, no significant influence of 0.5 μg 20α-hydroxycholesterol, which was the usual amount used for an incubation for enzyme activity, was observed. Thus it was determined that the trapping agent, 0.5 μg 20α-hydroxy[7α-^{3}H]cholesterol, should be added 1 min after the initiation of incubation. At the end of the incubation period, 10 ml of diethyl ether–benzene mixture (4:1, v/v) was added to stop the reaction and to extract the products. The extraction was repeated twice and the combined extract was evaporated to dryness. The residue was subjected to thin-layer chromatography on silica gel in hexane–ethyl acetate (6:1, v/v). 500 μg of unlabeled cholesterol and

TABLE I

RADIOCHEMICAL PURITY OF THE 20α-HYDROXYCHOLESTEROL ISOLATED AND PURIFIED FROM THE INCUBATION

Samples from the several incubations were pooled and further purified. An aliquot was taken after each purification and amounts of both ^{14}C and ^{3}H were determined.

Samples from	^{14}C *(counts/min)*	^{3}H *(counts/min)*	$^{14}C/^{3}H$
2nd thin-layer chromatography	2005	3439	0.583
Paper chromatography in Bush's A system	1507	2870	0.525
Paper chromatography in ligroin–propylene glycol system	581	1082	0.537
Paper chromatography in heptane–ethylene glycol monophenyl ether system	466	861	0.541

200 μg of unlabeled 20α-hydroxycholesterol were added to each sample before chromatography to ensure the separation of the sterols. After scraping the 20α-hydroxycholesterol area into a glass-stoppered tube, the silica gel was extracted twice with 10 ml of methylene chloride–methanol (2:1, v/v). 500 μg of unlabelled cholesterol was again added to each extract and the extracts were applied to another thin-layer chromatography plate developed in methylene chloride–diethyl ether (6:1, v/v). The 20α-hydroxycholesterol area was extracted as before and subjected to paper chromatography in Bush's A system. The zone corresponding to the authentic 20α-hydroxycholesterol was eluted with the methylene chloride–methanol mixture, transferred to a counting vial and evaporated. The radioactivity of ^{14}C and ^{3}H in the samples was determined in a Tri-Carb liquid-scintillation spectrometer after adding 10 ml toluene containing 4% 2,5-diphenyloxazole and 0.01 % 1,4-bis-(5-phenyloxazolyl)-benzene.

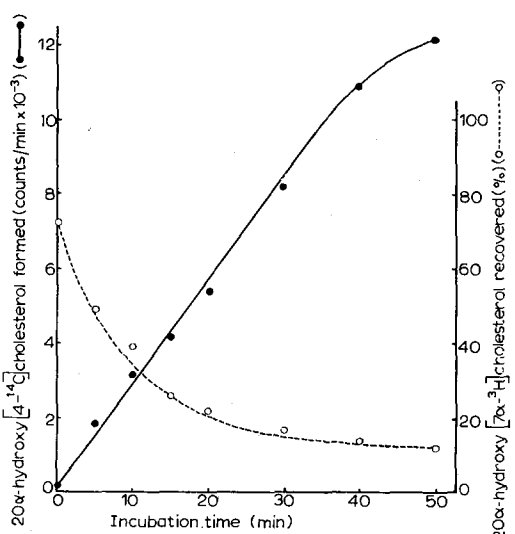


Fig. 3. Formation of 20α-hydroxy[4-^{14}C]cholesterol and the recovery rate of 20α-hydroxy[7α-^{3}H]-cholesterol added to the incubation mixture with time. Additions and incubation conditions are described in the text. ●—●, 20α-hydroxy[^{14}C]cholesterol formed; ○- - - -○, 20α-hydroxy[^{3}H]-cholesterol recovered.

The radiochemical purity of 20α-hydroxycholesterol thus isolated from the incubation mixture was determined by the following method: Pooled material from several incubations was subjected to paper chromatography first in ligroin–propylene glycol and then in heptane–ethylene glycol monophenyl ether system. The zone corresponding to the authentic 20α-hydroxycholesterol was eluted. Aliquots of the eluate were retained after each paper chromatography for measurements of radioactivity. No significant change in ^{14}C to ^{3}H ratio was observed between the samples before and after the second paper chromatography (Table I).

The linear relationship between the amount of enzyme and the rate of formation of 20α-hydroxy[^{14}C]cholesterol was observed up to 8 mg of acetone-dried mitochondria per incubation (Fig. 1). The time course of cholesterol 20α-hydroxylation and the rate of disappearance of added 20α-hydroxy[^{3}H]cholesterol are depicted in Fig. 3. It can be noted that the time course of the enzymatic reaction was almost linear at least for 30 min.

Determination of 22ξ-hydroxylase activity

The activity of 22ξ-hydroxylase was determined by measuring the rate of disappearance of the added substrate, 20α-hydroxy[7α-^{3}H]cholesterol during a 15-min incubation. At the end of the incubation, the mixture was extracted with 10 ml of diethyl ether–benzene (4:1, v/v) three times and the remaining 20α-hydroxy[^{3}H]cholesterol was purified by paper chromatography in Bush's A system. Control incubation without the addition of NADPH was performed at the same time and the difference in recovered 20α-hydroxy[^{3}H]cholesterol between these two incubations was taken as the 22ξ-hydroxylase activity. The incubation mixture was the same as that described for 20α-hydroxylase, except that 20 000 counts/min (10 μg) of 20α-hydroxy-[7α-^{3}H]cholesterol was used as the substrate.

RESULTS

Purification of 20α-hydroxylase

Acetone-dried mitchondria were extracted with ice-cold, 50 mM KCl containing 1 mM EDTA and centrifuged at 105 000 × *g* for 20 min. The resultant clear supernatant was fractionally precipitated with $(NH_4)_2SO_4$. The 40–60% $(NH_4)_2SO_4$ fraction was dissolved in a small volume of 10 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, and subjected to chromatography on a Sephadex column. 2 g of Sephadex G-200 was thoroughly washed with deionized water followed by 10 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and packed in a glass column (10 mm × 900 mm). The column was loaded with 40–60% $(NH_4)_2SO_4$ fraction, eluted with the phosphate buffer and 1-ml fractions were collected. Most of the 20α-hydroxylase activity was observed in the first few fractions. The fractions which contained most of the enzyme activity were combined and passed through a column of DEAE-cellulose (10 mm × 200 mm) which had been previously equilibrated with 10 mM phosphate buffer (pH 7.4). The column was eluted stepwise with increasing concentrations of phosphate buffer (pH 7.4) and KCl. The enzyme was eluted after washing the column with 100 mM phosphate buffer containing 200 mM KCl.

The results of a typical purification experiment are summarized in Table II. Usually, an overall purification of 40 to 50-fold could be obtained. The preparation

TABLE II

PURIFICATION OF CHOLESTEROL 20α- AND 22ξ-HYDROXYLASE FROM ACETONE-DRIED HOG ADRENAL MITOCHONDRIA

1.8 g acetone-dried mitochondria were used as the starting material.

Fraction	*Protein (mg)*	*20α-Hydroxylase (units*/mg protein)*	*22-Hydroxylase (units*/mg protein)*
1. 105 000 × *g* supernatant	1170	2.4	1.8
2. 40–60% $(NH_4)_2SO_4$ fraction	114	20.2	8.2
3. Sephadex G-200 fraction	43	42.2	23.5
4. DEAE-cellulose fraction	6.8	105.9	49.6

* A unit of activity of the enzymes was defined tentatively as the amount of enzyme which converts 1% of added substrate under the conditions described in the text.

still possessed 22ξ-hydroxylase activity and the specific activity of this enzyme was also increased throughout the purification.

Identification of products

To identify products formed after the incubation of the enzyme preparation with [4-^{14}C]cholesterol, 630 μg of DEAE-cellulose eluate and 1.8 mg of Sephadex G-200 eluate (in terms of protein) were incubated with [4-^{14}C]cholesterol (100 000 counts/min, 5.0 μg) in the presence of 2.0 mg of non-heme iron-protein fraction and 0.9 μmole NADPH for 1 h, respectively. After the incubation, the mixture was extracted with 10 ml of diethyl ether–benzene mixture (4:1, v/v), followed by 10 ml of methylene chloride. The extracts were combined and evaporated. The residue was applied to paper chromatography in Bush's A system. The radioscanograms of these incubation products are presented Figs. 4 and 5. When [4-^{14}C]cholesterol was incubated with the preparation from the Sephadex column, two major products were observed on the scanogram and these were identified as pregnenolone and progesterone by the isotope dilution method. Only one product, pregnenolone, was identified in the incubation with the preparation from the DEAE-cellulose column (Fig. 5). The

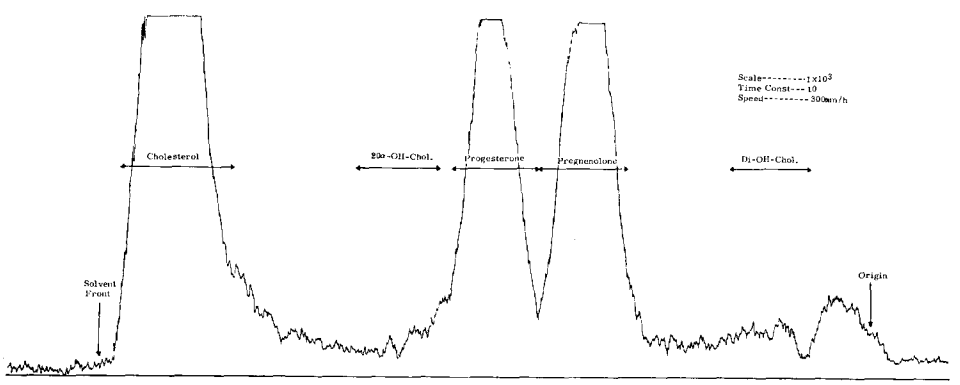


Fig. 4. Radioscanogram of products in the incubation of the Sephadex G-200 preparation with [4-^{14}C]cholesterol. The horizontal arrows in the figure indicate the location of authentic samples.

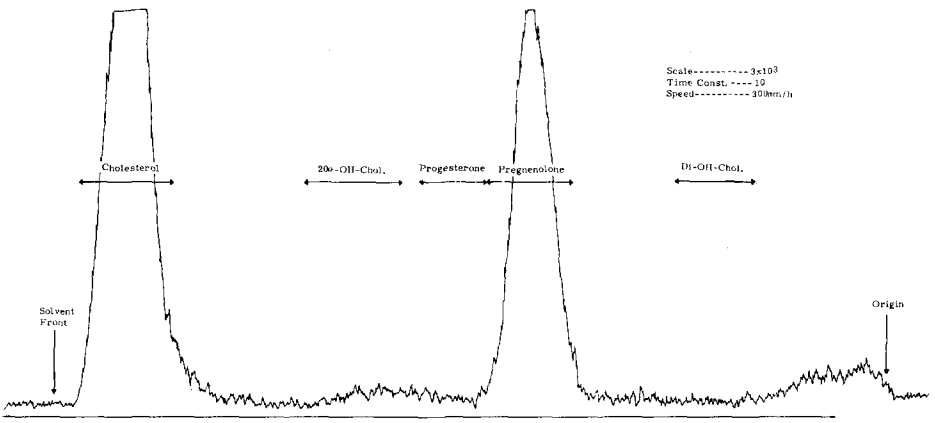


Fig. 5. Radioscanogram of products in the incubation of the DEAE-cellulose preparation with [4-^{14}C]cholesterol. The horizontal arrows in the figure indicate the location of authentic samples.

polar materials located close to the origin of the chromatogram in the case of the incubation with the Sephadex preparation was rerun in toluene–propylene glycol system. However, no peak of radioactivity corresponding to the authentic 11β-hydroxyprogesterone was observed. These results suggested that the Sephadex preparation contained 20α-hydroxylase, 22ξ-hydroxylase, C-20–C-22-lyase and Δ^5,3β-hydroxysteroid dehydrogenase (EC 1.1.1.51) but not steroid 11β-hydroxylase. The subsequent application of DEAE-cellulose column chromatography eliminated Δ^5,3β-hydroxysteroid dehydrogenase but not 22ξ-hydroxylase and C-20–C-22-lyase.

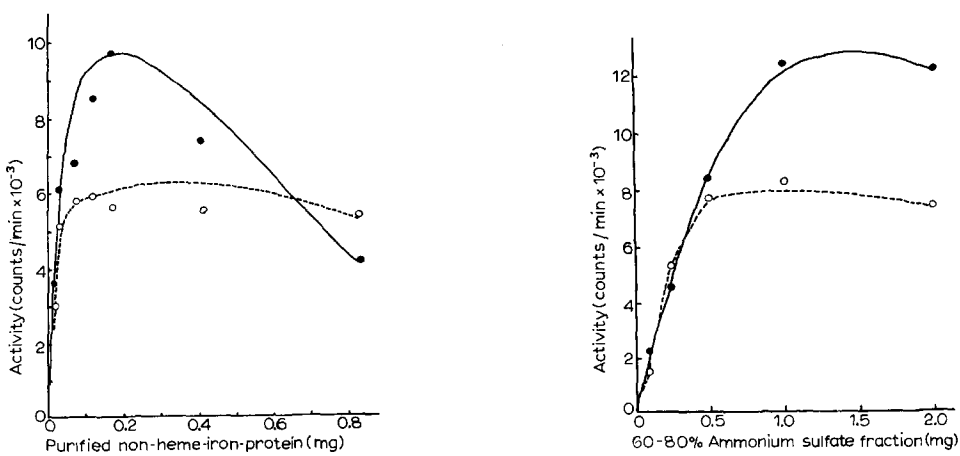


Fig. 6. Effect of various amounts of purified non-heme iron-protein on the activity of cholesterol 20α- and 22ξ-hydroxylase. ●—●, 20α-hydroxylase; ○- - - -○, 22ξ-hydroxylase.

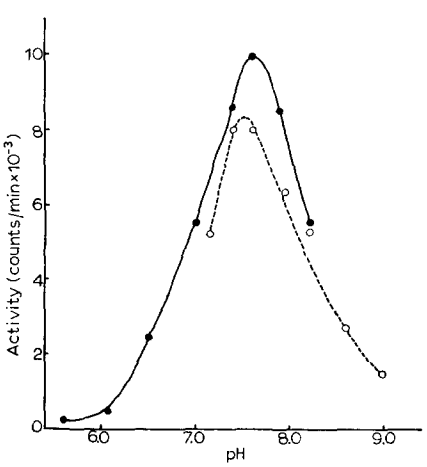
Fig. 7. Effect of various amounts of the 60–80% $(NH_4)_2SO_4$ fraction on the activity of cholesterol 20α- and 22ξ-hydroxylase. The 60–80% $(NH_4)_2SO_4$ fraction of the extract from acetone-dried mitochondria of adrenal cortex was desalted by passage through a Sephadex G-25 column. The additions and the incubation conditions are described in the text. ●—●, 20α-hydroxylase; ○- - - -○, 22ξ-hydroxylase.

The absence of 11β-hydroxylase in the preparation from DEAE-cellulose was further confirmed by incubating the preparation with deoxy[4-^{14}C]corticosterone in the presence of NADPH and non-heme iron-protein. No significant amount of corticosterone was observed.

Requirement of non-heme iron-protein for the activity of 20α- and 22ξ-hydroxylase

The influence of non-heme iron-protein purified by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose column chromatography[8] (adrenodoxin, kindly supplied by Dr. T. KIMURA, St. Paul's University, Tokyo) on the activity of these enzymes was examined. As shown in Fig. 6, complete dependence of the enzyme activity on the added non-heme iron-protein was observed. However, higher concentrations of non-heme iron-protein seemed to be somewhat inhibitory to the activity of 20α-hydroxylase but not to 22ξ-hydroxylase.

The non-heme iron-protein required for the enzyme activity should be replaced by a protein fraction from adrenal mitochondrial extract precipitated with 60–80% saturation of $(NH_4)_2SO_4$. The 60–80% fraction itself exhibited neither 20α- nor 22ξ-hydroxylase activity but the dependence of these enzymes on the presence of this fraction in the incubation system is evident (Fig. 7).



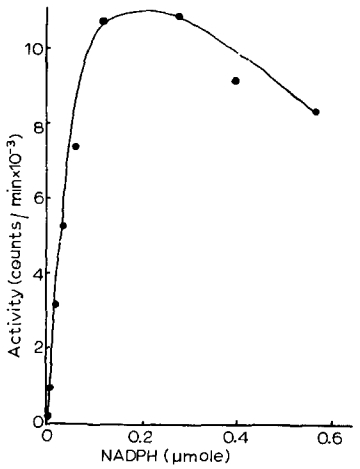


Fig. 8. Effect of pH on the activity of cholesterol 20α-hydroxylase. ●—●, 50 mM phosphate buffer; ○- - - -○, 50 mM Tris-HCl buffer.

Fig. 9. Effect of various concentrations of NADPH on the activity of cholesterol 20α-hydroxylase.

Effect of pH

The effect of pH on the activity of 20α-hydroxylase was determined in the pH range between 5.60 and 9.05, using phosphate buffer (pH 5.60–8.20) and Tris–HCl buffer (pH 7.15–9.05). The optimum pH for the enzyme activity was 7.4 (Fig. 8).

Effect of NADPH

The effect of various concentrations of NADPH on the activity of 20α-hydroxylase is shown in Fig. 9. Under the experimental conditions used here, the optimum

concentration of NADPH was 0.1 mM. Higher concentrations of NADPH were found to be inhibitory.

Effect of substrate concentration

Fig. 10 shows the LINEWEAVER–BURK plot[9] for 20α-hydroxylase. The K_m value obtained from the figure was $0.57 \cdot 10^{-5}$ M for cholesterol.

Effect of pregnenolone

The effect of the addition of pregnenolone to the incubation system on the activity of 20α-hydroxylase was examined. It may be seen in Fig. 10 that the addition of 10 μg pregnenolone inhibited the activity of the enzyme but did not alter its K_m value. Similar non-competitive-type inhibition by pregnenolone has also been observed by KORITZ AND HALL[3] in a cholesterol side-chain cleavage system from bovine adrenal. The addition of pregnenolone up to 40 μg per tube did not produce any in-

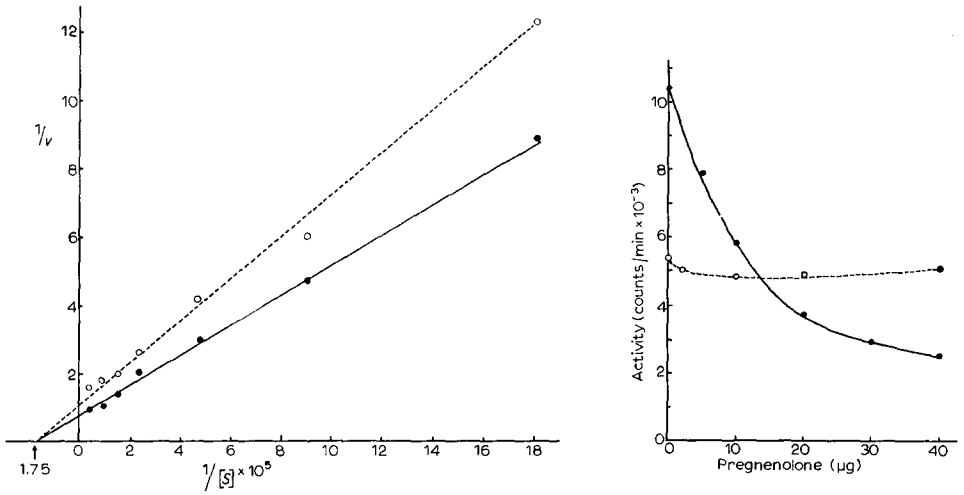


Fig. 10. LINEWEAVER–BURK plot for the 20α-hydroxylation of cholesterol. Pregnenolone was added to the incubation tube in benzene and evaporated to dryness prior to adding the other components for the incubation. ○- - - - -○, incubated in the presence of 10 μg pregnenolone per tube.

Fig. 11. Effect of pregnenolone added to the incubation mixture on the activity of cholesterol 20α- and 22ξ-hydroxylase. Pregnenolone was added to the incubation tube in benzene and evaporated to dryness prior to adding other components for the incubation. ●—●, 20α-hydroxylase; ○- - - - -, 22ξ-hydroxylase.

hibitory effect on the activity of 22ξ-hydroxylase, while about 70% of 20α-hydroxylase was inhibited by the same amount of pregnenolone (Fig. 11). Thus, the non-competitive inhibition of cholesterol side-chain cleavage by pregnenolone might be attributable to the inhibition of 20α-hydroxylase.

Effect of various metal ions and EDTA

The effect of several divalent cations on the enzyme activity was examined (Table III). Ca^{2+}, Mg^{2+} and Mn^{2+} were stimulatory, while Fe^{2+}, Hg^{2+}, Co^{2+}, Cu^{2+} and

TABLE III

EFFECT OF METAL IONS AND EDTA ON THE ACTIVITY OF 20α-HYDROXYLASE
Tris–HCl buffer (pH 7.4) was used.

Addition	*Concentration (M)*	*Effect**
Mg^{2+}	10^{-2}	+74
	10^{-3}	+101
Mn^{2+}	10^{-2}	+47
	10^{-3}	+128
Ca^{2+}	10^{-3}	+101
Co^{2+}	10^{-3}	−61
Hg^{2+}	$2 \cdot 10^{-3}$	−91
	$5 \cdot 10^{-4}$	−67
Zn^{2+}	$2 \cdot 10^{-3}$	−89
	$5 \cdot 10^{-4}$	−80
Fe^{2+}	$5 \cdot 10^{-4}$	−41
Fe^{3+}	$5 \cdot 10^{-4}$	−67
Cu^{2+}	$2 \cdot 10^{-4}$	−93
EDTA	$5 \cdot 10^{-3}$	−35
	$5 \cdot 10^{-4}$	+57
Cu^{2+} and EDTA	$2 \cdot 10^{-4}$ and 10^{-3}	−45
	$2 \cdot 10^{-4}$ and $5 \cdot 10^{-4}$	−11

* +, percent of stimulation of the activity over the control; −, percent of inhibition.

Zn^{2+} were very inhibitory at the concentration of $5 \cdot 10^{-4}$ M. Addition of EDTA at $5 \cdot 10^{-4}$ M enhanced the activity, but at $5 \cdot 10^{-3}$ M, EDTA was rather inhibitory. EDTA restored the activity of the enzyme from Cu^{2+} inhibition.

DISCUSSION

Many investigations have been performed to evaluate the nature of the side-chain cleavage of cholesterol in homogenates and soluble preparations from corpus luteum[1,10], adrenal[5,11], and testis[2,12]. However, none of these has been concerned with the purification and examination of properties of the individual enzymes in the system.

It has been shown that the activity of cholesterol 20α-hydroxylase in a system for the successive biotransformation of cholesterol can be assessed by adding 20α-hydroxy[7α-^{3}H]cholesterol to the incubation mixture as a trapping agent and as an internal standard for recovery correction. Using this method, partial purification and examination of some of the properties of cholesterol 20α-hydroxylase from hog adrenal mitochondria was possible.

The requirement of non-heme iron-protein for the activity of steroid 11β-hydroxylase in adrenal has been reported[4,13]. MASON[14] introduced the term mixed function oxidase for enzymes mediating this type of reaction and the participation of flavoprotein and cytochrome P450 as well as non-heme iron-protein and NADPH in a preparation from bovine adrenal mitochondria was described by OMURA *et al.*[4]. An attempt to purify the flavoprotein from bovine adrenal mitochondria was also made by the same authors[4]; however, the enzyme component which determines the carbon to be hydroxylated had not been investigated. In this study, the requirement

of non-heme iron-protein for the activity of both cholesterol 20α- and 22ξ-hydroxylase of hog adrenal mitochondria was established and thus the hydroxylation of cholesterol side-chain prior to the formation of C-21 steroids seems to be of the "mixed function" type.

The fact that the activity of 22ξ-hydroxylase and C-20–C-22-lyase was not separated from the 20α-hydroxylase fraction during the purification may suggest either that these components in the cholesterol side-chain cleaving system are very similar to each other in their protein nature or that they may possibly be very tightly associated with each other.

While this manuscript was in preparation, a brief report by BRYSON AND KAISER[15] appeared demonstrating the requirement of non-heme iron-protein and cytochrome P450 for the side-chain cleavage of cholesterol in bovine adrenal mitochondria. We did not obtain any evidence for the contribution of cytochrome P450 in cholesterol side-chain hydroxylation. However, possibilities that this substance adheres to some fraction in the system were not excluded, since the treatment with organic solvent solubilizes liver microsomal cytochrome P450 to form cytochrome P420 (ref. 16). but the behavior and functions of the soluble cytochrome P420 from adrenal mitochondria in the electron-transfer system of steroid hydroxylation, has not been clarified.

ACKNOWLEDGEMENTS

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