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CYTOCHROME P-450$_{scc}$-CATALYZED PRODUCTION OF PROGESTERONE FROM 22R-HYDROXYCHOLEST-4-EN-3-ONE BY WAY OF 20,22-DIHYDROXYCHOLEST-4-EN-3-ONE

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Summary—Transient accumulation of a dihydroxylated steroid was found when 22R-hydroxycholest-4-en-3-one was used as the substrate for a reconstituted cholesterol side-chain cleavage system derived from bovine adrenocortical mitochondria. The indications were that the accumulated steroid was an intermediate in the cytochrome P-450$_{scc}$-catalyzed reaction. The retention time of the accumulated intermediate was identical with that of authentic 20,22-dihydroxycholest-4-en-3-one on HPLC. When 22R-hydroxycholesterol and 22R-hydroxycholest-4-en-3-one were incubated simultaneously, the total amount of reaction products was essentially the same as that observed with 22R-hydroxycholest-4-en-3-one alone. Under the conditions employed, the apparent turnover number of cytochrome P-450$_{scc}$ for 22R-hydroxycholesterol was calculated to be 77 nmol/min/nmol P-450 from the amount of pregnenolone formed, whereas the apparent turnover number for 22R-hydroxycholest-4-en-3-one was 64 nmol/min/nmol P-450 with respect to the intermediate formation and 77 nmol/min/nmol P-450 with respect to the progesterone formation. The apparent turnover number for 20,22-dihydroxycholest-4-en-3-one was about 125 nmol/min/nmol P-450, which was not significantly different from that of 20,22-dihydroxycholesterol. The apparent K_m for 22R-hydroxycholesterol was about 20 μM and those for 22R-hydroxycholest-4-en-3-one and 20,22-dihydroxycholest-4-en-3-one were 50 and 40 μM, respectively. Thus, 22R-hydroxycholest-4-en-3-one was efficiently metabolized to progesterone by way of 20,22-dihydroxycholest-4-en-3-one by cytochrome P-450$_{scc}$.

INTRODUCTION

Cytochrome P-450$_{scc}$ of adrenocortical mitochondria is known to catalyze the side-chain cleavage of cholesterol to produce pregnenolone, which is the initial step in the biosynthesis of various steroid hormones [1–3]. The most probable mechanism of this reaction is hydroxylation, occurring first at the C-22 position of cholesterol, then at the C-20 position, and finally cleavage of the C20–C22 carbon bond ([4] and Scheme 1A). It is also widely accepted that this reaction is regioselective and stereospecific [4]. Hume *et al.* [5] and others [6, 7] have suggested that 22R-hydroxycholesterol and 20α,22R-dihydroxycholesterol are physiological intermediates in the cytochrome P-450$_{scc}$-catalyzed reaction.

However, since both intermediates are tightly bound to the enzyme and since the first hydroxylation is the rate-limiting step [8], these intermediates have not been detected so far in the reconstituted system. Furthermore, it is known that other hydroxylated cholesterol derivatives, such as 20α-hydroxycholesterol [9], 22S-hydroxycholesterol and 20α, 22S-dihydroxycholesterol [10], can be metabolized by cytochrome P-450$_{scc}$. Therefore, although the sequential monooxygenation reactions described above are the most probable, it remains to be established that 22R-hydroxycholesterol and 20α, 22R-dihydroxycholesterol are the physiological intermediates.

Use of substrate analogs has been found to be an efficient method to elucidate the cytochrome P-450$_{scc}$-substrate interactions and the catalytic mechanism. Using various cholesterol analogs with modified side-chains, the importance of the stereochemistry of the C-22R position for coordination to the heme has been underlined [11]. Sheets and Vickery [12] using 22-amino-23,24-bisnor-5-cholen-3β-ol specifically demonstrated that C-22R was positioned in closest proximity

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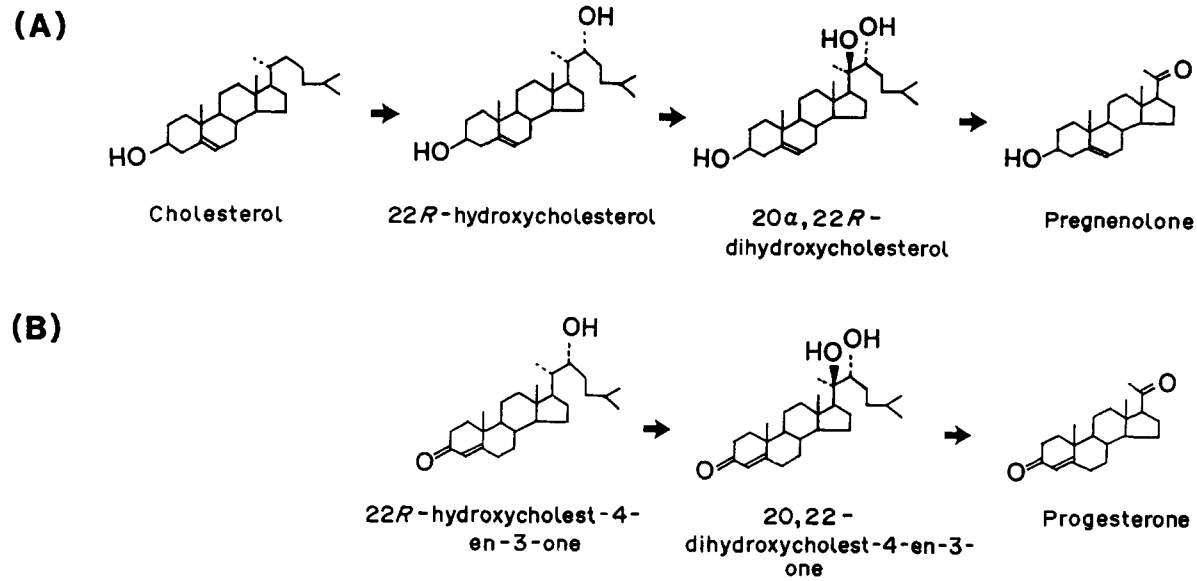


Scheme 1. Cytochrome P-450$_{scc}$-dependent metabolism of (A) cholesterol and (B) 22 R-hydroxycholest-4-en-3-one.

to the sixth coordination site of the heme-iron. Heyl *et al.* [13], using various cholesterol analogs with modified C-3 positions, showed that the 3β-hydroxyl group was critical for the binding of substrate to the enzyme. It may be assumed, therefore, that the 3β-hydroxyl group and the 5-androstene ring is recognized by the cholesterol binding site of cytochrome P-450$_{scc}$ and interacts by hydrogen bonding with a specific amino acid residue of this enzyme [13, 14].

Recently, we have developed a sensitive assay method for cytochrome P-450$_{scc}$ activity by HPLC, and this method made it easy to separate and collect the reaction products [15]. Furthermore, we found that 22R-hydroxycholest-4-en-3-one, a substrate analog, served as a good substrate for cytochrome P-450$_{scc}$ and that the 20,22-dihydroxyl derivative could be detected as a possible reaction intermediate in the reconstituted side-chain cleavage system.

In this report, we investigated the catalytic activity of cytochrome P-450$_{scc}$ for various substrate analogs modified to the corresponding 3-one-4-en forms. The results strongly suggest that, once the first hydroxylation has occurred at the C-22R position, the 3β-ol-5-en structure is not required for subsequent hydroxylation and lyase reactions. We also discuss the rationale for the accumulation of 20,22-dihydroxycholest-4-en-3-one.

EXPERIMENTAL

Materials

Most of the steroids used were purchased from Sigma (St Louis, Mo. U.S.A.). NADPH, glucose-6-phosphate (G6P), G6P dehydrogenase and cholesterol oxidase from *Nocardia* sp. were from Oriental Yeast Company (Japan). Other chemicals used were of the highest available grade from commercial sources. All solvents were of HPLC grade.

Preparation of enzymes

Cytochrome P-450$_{scc}$ was purified from bovine adrenocortical mitochondria in the presence of added cholesterol as described previously [16]. Adrenodoxin and adrenodoxin reductase were purified from bovine adrenal cortex according to the methods described by Suhara *et al.* [17] and Sugiyama and Yamano [18], respectively.

Instrumentation

The HPLC system for analysis of steroids employed was the same as that described previously [14]. For normal-phase HPLC, we used a TSK-gel silica-150 column (4 × 250 mm, Tosoh) with a mobile phase of n-hexane:isopropanol (100:2, v/v) at a flow rate of 1.5 ml/min. For reverse-phase HPLC, a TSK-gel ODS-80TM column (4 × 250 mm, Tosoh) was used with a solvent system of acetonitrile: isopropanol (15:1, v/v) at a flow rate of 0.8 ml/min.

Preparation of 3-one-4-en steroids

Cholesterol, 20α-hydroxycholesterol, 22S-hydroxycholesterol and 22R-hydroxycholesterol were converted into corresponding 3-one-4-en steroids by cholesterol oxidase treatment as

described previously [15]. The steroids were extracted from the reaction mixture with dichloromethane and applied to the normal phase HPLC system monitoring absorbance at 214 nm. The converted 3-one-4-en steroids were collected and purified by rechromatography to obtain a single peak. The amounts of steroid were estimated by the peak area with the integrator.

Assay for the activity of cytochrome P-450$_{scc}$

The cholesterol side-chain cleavage activity was measured in the reconstituted system fortified with a NADPH-generating system and electron transfer components according to the method reported previously [15] with the following modifications. The reaction was allowed to proceed for 5 min at 37°C. Steroids were extracted with dichloromethane and analyzed by normal-phase HPLC without pretreatment with cholesterol oxidase. The amount of each steroid was estimated by monitoring absorbance at 214 nm with deoxycorticosterone acetate as an internal standard. When the turnover number was measured, the addition of substrate was adjusted so as to obtain more than twice the concentration of the apparent K_m value for the respective substrate.

Preparation of the intermediate in the side-chain cleavage reaction

In order to obtain a sufficient amount of the intermediate, a large scale preparation was performed as follows: 22R-hydroxycholest-4-en-3-one (3 μmol) was incubated with cytochrome P-450$_{scc}$ (1 nmol) for 5 min at 37°C in 18 ml of 20 mM K-phosphate buffer (pH 7.4), containing 0.3%(v/v) Tween 20, NADPH (2 μmol), G6P (100 μmol), G6P dehydrogenase (10 units), $MgCl_2$ (80 μmol), adrenodoxin (20 nmol) and adrenodoxin reductase (20 nmol). The products were extracted three times with 70 ml of dichloromethane and then the solvent was evaporated off. The extracted material was applied to the normal-phase HPLC with a mobile phase of dichloromethane: ethanol: water (98: 1.8: 0.2, v/v/v) and the fraction showing a retention time (5.5 min) identical to that of 20,22-dihydroxycholest-4-en-3-one was collected. This intermediate was further purified by rechromatography to show a single peak using the normal-phase HPLC with a solvent system of n-hexane and isopropanol.

Analytical methods

Estimation of cytochrome P-450$_{scc}$ was carried out spectrophotometrically as described by Omura and Sato[19]. Concentrations of adrenodoxin and adrenodoxin reductase were calculated on the basis of ϵ mM 414 = 10 mM^{-1} cm^{-1} and ϵ mM 450 = 11 mM^{-1} cm^{-1}, respectively.

RESULTS

Formation of 20,22-dihydroxycholest-4-en-3-one as an intermediate in cytochrome P-450$_{scc}$-catalyzed reaction

When 22R-hydroxycholest-4-en-3-one was incubated with purified cytochrome P-450$_{scc}$ in the presence of adrenodoxin, adrenodoxin reductase and a NADPH-generating system, two product peaks appeared on the chromatogram of the normal-phase HPLC with n-hexane 100: isopropanol 2 as the mobile phase (Fig. 1A). On the basis of its retention time, one product (peak 2 in Fig. 1A) was identified as

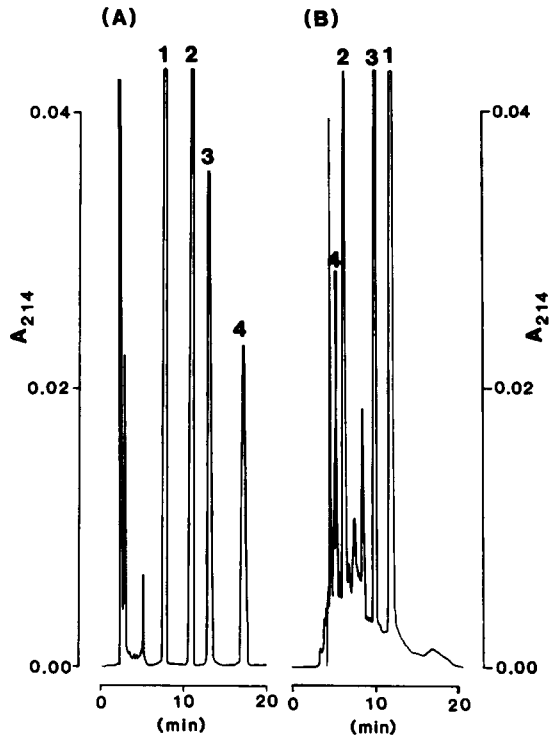


Fig. 1. HPLC profiles of the reaction products obtained with 22R-hydroxycholest-4-en-3-one as the substrate in the reconstituted cytochrome P-450$_{scc}$ system. (A) Normal-phase HPLC and (B) reverse-phase HPLC. 22R-Hydroxycholest-4-en-3-one (100 nmol) was incubated with cytochrome P-450$_{scc}$ (35 pmol) in the reconstituted system at 37°C for 5 min. (1) 22R-Hydroxycholest-4-en-3-one (2) progesterone, (3) 20,22-dihydroxycholest-4-en-3-one and (4) deoxycorticosterone acetate (the internal standard). The steroids were detected by monitoring at 214 nm and identified on the basis of their retention times.

progesterone, which was the final reaction product of side-chain cleavage of 22*R*-hydroxycholest-4-en-3-one. The retention time of the other product (peak 3 in Fig. 1A) was found to be identical with that of authentic 20,22-dihydroxycholest-4-en-3-one. When analyzed by reverse-phase HPLC with acetonitrile 15:isopropanol 1 as the mobile phase (Fig. 1B) and also by normal-phase HPLC with a mobile phase of dichloromethane:ethanol:water (98:1.8:0.2, v/v/v) (data not shown), the other product (peak 3) showed the same retention time as authentic 20,22-dihydroxycholest-4-en-3-one on the chromatograms. To confirm that this 20,22-dihydroxyl derivative is an intermediate of the cytochrome P-450$_{scc}$-catalyzed reaction, the following experiments were performed.

First, we examined the time courses of formation of 20,22-dihydroxycholest-4-en-3-one and progesterone. As shown in Fig. 2A, both of them linearly accumulated up to 2 min at the ratio of about 1:1. After that, the amount of 20,22-dihydroxycholest-4-en-3-one decreased in the reaction mixture and that of progesterone increased progressively. Therefore, it seemed probable that 20,22-dihydroxycholest-4-en-3-one was further metabolized to progesterone and the amount of the former decreased as the substrate was exhausted under the conditions employed. Also, both 20,22-dihydroxycholest-4-en-3-one and progesterone were formed within 30 s of incubation time and thus, a time lag in progesterone formation was not observed (data not shown).

Secondly, we examined the dependency of the formation of 20,22-dihydroxycholest-4-en-3-one and progesterone on the concentration of cytochrome P-450$_{scc}$. The accumulation of both steroids in 5 min was almost linear relative to the amount of cytochrome P-450$_{scc}$ when a relatively small amount of cytochrome P-450$_{scc}$ was added (Fig. 2B). When larger amounts of cytochrome P-450$_{scc}$ were added to the reaction mixture, however, the accumulation of 20,22-dihydroxycholest-4-en-3-one decreased and the total amount of the reaction products reached a plateau. This suggested that the substrate, 22*R*-hydroxycholest-4-en-3-one, was exhausted in 5 min by larger amounts of cytochrome P-450$_{scc}$ and that 20,22-dihydroxycholest-4-en-3-one was further metabolized to progesterone. These observations indicate that 22*R*-hydroxycholest-4-en-3-one, a substrate analog with a modified C-3 position, was metabolized by cytochrome P-450$_{scc}$ and 20,22-dihydroxyl derivative accumulated as an intermediate in the reconstituted system.

In order to investigate whether 3β-ol-5-en steroid and 3-one-4-en steroid were catalyzed on the same active site of cytochrome P-450$_{scc}$, we

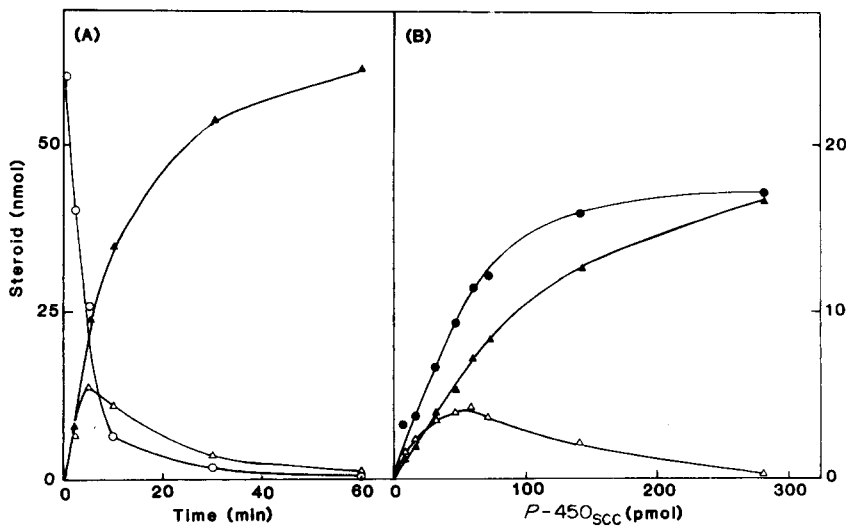


Fig. 2. (A) Time-courses of production of 20,22-dihydroxycholest-4-en-3-one and progesterone. 22*R*-Hydroxycholest-4-en-3-one (60 nmol) was incubated with cytochrome P-450$_{scc}$ (70 pmol) in the reconstituted system at 37°C for various periods as indicated. (B) Dependency of the formation of 20,22-dihydroxycholest-4-en-3-one and progesterone on the concentration of cytochrome P-450$_{scc}$. 22*R*-Hydroxycholest-4-en-3-one (18 nmol) was incubated for 5 min with varying amounts of cytochrome P-450$_{scc}$ in the reconstituted system. The products were analyzed by normal-phase HPLC as described in Experimental. The amounts of 22*R*-hydroxycholest-4-en-3-one (○), 20,22-dihydroxycholest-4-en-3-one (△), progesterone (▲) and the sum of 20,22-dihydroxycholest-4-en-3-one and progesterone (●) were estimated from the absorbance at 214 nm.

measured the activity with the simultaneous addition of 22R-hydroxycholesterol and 22R-hydroxycholest-4-en-3-one (Fig. 3). The addition of substrate was adjusted so as to obtain more than twice the concentration of the apparent K_m value for the respective substrate. When 22R-hydroxycholesterol (50 nmol) alone was incubated as a substrate for 5 min, the formation of pregnenolone was 10.6 nmol. When 22R-hydroxycholest-4-en-3-one (100 nmol) alone was incubated, the formation of 20,22-dihydroxycholest-4-en-3-one and progesterone was 7.8 nmol and 10.1 nmol, respectively. As shown in Fig. 3, however, the product formation was not additive when the two substrates, 22R-hydroxycholesterol (50 nmol) and 22R-hydroxycholest-4-en-3-one (100 nmol), were simultaneously incubated. Similarly, when equal amounts of 22R-hydroxycholesterol and 22R-hydroxycholest-4-en-3-one (80 nmol each)

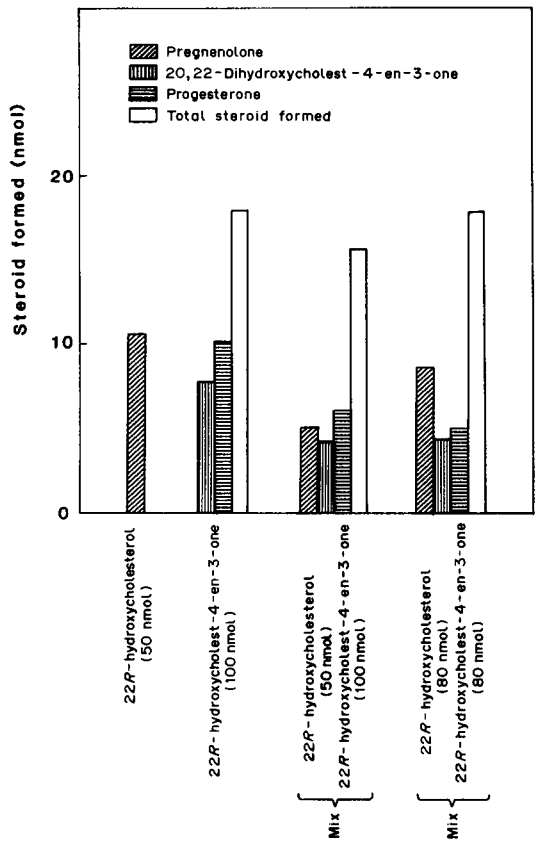


Fig. 3. Effect of simultaneous additon of two substrates in the cytochrome P-450$_{scc}$-catalyzed reaction. (1) 22R-Hydroxycholesterol (50 nmol) alone was incubated for 5 min with cytochrome P-450$_{scc}$ (35 pmol) in the reconstituted system. (2) 22R-Hydroxycholest-4-en-3-one (100 nmol) alone. (3) Mixture of 22R-hydroxycholesterol (50 nmol) and 22R-hydroxycholest-4-en-3-one (100 nmol). (4) Mixture of 22R-hydroxycholesterol (80 nmol) and 22R-hydroxycholest-4-en-3-one (80 nmol). The products were analyzed by normal-phase HPLC as described in Experimental.

were simultaneously incubated, the total amount of reaction products was essentially the same as that observed with 22R-hydroxycholest-4-en-3-one alone. These findings suggest that 22R-hydroxycholest-4-en-3-one was metabolized on the same active site of cytochrome P-450$_{scc}$ that 22R-hydroxycholesterol.

Effect of concentration of adrenodoxin on the metabolism of 22R-hydroxycholest-4-en-3-one

We further examined the rate of formation of 20,22-dihydroxycholest-4-en-3-one and progesterone when 22R-hydroxycholest-4-en-3-one was incubated with cytochrome P-450$_{scc}$ in the presence of a varying amount of adrenodoxin in the reconstituted system (Fig. 4). When adrenodoxin was at a subsaturating level, the accumulation of 20,22-dihydroxycholest-4-en-3-one was enhanced while the production of progesterone was reduced. This was due to the suppression of progesterone production as a result of the reduced rate of cytochrome P-450$_{scc}$ reduction and hence the accumulation of the intermediary dihydroxysteroid. Whereas when adrenodoxin was at a saturating level, the accumulation of 20,22-dihydroxycholest-4-en-3-one was reduced, being due to an enhancement of progesterone production.

These results (Figs 1–4) indicate that 22R-hydroxycholest-4-en-3-one was efficiently metabolized to progesterone by way of 20,22-dihydroxycholest-4-en-3-one by cytochrome P-450$_{scc}$ (Scheme 1B).

Catalytic activity of cytochrome P-450$_{scc}$ for various 3-one-4-en steroids substrates

We also carried out kinetic analyses to study the mechanism of the side-chain cleavage reaction and the rationale for the accumulation of the intermediate in the cytochrome P-450$_{scc}$-catalyzed reaction. Table 1 shows the catalytic activity of this enzyme for various substrates and corresponding 3-one-4-en forms. As described in the preceding paper [15], cholestenone and 20α-hydroxycholest-4-en-3-one were metabolized by only about 4% compared with cholesterol and 20α-hydroxycholesterol, respectively. 22S-Hydroxycholest-4-en-3-one was scarcely metabolized by cytochrome P-450$_{scc}$. However, 22R-hydroxycholest-4-en-3-one was metabolized rapidly into 20,22-dihydroxycholest-4-en-3-one and progesterone with the turnover numbers of 64 and 77 nmol/min/nmol P-450, respectively. Under the same conditions, 22R-hydroxycholesterol was metabolized to preg-

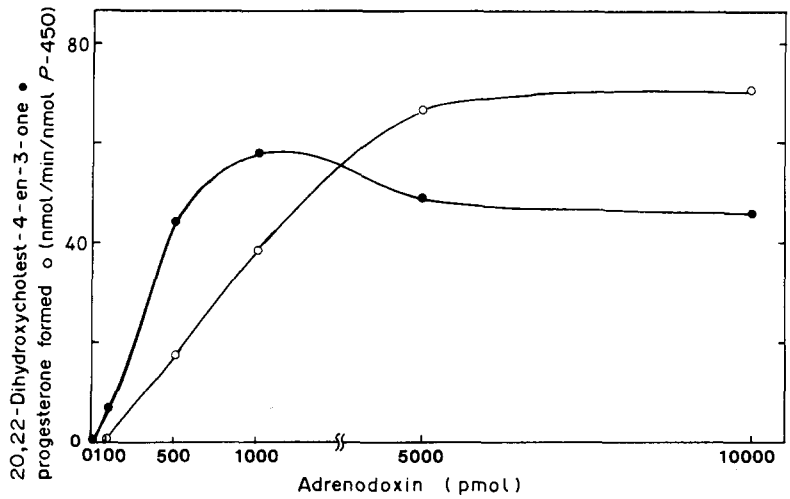


Fig. 4. Effect of concentration of adrenodoxin on the formation of 20,22-dihydroxycholest-4-en-3-one and progesterone. 22*R*-hydroxycholest-4-en-3-one (100 nmol) was incubated for 5 min with cytochrome $P\text{-}450_{scc}$ (35 pmol) in the reconstituted system containing the indicated amounts of adrenodoxin. The products were analyzed by normal-phase HPLC as described in Experimental. Closed and open circles show the rate of formation of 20,22-dihydroxycholest-4-en-3-one and that of progesterone, respectively.

nenolone with a turnover number of 77 nmol/min/nmol *P*-450. Thus, 22*R*-hydroxycholest-4-en-3-one as well as 22*R*-hydroxycholesterol was efficiently metabolized. Using a large scale preparation and HPLC, we prepared 20,22-dihydroxycholest-4-en-3-one and also estimated the 20,22-lyase activities for 20,22-dihydroxycholest-4-en-3-one and authentic 20,22-dihydroxycholesterol. The turnover number for 20,22-dihydroxycholest-4-en-3-one was calculated to be 125 nmol/min/nmol *P*-450 and that for 20,22-dihydroxycholesterol to be 121 nmol/min/nmol *P*-450, indicating that cytochrome $P\text{-}450_{scc}$ showed almost the same activity for these two steroids. These results indicate that, once the first hydroxylation has occurred at the C-22*R* position, subsequent 20-hydroxylation and 20,22-lyase reaction do not require the 3β-ol-5-en structure of natural substrate.

We performed estimation of Michaelis constants by Lineweaver–Burk plots. As illustrated in Fig. 5, the apparent K_m for 22*R*-hydroxycholesterol was estimated to be 20 μM and that for 22*R*-hydroxycholest-4-en-3-one to be 50 μM, indicating a 2.5 times lower affinity of the 3-one-4-en form to the enzyme than that of the natural substrate. The apparent K_m for 20,22-dihydroxycholest-4-en-3-one was estimated to be 40 μM and this value was not so different from that obtained for 22*R*-hydroxycholest-4-en-3-one. Since the K_m for 20,22-dihydroxycholesterol could not be measured because of the limited amount of this steroid available, a direct comparison of the affinities of 20,22-dihydroxycholest-4-en-3-one and 20,22-dihydroxycholesterol was not possible in the present study.

The result seem to suggest that the conversion of 22*R*-hydroxycholest-4-en-3-one to proges-

Table 1. Catalytic activity of cytochrome $P\text{-}450_{scc}$ for various steroids as substrates

Substrate	Product	Activity (nmol/min/nmol *P*-450)
Cholesterol	Pregnenolone	16
Cholestenone	Progesterone	0.6
20α-Hydroxycholesterol	Pregnenolone	27
20α-Hydroxycholest-4-en-3-one	Progesterone	1.0
22*S*-Hydroxycholesterol	Pregnenolone	10
22*S*-Hydroxycholest-4-en-3-one	Progesterone	Trace
22*R*-Hydroxycholesterol	Pregnenolone	77
22*R*-Hydroxycholest-4-en-3-one	20,22-Dihydroxycholest-4-en-3-one	64
	progesterone	77
20α,22*R*-Dihydroxycholesterol	Pregnenolone	125
20,22-Dihydroxycholest-4-en-3-one	Progesterone	121

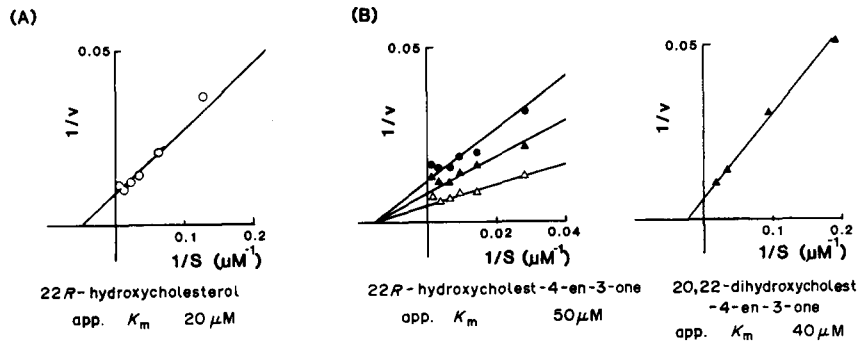


Fig. 5. Lineweaver–Burk plots of the metabolism of (A) 22R-hydroxycholesterol and (B) 22R-hydroxycholest-4-en-3-one by cytochrome P-450$_{scc}$. The substrates were incubated for 5 min with cytochrome P-450$_{scc}$ (35 pmol) in the reconstituted system. The product of 22R-hydroxycholesterol metabolism is pregnenolone (○) and the products of 22R-hydroxycholest-4-en-3-one metabolism are 20,22-dihydroxycholest-4-en-3-one (●) and progesterone (▲). Sum of 20,22-dihydroxycholest-4-en-3-one and progesterone is shown by (△). The product of 20,22-dihydroxycholest-4-en-3-one metabolism is progesterone (▲). The products were analyzed by normal-phase HPLC as described in Experimental.

terone catalyzed by cytochrome P-450$_{scc}$ takes place by successive reactions.

DISCUSSION

We have demonstrated that artificial 3-one-4-en derivatives of the natural substrates are metabolized by a reconstituted cytochrome P-450$_{scc}$ system and that transient accumulation of a steroid other than the final reaction product, progesterone, can be observed by using 22R-hydroxycholest-4-en-3-one as the substrate. The time-course of accumulation, the dependency of accumulation on the dose of cytochrome P-450$_{scc}$ (Fig. 2) and the requirement of the electron transport components for the accumulation (data not shown) indicate that the accumulated steroid was an intermediate in the side-chain cleavage reaction of 22R-hydroxycholest-4-en-3-one. To our knowledge, this is the first demonstration that an intermediate of the side-chain cleavage reaction accumulated in the reaction mixture, although Hume *et al.* [5] detected a small amount of the intermediates by GC–MS analysis on a single turnover study. The structure of the intermediate obtained in the present study was strongly suspected to be 20,22-dihydroxycholest-4-en-3-one on the basis of the retention time on HPLC (Fig. 1). Since natural cholesterol has only one proton at the 20α position which can be substituted by cytochrome P-450$_{scc}$-catalyzed reaction, this intermediate is probably a 20α,22R-dihydroxyl derivative. Analysis of the stereochemical conformation of the intermediate by NMR is now in progress.

The reaction sequence, cholesterol→22R-hydroxycholesterol → 20α,22R-dihydroxycholesterol→pregnenolone, has been generally accepted as that of cholesterol side-chain cleavage ([4–7] and Scheme 1) and this reaction sequence has been assumed to require the 3β-ol-5-en structure of substrate. Heyl *et al.* [13] reported that the 3β-hydroxyl group was important for the initial enzyme-substrate binding since cholestenone showed no detectable spin-state change of cytochrome P-450$_{scc}$ on spectral examination. Sheets and Vickery [14] also showed the importance of the 5,6-double bond of the steroid B-ring for enzyme binding by measuring the spectral dissociation constant of 22-amino-23,24-bisnor-5α-cholan-3β-ol. On the other hand, Morisaki *et al.* [20] described cholestenone being converted to progesterone by the action of cytochrome P-450$_{scc}$. Like Morisaki *et al.* we observed that cholestenone was metabolized by cytochrome P-450$_{scc}$ although the rate of side-chain cleavage was very slow (Table 1). Many other authors have reported on the significance or insignificance of the free 3β-ol-5-en structure in the side-chain cleavage reaction and no consensus has been reached so far. With the aim of providing additional insight into this problem, we further examined the side-chain cleavage activity of cytochrome P-450$_{scc}$ for various hydroxylated 3-one-4-en steroids.

We observed that, although the activities for the side-chain cleavage reaction of cholestenone, 20α-hydroxycholest-4-en-3-one and 22S-hydroxycholest-4-en-3-one were small compared with natural substrates, 22R-hydroxycholest-4-en-3-one as well as 22R-hydroxycholesterol were efficiently metabolized (Table

1). The results suggest that the 3β-ol-5-en structure of the substrate was important for both the 22-hydroxylation of cholesterol and the 22-hydroxylation of 20α-hydroxycholesterol. However, once the first hydroxylation had occurred correctly at the C-22*R* position, subsequent 20-hydroxylation and 20,22-lyase reaction seemed not to require the 3β-ol-5-en structure of natural substrate. These findings of ours seem to support the idea that cytochrome *P*-450$_{scc}$ initially recognizes the 3β-hydroxyl group, combines with the substrate and the first hydroxylation occurs at the C-22*R* position in the closest proximity to the heme-iron [12, 21]. However, we could not observe any detectable change in the spin-state of cholesterol-bound cytochrome *P*-450$_{scc}$ on binding with 22*R*-hydroxycholest-4-en-3-one. This observation may indicate that this steroid did not significantly alter the spin-state equilibrium of cytochrome *P*-450$_{scc}$ although it was metabolized by cytochrome *P*-450$_{scc}$, as is known from the metabolism of 24,25-dihydrolanosterol by cytochrome *P*-450$_{14DM}$ [22]. Further studies with the cholesterol-free or reduced types of cytochrome *P*-450$_{scc}$ are necessary to clarify whether 3-one-4-en steroids are metabolized without spin-state change of cytochrome *P*-450$_{scc}$.

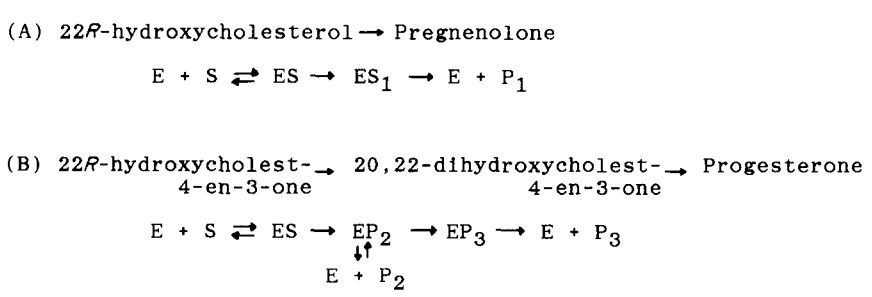
We carried out kinetic analyses to study the reaction mechanism of cytochrome *P*-450$_{scc}$. The apparent K_m values of cytochrome *P*-450$_{scc}$ for 22*R*-hydroxycholest-4-en-3-one and 20,22-dihydroxycholest-4-en-3-one were 50 and 40 μM, respectively (Fig. 5), indicating little difference in affinity for cytochrome *P*-450$_{scc}$. Furthermore, the amount of progesterone formed was almost the same as that of pregnenolone formed from 22*R*-hydroxycholesterol under the conditions where the turnover number of the lyase reaction for 20,22-dihydroxycholest-4-en-3-one was not significantly different from that for 20,22-dihydroxycholesterol. No lag in progesterone formation was observed within 30 s of incubation time. These observations appear to indicate that cytochrome *P*-450$_{scc}$ metabolized 22*R*-hydroxycholest-4-en-3-one with successive monooxygenations, as is assumed in the side-chain cleavage of natural substrate by cytochrome *P*-450$_{scc}$ [8, 23] and other cytochrome *P*-450-catalyzed reactions [24–26].

The rationale for the accumulation of the intermediate, 20,22-dihydroxycholest-4-en-3-one, is an interesting problem to be studied. As shown in Scheme 2, the accumulation of the intermediate may be due to the increased rate of 20-hydroxylation, the decreased rate of the lyase reaction or the loose binding of the enzyme-intermediate complex.

Our results showed that the turnover number of the lyase reaction for 20,22-dihydroxycholest-4-en-3-one and that for 20,22-dihydroxycholesterol were nearly the same. The formation of the intermediate was enhanced by substitution of the 3β-hydroxyl group by a 3-keto group (Table 1, Scheme 2B) and the 20,22-lyase reaction was the rate-limiting step of the side-chain cleavage for 22*R*-hydroxycholest-4-en-3-one.

It seems likely, therefore, that the 3-keto group interacts weakly with cytochrome *P*-450$_{scc}$ at a slightly shifted position and that the 20-hydroxylation occurs at a high rate since the intermediate is weakly bound to the active center of cytochrome *P*-450$_{scc}$. Furthermore, it is also possible that the rate of 20-hydroxylation itself is increased since the C-20 position of 3-one-4-en substrate comes closer to the heme-iron than the C-22*R* position. However, we could not directly measure the velocity of 20-hydroxylation since specific inhibition of 20,22-lyase reaction was not possible. We could not therefore further explore the rationale for the accumulation of the intermediate.

We would be able to evaluate the possible physiological significance of 3-one-4-en deriva-



Scheme 2. Reaction schemes of the metabolism of (A) 22*R*-hydroxycholesterol and (B) 22*R*-hydroxycholest-4-en-3-one by cytochrome *P*-450$_{scc}$.

tives of cholesterol by examination of their biosynthesis and distribution. If administered 3-one-4-en derivatives are utilized by animals, progesterone and other steroid hormones may be produced in the steroid hormone-producing tissues of a 3β-hydroxysteroid dehydrogenase/isomerase-deficient animal.

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