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Identification of Intermediates in the Conversion of Cholesterol to Pregnenolone with a Reconstituted Cytochrome P-450$_{scc}$ System: Accumulation of the Intermediate Modulated by the Adrenodoxin Level

Sachiko Sugano,*[,1] Retsu Miura,[†] and Naohiko Morishima*

*Department of Biochemistry, School of Nursing, Kitasato University, Sagamihara, Kanagawa 228; and [†]Department of Biochemistry, Kumamoto University School of Medicine, Kumamoto 860

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Dihydroxycholesterol and pregnenolone were clearly detected on HPLC when 22*R*-hydroxycholesterol was incubated with a reconstituted P450$_{scc}$ system containing equimolar amounts of P450$_{scc}$ and adrenodoxin. The dihydroxycholesterol, which has been accepted to be an intermediate in the conversion of 22*R*-hydroxycholesterol to pregnenolone, accumulated when adrenodoxin was at a subsaturating level with respect to P450$_{scc}$. The formation of the intermediate increased with increasing pH in the range of 7.2 to 8.1, and the ratio of the intermediate to the product, pregnenolone, increased with increasing pH. When the binding of P450$_{scc}$ to adrenodoxin was weakened by elevation of the ionic strength, the formation of the intermediate relative to the product increased. The apparent K_m for dihydroxycholesterol at a subsaturating level of adrenodoxin was about 7 μM, in contrast to 4 μM at a saturating level of adrenodoxin, implying that the affinity of dihydroxycholesterol is lower at a subsaturating level of adrenodoxin than at a saturating one. These results suggest that a subsaturating level of adrenodoxin weakened the binding of dihydroxycholesterol to P450$_{scc}$ and thus the intermediate, dihydroxycholesterol, was released. An intermediate other than dihydroxycholesterol, obtained when cholesterol was used as the substrate, was identified as 22*R*-hydroxycholesterol by HPLC and mass spectroscopic analysis. The intermediate obtained when 22*R*-hydroxycholesterol was used as the substrate was identified as 20*R*,22*R*-dihydroxycholesterol by HPLC, mass, and ^{1}H-NMR spectroscopic analyses. These results provide direct evidence that cholesterol is metabolized to pregnenolone by way of 22*R*-hydroxycholesterol and 20*R*,22*R*-dihydroxycholesterol by P450$_{scc}$.

Key words: adrenodoxin, cytochrome P-450$_{scc}$, 20*R*,22*R*-dihydroxycholesterol, 22*R*-hydroxycholesterol, intermediate.

Cytochrome P-450$_{scc}$ (CYP11A1) of bovine adrenocortical mitochondria catalyzes the side-chain cleavage of cholesterol to produce pregnenolone, the first and rate-limiting step in the biosynthesis of steroid hormones. The reaction occurs in three sequential monooxygenation steps, and requires two electrons and one molecule of oxygen at each step (Ref. *1* for review). Electrons are donated by NADPH and transferred to P450$_{scc}$ *via* NADPH-adrenodoxin reductase and adrenodoxin. Adrenodoxin (ferredoxin) forms a 1:1 complex with P450$_{scc}$ (*2*) and functions as a mobile electron shuttle (*3-5*). Electrostatic interactions are important for the binding of the complex (*3, 6, 7*).

Steady state and transient state kinetic studies have demonstrated that cholesterol is hydroxylated initially at the 22*R* position and then at the 20*R* position, followed by C20-C22 bond cleavage to yield pregnenolone; both 22*R*-hydroxycholesterol and 20*R*,22*R*-dihydroxycholesterol have been deduced to be natural intermediates in the P450$_{scc}$-catalyzed side-chain cleavage of cholesterol (*8, 9*).

[1] To whom correspondence should be addressed. E-mail: sugano@kitasato-u.ac.jp

Abbreviations: ADX, adrenodoxin; EI, electron impact; G6P, glucose-6-phosphate; P450$_{scc}$, cytochrome P-450$_{scc}$.

It has been reported that 20*R*,22*R*-dihydroxycholesterol accumulates following its production in the incubation mixture of 22*R*-hydroxycholesterol with mitochondria from the bovine adrenal cortex or from the human placenta (*10-12*). However, since the reaction proceeds successively without the release of intermediates due to the enhanced affinity of the intermediates to P450$_{scc}$ (*13, 14*), the intermediates do not usually accumulate in the reconstituted system and therefore we still lack direct evidence as to their structures. We present herein direct proof of the structure of the intermediate, 20,22-dihydroxycholesterol, offering valuable information for elucidation of the mechanism of cleavage of the C20-C22 bond. With regard to the dissociation of intermediates, Kominami *et al.* (*15*) discussed the physiological significance of intermediate release in the P450$_{17\alpha,lyase}$ (CYP17A1)- and P450$_{11\beta}$ (CYP11B1)-catalyzed reactions with reference to the regulatory mechanism of these reactions.

We previously developed an assay procedure for P450$_{scc}$ activity by HPLC, and this method has enabled easy analysis and isolation of reaction intermediates, if present (*16*). In the present study, we found that dihydroxycholesterol could be detected on HPLC when 22*R*-hydroxychole-

sterol was incubated with a reconstituted $P450_{scc}$ system containing equimolar amounts of $P450_{scc}$ and adrenodoxin. We were able to observe the accumulation of the intermediate in the successive reactions upon decreasing the amount of adrenodoxin. We further analyzed the factors that influenced the release of the intermediate during the reactions. We also elucidated the structure of the intermediate by mass and NMR spectroscopic methods. The rationale for the accumulation of the intermediate in the side-chain cleavage of 22*R*-hydroxycholesterol is also presented.

MATERIALS AND METHODS

Materials—Cholesterol, 22*R*-hydroxycholesterol, and Tween 20 were purchased from Sigma (USA). NADPH, glucose-6-phosphate (G6P), and G6P dehydrogenase were obtained from Oriental Yeast (Tokyo). 20*R*,22*R*-Dihydroxycholesterol was generously provided by Dr. Kyutaro Shimizu. Other chemicals used were of the highest grade available from commercial sources and were used as supplied.

Preparation of Enzymes—$P450_{scc}$ was purified to homogeneity from bovine adrenocortical mitochondria as described previously (*17*), and was in the high spin form ($A_{278}/A_{393}=1.2$). Its concentration was determined spectrophotometrically as described by Omura and Sato (*18*). Adrenodoxin and NADPH-adrenodoxin reductase were purified from bovine adrenal cortex according to the methods described by Suhara *et al.* (*19*), and Sugiyama and Yamano (*20*), respectively, and the concentrations were based on the extinction coefficients of 11 $mM^{-1}\cdot cm^{-1}$ at 414 nm (*21*) and 11 $mM^{-1}\cdot cm^{-1}$ at 450 nm (*22*), respectively. Adrenodoxin was purified to $A_{414}/A_{280}=0.6$.

Assay of the Intermediate and the Product Formation by $P450_{scc}$—The formation of the intermediate and the product in the $P450_{scc}$-catalyzed reaction with the reconstituted $P450_{scc}$ system was assayed by HPLC basically as described previously (*16*). 22*R*-Hydroxycholesterol (60 nmol) was incubated with 0.9 ml of a reaction mixture containing $P450_{scc}$ (0.14 nmol), adrenodoxin (0.14 nmol), NADPH-adrenodoxin reductase (1 nmol), G6P (5 μmol), G6P dehydrogenase (0.5 U), $MgCl_2$ (4 μmol), and NADPH (100 nmol) in 20 mM K-phosphate buffer, pH 7.4, 0.3% (w/v) Tween 20 at 37°C for 10 min. The adrenodoxin/$P450_{scc}$ molar ratio was adjusted as indicated by varying the amount of adrenodoxin when necessary. Deoxycorticosterone acetate was added to the assay mixture as an internal standard. Steroids were extracted with dichloromethane and then analyzed by normal-phase HPLC, with the absorbance detector set at 214 nm. A TSK-gel silica-150 column (4×250 mm, Tosoh) was used with a mobile phase of *n*-hexane:isopropanol (100 : 2, v/v) at the flow rate of 1.5 ml/min.

Mass Spectroscopic Analysis—The HPLC fractions containing the intermediate were collected and evaporated under nitrogen flow. The steroid (about 0.4 μg), dissolved in a small amount of ethanol, was analyzed by mass spectrometry in the electron impact mode with a Shimadzu QP-1000EX quadrupole mass spectrometer fitted with a direct insertion probe. Mass spectra were recorded at 70 eV at an ion source temperature of 160°C. The probe temperature was raised from 220 to 280°C at the rate of 80°C/min.

NMR Analysis—For the isolation of the second intermediate, 22*R*-hydroxycholesterol was incubated for 30 min with the reaction mixture described above, except for the use of 20 mM K-phosphate buffer, pH 8.0. The volume of the incubation mixture was scaled up to 9 ml. The steroids were extracted from the total of 180 ml of the reaction mixture with dichloromethane, and then the intermediate was purified to homogeneity by normal-phase and reverse-phase HPLC. The intermediate (about 0.1 mg) was subjected to ^{1}H-NMR analysis. ^{1}H-NMR spectra were recorded at 400 MHz with a Varian XL-400 spectrometer. Chemical shifts (δ) in $CDCl_3$ solution were measured in ppm with residual $CHCl_3$ from the solvent as a reference (δ 7.26).

RESULTS AND DISCUSSION

Detection of an Intermediate in the Side-Chain Cleavage of 22R-Hydroxycholesterol—When 22*R*-hydroxycholesterol was incubated with the usual $P450_{scc}$-reconstituted system fortified with excess adrenodoxin, NADPH-adrenodoxin reductase and an NADPH-regenerating system, pregnenolone, the final reaction product, appeared in the chromatogram (Fig. 1A). However, when the amount of adrenodoxin was limited, an extra peak in addition to that of pregnenolone was clearly detected in the HPLC chromatogram (Fig. 1B). The retention time of peak 3 was identical with that of authentic 20*R*,22*R*-dihydroxycholesterol. We assume that 20,22-dihydroxycholesterol, which has been accepted to be an intermediate in the conversion of 22*R*-hydroxycholesterol to pregnenolone, was detected as a consequence of decreasing the amount of adrenodoxin.

Thus, we examined the effect of the concentration of

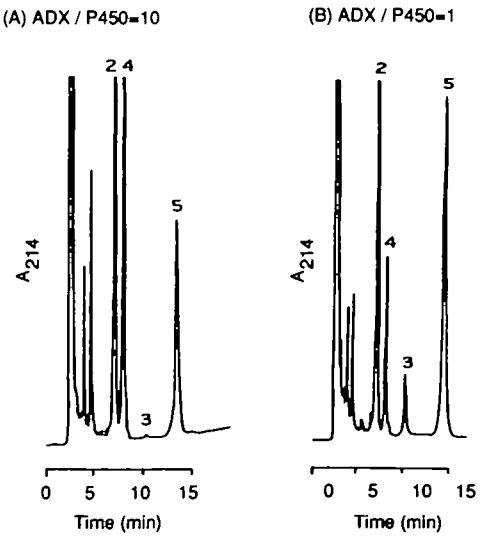

Fig. 1. **HPLC profiles of the reaction products obtained with 22*R*-hydroxycholesterol as the substrate.** 22*R*-Hydroxycholesterol (60 nmol) was incubated with the $P450_{scc}$ (140 pmol)-reconstituted system containing (A) 1,400 pmol of adrenodoxin (adrenodoxin/$P450_{scc}$ molar ratio, 10) and (B) 140 pmol of adrenodoxin (ADX, adrenodoxin/$P450_{scc}$ molar ratio, 1) at 37°C for 10 min. Peaks 2, 3, 4, and 5 correspond to 22*R*-hydroxycholesterol, 20,22-dihydroxycholesterol, pregnenolone, and deoxycorticosterone acetate (internal standard), respectively. The steroids were detected by monitoring the absorbance at 214 nm and identified on the basis of their retention times.

adrenodoxin relative to that of $P450_{scc}$ on the formation of 20,22-dihydroxycholesterol and pregnenolone. As shown in Fig. 2A, the formation of both steroids showed dependency on the concentration of adrenodoxin. Note that the concentration of adrenodoxin in Fig. 2A is presented as the molar ratio to $P450_{scc}$, the concentration of which was held constant. The amount of pregnenolone increased with increasing amount of adrenodoxin and reached a plateau. On the other hand, 20,22-dihydroxycholesterol accumulated when adrenodoxin was at a subsaturating level with respect to $P450_{scc}$, but did not accumulate with a saturating level of adrenodoxin. Since the accumulation of 20,22-dihydroxycholesterol was maximum in the presence of equimolar amounts of $P450_{scc}$ and adrenodoxin, the experiments described below were carried out under these conditions. Hanukoglu and Hanukoglu (*23*) have reported that the concentrations of $P450_{scc}$ and adrenodoxin are similar in adrenal cortex mitochondria.

To confirm that the 20,22-dihydroxycholesterol is an intermediate in the side-chain cleavage of 22*R*-hydroxycholesterol, we examined the time courses of the formation of 20,22-dihydroxycholesterol and pregnenolone (Fig. 2B). The accumulation of both steroids in the early stage of the reaction occurred concomitantly with the consumption of both NADPH and O_2. While pregnenolone accumulated progressively with time, 20,22-dihydroxycholesterol accumulated progressively up to 30 min, after which its amount gradually decreased. When a low level of substrate was used, the amount of 20,22-dihydroxycholesterol decreased as the substrate became exhausted (data not shown). Furthermore, when the peak 3 material in Fig. 1 was isolated and used as the substrate, it was metabolized to pregnenolone. These observations suggest that the accumulation of 20,22-dihydroxycholesterol, corresponding to peak 3 of Fig. 1, was transient and that this compound was further metabolized to pregnenolone. Taking these results into account, we conclude that an intermediate in the side-chain cleavage of 22*R*-hydroxycholesterol by $P450_{scc}$ accumulated in a reconstituted reaction mixture with a subsaturating level of adrenodoxin.

Effects of pH on the Formation/Accumulation of the Intermediate and the Product—We examined the effect of pH on the formation/accumulation of the intermediate, 20,22-dihydroxycholesterol, and the product, pregnenolone (Fig. 3A). The formation/accumulation of pregnenolone was maximum at pH 7.6 and progressively decreased above pH 7.6. The pH profile of the product formation is consistent with the result of Takikawa *et al.* (*24*) on the side-chain cleavage of cholesterol in Tris-HCl buffer. However, the formation/accumulation of 20,22-dihydroxy-

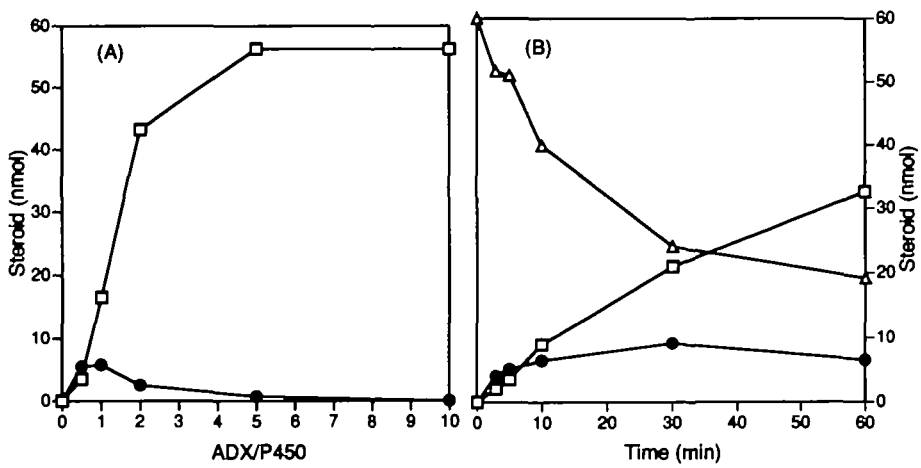

Fig. 2. **(A) Effect of the adrenodoxin/$P450_{scc}$ ratio on the formation of 20,22-dihydroxycholesterol and pregnenolone with 22*R*-hydroxycholesterol as the substrate.** 22*R*-Hydroxycholesterol (60 nmol) was incubated with the $P450_{scc}$ (140 pmol)-reconstituted system at varying values of the adrenodoxin/$P450_{scc}$ molar ratio (ADX/P450) as indicated. **(B) Time courses of the formation of 20,22-dihydroxycholesterol and pregnenolone.** 22*R*-Hydroxycholesterol (60 nmol) was incubated with the $P450_{scc}$ (140 pmol)-reconstituted system containing 140 pmol of adrenodoxin for various periods of time as indicated. The products were analyzed by normal-phase HPLC as described under "MATERIALS AND METHODS." The amounts of 22*R*-hydroxycholesterol (△), 20,22-dihydroxycholesterol (●), and pregnenolone (□) were estimated from the absorbance at 214 nm.

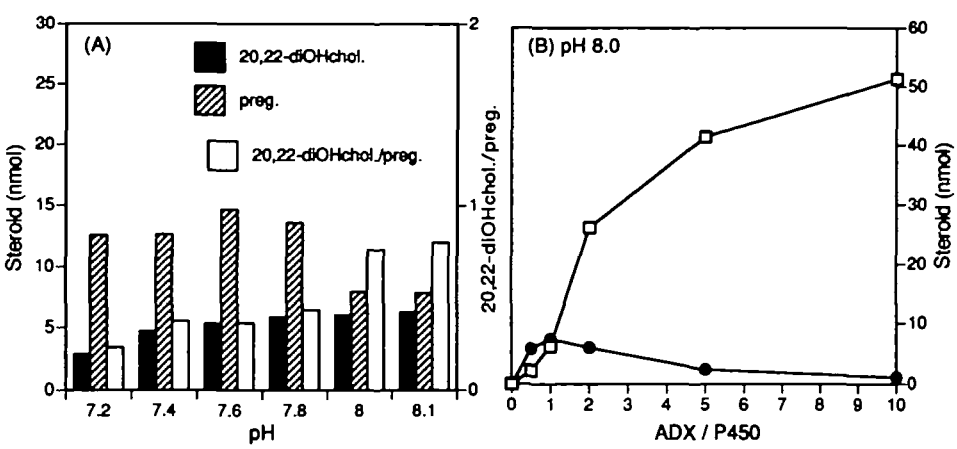

Fig. 3. **(A) Effect of pH on the formation of 20,22-dihydroxycholesterol and pregnenolone with 22*R*-hydroxycholesterol as the substrate.** 22*R*-Hydroxycholesterol (60 nmol) was incubated with the $P450_{scc}$ (140 pmol)-reconstituted system containing 140 pmol of adrenodoxin for 10 min at the indicated pHs. The buffer was 30 mM Tris-HCl at pHs ranging from 7.2 to 8.1 at 37°C. Closed bars, 20,22-dihydroxycholesterol (20,22-diOHchol); hatched bars, pregnenolone (preg). **(B) Effect of the adrenodoxin/$P450_{scc}$ ratio on the formation of 20,22-dihydroxycholesterol and pregnenolone at pH 8.0.** The amounts of 20,22-dihydroxycholesterol (●) and pregnenolone (□) were estimated from the absorbance at 214 nm. Experiments were carried out as in Fig. 2A except for the use of 20 mM K-phosphate buffer, pH 8.0.

cholesterol increased on raising the pH from 7.2 to 8.1. This tendency was more prominent when the ratio of 20,22-dihydroxycholesterol to pregnenolone was examined at different pHs, ranging from 7.2 to 8.1. Similar results were obtained in potassium phosphate buffer (results not shown), indicating that the pH-profile obtained is independent of the buffer used. Figure 3B shows the effect of the concentration of adrenodoxin relative to that of $P450_{scc}$ on the formation/accumulation of the intermediate and the product at pH 8.0. At pH 8.0 the formation/accumulation of the intermediate relative to the product is greater than that at pH 7.4, and the maximum level of the intermediate observed declined more gradually with increasing adrenodoxin/$P450_{scc}$ ratio at pH 8.0 than at pH 7.4 (compare Figs. 2A and 3B).

Lambeth *et al.* (*14*) showed that the binding of 20,22-dihydroxycholesterol to $P450_{scc}$ became progressively weaker above pH 6.8. Thus, the pH profile in Fig. 3B can be explained by the weaker affinity of 20,22-dihydroxycholesterol to $P450_{scc}$ at higher pH and/or by the weaker affinity of 20,22-dihydroxycholesterol to $P450_{scc}$ when $P450_{scc}$ is not complexed with adrenodoxin than when $P450_{scc}$ is complexed with adrenodoxin.

Effects of Ionic Strength on the Formation/Accumulation of the Intermediate and the Product—In order to find out the rationale for the accumulation of the intermediate, we investigated the influence of the adrenodoxin level with respect to $P450_{scc}$ on the reaction. It has been demonstrated that the binding of the adrenodoxin-$P450_{scc}$ complex is dependent on the ionic strength of the reaction medium (*25, 26*), and that the binding of steroids such as cholesterol with $P450_{scc}$ is independent of the ionic strength (*25, 14*). Lambeth *et al.* (*14*) also pointed out that adrenodoxin enhanced the binding of not only cholesterol, but also dihydroxycholesterol, to $P450_{scc}$, although to a lesser extent than cholesterol. Thus, we examined the formation/accumulation of the intermediate and the product when the binding of the adrenodoxin-$P450_{scc}$ complex was altered by varying the ionic strength (Fig. 4, A and B). At both pH 7.4 and 8.0, 20,22-dihydroxycholesterol and pregnenolone decreased with increasing ionic strength; this can be explained by the weaker complexation of adrenodoxin-$P450_{scc}$ at higher ionic strength, resulting in less efficient electron transfer from adrenodoxin to $P450_{scc}$. To normalize the decreased efficiency of the electron transfer, the ratio of 20,22-dihydroxycholesterol to pregnenolone was plotted against the ionic strength (Fig. 4C). The ratio of the intermediate to the product increased with increasing ionic strength, and the ratios at pH 8.0 were higher than those at pH 7.4. Figure 5 shows the effects of the concentration of adrenodoxin relative to that of $P450_{scc}$ on the formation/accumulation of the intermediate and the product with and without 80 mM NaCl. The accumulation of the intermediate was higher with 80 mM NaCl than without it at each

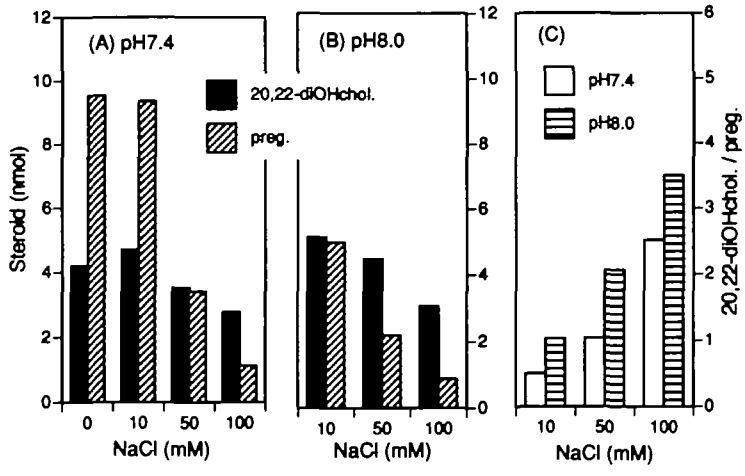

Fig. 4. **Effect of ionic strength on the formation of 20,22-dihydroxycholesterol and pregnenolone with 22*R*-hydroxycholesterol as the substrate at (A) pH 7.4 and (B) pH 8.0.** 22*R*-Hydroxycholesterol (60 nmol) was incubated with the $P450_{scc}$ (140 pmol)-reconstituted system containing 140 pmol of adrenodoxin. The ionic strength was varied by adding 10, 50, and 100 mM NaCl (final concentrations). Closed bars, 20,22-dihydroxycholesterol (20,22-diOHchol); hatched bars, pregnenolone (preg). (C) **Effect of ionic strength on the ratio of 20,22-dihydroxycholesterol and pregnenolone at pH 7.4 and 8.0.**

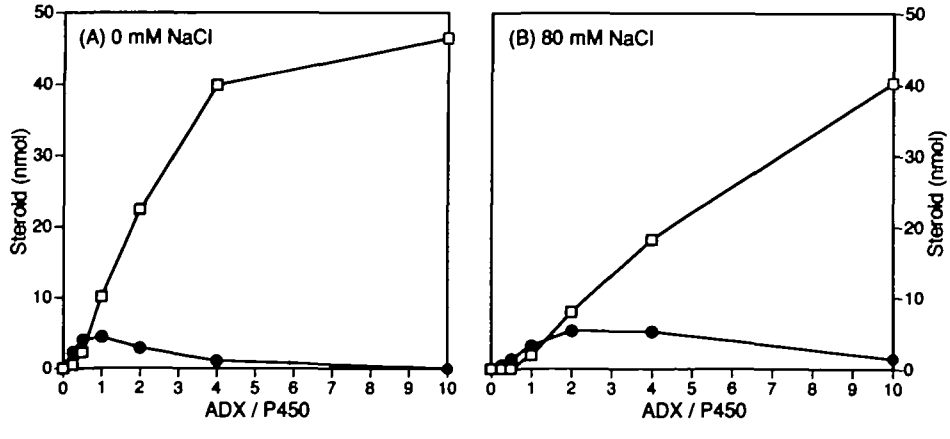

Fig. 5. **Effect of the adrenodoxin/P-450_{scc} ratio on the formation of 20,22-dihydroxycholesterol and pregnenolone with 22*R*-hydroxycholesterol as the substrate in the absence (A) or presence (B) of 80 mM NaCl.** The amounts of 20,22-dihydroxycholesterol (●) and pregnenolone (□) were estimated from the absorbance at 214 nm. Experiments were carried out as in Fig. 2A except for the use of 80 mM NaCl.

adrenodoxin/P450$_{scc}$ ratio, and the ratio giving the maximum accumulation shifted to a greater adrenodoxin/P450$_{scc}$ ratio in the presence of NaCl than in its absence. The results indicate that weakening of the binding of adrenodoxin to P450$_{scc}$ increases the accumulation of the intermediate, implying that the intermediate is released when P450$_{scc}$ is not saturated with adrenodoxin because the affinity of the intermediate to P450$_{scc}$ is weaker when it is not complexed with adrenodoxin than when it is complexed.

Binding of the Intermediate to P450$_{scc}$—To investigate whether the level of adrenodoxin with respect to that of P450$_{scc}$ affects the binding of the intermediate to P450$_{scc}$, we measured the apparent K_m for 20,22-dihydroxycholesterol in the presence of varying levels of adrenodoxin. For this experiment a sufficient amount of 20,22-dihydroxycholesterol was isolated in a large-scale preparation. The apparent K_m for 20,22-dihydroxycholesterol at a subsaturating level of adrenodoxin (see the legend to Fig. 6) was estimated to be 7 μM, whereas that at a saturating level of adrenodoxin was 4 μM (Fig. 6), indicating clearly that the level of adrenodoxin with respect to P450$_{scc}$ indeed influences the affinity of the intermediate to P450$_{scc}$. This result supports the weaker binding of the intermediate to P450$_{scc}$ uncomplexed to adrenodoxin than to that complexed to adrenodoxin. Taking all the experimental results presented above into consideration we conclude that the intermediate accumulated at a subsaturating level of adrenodoxin due to the weakened binding of 20,22-dihydroxycholesterol to P450$_{scc}$ and to dissociation of the intermediate from P450$_{scc}$.

Identification of the First Intermediate in the Conversion of Cholesterol to Pregnenolone—Using the reaction intermediates obtained with a subsaturating level of adrenodoxin, we carried out the identification of the intermediates in the conversion of cholesterol to pregnenolone by P450$_{scc}$.

To detect the first intermediate in the conversion of cholesterol to pregnenolone, cholesterol was incubated as the substrate. As shown in Fig. 7A, two minor peaks (peaks 2 and 3) in addition to that of pregnenolone appeared on HPLC. Peaks 2 and 3 showed the same retention times as authentic 22*R*-hydroxycholesterol (at 7.1 min) and 20*R*, 22*R*-dihydroxycholesterol (at 10.4 min), respectively. Other peaks corresponding to 20α-hydroxycholesterol (at 6.3 min) and 22*S*-hydroxycholesterol (at 9.0 min) were not detected in the chromatogram under the conditions employed. Because 22*R*-hydroxycholesterol is metabolized to pregnenolone by way of 20,22-dihydroxycholesterol (Fig. 2B), it was suggested that if the peak 2 material detected on HPLC with cholesterol as the substrate is 22*R*-hydroxycholesterol, peak 2 is the first intermediate in the side-chain cleavage of cholesterol. As there have been reports that 22*R*-hydroxycholesterol is bound 1 order of magnitude

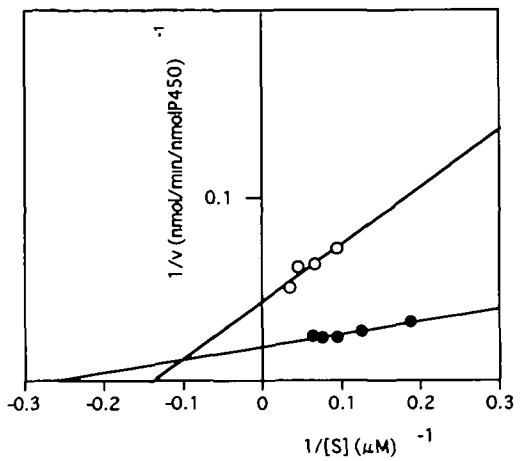

Fig. 6. **Double reciprocal plots of the rate *versus* 20,22-dihydroxycholesterol at a saturating or subsaturating level of adrenodoxin.** Open symbols represent the rate of pregnenolone formation when 20,22-dihydroxycholesterol was incubated with the P450$_{scc}$ (70 pmol)-reconstituted system containing 70 pmol of adrenodoxin for 4 min. Closed symbols represent the rate of pregnenolone formation when 20,22-dihydroxycholesterol was incubated with the P450$_{scc}$ (35 pmol)-reconstituted system containing 350 pmol of adrenodoxin for 4 min.

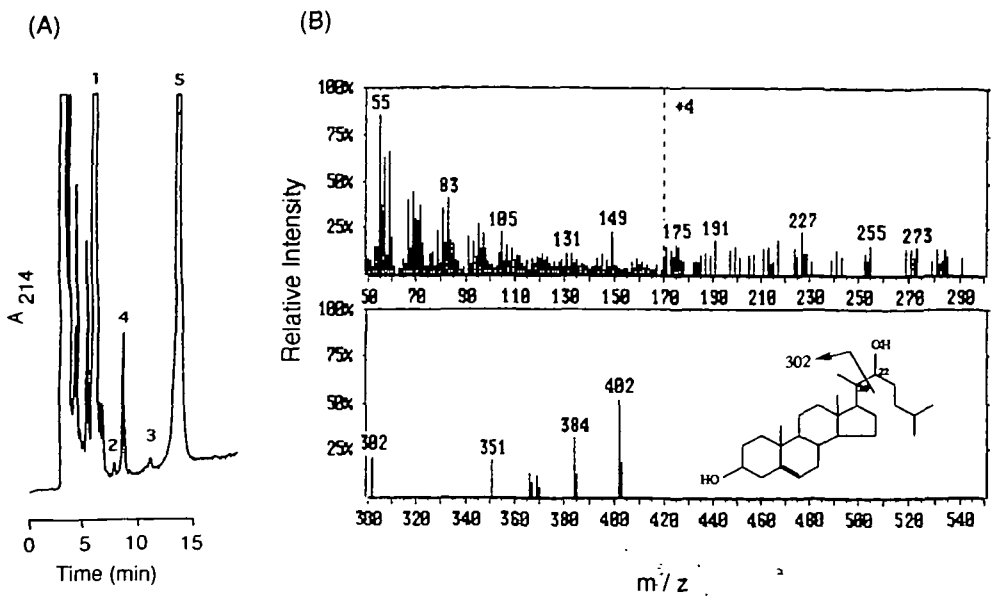

Fig. 7. **(A) HPLC profile of the reaction products obtained with cholesterol as the substrate.** Cholesterol (60 nmol) was incubated with the P450$_{scc}$ (140 pmol)-reconstituted system containing 140 pmol of adrenodoxin for 30 min. Peaks 1, 2, 3, 4, and 5 correspond to cholesterol, 22*R*-hydroxycholesterol, 20,22-dihydroxycholesterol, pregnenolone, and deoxycorticosterone acetate (internal standard), respectively. The steroids were detected by monitoring at 214 nm and identified on the basis of their retention times. **(B) EI mass spectrum of the first intermediate in the side-chain cleavage of cholesterol.** The peak 2 material in (A) was collected and analyzed by EI mass spectrometry. The structure and the major cleavage site of 22*R*-hydroxycholesterol are shown.

more tightly than 20*R*,22*R*-dihydroxycholesterol (*13, 14*), peak 2 may well be small.

To clarify the structure of the first intermediate, the peak 2 fractions were collected manually from several HPLC operations and then subjected to mass spectrometric analysis without trimethylsilyl derivatization. The electron-impact (EI) mass spectrum of the substance (Fig. 7B) was essentially identical to that of authentic 22*R*-hydroxycholesterol (data not shown). The molecular ion was detected at *m/z* 402, with a relatively low ion-source temperature. The ion at *m/z* 384 (M − H_2O) was due to the elimination of a hydroxyl group, and the ion at *m/z* 302 was due to the cleavage of the C20-C22 bond, suggesting the presence of a hydroxyl group at the C-22 position. Other significant ions (*m/z* 369, 384 − CH_3; *m/z* 366, M − 2H_2O; *m/z* 351, 366 − CH_3; *m/z* 284, 302 − H_2O) in this spectrum were consistent with the structure of 22-hydroxycholesterol. The stereochemistry of 22-hydroxycholesterol was

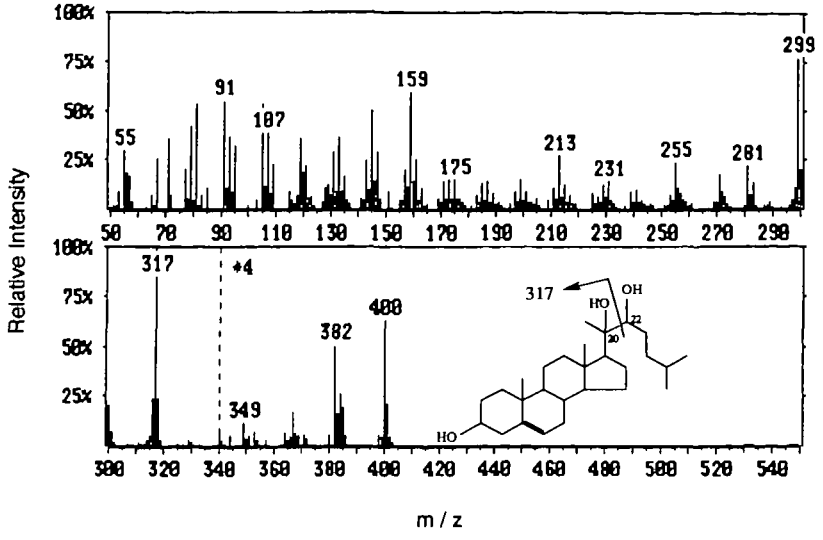

Fig. 8. **EI mass spectrum of the reaction intermediate in the side-chain cleavage of 22*R*-hydroxycholesterol.** The intermediate obtained with 22*R*-hydroxycholesterol as the substrate was isolated and analyzed by EI mass spectrometry. The structure and the major cleavage site of 20,22-dihydroxycholesterol are shown.

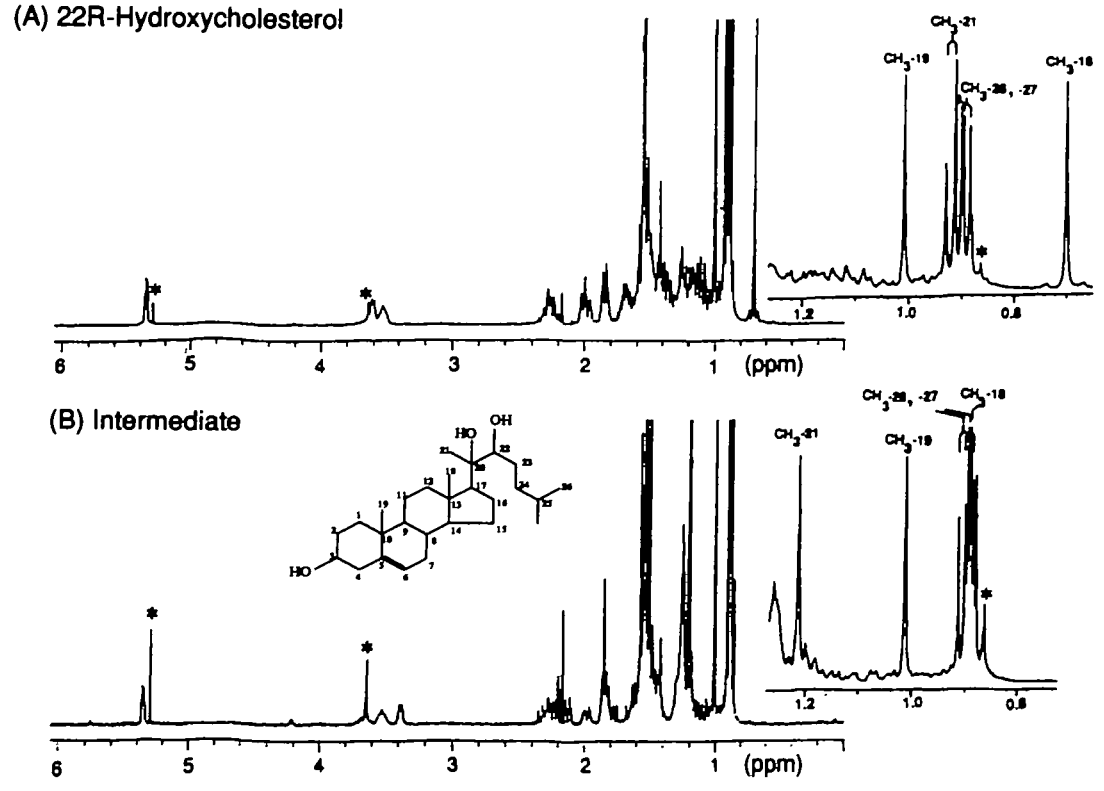

Fig. 9. **^{1}H-NMR spectra of 22*R*-hydroxycholesterol and the reaction intermediate in the side-chain cleavage of 22*R*-hydroxycholesterol.** (A) 22*R*-Hydroxycholesterol, which was used as the substrate, and (B) the reaction intermediate were purified by HPLC. The peaks indicated by asterisks are due to contaminants arising from the solvent system used for HPLC.

confirmed by comparing the HPLC retention times of authentic 22*S*- and 22*R*-isomers, which were distinguishable on this HPLC. As shown in Fig. 7A, the retention time of peak 2 coincided with that of 22*R*-hydroxycholesterol (at 7.1 min) and did not coincide with that of 22*S*-hydroxycholesterol (at 9 min). Based on the HPLC retention time and EI mass spectrum, the substance in peak 2 was identified as 22*R*-hydroxycholesterol, the first intermediate.

Identification of the Second Intermediate in the Conversion of Cholesterol to Pregnenolone—To clarify the structure of the second intermediate, the intermediate obtained with 22*R*-hydroxycholesterol as the substrate was isolated and similarly analyzed by mass spectrometry (Fig. 8). The EI mass spectrum of the intermediate (Fig. 8) was essentially identical to that of authentic 20*R*,22*R*-dihydroxycholesterol (data not shown). A molecular ion (*m/z* 418) was not detected. The ions at *m/z* 400 ($M-H_2O$) and 382 ($M-2H_2O$) were detected due to the successive loss of two hydroxyl groups. A base peak was observed at *m/z* 317 due to the cleavage of the C20-C22 bond and a prominent peak was also observed at *m/z* 299 due to the loss of a hydroxyl group from *m/z* 317, suggesting that a hydroxyl group is located at the C-20 position in addition to the C-22 position. Other significant ions (*m/z* 367, $382-CH_3$; *m/z* 349, $367-H_2O$; *m/z* 281, $299-H_2O$) in this spectrum were consistent with the structure of 20,22-dihydroxycholesterol.

To identify the structure, including the stereochemistry, of this intermediate, proton NMR spectra of 22*R*-hydroxycholesterol and the intermediate were obtained (Fig. 9). The spectrum of 22*R*-hydroxycholesterol used as the substrate coincided with that of authentic 22*R*-hydroxycholesterol, and the resonance peaks at 3.52 and 5.35 ppm were assigned as due to H-3 and H-6, respectively. The signal of the methyl protons of C-19 was observed as a singlet at 1.01 ppm, and the signals of the methyl protons of C-26 and C-27 were observed at 0.89 and 0.90 ppm, as two doublets coupled with H-25, characterized by a coupling constant of 6 Hz. These signals for 22*R*-hydroxycholesterol were found at almost the same positions as those for the intermediate. The fact that the doublet signal of the methyl protons of C-21 at 0.92 ppm coupled with H-20 in 22*R*-hydroxycholesterol became a singlet at 1.21 ppm for the intermediate indicates the disappearance of H-20. In addition, the up-field shift (from 3.61 to 3.39 ppm) of the H-22 signal may be correlated to the substitution of H-20 with a hydroxyl group. The absolute configurations of the hydroxyl groups at C-20 and C-22 of the intermediate were established by comparing the NMR data for four synthetic isomers reported by Morisaki *et al.* (*27*). They reported that the signals of the methyl protons of C-21 of 20*R*,22*R*-dihydroxycholesterol, the 20*R*,22*S*-isomer, and the 20*S*,22*S*-isomer appeared at 1.20, 1.26, and 1.05 ppm, respectively, but no data for the 20*S*,22*R*-isomer were presented. The chemical shift for the methyl protons of the 20*S*,22*R*-isomer was expected to be close to that of the 20*S*,22*S*-isomer, since it was reported that the chemical shift of the methyl protons of C-21 in 20α-hydroxycholesterol exhibited a downfield shift of 0.17 ppm for these protons relative to the 20β-epimer (*28*). Therefore, the absolute configuration at C-20 of the intermediate was established to be *R*. It was considered that monooxygenation occurred at the C-20 position of 22*R*-hydroxycholesterol with retention of the configuration, and consequently, the intermediate is 20*R*,22*R*-dihydroxycholesterol. Thus, the second intermediate was identified as 20*R*,22*R*-dihydroxycholesterol on the basis of the results of HPLC, mass and ^{1}H-NMR spectroscopic analyses.

These results provided direct evidence for the structures of the intermediates in the P-450$_{scc}$-catalyzed side-chain cleavage of cholesterol. Therefore, based on the direct experimental evidence, we conclude that cholesterol is metabolized to pregnenolone by way of 22*R*-hydroxycholesterol and 20*R*,22*R*-dihydroxycholesterol by P450$_{scc}$, in agreement with the generally accepted reaction pathway deduced from various studies (*8, 9, 29, 30*). We have also demonstrated that an intermediate in the side-chain cleavage of 22*R*-hydroxycholesterol accumulated in a reconstituted P450$_{scc}$ system when adrenodoxin was at a subsaturating level with respect to P450$_{scc}$.

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