CHOLESTEROL 20,22-EPOXIDES : NO CONVERSION TO PREGNENOLONE BY ADRENAL CYTOCHROME P-450$_{scc}$

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Received January 26,1976

SUMMARY All of the four 20,22-epoxycholesterols and (E)-20(22)-dehydrocholesterol were chemically synthesized and incubated with purified adrenocortical cytochrome P-450$_{scc}$ in the presence of an appropriate electron-supplying system. None of these cholesterol derivatives were significantly converted to pregnenolone by the enzyme. A slight inhibition of the side-chain cleavage of radioactive cholesterol was observed by the addition of the cholesterol derivatives, but there occurred no trapping of the radioactivity by these compounds. It may be concluded that the side-chain cleavage of cholesterol by the adrenal cytochrome P-450 does not operate through olefin and epoxide formation as the intermediates.

INTRODUCTION

The first step of steroid hormone production from cholesterol is the carbon-carbon chain cleavage between C-20 and C-22. The reaction is catalized by a specific cytochrome P-450 and requires three moles of molecular oxygen per mole of cholesterol cleaved (1, 2). Although it is widely documented that hydroxycholesterols occur as the intermediates between cholesterol and pregnenolone, there has been much controversy about the role of these hydroxycholesterols in the pathway. Kraaipoel et al.(3 - 5) have recently proposed a reaction sequence which includes olefin, epoxide and dihydroxy compound as the intermediates. This pathway looks pertinent in view of our previous observation that an epoxide occurs as the intermediate in dealkylation of sitosterol by silkworm (6,7).

Abbreviation : P-450$_{scc}$= P-450 specific for side-chain cleavage of cholesterol.

We have synthesized all the four stereoisomers of 20,22-epoxycholesterol as well as (E)-20(22)-dehydrocholesterol in order to examine whether or not these compounds can be an intermediate of the side-chain cleavage of cholesterol by the cytochrome P-450. On the contrary to our expectation, none of these compounds was effectively converted to pregnenolone by the enzyme. This observation does not support the pathway proposed by Kraaipoel et al (3-5).

EXPERIMENTAL PEOCEDURES

Synthesis of Cholesterol Derivatives

(E)-20(22)-Dehydrocholesterol was prepared by the slight modified method of Schmit et al (8). Oxidation of the 20(22)-olefin acetate with m-chloroperbenzoic acid followed by saponification gave 20R,22R-epoxycholesterol and 20S,22S-epoxycholesterol. Osmium tetroxide oxidation of the 20(22)-olefin acetate afforded the 22-glycols, which in turn were reacted with methanesulfonyl chloride. Treatment of the 22-mesylates with potassium carbonate yielded 20S,22R-epoxycholesterol and 20R,22S-epoxycholesterol. Details of these synthesis will be reported elsewhere (9).

Preparation of Cytochrome P-450$_{scc}$

Bovine adrenal glands were collected at a slaughter house and stored at -80°C. The glands were thawed at 5°C and mitochondrial pellets were prepared and sonicated as described by Suhara et al (10). The sonicate was ultracentrifuged to remove a major amount of adrenodoxin reductase and the supernatant fluid was treated with sodium cholate (0.45 mg per mg of protein). Methods of further purification of the cytochrome were same as those reported previously (2), except for the use of a different brand (Bio-Rad Lab., Richmond, California) and Sepharose 4B (Pharmacia Fine Chem., Uppsala) in the place of Bio-Gel A-15m. The final preparation contained 11 nmoles of hem and 28 nmoles of cholesterol per mg of protein and exhibited a sedimentation coefficient of 8.4 on a sucrose gradient of 5 to 20%. It contained no 11β-hydroxylase activity. The preparation was stored at -20°C in 50% glycerol.

Incubation of Cholesterol and Cholesterol Derivatives

Cholesterol and cholesterol derivatives were dissolved in dimethylformamide to a concentration of 0.85 mM, and 10μl(8.5 nmole)of each was added to 1 ml of the reaction mixture containing potassium phosphate (50 μmoles, pH 7.0), cysteine hydrochloride (5 μmoles), EDTA Na_2 (1 μmole), NADP Na_4 (300 nmoles), sodium glucose-6-phosphate (4.6 μmoles), glucose-6-phosphate dehydrogenase (10 ng protein), adrenodoxin (2 nmoles), adrenodoxin reductase (5 DCPLP units) and bovine serum albumin (5 mg). The side-chain cleavage reaction was initiated by the addition of cytochrome P-450 (0.09 nmole, 20 μl in volume) and terminated by adding dichloromethane after 60 min incubation at 37°C. The incubation mixture endogenously contained cholesterol (0.45 nmole per ml) which amounts about 5 % of total sterol in the mixture.

Determination of Pregnenolone

The dichloromethane extracts were treated with trimethylsilylimidazole

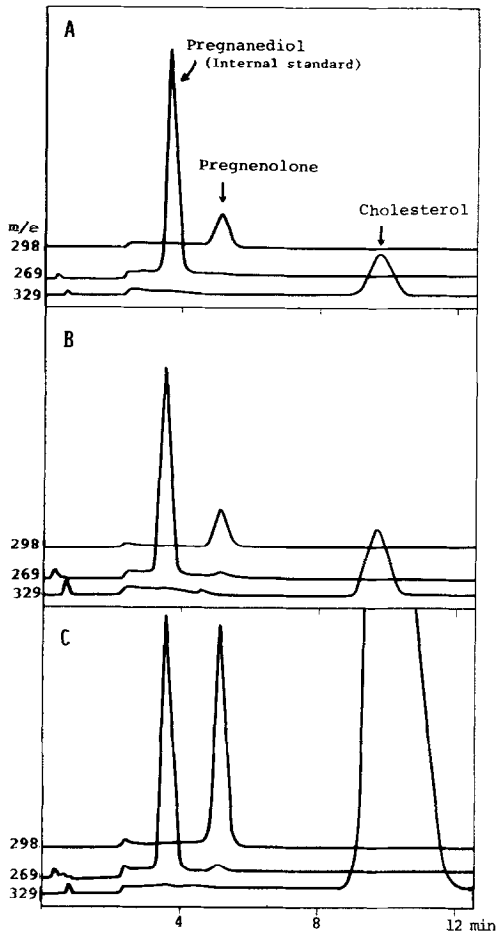

Fig. 1 Mass fragmentograms of steroids as their trimethylsilyl (TMS) ethers obtained from (A) incubation without adding substrate, (B) with 20R,22R-epoxycholesterol, and (C) with cholesterol. A Shimadzu-LKB 9000S gas-chromatograph-mass specrometer equipped with a multiple ion detector MID-PM was used. The column was 1.0 m x 4 mm i.d., glass coil packed with 1.5 % OV-17 on Shimalite W (80-100 mesh). The column temperature was 260°C ; the flow rate of carrier gas (He) was 25 ml/min.; the ionization current was 60 μA ; the voltage was 20 eV ; the ion source temperature was 290°C. Channels 1,2, and 3 of the multiple ion detector were set to detect m/e 298 (pregnenolone TMS - TMSOH), 269 (pregnanediol 2TMS - 2TMSOH - CH_3) and 329 (cholesterol TMS - 129), respectively. Sensitivities of each channel were appropriately set and the size of the peak of steroids was calibrated for a series of known amounts of reference steroid.

(20°C, 10 min) and aliquots were injected into a gas chromatograph - mass spectrometer equipped with a multiple ion detector MID-PM. A known amount of 5β-pregnane-3α,20α-diol bis-trimethylsilylether was used as an internal standard for calibration of the amount injected. Pregnanediol, pregnenolone and cholesterol were determined by mass fragmentography. For details see the legend of Fig. 1.

Inhibition of the Side-Chain Cleavage of [4-^{14}C]Cholesterol

[4-^{14}C]Cholesterol (Cea Ire Sorin, France) was diluted to a specific activity of 52,000 cpm per nmole and purified by thin-layer chromatography. The radioactive cholesterol (1.8 nmoles in 20µl dimethylformamide) was mixed with the cholesterol derivatives (17 or 52 nmoles each in 30 µl of dimethylformamide) and then dissolved in 1.0 ml of the reaction mixture as described above. After addition of 20 µl of the P-450 solution, the mixture was incubated at 37°C for 30 min. Steroids were extractes with dichloromethane and separated by thin-layer chromatography by the method of Simpson and Boyde (11). After autoradiography the radioactive compounds were conventionally determined by a liquid scintillation spectrometer.

RESULTS AND DISCUSSIONS

It will be seen from Figure 1 that both endogenous and exogenous cholesterols were effectively converted to pregnenolone by the incubation with the cytochrome P-450. Incubation of 20R,22R-epoxycholesterol caused no apparent increase of the pregnenolone production over that from endogenous cholesterol. Similar mass fragmentograms were obtained by the incubations of the other three 20,22-epoxides as well as (E)-20(22)-dehydrocholesterol. Figure 2 shows the chemical structures of these cholesterol derivatives and the increment of pregnenolone production by the incubation. Incomplete

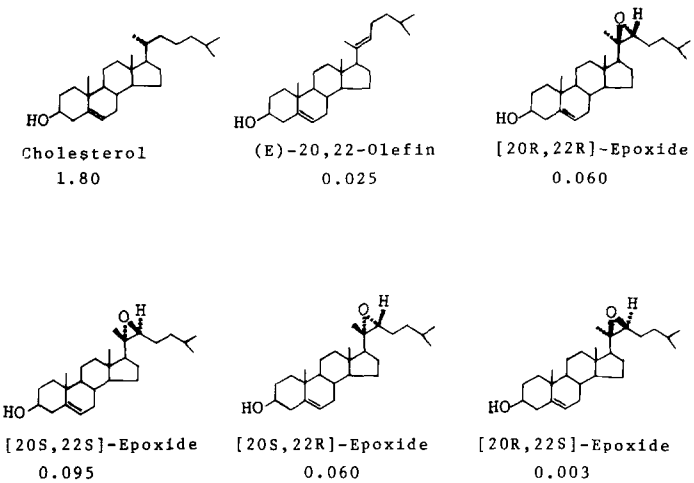

Fig. 2 Chemical structures of cholesterol derivatives used for the side-chain cleavage by adrenocortical cytochrome P-450$_{scc}$. Figures represent nmoles of pregnenolone produced by the incubations of these compounds (8.5 nmoles). The amount of pregnenolone (0.26 nmoles) produced from endogenous cholesterol was subtracted.

TABLE 1

Inhibition of the Side-chain Cleavage of [4-^{14}C]Cholesterol (1.8 nmoles) by Cholesterol Derivatives (17 nmoles).

Cholesterol derivatives added	Inhibition observed*
[20R,22R]-Epoxide	6 %
[20S,22S]-Epoxide	24
[20S,22R]-Epoxide	1
[20R,22S]-Epoxide	30
(E)-20(22)-Olefin	0

*In the control incubation where dimethylformamide containing no inhibitor was added, 1.1 nmoles of pregnenolone was produced.

conversion of endogenous cholesterol (Fig. 1, A) despite greater conversion of exogenous cholesterol (Fig. 1, C) may be due to the fact that the rate of the enzyme reaction depends on the concentration of the substrate. Since exogenous cholesterol was effectively transformed to pregnenolone, the presence of endogenous cholesterol does not hamper the conclusion on the lesser reactivity of the 20,22-epoxides or the 20(22)-olefin. It may be concluded therefore, that none of the four epoxides nor the olefin can be a good substrate for the side-chain cleavage enzyme. This was repeatedly observed by separate experiments where cholesterol and its derivatives were incubated with the enzyme in different amounts for various periods of time.

If the epoxides or the olefin were occurring as the intermediates of the overall enzyme reaction, the conversion of cholesterol to pregnenolone should be inhibited by the existence of these cholesterol derivatives in the incubation mixtures. As shown in Table 1, only a slight inhibition of the side-chain cleavage of [4-^{14}C]cholesterol was observed, by the addition of any of the 20,22-epoxides or the 20(22)-olefin even at such a high concent-

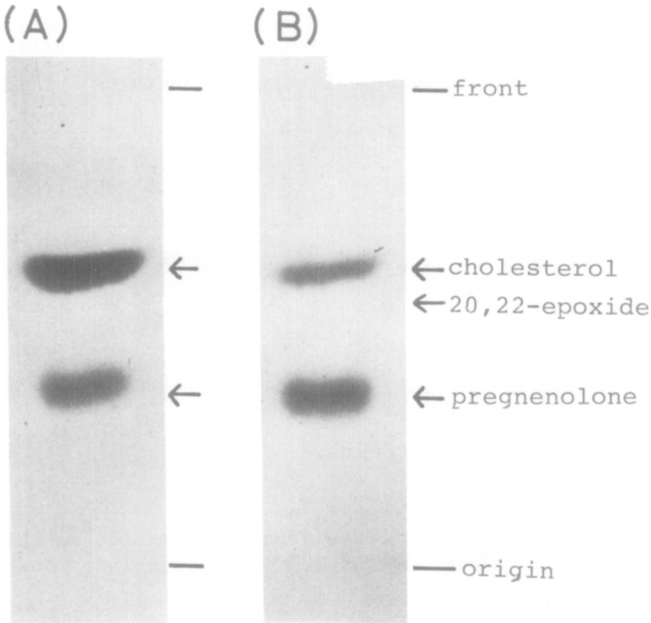

Fig. 3 Autoradiographic detection of the reaction products separated by thin layer chromatography after incubation of [4-^{14}C]cholesterol (1.8 nmoles) with adrenocortical cytochrome P-450$_{scc}$ (A) with or (B) without addition of 20R,22S-epoxycholesterol (52 nmoles). Similar autoradiograms were obtained with other cholesterol derivatives. Merck silica gel 60 F_{254} was used with the solvent system of n-pentane-diethyl ether-glacial acetic acid (60 : 40 : 2).

ration as 10 times of the substrate cholesterol. Furthermore, even when strong inhibition occurred by the addition of a large amount of the epoxides or the olefin, the radioactivity of ^{14}C-cholesterol was not trapped by these compounds. An example of the autoradiograms was shown in Fig. 3. These data suggest that the slight inhibitory effects of the cholesterol derivatives may not be due to the intermediate between cholesterol and pregnenolone.

Both conversion and trapping experiments with the cholesterol derivatives mentioned above, failed to support the pathway proposed by Kraaipoel et al. (3-5). They have supposed (4) that a substance of Rf value of 0.37 on a thin layer chromatogram to be 20,22-epoxycholesterol. This is highly improbable because all of the four 20,22-epoxides chemically

synthesized have Rf values of about 0.56 (Fig. 3) in the chromatography under the conditions of Kraaipoel et al. (4). They have also postulated (4) the presence of an epoxide hydrase in their preparations of the adrenal mitochondria, in order to explain their data. However, it is evident that the purified adrenocortical cytochrome P-450 we used here, did not contain such an epoxide hydrase, since 20,22-dihydroxycholesterol which could possibly be produced from the epoxide by the action of the hydrase is a good substrate of the P-450 (1).

Kraaipoel et al. claimed (3) that 20(22)-dehydrocholesterol, whose stereochemistry of the double bond was undefined, was converted to prengnenolone by the incubation of the adrenal mitochondria. We were unable to observe noticeable conversion of the (E)-20(22)-olefin to pregnenolone by the adrenal enzyme. Kraaipoel et al. (3) assumed that poor cleavage of the olefin was due to the contamination of the sterol preparation by an inhibitory substance, 17(20)-olefin. However, no such contaminant was present in our (E)-20(22)-olefin (9). A possibility is remained that only (Z)-20(22)-dehydrocholesterol which might be contained in Kraaipoel's sample, could be a good substrate for the cholesterol side-chain cleavage system.

Acknowledgment This work was supported by a grant from the Ministry of Education, for which we greatly appreciate.

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