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THE FINAL STEP OF SIDE-CHAIN CLEAVAGE OF CHOLESTEROL BY ADRENOCORTICAL CYTOCHROME P-450(scc) STUDIED WITH [22-^{18}O] 20, 22-DIHYDROXYCHOLESTEROLS, [^{18}O] ISOCAPROALDEHYDE, [^{18}O] WATER AND ATMOSPHERIC [^{18}O] OXYGEN

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SUMMARY. Side-chain cleavage by purified P-450(scc) of either [20R, 22R]-20, 22-dihydroxycholesterol under [18-O] oxygen or of [22-18-O] [20R,22R]-20, 22-dihydroxycholesterol under air produced isocaproaldehyde with no significant amount of 18-O. The result suggested that the aldehydic oxygen is all derived from water in the medium. However, incubation of the dihydroxycholesterol in [18-O] water showed that only 58 % of the oxygen atom of the aldehyde came from water. On the other hand, the extent of oxygen exchange between exogenously added isocaproaldehyde and water never exceeded 32 % in the same conditions as above. These results suggest that the atomospheric oxygen which is once incorporated into the aldehyde is lost from the aldehyde by the exchange with water oxygen, and that a process catalyzed by the enzyme is involved in this exchange reaction.

INTRODUCTION

Although many workers have studied the biosynthesis of pregnenolone and shown [20S]-20-hydroxycholesterol, [22 R]-22-hydroxycholesterol and [20R, 22R]-20, 22-dihydroxycholesterol as the intermediates of the reaction [1, 2, 3], precise mechanism of the carbon-20, 22 bond cleavage has not yet been elucidated. It has been reported that P-450 is responsible for the carbon bond cleavage of [20R, 22R]-20, 22-dihydroxycholesterol [4], and that 1 mole each

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of NADPH and oxygen are required [5] whereas the molecular oxygen is not incorporated into pregnenolone in this reaction [6]. Constantopoulos et al. [7] previously proposed the formation of hemiacetal as an immediate product of the reaction, while we postulated that the oxygen atom is incorporated into the aldehyde in the form of its hydrate [8]. In the present study, we used [^{18}O] molecular oxygen, [^{18}O] water and [22-^{18}O] 20, 22-dihydroxycholesterols to find the role of oxygen at the final step of side-chain cleavage of cholesterol.

MATERIALS AND METHODS

Compounds : [20R, 22R]-20, 22-Dihydroxycholesterol and [22S]-20-hydroxycholesterol were synthesized as reported previously [9]. Isocaproaldehyde was prepared by reduction of isocaproic acid with $LiAlH_4$ and subsequent oxidation of the alcohol by pridinium chlorochromate reagent [10].

^{18}O-Labeled compounds : [^{18}O] Water (96.5 atom % ^{18}O) and [^{18}O] oxygen (96.95 atom % 18-O) were purchased from Merck-Sharp & Dohme Canada Ltd., Montreal and from Miles Lab., Inc., Elkhart, Indiana, respectively.

[22-18-O] 20, 22-Dihydroxycholesterols were synthesized by heating (90-100°, 48 hr) of [20R]-22-oxocholest-5-ene-3β, 20-diol [11] (15 mg) in a mixture of sodium metal (3 mg), dioxane (0.3 ml) and [18-O] water (60 µl) and then reducing the products with $LiAlH_4$ (15 mg) in tetrahydrofuran (1.5 ml) at 25° for 15 min. The crude product (11 mg) was applied to a silica gel plate (0.25 mm thick, Merck) and developed 5 times with benzene-ethyl acetate (5:1). The upper and lower bands were separately eluted with ethyl acetate to give [20R, 22R] compound (2 mg) and [20R, 22S] compound (8 mg). The 18-O content of these dihydroxycholesterols was determined by analyses of their tris-TMS ether derivatives on GC-MS (Shimadzu-LKB 9000S, 1.5 % OV-17, 270°).

To prepare [18-O] isocaproaldehyde, the aldehyde (5 mg) was left in a mixture of [18-O] water (100 ul), dioxane (20 µl) and conc. HCl (0.5 µl) for 48 hr at 20°. It was then extracted with ether (0.5 ml). After washed with water, the extract was dried over $MgSO_4$ at 0° for 1 hr and diluted to 5 ml with dioxane. For the determination of 18-O content, the aldehyde (about 40 µg) was reduced with $LiAlH_4$ (10 mg), converted to phenyl urethane derivative with 40 µl of phenyl isocyanate and analyzed on GC-MS (1.5 % OV-17, 180°).

Enzyme preparation : Adrenodoxin, adrenodoxin reductase as well as cytochrome P-450(scc) were highly purified according to the described methods (12, 13, 14).

C_6 Fragment product analyses : [20R, 22R]-20, 22-Dihydroxycholesterol (50 µg dissolved in 50 µl of N, N-dimethyl formamide) was placed in 1.8 ml of Solution A (50 mM potassium phosphate buffer of pH 7.0 containing 2.5 nmols of P-450, 10 nmols of adrenodoxin, and 30 DCPIP units of adrenodoxin reductase). After 15 min preincubation, 0.6 ml of the phosphate buffer containing 11.5 µmols of glucose-6-phosphate, 750 nmols of NADP, and 25 ng of glucose-6-phosphate dehydrogenase was added and the mixture was incubated

for 6 min at 37°. The incubation mixture was then partitioned between ether and 2 N NaOH. The ether layer was divided into two portions (I and II), while the aqueous layer was acidified and then extracted with ether (III). The ether extract (I) was directly derivatized, while the extracts (II) and (III) were reduced with $LiAlH_4$ and then derivatized. The resulting isocapryl alcohol phenyl urethanes were analyzed by GC-MS to quantify isocapryl alcohol (I), isocaproaldehyde (II), and isocapric acid (III).

Incubation of the labeled substrates : Seventy five μg of either [22-^{18}O] [20R, 22R]-20, 22-dihydroxycholesterol or [22-18-O][20R, 22S]-20, 22-dihydroxycholesterol were added in 1.8 ml of Solution A and incubated with the NADPH-generating system as described above. The ether extract of the incubation mixture was dried over $MgSO_4$ at 0° for 1 hr and then distilled azeotropically with benzene, while isocaproaldehyde was trapped at -50°. Reduction, derivatization and analyses were followed as mentioned above

[18-O]Isocaproaldehyde (40 μg) was incubated also with the enzyme mixture for 6 min. In a series of separate experiments, the extent of 18-O exchange between water and the labeled aldehyde was measured every 10 min for 1 hr in the same manner as above.

Incubation in ^{18}O-atomosphere : A specially designed long-necked glass flask was used. The flask was equipped with a 3-way stop cock on its arm and with a tightly shielded rubber stopper on its top through which solutions were injected in. The incubation mixture contained 75 μg of either [20R, 22R]-20, 22-dihydroxycholesterol or [20S]-20-hydroxycholesterol and other components as above in a final volume of 2.4 ml, and was degassed three times by evacuation followed by introduction of pure argon. Finally, about 20 ml of 18-oxygen: nitrogen (1:4) mixture was introduced and the pressure was adjusted to 1 atm. The reaction was started by the addition of the NADPH generating system and stopped after 6 min incubation at 37°. The 18-O content of the atomospheric oxygen was 94 atom % excess when aliquots of the gas were analyzed by the mass spectrometer at the end of the reaction

Incubation in [^{18}O]water : Fifty μg of [20R, 22R]-20, 22-dihydroxycholesterol or 40 μg of isocaproaldehyde were incubated in the enzyme mixture as above, but the solution contained [18-O]water (24 atom % excess 18-O). The 18-O content of the solution was determined by GC-MS at the end of the reaction.

RESULTS

Syntheses of ^{18}O-labeled dihydroxycholesterol isomers and ^{18}O-labeled isocaproaldehyde

Two stereoisomers of [22-^{18}O]20, 22-dihydroxycholesterol have been synthesized by the reaction of [20R]-22-oxocholest-5-ene-3β, 20-diol with sodium metal in a mixture of dioxane and [^{18}O]water followed by reduction with $LiAlH_4$. The [20R, 22R] and [20R, 22S]-isomers were produced in a ratio of 4 : 1 and separated by preparative thin layer chromatography. The mass

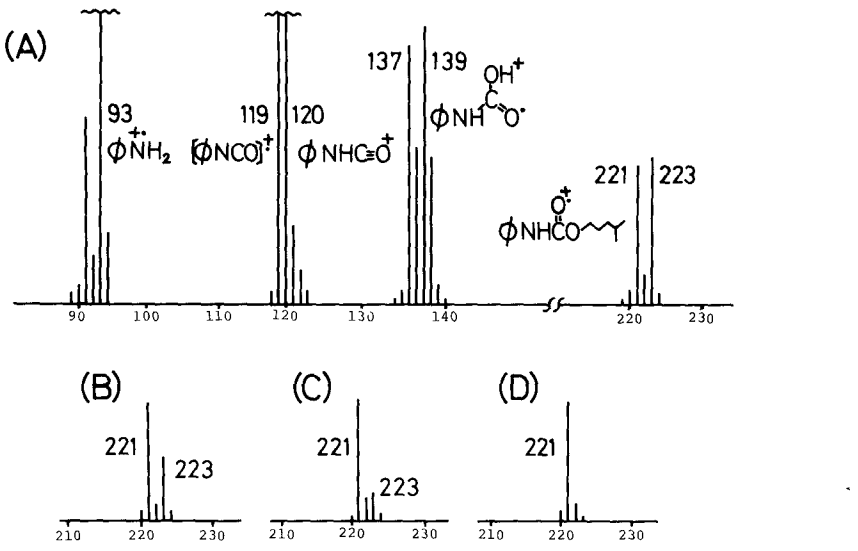

Fig. 1. Partial mass spectra of isocapryl alcohol phenyl urethane derived from isocaproaldehyde.

(A) Standard [18-O]isocaproaldehyde; (B) [18-O]Isocaproaldehyde incubated with P-450(scc); (C) Isocaproaldehyde obtained by the incubation of [20R, 22R]-20, 22-dihydroxycholesterol with P-450(scc) in [18-O]water; (D) Isocaproaldehyde after incubation with P-450(scc).

spectra obtained as tris-trimethyl silyl ethers showed the molecular ion peaks 634 and 636 in a relative intensity of 1.4 : 8.6. The height of the fragment ions m/e 619 (M^+ - 15) and 289 (the sterol C_{20}-C_{27} side chain) and their respective isotopic peaks 621 and 291 also showed that the ^{18}O content is about 86 % and consistent for both stereoisomers. Location of ^{18}O at C_{22} was indicated by the presence of fragment ion m/e 461 (the sterol C_1-C_{21} nucleus) accompanying no significant peak at 463.

Isocaproaldehyde was labeled with ^{18}O by acid catalyzed exchange reaction in [^{18}O]water. The ^{18}O content was determined by GC-MS after the aldehyde was converted to isocapryl alcohol phenyl urethane (Fig. 1-A) and estimated to be 52 % on the basis of the intensity of the molecular ion peak m/e 221 and its isotopic peak 223. The isotopic McLafferty ion m/e 139 also confirmed the presence of the ^{18}O in the molecule.

Identification and ^{18}O content of isocaproaldehyde as the product of cleavage of dihydroxycholesterols

After incubation of [20R, 22R]-20, 22-dihydroxycholesterol with purified P-450(scc), the analyses of the acidic and neutral fractions showed that as much as 95 % of the total C_6 fragments was isocaproaldehyde. The aldehyde was identified in the form of isocapryl alcohol phenyl urethane by comparing its GC retention time and mass spectrum with those of the standard compound

Table I shows the ^{18}O content of isocaproaldehyde which was produced by P-450(scc) under a variety of conditions. The aldehyde contained no significant amount of ^{18}O in the incubation of unlabeled [20R, 22R]-20, 22-dihydroxycholesterol or [20S]-20-hydroxycholesterol under [^{18}O]oxygen (Exps. 3 and 5) When [22-^{18}O][20R, 22R] or [22-^{18}O][22R, 22S]-20, 22-dihydroxycholesterol was cleaved under air (Exps. 6 and 7), the produced aldehyde was also devoid of significant amount of ^{18}O. However, it was revealed by the incubation of 20, 22-dihydroxycholesterol in the labeled water (Exp 4 and Fig 1-C) that fifty eight percent of the aldehyde incorporated oxygen from water On the other hand, the results of Exps. 1 (Fig. 1-B) and 2 showed the extent of non-enzymic exchange of oxygen between the aldehyde and water during the incubation and separation procedures. It will be seen that there is no discrepancy of observations between the two experiments regardless of whether the label was placed on the aldehyde or on water.

DISCUSSION

The C_6 fragments of the side chain cleavage of cholesterol are isocaproic acid, isocapryl alcohol or isocaproaldehyde, depending on the purity of the enzyme preparation used [15]. In the present work, we used purified P-450 and found that isocaproaldehyde was an exclusive product of the reaction of [20R, 22R]-20, 22-dihydroxycholesterol.

Table I

^{18}O CONTENT IN ISOCAPROALDEHYDE RELEASED DURING SIDE CHAIN CLEAVAGE OF DIHYDROXYCHOLESTEROL

Exp.	Compound	Conditions	^{18}O corrected content of isocaproaldehyde % (a)
1	[^{18}O]-Isocaproaldehyde	$^{16}O_2$ + $H_2{}^{16}O$	68 ± 3
2	Isocaproaldehyde	$^{16}O_2$ + $H_2{}^{18}O$	32 ± 2
3	[20R,22R]-20,22-Dihydroxy-cholesterol	$^{18}O_2$ + $H_2{}^{16}O$	2 ± 1
4	[20R,22R]-20,22-Dihydroxy-cholesterol	$^{16}O_2$ + $H_2{}^{18}O$	58 ± 4
5	[20S]-20-Hydroxycholesterol	$^{18}O_2$ + $H_2{}^{16}O$	5 ± 1
6	[22-^{18}O]-[20R,22R]-20,22-Dihydroxycholesterol	$^{16}O_2$ + $H_2{}^{16}O$	2 ± 1
7	[22-^{18}O]-[20R,22S]-20,22-Dihydroxycholesterol	$^{16}O_2$ + $H_2{}^{16}O$	4 ± 1

(a) The figure in the table represents the corrected value (± S. E) of 18-O content of isocaproaldehyde which was analyzed in the form of phenyl urethane derivative. Corrections were done as follows : 18-O corrected content = $A \times 10^4 / (A+B) \times C$; where A = peak height of m/e 223 corrected for the blank and naturally occurring heavy isotopes, B = peak height of m/e 221 corrected for the blank and naturally occurring heavy isotopes; C = 18-O content of the substrates or aqueous phase or gas phase expressed in %. Standard isocaproaldehyde was incubated in each experiment in the same conditions as used for the substrate, and was used as the blank in each case.

It is unquestionable that molecular oxygen is required for the final step of the side chain cleavage reaction, because the formation of pregnenolone from dihydroxycholesterols was markedly inhibited under anoxic atomosphere (data not shown). According to the mechanism previously proposed by us (Fig 2), the produced isocaproaldehyde should contain 50 atom % excess of ^{18}O. However no significant excess amount of ^{18}O was detected in the aldehyde, either when [20R, 22R]-20, 22-dihydroxycholesterol was cleaved under [^{18}O]oxygen or [22-^{18}O][20R, 22R]-20, 22-dihydroxycholesterol was cleaved under air. We suspected that rapid exchange of oxygen between water and the aldehyde caused

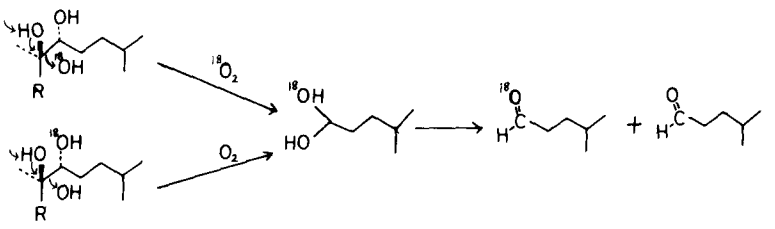

Fig. 2. The mechanism of the last step of side chain cleavage of cholesterol previously proposed by Morisaki et al. (FEBS Letters, 72, 337-340, 1976).

the loss of the once-incorporated oxygen from the aldehyde. However, the results of Exps. 1 and 2 showed that the chemical exchange of oxygen never exceeded the level of 32 %. We believe that this unavoidable exchange occurs during the extraction or derivatization procedure rather than during the side-chain cleavage reaction, because there was no increase of exchange with time in 1 hr of incubation (data not shown). It was then suspected that the exogenously added dihydroxycholesterol and the enzymically produced dihydroxycholesterol behave differently. However, the result of incubation of [20S]-20-hydroxy-cholesterol which is the immediate precursor of the dihydroxycholesterol (Exp 5) demonstrated that this is not the case. The possibility of stereospecific OH addition to C_{22} followed by stereospecific removal of [^{18}O] water from isocapro-aldehyde hydrate was also excluded by the result of incubation of [20R, 22S]-20, 22-dihydroxycholesterol (Exp. 7). All these data suggest that the oxygen of isocaproaldehyde comes from water by the exchange reaction, while the result of Exps. 1 and 2 indicates that an enzyme-catalyzed process is involved in this exchange reaction. However, incubation of [20R, 22R]-20, 22-dihydroxy-cholesterol in [^{18}O] water produced isocaproaldehyde with only 58 atom % excess of ^{18}O (Exp. 4). It should be questioned now why the oxygen exchange did not complete in Exp 4, while all the ^{18}O was supposedly lost from the aldehyde in Exps. 3, 5, 6, and 7. This discrepancy of observations could be

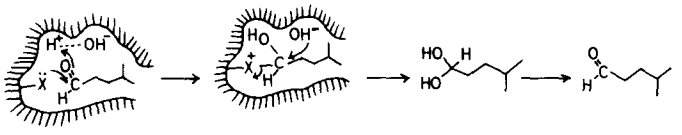

Fig. 3. Hypothetical mechanism of the exchange of oxygen of isocaproaldehyde with water oxygen at the catalytic site of P-450(scc). X may be NH_2, OH, or SH group of the enzyme.

interpreted, if we assume that there is a compartment of bound water around the catalytic site of the enzyme and that substitution of oxygen of the produced aldehyde with water oxygen occurs only there in such a fashion as illustrated in Fig. 3. Further studies on this line would be interesting to understand the dynamics of the hydrophobic enviroment around the active site of the enzyme.

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