STEREOCHEMICAL SPECIFICITY AT CARBON-20 AND -22 OF HYDROXYLATED CHOLESTEROLS FOR SIDE-CHAIN CLEAVAGE BY ADRENOCORTICAL CYTOCHROME *P*-450$_{scc}$

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1. Introduction

The enzymatic conversion of cholesterol to pregnenolone is the initial step of biosynthesis of various steroid hormones. The mechanism of this important biological reaction has been studied by many workers mostly with crude enzyme preparations such as adrenocortical mitochondrial acetone-dried powders. Recently, a cytochrome *P*-450 which has an enzyme activity of converting cholesterol to pregnenolone has been purified from bovine adrenocortical mitochondria [1,2]. By the use of this purified cytochrome *P*-450$_{scc}$ (cholesterol 20,22-lyase), we have previously shown [3] that, in contradiction to Kraaipoel's hypothesis [4,5], neither 20,22-epoxycholesterol nor 20 (22)-dehydrocholesterol could be the intermediate between cholesterol and pregnenolone. A similar conclusion was independently obtained by Burstein et al. [6]. On the role of hydroxycholesterols which have been postulated as the intermediates of the cleavage reaction, some controversy was recently raised [7–9].

We have now synthesized all the C-20 and/or C-22 stereoisomers of 20-hydroxycholesterol, 22-hydroxycholesterol and 20,22-dihydroxycholesterol (fig.1), and incubated them with cytochrome *P*-450$_{scc}$ in the presence of NADPH generating and electron transport systems. Our aim was to know stereochemical specificity of the substrate for the cleavage between C-20 and C-22.

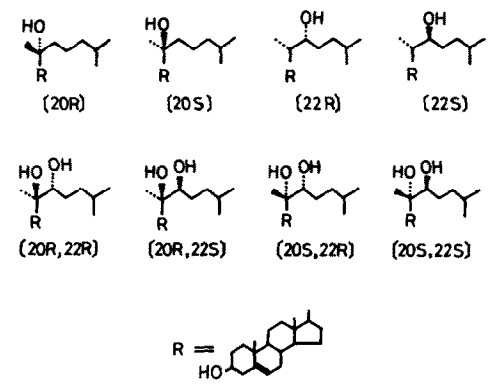

Fig.1. Hydroxycholesterol isomers synthesized and tested for the side-chain cleavage by cytochrome *P*-450$_{scc}$.

2. Experimental

2.1. *Substrate*

[20 S]-20-*p*-Tolylpregn-5-ene-3β, 20-diol was prepared by the published method [8]. [20 R]-20-Hydroxycholesterol and its [20 S]-isomer were obtained by the reduction of [20 S, 22 S]- and [20 R, 22 R]-20,

22-epoxycholesterols [10] with lithium aluminum hydride. [22 S]-22-Hydroxycholesterol [11] was synthesized by Grignard reaction of 22,23-bisnorchol-5-en-22-al with isoamyl magnesium bromide. [22 R]-22-Hydroxycholesterol was synthesized through hydroboration of (E)-6β-methoxy-3α, 5-cyclo-cholest-20 (22)-ene [12]. Treatment of [20 R, 22 R]-20,22-epoxycholesterol [10] with perchloric acid yielded [20 S, 22 R]-20,22-dihydroxycholesterol. [20 S, 22 S]-20,22-Dihydroxycholesterol was obtained by the oxidation of (E)-20 (22)-dehydrocholesterol acetate with osmium tetroxide [10] followed by alkaline hydrolysis. [20 R, 22 S]-20,22-Dihydroxycholesterol and its [20 R, 22 R]-isomer were synthesized from 6β-methoxy-3α,5-cyclo-pregnan-20-one by the following reactions in sequence:

(1) Reaction with vinyl magnesium bromide affording the [20 S]-20-vinyl-20-ol,

(2) Oxidation with *m*-chloroperbenzoic acid to give a diastereoisomeric mixture of the 22, 23-epoxides,

(3) Coupling reaction with lithium diisobutyl cuprate yielding [20 R, 22 S]- and [20 R, 22 R]-20,22-glycols which were subsequently separated by chromatography on a silica gel column,

(4) Acid treatment to recover the 3β-ol-5-ene system. Details of these syntheses will be described elsewhere [12].

2.2. *Incubation and product determination*

Incubations and extraction of the products were carried out as previously described [3] except for omission of bovine serum albumin from the incubation medium. The cholesterol side-chain cleavage enzyme was purified as the tetrameric species of mol. wt 200 000 [2]. Pregnenolone was determined as described in the previous paper [3]. 20-Hydroxycholesterol, 22-hydroxycholesterol and 20,22-dihydroxycholesterol were determined as their trimethylsilyl ethers by mass fragmentography with a Shimadzu-LKB 9000 S gas chromatography–mass spectrometer (GC–MS). The column (1 m × 3 mm inner diameter) was packed with 1.5% OV-17 on Shimalite W (80–100 mesh) and operated at 262°C with helium carrier gas at a flow rate of 30 ml/min. The injection port and separator temperatures were 280°C and 290°C, respectively. Spectra were taken at 70 eV. The products of the above-mentioned incubation were heated with trimethylsilylimidazole (20 μl) at 80°C for 1 h in a sealed tube and the solution directly injected into the GC–MS system. The trimethylsilyl ethers of the hydroxycholesterols were analyzed by monitoring fragment ions of m/e 173, 201, 461 and 475.

3. Results and discussion

Results of incubations of the 20,22-dihydroxycholesterols are presented in fig.2. It is evident that [20 R, 22 R]- and [20 R, 22 S]-dihydroxycholesterols were transformed to pregnenolone nearly by 100% in one hour incubation, while the [20 S, 22 R]- and [20 S, 22 S]-isomers were less effectively cleaved than cholesterol. These results suggest that the cytochrome *P*-450 requires strict stereospecificity around C-20 of the substrate, while it is generous about the configuration at C-22. In accord with this supposition, [20 R]-20-hydroxycholesterol was completely inactive as the substrate of the enzyme, whereas [20 S]-20-hydroxycholesterol was greatly reactive (fig.3). The loose requirement for the stereochemical specificity at C-22 was further indicated by the incubation of the 22-hydroxycholesterol isomers: both [22 R]- and [22 S]-isomers were much more efficiently transformed to pregnenolone than cholesterol, although the [22 R]-isomer seems to be a better substrate than the [22 S]-isomer. A small preference for the [22 R]-configuration over the [22 S]-configuration was also observed when 20,22-dihydroxycholesterols were incubated for shorter periods of time (fig.4). These observations allow us to suppose that among these

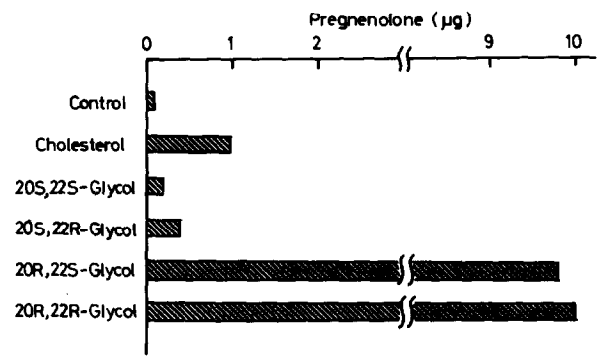

Fig.2. Pregnenolone formed by incubation (1 h) of 20,22-dihydroxycholesterols (10 μg each) with cytochrome *P*-450$_{scc}$.

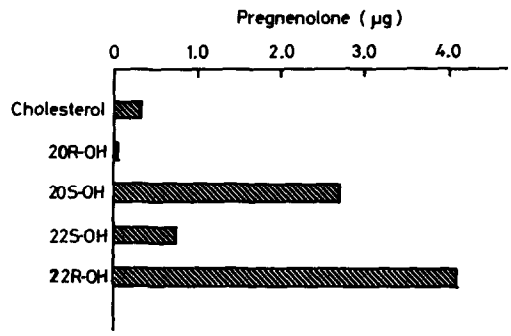

Fig.3. Pregnenolone formed by incubation (10 min) of 20- and 22-hydroxycholesterols (10 μg each) with cytochrome $P\text{-}450_{scc}$.

diastereoisomers, [20 S]-20-hydroxycholesterol, [22 R]-22-hydroxycholesterol and [20 R, 22 R]-dihydroxycholesterol are the most probable intermediates between cholesterol and pregnenolone. Figure 4 also indicates that relative rates of conversion of these sterols to pregnenolone were: 20, 22-diol> 22-ol>20-ol≫cholesterol. This result supports the view that the dihydroxycholesterol is proximate to pregnenolone in the pathway and that the first step of hydroxylation at C-20 or C-22 is ratelimiting for the over-all reaction of the side-chain cleavage of cholesterol. In contrast to the results obtained by Hochberg et al. [8], [20 S]-20-tolylpregn-5-en-3β, 20-diol was not converted to pregnenolone in significant amounts by the incubation with the purified cytochrome $P\text{-}450_{scc}$.

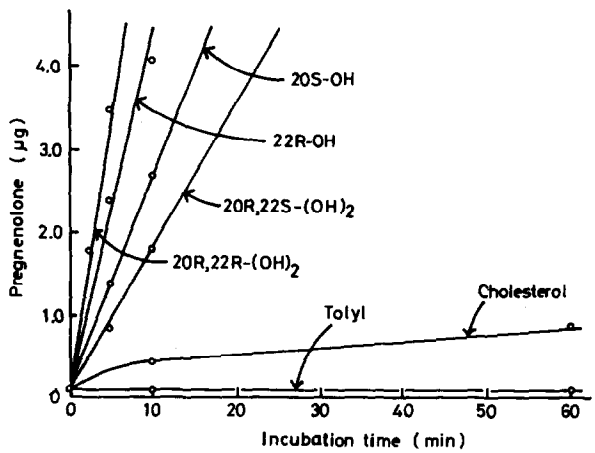

Fig.4. Time-course of the production of pregnenolone when various hydroxylated cholesterol derivatives (10 μg each) were incubated with cytochrome $P\text{-}450_{scc}$.

If these hydroxycholesterols are actually the intermediates of the cholesterol side-chain cleavage reaction, these compounds must be detected in the incubation mixture, but only in minute amounts because they are instantly transformed to pregnenolone. We have developed a highly sensitive mass fragmentographic method to detect these hydroxycholesterols as their trimethylsilyl ether. 20,22-Dihydroxycholesterol tris-trimethylsilyl ether gives a characteristic mass fragment ion of m/e 461 due to the cleavage of C-20,22 bond, as reported by other workers [13,14]. The same ion was also observed with 20-hydroxycholesterol bis-trimethylsilyl ether. The latter compound showed another prominent ion peak at m/e 201 comprising C-20-27. For mass fragmentography of 22-hydroxycholesterol bis-trimethylsilyl ether, the mass ion of m/e 475 produced by the cleavage of C-22,23 and the ion of m/e 173 comprising C-22-27 proved useful. By monitoring these four fragment ions (m/e 173, 201, 461 and 475) we were able to detect the three hydroxycholesterols in amounts as small as 10^{-10} g.

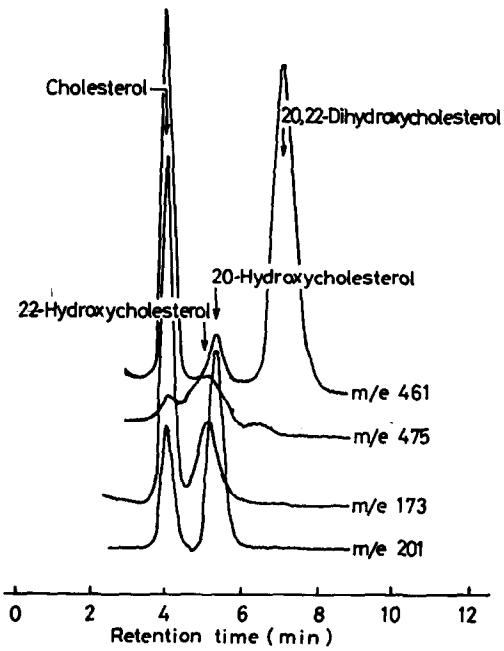

Fig.5. Mass fragmentographic detection of 20- and 22-hydroxy- and 20,22-dihydroxycholesterols which were formed by the incubation (2.5 min) of cholesterol (10 μg) with cytochrome $P\text{-}450_{scc}$. The hydroxycholesterols were injected into the GC–MS system after conversion to their TMS ethers (see Experimental for further details).

Thus, we were able to demonstrate the formation of these hydroxycholesterols by incubating cholesterol with the enzyme for 2.5 min as shown in fig.5. We also revealed by the mass fragmentographic technique that both [20 S]-20-hydroxycholesterol and [22 R]-22-hydroxycholesterol yielded 20,22-dihydroxycholesterol in 5 min incubation with the enzyme (data not shown). At the present moment, our gas chromatographic system is not able to complete separation of the stereoisomers of these hydroxycholesterols. Further studies are needed for the stereochemistry of these compounds as well as for their quantitative determination as a function of incubation time.

Burstein et al. [13,15] have been sceptical about the intermediary role of 20-hydroxycholesterol mainly because of their failure in detecting this compound after incubation of cholesterol. However, our positive detection of this compound (fig.5) and the observation of its faster conversion to pregnenolone as compared with cholesterol (fig.4) reconcile the assumption that some significant aliquot of cholesterol is transformed to pregnenolone by the route of [20 S]-20-hydroxycholesterol.

All these results reported here support the view that 'the classical hydroxycholesterol pathway' (fig.6) acts even with the purified cytochrome *P*-450$_{scc}$. The precise mechanism of the last step of the reaction, i.e., carbon–carbon chain cleavage, is not clear, while it has been known that molecular oxygen and NADPH are required [16] and that a cytochrome *P*-450 participated in the reaction [17]. The scheme proposed in fig.6 would predict that the last step involves insertion of one 'activated oxygen atom' to C-22 to release the hydrate of isocapraldehyde as the immediate C_6 product. According to this scheme, the mechanism of carbon–carbon chain cleavage is not intrinsically different from that of C-20 and C-22 hydroxylation and it is not strange to assume that all the three steps of the reaction are undertaken by a single species of cytochrome *P*-450.

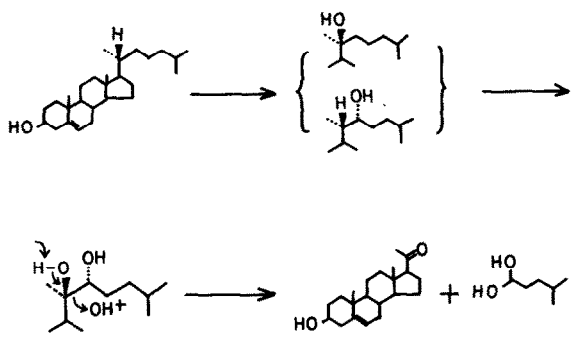

Fig.6. Presumable mechanism of cholesterol side-chain cleavage by cytochrome *P*-450$_{scc}$. Alternatively, OH^+ may attack C-20 of the diol yielding pregnenolone hydrate and isocapraldehyde.

Acknowledgement

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