EVIDENCE FOR 20, 22-EPOXYCHOLESTEROL AS AN INTERMEDIATE IN SIDE-CHAIN CLEAVAGE OF 22R-OH CHOLESTEROL BY ADRENAL CORTEX MITOCHONDRIA

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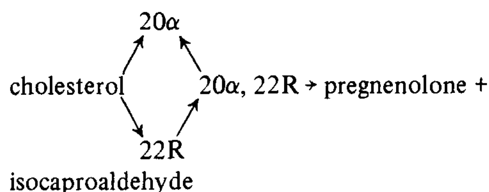
1. Introduction

The cholesterol side-chain cleavage enzymesystem occurring in the adrenal cortex is able to convert cholesterol, 20α-OH cholesterol (20α)*, 22R-OH cholesterol (22R) and 20α, 22R-di-OH cholesterol (20α, 22R) (inter alia) into pregnenolone and isocaproaldehyde. According to the 'classical scheme' the reactions are:



A direct conversion of cholesterol into 20α, 22R has also been proposed [1,2]. However evidence for two sequential mono-oxygenases or for a concerted attack of oxygen producing the dihydroxy cholesterol is still lacking. There are several other models of cholesterol side-chain cleavage which also lack good experimental evidence [3–5].

We have studied the conversion of 22R into pregnenolone and isocaproaldehyde. In this paper the identity of the intermediate 20α, 22R will be established by a combination of gas chromatography and mass spectrometry. In addition we found that 20α, 22R is not the only intermediate. The results strongly suggest 20,22-epoxycholesterol to be an intermediate in the same reaction. The experiments support our previously presented hypothesis, according to which 22R is converted into pregnenolone and isocaproaldehyde via Δ^{20-22} cholesterol, 20,22-epoxycholesterol and 20α, 22R-di-OH cholesterol [6].

2. Materials and methods

Bovine adrenal cortex mitochondria were prepared according to standard procedures and stored directly in liquid nitrogen. Conversion of 22R-OH cholesterol into pregnenolone was estimated in a medium containing 154 mM KCl, 11.5 mM NaCl, 50 mM nicotinamide, 20 mM HEPES (pH 7.3), 5 mM $CaCl_2$, 4 mM sodium azide, 1% bovine serum albumin (w/v) with a final vol of 25 ml. Incubations were carried out at 37°C in a thermostatically controlled vessel, the contents of which were magnetically stirred. The above 'freeze-damaged' mitochondria (23 mg protein) were used. Additions were made to achieve a final concentration of 30 μM cyanoketone [7], 5 μM antimycin A, 0.1 mM NADP, 3 mM glucose 6-phosphate and

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; Cyanoketone, 2α-cyano-4,4,17α-trimethyl-17β-hydroxyandrost-5-en-3-one; Cholesterol, 5-cholesten-3β-ol; Epi-cholesterol, 5-cholesten-3α-ol; 20α-OH cholesterol, 5-cholesten-3β,20α-diol; 22R-OH cholesterol, (22R)-5-cholesten-3β,22-diol; 20α,22R-di-OH cholesterol, (22R)-5-cholesten-3β,20α,22-triol; 20α,22S-di-OH cholesterol, (22S)-5-cholesten-3β,20α,22-triol; Δ^{20-22} cholesterol, 5,20(22)-cholestadien-3β-ol; 20,22-epoxycholesterol, 20,22-epoxy-5-cholesten-3β-ol; Pregnenolone, 5-pregnen-3β-ol-20-one.

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0.6 U/ml glucose 6-phosphate dehydrogenase.

After a 5 min preincubation at 37°C a sample was taken to correct for the pregnenolone formation from endogeneous cholesterol and 1300 nmol 22R-OH cholesterol (Ikapharm, Israel) were added to the medium.

Exp. 1-A: Every minute a 0.5 ml sample was taken and extracted with ice-cold ethylacetate. The first sample was taken 10 sec after addition of 22R-OH cholesterol.

Exp. 1-B: Samples of 2 ml were taken from the *same* incubation at intervals as indicated in fig. 1-B, added to 25 ml erlenmeyers and immediately flushed with 100% carbonmonoxide (500 ml/min) for 120 sec. The contents were sealed off from air and incubated in a shaking incubator at 37°C for 20 min. Reactions were also stopped by cold ethylacetate. Since it took CO 1 min. to stop 22R conversion and pregnenolone formation, the indicated time at the abscissa of fig. 1-B is the time the sample was taken from the experiment (shown in fig. 1–A) plus 1 minute.

To all samples 20 μg/ml (50 μM) epi-cholesterol was added as an internal standard. Steroids were extracted with 3 X 6 ml distilled ethylacetate. The solvent was evaporated at room temperature with nitrogen. The extract was silylated and the steroids were quantified by gas chromatography as described [6]. All glassware had been cleaned with a potassium bichromate–sulfuric acid mixture and siliconized with Siliclad (Clay Adams).

Gaschromatographic analysis (fig.2) was performed at 250°C on a capillary column coated with Se-30, length 60 m [8], with a solid state injector [9], equipped with a flame ionizing detector (full scale in fig.2 1×10^{-11} A) and with nitrogen as a carrier gas.

The mass spectra were obtained by a GC-MS combination as described by Leferink et al. [10]. The conditions were as follows: temperature of the column 230°C, GC-MS interface 260°C, source 230°C, accelerating voltage 4 KeV, ionizing voltage 70 eV and ionizing current 500 μA.

Thin-layer chromatography was performed on silicagel (Merck 60 F_{254}) with pentane : ether : glacial acetic acid (60 : 40 : 2). 20α, 22S-di-OH cholesterol was synthesized according to Chaudhuri et al. [11] using sodium borohydride reduction. Protein was estimated by the biuret method.

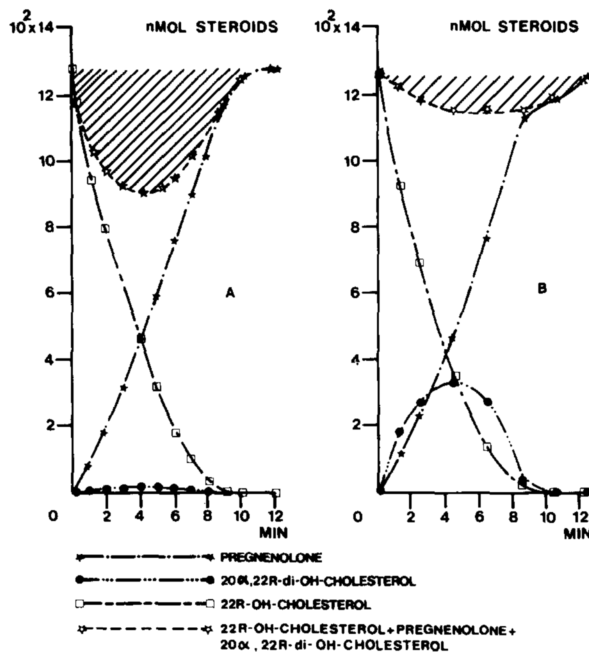


Fig.1. (A) Conversion of 22R-OH cholesterol into pregnenolon by damaged bovine adrenal cortex mitochondria supported by a NADPH-generating system. 20α, 22R is formed as an intermediate. The shaded area represents the apparent 'mass-defect'. The steroids were quantified after trimethylsilylation by GLC. (B) Samples taken during the reaction of the experiment shown in fig.1(A) were immediately flushed with 100% carbon monoxide (methods). They were incubated air-free in a shaking incubator at 37°C for 20 min. The shaded area decreases and 20α, 22R-OH cholesterol increases. The indicated time at the abscissa has been corrected for the time it took CO to stop the reaction ($t_{1\text{-}B} = t_{1\text{-}A} + 1$ min.).

3. Results

In fig.1-A it can be seen that 22R (1300 nmol) disappears in 10 min and 1300 nmol pregnenolone are formed. Assuming 20α, 22R to be the only intermediate in the conversion from 22R into the products pregnenolone and isocaproaldehyde, the sum (nmol) of 22R, 20α, 22R and pregnenolone must be constant during the reaction. However the sum of the nmol substrate, intermediate and product is not constant. At t=4 min it is only 70% of the initial amount of substrate. Therefore 30% must be present in a form not detected by our gaschromatographic

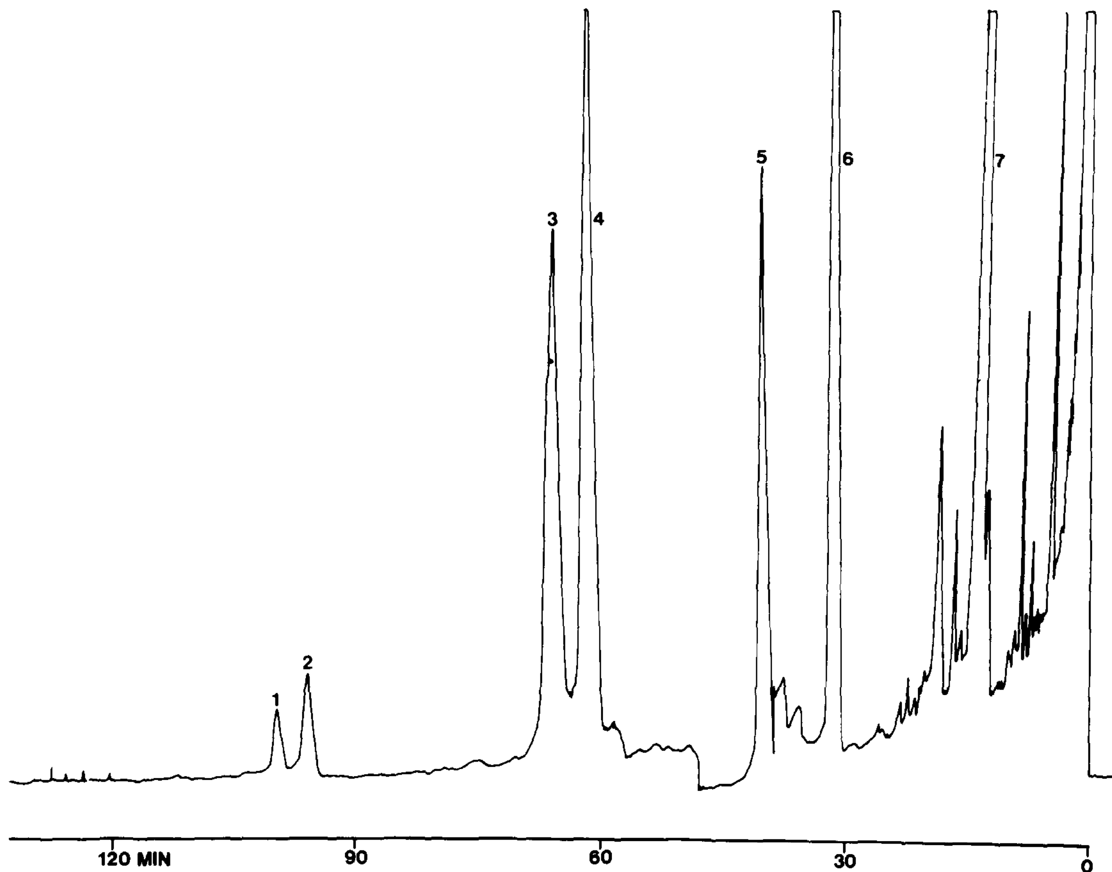


Fig.2. Gas-liquid chromatography of trimethylsilylated steroids on a capillary column (Methods): 1) 20α, 22R-di-OH cholesterol, 2) 20α, 22S-di-OH cholesterol, 3) 20α-OH cholesterol, 4) 22R-OH cholesterol, 5) cholesterol, 6) epi-cholesterol, 7) pregnenolone.

method. The time course of formation and disappearance of this or these compounds is represented by the shaded area of fig.1-A. Analysis of the samples (taken from the experiment shown in fig.1-A), incubated for 20 min under carbon monoxide (see Methods) and thus with complete inhibition of cytochrome *P*-450, shows a marked increase in 20α, 22R and a proportional decrease in the shaded area (fig.1-B). Apparently the compound(s) not measured by our method are in the presence of ⩾ 99% CO converted into 20α, 22R.

Fig.2 shows a gas chromatogram obtained with a capillary column. The sample was taken at t=4 min (fig.1-B) and injected together with a mixture of pregnenolone, epi-cholesterol, cholesterol, 22R-OH cholesterol, 20α-OH cholesterol and 20α, 22S-di-OH cholesterol. The biological intermediate 20α, 22R is clearly separated from the synthetically prepared 20α, 22S-di-OH cholesterol, while the mass spectra of both compounds are almost identical (fig.3).

Thin-layer chromatography (fig.4) of samples taken from an incubation analogous to the one shown in fig.1-A is in accord with the gaschromatographic analysis and supplements it. 22R decreases and pregnenolone is formed. Cholesterol from mitochondria origin is detected in all samples. However a compound with R_f=0.37 is detectable during the reaction. It is neither present at the start of the reaction (t=0 min) nor after the reaction has come to an end (t=11 min).

Isolation of the unknown substance from thin-layer plates followed by silylation and gas chromatography showed the presence of a neglible amount of 20α, 22R as might be expected from the results, represented

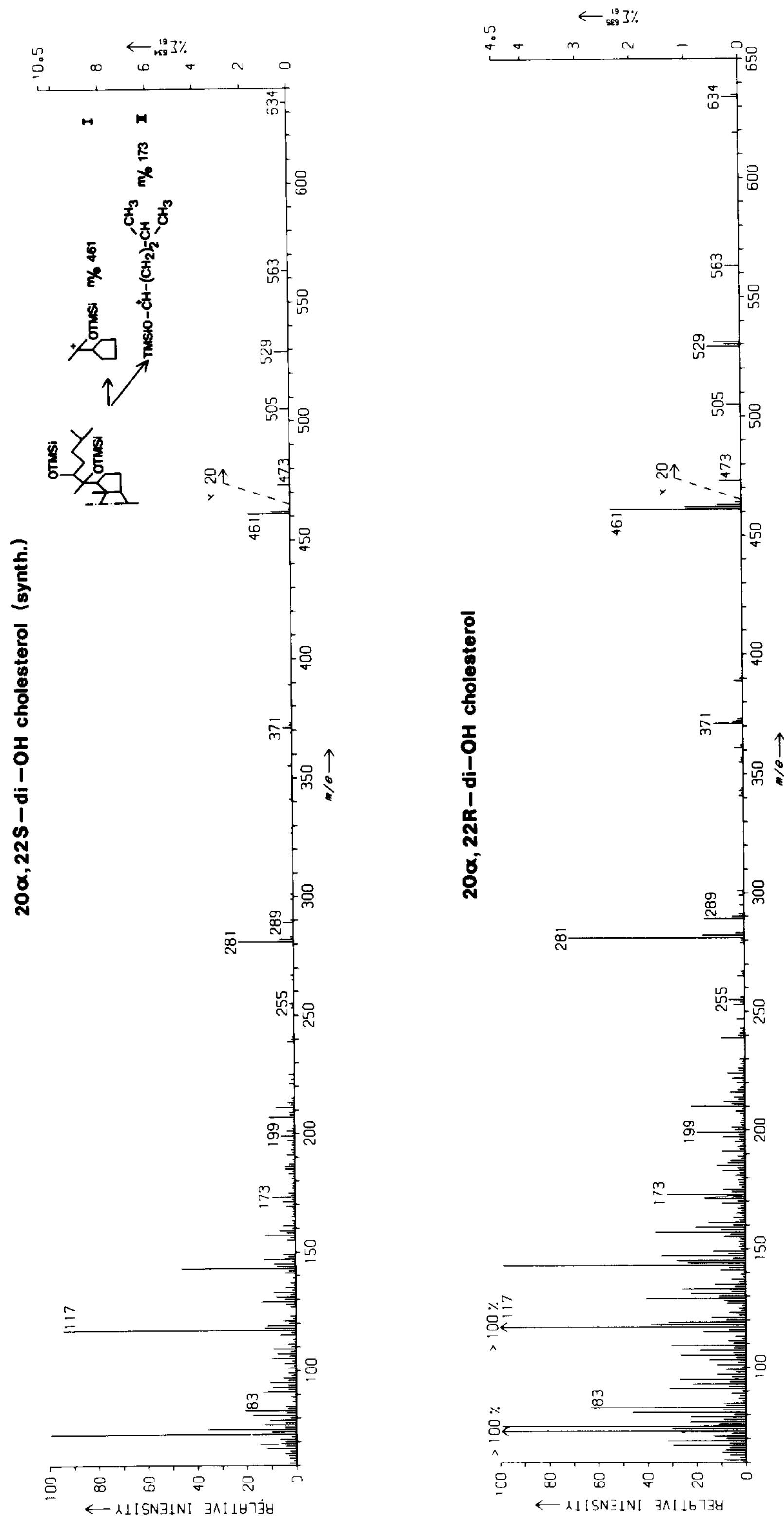


Fig.3. Mass spectra from synthetic 20α, 22S-di-OH cholesterol and from the biological intermediate 20α, 22R-di-OH cholesterol.

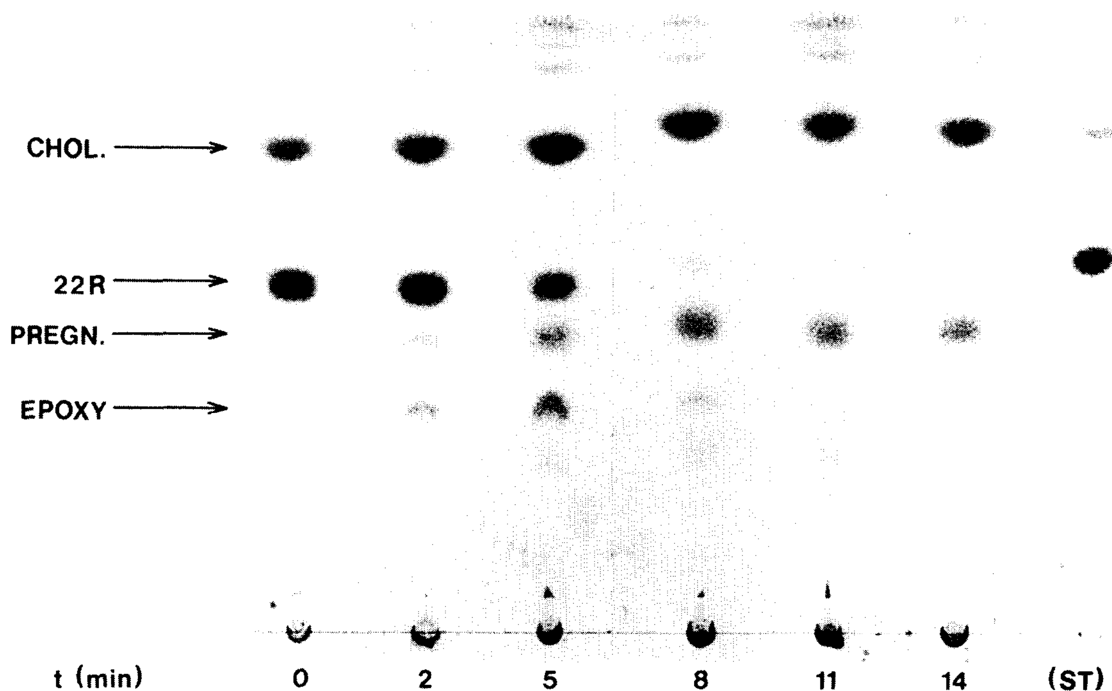


Fig.4. Thin-layer chromatography of samples taken from an experiment analogous to the one described in fig.1. (A). Cholesterol of mitochondrial origin is present in all samples. At t=2, 5 and 8 min a spot with R_f=0,37 is visible, preliminarily identified as 20, 22-epoxycholesterol.

by fig.1-A. Chemical hydrolysis with perchloric acid [12] converted the unknown compound into 20, 22-di-OH cholesterol.

3.1. *Discussion of the mass spectra*

The upper part of fig.3 is the mass-spectrum of persilylated 20α, 22S-di-OH cholesterol. The molecular weight of the compound is 634. Characterisation of some important fragments demonstrates the location of the hydroxylgroups in the side-chain.

The fragment at m/e 563 indicates a simple fission of the 22–23 bond, and loss of an additional $(CH_3)_3$ Si-OH group produces a fragment at m/e 473. The peaks at m/e 461 (fragment I) and m/e 173 (fragment II) are due to fission of the 20–22 bond with the charge located on either the large or the small fragment (see insert of fig.3). The fragments at m/e 371 and m/e 281 are formed from fragment I by losing one or two $(CH_3)_3$ Si-OH groups respectively. Analogous to this fragment II will give a peak at m/e 83. The peak at m/e 117 could be explained by production of another small fragment by cleavage of the C17-20 bond in fragment I after hydrogen transfer from the steroid skeleton with the charge remaining at the C-20 site. Cleavage of the total side-chain is responsible for the fragment at m/e 289; it will also lose one $(CH_3)_3$ Si-OH group, yielding a peak at m/e 199.

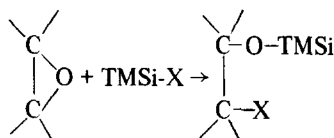
The lower mass spectrum in fig.3 is that of the biological intermediate and compares extremely well with the upper spectrum. From these spectra and the gaschromatogram the conclusion is justified that the intermediate is 20α, 22R-di-OH cholesterol. The mass spectrum is similar to the spectrum from the same compound isolated from meconium [13] with slight changes for the underivatised 20α-OH group.

4. Discussion

According to the experiments described an intermediate between 22R and 20α, 22R must be present when 22R is converted into pregnenolone and isocaproaldehyde. A peroxide as a physiological intermediate is highly improbable. All tests on peroxides as described in Feigl [14] and Stahl [15] indicated the presence of only traces of peroxides. In addition, the concentration of these trace amounts did not vary significantly in time.

The following evidence strongly suggests that an epoxide is an intermediate.

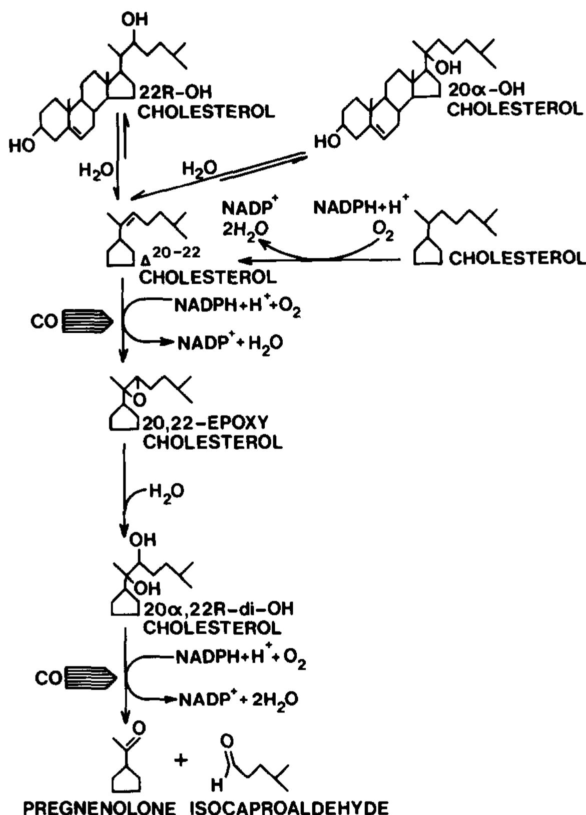
1. Both chemical ($HClO_4$) and enzymatic hydrolysis (fig.1-A, 1-B) yield 20,22-di-OH cholesterol.
2. ^{18}O from H_2 ^{18}O is incorporated in 20α, 22R with 22R as starting product [6]. Assuming 20,22-epoxycholesterol is an intermediate in the side-chain cleavage reaction this could easily be explained. It is incompatible with the consecutive or concerted action of two hydroxylases (mono-oxygenases).
3. Epoxides can react with silylation agents in the following way [16]:



(X = Cl, *N*-methylacetamide, imidazole)

The products of such silylation reactions are known to have low volatility [17]. Thus on considering the reaction between 20,22-epoxycholesterol and trimethylsilylimidazole one would expect a similar derivative, unsuitable for gas chromatography owing to its low volatility.

It can be seen that under the conditions of exp. 1-A the epoxy-hydrase is rate limiting. In the presence of ⩾ 99% CO (fig.1-B) neither is an epoxide formed nor is 20α, 22R converted; however the epoxy-hydrase is able to catalyze the conversion of 20,22-epoxycholesterol into 20α, 22R. The following explanation can now be given. The conversion of 20α, 22R into pregnenolone is known to require NADPH and O_2; cytochrome *P*-450 is involved and the reaction is inhibited by CO [18,19]. Epoxidation of double bonds in the liver is also known to be cytochrome *P*-450- or *P*-448-dependent; NADPH and O_2 are



required [20–22]. By analogy one could therefore expect in exp. 1-B both the formation of the cholesterol epoxide and the conversion of 20α, 22R to be inhibited by CO. The data obtained support our recently proposed hypothesis [6] concerning cholesterol side-chain cleavage (fig.5).

One might expect 20α-OH cholesterol to behave in a manner similar to 22R, since conversion of the sterol in the presence of H_2 ^{18}O gave rise to ^{18}O containing pregnenolone [6]. However the phenomena shown in fig.1-A have not been found with 20α as a substrate (unpublished experiments, R. J. Kraaipoel), probably because the conversion 20α → Δ^{20-22} is slower than 22R → Δ^{20-22}. The epoxy-hydrase reaction therefore is no longer the rate limiting step. It is also possible to explain the different behaviour of 20α and 22R with respect to the apparent 'mass-defect' by postulating the formation of two different epoxides (20α, 22α-epoxycholesterol and 20β, 22β-epoxycholesterol). This would agree with our earlier suggestior

[6] that Δ^{20-22} cholesterol originating from 20α is attached to the enzyme surface in a stereochemically different way than Δ^{20-22} cholesterol originating from 22R.

It is of some interest that several epoxy-hydrases have been described [22,23]. Recently it was reported that 5α, 6α-epoxy-cholesterol, a known carcinogen, is formed continuously in the skin under the influence of light. It is transformed by a 5α, 6α-epoxy-hydrase into cholestane-3β, 5α, 6β-triol [24].

Burstein et al. [25], using acetone extracted bovine adrenal cortex mitochondria, tentatively identified 20α, 22R as an intermediate by recrystallisation techniques. This has now been confirmed by our experiments. The time course of their reaction [1,2], using this material, was similar to the one shown in fig.1-B but no 'mass-defect' was found. An explanation for the discrepancy was found when we used mitochondrial preparations stored under poor conditions (the storage container was not refilled in time with liquid nitrogen). A conversion of 22R into pregnenolone according to fig.1-B was repeatedly demonstrated. We found earlier that the enzymatic conversion of 20α, 22R into pregnenolone and isocaproaldehyde is very susceptible to inhibition. Contact with air when mitochondria are in a freeze-dried condition also selectively damages this last step [6]. The epoxy-hydrase is no longer rate limiting and thus no epoxide but 20α, 22R accumulates. This may be the reason why some investigators found 20α, 22R as an intermediate and others did not.

It is tempting to speculate about the four enzymatic activities, needed to convert cholesterol into pregnenolone. According to our scheme these are an oxidative desaturase [26], an epoxidase, an epoxy-hydrase and a lyase (desmolase). We suggest that the epoxidase and lyase activities belong to the group of cytochromes *P*-450. It is interesting to compare this with the cytochrome *P*-450 preparation isolated by Shikita and Hall [27,28], containing 8 heme groups per molecule *P*-450. It appears that this adrenocortical *P*-450 (mol. wt 850 000) is isolated in a form consisting of 16 subunits and can exist in forms of 8 (mol. wt 470 000) and 4 (mol. wt 200 000) subunits. Drastic treatments result in the formation of single units. The fragment of 4 subunits (after addition of the specific flavoprotein and non-heme iron protein) was still able to convert cholesterol into pregnenolone [19]. This is clearly an area that requires further research and elucidation.

Acknowledgements

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