INCORPORATION OF H_2 ^{18} O INTO 20α, 22R-di-OH CHOLESTEROL: EVIDENCE FOR AN EPOXIDE–DIOL PATHWAY IN THE ADRENOCORTICAL CHOLESTEROL SIDE-CHAIN CLEAVAGE MECHANISM

R. J. KRAAIPOEL, H. J. DEGENHART, J. G. LEFERINK*

*Department of Pediatrics, Erasmus University and Academic Hospital Rotterdam/Sophia Children's Hospital and Neonatal Unit, Gordelweg 160, Rotterdam, The Netherlands and *Laboratory for Toxicology, State University Utrecht, The Netherlands*

1. Introduction

It is generally accepted that 20α,22R-di-OH cholesterol (20α,22R)* is an intermediate in the conversion of cholesterol into pregnenolone. There exist two main hypotheses concerning the biosynthesis of this intermediate in cholesterol side-chain cleavage, namely (a) a sequential hydroxylation of cholesterol and (b) a concerted attack of oxygen [1–6]. Both mechanisms imply that the two hydroxyl groups in the side-chain of 20α,22R contain oxygen atoms originating from molecular oxygen (O_2).

In our previous communications [7–9] it was suggested that in the adrenal cortex cholesterol is converted into pregnenolone and isocaproaldehyde via the intermediates Δ^{20-22} cholesterol, 20,22-epoxycholesterol and 20α,22R. This epoxide–diol pathway implies that the side-chain of 20α,22R contains one oxygen atom originating from molecular oxygen (O_2) and another from water (H_2O).

In this paper we will present evidence, obtained by GC-mass spectrometry and GC-mass fragmentography, that supports our previously advanced hypothesis. Since it is suggested that both cholesterol and 22R-OH cholesterol are converted into pregnenolone via

the same intermediates, the evidence for and against the rôle of 22R-OH cholesterol as an intermediate in the side-chain cleavage of cholesterol is discussed.

2. Materials and methods

The conversion of cholesterol into pregnenolone and isocaproaldehyde in the presence of 18-oxygen enriched water (H_2 ^{18} O) was essentially carried out as described before [8]. Freeze-dried bovine adrenal cortex mitochondria (26 mg protein, estimated by the biuret method) were added to the incubation medium containing 50% H_2 ^{18} O. Cholesterol, 600 µg (= 1.56 µmol) dissolved in 0.1 ml acetone, was added to the reaction mixture. The final vol was 4.2 ml. Caproaldehyde, 100 µg (= 1.0 µmol) dissolved in 0.01 ml ethanol, was added to the reaction mixture and the conversion was started by the addition of a NADPH-generating system [8].

After 30 min. incubation at 37°C with continuous stirring the reaction mixture was divided into three equal parts. Each of these was added to 3 ml pentane–ether (1:1, v/v). Epicholesterol (100 µg/ml) was added as an internal standard. The water layer was made alkaline (pH 8) with sodium bicarbonate and was extracted three times. The combined organic layers contained the sterol fraction. The water layer was acidified (pH 2) with $H_2 SO_4$ and isocaproic acid and caproic acid were extracted. The pentane–ether extract containing the acids was concentrated by evaporation under nitrogen. Gas chromatographic analysis was performed on a Varian 2100 instrument, equipped

with a flame ionisation detector. The stainless steel column (length 2 m, i.d. 3.5 mm) was packed with Chromosorb WHP, coated with 25% DEGA, 2% H_3PO_4. The temperature of the injector was $160°C$, of the oven $145°C$ and of the detector $190°C$. Nitrogen was used as the carrier gas. Retention times of isocaproic acid and caproic acid were 10 and 12 min respectively. Under these conditions caproaldehyde was located in the solvent peak.

The extract containing the sterol fraction was evaporated under nitrogen and derivatised with trimethyl-silylimidazole and analysed by gas chromatography [8]. The sterols were quantified by comparison of the areas of their GC-peaks with the area of that of epicholesterol. ^{18}O-incorporation into pregnenolone was measured by GC-mass spectrometry [8], using a Finnigan 1015D-6000 MS-computer system. ^{18}O-incorporation into 22R-OH cholesterol, 20α,22R, isocaproic acid and caproic acid was measured by GC-mass fragmentography [10], using the same instrument. In this technique the mass spectrometer functions as a specific detector for the gas chromatograph. The areas of the GC-peaks of the fragment ions A and A + 2 were calculated by computer. Incorporation of ^{18}O into fragment A should increase the area of the ion A + 2 with respect to the area of fragment A.

GC conditions used in combination wtih MS were similar to the conditions described above but helium was used as the carrier gas.

3. Results and discussion

Bovine adrenal cortex mitochondria (with endo-geneous and exogeneous cholesterol as a substrate) were incubated in the presence of 50% $H_2{}^{18}O$ as described in the methods. After 30 min at $37°C$ the sterols were extracted from the incubation medium and analysed by gas chromatography. The gas chromatogram of the persilylated sterol fraction revealed peaks with retention times of pregnenolone (110 μg), epicholesterol, cholesterol, 22R-OH cholesterol (8 μg) and 20α,22R (2 μg). The identity of these compounds was confirmed by comparing their mass spectra with the mass spectra of the authentic compounds. Furthermore ^{18}O-incorporation from $H_2{}^{18}O$ into pregnenolone, 22R-OH cholesterol, 20α,22R, isocaproic acid and caproic acid was measured.

3.1. 20α,22R-di-OH cholesterol

The mass spectrum of persilylated 20α,22R obtained from the incubation described above was similar to the one recently described [9]. In order to measure ^{18}O-incorporation into the hydroxyl groups of the side-chain of 20α,22R, two fragment ions from the mass spectrum were chosen. Fig. 1 shows that the C-8 fragment gives rise to m/e 289. This fragment ion contains both hydroxyl groups of the side-chain of 20α,22R. Cleavage between C_{20} and C_{22} results in the formation of a fragment ion at m/e 461. This C-21 fragment contained only one hydroxyl group attached to C_{20} of the steroid skeleton. In order to measure ^{18}O-incorporation into these fragments a gas chromatogram was made with monitoring of the fragment ions at m/e 289, 291, 461 and 463. The intensities of the ions at m/e 289 and at m/e 461, calculated from the areas under their GC-peaks, were arbitrarily taken as 100. Table 1 shows that in the experiment performed in the presence of $H_2{}^{18}O$ there was a marked increase in the abundance of the ion at m/e 291 compared to that of the same fragment ion obtained in the presence of $H_2{}^{16}O$. In contrast to this the abundance of the fragment ion at m/e 463 (C-21 fragment) did not change in the presence or absence of $H_2{}^{18}O$. From these results it was concluded that incorporation of ^{18}O from $H_2{}^{18}O$ is restricted to the hydroxyl group attached to C_{22}. This incorporation of water into a vicinal diol strongly supports the concept of an epoxide—diol pathway. The epoxy-hydrase catalysing
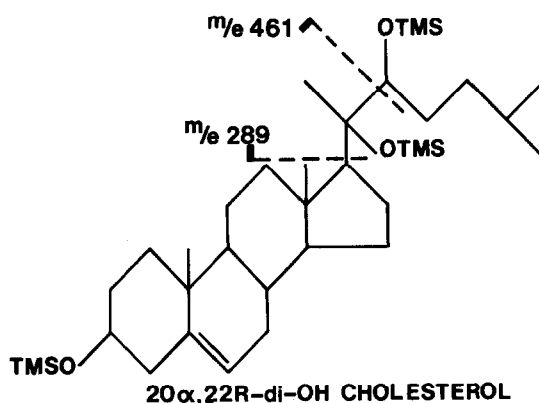

Fig.1. The trimethylsilyl derivative of 20α,22R-di-OH cholesterol. The C-8 fragment at m/e 289 and the C-21 fragment at m/e 461 are indicated.

Table 1

^{18}O-Incorporation into the compounds above, isolated from the $H_2\,^{18}$O-containing incubations, was measured by GC-mass fragmentography

Compound	m/e	m/e	m/e	m/e
20α,22R	461	463	289	291
$H_2\,^{16}$O	100	11.6	100	8.5
$H_2\,^{18}$O	100	11.7	100	52.8[a]
22R-OH cholesterol	173	175		
$H_2\,^{16}$O	100	20.0[b]		
$H_2\,^{18}$O	100	21.1[b]		
Pregnenolone	298	300	388	390
$H_2\,^{16}$O	100	3.0	100	7.2
$H_2\,^{18}$O	100	4.0	100	8.0
Isocaproic acid	60	62	64	
$H_2\,^{16}$O	100	0.7	0.0	
$H_2\,^{18}$O	100	72.4	16.3	
Caproic acid	60	62	64	
$H_2\,^{16}$O	100	0.7	0.0	
$H_2\,^{18}$O	100	72.8	21.7	

Control values were obtained from similar experiments in the presence of $H_2\,^{16}$O, except for 20α,22R which was biosynthesised [9].

[a] The percentage incorporation of H_2O from the medium was calculated as follows:

$$\frac{52.8 - 8.5}{100 + (52.8 - 8.5)} \cdot \frac{100}{50} \cdot 100\% \cong 60\%$$

[b] The high abundance of the ion 175 is due to other fragments adding to the abundance of the isotope fragment.

this reaction selectively opens 20,22-epoxycholesterol in such way that the oxygen atom from the epoxide is transferred to the hydroxyl group attached to C_{20} from 20α,22R. As shown above, the oxygen atom from water will be transferred to the hydroxyl group attached to C_{22} from 20α,22R.

The enzymic formation of an epoxide requires both an epoxidase and a substrate containing a double bond (Δ^{20-22} cholesterol). In addition to this NADPH and O_2 are required [9]. We therefore suggest that cholesterol is converted into pregnenolone and iso-caproaldehyde via Δ^{20-22} cholesterol, 20,22-epoxy-cholesterol and 20α,22R.

From our results (table 1) it can be calculated that 60% of 20α,22R is formed with water from the $H_2\,^{18}$O-containing medium. Therefore 40% is formed with

water from another source. Side-chain cleavage of cholesterol requires 3 mol NADPH + H^+ and 3 mol O_2 [11]. Besides pregnenolone and isocaproaldehyde, 3 mol $NADP^+$ and 5 mol H_2O are formed. The conversion of 20,22-epoxycholesterol into 20α,22R, catalysed by an epoxy-hydrase, requires 1 mol H_2O. The net water production of cholesterol side-chain cleavage therefore is 4 mol H_2O. The finding that only 60% of 20α,22R is synthesised with water from the medium indicates that only a limited exchange exists between water formed during the side-chain cleavage of cholesterol and water from the $H_2\,^{18}$O containing medium. As a result of this 40% of 20α,22R is synthesised with water that has been formed by the side-chain cleavage reaction.

If one calculates the ^{18}O-content of the two hydro-

xyl groups attached to C_{20} and C_{22} of 20α,22R (assuming the experiment was carried out in the presence of 100% $H_2{}^{18}O$) one finds 0% (C_{20}) and 60% (C_{22}) resulting in a mean content of 30%. On calculating the ^{16}O-content of the two hydroxyl groups one finds 100% (C_{20}) and 40% (C_{22}) giving a mean content 70%. The oxygen atom attached to C_{20} originated from 20,22-epoxycholesterol. The epoxide in its turn was formed from Δ^{22-22} cholesterol with molecular oxygen ($^{16}O_2$). The oxygen atom attached to C_{22} originated from water. As shown above 40% of the water used in the biosynthesis of 20α,22R has been formed as a product of the cholesterol side-chain cleavage reaction. This water will be $H_2{}^{16}O$.

Assuming this experiment was carried out with water ($H_2{}^{16}O$) and 18-labeled molecular oxygen (100% $^{18}O_2$) the same calculations would result in 30% ^{16}O-content and 70% ^{18}O-content.

Recently Burstein et al. [12] described an experiment in which [^{14}C] cholesterol was incubated with an acetone powder of bovine adrenal cortex mitochondria in the presence of $^{18}O_2$. They reported a 68% incorporation (mean content) of ^{18}O in the two hydroxyl groups of the side-chain of [^{14}C] 20α,22R. Their result agrees nicely with our calculated 70%. When they doubled the amount of glucose 6-phosphate dehydrogenase the incorporation of ^{18}O into the side-chain increased up to 90.5%. Apparently a NADPH-oxidase not tightly coupled to the side-chain cleavage reaction increases the endogenous production of $H_2{}^{18}O$.

The hypothesis that the vicinal diol (20α,22R) is formed as a result of the reaction of an epoxide (20, 22-epoxycholesterol) with water is supported by both our experiments and those of Burstein et al. [12]. The suggestion [12] that cholesterol is converted into 20α,22R by the concerted attack of *separate* molecules of oxygen must be rejected.

3.2. *22R-OH cholesterol*

The mass spectrum of the trimethylsilyl derivative of 22R-OH cholesterol showed a fragment ion at m/e 173, due to fission between C_{20} and C_{22}. The side-chain, containing the trimethylsilyl group at C_{22}, is present as a fragment ion. Moreover this ion at m/e 173 was the most abundant ion from the mass spectrum. Incorporation of ^{18}O into the hydroxyl group attached to C_{22} is expected to give an increase of the abundance of the ion at m/e 175. In order to measure ^{18}O incorporation into 22R-OH cholesterol a gas chromatogram was made while the fragment ions at m/e 173 and 175 were monitored. Table 1 shows that only a negligible difference existed between the abundance of the ion at m/e 175 from either synthetic 22R-OH cholesterol or the same compound formed from cholesterol in the presence of $H_2{}^{18}O$. From these data we conclude that no incorporation of ^{18}O from $H_2{}^{18}O$ into 22R-OH cholesterol took place.

Recently it was reported [13] that incubation of [^{14}C] cholesterol with an acetone powder of bovine

Table 2
Summary of the labeling studies performed with $^{18}O_2$ and $H_2{}^{18}O$

Substrate	22R-OH cholesterol		20α,22R-di-OH cholesterol				Pregnenolone	
			C_{20}		C_{22}			
	$^{18}O_2$	$H_2{}^{18}O$	$^{18}O_2$	$H_2{}^{18}O$	$^{18}O_2$	$H_2{}^{18}$	$^{18}O_2$	$H_2{}^{18}O$
Cholesterol	+[a]	—	+[b]	—	—[b]	+	+[c]	—
22R-OH cholesterol			nm	—[d]	nm	+[d]	+[c]	—[d]
20α-OH cholesterol			nm	nm	nm	nm	—[c]	+
20α,22R-di-OH cholesterol							—[e]	nm

Incorporation of ^{18}O into the side-chain of 20α,22R is expressed separately for the hydroxyl groups attached to C_{20} and C_{22}. (+) ^{18}O-incorporation. (−) No ^{18}O-incorporation. (nm) Not measured.
[a] Burstein et al. [13],
[b] See comments on the results of Burstein et al. [12], in this paper,
[c] Takemoto et al. [17],
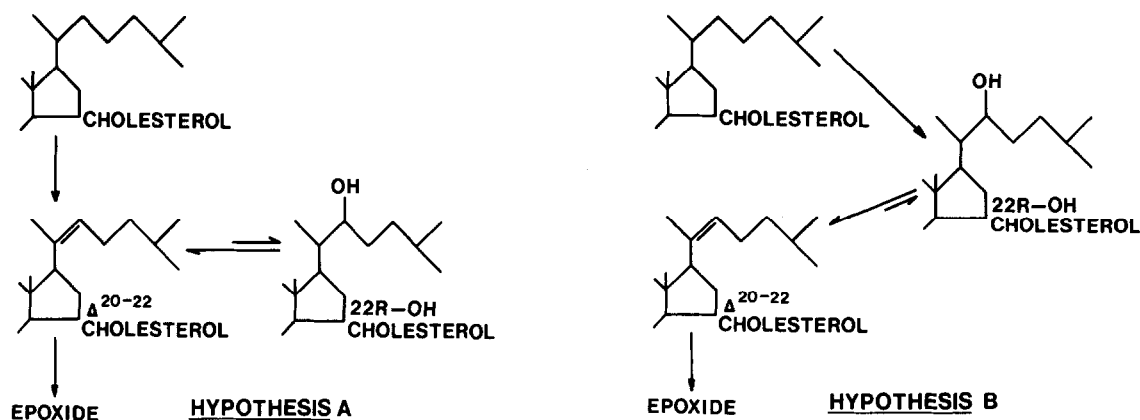[d] Kraaipoel et al. [8], Nakano et al. [18].

Fig.2. Two hypotheses for the conversion of cholesterol into Δ^{20-22} cholesterol.

adrenal cortex mitochondria in the presence of $^{18}O_2$ resulted in the formation of ^{18}O-incorporated [^{14}C] 22R-OH cholesterol. We suggest that both cholesterol and 22R-OH cholesterol [9] are converted into pregnenolone and isocaproaldehyde via Δ^{20-22} cholesterol, 20,22-epoxycholesterol and 20α,22R. Moreover both substrates in the presence of H_2 ^{18}O are converted into 20α,22R with ^{18}O attached to C_{22} (table 2). Therefore it might be concluded that 22R-OH cholesterol is an intermediate in the conversion of cholesterol into Δ^{20-22} cholesterol (Hypothesis B; fig.2). However other experiments suggest that cholesterol is converted directly into Δ^{20-22} cholesterol (Hypothesis A). On closer examination of both these hypotheses one must make additional assumptions.

Hypothesis A

$$\text{cholesterol} \xrightarrow{\text{desaturase}} \Delta^{20-22} \text{ cholesterol}$$

(de)hydratase
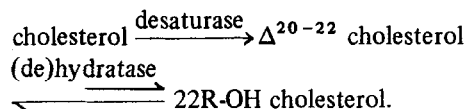
22R-OH cholesterol.

Thus 22R-OH cholesterol would be formed by the reaction of Δ^{20-22} cholesterol with water. Our experiments show that water from the H_2 ^{18}O containing medium was not used. Therefore the assumption has to be made that water formed by the desaturase reaction is retained by the enzyme system and transferred to 22R-OH cholesterol without exchange with water from the medium.

Hypothesis B

$$\text{cholesterol} \xrightarrow{\text{mono-oxygenase}} \text{22R-OH cholesterol}$$

(de)hydratase
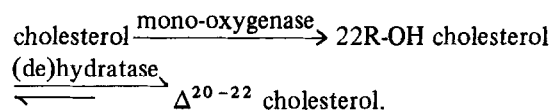
Δ^{20-22} cholesterol.

The studies of Hochberg et al. [14] show that a cholesterol analog (20S)-20-(p-Tolyl)-5-pregnen-3β-ol is converted into a 20-hydroxyl derivative, which is further converted into pregnenolone. In order to explain these results in terms of Hypothesis B one must assume that the 22R-mono-oxygenase is non-specific and therefore also can act as a 20-mono-oxygenase.

(20S)-20 (p-Tolyl)-5-pregnen-3β-ol [14], 23,24-di-nor-chol-5-en-3β-ol [15], and 25-OH cholesterol [16] can all be converted to their 20-hydroxy-derivatives and can also be converted into pregnenolone. However the formation of these 20-hydroxylated compounds can also be explained by a desaturase reaction followed by a hydratase reaction (Hypothesis A).

The calculations made by Bustein et al. [6] led them to the conclusion that the pathways via the mono-hydroxylated sterols contributed only a small part to pregnenolone formation from cholesterol and that the most important pathway consisted of a direct conversion of cholesterol into 20α,22R. Very recently [13] these authors however have drastically altered the interpretation of their experiments.

We believe the current experimental evidence does

not permit one to distinguish which of these two mechanisms is correct.

3.3. Pregnenolone

From table 1 it can be seen that no ^{18}O has been incorporated into pregnenolone formed by cholesterol side-chain cleavage in the presence of $H_2{}^{18}O$. This result is consistent with the finding that no ^{18}O-labeling occurred at C_{20} of 20α,22R. Table 2 shows that cholesterol in the presence of $^{18}O_2$ is converted into ^{18}O-labeled pregnenolone [17].

3.4. (Iso)caproaldehyde and (iso)caproic acid

Freeze-dried bovine adrenal cortex mitochondria are able to convert both isocaproaldehyde and capro-aldehyde into their acids. Incubation of 100 μg (1.0 μmol) caproaldehyde for 20 min at 37°C in a medium (see Methods) containing denatured mitochondria (3 min at 95°C), or a similar incubation in medium alone resulted in a 15% conversion of caproaldehyde into acid. Also addition of the same aldehyde to a pentane—ether—water mixture, followed by the extraction procedure (see Methods) gave a similar conversion (15%). Considering the oxidative capacity of the freeze-dried bovine adrenal cortex mitochondria, one might expect the isocaproaldehyde formed by cholesterol side-chain cleavage to be converted into isocaproic acid. The time course of the formation of pregnenolone and isocaproic acid was followed by taking samples every 20 min (fig.3). The results of this experiment show a stoichiometric relationship between pregnenolone and isocaproic acid. It was concluded that all the caproaldehyde formed in the side-chain cleavage reaction by damaged mitochondria is transformed into acid.

Incorporation of $H_2{}^{18}O$ into 20α,22R with the 18-oxygen atom attached to the C_{22} makes it plausible that the 18-oxygen atom was transferred to isocapro-aldehyde and isocaproic acid. However oxygen atoms from aldehydes and acids are exchangeable with the same atoms from water. Incorporation can be measured if such an exchange is slow. Caproaldehyde was therefore added as an internal standard, and oxidation of this aldehyde to isocaproic acid provided a control for oxygen exchange. The mass spectra of both isocaproic and caproic acid present an abundant fragment at m/e 60 $\{CH_2{=}C(OH)_2\}$, containing both oxygen atoms from the acids. The intensity of m/e 60, calculated
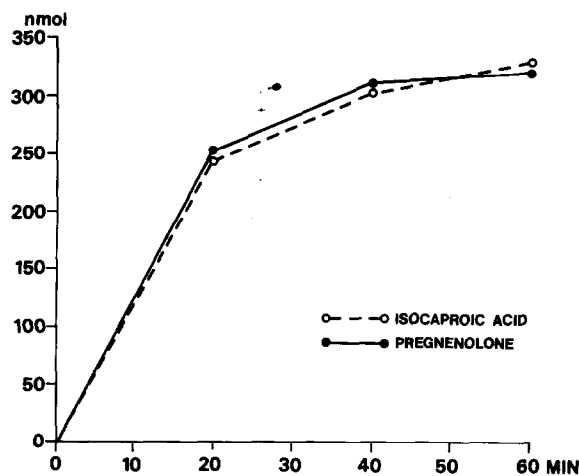

Fig.3. Freeze-dried bovine adrenal cortex mitochondria (30 mg protein), supported by a NADPH-generating system, converted cholesterol into pregnenolone and isocaproic acid (see Methods). Epicholesterol (20 μg/ml) and caproic acid (10 μg/ ml) were added as internal standards. Both pregnenolone and isocaproic acid were estimated by gas chromatography.

from the area under the GC-peak of the ion at m/e 60, was arbitrarily taken as 100. The fragment ions at m/e 62 and at m/e 64 (two ^{18}O atoms incorporated) were monitored at the same time. The observations shown in table 1 clearly indicate the existence of an exchange between ^{18}O from $H_2{}^{18}O$ and the oxygen atoms from the internal standard caproic acid. Equilibrium has almost been nearly reached in respect to the ^{18}O-enrichment of both the water from the medium and caproic acid. Therefore it is not possible in this way to measure ^{18}O transfer from 20α,22R to isocaproic acid.

Combining all the experiments carried out in the presence of $^{18}O_2$ and $H_2{}^{18}O$ it can be seen from table 2 that the results are complementary. ^{18}O-incorporation from $H_2{}^{18}O$ into the common intermediate 20α,22R is the same for both cholesterol and 22R-OH cholesterol. The reverse holds for 20α-OH cholesterol and so it cannot be considered as an intermediate in cholesterol side-chain cleavage.

Acknowledgements

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