STUDIES ON CHOLESTEROL SIDE-CHAIN CLEAVAGE IN ADRENAL CORTEX MITOCHONDRIA: PROPOSAL OF A NEW MECHANISM

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PROEFSCHRIFT

Ter verkrijging van de graad van doctor in de geneeskunde aan de Erasmus Universiteit te Rotterdam, op gezag van de rector magnificus Prof. Dr. B. Leijnse en volgens besluit van het college van dekanen.
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Door

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GEBOREN TE AMSTERDAM

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Dit proefschrift werd onder leiding van Dr. H.J. Degenhart bewerkt op het laboratorium van de afdeling Kindergeneeskunde, Erasmus Universiteit Rotterdam, Academisch Ziekenhuis Rotterdam/Sophia Kinderziekenhuis en Zuigelingenkliniek. Het onderzoek werd mogelijk gemaakt door belangrijke steun van de Stichting voor Medisch Wetenschappelijk Onderzoek FUNGO, een dochter van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek ZWO.

voor Zeljka en Milan Damir

Wij gingen uit om de wereld te veroveren, alleen Hoyer geloofde daar niet aan, die wist niet beter dan datti op den Zeeburgerdijk liep, bij de slachtplaats.

Nescio.

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LIST OF TRIVIAL NAMES AND ENZYMES

Trivial names used in this work	Systematic names
cholesterol	- 5-cholesten-3β-ol
epi-cholesterol	- 5-cholesten-3α-ol
20α-OH cholesterol	- (20S)-5-cholesten-3β,20-diol
22R-OH cholesterol	- (22R)-5-cholesten-3β,22-diol
20α,22R-di-OH cholesterol	- (20R,22R)-5-cholesten-3β-20, 22-triol
20α,22S-di-OH cholesterol	- (20R,22S)-5-cholesten-3β,20, 22-triol
17α,20α-di-OH cholesterol	- (17S,20S)-5-cholesten-3β,17, 20-triol
Δ^{20-22} cholesterol	- 5,20(22)-cholestadien-3β-ol
Δ^{17-20} cholesterol	- 5,17(20)-cholestadien-3β-ol
20,22-epoxy cholesterol	- (20ξ,22ξ)-20,22-epoxy-5-cholesten-3β-ol
corticosterone	- 11β,21-dihydroxy-4-pregnene-3,20-dione
cyanoketone	- 2α-cyano-4,4,17α-trimethyl-17β-hydroxy-5-androsten-3-one
deoxycorticosterone	- 21-hydroxy-4-pregnene-3,20-dione
dehydroepiandrosterone	- 3β-hydroxy-5-androsten-17-one
20α-hydroperoxycholesterol	- (20S)-20-hydroperoxy-5-cholesten-3β-ol
flavoprotein (adrenodoxin reductase)	- NADPH: ferredoxin oxido-reductase E.C. 1.6.7.1.
glucose-6-phosphate dehydro-genase	- D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase E.C. 1.1.1.49.
steroid 11β-hydroxylase	- steroid, reduced-adrenal-ferredoxin: oxygen oxido-reductase (11β-hydroxylating) E.C. 1.14.15.4.
steroid 17α-hydroxylase	- steroid, hydrogen-donor: oxygen oxidoreductase (17α-hydroxylating) E.C. 1.14.99.9.

steroid 21-hydroxylase	- steroid, hydrogen-donor: oxygen oxidoreductase (21-hydroxylating) E.C. 1.14.99.10.
cholesterol side-chain cleaving enzyme system	- cytochrome P-450 containing enzyme complex catalyzing the conversion of cholesterol to pregnenolone and isocaproaldehyde (NADPH dependent)

LIST OF ABBREVIATIONS

AGI	- aminoglutethimide phosphate (Elipten ®) 2-ethyl,2-(4-aminophenyl)-glutarimide phosphate
a.m.u.	- atomic mass unit
dpm	- desintegrations per minute
E	- extinction
EGTA	- ethylene-glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetra-acetic acid
EPR	- electron paramagnetic resonance
g	- relative centrifugal force
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
i	- total concentration inhibitor
IUPAC	- International Union of Pure and Applied Chemistry
K_i	- inhibitor constant
K_m	- Michaëlis constant
NADPH	- nicotinamide-adenine-dinucleotide phosphate: reduced form
m/e	- mass to charge ratio
mg	- milligram
v	- rate of reaction catalysed by an enzyme
V	- value of v when the enzyme is saturated with substrate, as given by the Michaëlis equation

CHAPTER 1.

INTRODUCTION

1.1 General

The enzymic conversion of cholesterol into pregnenolone involves a cleavage of the cholesterol side-chain, yielding two fragments: pregnenolone and isocaproaldehyde (fig. 1.1). This process, the rate limiting step in the biosynthesis of the steroid hormones, is generally known as the "cholesterol side-chain cleavage" reaction. All the enzymes required for this conversion are located in the mitochondria of the adrenal cell, while the main regulating factor is the adrenocorticotropic hormone (ACTH).

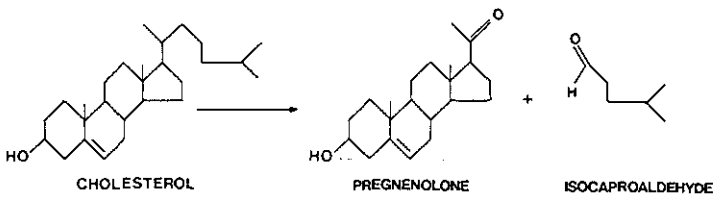


Fig. 1.1

In the congenital lipoid adrenal hyperplasia, an inborn error of metabolism, described by Prader and Siebenmann (1957), the adrenal cortex synthesizes almost no steroids, but large quantities of cholesterol and cholesterol-esters are accumulated in the adrenals. Apparently cholesterol is not converted into pregnenolone. To understand the exact nature of this defect in pregnenolone synthesis, an understanding of the mechanisms involved in cholesterol side-chain cleavage is indispensable.

At the start of this investigation (1971), it was generally assumed that cholesterol was converted into pregnenolone via the intermediates 20α-OH cholesterol, 22R-OH cholesterol and

20α,22R-di-OH cholesterol (fig. 1.2). The accumulation of cholesterol seen in congenital lipoid adrenal hyperplasia suggested that one or more of the enzymes involved in the conversion of cholesterol into pregnenolone was defect.

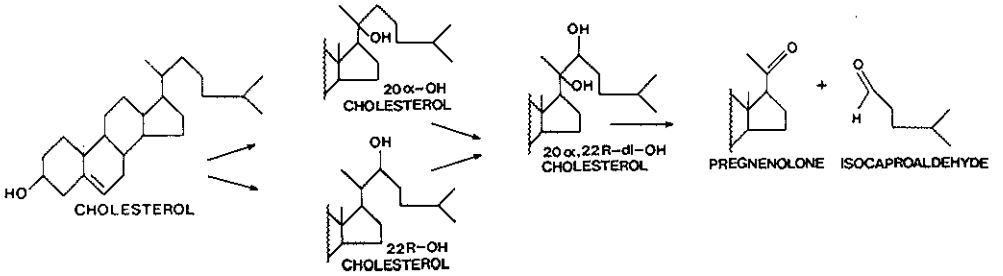


Fig. 1.2

With an adrenal preparation, obtained post mortem from a patient with congenital lipoid adrenal hyperplasia, Degenhart et al. (1972) showed that 20α-OH cholesterol was converted into pregnenolone but cholesterol was not. This experiment supported the hypothesis that the inborn error was localized in the cholesterol 20α-hydroxylase. In contrast to these conclusions, Burstein et al. (1971), van Lier and Smith (1970) and Lieberman et al. (1969) have presented evidence that cholesterol may not be converted into pregnenolone via 20α-OH cholesterol.

This conflicting situation prompted us to investigate the pathway by which cholesterol is converted into pregnenolone.

1.2 Methods

The cholesterol side-chain cleaving system consists of an NADPH-dependent flavoprotein (adrenodoxin reductase), an iron-sulphur protein (adrenodoxin) and a hemeprotein (cytochrome P_{450}). The latter serves as the terminal oxidase in the electron transport from NADPH to oxygen.

Damaged bovine adrenal cortex mitochondria were generally

used to study the conversion of cholesterol and other sterols into pregnenolone. In the present study the reaction was supported by a NADPH-generating system, while substrates, intermediates and products were estimated by gas-liquid chromatography.

The objective of this investigation, as mentioned earlier, was to investigate the pathway(s) by which cholesterol can be converted into pregnenolone and isocaproaldehyde. Reaction conditions were chosen in such a way that a maximum amount of substrate was converted into pregnenolone in a minimum amount of time; hence a maximum amount of intermediates could be expected. Possible intermediates which can be measured by gas-liquid chromatography, could also be indentified by the combination of gas-liquid chromatography and mass-spectrometry. Identification of such intermediates might be of help in the elucidation of the complicated cholesterol side-chain cleavage reaction.

Once the number of O_2 and NADPH molecules required for the conversion of 1 molecule cholesterol (or 22R-OH cholesterol) into 1 molecule pregnenolone is known, the number of theoretically possible pathways for the side-chain cleavage reaction would be considerably reduced. To calculate the stoichiometry of this reaction with respect to oxygen, intact bovine adrenal cortex mitochondria were used, supported by succinate and malate.

It has been reported that administration of aminoglutethimide phosphate (Elipten) to animals mimics in vivo the congenital lipoid adrenal hyperplasia to a certain degree (Goldman, 1970), while in vitro Elipten inhibits the cholesterol side-chain cleaving system (Kant and Neher, 1969). Therefore the interaction between this drug and the enzyme-system was studied.

1.3 Nomenclature

Several excellent articles and books exist (Träger, 1977) explaining "classical" steroid nomenclature. No attempt will therefore be made to review here the matter of axial and equatorial bonds, cis and trans joining of rings, etc. There is, however, less information available to workers in the steroid field concerning the more recent Cahn-Ingold-Prelog system. This system, specifying 3-dimensional configurations is widely used in organic chemistry, but many biochemists and endocrinologists educated at a medical school are not yet accustomed to its formalism.

We take as an example the (unstable) compound

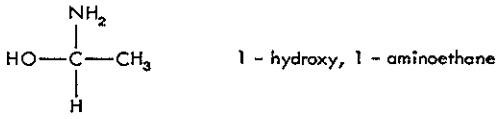


Fig. 1.3

The four substituents -H, $-CH_3$, $-NH_2$ and -OH are given a priority, depending upon the atomic numbers of the atoms to the central C-atom.

H = 1; C = 6; N = 7 and O = 8. The priority sequence becomes:

O = I

N = II

C = III

H = IV (lowest priority)

Now the molecule is positioned in such a way that the substituent with the lowest priority, in this case the H-atom, is the farthest away from the observer.

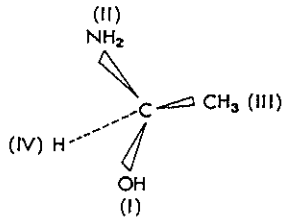


Fig. 1.4

Going from priority I (-OH) to priority II ($-NH_2$) and next to priority III($-CH_3$) corresponds with a clockwise movement. This configuration will be called R (rectus = right). If the configuration had been as shown in fig. 1.5,

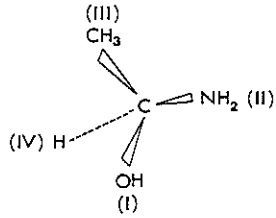


Fig. 1.5

the movement I → II → III is counter-clockwise; the configuration is called S (sinister = left). When two or more of the atoms attached to the central C are identical, one has to consider the atoms attached to the first ones. This process can be continued until the priority is settled.
Some examples:

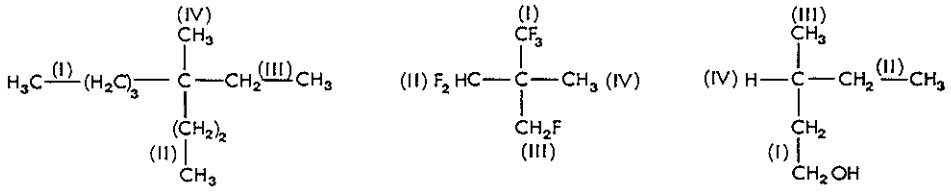


Fig. 1.6

In order to assign an R or S configuration to the central C-atom without constructing 3-dimensional pictures, one has to write the common Fischer projection formulae in such a way, that the group with the lowest priority is at the bottom, for this group points to the rear.
Remember that interchanging any two groups in a molecule yields

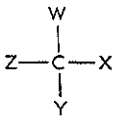
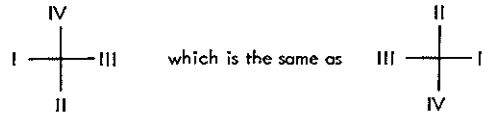


Fig. 1.7

a mirror image, so that two consequent interchanging procedures yield the original structure.
Compound A has the configuration



I→II→III is counterclockwise: A has the S configuration

Fig. 1.8

On manipulating the molecule B, one finds that it has the R configuration. The same is true for compound C.

In the case of 20α-hydroxycholesterol the priority sequence for the groups attached to C_{20} is as follows:

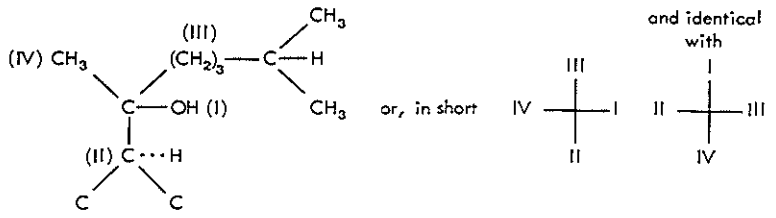
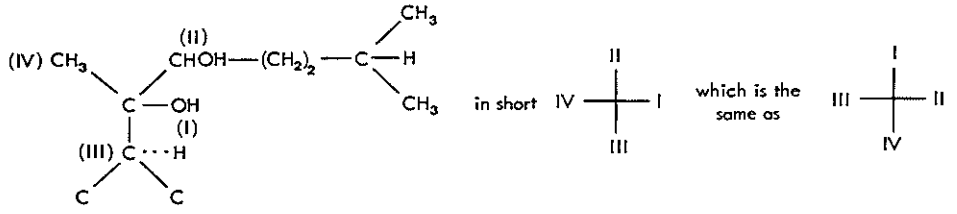


Fig. 1.9

Using the new nomenclature the compound 20α-hydroxycholesterol should be called 20S-hydroxycholesterol. When, however, C_{22} carries an additional hydroxylgroup (orientation not important; we'll call it ξ) the priority changes.



I→II→III is clockwise.

Fig. 1.10

The systematic name of 20α,22ξ-di-hydroxycholesterol is therefore 20R,22ξ-di-hydroxycholesterol.

Using the same priority rules it is also possible to designate the configuration about double bonds. The expressions "cis" and "trans" are ambiguous and should no longer be used.

A plane P, perpendicular to the molecule plane, is thought to divide the molecule in two halves.

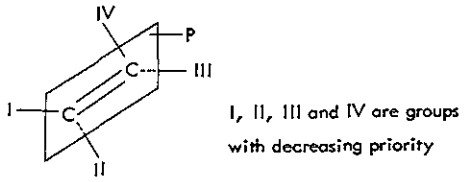


Fig. 1.11

This plane P bisects the angles between groups I and II and the angle between groups IV and III. In this example the first C-atom has its highest priority group (I) on the left side of plane P and the second C-atom has its highest priority group (III) at the right side. This configuration is called E (from "entgegen"). If group III had been at the left side, the groups with highest priority had been at the same side of plane P. In this case the configuration would have been Z (from "zusammen").

Example:

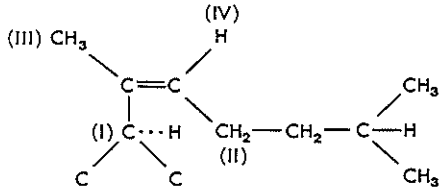


Fig. 1.12

(I) and (II) are at the same side of the reference plane; this structure has the Z-configuration. The reader who wants an explanation for more complicated cases, finds a good start in

the book by Allinger et al. (1976).

A major disadvantage of the R-S and E-Z systems is the fact that minor changes in a molecule can dictate whether the configuration receives another name.
Example:

20α-hydroxycholesterol	=	20S-hydroxy-cholesterol
20α,22ξ-di-hydroxycholesterol	=	20R,22ξ-di-hydroxy-cholesterol
17α,20α,22ξ-tri-hydroxycholesterol	=	17S,20S,22ξ-tri-hydroxy-cholesterol
16ξ,20α,22ξ-tri-hydroxycholesterol	=	16ξ,20R,22ξ-tri-hydroxy-cholesterol

etc.

On the other hand it is possible to construct an exact 3-dimensional model once the configuration is known to be R or S.

As many authors (and suppliers of biochemicals) still use a mixture of the Fischer (α;β) and Prelog (R,S) conventions, we decided to conform ourselves to the most common nomenclature. Therefore names like 20α-hydroxycholesterol will be used throughout this thesis. In the list of trivial names and abbreviations all the IUPAC names can be found.

CHAPTER 2.

REVIEW AND STATEMENT OF THE PROBLEM

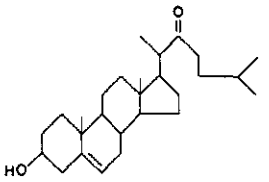
2.1 Introduction

The presence of an 11β-, 17α- and 21-hydroxylase system in the adrenal cortex caused several authors to suggest that similar hydroxylases are involved in the cholesterol side-chain cleavage. Much energy has been invested in the isolation and identification of possible intermediates of the cholesterol side-chain cleaving system, because knowledge of the identity of an intermediate may provide information about the enzymic activity required for the formation of the intermediates.

2.2 Review of the literature from 1954 till 1972 concerning cholesterol side-chain cleavage

Over 20 years ago Stone and Hechter (1954) showed that adrenocorticotropic hormone stimulated steroidogenesis at a site between cholesterol and progesterone. In the same year this group found that bovine adrenal gland homogenate, supported by NAD^+ and ATP, converted labeled cholesterol into labeled pregnenolone (Saba et al. 1954). One month later Lynn et al. (1954) reported that isocaproic acid was formed from cholesterol with enzyme preparations from beef adrenals, ovaries, testes and rat liver. They found neither pregnenolone nor progesterone. These two short communications in the Journal of the American Chemical Society were followed by a full paper in the Journal of Biological Chemistry (Staple et al. 1956), describing the conversion of cholesterol into pregnenolone and isocaproic acid. Addition of 22-keto-cholesterol (fig. 2.1) and 22-hydroxycholesterol (a mixture of 22R and 22S-hydroxy-cholesterol) did not interfere with the side-chain cleaving system. These substances were not radioactive on isolation after incubation with labeled cholesterol and the authors concluded (Staple et al. 1956): "These two compounds are there-

fore not intermediates. For this reason it does not appear that the initial oxidative attack begins at carbon atom 22 in the side-chain".



22 KETO-CHOLESTEROL

Fig. 2.1

The finding that cholesterol was converted into pregnenolone and isocaproic acid led Solomon et al. (1956) to investigate the possibility that 20α-hydroxycholesterol is an intermediate in the process. These investigators tried to trap the isotopically labeled hydroxysterol in an in vitro experiment using homogenates of bovine adrenal glands, (4-^{14}C) cholesterol as a substrate and 20α-hydroxycholesterol as trapping agent. Although 20α-hydroxycholesterol isolated from the incubation mixture appeared to be associated with ^{14}C radioactivity, the authors were unable to ensure the radiochemical homogeneity with certainty since the small quantity of available material precluded further manipulation.

According to Dorfman (1957) the studies of Solomon et al. (1956) suggested that 20α-hydroxycholesterol could be an intermediate. He formulated the following hypothesis: the formation of pregnenolone from cholesterol involves two prior hydroxylations; one at carbon 22 and the other at the 20α position. The 20α,22-dihydroxy derivative of cholesterol is then degraded by a desmolase to form pregnenolone. Dorfman (1957) further speculated that 20α-hydroxycholesterol might be a key intermediate in the formation of C_{19} steroids from cholesterol. The 17α-hydroxylation of 20α-hydroxycholesterol would lead to 17α,20α-di-OH cholesterol which under influence of a proper desmolase might yield dehydro-epiandosterone directly (fig. 2.2). The latter suggestion has been

investigated by Jungman (1968a,b) but was later disproved by Hochberg (1971) and Burstein (1971).

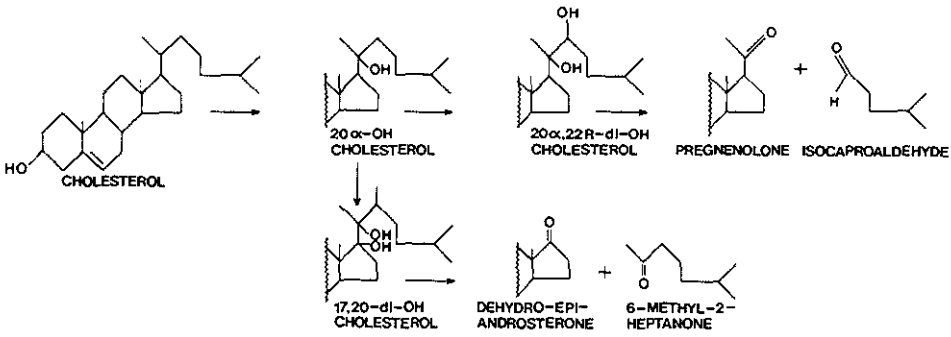


Fig. 2.2

Two groups of workers, Constantopoulos and Tchen (1961a) and Shimizu and his colleages (1960) reported further attempts to clarify the possible intermediacy of 20α-OH cholesterol. The latter group incubated labeled 20α-OH cholesterol with a preparation made from bovine adrenals and showed that it was efficiently converted into pregnenolone and isocaproic acid (Shimizu et al. 1960, 1961). The yield of labeled isocaproic acid was 15 to 20%. Under identical conditions 0.8% of labeled cholesterol was converted to the same acid. The more efficient cleavage of the hydroxylated cholesterol by the tissue preparation was interpreted as strong evidence for the intermediacy of 20α-OH cholesterol in the biosynthetic pathway leading from cholesterol to the C_{21} steroid (Shimizu et al. 1960). In the presence of relatively large amounts of carrier pregnenolone a compound X was seen to accumulate during incubations of 20α-OH cholesterol with adrenal cortex preparations. Incubations of compound X with an adrenal supernatant, supported by NAD^+ and ATP, resulted in the formation of pregnenolone and isocaproic acid. The yield of isocaproic acid from compound X was significantly higher than from 20α-OH cholesterol incubated simultaneously (Shimizu et al. 1961). It was speculated that

compound X was identical with 20α,22-keto-cholesterol. The immediate precursor of compound X probably was the 20α,22R-di-hydroxylated derivative which could arise from the enzymic hydroxylation of 20α-OH cholesterol. According to the authors (Shimizu et al. 1961) the rate limiting step in the overall sequence as observed in the experiments was the C-20-22 desmolase activity; hence the accumulation of compound X. In fig. 2.3, the biochemical sequence is shown: Cholesterol → 20α-OH cholesterol → 20α,22R-di-OH cholesterol → 20α,22-keto-cholesterol → pregnenolone + isocaproic acid.

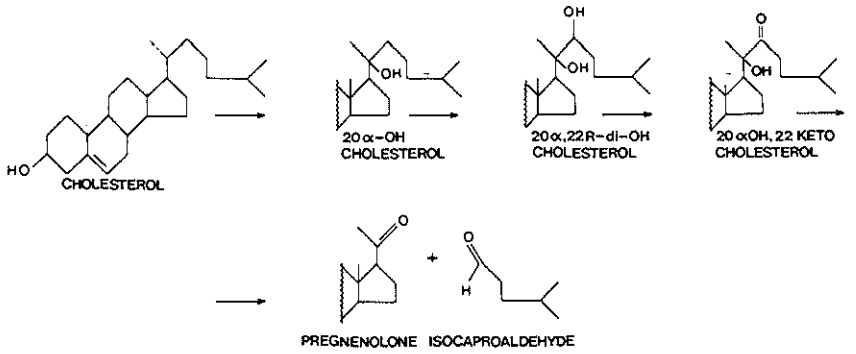


Fig. 2.3

In 1961 Constantopoulos and Tchen (1916a) synthesized isocaproic aldehyde and tested this aldehyde as a trapping agent. The aldehyde inhibited the formation of isocaproic acid from 26-^{14}C cholesterol by more than 80% and led to the accumulation of labeled isocaproic aldehyde. The experiment established the primary product of cholesterol metabolism as isocaproic aldehyde and not isocaproic acid. In the same year the authors reported that NADH or NADPH was required for the conversion of 20α-OH cholesterol into pregnenolone (Constantopoulos and Tchen 1961b). The obligatory role of NADPH had also been observed by Halkerston (1959) for the formation of labeled isocaproic acid from (26 -^{14}C) cholesterol.

Constantopoulos and Tchen (1961b) discussed a number of possible pathways for the conversion of cholesterol into pregnenolone. One of these pathways implicated the formation of a double bond between carbon atoms 20 and 22 and a subsequent cleavage by molecular oxygen. According to the authors the cleavage should not require NADPH and the formation of a double bond was therefore rejected. For this concept the authors referred to a review by Mason (1957). The observed requirement of a reduced pyridine nucleotide, which is characteristic for hydroxylation reactions, indicated to the authors that 20,22-di-hydroxy- and 20-hydroxy-22-keto-cholesterol were involved in the biogenesis of pregnenolone. (fig. 2.3).

Shimizu et al. (1962) described the identification of the earlier mentioned compound X. 20α,22-keto cholesterol, 20α, 22R-di-OH and 20α,22S-di-OH cholesterol were synthesized. These compounds were chromatographed on paper in a toluene-propyleneglycol system. Compound X showed exactly the same chromatographic behaviour as 20α,22R-di-OH cholesterol. After elution extraction and evaporation to dryness the residue was recrystallized twice from aqueous methanol. The specific activity remained constant. The formation of pregnenolone from 20α,22R-di-OH cholesterol (compound X) by incubation with adrenal enzyme preparations had already been demonstrated (Shimizu et al. 1961). Incubation of the three synthesized compounds showed that 20α,22R-di-OH cholesterol was the most efficient precursor of pregnenolone. Reduced pyridine nucleotide was necessary for, or at least accelerated, this conversion. It was concluded that since 20α,22R-di-OH cholesterol (compound X) was the most efficient precursor of pregnenolone, it was likely that pregnenolone arised directly from compound X and not from 20α,22-keto-cholesterol. The following scheme for the pathway from cholesterol to pregnenolone was suggested: Cholesterol → 20α-hydroxycholesterol → 20α,22R-di-hydroxycholesterol → pregnenolone + isocaproic aldehyde (fig. 1.2).

Constantopoulos et al. (1962) reached a similar conclusion.

A polar intermediate was found by incubating (4-^{14}C) or (26-^{14}C) cholesterol in the presence of NADPH, pregnenolone and progesterone. Re-incubation of this polar material with soluble enzyme and NADPH resulted in the formation of labeled pregnenolone or isocaproic acid, depending on the original position of ^{14}C in the labeled cholesterol. When a mixture of (4-^{14}C) and (26-^{14}C) labeled polar intermediate was treated overnight at room temperature with lead tetra-acetate in methanol, labeled pregnenolone and isocaproaldehyde were formed. According to the authors, these results clearly established the nature of the polar intermediate as 20,22-dihydroxycholesterol, presumably with the 20-OH group still in the α-configuration.

Another possibility for the sequence of the hydroxylations in cholesterol side-chain cleavage was suggested by Chaudhuri et al. (1962). (3-^{3}H) 22-keto-cholesterol (fig. 2.1) was synthesized, which upon reduction with sodium borohydride produced an isomeric mixture of 22-hydroxycholesterols. When incubated with an adrenal enzyme preparation (3-^{3}H) 22-hydroxycholesterol gave rise to (3-^{3}H) pregnenolone in a yield exceeding the one obtained from cholesterol. 22-Keto-cholesterol was not converted into pregnenolone. It was suggested that in addition to the reaction sequence via 20α-OH cholesterol another sequence might exist: Cholesterol → 22-hydroxycholesterol → 20α,22-hydroxycholesterol → pregnenolone (fig. 1.2).

According to my opinion this scheme, proposed by Chaudhuri, represents the end of a chapter in the history of the studies concerned with cholesterol side-chain cleaving system. This scheme, also known as the "classical" scheme, is still supported by many investigators and it is depicted in most textbooks.

Let us summarize the evidence for the classical scheme. The first intermediate was isolated by Solomon et al. (1956). The proof of its identity was restricted to consideration of chromatographic properties, which were similar to those of 20α-OH cholesterol in two chromatographic systems. An analogous finding was reported by Halkerston et al. (1961) who referred

to unpublished experiments. From this time till 1963 no other investigator reported the identification of 20α-OH cholesterol as an intermediate. However, 20α-OH cholesterol was accepted as an intermediate by almost every author at that time. A second intermediate, 20α,22R-di-OH cholesterol, was found as a metabolic product of 20α-OH cholesterol. Here paper chromatography and twice recrystallisation to constant specific activity had to prove the identity. The polar intermediate of Constantopoulos was "identified" via treatment with lead tetraacetate, which yielded pregnenolone and isocaproaldehyde. Arguments that were used to support the role as an intermediate, were: 20α,22R-di-OH cholesterol was more efficiently converted into pregnenolone and isocaproic aldehyde than 20α-OH cholesterol; 20α-OH derivative was more efficiently converted than cholesterol. These latter arguments were in fact based on enzyme kinetics. Other pathways were ruled out in view of the fact that the conversions were NADPH dependent, which fitted in the ideas of mixed function oxygenase held at that time. For the same reason Δ^{20-22} cholesterol was ruled out as an intermediate.

In conclusion, we want to state that no firm evidence for the intermediacy of 20α-OH cholesterol or 20α,22R-di-OH cholesterol was presented, but it was made less likely that 20α,22-keto cholesterol was an intermediate.

After 1963 one can distinguish two main groups of investigators: Those who isolated labeled 20α-OH cholesterol from incubations with labeled cholesterol as a substrate, and those who did not.

Ichii et al. (1963) reported that 20α-OH cholesterol and 20α,22R-di-OH cholesterol accumulated when an acetone powder of bovine corpus luteum was incubated in the presence of added pregnenolone.

Hall and Koritz (1969) reported that both 20α-OH cholesterol and pregnenolone inhibited the conversion of labeled cholesterol into pregnenolone. 20α-OH cholesterol used as a trapping agent yielded the result that no radioactivity was found in the 20α-OH fraction. The authors explained their findings with the assumption that there might be a specific binding between the enzyme and 20α-OH cholesterol,

preventing the mixing with added 20α-OH cholesterol. In contrast to the above report, Menon et al. (1965) claimed the isolation of the intermediate 20α-OH cholesterol from a mitochondrial fraction prepared from rat testis. The labeled intermediate behaved like authentic 20α-OH cholesterol on several paperchromatograms. After repeated paperchromatography and dilution with carrier 20α-OH cholesterol, the material was crystallized to constant specific activity.

The purification of a "cholesterol-20α-hydroxylase" from hog adrenal mitochondria was reported by Ichii et al. (1967). They claimed that addition of adrenodoxin (non-heme iron protein) to their acetone powder preparation was obligatory for side-chain cleaving activity. The identity of the intermediate (in the presence of labeled carrier 20α-OH cholesterol) was established by repeated paper- and thin layer chromatography. No significant change in ^{14}C to ^{3}H ratio was observed as a result of these chromatographic treatments.

Simpson and Boyd (1967) reported that the inhibition of cholesterol side-chain cleavage by carbon monoxide was reversed by light (450 nm), suggesting the involvement of the heme-containing protein cytochrome P-450. Working with sonicated bovine adrenal cortex mitochondria, they were unable to find hydroxylated derivatives of cholesterol. The inhibition of cholesterol side-chain cleavage by 20α-OH cholesterol (Hall & Koritz,1964) was confirmed and extended to 24-OH, 25-OH and 26-OH cholesterol. Simpson and Boyd (1967) were the first (at least in this field) in critizising the criteria for intermediacy of a certain compound. They stated that the strongest evidence for the involvement of 20α-OH cholesterol and 20α,22R-di-OH cholesterol as intermediates in the reaction was the fact that they are more efficient precursors of pregnenolone than cholesterol. However, the same had been shown to be true for 22-hydroxycholesterol (Chaudhuri, 1962), while 20α-OH,22-keto-cholesterol (Shimizu et al. 1962) and 25-OH cholesterol (Burstein and Gut, 1971) are also converted into pregnenolone but had been ruled out as obligatory intermediates (Burstein and Gut, 1971; Constantopoulos et al. 1962, 1966). Simpson and

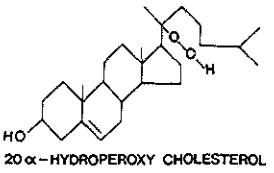
Boyd (1967) stated: "Thus if ability to be metabolized to pregnenolone is accepted as evidence for involvement as an intermediate, it is necessary to assume several possible pathways, which seems somewhat unlikely". A possible role of 20α-OH cholesterol and 20α,22R-di-OH cholesterol as intermediates was not ruled out by Simpson and Boyd. They assumed that the cleavage of the cholesterol side-chain was a concerted reaction in which the sterol molecule is bound to the enzyme along with molecular oxygen in a transition-state complex until the side-chain is liberated.

With double-isotope dilution techniques Roberts et al. (1969) showed that bovine adrenal glands contained 100 µg/kg unesterified and esterified 20α-OH cholesterol. With similar techniques Dixon et al. (1970) showed that bovine adrenal glands contained 1500 µg/kg 22R-OH cholesterol and 2150 µg/kg 20α,22R-di-OH cholesterol.

A breakthrough was reported by Burstein et al. (1970a,b; 1971). At relatively low substrate concentrations the conversion of cholesterol to 22R-OH cholesterol and 20α,22R-di-OH cholesterol (without the use of trapping agents in the incubation media) was established using reverse-isotope dilution techniques involving chromatographic separation, derivative formation, specific reactions and crystallisation. The amount of radioactivity found in 20α-OH cholesterol was exceedingly small, precluding a significant formation rate of this sterol from cholesterol. According to Burstein et al. (1970b; 1971), their studies using acetone powder preparations from bovine adrenal cortex, established the existence of the following pathways: 20α-hydroxycholesterol → 20α,22R-dihydroxycholesterol → pregnenolone and cholesterol → 22R-OH cholesterol → 20α,22R-di-OH cholesterol → pregnenolone (fig. 1.2). The rate of disappearance was measured for cholesterol and its hydroxylated derivatives. Using first order Michaëlis-Menten kinetics the first order rate constants were calculated, assuming overall irreversibility. Comparing the experimental results with their theoretical models it was suggested that the major fraction of pregnenolone arose by an

one-step (or direct) enzymatic reaction: Cholesterol → 20α, 22R-di-OH cholesterol. The second most important sequence was the one involving the conversion of cholesterol into 22R-OH cholesterol followed by an one-step transformation of 22R-OH cholesterol into pregnenolone. From their calculations it was concluded that the sequences, cholesterol → 20α-OH cholesterol → 20α,22R-di-OH cholesterol → pregnenolone and cholesterol → 22R-OH cholesterol → 20α,22R-di-OH cholesterol → pregnenolone accounted for a relatively small fraction of the experimentally found pregnenolone. The pathways involving 20α-OH cholesterol were quite insignificant.

Another pathway for the conversion of cholesterol into pregnenolone has been suggested by van Lier (1970) who showed that 20α-hydroperoxycholesterol (fig. 2.4) was converted into 20α,22R-di-OH cholesterol. This reaction was catalysed by an acetone powder of bovine adrenal cortex and proceeded in the absence of NADPH and oxygen. The enzymatic conversion of cholesterol into the 20α-hydroperoxy derivative has however, not been reported. According to the proposal only a single molecule of oxygen was involved in the formation of 20α,22R-di-OH cholesterol from cholesterol.



20α-HYDROPEROXY CHOLESTEROL

Fig. 2.4

A synthetic analog of 20α-OH cholesterol, completely substituted with CH_3-groups at C-22 has been synthesized by Luttrell et al. (1972). According to these authors oxygenation at C-22, if necessary for cleavage, could only occur simultaneously with fission of the C20-22 bond. Hence the involvement of stable, di-oxygenated intermediates analogous to 20α, 22R-di-OH cholesterol was not possible in these transformations.

Also the hydroxylated compounds 20α-OH cholesterol and 22R-OH cholesterol were not obligatory intermediates. The hydroxy sterols were considered to be by-products from as yet undefined reversible transformations of the true intermediates. The mechanism of the cleavage reaction involved reactive, transient, ill-defined intermediate complexes, which could be represented either as radical or ionic species.

In summary, it is concluded that in 1972 the possible pathways for the conversion of cholesterol into pregnenolone included:

a) The classical scheme involving hydroxylations at C-20 and C-22 with formation of 20α,22R-di-OH cholesterol. The intermediates were isolated from the incubation medium. (Burstein et al. 1970b; 1971).

b) A cleavage mechanism involving ionic or radical intermediates. These intermediates could not be isolated from the medium. Compounds that were isolated were merely by-products from the true intermediates. (Lieberman et al. 1969; Luttrell et al. 1972).

c) A cleavage mechanism involving the formation of 20α-hydroperoxycholesterol. This compound was further converted into 20α,22R-di-OH cholesterol. (van Lier and Smith 1968, 1970, 1972).

2.3 Statement of the problem

In this literature survey up to 1972, when we started our investigation, several mechanisms for the conversion of cholesterol into pregnenolone had been suggested. Firm evidence had not been presented for any of these hypotheses. A satisfactory model for cholesterol side-chain cleavage could not be constructed at that time. Such a model was considered essential for any study concerning inborn errors in steroid biosynthesis of the congenital lipoid adrenal hyperplasia type. Therefore it was attempted to construct a model, fitting all well established observations.

CHAPTER 3.

METHODS

3.1 Introduction

For a quantitative evaluation of the cholesterol side-chain cleaving activity radioactive (exogenous) substrate is often used. However, incubation studies with tracer amounts of radioactive cholesterol are complicated by the possibility that the radioactive precursor is not taken up or does not completely mix with the endogenous cholesterol pool and therefore the results obtained may not reflect true production rates. To circumvent this problem the mitochondria are often treated with acetone which removes most of the cholesterol, but by this treatment the activity of the enzyme(s) is considerably diminished, while always some cholesterol remains in the residue.

An additional problem arises when the formation of intermediates of the side-chain cleavage reaction is studied, using an acetone powder of bovine adrenal cortex mitochondria. The isolation of the very low amounts of radioactive intermediates requires laborious techniques.

Using an acetone powder, prepared from bovine adrenal cortex mitochondria, Burstein et al. (1971) reported the isolation of radioactive labeled 22R-OH cholesterol and labeled 20α,22R-di-OH cholesterol upon the incubation of labeled cholesterol with an adrenal preparation. Their technique depended on the isolation of small quantities of steroids by column chromatography. Identification of the labeled compounds was performed via crystallisation to constant specific activity. If the radioactive labeled compound and the added unlabeled compound are identical, the specific activity (dpm/mg) must remain constant during the recrystallisation steps. Unfortunately this method is not fully reliable (Hochberg et al. 1976b). Moreover, the assumed intermediate must be commercially available or has to be synthesized in a purity exceeding 99%. In addition, the

isolation method via column chromatography is very laborious. For the present studies gas-liquid chromatography was used for isolation and characterisation of reaction products. The major advantages of this technique are:

A) a high resolution, which enables one to detect several sterols and steroids in one gaschromatographic run.
B) the possibility to couple the gaschromatograph to a mass-spectrometer.

In this way a compound can be identified by its retention time on the gaschromatographic column as well as by its mass-spectrum. From the information contained in the mass-spectrum it is often possible to locate the site of incorporation of a label. In the present study (non-radioactive) $H_2{}^{18}O$ was used. The labeled oxygen atom is two mass units heavier than the ^{16}O present in air and in water. With the combination of GC-MS the site of ^{18}O incorporation in pregnenolone and in 20α,22R-di-OH cholesterol upon incubation of mitochondria in the presence of $H_2{}^{18}O$ was determined.

Compounds that are not volatile or compounds that decompose on heating at 200 - 300°C elude gaschromatographic detection, but these disadvantages can sometimes be useful as will be shown in chapter 5.

3.2 Stoichiometry

The stoichiometry of the side-chain cleavage reaction with respect to oxygen was used in the present investigation as a useful tool to construct a model for the conversion of cholesterol into pregnenolone. Once the stoichiometry of the reactions with respect to oxygen is known, the theoretical number of possible pathways (and intermediates) is considerably reduced. If an experimentally obtained stoichiometric relationship is not compatible with that of the hypothetical scheme, the scheme should be rejected.

Introduction of a hydroxyl group at C-11 of deoxycorticosterone converts this compound into corticosterone. The monooxygenase reaction is catalyzed by cytochrome $P\text{-}450_{11\beta}$ and is

located in the inner membrane of the bovine adrenal cortex mitochondria. The reaction requires NADPH and oxygen:

$$1 \text{ steroid-H} + 1 \text{ NADPH} + 1 \text{ H}^+ + 1 \text{ O}_2 \rightarrow$$
$$1 \text{ steroid-OH} + 1 \text{ NADP}^+ + 1 \text{ H}_2\text{O}.$$

According to the classical scheme, which involves two consecutive mono-oxygenase reactions and a cleavage between C_{20} and C_{22}, side-chain cleavage of cholesterol would require 3 mol oxygen. Also according to the epoxide-diol hypothesis (see chapter 6) 3 mol oxygen are required for the conversion of cholesterol into pregnenolone.

During our investigations the oxygen requirement for side-chain cleavage of cholesterol, 20α-OH cholesterol and 20α,22R-di-OH cholesterol was reported (Shikita and Hall, 1974). Therefore we investigated the same relationship for 22R-OH cholesterol, which in our opinion is a more likely intermediate than 20α-OH cholesterol.

3.3 ^{18}O incorporation

The incorporation of ^{18}O can be studied with a mass spectrometer. If the reaction equation, shown in 3.2, is valid, addition of $H_2{}^{18}O$ will not result in incorporation of ^{18}O in the hydroxylated product, for this reaction requires molecular oxygen. If, however, ^{18}O incorporation does occur with $H_2{}^{18}O$ as oxygen donor, the reaction equation, shown in 3.2, is not valid. Hence the incorporation experiment is of importance in studying the enzymes involved in the reaction.

The use of ^{18}O has some drawbacks that have not generally been recognized up till now. According to the reaction equation NADPH and H^+ produce in the presence of $^{18}O_2$ and adrenal mitochondria, $NADP^+$ and $H_2{}^{18}O$. As a result of this oxidation reaction both $H_2{}^{18}O$ and $^{18}O_2$ are at the same time present in the reaction medium and evidently such a situation should be avoided. Therefore in our experiments $H_2{}^{18}O$ was used as ^{18}O-donor.

If necessary, $^{18}O_2$ can be prepared from $H_2{}^{18}O$ via $Hg(^{18}OH)_2$.

PREGNENOLONE FORMATION FROM CHOLESTEROL IN BOVINE ADRENAL CORTEX MITOCHONDRIA: PROPOSAL OF A NEW MECHANISM

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1. Introduction

Several models of cholesterol side-chain cleavage have been proposed, but none of them has found general acceptance [1–3]. The schemes favoured by most authors presently working in this field are (a) two consecutive hydroxylations of cholesterol at carbon atoms 20 and 22 (the classical scheme) and (b) a concerted attack of oxygen with the formation of 20α, 22R-di-OH cholesterol. The vicinal glycol is cleaved to yield pregnenolone and isocaproaldehyde.

It has been found that 20α-OH cholesterol, 20α, 22R-di-OH cholesterol and 22R-OH cholesterol are all effective precursors of pregnenolone in adrenal tissue [1,2]. Burstein and Gut [2,4,5] were able to isolate labelled 20α, 22R-di-OH cholesterol and 22R-OH cholesterol when labelled cholesterol was added to an acetone powder of bovine adrenal cortex mitochondria. Their calculations suggest that 20α, 22R-di-OH cholesterol is an intermediate of the cholesterol side-chain cleavage reaction. They also presented evidence that pregnenolone formation via the 'classical pathway' is insignificant and their results strongly indicate the existence of other (as yet unidentified) pathways.

From the experiments of Wilson [6] and Burstein [2] it is evident that a correlation exists between the rate of oxygen uptake and the rate of pregnenolone formation when 22R-OH cholesterol, 20α, 22R-di-OH cholesterol and 20α-OH cholesterol are added to adrenal cortex mitochondria. This correlation was used as a screening method for potential intermediates of the cholesterol side-chain cleavage reaction.

The results of our studies cannot be explained by the models proposed until now. Therefore a new model will be suggested in this paper. Preliminary results of this work have been presented elsewhere [7–9].

Nomenclature and abbreviations:
HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid.
EGTA: Ethylene glycol-bis-(β-amino-ethylether)*N,N'*-tetraacetic acid.
Cyanoketone: 2α-cyano-4,4,17α-trimethyl-17β-hydroxyandrost-5-en-3-one.
Cholesterol: 5-cholesten-3β-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.
Δ^{20-22} cholesterol: 5,20(22)-cholestadien-3β-ol.
Δ^{17-20} cholesterol: 5,17(20)-cholestadien-3β-ol.
Δ^{20-21} cholesterol: 5,20(21)-cholestadien-3β-ol.
20,22-epoxycholesterol: 20,22-epoxy-5-cholesten-3β-ol.

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2. Materials and methods

Bovine adrenal cortex mitochondria were prepared according to standard procedures. Only mitochondria with a respiratory control ratio (succinate) ⩾ 3 were used.

Oxygen uptake was measured in a thermostated (37°C) vessel (vol 1.3 ml) equiped with a Clark electrode. The medium used contained: 200 mM sucrose, 20 mM KCl, 10 mM potassiumphosphate, 20 mM

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HEPES (pH 7.3), 1 mM EGTA, 1% bovine serum albumin (w/v). Further additions are mentioned in the legend to fig. 1.

$H_2{}^{18}O$ incorporation experiments were performed in a medium ($H_2{}^{18}O/H_2{}^{16}O$ = 1:2) containing 154 mM KCl, 11.5 mM NaCl, 50 mM nicotinamide, 20 mM HEPES (pH 7.3), 5 mM $CaCl_2$, 4 mM Na-azide, 1% bovine serum albumin (w/v) with a final vol of 5 ml. Bovine adrenal cortex mitochondria (stored under liquid nitrogen) were added to the $H_2{}^{18}O$ containing medium immediately after freeze-drying and kept for 30 min at 0°C (procedure *a*). Procedure *b* consisted of storing freeze-dried mitochondria in contact with the air during 30 min at 0°C, followed by addition to the $H_2{}^{18}O$ containing medium. These media containing mitochondria (15 mg protein) were preincubated at 37°C for 20 min. Additions were made to achieve a final concentration of 30 μM cyanoketone, 5 μM antimycin A, 200 μM 20α-OH cholesterol or 22R-OH cholesterol. The reactions were started by adding NADPH, glucose-6-phosphate and glucose-6-phosphate-dehydrogenase to give a final concentration of 0.1 mM, 3 mM and 0.6 U/ml respectively. Samples taken at 0, 10 and 20 min were extracted with ethylacetate, evaporated under nitrogen, persilylated with *N*-trimethylsilylimidazole by heating for 1 hr at 95°C in the presence of pyridine hydrochloride.

Gaschromatography: Analytical separations were carried out with a Hewlett-Packard 5700A instrument equiped with dual flame ionization detectors. The glass columns (2 m X 1.8 mm I.D.) were packed with 3% SP-2250 (Supelco) on 100–120 mesh Chromosorb WAW-DMCS H.P. The conditions were: injector

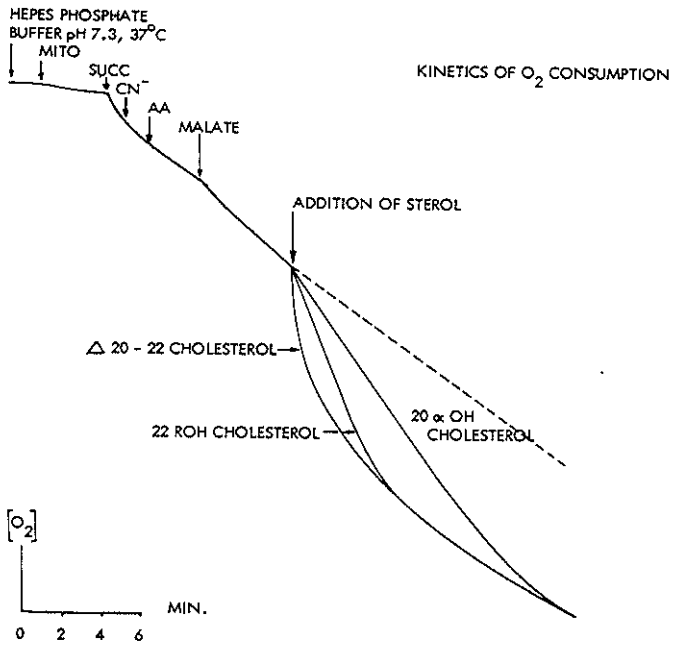


Fig. 1. Effect of the addition of sterols on the rate of oxygen uptake by bovine adrenal cortex mitochondria. Mitochondria (0.9 mg protein) were added to 1.25 ml medium (methods). Additions were made as indicated. Final concentrations were: 10 mM succinate, 1 mM potassium cyanide, 5 μM antimycin A, 10 mM malate, 20 μM 20α-OH cholesterol, 20 μM 22R-OH cholesterol, 20 μM Δ^{20-22} cholesterol. Sterols were added as their ethanolic solutions.

250°C, detector 300°C, nitrogen flow rate 30 ml/min. The oven was programmed from 236°C to 280°C at 2°C/min. Peak areas and retention times were measured with an electronic integrator (Infotronics CRS-100A).

Mass-spectrometry: The mass-spectra were recorded on a Finnigan 1015D-6000 GC-MS-computer system. The GLC-conditions were equivalent to those described above. Helium was used as a carrier-gas. The MS-conditions were: jetseparator at 250°C, source temperature 170°C, ionizing voltage 70 eV and ionizing current 500 μA.

Δ^{20-22} cholesterol was synthesized according to Sheikh and Djerassi [10]. Protein was estimated using the biuret method.

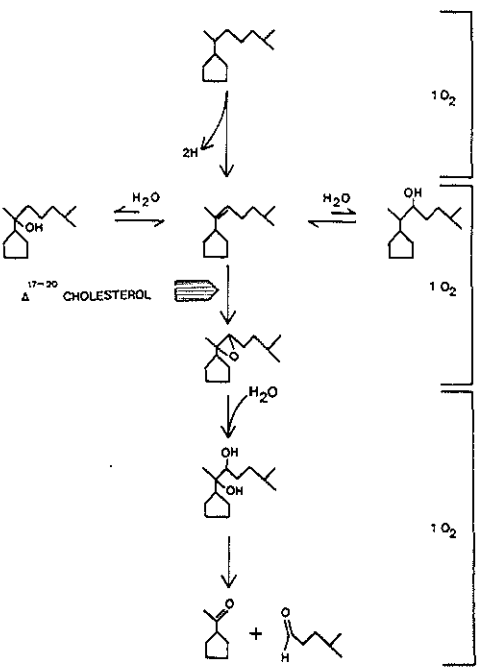
3. Results and discussion

The rate of oxygen uptake of intact bovine adrenal cortex mitochondria was dramatically increased by adding Δ^{20-22} cholesterol in comparison to the addition of 22R-OH cholesterol and 20α-OH cholesterol (fig. 1). All three substrates were converted into pregnenolone.

Dehydration of 20α-OH cholesterol produces a mixture of Δ^{17-20}, Δ^{20-22} and Δ^{20-21} cholesterol [10]. Δ^{17-20} cholesterol proved to be a very efficient inhibitor of the cholesterol side-chain cleavage system. Contamination by this inhibitor might be the reason why Δ^{20-22} cholesterol was reported earlier as a very poor substrate [2].

Combination of our results with the evidence available in the literature, allowed us to propose a new scheme (fig. 2), whereby cholesterol is converted into pregnenolone and isocaproaldehyde via Δ^{20-22} cholesterol, 20,22-epoxycholesterol, 20α, 22R-di-OH cholesterol. 20α-OH cholesterol and 22R-OH cholesterol will lose water to form Δ^{20-22} cholesterol.

According to this proposal $H_2{}^{18}O$ should be incorporated in 20α,22R-di-OH cholesterol and the ^{18}O-label must be found in pregnenolone or isocaproaldehyde. To check this hypothesis 1000 nmol 20α-OH cholesterol and an equal amount 22R-OH cholesterol were incubated in parallel in a $H_2{}^{18}O$ containing medium ($H_2{}^{18}O/H_2{}^{16}O = 1:2$) in the presence of freeze-dried bovine adrenal cortex mitochondria, supported by a NADPH generating system



(methods). After 20 min of incubation at 37°C (procedure *a*) 20α-OH cholesterol was partially converted; 330 nmol pregnenolone were formed. 22R-OH cholesterol was totally converted; 890 nmol pregnenolone were produced. Samples from these $H_2{}^{18}O$ incubations were extracted, trimethylsilylated and analyzed by GC-MS. Their mass-spectra were compared with that of synthetic pregnenolone and with those obtained in identical experiments in presence of unlabelled water (fig.3). The relative abundance of the M+2 peak of the ^{18}O incorporated pregnenolone shows an increase of 170% over the pregnenolone-standard (inaccuracy of the measurement: less than 10%). It could be concluded that water from the medium was incorporated in 20% of the product originating from 20α-OH cholesterol. No ^{18}O could be detected in the synthesized pregnenolone (standard), pregnenolone

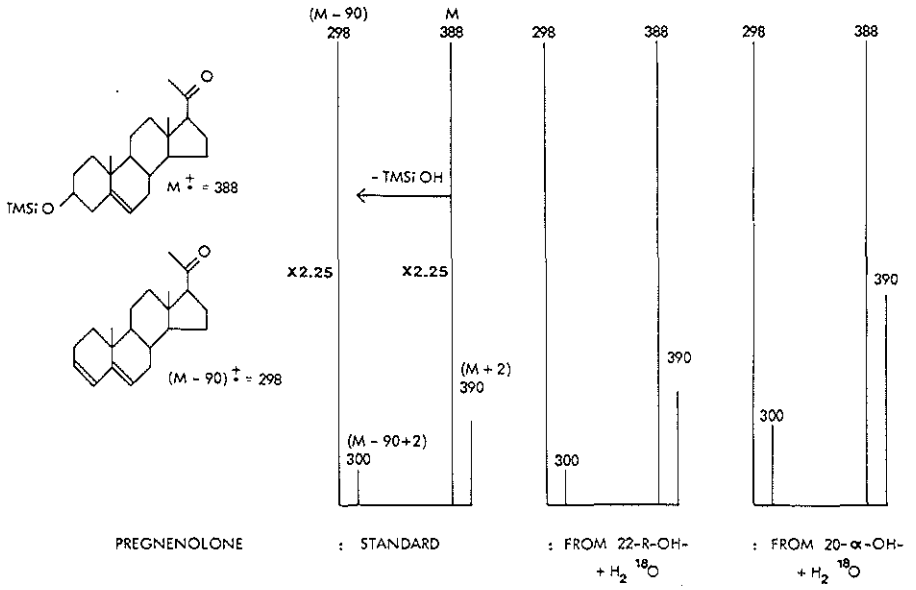


Fig. 3. The measured relative abundances of M-90+2 and M+2 of the pregnenolone-standard are in accord to the calculated natural abundances. The mass-spectrum of pregnenolone originating from 20α-OH cholesterol shows an incorporation of ^{18}O that can be seen from the marked increase in the relative abundances of M-90+2 and M+2. No incorporation was demonstrated in pregnenolone formed from 22R-OH cholesterol.

originating from 22R-OH cholesterol ($H_2{}^{18}O$) and pregnenolone from the control experiments. We could not demonstrate exchange of ^{18}O between the substrates 20α-OH cholesterol, 22R-OH cholesterol and $H_2{}^{18}O$. The low percentage of incorporation may be explained by the amount of water still present in the freeze-dried mitochondria and the water produced during the reaction (fig. 2). Pregnenolone originating from endogenous substrates contributes $\leq 5\%$ of the total pregnenolone production, causing only insignificant isotope dilution.

In measuring ^{18}O incorporation in 20α,22R-di-OH cholesterol, reaction conditions had to be changed (procedure *b*; methods) in order to isolate sufficient material for mass spectrometric identification. We found that 22R-OH cholesterol was partially converted and that 180 nmol pregnenolone and 157 nmol 20α,22R-di-OH cholesterol were formed. No detectable incorporation of ^{18}O could be found in pregnenolone; however ^{18}O was incorporated in 20α, 22R-di-OH cholesterol. Analysis of the mass-spectrum showed that ^{18}O was attached to the carbon-22 atom of 20α,22R-di-OH cholesterol. 20α-OH cholesterol incubated according to procedure *b* was partially converted; 70 nmol ^{18}O-incorporated pregnenolone were formed. The results are summarized in fig. 4.

Our results complement those of Takemoto et al. [11] and Nakano et al. [12] who performed incorporation studies with molecular oxygen ($^{18}O_2$). Incorporated ^{18}O in pregnenolone originating from cholesterol and 22R-OH cholesterol was found to be attached to the C-20 atom. In the presence of $^{18}O_2$ neither 20α-OH cholesterol nor 20α,22R-di-OH cholesterol was converted into pregnenolone containing labelled oxygen.

An explanation for the two different ways of the reaction of 20,22-epoxycholesterol with water (fig. 4) might be found by considering the aconitase reaction.

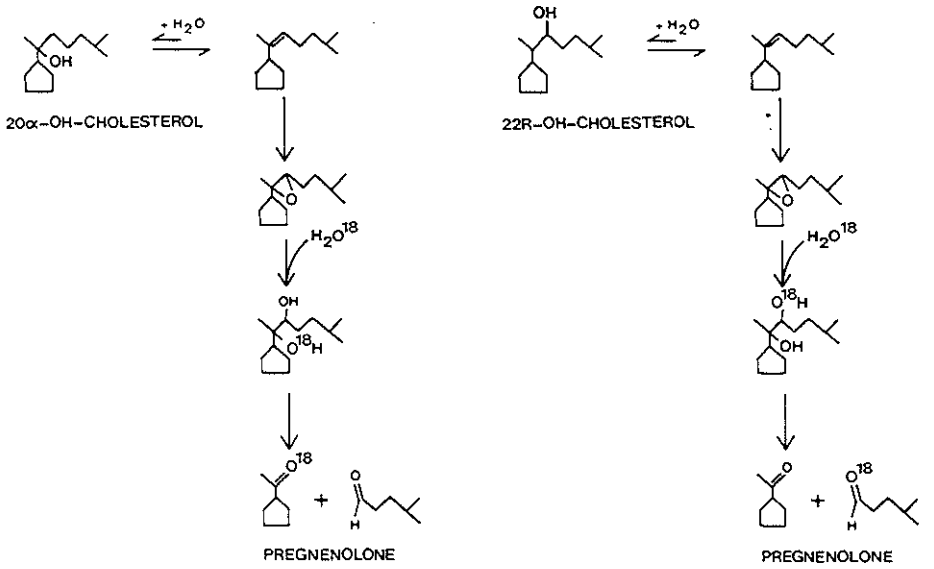


Fig. 4. Incorporation of ^{18}O after incubation of 20α-OH cholesterol and 22R-OH cholesterol with freeze-dried bovine adrenal cortex mitochondria supported by a NADPH-generating system in the presence of $H_2{}^{18}O$ (methods).

We postulate that Δ^{20-22} cholesterol originating from 20α-OH cholesterol is attached to the enzyme surface in a stereochemically different way than Δ^{20-22}cholesterol originating from 22R-OH cholesterol. This situation would be analogous to the aconitase reaction, where cis-aconitate formed by dehydration of citrate and cis-aconitate formed by dehydration of threo-D_s-isocitrate are bound in a stereochemically different way [13].

The proposed scheme (fig. 2) is also in accord with the stoichiometry of the reactions with respect to O_2 and NADPH, as reported by Shikita and Hall [14], and Kraaipoel et al. [7,8]. The conversion of 1 mol of cholesterol into Δ^{20-22} cholesterol would use at least $\frac{1}{2}$ mol of O_2 to form H_2O with the removed hydrogen. Activation of cytochrome P-450 [15,16] with 1 mol NADPH + H^+ probably needs another $\frac{1}{2}$ mol O_2. Since the conversion of both 20α-OH and 22R-OH cholesterol to pregnenolone and isocaproaldehyde requires 2 mol O_2 and 2 mol NADPH + H^+ per mol substrate, and the conversion of 20α,22R-di-OH cholesterol needs 1 mol O_2 plus 1 mol NADPH + H^+, it can be concluded that 1 mol O_2 plus 1 mol NADPH + H^+ are necessary for the conversion of Δ^{20-22} cholesterol into 20,22-epoxycholesterol.

20α,22R-di-OH cholesterol and 20,22-epoxycholesterol could be isolated from reaction media [20]. The high conversion rate of Δ^{20-22} cholesterol as an intermediate has hitherto prevented its isolation.

Virtually all the data found in the literature concerning cholesterol side-chain cleavage fit nicely into this new scheme [2,17,18]. Its consequences for the study of inborn errors in pregnenolone biosynthesis [19] will be discussed elsewhere [20].

Acknowledgements

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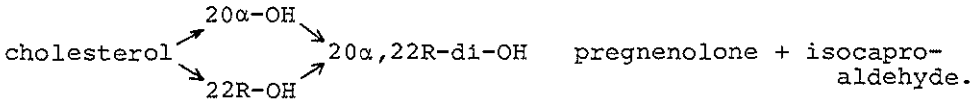
CHAPTER 5.

SIDE-CHAIN CLEAVAGE OF 22R-OH CHOLESTEROL BY BOVINE ADRENAL CORTEX MITOCHONDRIA *

5.1 Introduction

Bovine adrenal cortex mitochondria contain an enzyme complex which catalyzes the cleavage of the C_{20}-C_{22} bond of cholesterol, 20α-OH cholesterol, 22R-OH cholesterol and 20α,22R-di-OH cholesterol to form pregnenolone. This complex is usually called cytochrome P-450_{scc}. According to the "classical scheme" the mechanism of this side-chain cleavage involves hydroxylation of C_{20} and C_{22} followed by cleavage of the vicinal glycol to yield pregnenolone and isocaproaldehyde:



A direct conversion of cholesterol into 20α,22R-di-OH cholesterol has also been proposed (Burstein et al. 1970b; Burstein and Gut, 1971). However, evidence for two sequential mono-oxygenases or for a concerted attack of oxygen producing the dihydroxycholesterol is still lacking. There are several other models of cholesterol side-chain cleavage which also lack good experimental evidence (van Lier and Smith, 1970; Luttrell et al. 1972).

We have studied the conversion of 22R-OH cholesterol into pregnenolone and isocaproaldehyde. In this chapter we will present results on the identity of the intermediate 20α-22R-di-OH cholesterol which was established by a combination of gas-chromatography and mass-spectrometry. In addition we found that 20α,22R-di-OH cholesterol is not the only intermediate. The results suggest 20,22-epoxycholesterol to be an intermediate in the same reaction. The experiments support our previously presented hypothesis, according to which 22R-OH cholesterol is

*The results of this chapter have been published in part, Kraaipoel et al. (1975b).

converted into pregnenolone and isocaproaldehyde via Δ^{20-22} cholesterol, 20,22-epoxycholesterol and 20α,22R-di-OH cholesterol (chapter 4).

5.2 Methods and materials

Bovine adrenals were obtained from a local slaugtherhouse and transported on ice to the laboratory within 30-45 min. The glands were trimmed to remove fat and cut in half longitudinally. The central medulla and a minor portion of the adjacent zone reticularis were scraped away and discarded. The remainder of the cortex, mainly zona fasciculata, was scraped off and collected. A 10% (w/v) homogenate in 250 mM sucrose, 0.5 mM EGTA and 10 mM HEPES at pH 7.35 was prepared using a Potter-Elvehjem homogeniser with a power-driven Teflon pestle. The homogenate was centrifuged at 900xg for 10 minutes at 4°C to remove nuclei, erythrocytes and unbroken cells. The supernatant was centrifuged at 9000 g for 10 minutes to give the mitochondrial pellet. This pellet was resuspended in 250 mM sucrose (one half of the original homogenate volume) and centrifuged again at 10.000 g for 10 minutes. This washing procedure was repeated once. The final pellet was resuspended to obtain a protein concentration of 30-50 mg/ml. Throughout the procedure any visible sedimented hemoglobin was carefully removed. The mitochondria were stored in liquid nitrogen.

Conversion of 22R-OH cholesterol (Ikapharm, Israel) 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 volume 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 (Goldman, 1967), 5 µM antimycin A, 0.1 mM NADP, 3 mM glucose-6-phosphate and 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 endogenous cholesterol and 1300 nmol 22R-OH cholesterol were added to the medium. The reaction was started by addition of the substrate 22R-OH cholesterol and stopped by addition of 6 ml cold ethyl acetate to a sample drawn from the incubation, and shaking vigorously. To all samples 20 µg/ml (50 µM) epi-cholesterol (Steraloids) was added as an internal standard. The solvent was evaporated at room temperature with nitrogen. The extract was silylated and the steroids were quantified by gas-chromatography as described in chapter 4. The experiments A and B were conducted as shown in fig. 5.1. All glassware had been cleaned with a potassium bichromate-sulphuric acid mixture and siliconized with Siliclad (Clay Adams).

Gaschromatographic analysis (fig. 5.7) was performed at 250°C on a capillary column coated with SE-30, lenght 60 m (Rutten, 1972), with a solid state injector (van den Berg, 1972), equipped with a flame ionisation detector" (full scale in fig. 5.2, 1 x 10^{-11} A) and with nitrogen as a carrier gas.

Gaschromatograms obtained with a packed column (3% SP-2250 on chromosorb WHP, 100-120 mesh) are shown in fig. 5.3, 5.4, 5.5 and 5.6.

The mass spectra were obtained by a GC-MS combination as described by Leferink et al. (1974). 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.

20α,22S-di-OH cholesterol was synthesized according to Chaudhuri et al. (1970) using sodium borohydride reduction. Protein was estimated by the biuret method.

Fig. 5.1

CONVERSION OF 22R-OH CHOLESTEROL INTO PREGNENOLONE

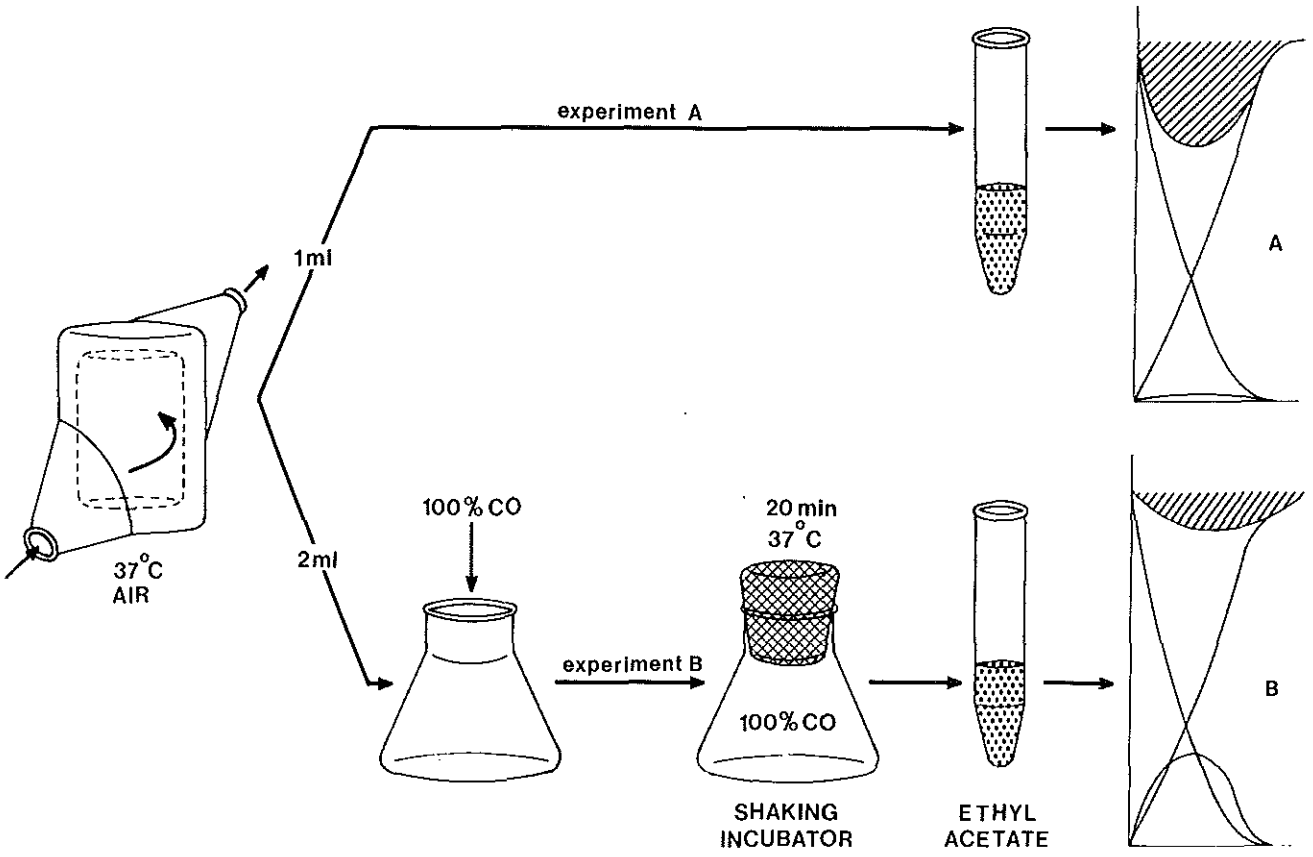


Fig. 5.1

Experiment 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.
Experiment B: Samples of 2 ml were taken from the same incubation at intervals as indicated in fig. 2-B, added to 25 ml erlenmeyers and immediately flushed with 100% carbonmonoxide (5oo 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.

5.3 Results

In fig. 5.2 it can be seen that 22R-OH cholesterol (1300 nmol) disappears in 10 min and 1300 nmol pregnenolone are formed. Assuming 20α,22R-di-OH cholesterol to be the only intermediate in the conversion from 22R-OH cholesterol into the products pregnenolone and isocaproaldehyde, the sum (nmol) 22R + 20α,22R + 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 method. The time course of formation and disappearance of this or these compound(s) is represented by the shaded area of fig. 5.2. Analysis of the samples taken from the experiment shown in fig. 5.2a, incubated for 20 min. under carbon monoxide, (see fig. 5.1) and thus with complete inhibition of cytochrome P-450, shows a marked increase in 20α,22R-di-OH cholesterol and a proportional decrease in the shaded area (fig. 5.2b). Apparently the compounds not measured by our method are converted into 20α,22R-di-OH cholesterol in the presence of >99% CO.
In fig. 5.4, 5.5 and 5.6 the gaschromatographic analysis of samples taken from an incubation similar as described in fig. 5.2 is shown. A standard mixture, containing equivalent amounts of steroids, is shown in fig. 5.3.

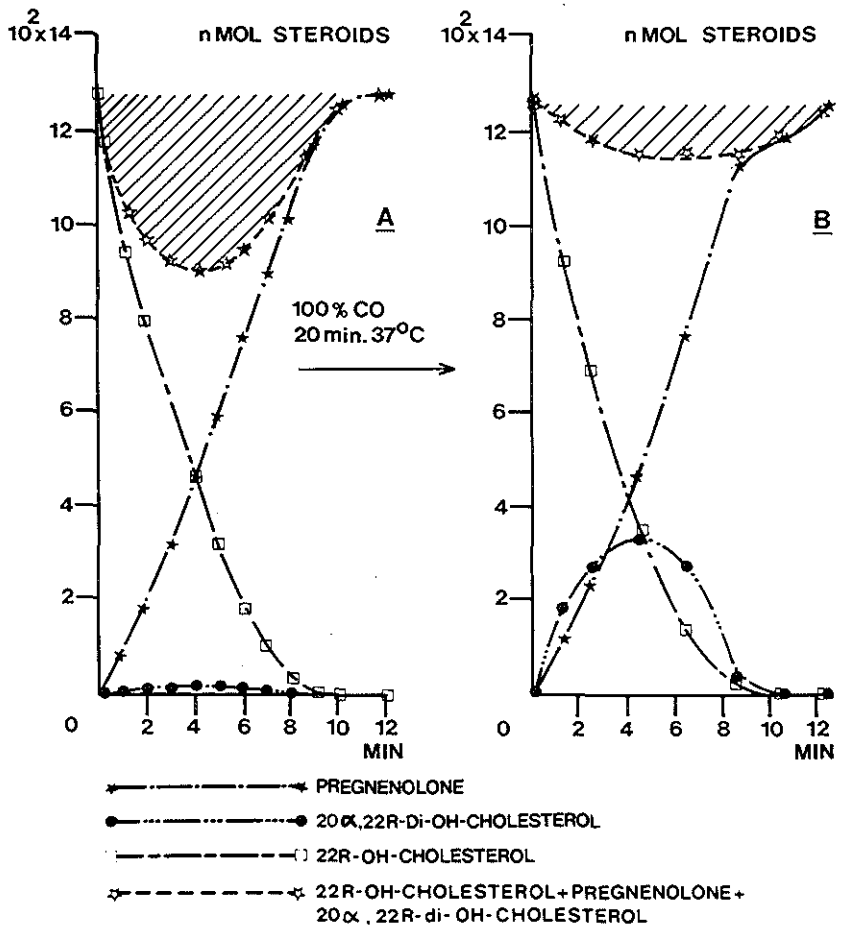


Fig. 5.2.

Conversion of 22R-OH cholesterol into pregnenolone by damaged bovine adrenal cortex mitochondria supported by a NADPH-generating system. 20α,22R-di-OH cholesterol is formed as an intermediate. The shaded area represents the apparent "mass-defect". After trimethylsilylation the steroids were quantified by GLC. Samples taken during the experiment, shown in fig. 5.2a, were immediately flushed with 100% carbon-monoxide (see fig. 5.1). They were incubated air-free in a shaking incubator at 37 ^{0}C for 20 min. After gaschromatographic analysis of these samples, a marked increase of 20α,22R-di-OH cholesterol and a decrease of the shaded area is shown (fig. 5.2b). The indicated time at the abscissa has been corrected for the time it took CO to stop the reaction ($t_{1-B} = t_{1-A} + 1$ min).

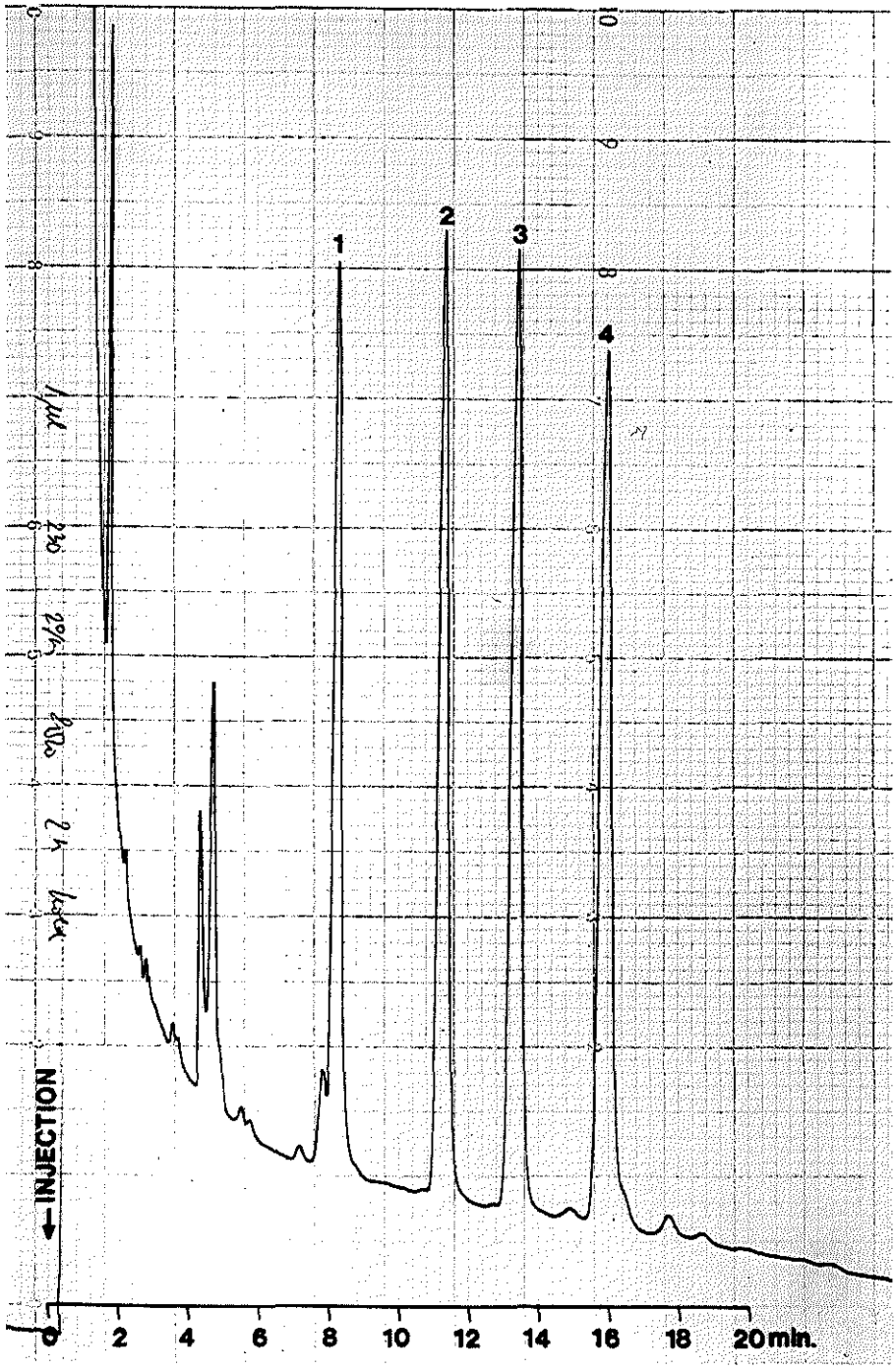


Fig. 5.3 (See legend on page 49)

Fig. 5.4 (See legend on page 49)

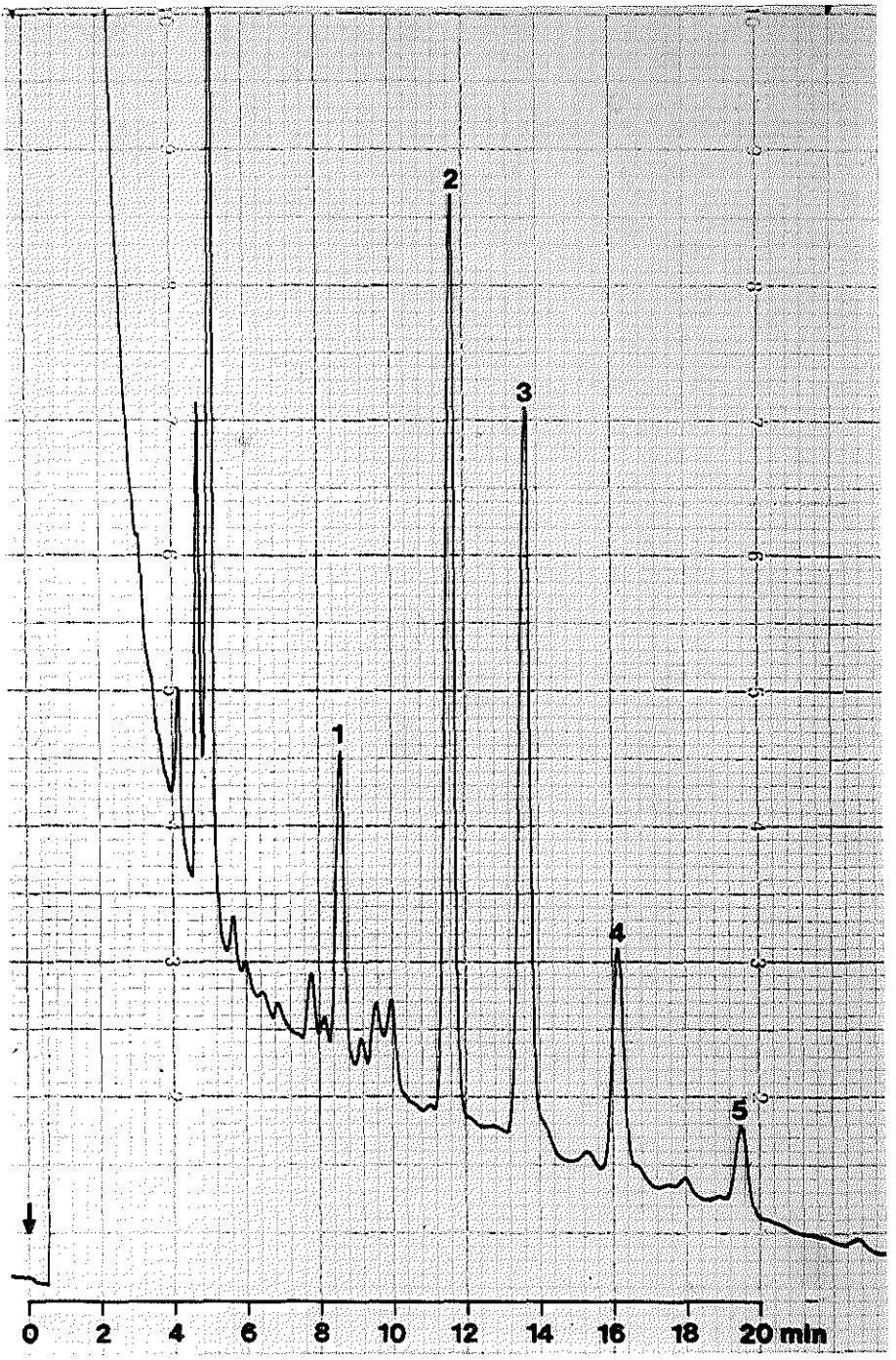


Fig. 5.5 (See legend on page 49)

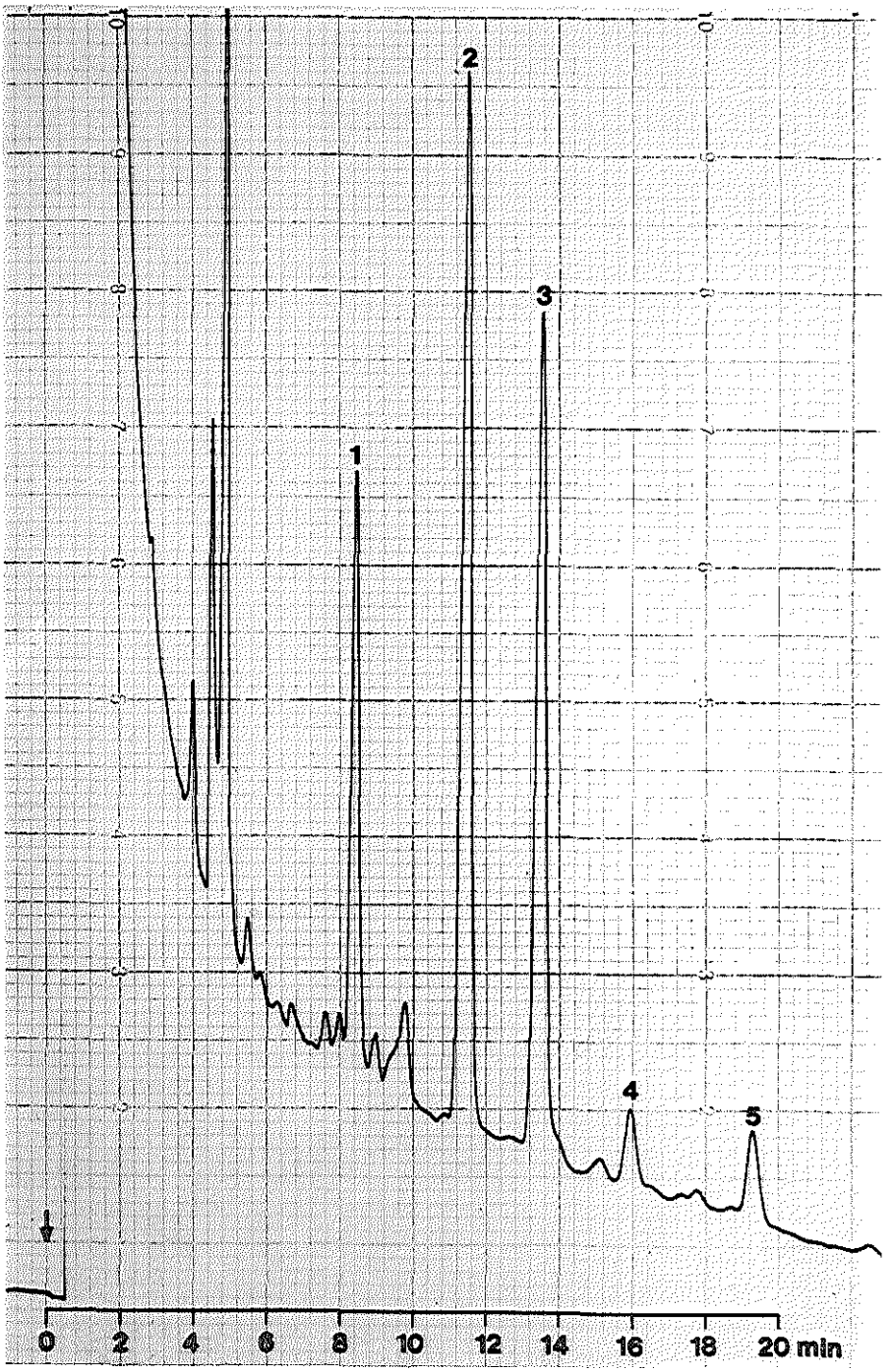


Fig. 5.6 (See legend on page 49)

Fig. 5.3

Gaschromatographic separation on a packed column (3% SP-2250 on chromosorb WHP, 100-120 mesh, length 1.8 m) of 200 ng each of the following trimethylsilylated steroids:
1) pregnenolone, 2) epi-cholesterol, 3) cholesterol and 4) 22R-OH cholesterol. Starting at 230°C, the oven temperature was programmed at 2°C/min to 280°C. The attenuation was 4 x 10.

Fig. 5.4

Gaschromatographic tracing of a sample taken from an incubation similar to the one described in fig. 5.2, 20 sec. after addition of 22R-OH cholesterol. Extraction, derivatisation, internal standard and gaschromatographic conditions are as described in fig. 5.3. Peak number 5 represents 20α,22R-di-OH cholesterol.

Fig. 5.5

Gaschromatographic tracing of a sample at t = 5 min.

Fig. 5.6

Gaschromatographic tracing of a sample at t = 7 min.

Fig. 5.8

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

Fig. 5.9

Hypothetical scheme for the conversion of 22R-OH cholesterol into pregnenolone and isocaproaldehyde.

Fig. 5.7 shows a gaschromatogram obtained with a capillary column. The sample was taken at t=4 min. (fig. 5.2b) 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-di-OH cholesterol is clearly separated from the synthetically prepared 20α,22S-di-OH cholesterol, while the mass-spectra of both compounds are almost identical (fig. 5.8).

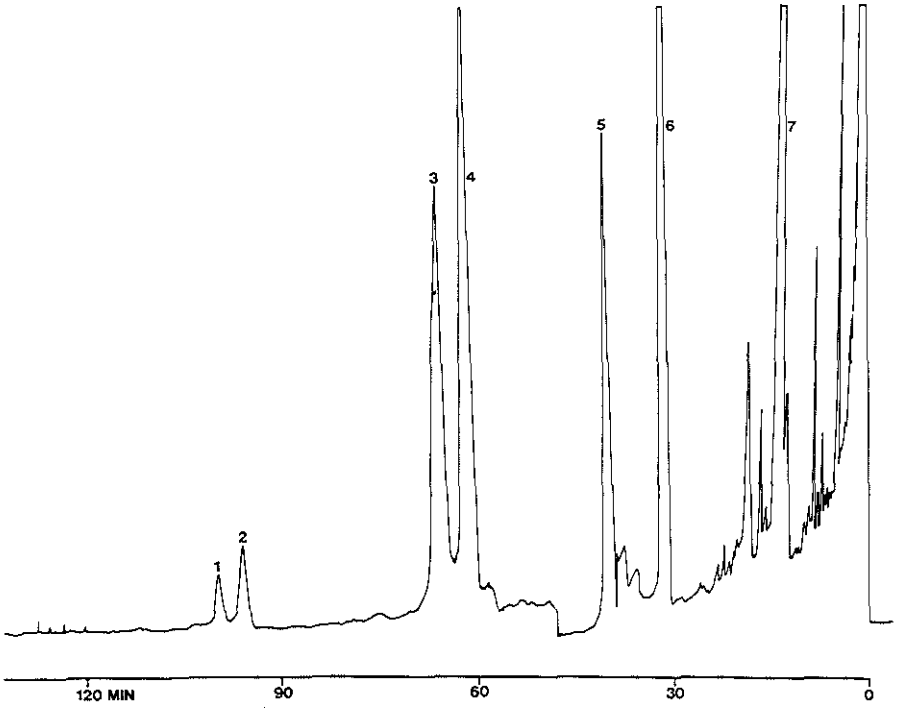


Fig. 5.7

Gas-liquid chromatography of trimethylsilylated steroids on a capillary column (see 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.

5.4 Discussion of the mass-spectra

The upper part of fig. 5.8 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.5.8). 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. 5.8 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 (Eneroth, 1969) with slight changes for the 20α-OH group.

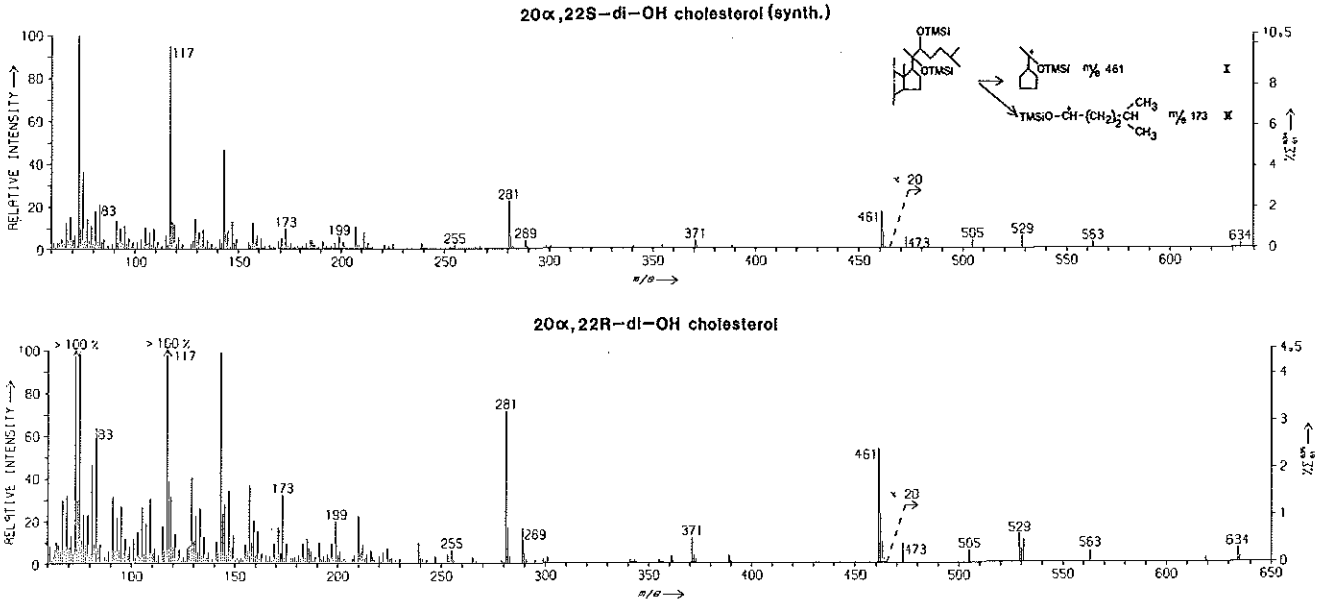


Fig. 5.8 (See legend on page 49)

5.5 Discussion

According to the experiments described an intermediate between 22R-OH cholesterol and 20α,22R-di-OH cholesterol must be present when 22R-OH cholesterol is converted into pregnenolone and isocaproaldehyde. A peroxide as a physiological intermediate is highly improbable. All tests on peroxides (Feigl, 1971; Stahl, 1967) indicated the presence of only traces of peroxides. In addition, the concentration of these trace amounts did not vary significantly in time.

It has been suggested that in addition to 20α,22R-di-OH cholesterol two other intermediates (Δ^{20-22} cholesterol and 20,22-epoxy cholesterol) are involved in the side-chain cleaving mechanism of 22R-OH cholesterol (chapter 4). In fig. 5.2 it is shown that not all the intermediates of the reaction are detected by the method used. The possibility exists that one (or more) of the intermediates (i.c. 20,22-epoxy cholesterol) is not extracted by ethylacetate. In addition, derivative formation of this intermediate may not yield a compound that can be detected by the gaschromatographic method. A sample from the same incubation mixture, treated in a different way (exp. B), shows a relatively high amount of the intermediate 20α,22R-di-OH cholesterol. Moreover the apparent mass-defect is less than in experiment A. Apparently the incubation under 100% CO, and thus with complete inhibition of cytochrome P-450, is able to convert a compound, undetectable in experiment A, into 20α,22R-di-OH cholesterol. The reaction conditions preclude a conversion involving cytochrome P-450, NADPH or oxygen. This experiment may be explained by the involvement of an epoxyhydrase catalyzing the conversion of 20,22-epoxy cholesterol to 20α, 22R-di-OH cholesterol (fig. 5.9). This enzyme requires a substrate and H_2O to react and is not inhibited by carbonmonoxide. Support of this hypothesis has been obtained from our studies describing the incubation of 22R-OH cholesterol in the presence of $H_2{}^{18}O$. The ^{18}O-label is incorporated in the intermediate 20α,22R-di-OH cholesterol (chapter 4), but

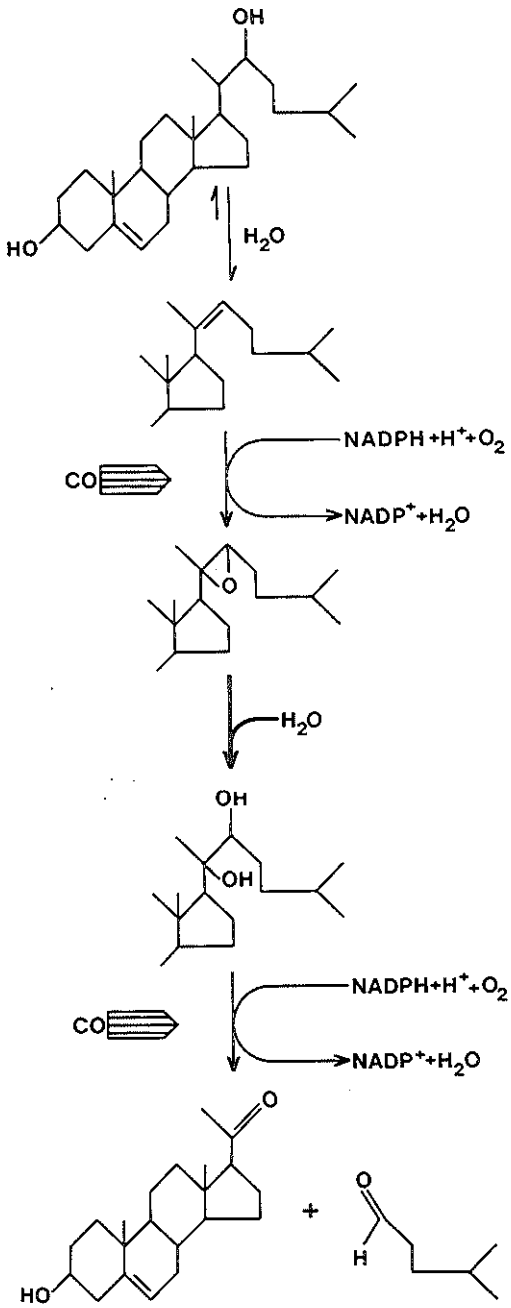


Fig. 5.9

(See legend on page 49)

not in pregnenolone. The incorporation of a molecule $H_2{}^{18}O$ into a diol suggests the involvement of an epoxyhydrase in the reaction.

One might expect 20α-OH cholesterol to behave in a manner similar to 22R-OH cholesterol since conversion of the sterol in the presence of $H_2{}^{18}O$ gave rise to ^{18}O containing pregnenolone (chapter 4). However, the phenomena shown in fig. 5.2 have not been found with 20α-OH cholesterol as a substrate (unpublished experiments, R.J. Kraaipoel), probably because the conversion 20α → Δ^{20-22} is slower than 22R → Δ^{20-22}. The epoxyhydrase reaction therefore is no longer the rate limiting step.

It is also possible to explain the different behaviour of 20α-OH cholesterol and 22R-OH cholesterol with respect to the apparent "mass-defect" by postulating the formation of two different epoxides (20α,22α-epoxycholesterol and 20β,22β-epoxycholesterol). This would support our earlier suggestion (chapter 4) that Δ^{20-22} cholesterol originating from 20α-OH cholesterol and Δ^{20-22} cholesterol originating from 22R-OH cholesterol are attached to the enzyme surface in stereochemically different ways.

Burstein et al. (1970b), using acetone extracted bovine adrenal cortex mitochondria, with recrystallisation techniques tentatively identified 20α,22R-di-OH cholesterol as an intermediate. This has now been confirmed by our experiments, which are the first demonstration of the formation of the glycol from 22R-OH cholesterol by mass-spectrometry. The time course of the reaction described by Burstein et al. (1970b) and Burstein and Gut (1971), using this material, was similar to the one shown in fig. 5.2b but no "mass-defect" was found. An explanation for the discrepancy was suggested when we used mitochondrial preparations stored under poor conditions (the storage container was not refilled in time with liquid nitrogen). Under those conditions a conversion of 22R-OH cholesterol into pregnenolone according to fig. 5.2b was repeatedly demonstrated. We found earlier that the enzymic conversion of 20α,22R-di-OH cholesterol 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 (chapter 4). The epoxyhydrase is no longer rate limiting and thus no epoxide but 20α,22R-di-OH cholesterol accumulates. This may be the reason why some investigators found 20α,22R-di-OH cholesterol as an intermediate and others did not.

It is tempting to speculate about the four enzymatic activities, that are involved in the conversion of cholesterol into pregnenolone. According to the scheme in chapter 4 this includes an oxidative desaturase (Fulco, 1974), an epoxidase, an epoxyhydrase and a lyase (desmolase). We suggest that the epoxidase and lyase activities belong to the group of cytochromes P-450 (fig. 5.9). It is interesting to compare this with the cytochrome P-450 preparation isolated by Shikita and Hall (1973a,b), 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 treatment results in the formation of single units. The fragment of 4 subunits (after addition of the specific flavo-protein and non-heme iron protein) was still able to convert cholesterol into pregnenolone (Shikita and Hall, 1974). This clearly is an area that requires further research and elucidation.

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

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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.

Nomenclature and abbreviations: Cholesterol : 5-cholesten-3β-ol. Epicholesterol : 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. 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.

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_2SO_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

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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 trimethylsilylimidazole 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 endogeneous 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

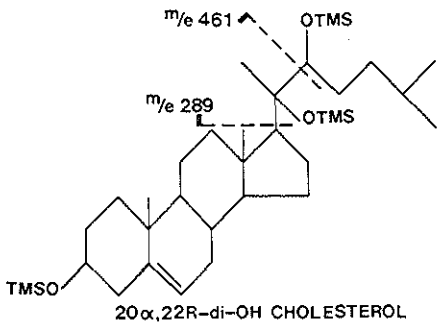

Fig. 1. The trimethylsilyl derivative of 20α,22R-di-OH cholesterol. The C-8 fragment at m/e 289 and the C-21 fragment at m/e 461 are indicated.

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:

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

[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 isocaproaldehyde via Δ^{20-22} cholesterol, 20,22-epoxycholesterol 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 $H_2{}^{18}O$

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

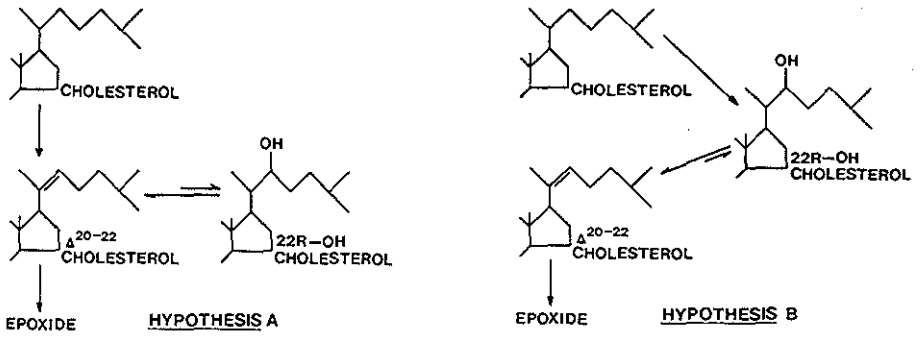

Fig.2. Two hypotheses for the conversion of cholesterol into Δ^{20-22} cholesterol.

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

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

(de)hydratase $\rightleftharpoons$ 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

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

(de)hydratase $\rightleftharpoons$ 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 caproaldehyde into their acids. Incubation of 100 μg (1.0 μmol) caproaldehyde for 20 min at 37°C in a medium (see Methods) containing denaturated 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 isocaproaldehyde 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

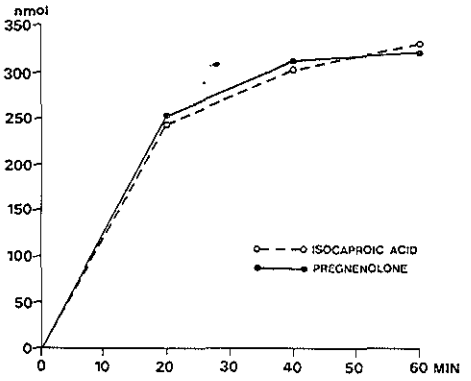

Fig.3. Freeze-dried bovine adrenal cortex mitochondria (30 mg protein), supported by a NADPH-generating system, converted cholesterol into pregnenolone and isocaproic acid (see Methods). Epicholesterol (20 μg/ml) and caproic acid (10 μg/ml) were added as internal standards. Both pregnenolone and isocaproic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

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CHAPTER 7.

STOICHIOMETRY OF THE ADRENOCORTICAL SIDE-CHAIN CLEAVAGE REACTION, 22R-OH CHOLESTEROL INTO PREGNENOLONE, ESTIMATED WITH RESPECT TO OXYGEN

7.1 Introduction

The stoichiometry of the side-chain cleavage of cholesterol, 20α-OH cholesterol and 20α,22R-di-OH cholesterol has been previously examined with respect to oxygen by Shikita and Hall (1974). In our opinion it is highly questionable if 20α-OH cholesterol is an essential intermediate in cholesterol side-chain cleavage (chapter 6). A more likely candidate is 22R-OH cholesterol. We therefore have estimated the stoichiometric relationship between oxygen and side-chain cleavage of 22R-OH cholesterol. In addition, we have estimated the oxygen requirement of 20α-OH cholesterol conversion for comparison of our results with those of Shikita and Hall (1974).

7.2 Materials and methods

7.2a Preparation of the mitochondrial fraction

Bovine adrenals were obtained from the local slaughterhouse and transported on ice to the laboratory within 30-45 min. After removal of the medulla and the capsule, a 10% (w/v) homogenate of the cortex in 250 mM sucrose, 10 mM Hepes (pH 7.35) and 0.5 mM EGTA was prepared by the use of a Potter-Elvehjem homogenizer with a power-driven teflon pestle. Mitochondria were isolated from the supernantant of a 800 g 10 min spin by centrifugation at 9000 g for 10 min. The mitochondrial pellet was washed twice by resuspending in the isolation medium and resedimenting at 10.000 g for 10 min. The final pellet was suspended at a protein concentration of 30 mg/ml. The respiratory control rations with succinate were 2-3. The whole isolation procedure was performed at 4^{0}C in a cold room.

7.2b Estimation of the stoichiometry

The oxygen consumption corresponding with the side-chain cleavage of the steroid-substrates was measured with an oxygen electrode in a magnetically stirred vessel, at 37°C. The oxygen concentration of the incubation medium at 37°C saturated with air was calculated as described by Chappell (1964). Intact bovine adrenal cortex mitochondria (0.63 mg) were suspended in a medium containing: 100 mM sucrose, 40 mM KCl, 10 mM potassium-phosphate buffer, 20 mM Hepes-buffer pH 7.35, 1 mM EGTA, 1% bovine serum albumin, 4 mM NaN_3, 2 µg/ml cyanoketone, 0.5 µg/ml oligomycine and 10 mM succinate. One minute after the addition of the mitochondria 2 µg/ml antimycine was added, followed by 10 mM malate. After 6 min incubation 22R-OH cholesterol or 20α-OH cholesterol (50 nmol; yielding a concentration of 34.5 µM) was added. The final volume was 1.45 ml.

Conversion of steroid-substrates and the formation of intermediates and products were measured in a parallel incubation experiment, using the same medium, but with a final volume of 30 ml. Every 30 sec a sample of 1 ml was taken and added to 6 ml cold ethylacetate. Before the addition of the steroid-substrate a sample was taken to correct for the pregnenolone production from endogenous cholesterol. 35 µM epi-cholesterol was added to the sample as an internal standard. Steroids were extracted with 3 x 6 ml distilled ethylacetate. The solvent was evaporated at room temperature under nitrogen. The extract was silylated with N-trimethylsilylimidazole by heating at 100°C for one hour in the presence of dry pyridine hydrochloride. The analytical separations were carried out by gaschromatography as described in chapter 4. Peak areas and retention times were calculated by a Hewlett-Packard 21 MX computer. The stoichiometry of the reactions was calculated from the rate of oxygen consumption and the rate of pregnenolone production.

7.3 Results

7.3a Estimation of the stoichiometry of the side-chain cleavage reaction with respect to oxygen

22R-OH cholesterol

The conversion of 22R-OH cholesterol by intact mitochondria was complete within 6 min (fig. 7.1). Only a small amount of the intermediate 20α,22R-OH cholesterol could be detected. Its identity was confirmed by GLC-mass-spectrometry (see chapter 5).

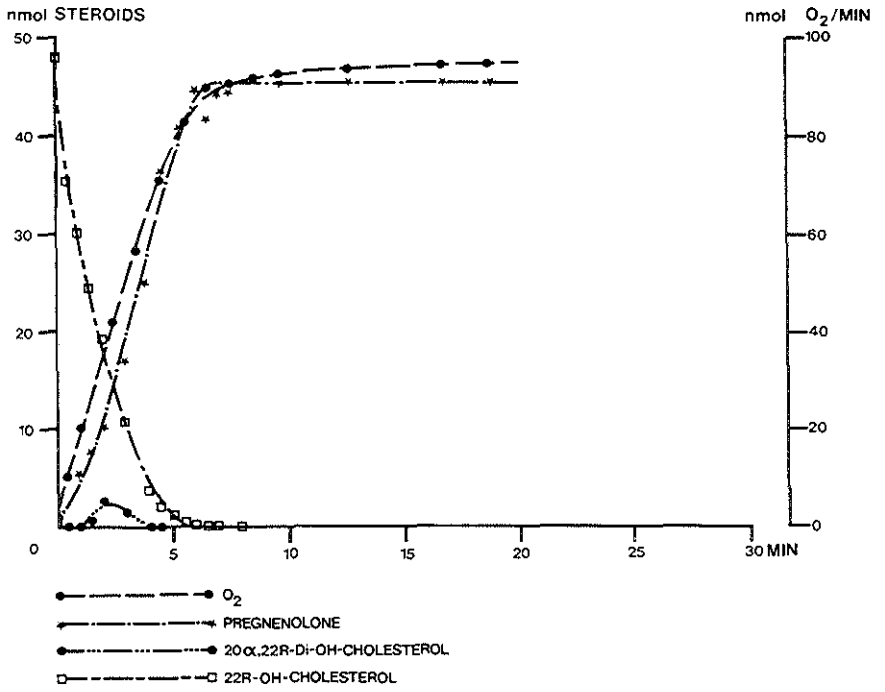

Fig. 7.1

Conversion of 50 nmol (34 µM) 22R-OH cholesterol via 20α,22R-di-OH cholesterol into pregnenolone by intact bovine adrenal cortex mitochondria. Oxygen consumption was measured with an O_2-electrode; final volume 1,45 ml. Steroids and sterols were estimated in samples taken from a parallel-incubation (see 7.2b) and quantitated by gas-liquid chromatography.

Parallel to this incubation in a large volume, an incubation was performed at the same time in an oxygraph with a volume of 1.45 ml. It is shown in fig. 7.1 that 48 nmol 22R-OH cholesterol is converted into 46 nmol pregnenolone with the consumption of 96 nmol oxygen. The stoichiometry was calculated by taking the ratio of 75 nmol oxygen consumed/5 min and 36.5 nmoles pregnenolone produced/ 5 min. This resulted in a stoichiometry: 2.06. After completion of the reactions, catalase was added to the oxygraph to detect any H_2O_2 formed. There was, however, no increase of the oxygen concentration in the incubation vessel after addition of catalase.

The oxidation of isocaproaldehyde (a product of the side-chain cleavage) to isocaproic acid might also consume oxygen. Therefore isocaproaldehyde was added to the incubation vessel. The addition of various concentrations of this aldehyde to the intact mitochondria in the oxygraph did not result in a measurable oxygen consumption. As further metabolism of pregnenolone and therefore the oxygen consumption required for these reactions were inhibited by cyanoketone (Goldman, 1967) it can be stated that 2 mol oxygen are required to convert 1 mol 22R-OH cholesterol into 1 mol pregnenolone and 1 mol isocaproaldehyde. Three additional experiments resulted in a stoichiometry of 1.90, 1.99 and 2.05.

20α-OH cholesterol

Intact bovine adrenal mitochondria from the same batch converted 20α-OH cholesterol into pregnenolone within 12 min (fig. 7.2). Similar control experiments as described for 22R-OH cholesterol were performed. The amount of pregnenolone formed by the cleavage reaction was only 60% of the expected amount and in addition to the intermediate 20α,22R-di-OH cholesterol another compound, tentatively identified as 20α,25-di-OH cholesterol, was formed during the reaction. The mass-spectrum of this compound is shown in fig. 7.3. The formation of by-products precluded an exact calculation of the stoichiometry with respect to oxygen. The estimated stoichiometry, obtained from the lineair parts of the curves shown in fig. 7.2 (t = 0 to t = 5 min), was 2.6.

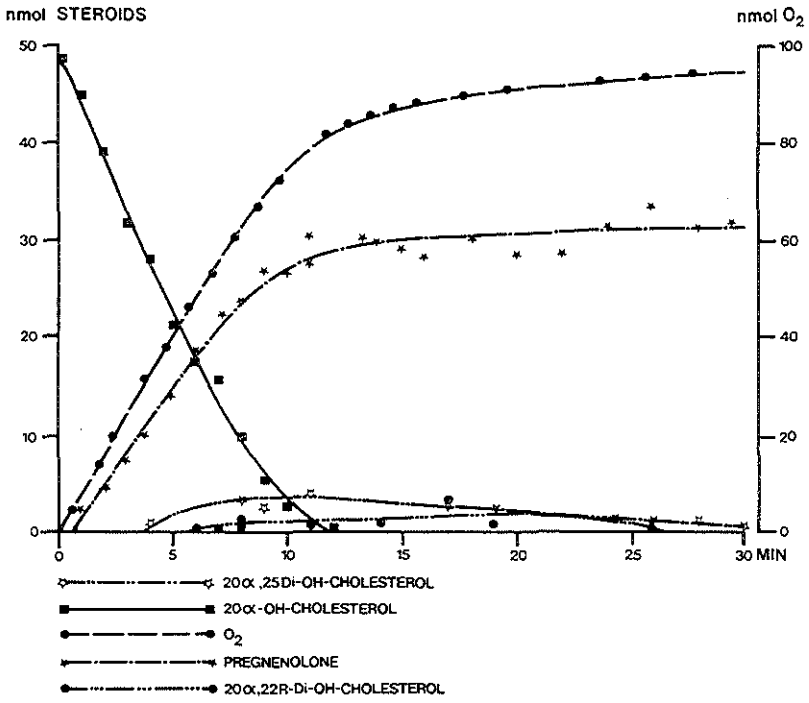

Fig. 7.2

Conversion of 50 nmol (43 μM) 20α-OH cholesterol into pregnenolone. Both 20α,22R-di-OH cholesterol and 20α,25-di-OH cholesterol (tentatively identified) were formed during the reaction. The reaction conditions were similar to those described for fig. 7.1.

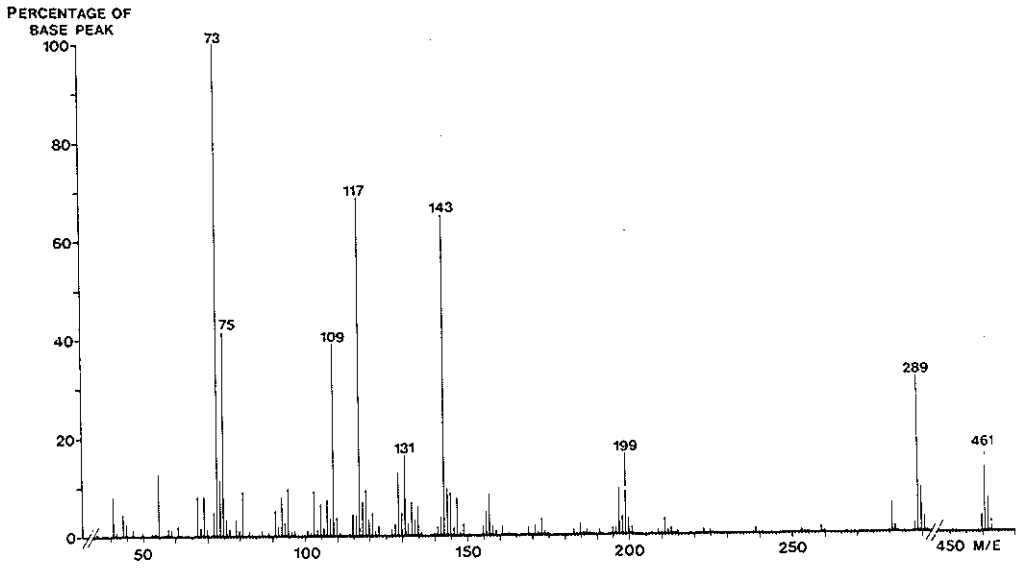

Fig. 7.3

Mass-spectrum of the compound detected in the experiment shown in fig. 7.2.
The compound was tentatively identified as 20α,25-di-OH cholesterol.

7.4 Discussion

7.4a Discussion of the mass-spectrum of 20α,25-di-OH cholesterol

The mass-spectrum of the compound detected showed some resemblance with the previously reported spectrum of 20α, 22R-di-OH cholesterol (chapter 5). Although no molecular ion was recorded, the fragments with m/e 461 and m/e 117 and also m/e 289 and m/e 199 indicate the presence of a side-chain with two hydroxylgroups. The location of one of these hydroxyl-groups is evident as a 20α-OH group from the fragments 461 and 117.

The location of the second group is found from a fragment at 131 amu, indicating a cleavage between C_{24} and C_{25}. The

same fragment was also observed in the mass spectrum of 25-OH cholesterol. From these data the compound was tentatively identified as 20α,25-di-OH cholesterol.

7.4b Discussion of the stoichiometry

A stoichiometric relationship with respect to oxygen has been calculated for cholesterol, 20α-OH cholesterol and 20α, 22R-di-OH cholesterol (Shikita and Hall, 1974). These substrates required 3,2 and 1 mol oxygen respectively for conversion into pregnenolone and isocaproaldehyde. According to the experiment shown in fig. 7.1, 1 mol 22R-OH cholesterol required 2 mol oxygen (O_2) for conversion into pregnenolone and isocaproaldehyde. The estimated stoichiometry fitted both the classical scheme of cholesterol side-chain cleavage (22R-OH cholesterol, 20α,22R-di-OH cholesterol, pregnenolone) and the epoxide-diol hypothesis (22R-OH cholesterol, Δ^{20-22} cholesterol, 20,22-epoxy-cholesterol, 20α,22R-di-OH cholesterol, pregnenolone).

In fig. 7.2 it can be seen that using intact bovine adrenal cortex mitochondria, an unequivalent amount of pregnenolone was formed from 20α-OH cholesterol. Apparently other enzymatic activities (i.e. a 25-hydroxylase) were present in the mitochondrial preparation (Bosisio et al. 1976). Using purified cytochrome $P\text{-}450_{scc}$ (Shikita and Hall, 1974) 20α-OH cholesterol was converted into an equivalent amount of pregnenolone. In this way the formation of 1 mol pregnenolone required 2.1 ± 0.3 mol oxygen. The advantage of using purified cytochrome $P\text{-}450_{scc}$ is evident; no other products than pregnenolone are formed and a more exact stoichiometry can be calculated. However, a disadvantage of using purified $P\text{-}450_{scc}$ is the high background consumption of oxygen and NADPH not coupled to the side-chain cleavage reaction. The high background oxygen consumption decreases the accuracy of the calculated stoichiometry.

Using intact bovine adrenal cortex mitochondria the formation of both pregnenolone and possibly 20α,25-di-OH

cholesterol from 20α-OH cholesterol precluded an exact calculation of the stoichiometry. However, the relative fast conversion of 22R-OH cholesterol, the low background oxygen consumption and the nearly equivalent amount of pregnenolone formed, allowed us to calculate a stoichiometry with respect to oxygen.

CHAPTER 8

THE EFFECTS OF AMINOGLUTETHIMIDE PHOSPHATE ON THE CHOLESTEROL SIDE-CHAIN CLEAVING SYSTEM IN BOVINE ADRENAL MITOCHONDRIA

8.1 Introduction

Aminoglutethimide phosphate (AGI) is a well-known inhibitor of steroidogenesis in adrenals, ovaries and testes (Gower, 1974). AGI can inhibit pregnenolone formation from cholesterol by adrenal preparations in vitro (Kahnt and Neher, 1966). After administration of AGI in vivo a syndrome resembling congenital lipoid adrenal hyperplasia has been reported (Goldman, 1970, Prader and Siebenman, 1957).

Initially it was thought that AGI inhibited the first enzymic step of the cholesterol side-chain cleaving system, the conversion of cholesterol into 20α-OH cholesterol (Kahnt and Neher, 1966). More recently, it has been suggested that AGI inhibits the side-chain cleavage of cholesterol at the site of cytochrome $P\text{-}450_{scc}$ reduction (McIntosh and Salhanick, 1969). This suggestion has been supported by Bell and Harding (1974) who reported that AGI inhibited cholesterol side-chain cleavage non-competitively.

Evidence for another mechanism of inhibition was found by Paul et al. (1976) who reported that AGI inhibited the (ACTH induced) formation of an enzyme-substrate complex between cholesterol and cytochrome $P\text{-}450_{scc}$.

In this chapter we will describe the effects of AGI on

a) pregnenolone formation from several possible intermediates of cholesterol side-chain cleavage,
b) the rate of cytochrome $P\text{-}450_{scc}$ reduction in the presence of several sterols and
c) the pH-dependent formation of an enzyme-substrate complex between cholesterol and cytochrome $P\text{-}450_{scc}$.

For details about Type I, reverse Type I and Type II spectra, the reader is referred to the appendix of chapter 8.

8.2 Materials and methods

8.2a Preparation of the mitochondrial fraction

Mitochondria were prepared as described in chapter 7. Damaged mitochondria were prepared by suspending the intact mitochondria for 5 minutes in distilled water. After centrifugation for 10 minutes at 10.000 x g the mitochondrial pellet was suspended in 250 mM sucrose and this suspension was stored in liquid nitrogen.

8.2b Estimation of pregnenolone production and oxygen consumption

The rate of the side-chain cleavage reactions and the corresponding oxygen consumption were measured as reported in chapter 7.

8.2c Estimation of V and K_m

The integrated form of the Michaëlis-Menten equation* was used. This equation can, using initial condition:

$$S = S_o \text{ at time } = 0,$$

be written as:

$$K_m \ln (S/S_o) + S - S_o + V.t = 0.$$

Using standard mathematical techniques for the calculation of the least square fit and the set of experimental data (s_i, t_i), explicit expressions for V and K_m are obtained in case S_o is known. In case S_o is not known a simple extension of the procedure is sufficient to obtain S_o (Cornish-Bowden, 1976).

*The Michaëlis-Menten equation is: $v = dS/dt = \frac{V.S}{K_m + S}$

8.2d Kinetics of NADPH supported cytochrome P-450 reduction

The rate of the NADPH-supported cytochrome P-450 reduction was assayed as described by Gigon et al. (1969). Damaged mitochondria, equivalent to 3 mg protein, were suspended in 2.5 ml of the medium described in the legend of figure 7.2. In order to remove traces of oxygen, the N_2 over the reaction medium was first bubbled through an alkaline dithionite solution. A stopcock-plunger assembly provided with 50 µl of NADPH-solution (final concentration 0.4 mM) was fitted to the cuvette and CO was led over the suspension for one additional minute. Then the cuvette was closed and placed into an Aminco DW-2 UV-VIS spectrophotometer, used in the dual wavelength mode. The cuvette was kept at 30°C and a baseline absorbance was recorded. After one minute the plunger was quickly depressed and ($E_{450\ nm} - E_{490\ nm}$) was recorded (chart speed of 2 inch/sec). The initial rate of reduction was estimated by determining the change in ($E_{450\ nm} - E_{490\ nm}$) during the first 1,5 sec after mixing. The rate was expressed as nmol cytochrome P-450 reduced/min/mg protein using 91 cm^{-1}. mM^{-1} as the value of the absorption coefficient (Omura and Sato, 1963). The total amount of cytochrome P-450 present was determined by adding a few crystals of sodium dithionite to the cuvette and allowing the reaction to proceed for about one minute to obtain at least 99% reduction.

Bovine adrenal cortex mitochondria contain at least three different cytochromes P-450 respectively for cholesterol side-chain cleavage, 11β-hydroxylation and 18-hydroxylation. These cytochromes contribute to the estimated rate of cytochrome P-450 reduction.

8.2e Estimation of the pH dependent change in spin-state

Damaged mitochondria were added to a medium containing 125 mM sucrose, 50 mM phosphate buffer and 50 mM Tris-HCl buffer (pH 6.40). The difference spectra were recorded on a Shimadzu double beam spectrophotometer, slit 2 nm. The

suspension was divided equally over two cuvettes and a base line of constant absorbance was obtained. Both cuvettes contained mitochondria equivalent to 0.45 mg protein/ml. A difference spectrum was obtained by adding aminoglutethimide phosphate to the sample cuvette (final concentration 400 µM). The extinction difference between 390 and 410 nm was calculated from the difference spectrum. Full scale of the recorder was 0.1 E. pH-induced changes in the difference spectrum were measured after 15 minutes.

8.3 Results

8.3a Inhibition of the side-chain cleavage reactions by aminoglutethimide phosphate

To study the effect of aminoglutethimide on the side-chain cleaving system, 22R-OH cholesterol, 20α-OH cholesterol and Δ20-22 cholesterol were incubated both in presence and absence of the inhibitor. First the concentration of aminoglutethimide phosphate needed for a complete inhibition of side-chain cleavage of cholesterol was estimated.

Damaged bovine adrenal cortex mitochondria, supported by an NADPH-generating system, were used (fig. 8.1). 50% inhibition was obtained at a concentration of 30 µM AGI, and no pregnenolone at all was produced at 120 µM. In order to completely prevent pregnenolone production in the following experiments 150 µM AGI was used to inhibit cholesterol side-chain cleavage.

22R-OH cholesterol

The conversion of 22R-OH cholesterol by intact mitochondria in the presence of 150 µM aminoglutethimide phosphate is shown in fig. 8.2. All 22R-OH cholesterol had been converted after 8 minutes. However, only 75% of the expected amount of pregnenolone was formed. The conversion in the absence of AGI is shown in fig. 7.1.

20α-OH cholesterol

In the presence of 150 µM AGI a marked decrease occurred in

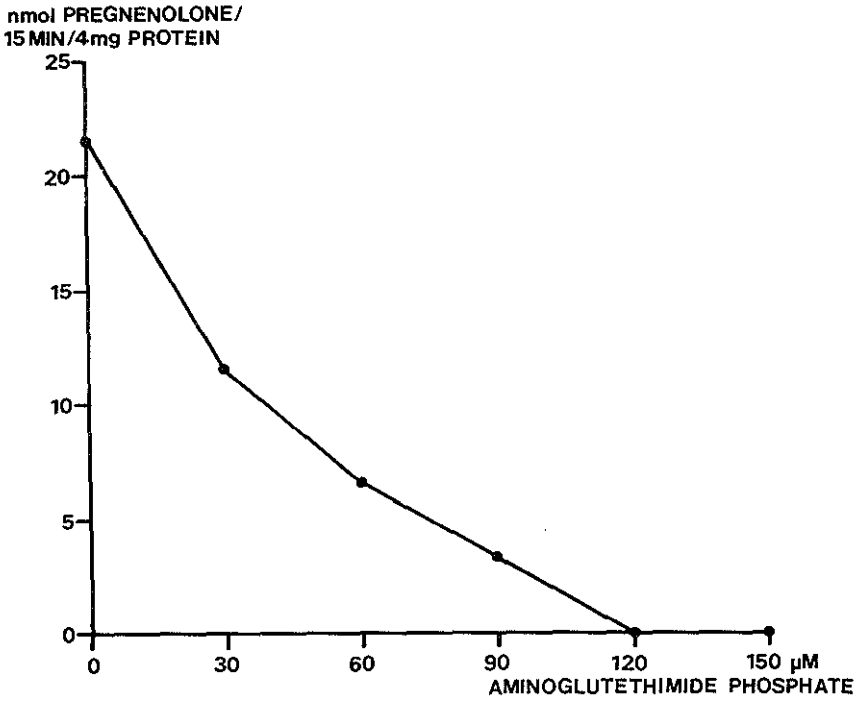

Fig. 8.1

Inhibition of cholesterol side-chain cleavage by aminoglutethimide phosphate. 4 mg damaged mitochondria, containing 45 nmol endogenous cholesterol/mg protein were incubated in 2 ml medium (final volume) at 37 °C. The medium contained: 150 mM KCl, 11 mM NaCl, 50 mM nicotinamide, 20 mM Hepes (pH 7.30), 6 mM $CaCl_2$, 4 mM sodium-azide, 1% bovine serum albumen, 4 µg cyanoketone/mg mitochondrial protein, 2 µg/ml antimycine. The reaction was started by the addition of 0.1 mM $NADP^+$, 1.2 U glucose-6-phosphate dehydrogenase and 0.6 mM glucose-6-phosphate. After 15 min. in a shaking incubator the reactions were terminated by shaking with 6 ml cold ethylacetate.

the rate of conversion of 20α-OH cholesterol into pregnenolone (fig. 8.3), when compared with the experiment in the absence of AGI (fig. 7.2). Only 40% of the expected amount of pregnenolone was formed. Besides pregnenolone the same compound as described in chapter 7, (tentatively identified as 20α,25-di-OH cholesterol) was formed. Complete conversion of all 20α-OH cholesterol took about 30 minutes. The total oxygen consumption

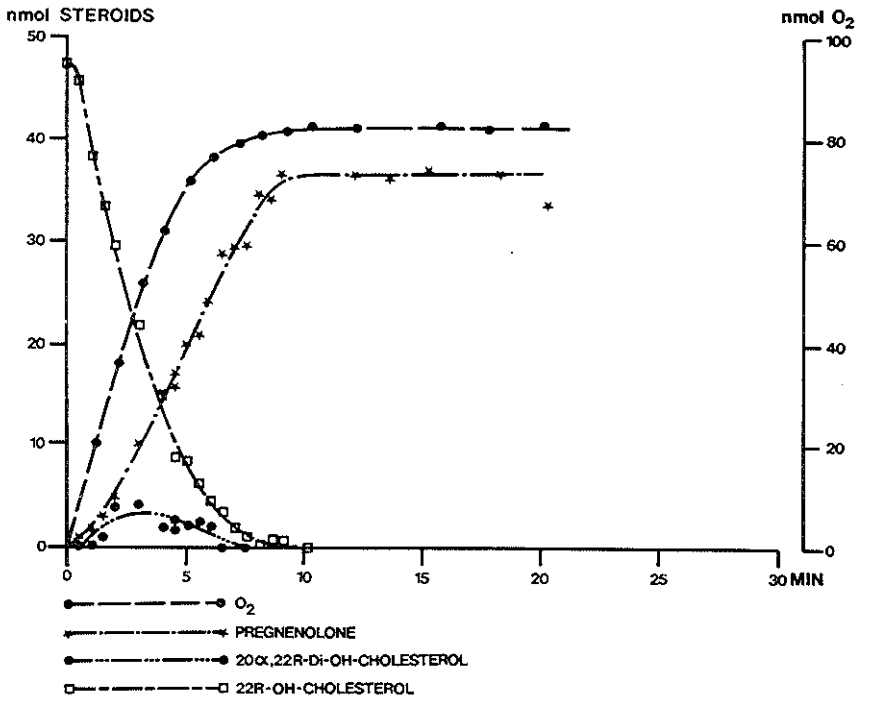

Fig. 8.2

Conversion of 50 nmol (34 µM) 22R-OH cholesterol into pregnenolone. The reaction conditions were similar as described in fig. 7.1 except for the presence of 150 µM aminoglutethimide phosphate.

after 30 minutes was 80% of what could be expected.

Δ^{20-22} cholesterol

In the presence as well as in the absence of 150 µM AGI a marked increase in the oxygen uptake occurred after addition of succinate (fig. 8.4). The oxygen consumption of the respiratory chain was inhibited by antimycine and sodium azide. Addition of malate stimulated the side-chain cleavage of endogenous cholesterol and the associated oxygen uptake. AGI inhibited both malate stimulated cholesterol side-chain cleavage and oxygen uptake. The addition of the possible intermediate Δ^{20-22} cholesterol to the intact mitochondria resulted in an immediate oxygen uptake, which was not inhibited

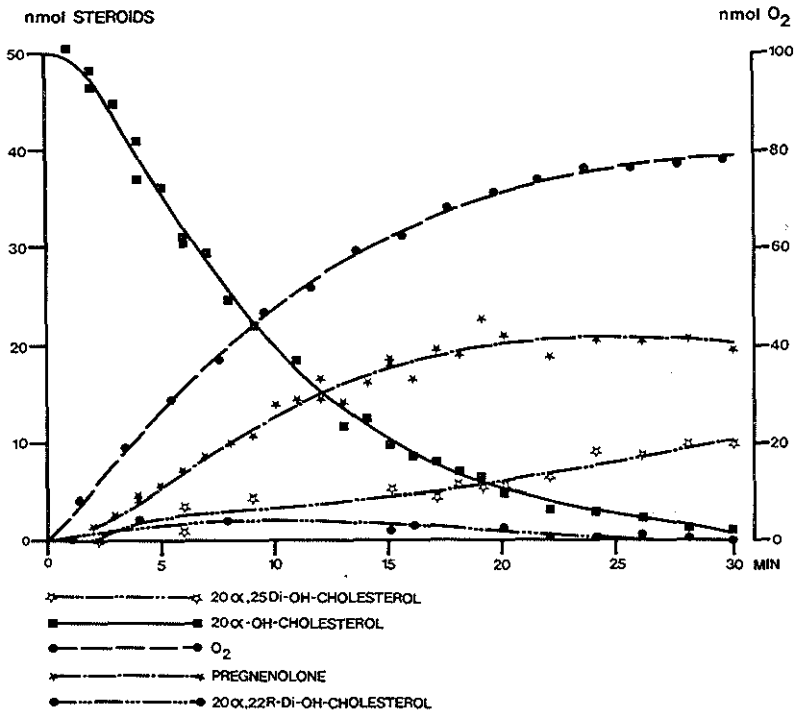

Fig. 8.3

Conversion of 20α-OH cholesterol into pregnenolone in the presence of 150 μM aminoglutethimide phosphate. 20α,22R-di-OH cholesterol and 20α,25-di-OH cholesterol (tentatively identified) were formed during the reaction. Reaction conditions are similar as described for fig. 7.1.

by 150 μM aminoglutethimide phosphate.

The small amount of available Δ^{20-22} cholesterol precluded the estimation of a stoichiometric relationship with respect to oxygen.

8.3b The effect of AGI on the kinetics of side-chain cleavage

The effect of AGI on the kinetics of the side-chain cleavage reactions was estimated by calculation of the V and the apparent K_m from the progress curves shown in fig. 7.1, 7.2, 8.2 and 8.3. A least square curve-fitting method was used for

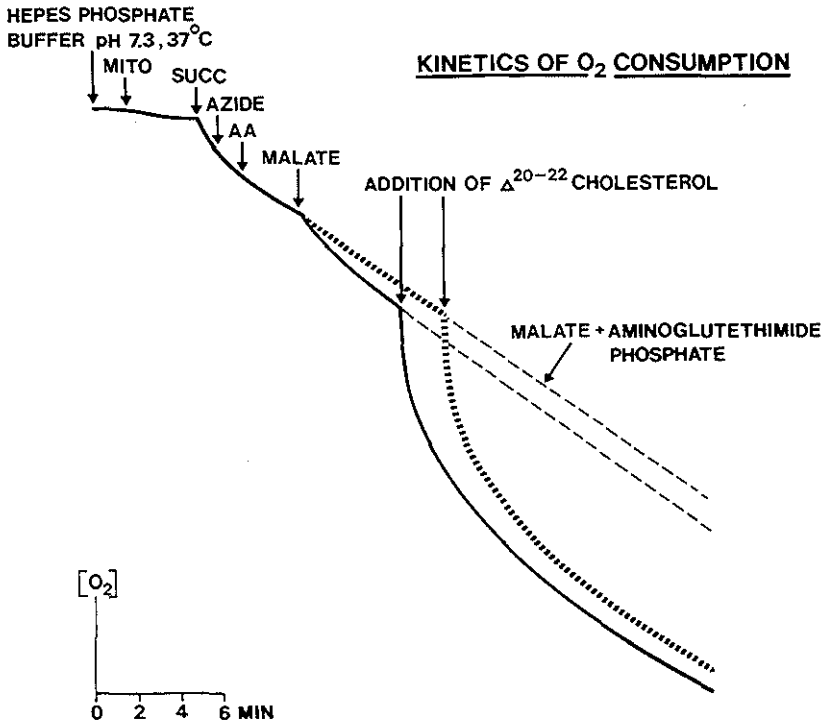

Fig. 8.4

The effect of the addition of Δ^{20-22} cholesterol on the rate of oxygen consumption by intact bovine adrenal cortex mitochondria, both in presence and absence of 150 µM aminoglutethimide phosphate. For experimental conditions, see fig. 4.1.

the integrated form of the Michaëlis-Menten equation as described (8.2c). The results of the calculations are shown in table 8.I.

Table 8.I

The effect of AGI on the kinetics of the conversion of 20α-OH cholesterol and 22R-OH cholesterol into pregnenolone.

Substrate	K_m (μM)	V (nmol/min/mg prot.)	K_i (μM)
22R-OH cholesterol	68	265	
id + 150 μM AGI	73	185	347
20α-OH cholesterol	24	94	
id + 150 μM AGI	166	92	25

V and the apparent K_m were calculated from the progress curves shown in fig. 7.2, 7.3, 8.2 and 8.3. The K_i values were calculated using the value i = 150 μM and the formulae:

$K_m' = K_m\ (1 + i/K_i)$ and $V' = V/(1 + i/K_i)$

150 μM AGI resulted in a slightly higher apparent K_m value for the conversion of 22R-OH cholesterol and markedly decreased the V. The same AGI concentration produced a marked increase in the apparent K_m of 20α-OH cholesterol. The V for this compound was not changed by the inhibitor.

8.3c The effect of AGI on the rate of cytochrome P-450 reduction

The effect of AGI on the kinetics of the NADPH-supported cytochrome P-450 reduction is shown in table 8.II. 150 μM inhibited the cholesterol side-chain cleavage reaction (fig. 7.3) and decreased the rate of reduction of cytochrome P-450 (table 8.II). Compounds producing a reverse type I absorbance spectrum with cytochrome P-450 (such as 22R-OH cholesterol, 20α-OH cholesterol and pregnenolone) decreased the rate of cytochrome P-450 reduction.

Compounds giving a type I absorbance spectrum with cytochrome P-450 (25-OH cholesterol), increased the rate of cytochrome P-450 reduction.

It is shown in table 8.II that adding 22R-OH cholesterol to mitochondria decreased the reduction rate to 73% of that

Table 8.II

The effect of several sterols and steroids on the rate of cytochrome P-450 reduction in the presence and absence of aminoglutethimide phosphate.

	$\frac{\text{substr.}}{\text{blank}} \times 100\%$	$\frac{\text{substr.+AGI}}{\text{blank}} \times 100\%$	inhibition (%) produced by 150 µM AGI
Blank	100	16	84
Cholesterol (type I)	98	15	85
20α-OH cholesterol (R I)	52	42	19
22R-OH cholesterol (R I)	73	73	0
Pregnenolone (R I)	52	12	76
deoxycorticosterone (type I)	97	34	65
25-OH cholesterol (type I)	126	37	71

Mitochondria (1.2 mg/ml) were incubated in the medium as described in fig. 8.1. Cytochrome P-450 reduction rates were obtained by adding 75 µM steroid (final concentration) to the medium. The initial rate found with the blank was 4.2 ± 0.1 nmol cyt. P-450 reduced/min/mg protein. Type I: this compound induces a type I difference spectrum. R I yields a reverse type I difference spectrum. See also the appendix 8.5.

rate observed in the blank. Addition of AGI did not further decrease the reduction rate, it remained constant. AGI (in the absence of 22R-OH cholesterol) reduced the reduction rate to 16%. Addition of 22R-OH cholesterol (a reverse type I substrate) under these conditions increased the reduction rate from 16% to 73%. Apparently AGI had no effect on the rate of reduction in the presence of 22R-OH cholesterol.

8.3d Effect of AGI on the formation of a complex between cholesterol and cytochrome P-450$_{scc}$

At low pH (pH = 6.40) cytochrome P-450$_{scc}$ and endogenous cholesterol form a complex (Mitani and Horie, 1969, Jefcoate and Boyd, 1971). From optical difference spectra and EPR-spectra it can be concluded that this complex is in a high spin state. At high pH (pH = 8.10) the heme iron atom of cytochrome P-450$_{scc}$ is converted into a low spin state.

We confirmed the findings of Jefcoate and Boyd (1971), who showed that lowering the pH from 8.10 to 6.40 produced a complete reversal of the above spectral changes. This experiment was repeated in the presence of 400 µM AGI. The inhibitor induced a type II-amine difference spectrum with a minimum at 390 nm and a maximum at 425 nm. The difference of the extinction at 390 nm and 410 nm, which is dependent on both the spin state and the concentration of cytochrome P-450$_{scc}$ (Jefcoate et al., 1973), was measured at different pH values. In fig. 8.5 it can be seen that in the presence of AGI the spin state could be changed from high to low spin and back to high spin under influence of the pH. Apparently, if cholesterol does form a complex with cytochrome P-450$_{scc}$ (high spin) under the influence of the pH changes, the presence of aminoglutethimide does not inhibit this interaction.

8.4 Discussion

8.4a Inhibition of the side-chain cleavage reaction

The concentration of aminoglutethimide phosphate needed in our study for a virtually complete inhibition of cholesterol side-chain cleavage was 120 µM. This is in reasonable agreement with the results of Cohen (1968) and Counsel et al. (1972).

150 µM AGI partially inhibit the conversion of 20α-OH cholesterol and 22R-OH cholesterol into pregnenolone and isocaproaldehyde. We have no quantitative data concerning the pregnenolone production from Δ^{20-22} cholesterol, but the oxygen

consumption, stimulated by the addition of this compound is not inhibited.

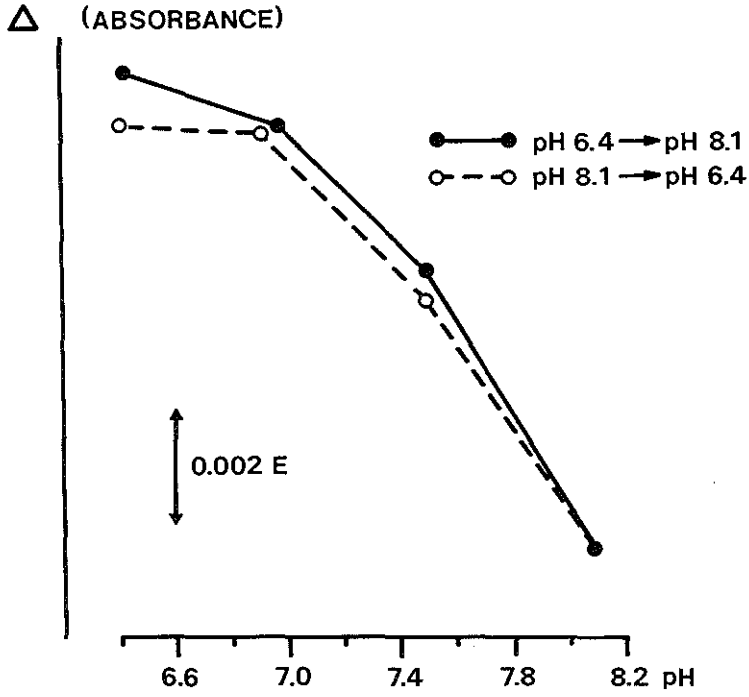

Fig. 8.5

pH-induced changes in the spin state of cytochrome P-450$_{scc}$. $E_{390nm}-E_{410nm}$ was calculated from the difference spectrum obtained in the presence of aminoglutethimide phosphate (0.4 mM).

8.4b Effects of AGI on the kinetics of the side-chain cleavage reactions

The side-chain cleavage of cholesterol is non-competitively inhibited by AGI (Bell and Harding, 1974). Our experiments show that the conversion of 22R-OH cholesterol into pregnenolone is also non-competitively inhibited by AGI. It has been suggested that AGI inhibits the cholesterol side-chain cleavage reaction by decreasing the rate of cytochrome P-450$_{scc}$

reduction (McIntosh and Salhanick, 1969). Our experiments (table 8.II) show that AGI inhibits the rate of cytochrome P-450 reduction in the presence of cholesterol by 85%. Using 22R-OH cholesterol as a substrate, AGI does not at all inhibit the rate of cytochrome P-450 reduction (table 8.II). This somewhat unexpected result may be explained by the fact that by inhibiting the conversion of 22R-OH cholesterol into pregnenolone, AGI can exert its influence not only on the substrate but also on every intermediate of that reaction. In fact the reduction of cytochrome $P\text{-}450_{scc}$ is only a part of the whole process. The rate of reduction is estimated in the absence of oxygen and in the presence of carbonmonoxide. Therefore no intermediates are formed by cytochrome $P\text{-}450_{scc}$. Considering the experimental conditions, AGI may inhibit the conversion of 22R-OH cholesterol into pregnenolone non-competitively (the V is decreased, the K_m remains constant) but it does not necessarily have to inhibit the rate of cytochrome $P\text{-}450_{scc}$ reduction. Apparently, either the reduction of cytochrome $P\text{-}450_{scc}$ is not a rate-limiting process with 22R-OH cholesterol as a substrate, or this sterol is not hydroxylated by cytochrome $P\text{-}450_{scc}$ at all.

The side-chain cleavage of 20α-OH cholesterol is competitively inhibited by AGI (table 8.I). Although the maximum velocity (V) of that reaction is not decreased, the rate of cytochrome P-450 reduction is inhibited by 15%. Apparently the latter inhibition is not a rate-limiting process in the side-chain cleavage of 20α-OH cholesterol. A possible explanation may be given when the fact is considered that adrenal mitochondria contain at least three different cytochromes P-450: one for cholesterol side-chain cleavage, one for 11β-hydroxylase and one for 18-hydroxylase. Each of these cytochromes P-450 contributes to the rate of cytochrome P-450 reduction measured by our method. A decrease in the rate of P-450 reduction does therefore not necessarily reflect a decrease in side-chain cleavage of 20α-OH cholesterol.

In contrast to the side-chain cleavage of cholesterol, the

side-chain cleavage of both 22R-OH cholesterol and 20α-OH cholesterol is not inhibited via a decrease in the rate of cytochrome P-450$_{scc}$ reduction.

8.4c Inhibition of the enzyme-substrate formation between cholesterol and cytochrome P-450$_{scc}$

Recently Brownie and Paul (1974) and Paul et al. (1976) suggested an alternative mechanism of inhibition by AGI, using intact adrenal cortex mitochondria from ACTH-treated rats. 2 μM AGI inhibited for about 50% the ACTH induced formation of an enzyme-substrate complex between cholesterol and cytochrome P-450$_{scc}$.

As the formation of a complex between cholesterol and P-450$_{scc}$ may also be induced by a change in pH, (Mitani and Horie, 1969; Jefcoate et al., 1971; Paul et al., 1976) we have investigated if AGI also inhibited the pH-induced formation of the cholesterol cytochrome P-450$_{scc}$ complex.

$$\underset{\text{low spin}}{\text{cholesterol + cyt. P-450}_{scc}} \underset{\text{high pH}}{\overset{\text{low pH}}{\rightleftharpoons}} \underset{\text{high spin}}{\text{(cholesterol cyt. P-450}_{scc}\text{ complex)}}$$

Fig. 8.5 shows that increasing the pH to 8.10 in the presence of 400 μM AGI caused a dissociation of the cholesterol P-450$_{scc}$ complex. It can be converted into its original state by decreasing the pH to 6.40.

From this experiment one may conclude that AGI does not inhibit the formation of a complex between cholesterol and cytochrome P-450$_{scc}$.

The results obtained in the experiments of Paul et al. (1976) differ from ours, but this may be explained by the different experimental conditions. Paul et al. (1976) used intact mitochondria from ACTH pretreated rats as the source of the cholesterol cytochrome P-450$_{scc}$ complex. In our experiments damaged bovine adrenal cortex mitochondria were used, while the enzyme-substrate complex was produced by a change in pH.

8.4d The possible mechanisms of inhibition by AGI

The available data are summarized in table 8.III.

Table 8.III

Author	AGI concentration	System	Inhibition	Parameter
Boyd et al. (1975)	1 μM	intact rat adrenal mitochondria	75%	O_2 uptake
Paul et al. (1976)	2 μM	intact rat adrenal mitochondria from ACTH pretreated animals	50%	heat generated type I absorbance change
This thesis	30 μM	damaged bovine adrenal mitochondria	50%	pregnenolone production
Bell and Harding (1974)	25 μM	damaged rat adrenal mitochondria	75%	pregnenolone production
Užgiris et al. (1977)	26 μM	damaged corpus luteum mitochondria	50%	isocaproate production
Falke et al. (1976) Falke (1977)	40 μM	isolated rat adrenal cells, stimulated with ACTH	50%	corticosterone production

From the results in table 8.III we tend to conclude that formation of a complex between cholesterol and cytochrome $P\text{-}450_{scc}$ can be inhibited at low AGI concentrations if the structure of the mitochondria is intact. In damaged mitochondria there is a greater availability of the substrate cholesterol. This may explain why AGI is not able to inhibit the formation of the cholesterol cytochrome $P\text{-}450_{scc}$ complex. At higher AGI concentrations (25-30 μM) side-chain cleavage of cholesterol is inhibited via a decrease in the rate of cytochrome $P\text{-}450_{scc}$ reduction. The high concentration of AGI (40 μM) required for the inhibition of corticosterone production by intact rat adrenal cells may be explained by a poor transport of the inhibitor through the adrenal cell membrane.

8.5 Appendix

25-OH cholesterol, 20α,22R-di-OH cholesterol and cholesterol produce so-called type I optical difference spectra (λ_{max} 385 nm, λ_{min} 420 nm) upon interaction with adrenal cytochrome P-450 (Burstein et al., 1972). The type I optical difference spectrum is a reflection of the formation of an enzyme-substrate complex between cytochrome P-450 and the added steroid (Cheng and Harding, 1973). EPR spectra show that the type I optical difference spectrum corresponds with the transition of a low - to a high spin-state of the heme-Fe in cytochrome P-450 (Whysner et al., 1970). Compounds inducing type I spectra increase the rate of cytochrome P-450 reduction (Gigon et al., 1969).

20α-OH cholesterol, 22R-OH cholesterol and pregnenolone produce reverse type I optical difference spectra (λ_{max} 420 nm, λ_{min} 385 nm) (Burstein et al., 1972) which are in fact mirror images of the type I spectral changes. They are supposed to reflect the displacement of endogenously bound type I compounds (Diehl et al., 1970). Reverse type I, and type II optical difference spectra, are associated with a dissociation of the enzyme-substrate complex and a transition of a high to a low spin state of the heme-Fe. Reverse type I and type II compounds decrease the rate of reduction of cytochrome $P\text{-}450_{scc}$ (Gigon et al., 1969). The binding of aminoglutethimide to cytochrome $P\text{-}450_{scc}$ results in a type II difference spectrum (McIntosh and Salhanick, 1969). EPR spectra show that this inhibitor changes the high spin complex for 100% into a low spin amine complex (Jefcoate et al. 1973). The spin state of cytochrome $P\text{-}450_{scc}$ is also sensitive to pH changes (Mitani and Horie, 1969). Increasing the pH from 6.0 to 8.0 brings about a reversible change from a high to a low spin state of the heme-Fe (Jefcoate et al. 1973).

CHAPTER 9.

DISCUSSION

9.1 Current hypotheses about cholesterol side-chain cleavage

At present (1977) three main hypotheses exist for the conversion of cholesterol into pregnenolone:

1-a: cholesterol → 22R-OH cholesterol → 20α,22R-di-OH cholesterol → pregnenolone + isocaproaldehyde.

1-b: cholesterol → 22R-OOH cholesterol → 22R-OH cholesterol → 20α-OOH,22R-OH cholesterol → 20α,22R-di-OH cholesterol → pregnenolone.

1-c: cholesterol → 20α-OH cholesterol → 20α,22R-di-OH cholesterol → pregnenolone.

2 : cholesterol → pregnenolone, via radical or ionic intermediates.

3-a: cholesterol → 22R-OH cholesterol → Δ^{20-22} cholesterol → 20,22-epoxy cholesterol → 20α,22R-di-OH cholesterol → pregnenolone.

3-b: cholesterol → Δ^{20-22} cholesterol → etc.

The mechanism with consecutive hydroxylations (hypothesis 1-a) is defended by Burstein et al. (1974, 1975, 1976a,b) and van Lier et al. (1977; hypothesis 1-b). According to these authors 22R-OH cholesterol, and not 20α-OH cholesterol is the main intermediate sterol.

Morisaki et al. (1976) detected 20ξ-OH cholesterol during the conversion of cholesterol to pregnenolone and they claim that a significant percentage of cholesterol is transformed into pregnenolone via 20α-OH cholesterol (hypothesis 1-c).

The involvement of radical or ionic intermediates (hypothesis 2) has been proposed by Luttrell et al. (1972), Hochberg et al. (1974, 1976a,b).

A side-chain cleaving mechanism involving Δ^{20-22} cholesterol and 20,22-epoxy cholesterol (hypotheses 3-a, 3-b) has been suggested in chapter 4, 5 and 6 (Kraaipoel et al. 1974a,

1974b, 1975a, b and c). The involvement of 22R-OH cholesterol (hypothesis 3-b) has been discussed in chapter 6.

9.2 The classical scheme and the epoxy-diol hypothesis.

The main difference between the hypotheses supported by Burstein et al. (1974, 1975, 1976a, b), van Lier et al. (1977), Morisaki et al. (1976) and us, can be found in the conversion of 22R-OH cholesterol into 20α,22R-di-hydroxycholesterol. According to Burstein, 22R-OH cholesterol is hydroxylated to 20α,22R-di-OH cholesterol via a mixed-function oxygenase. This reaction is catalyzed by cytochrome P-450_{scc} and requires NADPH and O_2. In contrast to this proposal, we suggest (chapter 5) that 22R-OH cholesterol is first converted into Δ^{20-22} cholesterol by a dehydratase. Δ^{20-22} cholesterol is then converted into 20,22-epoxy cholesterol by an epoxydase, probably a mixed-function oxygenase requiring NADPH and O_2. The epoxy-cholesterol is then converted into 20α,22R-di-OH cholesterol by an epoxyhydrase. The enzyme incorporates a molecule of water to give a dihydroxy-compound. The results of our experiments (chapter 6) suggest that two dihydroxyl-groups of 20α,22R-di-OH cholesterol are formed with oxygen atoms from molecular oxygen (O_2) and water (H_2O). The oxygen atom attached to C_{20} from the glycol originates from molecular oxygen, the oxygen atom attached to C_{22} originates from water.

The hypothesis advocated by Burstein is supported by a number of experiments. Burstein et al. (1974) have reported that incubation of cholesterol with an acetone powder of bovine adrenal cortex mitochondria, in the presence of $^{18}O_2$, resulted in the formation of 20α,22R-di-OH cholesterol. Both hydroxyl-groups contained ^{18}O. Burstein therefore concluded that both hydroxyl-groups were formed with two separate molecules of oxygen ($^{18}O_2$) and that two hydroxylation reactions, requiring molecular oxygen were involved in the synthesis of 20α,22R-di-OH cholesterol.

This experiment can however also be explained by the epoxide-diol hypothesis. NADPH and O_2, both required for the side-chain

cleavage reaction, are converted into $NADP^+$ and H_2O during the reaction. Evidently with $^{18}O_2$, 18-oxygen labeled water is formed. In an acetone powder and in purified cytochrome $P\text{-}450_{scc}$ (supported by flavoprotein and adrenodoxin) part of the NADPH oxidation is not coupled to the side-chain cleavage reaction (Shikita and Hall, 1974). In the presence of $^{18}O_2$, this NADPH-oxidase produces $H_2{}^{18}O$. Therefore synthesis of the glycol via an epoxide-diol pathway, in the presence of $^{18}O_2$ and $H_2{}^{18}O$, will result in the formation of 20α,22R-di-OH cholesterol with ^{18}O on both C_{20} and C_{22}.

The same experiments (Burstein et al. 1974) give further support for our explanation. The cholesterol side-chain cleavage reaction was supported by NADPH, generated by glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $NADP^+$. Two experiments were performed, with different concentrations glucose-6-phosphate dehydrogenase. With the lower enzyme concentration an ^{18}O abundance in both hydroxyl-groups of 60% was found. A twofold increase of the concentration glucose-6-phosphate dehydrogenase resulted in a 90% ^{18}O abundance in the same hydroxyl-groups.
It is likely that by increasing the glucose-6-phosphate dehydrogenase concentration, a higher concentration of NADPH is obtained. Therefore the rate of $H_2{}^{18}O$ formation from NADPH-oxidases not coupled to the side-chain cleavage reaction will be higher. The increased availability of $H_2{}^{18}O$, for the epoxyhydrase will result in an increased incorporation into the glycol. In this way the increased ^{18}O-content (90%) may be explained.

More recently Burstein and Gut (1976b) reported an experiment that can be explained in a similar way. Cholesterol and an acetone powder prepared from adrenal cortex mitochondria were incubated in a $^{18}O_2$ enriched atmosphere. When the gas phase was abruptly changed to air ($^{16}O_2$) and the incubation continued for a relatively short period, a drop occurred in the ^{18}O-content of 22R-OH cholesterol and 20α,22R-di-OH cholesterol. The ^{18}O-content of 20α,22R-di-OH cholesterol was lower at position C_{20} than at C_{22}. Burstein and Gut explained

this finding by assuming that cholesterol is first hydroxylated at C_{22} and than at C_{20}. Upon changing the gasphase abruptly from $^{18}O_2$ to $^{16}O_2$, the 22R-^{18}OH cholesterol present in the incubation mixture will be converted into 20α-^{16}OH,22R-^{18}OH cholesterol.
An alternative explanation can be given based upon the epoxide-diol hypothesis. In the presence of $^{18}O_2$, the enzyme preparation catalyzes the reduction of $^{18}O_2$ by NADPH to $NADP^+$ and $H_2{}^{18}O$. Upon changing the $^{18}O_2$ atmosphere for air, $H_2{}^{16}O$ will be formed but some $H_2{}^{18}O$ will still be present within the enzyme preparation. 20,22-epoxy cholesterol formed, will contain ^{16}O from molecular oxygen ($^{16}O_2$). The epoxyhydrase converts epoxy cholesterol and $H_2{}^{18}O$ into 20α,22R-di-OH cholesterol with ^{18}O at C_{22}. The oxygen atom originating from molecular oxygen is then attached to C_{20}. According to our explanation an abrupt change in the gasphase will also result in a lower ^{18}O-content at C_{20} than at C_{22}.

In the literature a few experiments are reported that can not easily be explained by the epoxide-diol hypothesis, but there are also experiments that can not be explained by a mechanism consisting of consecutive hydroxylation. Burstein et al. (1976a) reported the synthesis of 22R-^{18}OH cholesterol. Incubation of this ^{18}O labeled substrate with an acetone powder of bovine adrenal cortex mitochondria resulted in the formation of 20α,22R-di-OH cholesterol with the same ^{18}O abundance in the hydroxyl-group at C_{22} as the starting material. The hydroxyl-group at C_{20} did not contain ^{18}O and as expected, no ^{18}O was found in the pregnenolone formed. This retention of the ^{18}O label at C_{22} does not fit within the epoxide-diol hypothesis.

In the same paper the incubation of the two stereo-isomers of Δ^{20-22} cholesterol (E and Z) is reported. Both isomers did not yield significant amounts of 20α,22R-di-OH cholesterol or pregnenolone. A similar result has been reported by Morisaki et al. (1976a) who incubated one of the stereo-isomers of Δ^{20-22} cholesterol, (E)-20(22)-dehydrocholesterol. These results are in contrast to the conversion of Δ^{20-22} cholesterol as reported in chapter 4.

The following comments can be made, however:

1. The synthesis of E and Z Δ^{20-22} cholesterol by Burstein et al. (1976a) was performed under rather drastic conditions and the purity of the material used was not established.
2. Models of both stereo-isomers give the impression that only (Z)-20(22)-dehydrocholesterol may interact with the active site of the enzyme system.

Both Burstein et al. (1976a) and Morisaki et al. (1976a) synthesized the four stereo-isomers of 20,22-epoxy cholesterol. Only (20R,22S)-20,22-epoxy cholesterol formed pregnenolone in approximately 20% of the yield obtained with cholesterol (Burstein et al. 1974a). The same substrate produced the highest inhibition (30%) of side-chain cleavage of ^{14}C-cholesterol (Morisaki et al. 1976a). In contrast to the results of Burstein the Japanese group did however, not find a significant conversion of (20R,22S)-20,22-epoxy cholesterol to pregnenolone. According to Burstein et al. (1976b), the slight transformation of the epoxide to pregnenolone suggests that an epoxide hydrase may exist in their acetone-dried powder preparation although this was not proven.

According to Oesch (1972) liver epoxyhydrase has a very low affinity for its substrates. An inhibitor of several epoxyhydrases, 1,1,1-trichloropropene-2,3-oxide (TCPO), has been described (Oesch et al. 1971); 0.4 mM TCPO almost completely inhibited epoxyhydrase from human liver microsomal fractions (Oesch 1974). A slow penetration of this inhibitor was described by Croteau and Kopattukudy (1974), who preincubated one hour with 10 mM TCPO and obtained a 50% inhibition of a plant epoxyhydrase. In addition Oesch (1971) reported that the formation of a dihydrodiol from exogenous naphtalene oxide was effectively inhibited by TCPO, while the conversion of naphtalene oxide endogenously formed from naphtalene was not inhibited. In our experience the inhibitor requires one hour preincubation at room temperature with damaged bovine adrenal cortex mitochondria to obtain a marked inhibition of the epoxyhydrase activity without affecting the

cytochrome P-450$_{scc}$ catalyzed reactions.

Considering the low affinity of epoxyhydrase for their substrates and the slow penetration of the inhibitor TCPO, one may not expect a very efficient conversion of exogenous 20,22-epoxy cholesterol to 20α,22R-di-OH cholesterol. Moreover the availability of the added 20,22-epoxy cholesterol to the active site of the enzyme epoxyhydrase, may be lower than the availability of 20,22-epoxy cholesterol endogenously formed from cholesterol. These considerations may well explain the low yield of pregnenolone obtained from the incubation with (20R,22S)-20,22-epoxy cholesterol.

9.3 Cholesterol side-chain cleavage via ionic or radical intermediates.

A mechanism of cholesterol side-chain cleavage, involving radical or ionic intermediates, has been proposed by Lieberman et al. (1969), and Luttrell et al. (1972). By using an analog of 20α-OH cholesterol, with a tertiary C_{22}, a possible fission of the C_{20}-C_{22} bond could not occur via a stable 20-22 glycol. The authors found that incubation of (20R)-20-t-butyl-5-pregnene-3β,20-diol, with an acetone powder of bovine adrenal cortex mitochondria resulted in a pregnenolone production of 0.5 to 1.2%.

This artificial substrate is, however, difficult to synthesize in a 100% pure form. Re-arrangement of the t-butyllithium used in the synthesis might occur under formation of isobutyllithium and a compound with a secondary C_{22} will then be formed. Separation of these two products will be very difficult, if not impossible. The low conversion of the analog of 20α-OH cholesterol into pregnenolone may therefore be explained by side-chain cleavage of the impurity. Since the latter product has one free H-atom at C_{22}, the results fit both the hypotheses of the classical and the epoxide-diol pathways.

To circumvent these problems, a 20-p-tolyl analog of 20α-OH cholesterol was synthesized (Hochberg et al. 1974). This substrate was converted into pregnenolone and progesterone

with a good efficiency (23%). The 20-p-tolyl analog of cholesterol was converted into its 20α-hydroxy derivative in a yield of 21%, suggesting the existence of a 20α-hydroxylase system in the acetone powder of bovine adrenal cortex mitochondria. However, the cleavage of these two substrates can also easily be explained by the formation of a double bond between C_{20} and C_{22}, followed by the formation of a C_{20-22}-epoxide, which is further converted into a C_{20-22} glycol, analogous to 20α,22R-di-OH cholesterol.

The styrene 20-(p-tolyl)-5,20-pregnadien-3β-ol can not rearrange to a compound with a double bond between C_{20} and C_{22}. It can therefore not be cleaved via the epoxide-diol pathway. Interestingly, Hochberg et al. (1974) report that this styrene is not a substrate for the cholesterol side-chain cleaving system.

To preclude a possible rearrangement to a C_{20}-C_{22} double bond, Hochberg et al. (1976a) next synthesized the 20-phenyl-analog of 20α-OH cholesterol, (20R)-20-phenyl-5-pregnene-3β, 20-diol. This compound was cleaved by an acetone powder of bovine adrenal cortex mitochondria to form pregnenolone and phenol in a yield of 5%. Furthermore a C_{19}-fragment (17-methyl-18-norandrosta-5,13(17)-dien-3β-ol) and a C_8-ketone, 6-methyl-2-heptanone were found in a yield of 0.5%, suggesting a cleavage of the C_{17}-C_{20} bond. (Hochberg et al. 1976b).
The 20-phenyl analog of 20α-OH cholesterol can not easily rearrange to an isomer with a C_{20-22} double bond, but the involvement of transient radical or ionic intermediates is not a prerequisite if oxygen insertion between C_{20} and C_{22} (Constantopoulos et al. 1962) or between C_{17} and C_{20} (Träger p. 54) is taken into consideration.

Cleavage of the C_{17}-C_{20} bond of the 20-phenyl analog might be initiated by splitting off H_2O which results in the formation of a C_{17}-C_{20} double bond. After epoxidation the C_{17-20}-epoxide might rearrange to a four-membered ring 18,20 oxide with migration of the C_{18}-methyl group to C_{17}, as pointed out by Wendler, (1964).

It is interesting to note that on basis of the results of

the cleavage of the C_{17-20} bond of the phenyl analog, Hochberg et al. (1976b) incubated 20α-OH cholesterol with an acetone powder prepared from mitochondria obtained from the cortex of bovine adrenals. No C_8-ketone (6-methyl-2-heptanone) was formed. Neither did the incubation of 17α,20α-dihydroxy-cholesterol with a guinea pig adrenal preparation result in cleavage of the C_{17-20} bond (Burstein et al. 1968).

The existence of radical or ionic intermediates in the mechanism of cholesterol side-chain cleavage is compatible with the experiments of Hochberg et al. (1976a, b). Other explanations are possible, however, and it remains to be proven that the hydroxylated sterols 22R-OH cholesterol and 20α,22R-di-OH cholesterol, isolated during cholesterol side-chain cleavage, are merely by-products, resulting from as yet undefined reversible transformations of the true intermediates.

9.4 The congenital lipoid adrenal hyperplasia (CLAH) and the epoxide-diol hypothesis.

In CLAH (Prader and Siebenmann, 1957) large quantities of cholesterol and cholesterol-esters accumulate in the adrenals and almost no steroids are formed. Apparently cholesterol is not converted into pregnenolone. CLAH can be expected to have two main causes.

I. A defect in the transport of adrenal cholesterol to the active site of cytochrome P-450$_{scc}$.

At present not enough is known about the exact transport mechanisms to enumerate the possible defects.

II. Defects in the cholesterol side-chain cleavage proper.

The enzyme system, capable of pregnenolone formation from cholesterol, has, according to the epoxide-diol hypothesis, several activities.
In principle, each of these could be affected.

A_1 cholesterol → Δ^{20-22} cholesterol.
The conversion occurs via an oxidative desaturase, requiring NADPH and oxygen.
NADPH → flavoprotein (a) → non-heme iron protein (b) → desaturase/cytochrome P-450 (c).

A_2 cholesterol → 22R-OH cholesterol → Δ^{20-22} cholesterol.
The conversion occurs via 22R-hydroxylation followed by a dehydratase.
NADPH → Fp (a) → NHI (b) → cyt. P-450 (d) → dehydratase (e).

B. Δ^{20-22} cholesterol → 20,22-epoxycholesterol.
The reaction is catalyzed by an epoxydase, NADPH and O_2 are required.
NADPH → Fp (a) → NHI (b) → cyt. P-450 (f).

C. 20,22-epoxycholesterol → 20α,22R-di-OH cholesterol.
The reaction is catalyzed by an epoxyhydrase (g) and requires H_2O.

D. 20α,22R-di-OH cholesterol → pregnenolone + isocaproaldehyde.
The reaction is catalyzed by a lyase. NADPH and O_2 are

required.

NADPH → Fp (a) → NHI (b) → cyt. P-450 (h).

Damaged mitochondria or acetone powders, prepared from adrenals with defect(s) I, can be expected to have the capability of pregnenolone formation from cholesterol.

The sites of possible type II defects are A, B, C and D. Sequential testing of cholesterol and the intermediates in the side-chain cleavage reaction with a mitochondrial preparation should allow to distinguish between these defects in a case of CLAH.

Further differentiation (a, b, c, d, e, f, g and h) is theoretically possible, but technical difficulties are at present the limiting factors. The components (a and b) of the electron transport chains are, for lack of evidence, supposed to be non-specific. Furthermore it is not known whether the cytochromes c, d, f and h are different chemical entities. The possibility exists that only one kind of cytochrome P-450 catalyzes the whole cholesterol side-chain cleavage. In this case c, d, f and h are identical.

SUMMARY

The cleavage of the side-chain of cholesterol is one of the most important enzymatic reactions in adrenal steroidogenesis. The products are pregnenolone and isocaproaldehyde. The pregnenolone formed is the starting point in the biosynthesis of the adrenal steroids.

This thesis describes the results of an investigation of the enzyme system catalyzing this cleavage reaction. The results obtained can be used in studies concerning an almost always lethal disease, congenital lipoid adrenal hyperplasia. In this condition the cholesterol side-chain cleaving system is effected, resulting in a very low activity.

The aims of the investigation are given in chapter 1. The methods used are briefly described. After a discussion of the Cahn-Ingold-Prelog nomenclature it is explained why, in this thesis, the old nomenclature is used.

In chapter 2 an historical survey is given of the work leading to the generally accepted hypothesis of the mechanism of pregnenolone formation from cholesterol. A statement of the problem is given, based on the knowledge available in 1971.

The reasons for the use of gaschromatographic estimation of the various sterols and steroids are given in chapter 3. The combination of gaschromatography and mass spectrometry provided us with the possibility to study the mechanism of the enzyme system by means of ^{18}O incorporation into intermediate and end-products.

In chapter 4 experiments are described concerning the enzymatic conversion of 20α-OH cholesterol into pregnenolone. The reactions were performed in the presence of the non-radioactive $H_2{}^{18}O$. The pregnenolone formed was found to contain ^{18}O. The incorporation of a molecule of water, occurring during the biosynthesis of pregnenolone, cannot be explained by the existing hypotheses. To explain the results of the experiments, described in chapter 4, a new hypothesis is presented (see figure A). During the enzymatic conversion of 22R-OH cholesterol into pregnenolone and isocaproaldehyde (chapter 5)

an intermediate is formed, identified as 20α,22R-di-OH cholesterol. During the reaction the sum:
(substrate) + (intermediate) + (end-product) appeared to be smaller than the initial substrate concentration. Apparently (an) intermediate(s), which could not be measured, was/were present. In the presence of > 99% carbon monoxide this/these unknown intermediate(s) appeared to be converted into the intermediate 20α,22R-di-OH cholesterol. The sum of the amounts of substrate, intermediate and end-product was then approximately equal to the initial amount of substrate. It is concluded that, besides the CO-inhibited cytochrome $P\text{-}450_{scc}$, an enzyme is present, which is capable of converting the unknown intermediate(s) into 20α,22R-di-OH cholesterol. This experiment supports our hypothesis, that an epoxyhydrase is involved in the biosynthesis of pregnenolone. An epoxyhydrase could convert 20ξ,22ξ-epoxy cholesterol in the presence of water. None of the epoxyhydrases described in the literature, are inhibited by CO, where this has been studied.

The conversion of cholesterol into pregnenolone and isocaproaldehyde in the presence of $H_2{}^{18}O$ is described in chapter 6. The mass spectrometric analyses of the intermediate 20α,22R-di-OH cholesterol proved that ^{18}O was attached to C-22 only. A similar result was obtained with 22R-OH cholesterol (chapter 4).

The estimation of the number of molecules of oxygen, required for the reaction:
1 22R-OH cholesterol → 1 pregnenolone + 1 isocaproaldehyde
is described in chapter 7. The reaction required 2 molecules of oxygen. This result is in agreement with the accepted hypothesis of cholesterol side-chain cleavage, and with the epoxide-diol hypothesis, proposed in this thesis.

The effects of the inhibitor, aminoglutethimide phosphate, (abbreviated AGI, "commercial name" Elipten) on cholesterol side-chain cleavage are described in chapter 8. AGI inhibits the conversion of cholesterol into pregnenolone. It was examined whether the side-chain cleavage of the substrates

20α-OH cholesterol and 22R-OH cholesterol were inhibited via the same mechanism as found for cholesterol, i.e. by a decrease in the reduction rate of cytochrome $P\text{-}450_{scc}$. This is most probably not the case. The validity of another recent proposal was examined. It has been postulated that AGI inhibited the formation of an enzyme substrate complex at a site between cholesterol and cytochrome $P\text{-}450_{scc}$. Our findings combined with data from the literature allowed us to conclude that the formation of a complex can only be inhibited if the adrenal mitochondria, containing the cytochrome $P\text{-}450_{scc}$, are intact. In damaged mitochondria, cholesterol seems to be readily available for cytochrome $P\text{-}450_{scc}$, preventing inhibition by AGI.

The most important current hypotheses are discussed in chapter 9. In contrast to the earlier described hypotheses, the mechanism suggested in this thesis allows explanation of both the incorporation of a molecule of water ($H_2{}^{18}O$) and the lack of inhibition of the 20α,22R-di-OH cholesterol formation by carbon monoxide. The epoxide-diol hypothesis is also in accordance with the results of almost all pertinent experiments described in the literature. Based upon the epoxide-diol hypothesis the different enzyme defects able to cause congenital lipoid adrenal hyperplasia are discussed.
Using tissues obtained post mortem, it is possible to localize these enzymedefects.

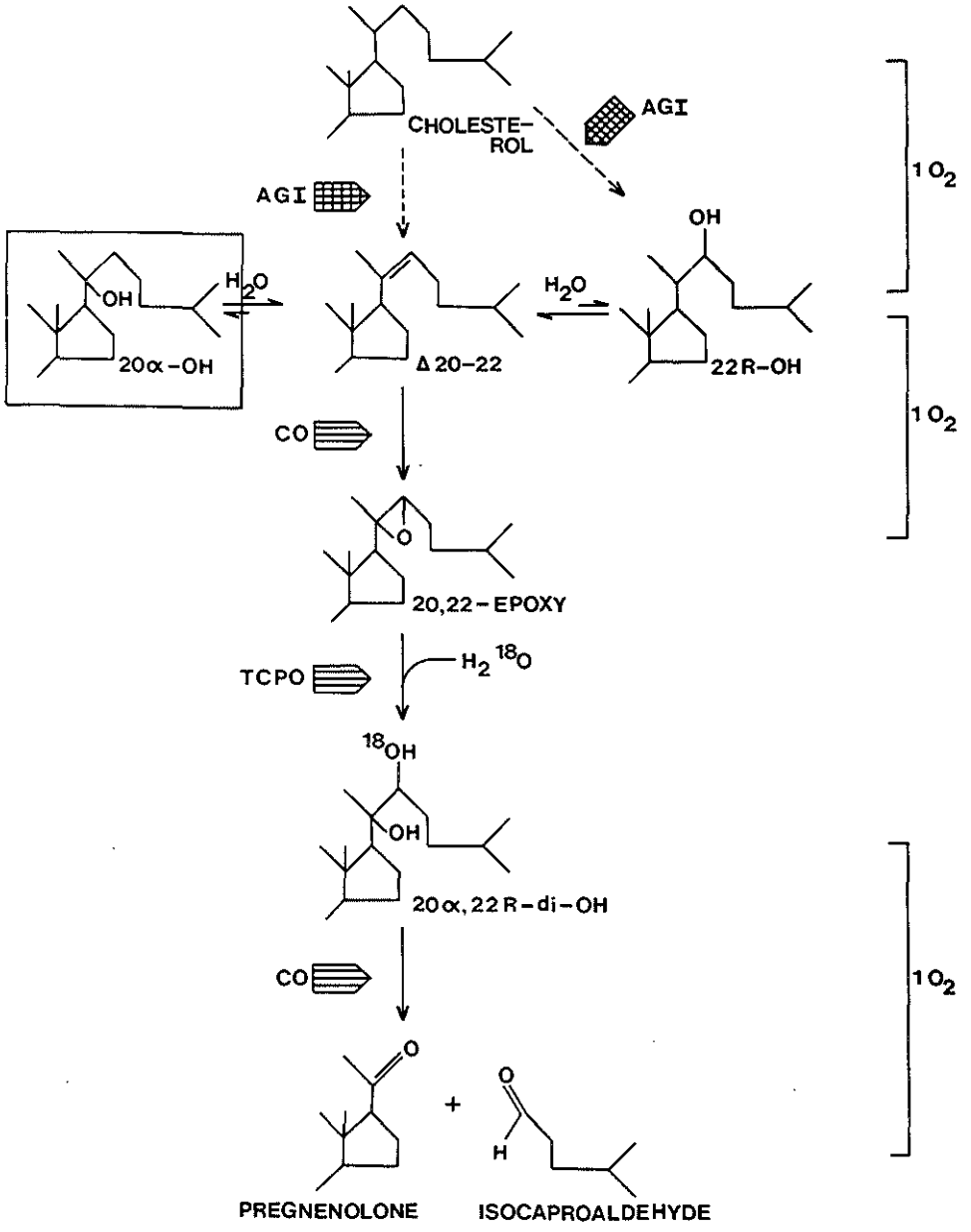
CHOLESTE-
ROL
AGI
AGI
OH
H_2O
20α-OH
Δ 20-22
H_2O
22R-OH
$1O_2$
$1O_2$
CO
O
20,22-EPOXY
TCPO
$H_2{}^{18}O$
^{18}OH
OH
20α,22R-di-OH
$1O_2$
CO
O
+
O
H
PREGNENOLONE
ISOCAPROALDEHYDE

Fig. A

Fig. A

The scheme for conversion of cholesterol and pregnenolone, as proposed in this thesis. The most likely point of inhibition in this scheme is indicated.
There are 2 pathways to Δ^{20-22} cholesterol possible in this scheme, shown here by dotted arrows.
20α-OH cholesterol is shown in a box to emphasize that this substrate is not a intermediate in the side-chain cleaving system.
CO: carbon monoxide. The point of inhibition is indicated.
TCPO: 1,1,1-trichloro-propene-2,3-oxide, an epoxyhydrase inhibitor.
$H_2{}^{18}O$: The labeled oxygen atom is bound only to the C-22 atom of 20α,22R-di-OH cholesterol.
O_2: The scheme indicates that the conversion of 1 molecule of cholesterol into 1 molecule of pregnenolone and 1 molecule of isocaproaldehyde requires 3 molecules of oxygen.
For the conversion into the same products, 20α-OH cholesterol, Δ^{20-22} cholesterol and 22R-OH cholesterol each require 2 molecules of oxygen.
20,22-epoxycholesterol and 20α,22R-di-OH cholesterol each require 1 molecule of oxygen.

SAMENVATTING

Als een van de eerste stappen in de steroidsynthese van de bijnieren wordt de zijketen van cholesterol afgesplitst, waarbij pregnenolon en isocaproaldehyde ontstaan. In dit proefschrift zijn de resultaten beschreven van een onderzoek over het enzym-systeem dat deze reaktie katalyseert. Het gevormde pregnenolon dient als uitgangspunt voor de steroidsynthese van de bijnieren. De via dit onderzoek verkregen kennis zou van toepassing kunnen zijn bij het zoeken naar de oorzaak (oorzaken) van een bijna altijd dodelijke aandoening, de congenitale bijnier lipoidhyperplasie. Bij deze aandoening is de werking van het zijketensplitsende enzym-systeem, dat cholesterol in pregnenolon en isocaproaldehyde omzet, gestoord.

In hoofdstuk 1 wordt het doel van het onderzoek weergegeven. In het kort is beschreven welke methoden zijn gebruikt. Na een beknopte behandeling van de Cahn-Ingold-Prelog nomenclatuur wordt uiteengezet waarom in dit proefschrift is gekozen voor de oude nomenclatuur.

In hoofdstuk 2 wordt een historisch overzicht gegeven van het werk dat geleid heeft tot de algemeen geaccepteerde hypothese over het mechanisme van de pregnenolon-vorming uit cholesterol. Uitgaande van de in 1971 beschikbare kennis van het betrokken enzym-systeem wordt vervolgens de probleemstelling geformuleerd.

De redenen voor het gebruik van een gaschromatografische bepaling van de verschillende sterolen en steroiden worden in hoofdstuk 3 gegeven. De kombinatie van gaschromatografie en massa-spectrometrie maakte het mogelijk om via de inbouw van ^{18}O in tussen- en eindprodukten, de werking van het enzymsysteem te bestuderen.

In hoofdstuk 4 zijn de experimenten beschreven over de enzymatische omzetting van 20α-OH cholesterol in pregnenolon. De reakties werden uitgevoerd in aanwezigheid van (het niet-radioaktieve) $H_2{}^{18}O$. Het gevormde pregnenolon bleek ^{18}O te bevatten. De inbouw van een molekuul water, zoals hier beschreven

voor de biosynthese van pregnenolon, is echter in strijd met de gangbare hypotheses. Om de resultaten van de in hoofdstuk 4 beschreven experimenten te kunnen verklaren wordt een nieuwe (epoxide-diol) hypothese voorgesteld (zie figuur A).

Bij de enzymatische omzetting van 22R-OH cholesterol in pregnenolon en isocaproaldehyde (hoofdstuk 5) werd een tussenprodukt gevormd, dat met behulp van massa-spectrometrie als 20α,22R-di-OH cholesterol geïdentificeerd kon worden. Gedurende de reaktie bleek de som van het aantal nmol substraat, tussenprodukt en eindprodukt kleiner te zijn dan het aantal nmol substraat waarmee de reaktie was gestart. Blijkbaar waren er tussenprodukten aanwezig die niet gemeten werden. Deze onbekende tussenprodukten bleken in aanwezigheid van > 99% koolmonoxide in het (geïdentificeerde) tussenprodukt 20α,22R-di-OH cholesterol omgezet te worden. De som van de hoeveelheden substraat, tussenprodukt en eindprodukt was dan bijna gelijk aan de hoeveelheid substraat waarmee de reaktie gestart werd. Hieruit is gekonkludeerd dat naast het cytochroom P-450$_{scc}$, dat door koolmonoxide wordt geremd, een enzym werkzaam is, dat in staat is om een onbekend tussenprodukt in 20α,22R-di-OH cholesterol om te zetten. Dit experiment ondersteunt onze veronderstelling dat een epoxyhydrase betrokken is bij de biosynthese van pregnenolon. Een epoxyhydrase zou een 20,22-epoxycholesterol in aanwezigheid van water (H_2O) in 20α,22R-di-OH cholesterol kunnen omzetten, terwijl ook bekend is dat de werking van de in de literatuur beschreven epoxyhydrases niet door koolmonoxide wordt geremd.

In hoofdstuk 6 is de omzetting van cholesterol in pregnenolon en isocaproaldehyde in de aanwezigheid van $H_2{}^{18}O$ beschreven. Uit de massa-spectrometrische analyse van het tussenprodukt bleek dat het ^{18}O uitsluitend aan het C-22 atoom van het tussenprodukt 20α,22R-di-OH cholesterol gebonden was. Eenzelfde resultaat werd bereikt bij een onder vergelijkbare omstandigheden uitgevoerde inkubatie van 22R-OH cholesterol (hoofdstuk 4).

De bepaling van het aantal molekulen zuurstof dat nodig is

voor de reaktie:
1 22R-OH cholesterol → 1 pregnenolon + 1 isocaproaldehyde
wordt in hoofdstuk 7 beschreven. Er bleken 2 molekulen zuurstof nodig te zijn om de reaktie te laten verlopen. Dit is in overeenstemming met zowel de in de literatuur meest gangbare hypothese omtrent de cholesterol zijketensplitsing, als met de door ons voorgestelde epoxide-diol hypothese.

In hoofdstuk 8 worden de effekten van de remmer aminoglutethimide fosfaat (afgekort AGI, merknaam Elipten) op de door cytochroom P-450$_{scc}$ gekatalyseerde zijketensplitsing beschreven. AGI remt de omzetting van cholesterol in pregnenolon. Nagegaan werd of de substraten van het bestudeerde enzymsysteem, 20α-OH cholesterol en 22R-OH cholesterol, op analoge wijze als cholesterol, d.w.z. door vertraging van de snelheid van de reduktie van het cytochroom P-450$_{scc}$, werden geremd. Dit bleek niet het geval te zijn. Tevens werd de juistheid van een recente veronderstelling onderzocht, waarbij werd gepostuleerd dat AGI de vorming van een enzymsubstraat komplex tussen cholesterol en cytochroom P-450$_{scc}$ verhindert. Mede op grond van literatuur gegevens werd gekonkludeerd dat remming van de komplex-vorming door AGI alleen mogelijk is indien de bijniermitochondriën, die het cytochroom P-450$_{scc}$ bevatten, intakt zijn. In niet-intakte mitochondriën is waarschijnlijk de beschikbaarheid van het cholesterol voor cytochroom P-450$_{scc}$ zo groot, dat AGI niet effektief kan remmen.

In hoofdstuk 9 worden de belangrijkste hypotheses van dit ogenblik besproken. In tegenstelling tot eerder beschreven hypotheses kan met het door ons voorgestelde mechanisme zowel de inbouw van een molekuul water ($H_2{}^{18}O$) als de niet door CO geremde vorming van 20α,22R-di-OH cholesterol verklaard worden. De epoxide-diol hypothese is toepasbaar bij de verklaring van de resultaten van bijna alle in de literatuur beschreven experimenten. Aan de hand van de epoxide-diol hypothese worden de verschillende enzymdefecten besproken die oorzaken van congenitale bijnier lipoid hyperplasie kunnen zijn. Het is mogelijk met geschikt autopsie materiaal de enzymdefecten te localiseren.

Fig. A

Het schema voor de omzetting van cholesterol in pregnenolon en isocaproaldehyde, zoals voorgesteld in dit proefschrift wordt in dit figuur weergegeven.

AGI: aminoglutethimide fosfaat. In dit schema is het meest waarschijnlijke aangrijpingspunt van deze remmer aangegeven. De gestippelde pijlen geven aan dat dit schema de keuze tussen de twee mogelijke wegen naar Δ^{20-22} cholesterol open laat. 20α-OH cholesterol is in een kader geplaatst om uit te laten komen dat dit substraat geen tussenprodukt is van het zijketen splitsende enzym-systeem.
CO: koolmonoxide. Het aangrijpingspunt van deze remmer is aangegeven.
TCPO: 1,1,1-trichloro-propene-2,3-oxide, een epoxyhydrase remmer.
$H_2{}^{18}O$: Het gelabelde zuurstof atoom wordt slechts gebonden aan het C-22 atoom van 20α,22R-di-OH cholesterol terug gevonden.
O_2: Uit het schema blijkt dat voor de omzetting van 1 molekuul cholesterol in 1 molekuul pregnenolon en 1 molekuul isocaproaldehyde 3 molekulen zuurstof nodig zijn.
Voor de omzetting in dezelfde produkten gebruiken 20α-OH cholesterol, Δ^{20-22} cholesterol en 22R-OH cholesterol elk 2 molekulen zuurstof.
20,22-epoxycholesterol en 20α,22R-di-OH cholesterol gebruiken volgens het schema elk 1 molekuul zuurstof.

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CURRICULUM VITAE

(op verzoek van de faculteit).

De schrijver van dit proefschrift behaalde in 1962 het diploma H.B.S.-B aan het Spinoza Lyceum te Amsterdam. In hetzelfde jaar liet hij zich inschrijven als student scheikunde aan de Universiteit van Amsterdam. Samen met A.H.J. van der Stap behaalde hij in 1965 het nederlands kampioenschap roeien in het nummer dubbel-twee. Het candidaatsexamen werd afgelegd in 1968 en het doctoraal-examen scheikunde in 1971 (hoofdvak biochemie, bijvak fysiologie en keuze richting radiochemie). Na het doctoraal-examen was hij als wetenschappelijk medewerker werkzaam op de afdeling kindergeneeskunde van de Erasmus Universiteit te Rotterdam, alwaar het in dit proefschrift beschreven onderzoek werd verricht. Gedurende het cursusjaar 1971/72 gaf hij enkele uren per week scheikunde-les aan de eind-examenklas van een MAVO. In december 1975 werd de schrijver van dit proefschrift aangesteld als hoofd van het klinisch-chemisch laboratorium van het Oogziekenhuis te Rotterdam. In december 1976 volgde een aanstelling als wetenschappelijk medewerker op de afdeling klinische microbiologie en anti-microbiële therapie van de Erasmus Universiteit.
In zijn huidige functie houdt de schrijver zich bezig met een onderzoek naar de metabole processen bij de ontwikkelings cyclus van het intracellulair levende en sexueel overdraag-bare micro-organisme, Chlamydia trachomatis.

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Nescio.