HYDROXYLATED STEROLS METABOLISM AND EFFECTS ON STEROID PRODUCTION AND STEROID UPTAKE

GEHYDROXYLEERDE STEROLEN METABOLISME EN EFFECTEN OP DE STEROID PRODUCTIE EN STEROID OPNAME

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Aan Nel, Saskia, Remco en Judith.
Aan mijn Moeder.
Ter nagedachtenis aan mijn Vader.

CONTENTS

LIST OF ABBREVIATIONS

A II	: angiotensin II
ACTH	: adrenocorticotropic hormone
ASF	: aldosterone stimulating factor
ATP	: adenosine-triphosphate
B	: corticosterone
cAMP	: adenosine-3',5'-cyclic monophosphate
cGMP	: guanosine-3',5'-cyclic monophosphate
CLIP	: corticotropin-like intermediate-lobe protein
CMO	: corticosterone methyloxidase
CO	: carbon monoxide
CTX	: cerebrotendinous xanthomatosis
DCM	: dichloromethane
DHEA	: dehydroepiandrosterone
DHEAS	: dehydroepiandrosterone sulfate
17S,20S-diOH	: 17S,20S-dihydroxy-cholesterol
DOC	: 11-deoxycorticosterone
F	: cortisol
GTP	: guanosine-triphosphate
hCC	: human chorionic corticotropin
hCG	: human chorionic gonadotropin
hCSM	: human chorionic somato-mammotropin
HDL	: high-density lipoprotein
HEPES	: 4-[2-hydroxyethyl]-1-piperazine-ethanesulfonic acid
hGH	: human growth hormone
HMG-CoA	: 3-hydroxy-3-methyl-glutaryl-Coenzyme A
hPRL	: human prolactin
3β-HSD	: 3β-hydroxysteroid dehydrogenase/Δ^{5-4}isomerase
11β-HSD	: 11β-hydroxysteroid dehydrogenase
17β-HSD	: 17β-hydroxysteroid dehydrogenase
KRBAG	: Krebs-Ringer-Bicarbonate buffer, supplemented with albumin and glucose
KRHAG	: Krebs-Ringer-HEPES buffer, supplemented with albumin and glucose
LDL	: low-density lipoprotein

LPH	: lipotropic hormone
MSH	: melanocyte-stimulating hormone
$NADP^+$	: nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	: nicotinamide adenine dinucleotide phosphate (reduced form)
22R-OH	: 22R-hydroxy-cholesterol
22S-OH	: 22S-hydroxy-cholesterol
25-OH	: 25-hydroxy-cholesterol
18-OH-DOC	: 18-hydroxy-11-deoxy-corticosterone
PA	: phosphatidic acid
PI	: phosphatidylinositol
PIP	: phosphatidylinositol monophosphate
PIP_2	: phosphatidylinositol diphosphate
SCC	: side-chain cleavage
SCP	: sterol carrier protein

LIST OF TRIVIAL NAMES

Aldosterone	: 18,11-hemiacetal of 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al
Androstenediol	: 3β,17β-dihydroxy-5-androstene
Androstenedione	: 4-androstene-3,17-dione
Cholestanol	: 3β-hydroxy-5α-cholestane
Cholestenone	: 4-cholesten-3-one
Cholesterol	: 3β-hydroxy-5-cholestene
17S,20S-diOH-cholesterol	: 3β,17S,20S-trihydroxy-5-cholestene
20R,22R-diOH-cholesterol	: 3β,20R,22R-trihydroxy-5-cholestene
22R-OH-cholesterol	: 3β,22R-dihydroxy-5-cholestene
22S-OH-cholesterol	: 3β,22S-dihydroxy-5-cholestene
20S-OH-cholesterol	: 3β,20S-dihydroxy-5-cholestene
25-OH-cholesterol	: 3β,25-dihydroxy-5-cholestene
7α-OH-cholesterol	: 3β,7α-dihydroxy-5-cholestene
7β-OH-cholesterol	: 3β,7β-dihydroxy-5-cholestene
7-keto-cholesterol	: 3β-hydroxy-5-cholesten-7-one
22-keto-cholesterol	: 3β-hydroxy-5-cholesten-22-one
Corticosterone	: 11β,21-dihydroxy-4-pregnene-3,20-dione
18-OH-corticosterone	: 11β,18,21-trihydroxy-4-pregnene-3,20-dione
Cortisol	: 11β,17α,21-trihydroxy-4-pregnene-3,20-dione
Cortisone	: 17α,21-dihydroxy-4-pregnene-3,11,20-trione
Dehydroepiandrosterone	: 3β-hydroxy-5-androsten-17-one
Deoxycorticosterone	: 21-hydroxy-4-pregnene-3,20-dione
18-OH-11-deoxycorticosterone	: 18,21-dihydroxy-4-pregnene-3,20-dione
Desmosterol	: 3β-hydroxy-5,24-cholestadiene
Epicholesterol	: 3α-hydroxy-5-cholestene
17α-OH-pregnenolone	: 3β,17α-dihydroxy-5-pregnen-20-one
17α-OH-progesterone	: 17α-hydroxy-4-pregnene-3,20-dione
Progesterone	: 4-pregnene-3,20-dione
Pregnenolone	: 3β-hydroxy-5-pregnen-20-one
Testosterone	: 17β-hydroxy-4-androsten-3-one

CHAPTER 1

GENERAL INTRODUCTION AND STATEMENT OF THE PROBLEM

Many hereditary enzyme defects lead to the accumulation of metabolites. Frequently the accumulated products are converted into other metabolites via pathways, that are normally of minor importance. These products or their metabolites may cause profound disturbances of physiological processes. Several inborn errors in steroid biosynthesis provide examples of this phenomenon: the steroid 21- and 11β-hydroxylase deficiencies lead to overproduction of androgens, which are responsible for extreme masculinization and abnormal growth.

Several clinical conditions are known, in which cholesterol or another sterol accumulates in tissues or body fluids, due to an enzymatic defect in sterol metabolism. Analogous to the situation found in other enzyme defects, production of "unusual sterols" might be expected in these conditions. In this context, "unusual sterols" are defined as sterols not normally occurring as a metabolite or in trace amounts only. This phenomenon has indeed been observed, as will be discussed below. Such sterols might contribute to the severity of the disease.

The existence of unusual sterols, having unexpected biological activity, is well-known (25-hydroxy-cholesterol, 3β,20S-dihydroxy-cholenic acid, toad poisons, etc.). However, much remains to be learned about their effects and mechanisms of action. For this reason we evaluated short-term effects of several sterols on steroid production and steroid uptake in isolated rat adrenal and liver cells and on steroid production in isolated human fetal adrenal cells. These sterols were selected according to two criteria:

1. not normally occurring as a metabolite or in trace amounts only,
2. being a potential metabolite of cholesterol.

Disturbances of sterol metabolism

The accumulation of sterols in tissue and body fluids probably plays a role in the etiology of several diseases.

One such condition is the **congenital lipoid adrenal hyperplasia** (Prader's syndrome; for a review see Degenhart, 1984). This inborn error of cholesterol side-chain cleavage results in a massive lipoid hyperplasia of both adrenals. The mortality rate is remarkably high. The absence of steroid production per se is not lethal, as children with a congenital absence of the adrenals can be treated with relative ease (Pakravan et al., 1974). The possibility of the production of unusual sterols has been discussed (Falke et al., 1975a).

In **cerebrotendinous xanthomatosis** (CTX) a deficiency of the 26-hydroxylase activity results in a diminished production of bile acids. As a consequence the cholesterol production rate is increased, leading to the formation of cholestanol (Wolthers et al., 1983). This sterol accumulates in plasma and several tissues like brain (leading to mental retardation), tendons (leading to swelling) and the eyes (giving cataract). A complex of cholestanol, cholesterol and 2 molecules of water (C-C-2W) might be responsible for the deposits in brain and tendons. C-C-2W exerts adverse effects, including inflammation, cell necrosis and destruction of cell membranes, on liver, intima tissue, eyes, lungs and other parts of the body.

In **familial hypercholesterolemia** a deficiency of cell surface receptors for low density lipoprotein (LDL) has been found, leading to a disturbance in the regulation of LDL degradation and cholesterol synthesis (Brown and Goldstein, 1974; Goldstein and Brown, 1984). The cholesterol concentration in plasma is greatly increased. This disease is clinically characterized by premature coronary heart disease and atherosclerosis. Fatty acid esters of 24-OH-cholesterol and 26-OH-cholesterol occur in human aortal tissue; these sterols accumulate with increasing severity of atherosclerosis (Teng and Smith, 1975). Also the free 26-OH-cholesterol concentration in aortal tissue increases with the advancement of this disease (Smith and Van Lier, 1970). Due to the high cholesterol concentration, C-C-2W (see above) may be an important factor in atherosclerosis.

In **Wolman's disease** (Wolman et al., 1961) and in the less severe **cholesteryl ester storage disease** (Sloan and Fredrickson, 1972) a lysosomal acid lipase is reduced in activity. This leads to an abnormal lysosomal accumulation of cholesteryl esters and triglycerides and an increased cholesterol synthesis. Patients suffering from Wolman's disease generally

die before the age of one year with symptoms like hepatosplenomegaly and adrenal calcification. The steryl ester fraction of the liver of a patient with Wolman's disease contained esters of 7α- and 7β-hydroxy-cholesterol, 7-keto-cholesterol and 5,6α- and 5,6β-epoxy-cholesterol in addition to cholesterol (Assmann et al., 1975).

There is a group of **peroxisomal disorders** (a.o. the cerebro-hepato-renal syndrome of Zellweger and adrenoleukodystrophy) in which a deficiency of peroxisomes or an impairment of peroxisomal metabolism leads also to an impaired conversion of cholesterol into bile acids by a deficient C_{24}-C_{25} cleavage (Schutgens et al., 1987). As a consequence bile alcohols with an extended side-chain accumulate in the liver and are excreted in the bile (Parmentier et al., 1979; Janssen et al., 1982). The clinical symptoms are muscular hypotonia, liver enlargement, renal cysts, craniofacial malformations and mental retardation. The Zellweger syndrome and the neonatal adrenoleuko-dystrophy are lethal at a very young age.

In **cholesterol cholelithiasis**, which most probably is not hereditary, enhanced hepatic cholesterol secretion is associated with a deficient bile acid pool. The amount of cholesterol is too great for the bile acid micelles to dissolve. As a result cholesterol crystals precipitate, which may aggregate to form gallstones. The initial compound deposited is the complex of cholesterol, cholestanol (which always accompanies cholesterol in our daily food) and water (C-C-2W).

In vitro occurrence of unusual sterols

In vitro the occurrence of unusual sterols has been shown under conditions in which the normal cholesterol side-chain cleavage is impaired. In bovine adrenal mitochondria Alsema et al. (1980) observed 25-hydroxylation of 20S-OH-cholesterol, besides the normal pregnenolone production, when the incubation was carried out at pH 7.8. Degenhart et al. (1984) observed a 25-hydroxylase activity, hydroxylating 20S-hydroxy-4-cholesten-3-one, when this sterol was incubated with bovine adrenal cortex mitochondria. Alsema et al. (1982a) also showed the conversion of several C_{24} and C_{27} 3β-hydroxy-5-ene sterols, some of which are normal side-chain cleavage intermediates, into 3-keto-Δ^4 products by bovine adrenal cortex 3β-hydroxysteroid dehydrogenase. Huijmans et al. (1982) showed the formation of 22-keto-cholesterol from 22S-OH-cholesterol in bovine adrenal mitochondria.

Falke et al. (1976a) discussed the possibility of a metabolite with inhibitory properties on the steroid synthesis in rat adrenal cells under conditions of a blocked cholesterol side-chain cleaving enzyme system.

Effects of exogenous sterols

Several in vivo and in vitro effects of sterols have been described. In vivo growth retardation and adrenal hyperplasia could be observed by feeding cholestenone to rats (Degenhart et al., 1981). Administration of 25-OH-cholestenone to pregnant rats caused severe malformations of head and spinal cord in the offspring (H.J. Degenhart, personal communication). In vivo administration of hydroxylated sterols to rabbits caused fibrotic lesions in the aorta and pulmonary arteries (Imai et al., 1980).
In vitro marked effects of hydroxylated sterols on cultured rabbit aorta smooth muscle cells (Peng et al., 1979), cell proliferation (Chen et al., 1974), endocytosis of peroxidase (Heiniger et al., 1976) and uptake and efflux of ions (Chen et al., 1978) are known. These long-term effects can be attributed to a depression of the cholesterol synthesis.
Some short-term in vitro effects of sterols on chemotaxis (Gordon et al., 1980), proliferation (Hoffmann et al., 1981), E-rosette formation of lymphocytes with erythrocytes (Streuli et al., 1979) and echinocyte formation of erythrocytes (Hsu et al., 1980) have also been described. These effects are most likely the consequence of insertion of the sterols into the cellular membrane.

The human fetal adrenal gland

The human fetal adrenal is an endocrine organ with a unique structure: a fetal zone is present, which fills the greater part of the adrenal volume but involutes after birth. An important function of the fetal adrenal is the production of precursors for estrogen synthesis. The adrenals of the human fetus may secrete up to 100 mg of steroids daily, with cholesterol as the main precursor. Part of the cholesterol (some 30%) is derived from the novo synthesis in the fetal adrenal gland (Carr and Simpson, 1981b). The other part is derived from the plasma LDL, produced mainly in the fetal liver. This means that in the fetal adrenal the cholesterol production and cholesterol influx is high. On the other hand the regular steroidogenic pathway is functionally blocked by a low 3β-hydroxysteroid dehydrogenase activity. Our hypothesis was, that the human fetal adrenal might be used as

a model system, in which the production of unusual sterols might occur.

The aims of this study

In view of the diseases just described and of the in vitro effects of hydroxylated sterols or products derived from them, we were interested in effects of sterols on cellular or subcellular processes. The aims of our studies were to investigate:

1. possible short-term effects of sterols on basal or stimulated steroid production in isolated rat adrenal cells,
2. possible short-term effects of sterols on steroid uptake in isolated rat adrenal and liver cells,
3. the possible production of unusual sterols in the human fetal adrenal.

For the latter purpose we studied:

   a. the applicability of the methods for rat adrenal cell isolation to the human fetal adrenal,
   b. the properties of hydroxylated sterols as steroid precursors in this system.

We restricted ourselves to sterols, which may be expected to occur in the human or rat adrenal system. Other sterols, like those occurring in plants and amphibians with a strong activity (e.g. digitonin, toad poisons), were not taken into consideration.

Design of the study

The experimental approach to the aims of this study will be described below.

Sterols and steroid production.

The isolated rat adrenal cell was used as a model system. The isolation technique for rat adrenal cells has been extensively studied in our laboratory (Falke et al., 1975a,b, 1976a,b). Starting with these cells we looked at the properties of hydroxylated sterols as a precursor for steroid production. In intact cells exogenous substrates have to pass through several membranes to reach the mitochondrion, where the side-chain cleavage occurs. In order to eliminate possible effects on transport through membranes, we also used damaged bovine adrenal mitochondria to investigate the conversion of hydroxylated sterols. We also studied the effects of hydroxylated sterols on short-term stimuli of steroid

production. Either the total cell suspension (Chapters 4 and 5) or suspensions containing largely glomerulosa cells or fasciculata/reticularis cells (Chapter 6) were used in these studies.

Sterols and steroid uptake.

Before exerting their action, steroid hormones have to be taken up by target cells and bound to receptors. It is commonly assumed that steroid hormones diffuse passively through the cell membrane, although there is some evidence for a mediated uptake mechanism (Rao, 1981). In the rat, corticosterone is the main glucocorticoid and the liver is an important target organ. To study possible effects of hydroxylated sterols on steroid uptake we used the uptake of corticosterone in isolated rat liver cells as a model system (Chapter 7). In addition the effects of several sterols on the uptake and conversion of pregnenolone in isolated rat adrenal cells were investigated (Chapter 8).

Sterols and the human fetal adrenal.

As will be discussed in Chapter 3, the method of cell isolation used for the rat adrenal can be applied to the human fetal adrenal, yielding viable cells. Also the method of separating cells of the fetal zone from those of the definitive zone by means of density gradient centrifugation can be used. With these cells the properties of hydroxylated sterols as steroid precursors were studied (Chapter 9).

CHAPTER 2

REVIEW OF THE LITERATURE

2.0 Introduction

As outlined in Chapter 1, several adrenal cell systems with their specific products and stimulating factors are used to study the effects of hydroxylated sterols. In this chapter some general features of the adrenal gland, like the morphology (2.1) and steroidogenesis (2.2) will be described. In the study of the effects of hydroxylated sterols on the steroid production in fasciculata/reticularis cells, ACTH was used as a stimulating factor. Therefore in section 2.3 the mechanism of action of ACTH will be reviewed. Similar studies were carried out in glomerulosa cells. Section 2.4 reviews the aldosterone production and its most important stimulating factors in this cell type. Section 2.5 gives a review of the literature on the occurrence and effects of hydroxylated sterols. Under special conditions metabolic pathways, leading to the formation of hydroxylated products, may become active. Examples will be given for the rat adrenal (2.5) and for the human liver (2.6). Section 2.7 deals with the human fetal adrenal gland and its morphology, steroidogenesis and stimulating factors.

2.1 The adrenal gland

Higher vertebrates possess two ovoid or bean-shaped adrenal glands, which are situated above the kidney, embedded in fat tissue. A cross-section of the gland reveals two distinct regions: the medulla as the inner region surrounded by the cortex (Bloom and Fawcett, 1968; Schulster et al., 1976). Despite their anatomical relationship the two regions are embryologically and functionally different. The medulla arises in the embryo from the neural crest of the ectoderm. The cortex emanates from the mesoderm at an early stage in the embryological development. In this region the steroid hormones are produced.

The structure of the adrenal cortex is complex and may change with age

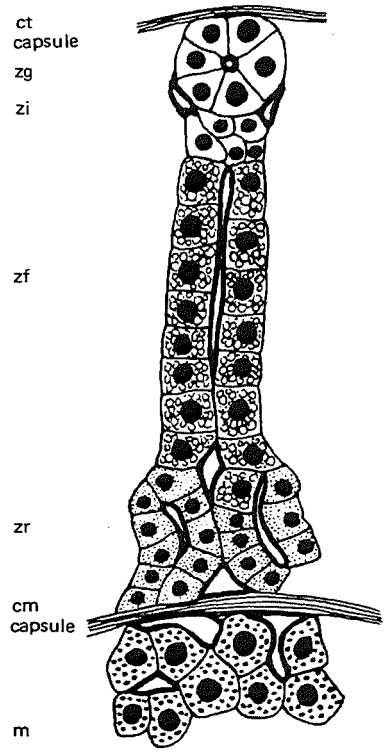


Figure 2.1: Schematic representation of the rat adrenal (derived from Schulster et al., 1976).
ct capsule: connective tissue capsule
zg: zona glomerulosa zi: zona intermedia
zf: zona fasciculata zr: zona reticularis
cm capsule: circum-medullary connective tissue capsule
m: medulla

and physiological condition. A schematic representation of the normal rat adrenal cortex is given in figure 2.1.
In the human and rat adrenal cortex three concentric zones can be distinguished:

a. The zona glomerulosa, directly beneath the capsule surrounding the gland. The cells are grouped in clusters that are continuous with the cell columns of the zona fasciculata. Lipid droplets are scarce.
This zone is the main site of production of mineralocorticoids with aldosterone as the most potent one (for a review see Tait et al., 1980a).
Angiotensin II is the most important regulating factor.

b. The zona fasciculata constitutes some 70% of the adrenal cortex volume and contains larger cells, arranged in columns radiating towards the center of the gland. The cells are crowded with lipid droplets and mitochondria, indicating high steroidogenic activity. The zona fasciculata is primarily responsible for the secretion of glucocorticoids like cortisol (not in the rat) and corticosterone in response to ACTH. In the rat a zona intermedia is present, which is located between the zonae glomerulosa and fasciculata. The cells of this zona intermedia are relatively free of lipid droplets.

c. The zona reticularis with cells arranged in a network, containing less cytoplasmatic lipid droplets.
The zona reticularis is the most probable site of adrenal androgen and estrogen biosynthesis. The androgen synthesis may be regulated by a pituitary factor, called Adrenal Androgen Stimulating Hormone or Cortical Androgen Stimulating Hormone (Grumbach et al., 1978). However in vivo the normal production rate is only minimal, exept for DHEA. The cells also have the capacity to produce corticosteroids after stimulation with ACTH (Bell et al., 1979).

The separation between the zonae fasciculata and reticularis is usually not so strict.

2.2 Steroidogenesis in the adrenal cortex.

Steroid production

An outline of the steroid production in the human adrenal is given in Figure 2.2. In the rat adrenal, 17α-hydroxylase activity is absent. Consequently the left column in Figure 2.2 represents the main metabolic route in the rat.

First of all the side-chain of cholesterol is cleaved to form pregnenolone. Via multiple hydroxylase and dehydrogenase reactions the three classes of steroid hormones (glucocorticoids, mineralocorticoids and androgens/ estrogens) are formed.

The localization of the enzymes involved in steroidogenesis is depicted in Figure 2.3. The steroid 21- and 17α-hydroxylases are microsomal. The 3β-hydroxysteroid dehydrogenase/isomerase occurs both in the microsomes and

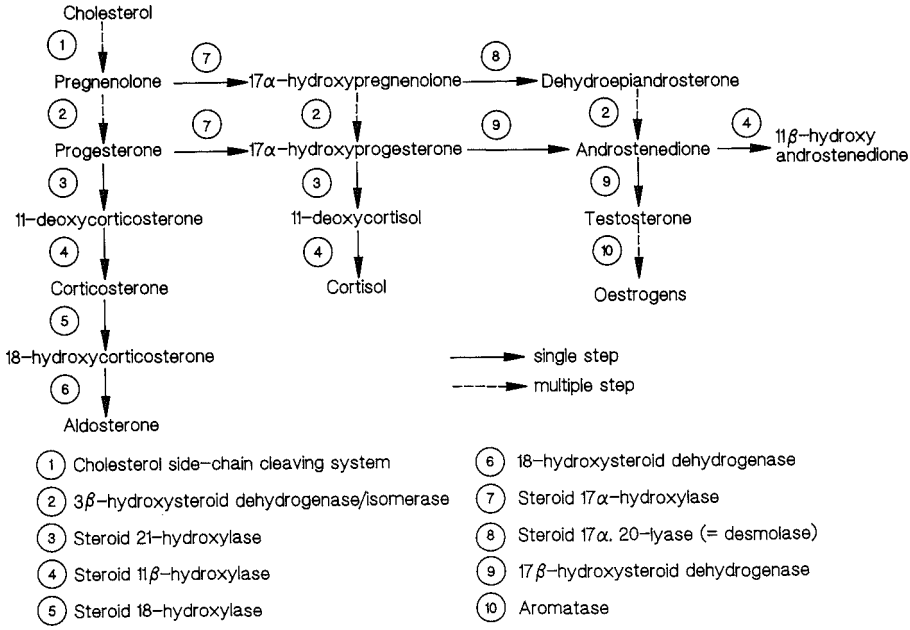


Figure 2.2: An outline of human adrenal steroidogenesis

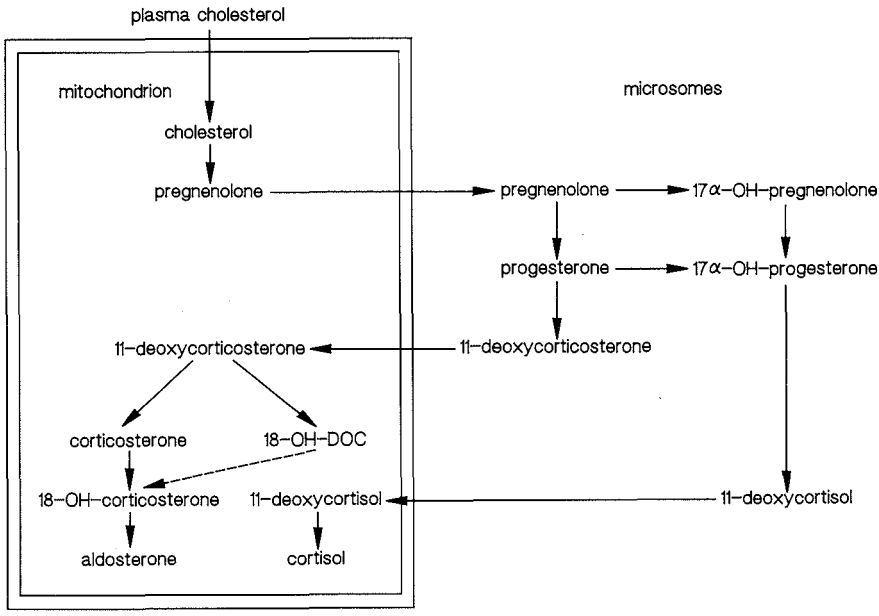


Figure 2.3: Intracellular localization of enzymes for adrenal steroidogenesis.

the mitochondria (Kream and Sauer, 1976). The other hydroxylases are exclusively mitochondrial.

Cholesterol

Steroidogenesis begins with cholesterol as the main precursor. Reports exist on other precursors, for example desmosterol (Diedrichsen et al., 1977), but these occur only in quantitatively minor pathways.
Cholesterol can be synthesized de novo from acetate (for extensive reviews see Schroepfer, 1981, 1982). De novo cholesterol synthesis is possible in e.g. liver and adrenal tissue. However, there is good evidence that, at

least in man and in the rat, plasma cholesterol is the main steroid precursor (Dexter et al., 1970; Borkowski et al., 1972a,b). This cholesterol is carried by the lipoproteins (Gwynne et al., 1976). In the rat ACTH stimulates the transfer of cholesterol from HDL but not from LDL. The HDL-apoproteins Apo-I and Apo-II markedly enhance the adrenal accumulation of cholesterol. In man LDL is the main source of cholesterol. In a cascade of enzymatic reactions cholesterol is converted into steroids. For a better understanding of the experimental results the subsequent steps in the production of steroids will be briefly discussed.

Cytochrome P-450

The cholesterol side-chain cleavage and all hydroxylation reactions have in common that they are cytochrome P-450 dependent. They have the characteristics of a mixed function oxidase.

A. Mitochondria

Components of this mixed function oxidase in the adrenal mitochondria include:

1. a flavoprotein dehydrogenase, specific for NADPH (adrenodoxin reductase),

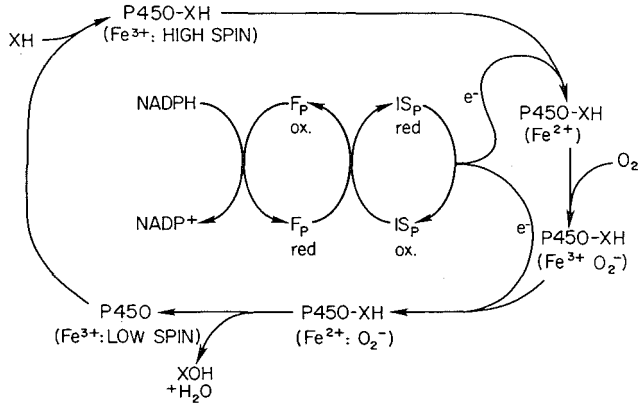


Figure 2.4: Reaction mechanism of cytochrome P-450 in adrenal cortex mitochondria. (From: Simpson, 1979)
XH: substrate XOH: product
Fp: flavoprotein ISp: iron-sulfur protein

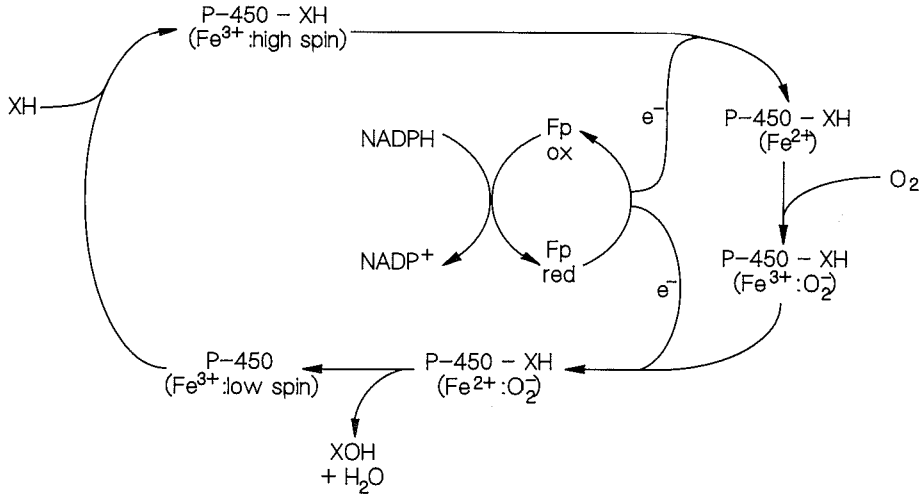


Figure 2.5: Reaction mechanism of cytochrome P-450 in adrenal cortex microsomes.
XH: substrate XOH: product
Fp: flavoprotein

2. a protein known as adrenodoxin, containing non-haem iron (also called non-haem iron protein or iron-sulphur protein),
3. a small particle containing the cytochrome P-450.

The mitochondrial cytochrome P-450 oxygenase cycle and coupled steroid hydroxylation is shown in figure 2.4.

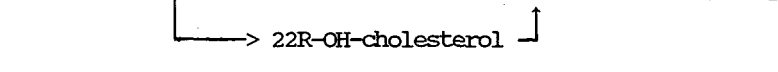
B. Microsomes

The microsomal hydroxylation systems have been shown to consist of a flavoprotein (NADPH-cytochrome P-450 reductase) and cytochrome P-450. The microsomal cytochrome P-450 oxygenase cycle and coupled steroid hydroxylation is shown in figure 2.5.

Cholesterol side-chain cleavage system

In the first step cholesterol is cleaved into pregnenolone and isocaproaldehyde. The latter is converted quickly into isocaproic acid. Several mechanisms have been proposed for this reaction, two of which I will mention.

1. The "classical scheme" with sequential hydroxylations, leading from cholesterol via 22R-OH-cholesterol to 20R,22R-diOH-cholesterol. This latter dihydroxysterol is cleaved by a 20,22-lyase into pregnenolone and isocaproaldehyde (Shimizu et al., 1962; Chaudhuri et al., 1962; Burstein and Gut, 1976; Shikita and Hall, 1974). The pathway:
   cholesterol —> 20S-OH-cholesterol —> 20R,22R-diOH-cholesterol —>
   —> pregnenolone
   is probably of minor importance.
2. "The epoxide-diol pathway" with the following reaction sequence (Kraaipoel et al., 1975a,b,c):



   —> 20R,22R-diOH-cholesterol —> pregnenolone + isocaproaldehyde

Although many of the experimental findings can be explained with both mechanisms, some reports made the proposal of an olefin and an epoxide as intermediates less probable (Morisaki et al., 1976; Burstein et al., 1976; Teicher et al., 1978).

Bovine adrenocortical cytochrome $P\text{-}450_{scc}$ was purified to homogeneity (Larroque et al., 1981). cDNA clones of bovine adrenal cortex $P\text{-}450_{scc}$ mRNA were isolated. Sequence analysis of the cloned cDNA's gave the complete structure of bovine cytochrome $P\text{-}450_{scc}$ (Matteson et al., 1984; Morohashi et al., 1984) and human cytochrome $P\text{-}450_{scc}$ (Morohashi et al., 1987a). Results of isoelectrofocussing and of kinetic studies of purified bovine adrenocortical cytochrome $P\text{-}450_{scc}$ have shown, that a single species of enzyme catalyzes both 20-hydroxylation and 22-hydroxylation and in addition the cleavage of the carbon 20—>22 bond (Duque et al., 1978; Nakajin et al., 1979).

Much work has been done on the role of cytochrome $P\text{-}450_{scc}$ in the mitochondrial side-chain cleavage (for reviews see Mitani, 1979; Simpson, 1979). The cytochrome $P\text{-}450_{scc}$ is firmly associated with the inner mitochondrial membrane. Topological studies by Churchill and Kimura (1979)

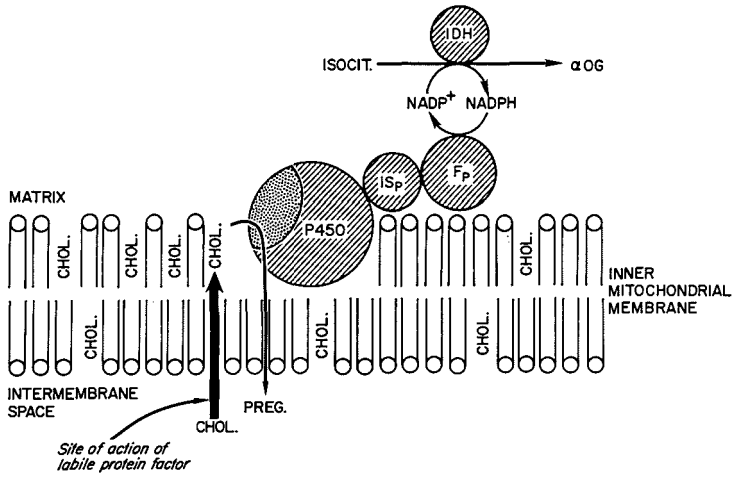


Figure 2.6: Schematic representation of the topology of the cytochrome P-450$_{scc}$ complex. (From: Simpson, 1979)
ISP=iron-sulfur protein Fp=flavoprotein
IDH=isocitrate dehydrogenase chol=cholesterol
preg=pregnenolone

suggest, that both cytochrome P-450$_{scc}$ and P-450$_{11\beta}$ are located on the matrix side of the inner mitochondrial membrane. They are probably deeply burried in the inner membrane with a portion extending outward into the mitochondrial matrix (see figure 2.6). The cytochrome P-450$_{scc}$, isolated from bovine adrenal mitochondria, has a molecular weight of 49,000 (Dubois et al., 1981).

Based on light and electron microscopy and using a peroxidase-labelled antibody technique, the adrenodoxin and adrenodoxin reductase could also be located around the inner mitochondrial membrane. Studies by Kido et al. (1979) revealed that the binding of adrenodoxin to cytochrome P-450$_{scc}$ requires the presence of membrane phospholipids and cholesterol.

The cholesterol side-chain cleavage is the rate-limiting step in steroidogenesis. Tropic hormones, that enhance adrenal steroidogenesis, have an effect on this step (see Simpson, 1979).

3β-hydroxysteroid dehydrogenase/Δ^{5-4}isomerase (3β-HSD)

3β-HSD mediates the conversion of 5-ene-3β-hydroxy steroids into 4-ene-3-oxo-analogues. Two enzymes can be distinguished in this complex: an NAD^+-linked dehydrogenase and an isomerase. In the adrenal 3β-HSD is found in mitochondria as well as in microsomes (Kream and Sauer, 1976; Headon et al., 1978).

steroid 21-hydroxylase

The 21-hydroxylase is a microsomal cytochrome P-450. The gene encoding the cytochrome $P\text{-}450_{21}$ is HLA linked (White et al., 1984, 1985). In the human, C_{21} hydroxylation preceeds $C_{11\beta}$-hydroxylation in the major pathway to cortisol biosynthesis (Maschler et al., 1977). However in the rat 11β-OH-progesterone proved to be an efficient substrate for 21-hydroxylation (De Nicola, 1975). ACTH acts to stimulate the synthesis of the 21-hydroxylase, but has no effect on the activity (Funkenstein et al., 1983).

steroid 11β-hydroxylase

The 11β-hydroxylase is a mitochondrial cytochrome P-450. All protein components of the hydroxylase (NADPH-adrenodoxin reductase, adrenodoxin and cytochrome P-450) are located on the matrix side of the inner mitochondrial membrane (Churchill et al., 1978). Cytochrome $P\text{-}450_{11\beta}$ can be separated from cytochrome $P\text{-}450_{scc}$ and also has different properties. The nucleotide sequence of DNA from bovine adrenal cytochrome $P\text{-}450_{11\beta}$ and $P\text{-}450_{scc}$ is also different (Morohashi et al., 1987b). Whipple et al. (1981) concluded, that both oxygenases are linked through their competition for a single electron source.

steroid 18-hydroxylase

The 18-hydroxylase (referred to as corticosterone methyloxidase I in Section 2.4) is also a mitochondrial cytochrome P-450. Conflicting results are presented about the similarity of the cytochrome P-450, involved in the 11β- and 18-hydroxylation (Rapp and Dahl, 1976; Sonino et al., 1980; Cheng

et al., 1976).

steroid 17α-hydroxylase

The 17α-hydroxylase is a microsomal cytochrome P-450 (for a review see Fevold, 1983). In the bovine adrenal cortex the affinity of this enzyme is 4-6 times higher for pregnenolone than for progesterone (Kremers, 1976).
The rat adrenal cortex produces little, if any, 17α-hydroxylated corticosteroids (Milewich and Axelrod, 1972). However Johnson (1979) demonstrated 17α-hydroxylase activity in the microsomal fraction in rat adrenals; the amount of enzyme appeared to be similar in rat and rabbit adrenals. So in vivo the 17α-hydroxylase is apparently inactive, as far as the corticosteroid production is concerned. The reason for this phenomenon is not clear.

Zonation

The localization of the cytochrome P-450-linked steroid hydroxylations within zones of the bovine adrenal was studied by Ichikawa et al. (1978). Cholesterol side-chain cleavage activity was found in the three zones, but the activity was particularly high in the zonae fasciculata and reticularis. 21-hydroxylation activity was much higher in the zona fasciculata than in the other zones. 11β-hydroxylation activity in the zona glomerulosa was a factor 8 higher than in the zona fasciculata and a factor 20 higher than in the the zona reticularis.
The components of the mixed-function oxidase were also unequally divided (Ichikawa et al., 1970). The zona fasciculata contained more cytochrome P-450 and adrenodoxin than the other zones. All results are in agreement with a considerably greater glucocorticoid production by the zona fasciculata, compared to the mineralocorticoid production by the zona glomerulosa.

2.3 MECHANISM OF ACTION OF ACTH.

Adrenal steroidogenesis is stimulated by Adreno Corticotropic Hormone (ACTH). This 39 amino acid peptide with a molecular weight of 4,500 is released from the anterior lobe of the pituitary. This release is under the control of the hypothalamus via the ACTH-releasing hormone (Antoni, 1986). ACTH is produced as a macromolecular precursor (pro-opiomelanocortin; a 240 amino acid protein), which is processed before gaining its biological activities (Lowry et al., 1984/85). Several neuropeptides with known biological activities are produced, as shown in figure 2.7.

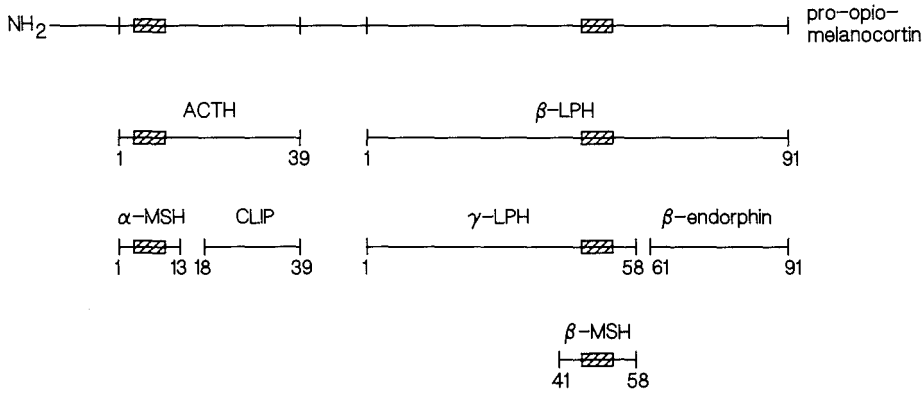


Figure 2.7: Mutual relationship between several pituitary peptide hormones.
▨ = common heptapeptide

Some physiological actions of ACTH on the adrenal are (Schulster et al., 1976):

a. enhancement of adrenal steroidogenesis; this effect is very rapid (within minutes),
b. increase in the adrenal blood flow,
c. increase in adrenal weight as a long term effect (Dallman, 1984/85).

The region of 11-20 amino acids from the N-terminal end of the ACTH molecule is important for binding of ACTH to its adrenocortical receptors. The estimation of the maximal response in the presence of several ACTH analogues shows, that the active center for steroid hormone stimulation is located at positions 4-10 of the ACTH molecule (Sayers et al., 1975). The synthetic analogue $ACTH^{1-24}$ shows full biological activity.
Stimulation of steroidogenesis in the adrenal cell is a complex process.To summarize, the following steps take place:

a. ACTH binds to its receptor and activates the adenylate cyclase,

b. the cAMP formed activates a protein kinase,

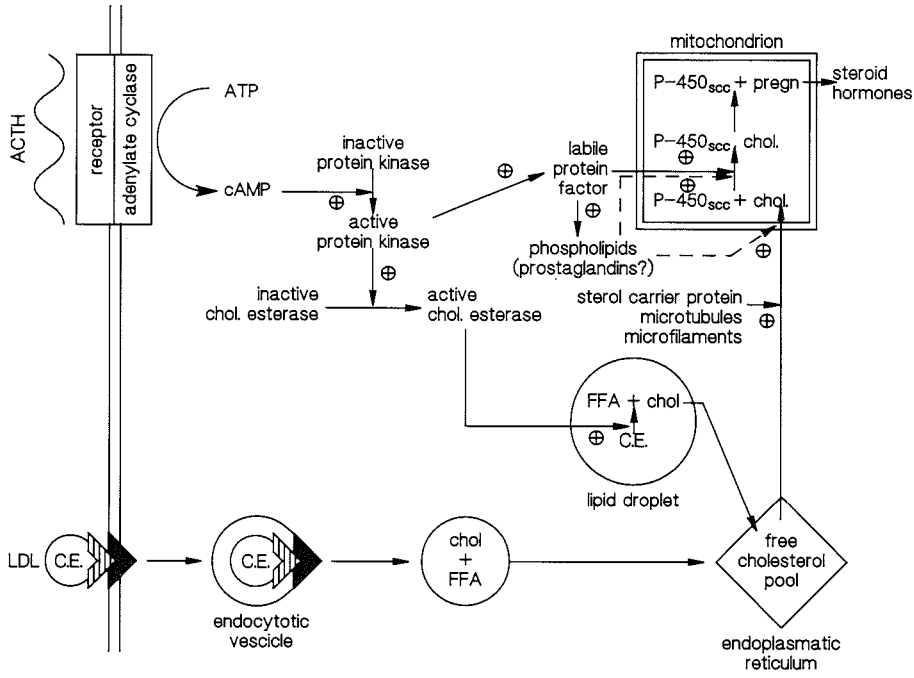


Figure 2.8: A schematic proposal for the action mechanism of ACTH in adrenal fasciculata cells.
chol=cholesterol pregn=pregnenolone
C.E.=cholesterol esters FFA=free fatty acids
+ =stimulation

c. this protein kinase activates a cholesterol esterase,
d. the activated cholesterol esterase liberates cholesterol in the lipid droplets, that moves to the mitochondrion,
e. a labile protein factor is produced, possibly under the influence of the active protein kinase, which is probably involved in the movement of cholesterol to the mitochondrion and/or binding of cholesterol to the cytochrome $P\text{-}450_{scc}$ on the matrix side of the inner mitochondrial membrane,
f. phospholipid metabolism is changed, yielding inositoltriphosphate which is probably also involved in the movement of cholesterol over the mitochondrial membrane,
g. cholesterol is converted into pregnenolone, which serves as a precursor of the other steroid hormones.

A schematic proposal is given in figure 2.8, adapted from Boggaram et al. (1984/85). The above-mentioned steps will now be discussed in more detail.

ACTH receptor

ACTH binds to receptors on the adrenocortical cell surface. In the rat adrenal there is evidence for the existence of two receptor sites, when using isolated cells from decapsulated rat adrenals (Yanagibashi et al., 1978):

1. a high-affinity, low-capacity receptor with a dissociation constant of 2.6×10^{-10} M and 7,350 sites per cell.
2. a low-affinity, high-capacity receptor with a dissociation constant of 7.1×10^{-9} M and 57,400 sites per cell.

Based on studies with $ACTH_{1-39}$ and two analogues ($ACTH_{5-24}$ and $ACTH_{6-24}$) Bristow et al. (1980) and Rybak and Ramachandran (1981) also concluded, that there are two different, steroidogenically responsive, receptors, one of which is coupled to the production of cAMP. Binding of ACTH to the other receptor may elicit steroidogenesis through another mechanism, probably involving calcium and/or cGMP (Yanagibashi et al., 1978; see also page 31). However, in a recent report Ramachandran (1984/85) described one class of receptors with an apparent K_d of 1.41 ± 0.21 nM. The number of sites was estimated to be 3840 ± 1045 per cell. Occupancy of a small fraction of the receptors was sufficient for inducing maximal steroidogenesis.

Adenylate cyclase

The ACTH-receptor-complex binds to the adenylate cyclase. Several factors like GTP, Mg^{2+}, Ca^{2+} and adenosine regulate the adenylate cyclase in the adrenal cortex (for a review see Glynn et al., 1979).

There is a great body of evidence to suggest that cAMP, produced by the adenylate cyclase, plays an intermediary role in the ACTH-stimulated steroidogenesis (Schimmer and Zimmerman, 1976; Rae et al., 1979; Hayashi et al., 1979; Podesta et al., 1979; Hyatt et al., 1980). Guillemant and Guillemant (1981) found a close correlation between plasma and adrenal corticosterone and adrenocortical protein-bound cAMP.

Different opinions exist on the role of guanosine nucleotide (cGMP) in the mechanism of action of ACTH. Hayashi et al. (1979) concluded that cGMP is unlikely to mediate the acute effects of ACTH on steroid production. Several authors found no positive relationship between the ACTH stimulation and the production of cGMP (Hayashi et al., 1979; Laychock and Hardman, 1978). In contrast others described an increase in cGMP after stimulation with ACTH (Sharma et al., 1976; Neri et al., 1978; Harrington et al., 1978; Hirai et al., 1980). In isolated adrenocortical carcinoma cells Perchellet and Sharma (1979a,b) described an increase in cGMP after stimulation by ACTH, which, however, rapidly declined to basal levels through an induction of a cGMP-phosphodiesterase. Calcium was obligatory for the activation of guanylate cyclase.

Protein kinase

The existence in the adrenal cell of a cAMP binding protein has been demonstrated (for a review see Schulster et al., 1976; Kimura, 1981). Garren and coworkers (1965) extensively studied this binding activity and proposed that cAMP binds to the receptor subunit of an inactive protein kinase. Upon binding the cAMP-receptor subunit dissociates from the catalytic subunit, which then becomes activated. A schematic drawing of this process is presented in figure 2.9. Sala et al. (1979) measured the fraction of cAMP bound to the receptor subunit of the protein kinase and demonstrated a close relationship with the ACTH concentration and with the ACTH-stimulated corticosterone production.

In isolated adrenal cells ACTH could activate the cAMP-dependent protein kinase within 2 minutes (Richardson and Schulster, 1973).

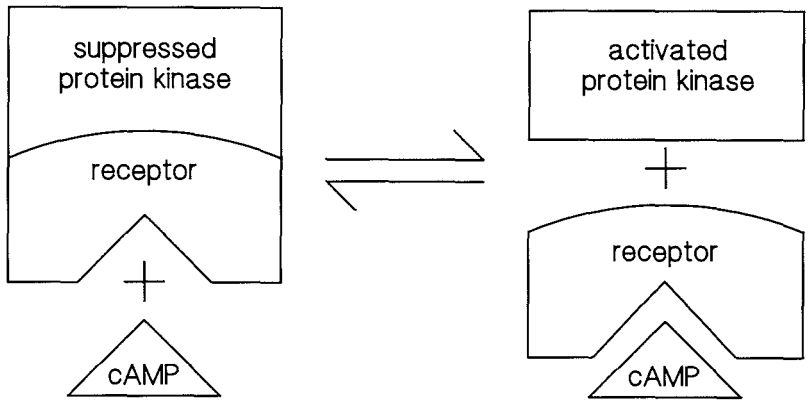


Figure 2.9: Activation of protein kinase by cAMP

Cholesterol esterase

Davis and Garren (1966) reported an in vivo stimulation of the conversion of cholesterol esters into cholesterol in the adrenal by ACTH. Trzeciak and Boyd (1974) postulated, that in the bovine adrenal cortex cholesterol esterase is activated by the cAMP-dependent protein kinase and that the activation involves a transfer of a terminal phosphate group from ATP onto the cholesterol esterase molecule (for a review see Vahouny et al., 1984/85). In vivo ACTH maintains the activity of the cholesterol esterase (Trzeciak et al., 1979).

Other protein factors

In addition to the cholesterol esterase, cAMP-dependent protein kinase may phosphorylate other proteins (Koroscil and Gallant, 1980). Bhargava et al. (1978) described a phosphorylated protein fraction, isolated from bovine adrenal mitochondria and separated from other proteins by gel electrophoresis. The concentration of this protein fraction was increased in the presence of ACTH and sharply decreased if an inhibitor of protein synthesis was present along with the ACTH. The changes in the extent of

phosphorylation were accompanied by corresponding changes in corticosteroid synthesis. Hofmann et al. (1978) found no protein kinase-mediated phosphorylation of a component or components of the cholesterol side-chain cleavage mixed-function oxygenase system.
Pon et al. (1986) described the production of a protein, the amount of which closely correlated with the steroid production in Leydig cells, adrenal cortex and corpus luteum. Glycosylation of this protein was a necessary step (Pon and Orme-Johnson, 1984/85).

Garren et al. (1965) proposed the involvement of a protein with a rapid turnover rate, stimulating the conversion of cholesterol into pregnenolone (labile protein factor). A protein was described with a molecular weight of 2200 (Pedersen, 1984/85). The lability of the protein may be caused by a very active ATP-dependent protease in adrenocortical mitochondria (Kimura, 1986). This protein factor is believed to stimulate steroidogenesis by regulating the availability of cholesterol to the cholesterol side-chain cleaving enzyme system, rather than having a direct effect on the mitochondrial enzyme system itself (Mahaffee et al., 1974; Farese and Prudente, 1977, 1978a). The function of this labile protein might be the transfer of cholesterol to the cholesterol side-chain cleaving enzyme complex (Mason et al., 1978b; Nakamura et al., 1980; Simpson et al., 1978; Williams-Smith et al., 1976).
Jefcoate et al. (1986) stated, that the labile protein factor is involved in the transport of cholesterol from the outer to the inner mitochondrial membrane. This transport may be facilitated by ACTH-inducible changes in the aqueous intermembrane space (Lambeth and Stevens, 1984/85) or by direct contacts between the two membranes (Wickner and Lodish, 1985).

Phospholipids

Studies on phospholipid metabolism in isolated rat adrenocortical cells revealed acute changes after ACTH treatment (for a review see Farese, 1984/85). These changes include:

a. an increased de novo synthesis of phosphatidic acid (PA), phosphatidylinositol (PI) and its mono- and di-phosphorylated derivatives (PIP and PIP_2),
b. hydrolysis of PIP_2 by phospholipase C or D yielding 1,2-diacylglycerol and inositoltriphosphate,
c. deacylation and reacylation of PI with release of arachidonic acid for

subsequent synthesis of prostaglandins. This process is calcium-dependent.

The action of ACTH on this phospholipid metabolism is probably mediated by cAMP. Cycloheximide blocks the increase in phopholipids. Most of the phospholipid increase is situated in the mitochondria (Igarashi and Kimura, 1984). Cholesterol itself does not easily pass across membranes (Jefcoate et al., 1986). Formation of a complex with polyphosphoinositides may enhance the permeation across the mitochondrial membrane.

Probably as a consequence of the release of arachidonic acid, mentioned before, prostaglandins seem to have a stimulatory effect on steroidogenesis in some species (Chavin et al., 1978; Hodges et al., 1978). In cat adrenal cells ACTH was able to stimulate prostaglandin synthesis from arachidonic acid (Laychock and Rubin, 1975, 1976).

Calcium

It is generally accepted, that calcium ions play a role in the ACTH-stimulated steroid production (for a review see Neher and Milani, 1976). Both cAMP and calcium could act as second messengers (Neher and Milani, 1978; Lymangrover and Martin, 1978). Podesta et al. (1980) found, that the calcium-induced steroidogenesis was accompanied by an increase in total intracellular and receptor-bound cAMP. In response to ACTH the increase in steroidogenesis always occurred concurrently with the uptake of extracellular calcium (Yanagibashi, 1979). An inhibitor of the calcium uptake (Verapamil) also inhibited the ACTH-stimulated steroid production. Calcium had no effect on the cAMP-stimulated steroidogenesis (Neher and Milani, 1978). Yanagibashi et al. (1978) suggested, that the two types of ACTH-receptors on the adrenal cell have different functions. The high-affinity receptor might be connected with increased calcium-influx to regulate steroidogenesis at physiological levels of ACTH, whereas the low-affinity receptor is coupled to the adenylate cyclase at supra-physiological concentrations of ACTH. Shima et al. (1979a,b) also suggested, that ACTH primarily increases intracellular calcium mobilization, thus stimulating directly the steroidogenesis, which is independent of the cAMP system. At higher ACTH concentrations the adenylate cyclase is activated, which depends on extracellular calcium.

Another effect of calcium may be situated at the level of the mitochondria. Mason et al. (1978a) found a stimulatory effect of calcium on

the adrenal mitochondrial pregnenolone synthesis in the presence of exogenous cholesterol. Farese and Prudente (1978b) described, that cholesterol-rich mitochondria from ACTH+cycloheximide-treated rats produced large amounts of pregnenolone, when a high concentration of calcium was present. Calcium may affect lateral displacement of cholesterol in the mitochondrial membrane into a compartment close to the cholesterol side-chain cleavage enzyme complex (Leaver and Boyd, 1981).

Other factors

Important factors in the ACTH-stimulation are the microtubules and microfilaments, probably involved in the transport of cholesterol from the lipid droplets to the mitochondrion (Hall, 1984/85).
The transport of cholesterol is also facilitated by the Sterol Carrier Protein (SCP), a basic protein with a molecular weight of 13,500 (Vahouny et al., 1984/85). This protein is synthesized in the liver and the intestine (Dempsey et al., 1986). The levels of SCP in various tissues correlate well with the capacity of each tissue to either synthesize or metabolize cholesterol. Adrenal mitochondria have a high level of SCP (Chanderbhan et al., 1986). ACTH has a stimulatory effect on the uptake of SCP in the adrenal cell (Dempsey et al., 1986). SCP enhances the transport of cholesterol from the cytosol to the outer mitochondrial membrane and probably also from the outer to the inner mitochondrial membrane (Vahouny et al., 1984/85; Chanderbhan et al., 1986; Jefcoate et al., 1986).

2.4 Aldosterone production and its regulation

Introduction

As already stated in Section 2.1, the adrenal gland consists of several zones, which are morphologically and functionally distinct. The outer zone, directly beneath the adrenal capsule, is the zona glomerulosa. This zone is the only site of production of aldosterone, the most important mineralocorticoid. The regulation of the sodium and potassium homeostasis is its main physiological function.

The main pathway for aldostrone synthesis is depicted in figure 2.10 (for a review see Veldhuis and Melby, 1981).
The corticosterone methyloxidase I (Ulick, 1976) is present in the zonae glomerulosa and fasciculata. 18-OH-corticosterone is considered as a side product, derived from a postulated C_{18}-oxygenated intermediate. Introduction of a second oxygen at the C_{18}-methyl group of the postulated intermediate, with subsequent loss of water, results in the formation of

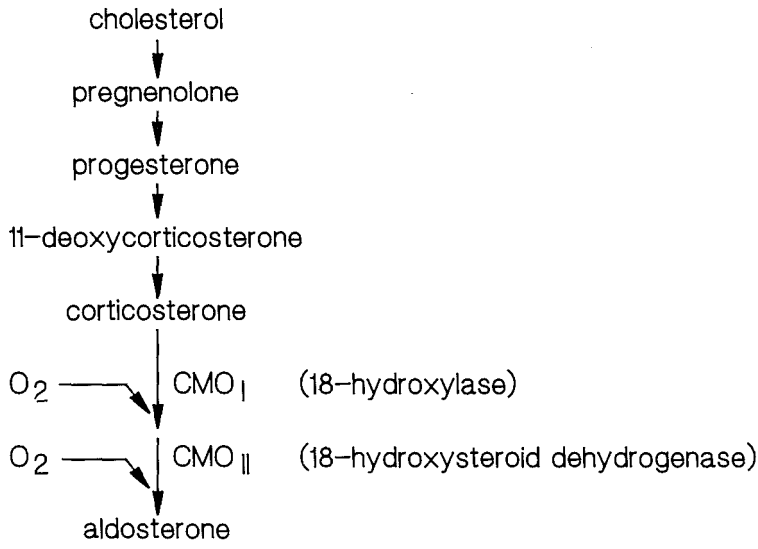


Figure 2.10: Main pathway for aldosterone synthesis.
C M O : corticosterone methyloxidase.

aldosterone. The enzyme corticosterone methyloxidase II is restricted to the zona glomerulosa.

In the rat an alternative pathway has been described for aldosterone biosynthesis with 18-OH-DOC and 18-OH-corticosterone as intermediates (Fattah et al., 1977; Aguilera and Catt, 1979). The relevance of this pathway is uncertain, as aldosterone production from DOC or corticosterone proceeds at a rate 10- to 20-fold faster than from the 18-hydroxylated intermediates. 18-OH-DOC has a weak mineralocorticoid activity. The close correlation between the plasma concentrations of 18-OH-DOC and corticosterone, whereas 18-OH-DOC and aldosterone do not correlate, suggests that in the rat 18-OH-DOC is an ACTH-dependent steroid (Tan and Mulrow, 1978). However Braley and Williams (1979) reported an increase in 18-OH-DOC after stimulating glomerulosa cells with angiotensin II.
The importance of this pathway in human adrenal steroidogenesis is not known.

In the regulation of aldosterone secretion, which is subject to multifactorial physiological control, the following factors have been described: angiotensin II, potassium, sodium, ACTH, serotonin, α-MSH, β-lipotropin and the central nervous system (for a review see Carey and Sen, 1986; Müller, 1988). Two regulatory sites in the aldosterone production can be distinguished. An "early step", being the cholesterol side-chain cleavage, and the "late steps", consisting of the conversion of corticosterone into aldosterone. The most important regulatory factors will be described in more detail.

ANGIOTENSIN II

Angiotensin II is recognized to play a major role in regulating aldosterone production under basal and salt-depleted conditions. Angiotensin II is able to stimulate the growth of the zona glomerulosa and its cells, which is mainly due to an increase in size of the smooth endoplasmatic reticulum and the mitochondrial compartment (Rebuffat et al., 1979; Mazzocchi et al., 1980).

The renin-angiotensin system is depicted in figure 2.11 (for a review see Ondetti and Cushman, 1982). The reaction is initiated by the release of renin, a 40,000 dalton protein, from the kidney. The output of renin increases, when the renal blood flow is reduced or following sodium

depletion. Renin acts on angiotensinogen, a 57,000 dalton plasma α_2 globulin and splits off a decapeptide, named angiotensin I. This decapeptide is converted into angiotensin II, an octapeptide. The conversion of angiotensin II into angiotensin III and further degradation of this heptapeptide is controlled by a group of enzymes, known as angiotensinases.

Angiotensin II in physiological and supraphysiological concentrations has a stimulatory effect on aldosterone production in isolated rat and canine glomerulosa cells (Fredlund et al., 1975; Braley et al., 1980; Tait et al., 1980b; Mendelsohn and Kachel, 1980). In fasciculata cells the corticosterone production did not increase in response to angiotensin II (Braley et al., 1980).

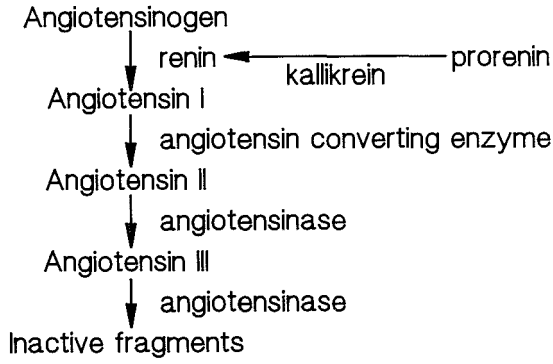


Figure 2.11: The Renin-Angiotensin system.

Specific receptors for angiotensin II have been demonstrated in zona glomerulosa cells (Douglas et al., 1978). The number of receptor sites for angiotensin II in the glomerulosa cell is regulated by angiotensin II itself, as demonstrated in rats (Hauger et al., 1978). An increase in the number of receptors is accompanied by an increased steroid response. Angiotensin II also has a stimulating effect on the cholesterol side-chain

cleavage and 21- and 11β-hydroxylase activities (Aguilera et al., 1980). In isolated rat glomerulosa cells angiotensin II and III are equipotent in stimulating aldosterone synthesis. However, angiotensin III is degraded more rapidly (Aguilera et al., 1979).

It is generally believed that cAMP plays no role in the action mechanism of angiotensin II (Bell et al., 1981; Müller, 1988). However one report described a close correlation between cAMP output and aldosterone production after stimulation with angiotensin II (Bing and Schulster, 1978). The steroidogenic response to angiotensin II and III could be blocked by inhibitors of calcium transport, suggesting a calcium-dependent mechanism.

Binding of angiotensin II to its receptor leads to the breakdown of a membrane phospholipid into 1,2-diacylglycerol and inositoltriphosphate by the membrane-bound phospholipase C. The first compound activates protein kinase C. The second compound releases calcium from the endoplasmatic reticulum; the calcium ions are bound to calmodulin, which leads to the activation of calmodulin-dependent protein kinase(s). Cycloheximide blocks the steroidogenic response to angiotensin II.

The effect of angiotensin II on early and late steps of aldosterone synthesis was studied in rat and dog zona glomerulosa cells (Aguilera and Catt, 1979). Both early and late steps were stimulated. Quantitatively the effect on the early step is much more pronounced than on the late steps. Kramer et al. (1980) found, that the late steps required chronic exposure to angiotensin II in order to contribute to an increase in aldosterone synthesis. This step thus seems to be important in the long term control. Angiotensin II enhanced the rate of cholesterol side-chain cleavage by enhancing the association of cholesterol with the cytochrome P-450 (Kramer et al., 1980). Cycloheximide had no effect on this step, suggesting there is no role for a labile protein in the mechanism of action of angiotensin II. These results show a divergence in the mechanisms, by which angiotensin II and ACTH promote steroidogenesis, as was already shown with respect to the involvement of cAMP (Müller, 1988). The action of angiotensin II on the late steps in aldosterone synthesis also seemed to be mediated by an effect on the cytochrome P-450 enzymes involved, i.e. by promoting the association of corticosterone with cytochrome $P\text{-}450_{18}$. However, cycloheximide could completely abolish the effect on the late steps, suggesting de novo synthesis of a protein, that promotes the association of

corticosterone with cytochrome P-450$_{18}$. Angiotensin II had no effect on the microsomal or mitochondrial cytochrome P-450 levels, nor on the 11β-, 21- or 18-hydroxylase activities.

As in the control of steroidogenesis by ACTH, Farese et al. (1981b) provided evidence that phospholipids might play a role in the stimulation of aldosterone synthesis by angiotensin II.

SODIUM ION

In a wide variety of animals and in man a correlation has been established between the sodium ion status and aldosterone secretion. Loss of sodium ions or decreased sodium intake stimulates aldosterone secretion. This regulation of aldosterone secretion is an important physiological control system in view of the effect of aldosterone itself on sodium ion retention. The mechanism of this regulation is very complex.
Sodium has no direct effect on the aldosterone production (Enyedi and Spät, 1981). In addition acute changes in extracellular sodium concentration do not modify the adrenal response to angiotensin II in vitro. However in vivo there is an increased sensitivity to angiotensin II during prolonged sodium deficiency (Aguilera et al., 1980), probably caused by angiotensin II itself via increased numbers of cellular receptors (Hauger et al., 1978). Studies of Aguilera et al. (1980) suggested, that effects on aldosterone production of changes in the sodium balance are mediated predominantly by angiotensin II. An additional factor in this process may be extracellular potassium. But other factors can not be excluded, e.g. ACTH. In normal subjects Kigoshi et al. (1980) noticed an enhancing effect of sodium depletion on the ACTH-induced aldosterone production under conditions in which the angiotensin II synthesis was blocked by converting enzyme inhibitors. The precise mechanism of this sensitization is not known.

Studies on rats (Kramer et al., 1979) indicated, that microsomal and mitochondrial cytochrome P-450 concentrations in the zona glomerulosa were not changed during sodium ion depletion. 11β- and 18-hydroxylation of DOC and 21-hydroxylation of progesterone were also unaffected. However the rate of cholesterol side-chain cleavage and the conversion of corticosterone into 18-OH-corticosterone and aldosterone were increased during sodium ion depletion. These increased enzyme activities were mediated, at least in part, by enhanced binding of cholesterol and corticosterone to mitochondrial cytochrome P-450$_{scc}$ and cytochrome P-450$_{18}$ respectively.

These changes after dietary sodium depletion were similar to the effects of angiotensin II on cytochrome P-450 enzymes (Kramer et al., 1980).

POTASSIUM ION

Many reports exist on the stimulatory effect of potassium on the aldosterone production in vivo and in isolated glomerulosa cells (Fredlund et al., 1975; Tait and Tait, 1976; McKenna et al., 1978; Komor and Müller, 1979). Both in rat and dog glomerulosa cells (Bell et al., 1978; Aguilera and Catt, 1979; Tait et al., 1980a,b) and in bovine and dog glomerulosa cells (McKenna et al., 1978) potassium stimulated the early and late steps in aldosterone biosynthesis.

The action mechanism of potassium is not exactly known. An increase in extracellular potassium caused a parallel increase in intracellular potassium and corticosterone output in isolated rat adrenal glomerulosa cells (Mendelsohn and Mackie, 1975). Intracellular potassium is important in modifying the acute response of glomerulosa cells to angiotensin II and ACTH (Braley and Williams, 1978). In rat adrenal cells 8.4 mM K^+ increased the output of cAMP (Bell et al., 1981). The fact that changes in potassium can still modify steroid output, when the cAMP has a maximal biologically effective concentration at the site of action, suggests another stimulating effect than through cAMP alone (Tait and Tait, 1976). An increased extracellular potassium concentration lowers the membrane potential and leads to the opening of potential-dependent calcium channels (Müller, 1988). In this way more calcium ions may enter the cell. These calcium ions may bind to calmodulin, a cytoplasmic protein containing 148 amino acids, which undergoes a conformational change that allows it to bind to other proteins and to modify enzymic activity, e.g. a protein kinase (Means and Chafouleas, 1982).

In rat adrenal glomerulosa cell cultures the pattern of DOC metabolism could be influenced by potassium; with high concentrations of potassium (>8 mM) the main products were 18-OH-B and aldosterone and at 4 mM of potassium 18-OH-DOC and corticosterone were the main products. The long-term action of potassium was not mediated by cAMP and was inhibited by glucocorticoids. Potassium may stimulate the activity of the enzymes of the late steps in aldosterone synthesis, possibly through a protein synthesis step (Hornsby and O'Hare, 1977). As found for angiotensin II, potassium affected the incorporation of phosphate into glomerulosa phospholipids

(Farese et al., 1981).

ACTH

ACTH is a potent short term stimulus of aldosterone secretion in vivo (Hilfenhaus, 1977; Komor and Müller, 1979; Kigoshi et al., 1980) and in vitro (Fredlund et al., 1975; Aguilera and Catt, 1979). ACTH is not important in the chronic maintenance of aldosterone secretion (Fraser et al., 1978). In isolated rat adrenal glomerulosa cells the production of pregnenolone in response to ACTH was higher, compared with the response to angiotensin II or potassium (Aguilera and Catt, 1979). ACTH exerts its effect on the early step in aldosterone biosynthesis. In dog adrenal glomerulosa cells no specific effect on the late steps was found (Aguilera and Catt, 1979). However in rat glomerulosa cells ACTH did affect also the late steps. This may be an effect which is secondary to the increase in corticosterone production, since corticosterone can induce its own conversion into aldosterone by a mechanism, which is still unknown (Bell et al., 1978; Tait et al., 1980b).

CALCIUM

Two inhibitors of calcium membrane transport (verapamil and lanthanum) completely abolished the ACTH, angiotensin II or potassium stimulated aldosterone output in rat glomerulosa cells (Schiffrin et al., 1981). The increased aldosterone synthesis seen at small doses of ouabain (Braley and Williams, 1978) was also inhibited by verapamil and lanthanum; probably the inhibition of the Na^+-K^+-ATPase by ouabain is accompanied by an increase in the calcium transport. These results suggest that calcium mobilization is critical for the stimulation of aldosterone biosynthesis by ACTH, angiotensin II, potassium and the action of ouabain.
In the ACTH stimulated aldosterone production by glomerulosa cells, calcium is required for the coupling of the ACTH-receptor-complex with the adenylate cyclase (Fakunding et al., 1979). However the differential effect of calcium transport inhibitors on the cAMP or aldosterone production in response to ACTH suggests that extracellular calcium is also able to stimulate steroidogenesis without involvement of the cAMP system (Shima et al., 1979b). This stimulation involves the potential-dependent calcium channels in the cell membrane, binding of calcium ions to calmodulin and activation of a calmodulin-dependent protein kinase (Müller, 1988).

In glomerulosa cells, stimulated with potassium at concentrations which gave a marked increase in steroid output, no effect was found on the rate of calcium efflux (Williams et al., 1981), in contrast with the effect of angiotensin II, which caused a dose-dependent increase in the rate of calcium efflux. This difference may be explained by the positive effect of potassium on the calcium influx via the potential-dependent calcium channels.
Calcium is not necessary for the binding of angiotensin II to its receptors.

OTHER FACTORS

Serotonin can stimulate aldosterone synthesis by means of the generation of cAMP, the early steps in the pathway being the main site of action (Bell et al., 1978; Tait et al., 1980b; Mendelsohn and Kachel, 1981).

In normal rat adrenal glomerulosa cells α-MSH stimulated corticosterone production, but had only a slight stimulatory effect on aldosterone production. However in sodium-restricted animals the glomerulosa cells were much more sensitive to α-MSH with regard to the production of aldosterone and 18-OH-corticosterone. Corticosterone production did not increase in the presence of α-MSH (Vinson et al., 1981a,b).

The glycoprotein fraction, isolated from normal human urine, contains a factor, which stimulates aldosterone production in rabbit glomerulosa cells (aldosterone-stimulating factor: ASF). ASF is produced in the anterior pituitary gland and can be distinguished from ACTH (it did not increase cAMP levels) and from angiotensin II (a competitive antagonist of angiotensin II had no effect on ASF) (Saito et al., 1981; Sen et al., 1981).

Other stimulators of aldosterone biosynthesis with physiological importance are: prolactin, vasopressin, prostaglandins and histamine (Müller, 1988).

2.5 Cholesterol and hydroxylated sterols

Introduction

Cholesterol is a very important sterol in nature. It serves several functions:

1. in eukaryotes it is one of the main components in the architecture of cellular membranes,
2. all steroid hormones produced in the adrenal, ovary and testis are derived from cholesterol,
3. cholesterol is the precursor of bile acids, necessary for the fat resorption in the intestine.

Oxygenated or hydroxylated sterols are structurally related to cholesterol but can have different and/or additional oxygen functions, double bonds or alkyl groups in the cholesterol nucleus or the side-chain.

Cholesterol or hydroxylated sterols play a role in the etiology of several hereditary or non-hereditary diseases, which are mentioned in Chapter 1. In this section the cholesterol synthesis will be briefly described. In addition the occurrence of sterols will be discussed, including the auto-oxidation of cholesterol. Several hydroxylated sterols have adverse effects on cellular and subcellular processes, some of which can be overcome by the addition of cholesterol or mevalonic acid. These effects and their possible action mechanisms will be reviewed.

Cholesterol synthesis and HMG-CoA reductase activity

Before turning to the occurrence and effects of hydroxylated sterols and their action mechanism the cholesterol synthesis will briefly be described. The HMG-CoA reductase is the key enzyme in the control of cholesterol synthesis. A schematic representation is given in figure 2.12. The main site of cholesterol synthesis is the liver. Other production sites are the adrenal, the testis and the ovary.

In the rat adrenal gland the HMG-CoA reductase activity is influenced by the demand for cholesterol to be used for steroid synthesis and the availability of a supply of exogenous cholesterol from plasma lipoproteins. In the presence of ACTH the HMG-CoA reductase activity remains low as long as cholesterol can be derived from plasma lipoproteins. However when hepatic lipoprotein secretion is blocked, the HMG-CoA reductase activity may rise by a factor of 200. This rise is

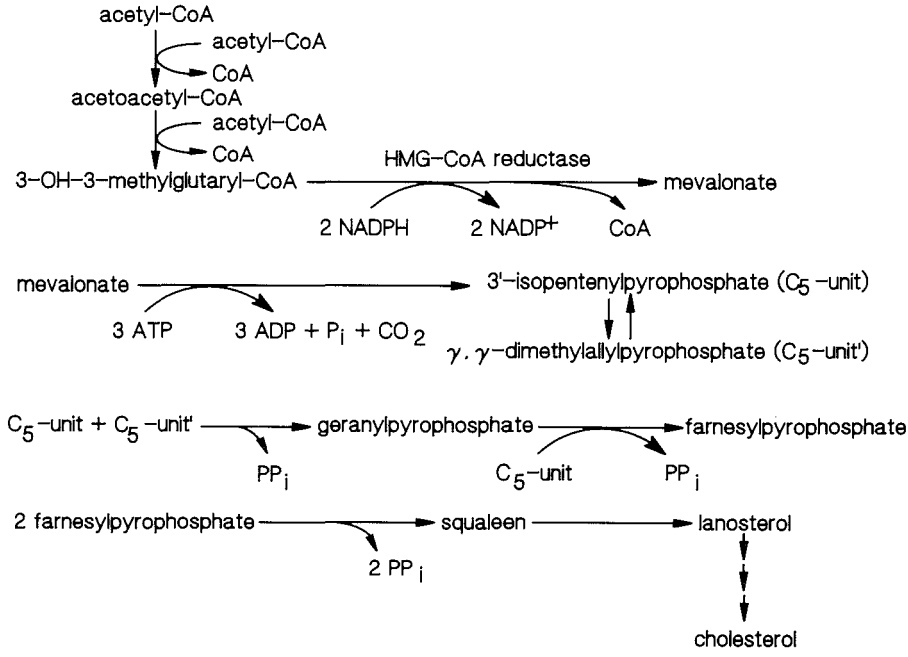


Figure 2.12: Schematic representation of the cholesterol biosynthesis.

associated with a parallel increase in cholesterol synthesis but occurs only if the adrenal content of cholesteryl-esters has declined by more than 75% (Balasubramaniam et al., 1977). Subsequent administration of cholesterol in the form of HDL or LDL restores the adrenal cholesteryl-ester content and at the same time suppresses HMG-CoA reductase activity to the normal value.

Occurrence of hydroxylated or oxygenated sterols in man and rat

An important sterol in human brain tissue is 24-OH-cholesterol (cerebrosterol) in addition to traces of 26-OH-cholesterol (Smith et al., 1973c). In the sulfate fraction of faeces from infants, aged 1-4 months, Gustafsson and Sjövall (1969) were able to demonstrate 22R-, 24-, 25- and 26-OH-cholesterol, 20R,22R-diOH-cholesterol and 22-keto-cholesterol in

addition to cholesterol. Using a gaschromatographic-mass spectrometric method tissue levels of oxygenated sterols in rat liver were estimated (Sanghvi et al., 1978). 7α-OH-colesterol, 7β-OH-cholesterol and 7-keto-cholesterol were present in the range of 1-2 μg/g of liver; the presence of 25-OH-cholesterol could not be demonstrated.
In rat adrenal extracts cholestenone levels were determined using a liquid chromatographic method; the cholestenone concentration ranged from 32 to 165 μg/g tissue (Tallova and Hakl, 1980).
The fatty acid esters of 24- and 26-OH-cholesterol have been detected in human aortal tissue (Teng and Smith, 1975).

Physiologically, many hydroxylated sterols are formed from cholesterol through the action of hydroxylases. In the adrenal gland steroid production is a process with many hydroxylation reactions. The same is true for bile acid synthesis in the liver. In addition to physiologically active hydroxylases, Alsema et al. (1980) observed a 25-hydroxylase activity in bovine adrenal cortex mitochondria, active on 20S-OH-cholesterol, when the normal side-chain cleavage was partially inhibited by elevation of the pH of the incubation medium. Degenhart et al. (1984) observed a 25-hydroxylase activity upon 20S-hydroxy-4-cholesten-3-one, when this sterol was incubated with bovine adrenal cortex mitochondria. Alsema et al. (1982b) described the conversion of 22S-OH-cholesterol into 22-keto-cholesterol in bovine adrenal cortex mitochondria.

Auto-oxidation of cholesterol

Auto-oxidation of cholesterol is a well-known phenomenon, especially in air-aged cholesterol. The major products of auto-oxidation are cholesterol-7α-hydroperoxide and cholesterol-7β-hydroperoxide. These initial products are converted into 7α-OH-cholesterol, 7β-OH-cholesterol and 7-keto-cholesterol. Similar reactions occur in the side-chain, yielding 20-, 24-, 25-, and 26-OH-cholesterol (van Lier and Smith, 1970a,b, 1971: van Lier and Kan, 1972: Smith et al., 1973a,b; Teng et al., 1973a,b; van Lier and Rousseau, 1976).
Air-aged samples of cholesterol contained components, which were mutagenic towards Salmonella Typhimurium. These mutagenic components were associated with cholesterol auto-oxidation products. Pure, non-mutagenic cholesterol, free from auto-oxidation products, became mutagenic upon heating in air or

following exposure to γ-radiation (Smith et al., 1979). The identification of the mutagenic components has not been achieved.

Cholestenone feeding in rat and man

Feeding studies with cholestenone in rat and man showed a significant decrease of serum cholesterol levels. However at the same time cholestanol accumulated in serum and tissues (Tomkins et al., 1957; Steinberg et al., 1958). Cholestanol has atherogenic properties, which rules out the use of cholestenone as a therapy in hypercholesterolemia. High doses of cholestenone (1% by weight in the basic diet) are toxic in rats, causing a suppression of adrenal function, accompanied by marked hypertrophy, arrest of growth, lethargy and a decreased food intake. The adrenal hypertrophy is caused by an increase in the number of cells and by accumulation of lipid-like material; the content of cholesterol decreased (Stárka et al., 1981). Cholestenone most probably affects cholesterol biosynthesis or utilization for steroidogenesis. However, cholestenone or a metabolite may also be inserted into the cellular membrane (unpublished observations).

Effects of sterols on HMG-CoA reductase activity

Looking at L-cells (mouse fibroblasts), growing in a chemically-defined, sterol-free medium several hydroxylated sterols are able to inhibit the cell culture growth. 25-OH-cholesterol is the most potent inhibitor, followed by 20S-OH-cholesterol, 7-keto-cholesterol and 7β-OH-cholesterol (Chen et al., 1974). Cholesterol or mevalonic acid, but not 3-hydroxy-3-methylglutaric acid can counteract this inhibitory effect of the sterols. The most likely explanation is a repression of the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity, the regulatory enzyme in cholesterol biosynthesis.

The same mechanism plays a role in the inhibition of endocytosis by hydroxylated sterols. 25-OH-cholesterol and 20S-OH-cholesterol have a marked inhibitory effect on the endocytosis of peroxidase in L-cells, a process which can be restored with mevalonic acid or cholesterol (Heiniger et al., 1976).

Chen et al. (1978) have described effects of 25-OH-cholesterol and 7-keto-cholesterol on the cellular ion transport. In sterol-treated L-cells the intracellular K^+ concentration decreases, whereas the concentration of

Na^+ increases. The uptake of $^{86}Rb^+$ is enhanced; also the Rb^+ efflux increases in the sterol depleted cells. All effects are counteracted in the presence of mevalonic acid. These authors find no effect on the (Na^+-K^+) ATPase activity in homogenates and plasma membrane preparations from treated cells. However Seiler and Fiehn (1976) describe an inhibition of the (Na^+-K^+) ATPase in erythrocyte membranes, when erythrocyte ghosts are treated with cholesterol oxidase, thus converting part of the membrane cholesterol into cholestenone.

Direct measurement of the HMG-CoA reductase activity reveals the inhibitory effect of hydroxylated sterols on the cholesterol synthesis. Sterols derived from cholesterol by hydroxylation in positions 6, 7, 15, 20S, 22R, 22S, 24 or 25 inhibit sterol synthesis from acetate and suppress the HMG-CoA reductase activity in mouse fetal liver and L-cell cultures (Kandutsch and Chen, 1974, 1975, 1978).

In human fibroblasts suppression of the HMG-CoA reductase activity can be achieved by incubating these cells in the presence of LDL, in which cholesterol is replaced by 25-OH-cholesteryl oleate. The cells stop growing and eventually die. These effects can be overcome by supplementation with cholesterol in ethanol or mevalonic acid. In fibroblasts of a patient with familial hypercholesterolemia, lacking LDL receptors, the [25-OH-cholesteryl oleate]-LDL has no effect on the reductase activity. These results suggest, that the toxicity of [25-OH-cholesteryl oleate]-LDL is due to its suppression of the HMG-CoA reductase activity, leading to cellular cholesterol deficiency (Krieger et al., 1978).

Lanosterol derivatives with an additional oxygen function at carbon 30 (an intermediate in the demethylation of the 14α-methyl group) strongly inhibit the incorporation of acetate into cholesterol in cultured Chinese hamster lung cells, L-cells and fetal mouse liver cells (Schroepfer et al., 1978, 1980; Gibbons et al., 1980). These sterols may therefore be involved in the regulation of cholesterol synthesis. Several reports describe an inhibitory effect of a number of 15-oxygenated sterols on sterol synthesis and HMG-CoA reductase activity (Schroepfer et al., 1977a,b, 1979). Some of these have been shown to be convertible into cholesterol in rat liver homogenates (e.g. 14α-methyl-5α-cholest-7-en-3β,15β-diol). Feeding rats or mice a diet containing 0.1-0.2% of a 15-oxygenated sterol [5α-cholest-8(14)-en-3β-ol-15-one], a profound hypocholesterolemic effect was shown

(Schroepfer et al., 1977c).

Another effect of hydroxylated sterols is the suppression of the rate of DNA synthesis. In L-cells the oxygenated sterols obviously block cell division in the G_1-phase of the cell cycle. It may be that a failure to produce cholesterol for membrane formation during an early stage of the cell cycle prevents further steps leading to mitosis.

Mechanism of inhibition of HMG-CoA reductase activity

The mechanism by which the sterols exert their effect may be a repression of the synthesis of HMG-CoA reductase (for a review see Kandutsch et al., 1978).

In vivo feeding studies with 25-OH-cholesterol show a decrease in the rat liver HMG-CoA reductase activity by 60%; this is less than reported for cultured cells (Erickson et al., 1978). This effect is reached after 18 hours and does not change up to 66 hours of feeding 25-OH-cholesterol. In the isolated liver of the rat, perfusion with 25-OH-cholesterol decreases the reductase activity to 54% of its initial value in 30 minutes. This is much more than can be accounted for by an inhibition of the HMG-CoA reductase synthesis. Consequently it seems probable, that 25-OH-cholesterol in the perfused liver inactivates HMG-CoA reductase to some degree. In isolated hepatocytes 25-OH-cholesterol induces the same rapid decrease in reductase activity within one hour. During the next two hours reductase activity rises again to 90% of the initial value only in the presence of low concentrations of 25-OH-cholesterol (<5 μg/ml). With higher concentrations the reductase activity remains low. The recovery of reductase activity may be explained by a rapid metabolism of 25-OH-cholesterol to inactive products, permitting synthesis of new HMG-CoA reductase. Since in cell-free systems 25-OH-cholesterol does not inhibit HMG-CoA reductase activity, the intact cell seems to be necessary for expression of the inhibitory action of this sterol.

The sterols do not affect the metabolism of acetate to fatty acids, nor the synthesis of RNA or protein. So, in addition to a repression of the synthesis of HMG-CoA reductase, a direct inactivation or an increased rate of degradation can not be excluded.

Three cell lines are isolated, two of which are completely and one of which is partially resistant to the killing effects of 25-OH-cholesterol (Chen et al., 1979; Cavenee et al., 1979; Sinensky, 1977). These cell

lines, selected for growth in the presence of 25-OH-cholesterol, are also resistant to the inhibitory effects of other sterols and serum upon HMG-CoA reductase and cholesterol synthesis, suggesting a common step in the suppression of cholesterol synthesis by these inhibitors. Structural changes in the HMG-CoA reductase are less likely, because several parameters, e.g. K_m, heat stability, susceptibility to inhibition by Mg^{2+} and ATP, are identical in the wild-type and mutant cells. The molecular basis of the resistence to 25-OH-cholesterol remains unknown.

Other effects of hydroxylated sterols

Not all effects of hydroxylated sterols can be attributed to a suppression of the sterol synthesis. The inhibition of human polymorphonuclear leukocyte chemotaxis by oxygenated sterol compounds is not likely to be mediated by a decreased sterol synthesis, because these cells lack certain enzymes necessary for sterol biosynthesis. In addition the effects are already visible at 5 minutes after the exposure of the cells to the sterols in a lipoprotein-depleted medium. Cholesterol causes a partial reversal of the sterol inhibition of chemotaxis. This effect may be the consequence of insertion of the sterol into the leukocyte plasma membrane (Gordon et al., 1980).

Hoffman et al. (1981) studied effects of hydroxylated sterols on human marrow granulocytic progenitor cells; 25-OH-cholesterol, 20S-OH-cholesterol, 7-keto-cholesterol and 6-keto-cholesterol had an inhibitory effect on the proliferation of these cells. This effect was reversible by cholesterol, but not by mevalonic acid. Effects different from inhibition of cholesterol synthesis were suggested, e.a. insertion of sterols into the cellular membrane.

Streuli et al. (1979) found an inhibition of the E-rosette formation of cultured human lymphocytes with sheep erythrocytes after exposure of the lymphocytes to several oxygenated sterols. 5α-OH-6-keto-cholestanol had an effect already after 15 minutes of exposure. With 25-OH-cholesterol, 20S-OH-cholesterol and 7α-OH-cholesterol a longer exposure time was necessary. The effect could be abolished by cholesterol, LDL or HDL, but not by mevalonic acid.

When erythrocytes are exposed to oxygenated sterol compounds, they become echinocytic. The initial stage of echinocyte formation is apparent within 2 minutes of exposure. The fraction of the sterols relative to

cholesterol, which is inserted into erythrocyte membranes, ranges from 2-4% in the case of 25-OH-cholesterol to 36% for 7β-OH-cholesterol. All insertion occurs within one hour of red cell exposure and remains constant over the next 23 hours (Hsu et al., 1980). Serum lipoproteins but not cholesterol may prevent the insertion of oxygenated sterols into the erythrocyte membrane (Streuli et al., 1981a). Following the insertion of oxygenated sterols into erythrocyte membranes a diminished osmotic fragility was observed. The shift of the osmotic fragility curve towards lower NaCl concentrations was proportional to the amount of sterol inserted (Streuli et al., 1981b). 7β-OH-cholesterol, 22-keto-cholesterol and 20S-OH-cholesterol were most effective of the sterols tested.

Taylor er al. (1977) investigated the effects of impurities (not further defined) from aged cholesterol on rabbit aortic smooth muscle cells. In vivo administration of these compounds induced focal fibrotic lesions. Intravenous injection of oxygenated sterols into rabbits caused cell death and inflammation in the aorta and pulmonary arteries within 24 hours after the third injection of the sterols (one injection per day; 5 mg/kg). 25-OH-cholesterol and cholestan-3β,5α,6β-triol were the most potent compounds (Imai et al., 1976, 1980). 7-Keto-cholesterol inhibited the in vivo cholesterol uptake in the arterial wall by 55%; there were only few pathological changes in the organs of animals injected with 7-keto-cholesterol (Sarma et al., 1976; Santillan et al., 1980).

In vitro studies with cultured rabbit aortic smooth muscle cells also showed a cytotoxic effect of auto-oxidation products of cholesterol: 25-OH-cholesterol and cholestan-3β,5α,6β-triol were the most toxic compounds among the sterols tested, causing 75-100% dead or dying cells within 24 hours (Peng et al., 1978, 1979). But also 20S-OH-cholesterol, cholestan-3β-ol-6-one and cholestan-3β,5α-diol-6-one were moderately toxic. Purified cholesterol and 5,6-epoxy-cholesterol had no effect, even up to a concentration of 100 μg/ml.

In vivo administration of 25-OH-cholesterol or 7-keto-cholesterol to mice led to a loss of weight and suppression of growth. Growth suppression due to 25-OH-cholesterol could only partially be relieved by cholesterol (Kandutsch et al., 1977). Sterol synthesis in the intestinal mucosa was inhibited by the sterols with marked suppression of food intake and growth, which were possibly secondary effects of alterations in the intestinal mucosa.

Cholesterol esterification

Differential effects of some sterols are found on cholesterol synthesis and cholesterol ester formation. 25-OH-cholesterol and 7-keto-cholesterol inhibit cholesterol synthesis via HMG-CoA reductase. However, these sterols stimulate cholesterol ester formation in cultured human fibroblasts (Brown et al., 1975; Goldstein et al., 1978). A structural analogue of 7-keto-cholesterol (with carbon atom 20 replaced by an oxygen and without a C-21 group) inhibits HMG-CoA reductase, but also inhibits cholesterol esterification. The precise mechanism of the stimulation of cholesterol ester formation remains to be established.

2.6 Hydroxylase activity in the liver

Several hydroxylases are involved in bile acid synthesis (see Björkhem and Danielsson, 1974; Salen and Shefer, 1983) and the detoxification of foreign toxic compounds (Pelkonen, 1977). It will be shown in this chapter that, under conditions of impaired bile acid synthesis, as in CTX, another hydroxylase becomes active.

The metabolic route for bile acid synthesis is depicted in figure 2.13. Several hydroxylases, most of them microsomal enzymes, but some also situated in the mitochondrion, are involved in this process. The key enzyme in the regulation of bile acid synthesis is the microsomal cholesterol 7α-hydroxylase. Its activity can be modulated by phosphorylation/dephosphorylation (Sanghvi et al., 1981). The enzyme system includes a cytochrome P-450 and a cytochrome P-450 reductase (Danielsson and Wikvall, 1981). The 12α-hydroxylase is the key enzyme to determine the relative amounts of cholic acid and chenodeoxycholic acid. It is also a microsomal enzyme.

Under normal conditions 5β-cholestan-3α,7α,12α-triol is 26-hydroxylated by both a mitochondrial and a microsomal enzyme. The mitochondrial 26-hydroxylase is a mixed function oxidase, bound to the inner mitochondrial membrane; cytochrome P-450 is involved. It has been shown that the mitochondrial cytochrome P-450 has a much higher potential for 26-hydroxylation than the microsomal cytochrome P-450 (Pedersen et al., 1979a). The mitochondrial 26-hydroxylase has a broad substrate specificity and hydroxylates many different C-27 sterols, including cholesterol (Björkhem and Gustafsson, 1973; Aringer and Nordström, 1981). The microsomal fraction of human liver has little capacity to 26-hydroxylate 5β-cholestan-3α,7α-diol and 5β-cholestan-3α,7α,12α-triol. The next step in the synthesis of bile acids is the introduction of a 24S-hydroxyl group. The localization of the enzyme is both microsomal and mitochondrial, the microsomal enzyme being more active (Gustafsson, 1975). This 24S-hydroxylation step is not catalyzed by a mixed function oxidase, but is analogous to fatty acid β-oxidation and involves an acyl-CoA dehydrogenase and hydratase.

Under conditions where the normal bile acid synthesis is blocked, alternative routes may become more important. One such condition is the hereditary disease Cerebrotendinous Xanthomatosis (CTX; see Chapter 1), in

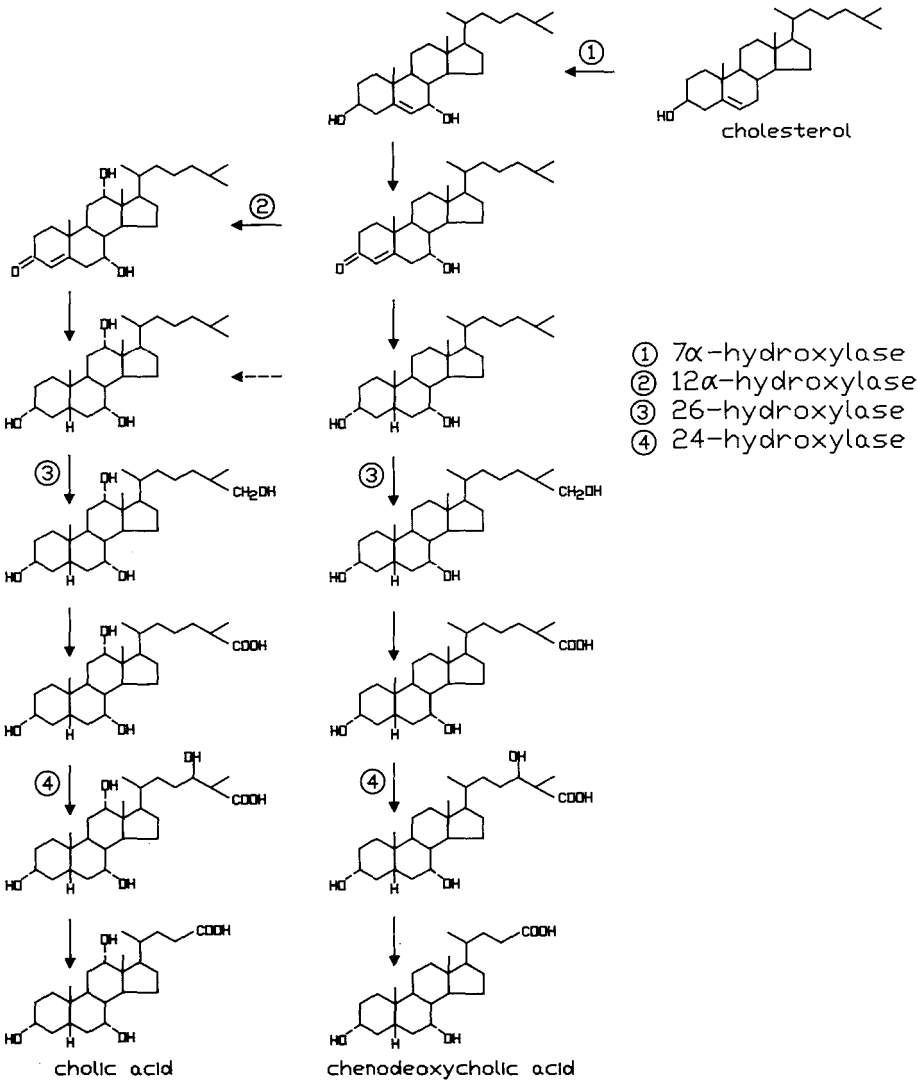


Figure 2.13: Major pathway for cholic acid and chenodeoxycholic acid synthesis in the liver.

which the 26-hydroxylase is most probably absent. An alternative route via 25-hydroxylation becomes prominent (see figure 2.14). This pathway is catalyzed by microsomal and soluable enzymes and is described in man and in rats (Shefer et al., 1976; Cheng et al., 1977; Wolthers et al., 1983). Some mitochondrial 25-hydroxylase activity of cholesterol has also been demonstrated in the rat liver (Björkhem and Gustafsson, 1973). It is possible, that this hydroxylase and the mitochondrial 25-hydroxylase active on vitamin D_3, are one and the same species of cytochrome P-450 (Björkhem and Holmberg, 1978; Pedersen et al., 1979b).

In patients with CTX a relative deficiency of the 24S-hydroxylase also contributes to the subnormal production of bile acids (Salen et al., 1979). The 23R-hydroxylase is an enzyme not being used in the regular bile acid synthesis. Only in conditions where the normal synthesis is blocked, as in CTX, does 23R-hydroxylation occur. Wolthers et al. (1983) found 5β-cholestan-3α,7α,12α,23R,25-pentol to be the most prominent bile alcohol in the urine of such a patient. This microsomal 23R-hydroxylase is a cytochrome P-450 containing mixed function oxidase.

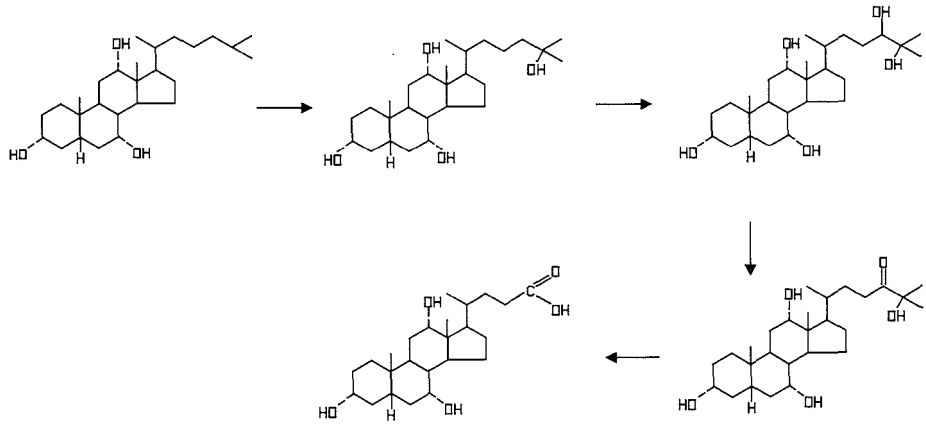


Figure 2.14: Alternative route for cholic acid synthesis in patients with CTX.

2.7 The human fetal adrenal gland

INTRODUCTION

The ultrastructure and function of the human fetal adrenal have been the objects of many studies (for extensive reviews see Benirschke et al., 1956; Lanman, 1961; Johannisson, 1979; Serón-Ferré and Jaffe, 1981; Carr and Simpson, 1981b).

At approximately 6 weeks of gestation the human fetal adrenal gland appears as a condensation of coelomic epithelium at the cranial end of the mesonephric kidney. The cells are large and have acidophilic cytoplasm. A second proliferation of epithelial cells takes place at about 8 weeks of gestation. These cells form a cap over the fetal zone and form the definitive or adult zone. The definitive zone cells are small and have basophilic cytoplasm. Neural elements penetrate the gland through the vascular pole from 7 weeks onwards and clumps of medullary cells can be observed in the central portion of the gland.

The fetal adrenal gland grows rapidly during gestation. An indication of this growth is given in table 2.1. The increase in weight can be attributed to both the fetal zone and the definitive zone. The volume of the fetal zone increases much more rapidly than the volume of the definitive zone (largely due to the greater size of the fetal zone cells). At term the fetal zone comprises approximately 80% of the total gland volume.

Weeks of gestation	combined adrenal weight
10	0.1 g
20	2.0 g
30	4.0 g
term	8.0 g
one month of age	5.0 g

Table 2.1: Weight of the combined adrenals during gestation and after birth.

Steroidogenesis

Many studies have been performed on the steroidogenic capacity of the fetal and definitive zones of the fetal adrenal. In this respect the properties and metabolic activities of the fetal adrenal and of the placenta should not be considered as separate entities. Both are incomplete steroidogenic systems, which complement each other. In table 2.2 the enzyme activities are given for the enzymes active in estrogen biosynthesis (from

Enzyme	Placenta	Adrenal Cortex
3β-hydroxysteroid dehydrogenase	+++	+
C_{17}-C_{20} desmolase	0	+++
17α-hydroxylase	0	+++
16α-hydroxylase	0 —> +	++
21-hydroxylase	0	+ —> ++
11β-hydroxylase	0	+ —> +++
aromatizing system	+++	0
sulfatase	+++	0
sulfokinase	0	+++

Table 2.2: Steroid enzymes in the human placenta and the human fetal adrenal cortex. The activity of the enzymes is expressed on a scale of 0 (generally not detectable) to +++ (very active enzyme in in vitro and in vivo studies).

Villee, 1969b). Most enzymes are present in either the placenta or the fetal adrenal cortex.
Schematically the steroidogenesis in the foeto-placental unit can be proposed as in figure 2.15.

An important precursor for steroidogenesis in the fetal adrenal is cholesterol. By measuring [^{14}C]-isocaproic acid production from [26-^{14}C]-cholesterol Gunasegaram et al. (1978) showed, that the fetal adrenals in mid-term pregnancy were able to convert cholesterol via a C_{20}-C_{22} desmolase into isocaproic acid and pregnenolone. Cholesterol can be produced de novo or obtained from plasma LDL-cholesterol. For fetal adrenals of 10-22 weeks of gestation Carr and Simpson (1981a) computed, that de novo cholesterol synthesis accounted for the synthesis of some 30% of the daily secretion of cortisol and DHEAS. Over 50% of the precursor cholesterol came from other sources, presumably lipoproteins (Simpson et al., 1979; Carr et al., 1980a).
The number of LDL binding sites in fetal adrenal cells of anencephalics (30-46 weeks of gestation) is low, probably due to chronic low levels of ACTH (Carr et al., 1981b). This suggests that in case of anencephaly, de

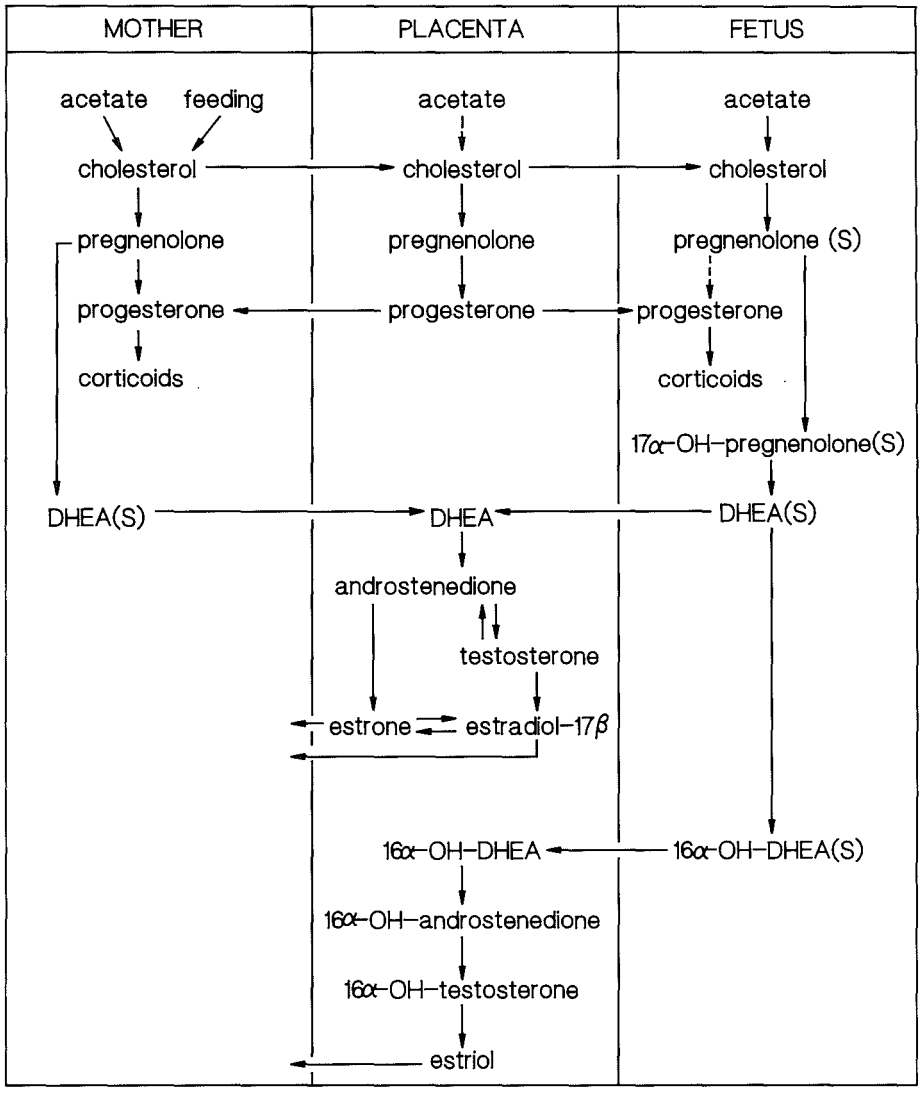


Figure 2.15: Steroid hormone interrelations in the materno-fetoplacental unit.

novo synthesis of cholesterol from acetate is the principal mechanism whereby the adrenals obtain cholesterol precursor for steroidogenesis.

Steroidogenesis, the number of LDL receptors and the degradation rate of LDL, as well as de novo synthesis of cholesterol are probably stimulated by ACTH via a cAMP-mediated pathway in the fetal adrenal (Carr et al., 1980b, 1981a; Ohashi et al., 1981).
Also in the placenta LDL from maternal plasma is preferentially used for placental biosynthesis of progesterone (Winkel et al., 1980). In vivo Lin et al. (1977) demonstrated, that cholesterol is transferable across the placenta from the mother into the fetus.

In the fetal adrenal the conversion of cholesterol into pregnenolone, 17α-OH-pregnenolone and DHEA is quantitatively an important pathway. In vitro incubation studies with fetal adrenal glands, obtained at mid-gestation, showed a basal production of pregnenolone and pregnenolone-sulfate (Yanaihara and Arai, 1981). In the presence of ACTH, DHEA and DHEAS production rose significantly. The conversion of pregnenolone into DHEA(S) may proceed via two routes. In vitro studies showed that with exogenous substrates the main route proceeds via the unsulfated intermediates pregnenolone, 17α-OH-pregnenolone and DHEA (Cooke et al., 1970; Cooke and Taylor, 1971). With endogenous substrates pregnenolone sulfate, 17α-OH-pregnenolone-sulfate and DHEAS were more important as intermediates (Telegdy et al., 1970; Huhtaniemi, 1977).

3β-Hydroxysteroid dehydrogenase/Δ^{5-4}isomerase (3β-HSD)

In vitro incubation of minced human fetal adrenals (22 weeks of gestation) with several precursor steroids showed a low 3β-HSD activity; virtually no Δ^4-3-oxo-compounds were produced from pregnenolone (Yoshida et al., 1978). In the presence of progesterone cortisol and corticosterone seemed to be the major end products (Voutilainen et al., 1979).
In the human fetal adrenal between 10-20 weeks of gestation corticoids were found to be primarily secreted by the definitive zone, whereas DHEAS was the main secretory product of the fetal zone (Serón-Ferré et al., 1978a). The corticoid production in the definitive zone was studied in the absence of exogenous substrates; this means that some 3β-HSD activity was present. These results are in accordance with studies of Goldman et al. (1966) on the histochemical distribution of 3β-HSD in the human fetal adrenal. In fetuses with a crown-rump length of 4-39 cm (10-39 weeks of gestation)

virtually no 3β-HSD activity could be shown in the fetal zone of the fetal adrenal. However in the definitive zone intense staining was present from 16 weeks onwards, suggesting a developing 3β-HSD activity.

Short term cultures of both fetal and definitive zones of the human fetal adrenal (9-18 weeks of gestation) showed a pattern of steroid secretion, characteristic for each zone, upon stimulation with ACTH, (Fujieda et al., 1981a; Simonian and Gill, 1981). Long term stimulation with ACTH (1-4 days) increased the steroidogenic capacity in cultures of both zones. The pattern of steroid production in the definitive zone cultures remained constant, but in the fetal zone cultures cortisol was preferentially increased (Kahri et al., 1976; Huhtaniemie, 1977). There is a close relationship between the morphological and functional changes in the adrenal cell (Voutilainen and Kahri, 1979). This change in steroid pattern was coupled with a 5-fold increase in 3β-HSD activity (Simonian and Gill, 1981). Similar results were obtained by Branchaud et al. (1978) with explants from the fetal and definitive zones kept in culture for 7-9 days. Voutilainen and Kahri (1980) found an inhibition of the ACTH-stimulated cortisol production in cultures of human fetal adrenals, when placental tissue was present. At the same time the amount of free DHEA increased; DHEAS did not rise, probably due to the high sulfatase activity in the placental tissue. They concluded that some placental factor(s), probably estrogens, inhibit the activity of adrenal 3β-HSD or prevent its synthesis and thus direct adrenal steroid synthesis towards DHEAS production in vivo.

Corticosteroids.

The synthesis of corticoids, like cortisol and corticosterone, has been the subject of many studies in mid-gestational human fetal adrenals. In tissue culture the ratio of cortisol to 11-deoxycortisol (on days 0-5) was the same as in the adult adrenal culture, suggesting a 11β-hydroxylase activity of the same magnitude (Voutilainen, 1979). The cortisol/corticosterone ratio was much higher in the fetal adrenal than in the adult adrenal, reflecting the high 17α-hydroxylase activity. No specific defect in aldosterone production was noted (higher aldosterone/corticosterone ratio than in the adult). Shibusawa et al. (1980) confirmed, that the 11β-hydroxylase was present in the mitochondrial fraction of the fetal adrenal at mid-gestation. These authors found that steroids may modify the 11β-hydroxylase activity, as was found for the 17α-hydroxylase and C_{17}-C_{20}

desmolase activity (Shibusawa et al., 1978).

In the human fetal adrenal the extent of the corticosteroid production in fetal adrenals is not exactly known. High levels of cortisol in early fetal life in many species can cause malformations and may have undesirable effects on development (Hall and Kalliecharan, 1976; Chen et al., 1977). On the other hand cortisol and especially cortisol-sulfate, produced by the fetal adrenal, have an important function in fetal lung maturation (Murphy, 1979). So the cortisol concentration in the human fetus is subject to careful regulation.
Cortisol can cross the placenta and is rapidly converted into cortisone by the placental 11β-hydroxysteroid dehydrogenase (11β-HSD) (Murphy, 1979). Amniotic fluid cortisol is mainly derived from the fetal adrenals.

Estrogens

The most important tissue for estrogen production is the placenta (for reviews see Villee, 1969a; Simpson and MacDonald, 1981). The placenta lacks steroid 17α-hydroxylase activity and consequently can not convert C_{21}-steroids into C_{19}-steroids. Since the estrogen excretion is low in women carrying an anencephalic fetus (lacking the fetal zone of the fetal adrenal), it seems likely that the fetal adrenal is involved in placental estrogen biosynthesis. Near term approximately half of the estradiol-17β, synthesized in the placenta, is derived from precursors in the fetal plasma and half from maternal precursors.

In figure 2.16 the metabolic pathway is given for estrogen biosynthesis (see also Villee, 1969a; Wolf et al., 1978). DHEAS, produced in the fetal zone of the fetal adrenal, is 16α-hydroxylated mainly in the fetal liver (Wynne and Renwick, 1976; Sano et al., 1980). Both DHEAS and 16α-OH-DHEAS are deconjugated in the placenta by a very active sulfatase.

Factors regulating the human fetal adrenal cortex

In many reports hormonal factors, regulating adrenal steroidogenesis, are described. These factors may be of placental (Simpson and MacDonald, 1981) or fetal origin. Much evidence has accumulated on the vital role of the fetal endocrine glands in the morphogenesis and functional maturation of the fetus. In this respect the fetal pituitary plays an important role (for a review see Kaplan and Grumbach, 1976). The capacity of the human fetal pituitary to secrete polypeptide hormones and of the hypothalamus to

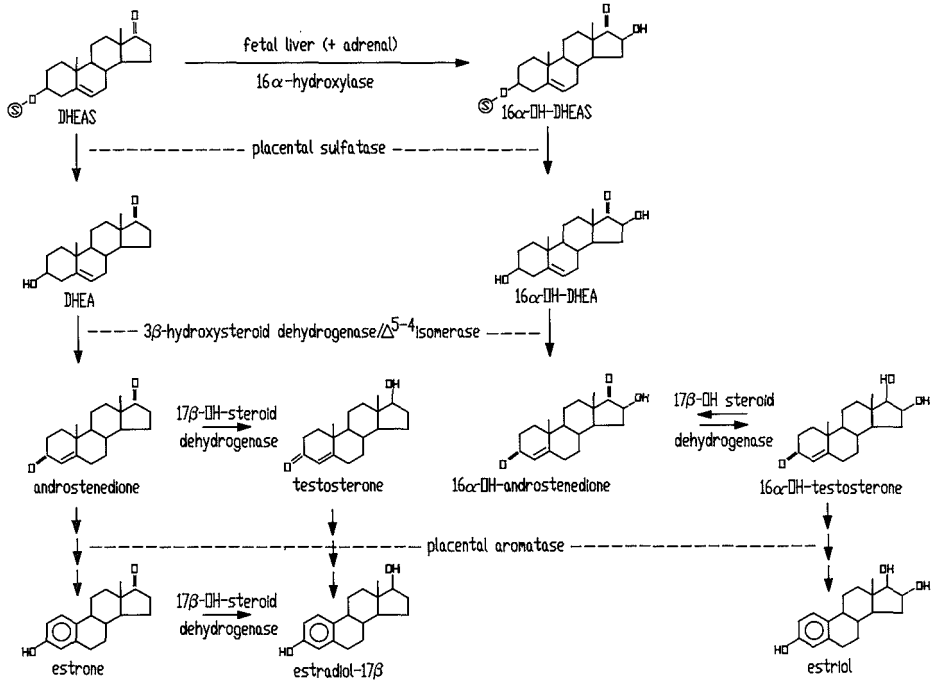


Figure 2.16: Metabolic pathways for conversion of androgens into estrogens in the human placenta.

store releasing factors has been established early in gestation. Maturation of the central nervous system regulation of the hypothalamic-pituitary complex occurs later.
The most important regulating factors will be described in more detail.

Using an immunocytological method it could be shown that in the fetal pituitary, corticotropic cells appeared at the end of the second month of gestation (Begeot et al., 1977). After the third month their normal development required the presence of brain (hypothalamus). This suggests that hypothalamic factors are needed for the normal development of ACTH-containing pituitary cells. Based on the amniotic fluid concentrations of

Δ^5- and Δ^4-steroids in fetuses with an 21-hydroxylase deficiency or anencephaly Pang et al. (1980) concluded, that in the mid-gestational human fetus there is a functional feedback in the hypothalamic-pituitary-adrenal axis. Determination of plasma ACTH by radioimmunoassay showed high levels during pregnancy (see table 2.3; Winters et al., 1974). These authors concluded that there is little transplacental passage of ACTH and that ACTH in fetal serum is probably secreted by the fetal pituitary.

The high fetal ACTH concentrations and the results with short-term tissue cultures fit in the hypothesis on fetal adrenal development, proposed by Fujieda et al. (1981a,b), who suggested that fetal pituitary ACTH is probably the primary regulator of fetal adrenal steroidogenesis. The characteristic fetal steroid pattern is due to the action of an inhibitor of the 3β-HSD complex, most likely of placental origin (Voutilainen and Kahri, 1980). Such a factor, coupled with a rapid clearance of fetal cortisol (conversion into cortisone) results in a state of relative cortisol deficiency. The intact hypothalamic-pituitary-adrenal axis increases ACTH secretion, causing compensatory adrenal hyperplasia and massive secretion of 3β-hydroxy-Δ^5 steroids.

	Plasma ACTH (pg/ml) (mean $\pm$ SEM)
12-19 weeks of gestation	249 $\pm$ 66 (n=7)
20-34 weeks of gestation	234 $\pm$ 29 (n=8)
34-42 weeks of gestation	143 $\pm$ 7 (n=376)
1-7 days postnatal	120 $\pm$ 8 (n=46)
normal adults (1-4 p.m.)	43 $\pm$ 4 (n=22)

Table 2.3: Fetal plasma ACTH concentrations in different periods of gestation and after birth. (From: Winters et al., 1974).

Several cases of low estrogen excretion during pregnancy have been described, which after birth appeared to be fetal ACTH deficiencies (Grimberg et al., 1978; Zachmann et al., 1979). So it seems that in the later stages of pregnancy urinary estrogen secretion not only depends on a well functioning fetal adrenal, but also on the entire hypothalamic-pituitary-adrenal axis. In spite of a fetal ACTH deficiency, the fetal adrenal develops normally in the cases just mentioned. One may conclude from these results, that there are other factors regulating the development of the fetal adrenal. Some reports describe the existence of a corticotropin-like placental hormone. This hormone was called human Chorionic Corticotropin (hCC) (Genazzani et al., 1975; Liotta et al., 1977), or human Chorionic Somato-mammotropin (hCSM) (Isherwood and Oakey, 1976).

The pituitary gland produces a family of related peptide hormones from the high molecular precursor pro-opiomelanocortin. Several of them have a heptapeptide in common. Figure 2.7 shows the mutual relationship between these peptides. β-lipotropin (β-LPH), γ-LPH and ACTH are produced in the pars distalis; α-Melanocyte-Stimulating Hormone (α-MSH), β-MSH and Corticotropin-like intermediate lobe protein (CLIP) originate in the pars intermedia. Silman et al. (1976) showed that the fetal pituitary (24 weeks of gestation) contained only small amounts of intact ACTH, but larger quantities of peptides, closely resembling α-MSH and CLIP. They suggested, that these smaller peptides are the dominant hormones in fetal life, being replaced by ACTH before parturition. However, a steroidogenic response to CLIP could not be demonstrated.

In table 2.4 the stimulatory properties of several hormones are summarized. In the presence of CLIP, fetal adrenal slices did not produce DHEA (Brown et al., 1981). The contradictory results with the other stimulating factors may be the consequence of the methodology used: explants of human fetal adrenals in tissue culture or fetal adrenal cells in cell culture may loose properties that were present in the original cells. ACTH seems the main tropic hormone for the human fetal adrenal. The in vivo contribution of other peptide hormones to the growth and function of the human fetal adrenal remains to be established.

	STIMULATION		NO STIMULATION	
α-MSH	Glickman et al.,1979	#	Fujieda et al.,1981c	+
CLIP			Brown et al.,1981	#
			Fujieda et al.,1981c	+
			Branchaud et al.,1978	*
hGH	Isherwood and Oakey,1976	#	Fujieda et al.,1981c	+
	Brown et al.,1978,1981	#		
hPRL	Taga et al.,1981	#	Fujieda et al.,1981c	+
hCG	Brown et al.,1978,1981	#	Voutilainen et al.,1979	*
	Lehmann and Lauritzen,1975	#	Fujieda et al.,1981c	+
	Jaffe et al.,1977	#		
	Serón-Ferré et al.,1978b	#		
β-LPH	Brown et al.,1981	#	Fujieda et al.,1981c	+
β-Endorphin			Fujieda et al.,1981c	+
hCSM	Isherwood and Oakey,1976	#		

Table 2.4: Summary of the stimulatory factors on steroidogenesis in the human fetal adrenal, as described in the literature.
= in vitro incubation studies.
* = explants from fetal adrenal tissue in organ culture.
+ = fetal adrenal cells in culture.

CHAPTER 3

METHODOLOGY

3A. TECHNICAL ASPECTS.

3A.1 Introduction

Working with isolated cells has several advantages over using tissue blocks, for example, better contact of all cells with the surrounding medium, containing nutrients and stimulatory factors, and an easier diffusion of products out of the cell. A disadvantage is, that the isolation procedure is rather laborious and may damage the cells.
Rat adrenal cell suspensions can be prepared successfully with collagenase/ DNase (for a review see Falke, 1977: thesis, Rotterdam). A detailed description of the isolation method will be given in this chapter. Part of this method has also been used for the isolation of rat adrenal glomerulosa cells, rat liver cells and human fetal adrenal cells.
Several analytical methods will be described in this chapter: the fluorometric assay of corticosterone and cortisol, gaschromatography of steroids and sterols, radioimmunoassay of aldosterone, protein binding assay of cAMP and the luminescence assay of ATP.
For some of the materials used in this study, reference can be made to Chapters 4-7.

3A.2 Isolation of cells

RAT ADRENAL CELLS

Cells from whole rat adrenals were prepared according to the method of Falke et al. (1975a,b) with minor modifications. A Krebs-Ringer-Hepes buffer (pH = 7.35), supplemented with 3% (W/V) bovine serum albumin and 0.2% (W/V) glucose, was used (=KRHAG). Male Wistar rats, weighing 190 - 210 grams, were obtained from the Centraal Proefdieren bedrijf TNO, Zeist. The animals were caged in groups of five with water and laboratory chow ad libitum. The rats were decapitated at approximately 8.30 a.m. The adrenals were removed and placed in cold saline. The collected adrenals of ten rats (mostly used in one experiment) were dissected free of adhering fat tissue and cut into 6-8 pieces per gland. The pieces were put in two 25 ml

Erlenmeyer flasks and washed twice with cold KRHAG. Then 5 ml of the enzyme solution, containing collagenase (5 mg/ml) and DNase (0.05 mg/ml) was added. The flasks were placed in a Dubnoff incubator, shaking at 100 rpm, at a temperature of 37°C. After 20 minutes the medium with the free cells was removed with a Pasteur pipette and replaced by 5 ml fresh enzyme solution. The combined free cell suspensions were allowed to stand for 2 minutes to sediment coarse particles. The supernatant with the cells was centrifuged for 10 minutes at 100xg. The cell pellet was resuspended in KRHAG containing DNase (0.05 mg/ml) and stored on ice. After the second incubation period the free cells were removed as described above. The remaining tissue particles were dissociated in the enzyme solution by sucking up and expelling several times in a Pasteur pipette, followed by a third 20 minutes incubation period. The free cells were removed as described. The three cell suspensions were centrifuged at 100xg for 10 minutes and washed once again in the DNase-containing KRHAG after a 5 minutes incubation period at 37°C. Finally the cells were resuspended in KRHAG and counted in a Bürker counting chamber under a light microscope (magnification 400x). Yields were 200,000-600,000 cells per adrenal. The cells were used immediately after isolation.

RAT ADRENAL GLOMERULOSA CELLS

Rat adrenal glomerulosa cells were prepared from male Wistar rats, weighing 190-210 grams. The glands were removed and dissected free of adhering fat tissue as just described. Incisions were made, followed by manual compression of each gland to extrude the zonae fasciculata and reticularis and the medulla from the capsule + zona glomerulosa. The capsules were cut into small pieces and washed with KRHAG. The further isolation procedure for the glomerulosa cells is similar to the procedure described above for the whole adrenal suspension except that the cells are centrifuged at 150xg for 10 minutes.
Average yields were 30,800 cells per adrenal. The purity of the glomerulosa cell suspension was checked by microscopic examination after staining with hematoxylin/eosin. Generally ≥95% of the cells was of the glomerulosa type, according to histological criteria.

RAT LIVER CELLS

For each experiment the liver of a 200 gram male Wistar rat was

removed, cut into pieces and washed with cold saline and KRHAG. The liver cells were prepared by collagenase/DNase digestion, as described for the whole adrenal cell suspension, except that the cells were centrifuged at 100xg for 5 minutes. The cells were finally resuspended in KRHAG and counted.

HUMAN FETAL ADRENAL CELLS

Human fetal adrenals were obtained from spontaneous or induced abortions. They were transported to the laboratory in icecold saline. Fat tissue was removed and the adrenals were weighed. The isolation procedure for the fetal adrenal cells is essentially the same as used for the rat adrenal cells (described earlier). Yields were 0.8-6.9 x 10^6 cells per adrenal.

3A.3 Incubation conditions

All incubations were done in siliconized glass tubes in a Dubnoff incubator, shaking at 100 rpm. Generally the incubation temperature was 37°C. ACTH was added in 0.9% NaCl, acidified to pH=3.75 and supplemented with 0.5% albumin. Angiotensin II was added in KRHAG. All sterols and steroids were added in 10 μl ethanol. To incubations not containing sterols vehicle only was added. The final incubation volume was 1 ml. The Na^+ and K^+ concentrations in KRHAG were 111.2 mM and 4.75 mM respectively. If other concentrations of potassium were required, the sodium and potassium concentrations were changed in such a way as to keep the sum of the concentrations of these ions unchanged.

3A.4 Fluorometry

Corticosterone and cortisol was estimated by fluorometry using the extraction procedure of Clark and Rubin (1969). Fluorescence was developed in ethanol/concentrated sulfuric acid (1:3) and measured at room temperature with a Baird Atomic fluorimeter (excitation wavelenth is 468 nm; fluorescence wavelenth is 520 nm). A corticosterone standard (mostly 500 ng/assay) was measured in duplicate in the same assay.
The variation coefficient due to methodological variations (measured for 15 corticosterone standards in duplicate) was 3.6%. The biological variation coefficient (measured for the ACTH-stimulated corticosterone production) was 5.1% (n=22).

LINEARITY

In the fluorometric assay of corticosterone the relationship between the fluorescence and the corticosterone concentration was checked in the range of 0-5 μg corticosterone per assay (see figure 3.1). Up to 2 μg corticosterone per assay the fluorescence is linear. A quadratic equation can be calculated for the curve with the following coefficients:

$$Y = 5 + 1061\ X - 32.97\ X^2 \qquad r = 0.9975$$

(X = μg corticosterone per assay)

The dilution of the sample was chosen in such a way as to have a corticosterone amount below 2 μg per assay.

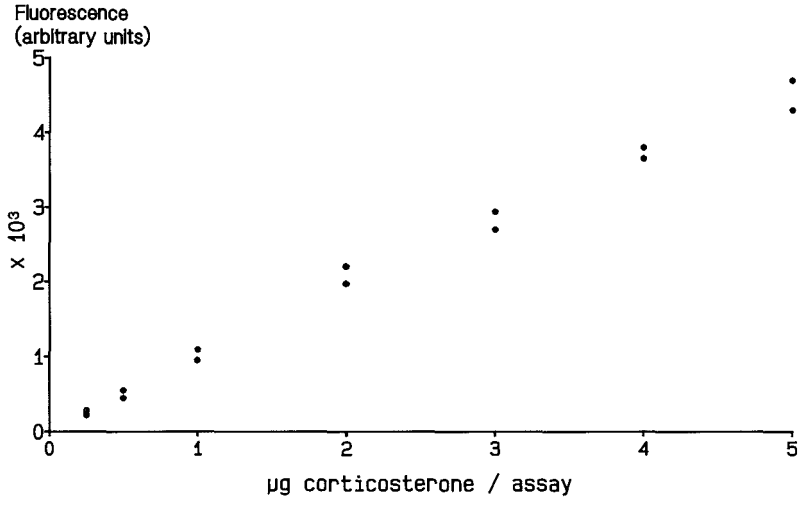


Figure 3.1: Relation between the fluorescence in arbitrary units and the corticosterone concentration in the range of 0-5 μg/assay.

FLUOROMETRIC CROSS-REACTIVITY

We determined the cross-reactivity of a number of sterols and steroids in this fluorometric assay. The results are given in table 3.1. When necessary the corticosterone values in incubations are corrected for the fluorescence caused by the sterols. The extent of the correction is based on gaschromatographic measurements of the sterol concentrations.

sterol/steroid	fluorescence	sterol/steroid	fluorescence
cholesterol	2.0	cortisol	437.0
22R-OH-cholesterol	4.2	21-deoxy-cortisol	102.3
22S-OH-cholesterol	4.2	11-deoxy-cortisol	18.5
25-OH-cholesterol	5.0	pregnenolone	2.4
20S-OH-cholesterol	4.9	17α-OH-pregnenolone	7.2
17R,20R-diOH-cholesterol	5.4	progesterone	0.7
22-keto-cholesterol	1.7	deoxycorticosterone	4.9
7-dehydrocholesterol	5.6	18-OH-deoxycorticosterone	0.1
7-keto-cholesterol	0.0	cortisone	0.3
7β-OH-cholesterol	0.4	21-deoxycortisone	5.0
desmosterol	3.0	18-OH-corticosterone	0.3
zymosterol	6.6	dehydroepiandrosterone-S	0.2
20S-OH-cholenic acid	2.0	dexamethason	2.0
3β,20S-diOH-cholenic acid	1.9	Δ^5-pregnene-3,11,17,21-tetrol-20-one	18.3

Table 3.1: Fluorescence of several sterols and steroids in a concentration of 1 μg/assay, relative to a corticosterone standard of 1 μg/assay, of which the fluorescence was arbitrarily set at 1,000.

3A.5 Gaschromatography

Gaschromatography was performed on a HP 5700 gaschromatograph, equipped with a flame ionization detector. Steroids were separated on a capillary column (length = 25 m; i.d. = 0.25 mm), coated with SE-30 (film thickness = 0.2 μm), or on a glass column (length 1.8 m; i.d. 0.3 cm), packed with 2% SP 2250 on chromosorb WHP (80-100 mesh). N_2 was used as carrier gas. Column temperature was 260°C and the detector and injector temperatures were 300°C. An internal standard (5-cholesten-3α-ol) was added to the incubation mixture before the extraction to correct for procedural losses and for identification purposes.

For the detection of both free and sulfo-conjugated steroids one ml of the incubated cell suspension was diluted 1:1 with H_2O, made up to 20% with sodium chloride and acidified to pH=1 with 6 N H_2SO_4. This mixture was extracted with 10 ml and 5 ml ethylacetate. The combined organic layers were kept overnight at 37°C. The next morning the ethylacetate was washed with 5 ml 0.1 M $NaHCO_3$ and 2 ml H_2O. The organic layer was dried over Na_2SO_4 and evaporated under nitrogen at 40°C. To the extract 100 µl 2% methoxyamine/HCl in pyridin was added and the mixture heated at 60°C for 1 hour. Then the pyridin was blown off with nitrogen. 100 µl of a silylating mixture, containing bis-trimethylsilylacetamide/trimethylchlorosilane/trimethylsilylimidazole (3/2/3; V/V/V) was added and the reaction completed at 80°C overnight. The steroids were purified with a Lipidex-5000 column, using 5 ml hexane/pyridin/hexamethyldisilazane/-dimethoxypropane (97/1/2/10; V/V/V/V) as eluting agent. After evaporation the residue was dissolved in 20 µl hexane.

For the detection of only the free steroids one ml of the incubated cell suspension was diluted with 1 ml acetone and kept overnight at 4°C. The protein precipitate was extracted once again with 1 ml acetone. To the combined acetone layers 5 ml of dichloromethane was added after which a phase separation occurred. The water layer was extracted with 1 ml of dichloromethane and the combined organic layers washed with 1 ml of water. After evaporation the extract was derivatized as described above.

3A.6 ATP

ATP was measured using the luciferin-luciferase method, as described by Guinn and Eidenbock (1972). The reaction can be written as:

$$\text{luciferin} + \text{ATP} + \tfrac{1}{2}O_2 \xrightarrow{\text{luciferase}} \text{dehydroluciferin} + \text{AMP} + PP_i + \text{light}$$

The enzyme solution was prepared from fireflies (Sigma Chem Comp).
For the measurement of ATP in whole cells, one ml of the cell suspension was poured into 3 ml of boiling water and left for 7 minutes in a boiling water bath. The precipitated protein was centrifuged and the supernatant stored frozen below -24°C until analysis. Before the analysis the supernatant was lyophilized and resolved in 0.5 ml distilled water. A standard series of ATP concentrations in water was prepared (0.5, 0.25, 0.125, 0.1, 0.05 and 0.025 µg/100 µl).

For the assay 1 ml of 0.05 M glycine buffer (pH=7.4) was mixed with 0.2 ml of the enzyme solution and 0.2 ml standard ATP or sample. The luminescence was measured for 5 minutes at 552 nm. At t=1 min, t=2 min, t=3 min and t=4 min the luminescence was read from the registrated curve. Each time a standard curve was measured. For t=1 minute and t=3 minutes the calibration curves are presented in figure 3.2. The luminescence of the unknown samples was also measured at all four times and the ATP concentration read from the appropriate standard curves. The mean of the 4 values was considered as the ultimate concentration. The mean variation coefficient for 7 measurements in quadruplo was 6.1% (range: 2.7% - 9.1%).

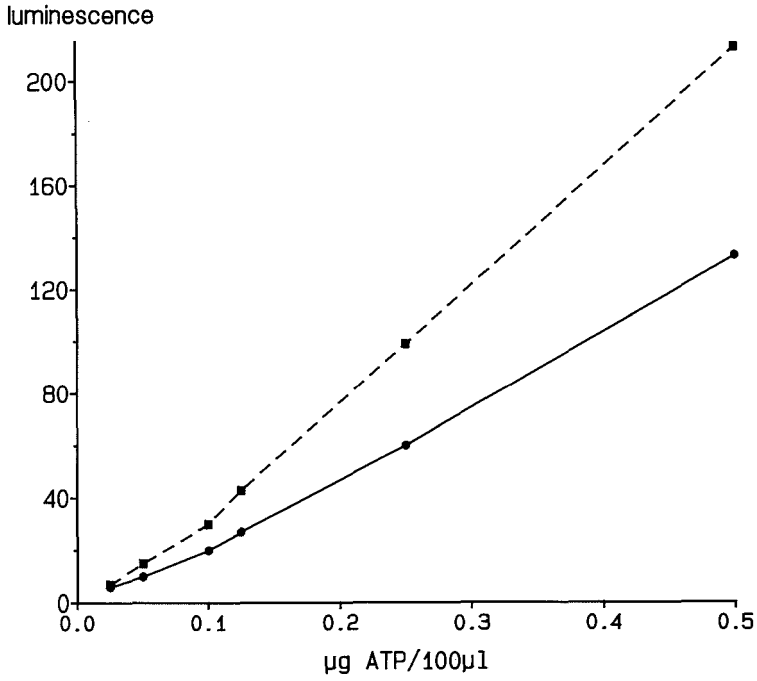


Figure 3.2: Calibration curves for the measurement of ATP, taken at t=1 min (■- - -■) and t=3 min (●——●).

3A.7 Cyclic AMP

Cyclic AMP was measured with a protein binding assay (The Radiochemical Centre, Amersham). The extraction procedure of Lavin et al. (1976) was used. Concentrations in unknown samples were read from a logit-log transformed standard curve. The best-fitting quadratic function was used as a calibration curve. Samples with a concentration out of the range (1-16 pg/tube) were measured again at a proper dilution. The recovery of cAMP added to the KRHAG buffer was 90%. The mean variation coefficient for 5 measurements in triplicate was 7.6% (range: 3.0% - 14.4%).

3A.8 Aldosterone

Aldosterone was estimated using a radioimmuno assay (CEA, Sorin) with prior extraction. The recovery for the extraction procedure is 97%. Concentrations in unknown samples were read from a logit-log transformed standard curve. The best-fitting quadratic function was used as a calibration curve.
The within-assay coefficient of variation was 9% (n=10) and the between-assay coefficient of variation 13.7% (n=15).

The specificity of the antiserum used in the aldosterone radioimmuno-assay is shown in table 3.2. The percentage of cross-reactivity was calculated as X.100/Y, where X and Y are respectively the weight of the aldosterone and the weight of the cross-reactant, that caused a 50% inhibition of the tracer binding. The antiserum is clearly very specific.

3A.9 Density gradient centrifugation

Sedimentation through a density gradient can be used to separate cells, cell fragments and viruses, either at unit gravity or after centrifugation. The density medium must fulfil several criteria:
a. easily adjustable to a suitable pH and osmolarity,
b. low viscosity even at high density,
c. a wide density range,
d. non-toxic to the cells,
e. impermeable to biological membranes.
Percoll^{R} (Pharmacia, Sweden) is a density gradient medium, consisting of a colloidal suspension of silica particles, coated with polyvinylpyrrolidone. It can be made isotonic by mixing 9 parts of Percoll with 1 part of 9% NaCl. The density gradients are formed spontaneously by centrifugation at

Aldosterone	100%
18-OH-deoxycorticosterone	0.03%
18-OH-corticosterone	0.01%
Corticosterone	0.0025%
Cortisol	0.00005%
Cortisone	0.0001%
Allotetrahydrocortisol	0.00005%
Tetrahydrocortisone	0.00005%
Progesterone	0.00005%
Tetrahydrocortisol	0.00005%
Pregnanetriol	0.00005%
Cortexolone	0.00005%
Testosterone	0.00005%
Estradiol	0.00005%
25-OH-cholesterol	0.0005%
22S-OH-cholesterol	0.0005%

Tabel 3.2: Percentage of cross-reactions of several steroids and sterols in the aldosterone radioimmunoassay.

25,000xg for 20 minutes. The shape of the gradient can be made visible by using density marker beads. These are coloured beads with each colour having its accurately known density. By varying the percentage of Percoll the shape of the gradient can be changed.

3B APPLICATION AND EVALUATION OF THE METHODS

3B.1 Introduction

With the rat adrenal cells, isolated as described in Section 3A, some general aspects of steroid metabolism were investigated in order to be informed about the steroidogenic capacity of the cells. The information obtained includes a dose-response curve for ACTH, effect of cell numbers on response, and effect of buffer and albumin concentration.

In many studies on fetal steroidogenesis tissue cultures of human fetal adrenal glands have been used. However under these conditions steroidogenesis changes from a "fetal pattern", with largely 3β-hydroxy-Δ^5 steroids like DHEA, into an "adult pattern" with cortisol as an important steroid (Branchaud et al., 1978; Fujieda et al., 1981a; Simonian and Gill, 1981). This change can be explained by the disappearance of the fetal zone cells from the cell culture and the simultaneous growth of the definitive zone cells (Voutilainen and Kahri, 1979). For this reason we wanted to use freshly prepared fetal adrenal cells. With the cell isolation technique described in Section 3A.2, human fetal adrenal cells were isolated. Two parameters for cell viability were studied: the production of cortisol and corticosterone from exogenous progesterone and the ATP content of the cells.

The human fetal adrenal consists of two morphologically and functionally different cell types: the fetal zone cells and the definitive zone cells. Two techniques for separating these cell types were compared: a manual dissection method of the whole fetal adrenal and a cell separation method by means of density gradient centrifugation on Percoll. Several parameters for steroid metabolism were investigated in both cell types and compared with data from the literature.

3B.2 Effect of ACTH stimulation

Figure 3.3 shows a typical dose-response curve for the ACTH-stimulated corticosterone production in isolated rat adrenal cells during a 2 hours incubation period at 37°C. The shape of the curve was always comparable to that, shown in figure 3.3. However, the responsiveness towards ACTH varied for each batch of cells. For this reason a limited dose-response curve was measured for all batches of cells.

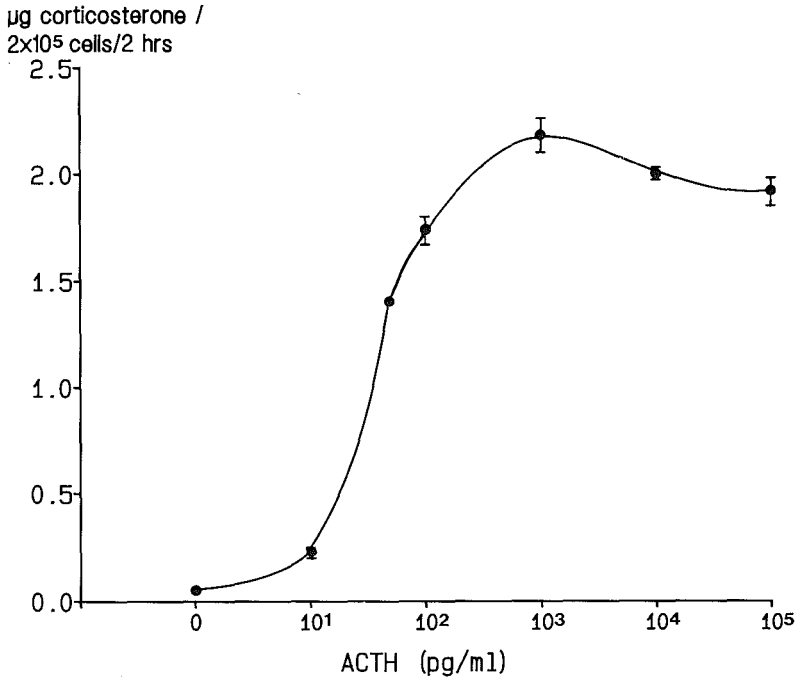


Figure 3.3: Corticosterone production by isolated rat adrenal cells (109,000/ml) in the presence of ACTH. Incubations are done in duplicate.

3B.3 Effect of cell numbers

We investigated the relationship between the corticosterone production and the number of incubated cells for several ACTH concentrations. The results are shown in figure 3.4. For all but the highest ACTH concentrations tested the corticosterone production was linear with the number of cells per incubation volume.

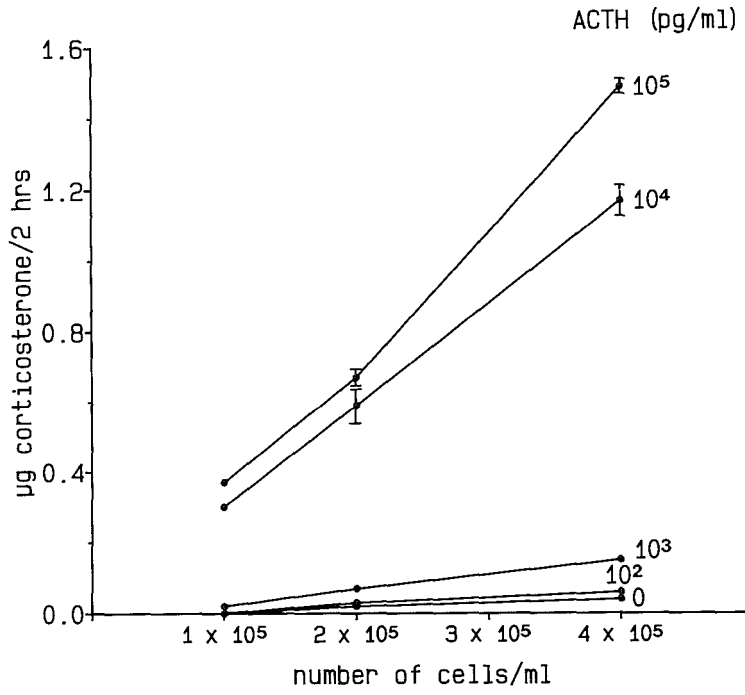


Figure 3.4: Corticosterone production by isolated rat adrenal cells in three concentrations in the presence of ACTH. The mean and range of duplicate incubations are plotted.

3B.4 Incubation buffer

Maintenance of pH with HEPES or bicarbonate

When working with a Krebs-Ringer-Bicarbonate buffer (KRBAG) one has to use a carbondioxide-enriched atmosphere to maintain a constant pH. This is not always possible (for example when cells are centrifuged). In addition the pK_a of bicarbonate at 37°C is 6.1; this results in sub-optimal buffering at the pH mostly used (7.35). We therefore used 4-[2-hydroxy-ethyl]-1-piperazine-ethane-sulfonic acid (HEPES), a hydrogen ion buffer, described by Good et al. (1966). The pK_a of Hepes is 7.31 at 37°C. We

investigated whether the results obtained with KRHAG were different from those with KRBAG, especially with respect to the effects of hydroxylated sterols on corticosterone production. Adrenal cells were prepared and suspended in both KRHAG and KRBAG. They were incubated in the presence of ACTH or a sterol (22S-OH). Results are given in figure 3.5.

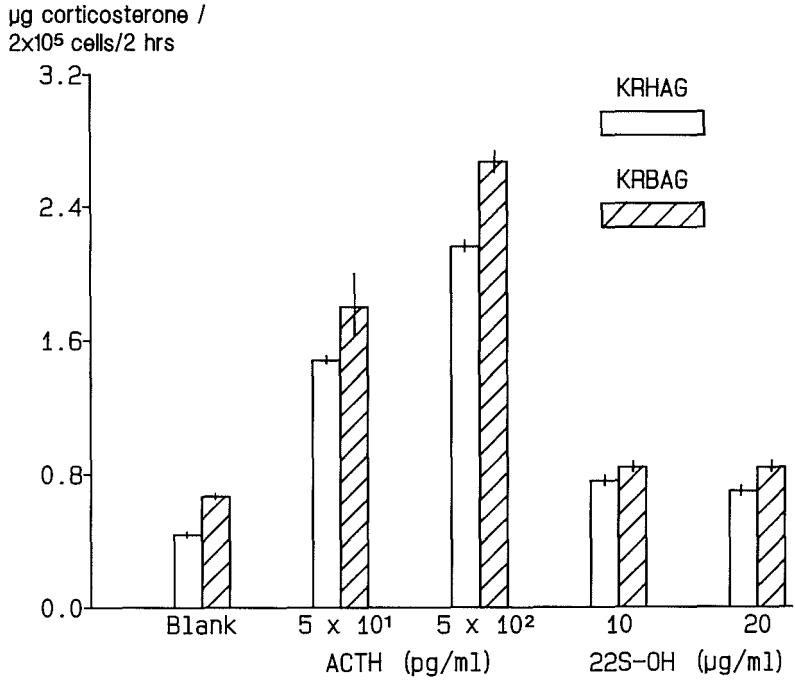


Figure 3.5: Influence of the incubation buffer with either HEPES (KRHAG) or bicarbonate (KRBAG) on the corticosterone production in isolated rat adrenal cells in the presence of ACTH or 22S-OH. Results are given as the mean and the range of duplicate incubations.

In general the corticosterone production, both basal and stimulated, was higher with KRBAG buffers than with KRHAG. The stimulation ratio in the presence of several ACTH concentrations is given in table 3.3.

	corticosterone production (stimulated/basal)	
addition	KRHAG	KRBAG
$5x10^1$ pg/ml ACTH	3.44	2.63
$5x10^2$ pg/ml ACTH	4.99	3.88
10^5 pg/ml ACTH	4.06	3.42

Table 3.3: Ratio of stimulated versus basal corticosterone production in isolated rat adrenal cells in the presence of three concentrations of ACTH. Incubations were carried out in Krebs-Ringer buffers, containing HEPES (KRHAG) or bicarbonate (KRBAG) in equimolar concentrations.

These results show that the use of HEPES in the incubation buffer only slightly influences corticosterone production in the presence of ACTH or 22S-OH. However, the use of HEPES has several practical advantages.

Effect of the albumin concentration.

The effect of two concentrations of albumin (3% and 0.5%) on the ACTH-stimulated corticosterone production in isolated rat adrenal cells was investigated. Results are presented in figure 3.6. Corticosterone production in adrenal cells, incubated in a medium containing 0.5% albumin, is somewhat lower, especially in the presence of maximally-stimulating ACTH concentrations. One explanation could be that 3% albumin is necessary to keep the cells in suspension without clotting and thus to optimalize the number of ACTH receptors, in contact with the surrounding medium. These results learned us to use 3% of albumin in all further studies.

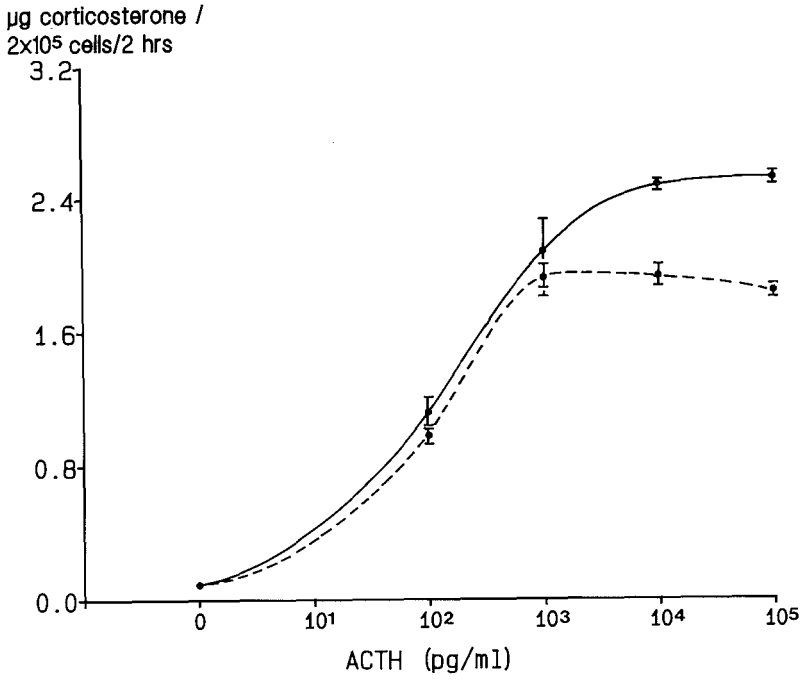


Figure 3.6: Dose-response curve of the ACTH-stimulated corticosterone production in isolated rat adrenal cells in the presence of 3% albumin (——) or 0.5% albumin (– – –). The mean and the range of duplicate incubations are plotted.

3B.5 Human fetal adrenals

3B.5.1 FETAL ADRENALS

We received human fetal adrenals from several obstetric departments of hospitals in and around the city of Rotterdam. The duration of the pregnancy and the time of death of the fetus were obtained from the obstetrician. A survey of the human fetal adrenals used in this study, is given in Table 3.4. In some cases the exact time of death was unknown; the estimated values of the time interval are minimal values.

Table 3.4: A survey of the human fetal adrenals, used in this study.

case number	weeks of gestation	adrenal weight mg/pair	time interval between death and start of the incubation (hours)	remark
1	15	300	4.50	
2	18	190	9.95	see 1
3	18	1220	6.65	
4	19	940	4.38	
5	20	1080	4.25	
6	22	1074	(6)[a]	
7	22	1230	4.75	
8	23	584	(7.5)[a]	
9	23	1717	(4)[a]	
10	24	1865	6.25	
11	26	1230	6.33	
12	27	860	4.33	
13	28	n.d.	5.5	see 2
14	(32)[a]	2240[b]	(6.25)[a]	see 3

a number in parentheses denotes uncertainty about the exact data

b only one adrenal received with the given weight

n.d. = not determined

1 anencephalic

2 several fetal malformations (a.o. meningomyelocele)

3 prematurely born; died after 8.5 hours

The regular supply of human fetal adrenals of the right gestational age has been a major problem. In fact it was the limiting factor in this study.

The adrenal weight increased with fetal age. These results are comparable to the data given by Winters et al. (1974). The adrenal weights in cases 8 and 12 were remarkably low.

The anencephalic fetus (case number 2) had a low adrenal weight, in accordance with the observation that in anencephaly a marked reduction in adrenal weight at birth exists (Lemire et al., 1978). Benirschke (1956) described a normal appearance of the adrenal in anencephalics up to 20 weeks of gestation with a reduction in weight during the second half of pregnancy. However a more recent report documented that the adrenal weight of anencephalics is reduced as early as 15 weeks of gestation (Carr et al., 1981b).

3B.5.2 CELL VIABILITY

Two parameters for the quality of the cells were measured: the ATP content of the cells as a general index of the cellular energy metabolism and the corticosterone plus cortisol production from exogenous progesterone, indicating the ability of the cells to convert steroid precursors into steroids by both microsomal and mitochondrial enzymes.

Figure 3.7 shows the correlation between the corticosterone plus cortisol production from exogenous progestrone and the ATP-content of the human fetal adrenal cells. The correlation coefficient is 0.82. These parameters can be used as an indication for the quality of the isolated human fetal adrenal cells.

It is striking that cases 2 and 8, with the longest interval between time of death of the fetus and start of the incubations, also show the smallest ATP content and corticosteroid production. No relationship between both parameters and the fetal age was found.

3B.5.3 3β-HSD ACTIVITY

Differences between the production of cortisol and corticosterone in the presence of pregnenolone or progesterone as substrates may yield information about the 3β-HSD activity in the fetal adrenal cells. The results for one fetal adrenal of 22 weeks of gestation are presented in Table 3.5. Pregnenolone appeared to be a poor substrate for cortisol and corticosterone production. The low glucocorticosteroid production from

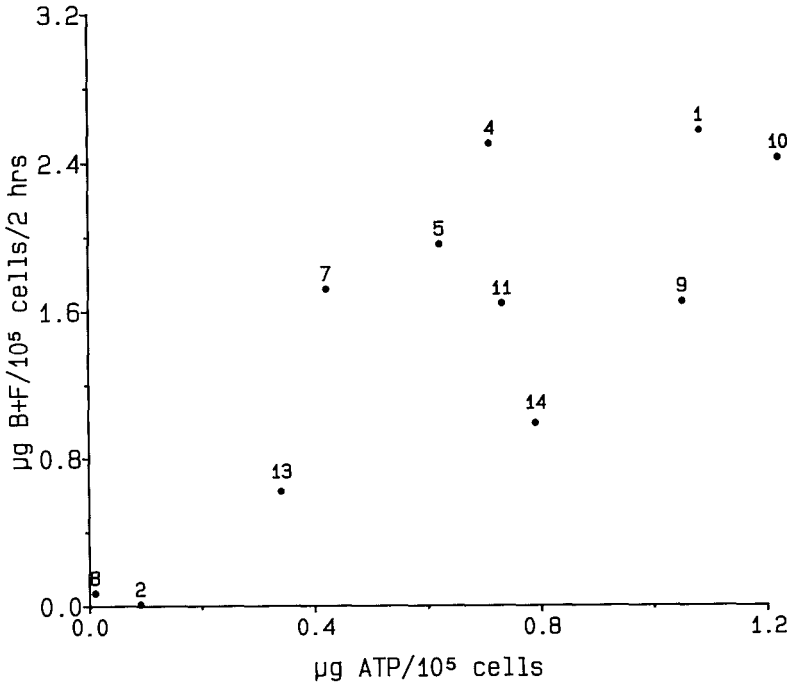


Figure 3.7: Relationship between the ATP content of the cells and the corticosterone (B) plus cortisol (F) production from 32 µM progesterone in isolated human fetal adrenal cells. Numbers refer to the case numbers in Table 3.4.

pregnenolone compared with that from progesterone suggests a low 3β-HSD activity, which is in agreement with the literature (Voutilainen and Kahri, 1980).

3B.5.4 FETAL ADRENAL CELL SEPARATION

Two methods for the separation of cells of the the fetal and definitive zones were used:

Table 3.5: Corticosterone and cortisol production by isolated human fetal adrenal cells (22 weeks of gestation; case number 6) in the presence of pregnenolone or progesterone.

Addition	corticosterone ($ng/10^5$ cells/2 hrs)	cortisol ($ng/10^5$ cells/2 hrs)
no addition	30	10
40 μg/ml pregnenolone	50	40
10 μg/ml progesterone	1340	1850

Separation method 1:

The adrenal from case number 14 was incised, followed by manual compression of the adrenal to extrude the fetal zone from the capsule plus definitive zone. The cells from both zones were further prepared as described in Section 3A.2.

Separation method 2:

a. Part of the human fetal adrenal cell suspension (case number 12) was layered on a density gradient (36% Percoll; starting density=1.05) and centrifuged for 15 minutes at 400 xg at 4°C. Figure 3.8 shows schematically the position of the cells. Two broad bands could be distinguished: an upper band with mean density of 1.035 and a lower band with mean density of 1.043. A third narrow band with a density of 1.061 seemed to contain erythrocytes among others. The upper and lower layers were isolated, washed and resuspended in KRHAG. Cells were counted in a Bürker counting chamber.

b. Part of the human fetal adrenal cell suspension from a second case (number 11) was treated in the same way, using a slightly different density gradient (30% Percoll; starting density = 1.04). An upper and a lower band of cells were obtained.

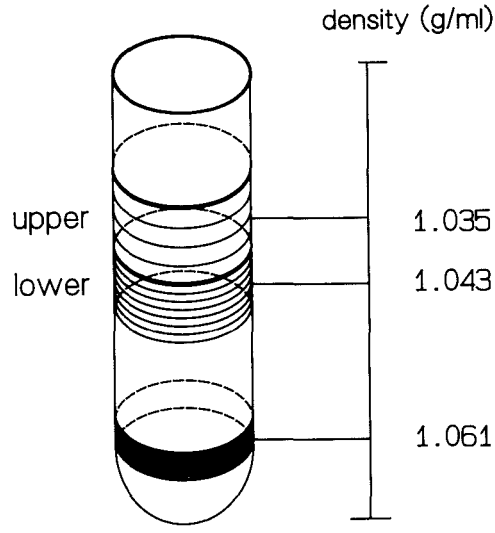


Figure 3.8: Sedimentation of human fetal adrenal cells on a Percoll density gradient.

Under the microscope the upper layer cells appeared to be much smaller, containing many cell fragments, whereas the lower layer cells contained larger cells and lumps of cells.

The two methods for the separation of cells of the fetal and definitive zones were compared with each other and with results from the literature. Two lines of evidence suggest that density gradient centrifugation does indeed give a separation between fetal zone and definitive zone cells:

1. On the Percoll density gradient two bands of cells were visible. Under the microscope the cells of the upper band were smaller than those of the lower band. Histological data indicate that the cells of the definitive zone are smaller than those of the fetal zone (Bloom and Fawcett, 1968; Johannisson, 1968).

2. The cells of the fetal and definitive zones of the human fetal adrenal, obtained with separation method one and two, were incubated in the presence of progesterone. The extent of conversion of progesterone into cortisol coincided with the presence of fetal zone cells in human fetal adrenal cell cultures (Voutilainen and Kahri, 1979; Voutilainen et al., 1979). We used the cortisol/corticosterone ratio as a measure for the 17α-hydroxylase activity and as a criterium for the presence of fetal zone cells. These ratios are presented in Table 3.6. The cortisol/corticosterone ratios in the lower layers of the density gradients had the same value, compared with the ratio in the fetal zone cells, while the cells in the upper layers had the same ratio as compared to the cells of the definitive zone. This is in agreement with the histological data, as mentioned above.

The results of this pilot experiment suggest that density gradient centrifugation is a suitable method for the separation of cells from the fetal and definitive zones of the human fetal adrenal.

Table 3.6: Ratio of corticosterone/cortisol production from exogenous progesterone (10 μg/ml) in human fetal adrenal cells, obtained according to separation methods 1 and 2.

Method	case number	Adrenal zone	Ratio cortisol/ corticosterone
1	3	definitive zone	0.60
		fetal zone	1.54
		Layer from Percoll gradient	
2	12	upper	0.50
		lower	1.15
2	11	upper	0.65
		lower	1.47

CHAPTER 4

EFFECTS OF 22S-HYDROXY-CHOLESTEROL AND OTHER HYDROXYLATED STEROLS ON THE ACTH-STIMULATED STEROID PRODUCTION IN RAT ADRENAL CELLS.

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Effects of 22S-hydroxy-cholesterol and other hydroxylated sterols on the ACTH-stimulated steroid production in rat adrenal cells

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Abstract. Several effects of hydroxylated sterols on cell cultures are known. Most of these can be explained by an inhibition of the cholesterol synthesis at the level of the 3-hydroxy-3-methylglutaryl CoA reductase.
When studying cholesterol metabolism in rat adrenal cells, an inhibitory action of some sterols on the ACTH-stimulated corticosterone production was observed. The effects of one sterol, 22S-OH-cholesterol, were investigated further.
The sterol had no effect on the ACTH-stimulated cyclic AMP production, suggesting an intact receptor-adenylate cyclase complex and cellular membrane. In the presence of ACTH and 22S-OH-cholesterol particularly the free cholesterol concentration was elevated; 22S-OH-cholesterol therefore probably exerts its inhibitory effect at a step located after hydrolysis of the cholesterol esters. 22S-OH-cholesterol had no effect on the conversion of exogenous pregnenolone into corticosterone.

These results make it probable, that the inhibitory effect of 22S-OH-cholesterol on the ACTH-stimulated corticosterone production is situated at the level of the cholesterol side-chain cleavage.

Several effects of oxygenated derivatives of cholesterol on liver cells and fibroblasts have been described.

They inhibit the 3-hydroxy-3-methylglutaryl CoA-reductase activity (Chen et al. 1974; Kandutsch & Chen 1975; Heiniger et al. 1976), are cytotoxic for smooth muscle cell cultures (Peng et al. 1979) and are angiotoxic and arteriosclerotic in rabbits (Imai et al. 1980). These effects become manifest after exposure to the agent for several hours or even days.

Whether these sterols affect steroid producing cells or influence steroid metabolism is not known. This matter is of considerable interest in view of the fact, that cholesterol is the main precursor for steroid production.

In a previous communication (Huijmans et al. 1977) we described an acute effect of several sterols on the ACTH-stimulated corticosterone production in isolated rat adrenal cells. One of these sterols, 22S-hydroxy-cholesterol (22S-OH) inhibited steroid production by more than 50%. At that time, the mechanism of this inhibition or the site of action of the sterol was still incompletely understood.

In this report we present data for a number of hydroxylated sterols as to their inhibitory effect on the ACTH-stimulated corticosterone production. The effects will be discussed of one of these sterols (22S-OH-cholesterol) on several steps in the ACTH-stimulatory mechanism and the steroidogenic pathway.

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Materials and Methods

Bovine serum albumin (Cohn fraction V), collagenase (type I) and DNAse were obtained from Sigma Chemical Company. The albumin was purified by extensive dialysis against 0.9% NaCl and finally against twice-distilled water. Hepes was from Calbiochem and synthetic $ACTH^{1-24}$ (synacthèn) from Ciba-Geigy. Zymosterol was a generous gift from Philips Duphar; 22S-OH was obtained from Ikapharm and desmosterol from Supelco Inc. Other sterols and steroids used were from Steraloids. ^{3}H-labelled pregnenolone (specific activity 54 mCi/mg) was bought from New England Nuclear.

All glassware coming in contact with the adrenal cells was siliconized (Siliclad, Clay Adams). Incubation media were sterilized before use by filtration through a Millipore filter (HAWP 02500; pore size 0.45 μm).

Preparation of the cell suspension

Cells were prepared, as described by Falke et al. (1975a) from adrenals of male Wistar rats, weighing 190–210 grams. A Krebs-Ringer Hepes buffer, pH = 7.35, supplemented with 3% (w/v) albumin and 0.2% (w/v) glucose was used (KRHAG; Falke et al. 1975b).

Incubation

Cells were incubated in a KRHAG medium at 37°C in a Dubnoff incubator, shaking at 100 r.p.m. ACTH was added in 0.9% NaCl, acidified with 1 N HCl to pH = 3.75 and supplemented with 0.5% (w/v) albumin. Sterols were added in 10 μl of ethanol. To control incubations (not containing ACTH or sterols) vehicle only was added. After the incubation period the tubes were rapidly frozen, tightly capped and stored at −20°C until further analysis.

The effect of 22S-OH on the conversion of exogenous pregnenolone by rat adrenal cells was measured as follows. Rat adrenal cells were pre-incubated for 1 h in the absence or presence of 22S-OH. Labelled pregnenolone was added and the incubation continued for 1 h. The incubation mixture was extracted with acetone/dichloromethane. Sterols and steroids were separated by thin layer chromatography on silicagel plates (Merck, Darmstadt) with chloroform/methanol 9:1 (v/v) as the eluting system. The radioactive spots were visualized with a radiochromatogram camera (LKB, Sweden), eluted with methanol and counted in a liquid scintillation counter. The recovery for this procedure was 73%.

Analytical procedures

Corticosterone was estimated by fluorometry using the extraction procedure of Clark & Rubin (1969). Fluorescence was developed in H_2SO_4/ethanol as described by Falke et al. (1975a). The specificity of this method was checked by measuring the fluorescence of many intermediates in steroidogenesis like pregnenolone, progesterone, deoxycorticosterone, 18-hydroxy-deoxycorticosterone, 18-hydroxycorticosterone. Compared to corticosterone their fluorescence was less than 0.5% for all substances tested. Corticosterone values were corrected for the intrinsic fluorescence of the sterols (< 11 ng corticosterone-equivalents per μg sterol). Cortisol, which gives a high fluorescence, is not produced in a significant amount in the rat adrenal cell (Slaga & Krum 1976; Milewich & Axelrod 1972).

Free cholesterol and 22S-OH were estimated with a gaschromatographic method. The incubation mixture was extracted with acetone-dichloromethane and the extract treated with trimethyl-silyl-imidazole (Pierce) for preparation of the trimethylsilyl-derivatives. Analyses were performed on a Hewlett Packard 5700 gaschromatograph, equipped with a flame ionization detector.

The glass column (length 1.8 m, i.d. 0.3 cm) was packed with 2% SP 2250 on chromosorb WHP (80–100 mesh). An internal standard (cholest-5-en-3α-ol) was added to the incubation mixture just before the extraction to correct for procedural losses and for identification purposes.

Cyclic AMP was measured with a protein binding assay (The Radio Chemical Centre, Amersham). For this purpose the incubation was terminated by adding one ml of a mixture containing 12% trichloroacetic acid and 1 N hydrochloric acid (10:1 v/v) to the adrenal cell incubation. The mixture was sonified for 20 s (Branson Sonifier) to disrupt all cells. The extraction procedure of Lavin et al. (1976) was used.

The recovery for a standard amount of cyclic AMP, added to the incubation medium, was 90%.

Results

Effect of hydroxylated sterols on ACTH-stimulation

The steroid production in isolated rat adrenal cells in the presence of several sterols and the effect of these sterols on the ACTH-stimulated corticosterone production is shown in Table 1. The ACTH concentration used in this experiment (1 ng per ml) was near-maximally stimulating.

25-hydroxy-cholesterol and 20S-hydroxy-cholesterol proved to be good substrates for steroid production. In addition, 22S-OH and desmosterol produced a small, but significant increase in corticosterone production. In the presence of ACTH steroid production with 25-OH-cholesterol and 20S-OH-cholesterol was partly additive.

However, 22S-OH, 17α, 20α-dihydroxy-cholesterol and 22-keto-cholesterol significantly inhibited the ACTH-stimulated steroid production. From these three 22S-OH was chosen as a model substrate for further studies.

Table 1.

Adrenal cells were incubated in the presence or absence of 1 ng/ml of ACTH and/or the sterol at the concentrations mentioned. Results are expressed as the mean and the range of two incubations. The mean ACTH-stimulated corticosterone production was put at 100%. Results were evaluated with the test, described by Kurtz et al. (1965).

Sterol added	Corticosterone production/2 h	
	Sterol (%)	Sterol + ACTH (%)
No sterol (control incubation)	5.2 ± 0.4	100.0 ± 5.3
Cholesterol 52 μM	1.7 ± 0.1*	90.3 ± 2.3*
Cholesterol 259 μM	–	76.7 ± 0.8*
Cholesterol sulphate 54 μM	9.9 ± 0.9*	117.9 ± 1.2*
Cholesterol phosphate 54 μM	8.1 ± 0.4*	110.1 ± 5.5*
Desmosterol 65 μM	11.8 ± 1.0**	110.4 ± 8.2*
Zymosterol 65μM	6.8 ± 1.8*	117.6 ± 2.8*
22-Keto-cholesterol 50 μM	1.7 ± 0.5*	68.9 ± 3.4**
7-Keto-cholesterol 62 μM	5.4 ± 0.3*	92.0 ± 6.9*
17α, 20α- diOH-cholesterol 62 μM	9.1 ± 1.8*	64.8 ± 5.0**
22S-OH-cholesterol 62 μM	15.3 ± 1.5**	43.8 ± 2.4**
20S-OH-cholesterol 50 μM	116.3 ± 5.6**	180.0 ± 2.3**
25-OH-cholesterol 50 μM	81.5 ± 3.9**	160.1 ± 3.5**

* Result not significantly different from control. ** $P < 0.05$.

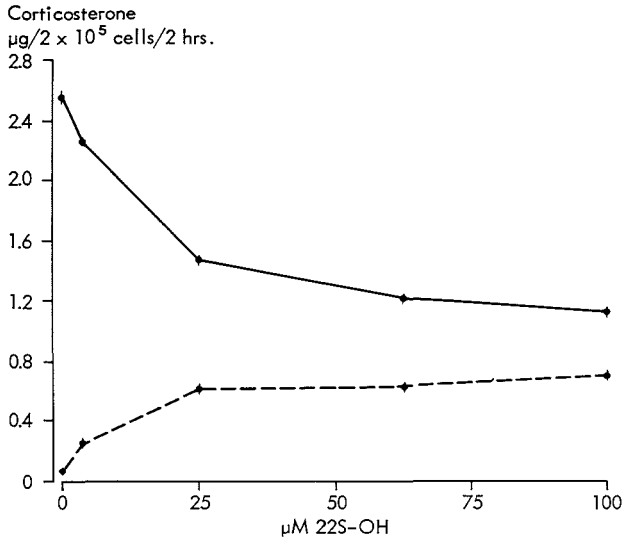

Fig. 1.

Effect of several concentrations 22S-OH on the corticosterone production in isolated rat adrenal cells in the absence (- - - -) or presence (——) of 1 ng of ACTH per ml. Incubations were done in triplicate; results are given as mean ± SEM.

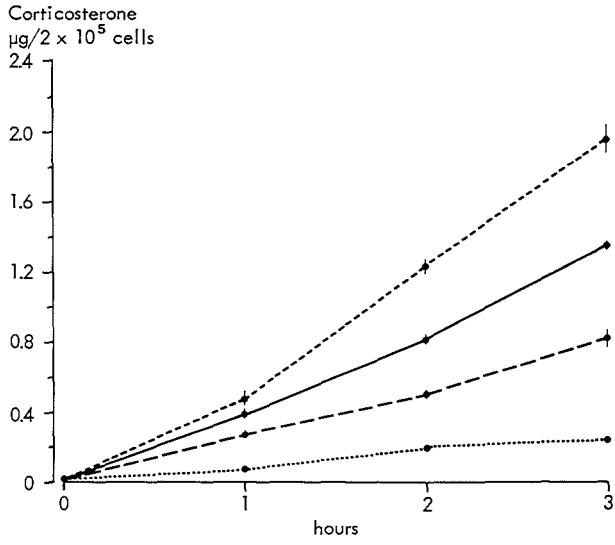

Fig. 2.
Time course of corticosterone production by isolated rat adrenal cells without additions (· · ·) and in the presence of 65 µM 22S-OH (– – –), 1 ng of ACTH per ml (- - - -) or both 22S-OH and ACTH (——). Incubations were done in triplicate; results are given as mean ± SEM.

Effect of several concentrations of 22S-OH on the ACTH-stimulation

A dose-response curve for the inhibitory effect of 22S-OH on the ACTH-stimulated corticosterone production is shown in Fig. 1. 22S-OH exerted a dose-dependent inhibition leveling off at higher concentrations.

The corticosterone production in the presence of 22S-OH increased up to sterol levels of 25 µM. At higher concentrations the steroid production was not further increased.

Time course of corticosterone production

The time course of corticosterone production in isolated rat adrenal cells in the absence or presence of ACTH and/or 22S-OH is shown in Fig. 2. The ACTH concentration was, again, near maximally stimulating in this experiment.

Corticosterone production was almost linear in time for all incubations. Even after 3 h no leveling off could be seen. The inhibition percentages for the effect of 22S-OH on the ACTH-stimulated corticosterone production were 71%, 70% and 69% at t = 1 h, t = 2 h and t = 3 h, respectively.

Effect of 22S-OH on the c-AMP concentration

The results of the cyclic AMP measurements in rat adrenal cells after a 20 min incubation period are listed in Table 2. No difference could be seen between a blank and a 22S-OH incubation; the cyclic AMP level did not rise above the background of the incubation buffer. ACTH significantly elevated the cyclic AMP levels. 22S-OH had no effect on this rise.

Table 2.
Adrenal cells were incubated for 20 min in the presence or absence of 1 ng ACTH per ml and/or 50 µM 22S-OH. Incubations were done in triplicate, results are given as mean ± SEM.

	pmol cAMP/10^5 cells/20 min
No additions	< 3.7
ACTH	47.5 ± 9.6
22S-OH	< 3.7
22S-OH + ACTH	53.7 ± 2.1

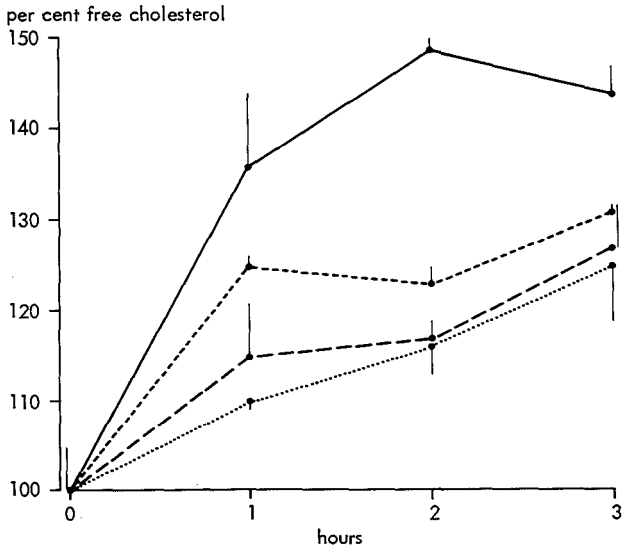

Fig. 3.

Time course of the free cholesterol concentration in isolated rat adrenal cells without additions (· · ·) and in the presence of 62 μM 22S-OH (– – –), 1 ng of ACTH per ml (- - - -) or 22S-OH plus ACTH (——). The free cholesterol concentration at t = 0 h is taken as 100%. Results are given as mean ± SEM of triplicate incubations.

Effect of 22S-OH on the free cholesterol concentration

The free cholesterol concentration in isolated rat adrenal cells was estimated after 1, 2, or 3 h respectively in the presence or absence of 22S-OH and/or ACTH. The results are shown in Fig. 3. Free cholesterol rose in the absence of an exogenous stimulus and/or substrate.

The presence of 62 μM 22S-OH had no effect on this small rise. Addition of 1 ng of ACTH per ml initially resulted in a small increase of the free cholesterol concentration; this difference with the blank incubation disappeared at longer incubation

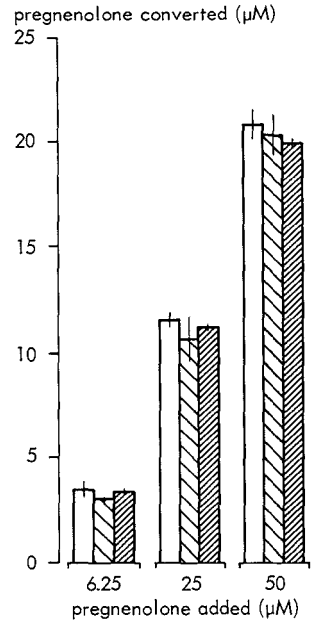

Fig. 4.

Conversion of exogenous (^{3}H-labelled) pregnenolone by rat adrenal cells without 22S-OH (open bars) and in the presence of 25 μM 22S-OH (hatched bars) or 50 μM 22S-OH (shaded bars). The pregnenolone concentration was 6.25, 25 and 50 μM. Incubations were done in duplicate; the range is indicated.

times. In the presence of both 22S-OH and ACTH a significant increase in free cholesterol is obvious, however, especially after two hours.

Effect of 22S-OH on the conversion of exogenous pregnenolone

The effect of two concentrations 22S-OH (25 μM and 50 μM) on the conversion of exogenous pregnenolone in three concentrations (6.25 μM, 25 μM and 50 μM) was measured as described under Materials and Methods. The results are given in Fig. 4. In the absence of 22S-OH pregnenolone was converted mainly into progesterone, deoxycorticosterone, corticosterone and 18-hydroxycorticosterone. An almost linear relationship exists between the amount of pregnenolone added and the amount of pregnenolone converted. None of the 22S-OH concentrations used had a significant effect on this conversion.

Discussion

Several long-term effects of hydroxylated sterols have been described in the literature (Chen et al. 1974; Kandutsch & Chen 1975; Peng et al. 1979; Imai et al. 1980) affecting de novo cholesterol synthesis at the level of the 3β-hydroxy-3-methylglutaryl CoA-reductase or through a direct toxic action.

In a previous report (Huijmans et al. 1977) a short-term inhibitory effect of some hydroxylated sterols on steroidogenesis in isolated rat adrenal cells was described. A diminished cholesterol synthesis cannot account for this effect because de novo cholesterol synthesis does not play an important role in adrenal steroidogenesis (Dexter et al. 1970; Gwynne et al. 1976; Balasubramaniam et al. 1977).

In addition the long term effects were only obvious after incubation with the sterol for several hours to days. In our studies the incubation time generally did not exceed two hours, and the inhibition of the corticosterone synthesis was already apparent after one hour (Fig. 2).

In Table 1 several additional sterols are listed that were investigated for an effect on steroid production. Neither cholesterol nor its sulphate or phosphate had much effect. The same is true for desmosterol and zymosterol, both precursors of cholesterol. Only when high cholesterol concentrations were used (Table 1, Sharma 1973) an inhibitory effect may be seen, probably due to purely physical effects (Falke et al. 1975b). 22-keto-cholesterol does not seem to be a substrate for steroid production, but it did inhibit the ACTH-stimulation. Both 20S-hydroxy-cholesterol and 25-hydroxy-cholesterol were found to be good substrates for steroid production. In the presence of ACTH corticosterone production was not completely additive, probably because the maximal steroidogenic capacity of the adrenal cell was reached.

A sterol with remarkable properties is 22S-OH, which seems to be a substrate, though not very effective, for steroid synthesis, while at the same time it inhibits the ACTH-stimulated corticosterone production (see Fig. 1). This inhibition is dose dependent. A direct adverse action of 22S-OH in concentrations up to 62 μM on the adrenal cells is less probable. This is suggested by the linearity of the corticosterone production in the presence of 22S-OH, as presented in Fig. 2. A similar conclusion for 25-hydroxy-cholesterol was reached by Falke et al. (1976).

The results with the cAMP-measurements (see Table 2) provide additional evidence that 22S-OH does not have non-specific (for example surfactant) effects on the cellular membrane. cAMP plays an intermediary role in the action of ACTH (Sala et al. 1979; Rae et al. 1979). 22S-OH had no effect on the cAMP concentrations after stimulation with ACTH. This indicates that the first part of the ACTH-stimulatory mechanism is probably unaffected. Analogous to our results Kandutsch & Chen (1975) found no effects of several sterols on the cAMP content of L-cell cultures, suggesting that the inhibitory sterols act through a mechanism not involving cAMP.

A further localization of the effect of 22S-OH could be accomplished by studying the liberation of cholesterol from its esters after stimulation with ACTH, as shown in Fig. 3. The effect of ACTH on the free cholesterol concentration is in agreement with the results of Vahouny et al. (1978), who found no change after stimulation with ACTH. The results obtained in the presence of both ACTH and 22S-OH suggest an inhibitory effect of 22S-OH on the further metabolism of cholesterol, once it is liberated from its esters by the esterase. In the rat a similar increase in free cholesterol after stimulation with ACTH was described when the further metabolism of cholesterol was impaired by treating the rat in vivo with the side-chain cleavage

inhibitor aminoglutethimide (Mahaffee et al. 1974) or the protein synthesis inhibitor cycloheximide (Davis & Garren 1966; Trzeciak & Boyd 1973).

Protein synthesis is required in the mechanism of action of ACTH; probably for the transport of cholesterol from the lipid droplets into the mitochondrion or the attachment to the side-chain cleaving enzyme system (Schulster et al. 1976). A direct effect of 22S-OH on protein synthesis cannot be excluded but is less probable. Kandutsch & Chen (1974) reported no effect of several hydroxylated sterols (a.o. 25-hydroxy-cholesterol and 20S-hydroxy-cholesterol) on protein synthesis or RNA- and fatty acid-synthesis. This suggests that the inhibitory action of 22S-OH resides in the cholesterol side-chain cleavage or the conversion of pregnenolone into its products. As shown in Fig. 4, 22S-OH, added in two concentrations (25 μM and 50 μM) had no effect on the conversion of exogenous pregnenolone.

The results presented here allow to locate the inhibitory effect of 22S-OH on the ACTH-stimulated corticosterone production in rat adrenal cells. The most probable site of action is the cholesterol side-chain cleavage. 22S-OH and cholesterol may compete for the active centers on the side-chain cleaving enzyme complex. Studies with bovine adrenal mitochondria support this conclusion; Simpson & Boyd (1967) showed that several cholesterol-derivatives, hydroxylated in positions 20, 24, 25 or 26 inhibited the conversion of cholesterol (the inhibition with 20S-hydroxy-cholesterol and 25-hydroxy-cholesterol being competitive). Probably 22S-OH not only competes for the active centers on the side-chain cleaving enzyme complex, but is being converted into pregnenolone too. This sterol proved to be a better substrate than cholesterol, when incubated with a purified bovine adrenal cytochrome P-450$_{scc}$. (Morisaki et al. 1976). The results in Figs. 1 and 2, obtained with rat adrenal cells, confirm the role of 22S-OH as a substrate for steroid production.

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CHAPTER 5

CONVERSION OF 22S-HYDROXY-CHOLESTEROL AND ITS EFFECT ON THE METABOLISM OF OTHER STEROLS IN RAT ADRENAL CELLS AND BOVINE ADRENAL MITOCHONDRIA.

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Conversion of 22S-hydroxy-cholesterol and its effect on the metabolism of other sterols in rat adrenal cells and bovine adrenal mitochondria

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Abstract. This study provides evidence that 22S-OH-cholesterol inhibits the conversion of 25-OH-cholesterol but has no effect on the conversion of 22R-OH-cholesterol. The latter sterol is an intermediate in the cholesterol side-chain cleavage, whereas for the conversion of 25-OH-cholesterol into pregnenolone the complete side-chain cleaving enzyme system is necessary. This complements a previous study in which it was shown, that 22S-OH-cholesterol has an inhibitory effect on the ACTH-induced conversion of cholesterol into corticosterone in isolated rat adrenal cells.

The available evidence thus suggests an inhibition by 22S-OH-cholesterol of the first step in the cholesterol side-chain cleavage.

The results, obtained from the experiments with rat adrenal cells and with bovine adrenal mitochondria, allow the hypothesis, that a causal relationship exists between conversion of 22S-OH-cholesterol and production of corticosterone, respectively pregnenolone.

We conclude, that 22S-OH-cholesterol is a substrate for steroid production in the adrenal cell. This sterol inhibits the ACTH-stimulated corticosterone production. The site of this inhibition is located at one of the first steps in the cholesterol side-chain cleavage, probably the binding of cholesterol to the cytochrome P_{450}-complex.

Several effects of hydroxylated sterols on cellular function, structure or metabolism are known (Kandutsch & Chen 1975; Heiniger et al. 1976; Peng et al. 1979; Streuli et al. 1979; Hsu et al. 1980; Imai et al. 1980; Huijmans et al. 1981). A possible role of these sterols in the regulation of the cholesterol synthesis has been suggested (Kandutsch & Chen 1974). In a previous report (Huijmans et al. 1981) we showed that some sterols inhibited the ACTH-stimulated corticosterone production in isolated rat adrenal cells. The studies with one sterol (22S-hydroxy-cholesterol (22S-OH)), suggested that this sterol probably exerts its action at the level of the cholesterol side-chain cleaving enzyme system. The exact mechanism of action as well as the role of 22S-OH as a substrate for steroid production in the adrenal cell is still not known.

In this report some additional studies will be presented in order to further localize the effect of 22S-OH and to evaluate the role of 22S-OH as a substrate for steroid production. We investigated a possible relationship between sterol conversion and steroid synthesis in rat adrenal cells and bovine adrenal mitochondria.

Materials and Methods

Materials

Bovine serum albumin (Cohn fraction V), collagenase (Type I) and DNase were obtained from Sigma Chemical Company. The albumin was purified by extensive dialysis and lyophilization. Hepes was from Calbiochem and synthetic $ACTH^{1-24}$ (Synacthen®) from Ciba-Geigy. 22S-Hydroxy-cholesterol was obtained from Ikapharm; all other sterols and steroids used were from Steraloids. Purity was checked by thin-layer chromatography and gas chromatography. All glassware coming in contact with the adrenal cells of ACTH-containing solutions was siliconized (Siliclad, Clay Adams).

Incubation media were sterilized before use by filtration through a Millipore filter (HAWP 02500; pore size 0.45 μm).

Methods

Preparation of the rat adrenal cell suspension. Cells were prepared from adrenals of male Wistar rats, weighing 190–210 g, as described by Falke et al. (1975a,b). A Krebs-Ringer-Hepes buffer, pH = 7.35, supplemented with 3% (w/v) albumin and 0.2% (w/v) glucose, was used.

Adrenal cell incubations. Cells were incubated at 37°C in a Dubnoff incubator, shaking at 100 r.p.m. The incubation time was 2 h, unless otherwise stated. The number of cells differed from experiment to experiment, but was kept between 100 000–150 000 per ml. ACTH was added in 0.1 ml of 0.9% NaCl, pH = 3.75, supplemented with 0.5% (w/v) albumin.

Sterols were added in 10 μl of ethanol. To incubations not containing ACTH or sterols, vehicle only was added. After the incubation period the tubes were immediately chilled in ice, tightly capped and stored at −20°C until further analysis.

Corticosterone was estimated by fluorometry, as described previously (Huijmans et al. 1981). With this method the minimal corticosterone concentration detectable is 30 ng per ml.

Preparation of the bovine adrenal metochondria. Bovine adrenals were obtained from the local slaughterhouse. The cortex was collected and homogenized in a Parr cell disruption bomb in a 10 mM Hepes buffer (pH = 7.30) containing 250 mM sucrose and 0.5 mM EDTA. The homogenate was centrifuged at 900 × *g* for 10 min at 4°C to remove unbroken cells, nuclei and erythrocytes. The mitochondria were obtained by centrifuging at 9000 × *g* for 10 min at 4°C. The pellet was washed two times and re-suspended to a protein concentration of 20 mg per ml. These mitochondria were frozen and stored in liquid nitrogen.

Mitochondrial incubations. Incubations were carried out at 37°C in a 20 mM Hepes buffer (pH = 7.40), containing 150 mM KCl, 11 mM NaCl, 6 mM $CaCl_2$, 4.3 mMNaN_3, 5 μM antimycine, 50 mM nicotinamide and 1% (w/v) albumin in a final volume of 5 ml. The initial sterol concentration was 50 mM. Cyanoketone (17 mM), an inhibitor of the 3β-hydroxy steroid dehydrogenase activity, was added to prevent further metabolism of pregnenolone. The incubation was started with the addition of 0.1 mM NADPH, 3 mM glucose-6-phosphate and 0.6 U per ml glucose-6-phosphate dehydrogenase. Aliquots of 1 ml were taken at t = 0, t = 8, t = 16 and t = 25 min and immediately deproteinated with 1 ml of cold acetone. The pellet was once again extracted with 1 ml of acetone. The combined acetone layer was shaken with 3 ml of dichloromethane. The organic layer was washed with 1 ml of water and dried. The extracts were derivatized and measured as described previously (Huijmans et al. 1981) except that a capillary column, coated with SE-30, was used. The minimal sterol concentration, detectable with this method, is 1 μM.

Results

Adrenal cell incubations

We examined the effects of 22S-OH on the conversion of 25-hydroxy-cholesterol (25-OH). The results are shown in Fig. 1. The corticosterone production in rat adrenal cells, incubated with 25-OH was virtually linearly dependent on the concentration of the sterol used. In the presence of 22S-OH the corticosterone production reached a maximum at 25 μM 22S-OH without further increment at 50 μM. With both 25-OH and 22S-OH the corticosterone synthesis was less than the sum of the sterol derived corticosterone production individually. This inhibitory effect became more clear in the presence of the high concentration of 25-OH (25 μM). Fig. 2 shows the effect of 22S-OH on the conversion of 25-OH in the presence of 100 pg ACTH per ml. This ACTH-concentration was maximally stimulating. The corticosterone production in the presence of ACTH and 12.5 μM 25-OH was almost additional; with 25 μM 25-OH cortico-

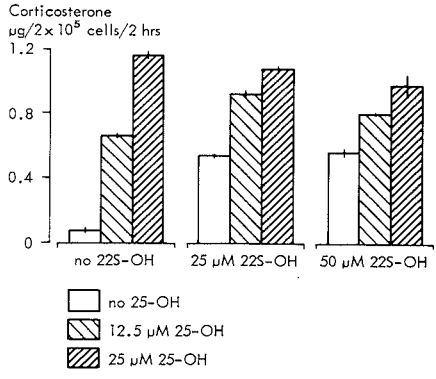

Fig. 1.
Effect of 22S-OH-cholesterol in two concentrations on the corticosterone production in isolated rat adrenal cells in the absence or presence of 25-OH-cholesterol. Results are presented as the mean of duplicate incubations and the individual values.

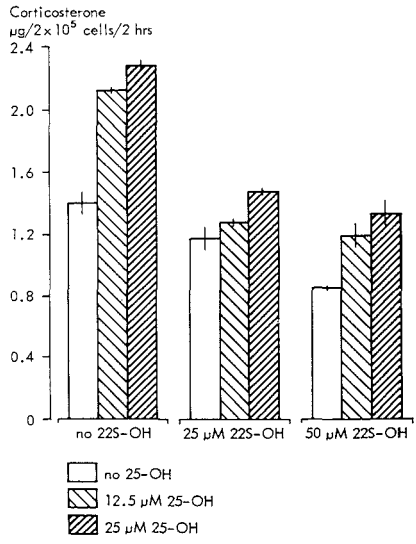

Fig. 2.

Effect of 22S-OH-cholesterol in two concentrations on the ACTH-stimulated corticosterone production in isolated rat adrenal cells in the absence or presence of 25-OH-cholesterol. The ACTH concentration was 100 pg per ml. Results are presented as the mean of duplicate incubations and the individual values.

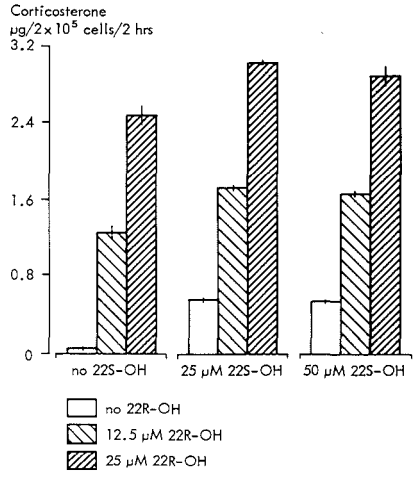

Fig. 3.

Effect of 22S-OH-cholesterol in two concentrations on the corticosterone production in isolated rat adrenal cells in the absence or presence of 22R-OH-cholesterol. Results are presented as the mean of duplicate incubations and the individual values.

sterone production was merely a fraction higher. In the presence of 25 µM 22S-OH, ACTH-stimulation was slightly inhibited; the inhibitory effect increased when both ACTH and 12.5 µM 25-OH were present. Again the higher 25-OH concentration did not change the effect of 22S-OH. In the presence of 50 µM 22S-OH, the ACTH-stimulated corticosterone production was inhibited by 39%. This 22S-OH concentration had no additional effects on the incubation with ACTH and 25-OH.

The same experiment was done with another hydroxylated sterol, 22R-hydroxy-cholesterol (22R-OH). Results are shown in Figs. 3 and 4. 22R-OH was also converted into corticosterone (Fig. 3); this conversion was linearly dose-dependent for the concentrations tested. The corticosterone production in the presence of 22S-OH and 22R-OH was completely additive for both concentrations. The results in the presence of 100 pg ACTH per ml are shown in Fig. 4. ACTH-stimulation and conversion of 22R-OH were addi-

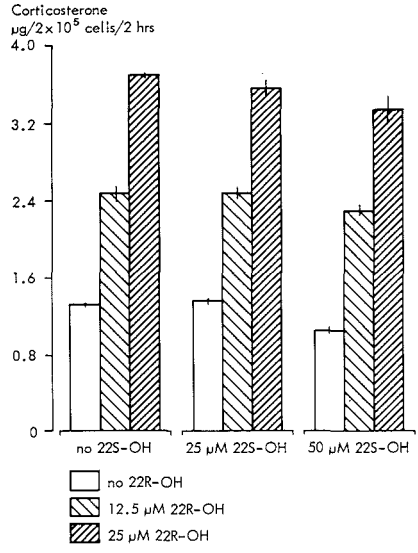

Fig. 4.

Effect of 22S-OH-cholesterol in two concentrations on the ACTH-stimulated corticosterone production in isolated rat adrenal cells in the absence or presence of 22R-OH-cholesterol. The ACTH concentration was 100 pg per ml. Results are presented as the mean of duplicate incubations and the individual values.

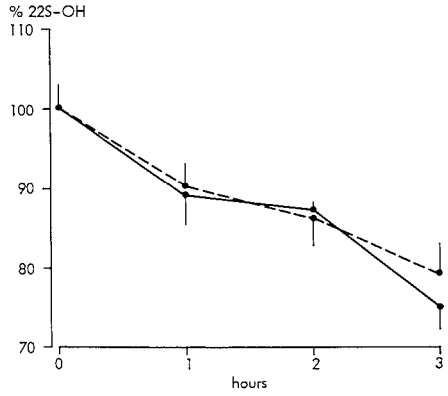

Fig. 5.
Conversion of 22S-OH-cholesterol in isolated rat adrenal cells, determined gas chromatographically, in the absence (----) or presence (——) of 1 ng ACTH per ml. The concentration measured at t = 0 was put at 100%. Results are given as mean ± SEM of triplicate incubations.

tional. Twenty-five μM 22S-OH had no effect on the corticosterone production. Fifty μM 22S-OH inhibited the ACTH-stimulated corticosterone production. This inhibition was the same with or without 22R-OH.

An additional experiment was done to investigate the course of the 22S-OH concentration upon incubation with adrenal cells. We measured the disappearance of 22S-OH after several incubation periods, the results of which are shown in Fig. 5. The concentration of 22S-OH decreased over a 3 h incubation period. The correlation-coefficient of the linear regression line was 0.84, which is significant at the 1% level. The slope of the fitted regression line differed significantly from zero. In the presence of 1 ng ACTH per ml (optimally stimulating in this batch of adrenal cells) the results were almost the same (correlation coefficient of the fitted regression line was 0.89).

Mitochondrial incubations

Several sterols were studied for their conversion into pregnenolone by damaged bovine adrenal mitochondria. We measured the sterol concentrations as a function of the incubation time. The results of a typical experiment are shown in Fig. 6. 22R-OH in a concentration of 50 μM was a good substrate. It had completely disappeared after 10 min and was almost quantitatively converted into pregnenolone. After a 25 min incubation period 9% of the initial 25-OH concentration (50 μM) was left. 22S-OH disappeared in time; after 25 min some 30% of the initial concentration

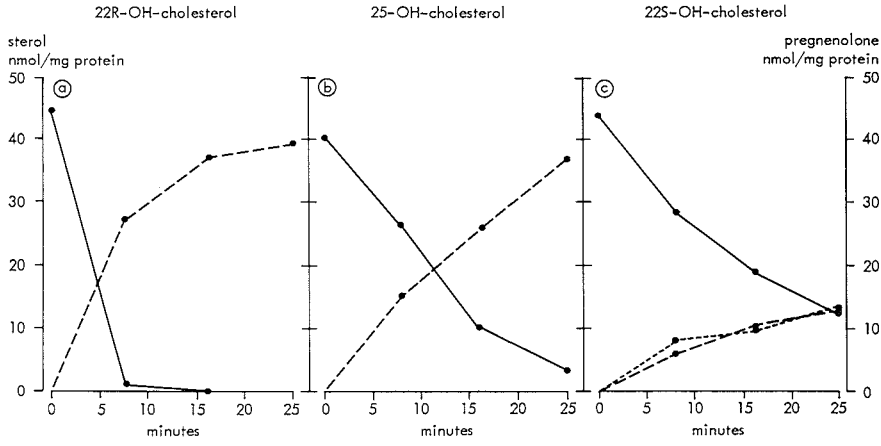

Fig. 6.
Production of pregnenolone from 22R-OH-cholesterol (a), 25-OH-cholesterol (b) and 22S-OH-cholesterol (c) during incubation with bovine adrenal mitochondria. The initial sterol concentrations were 50 μM. (——) Sterol concentration; (– – –) pregnenolone concentration; (----) 22-keto-cholesterol concentration.

(50 μM) was left. Parallel to this conversion pregnenolone was produced. However, besides pregnenolone another product appeared with (gas chromatographically) the same properties as 22-keto-cholesterol.

Discussion

In a previous report (Huijmans et al. 1981) we demonstrated an inhibitory effect of 22S-OH on the ACTH-stimulated corticosterone production in rat adrenal cells. The most probable site of action was the cholesterol side-chain cleaving enzyme system.

Using several substrates, entering the cholesterol side-chain cleaving enzyme system via different intermediates, the effect of 22S-OH can be localized.

1. 22S-OH inhibits the ACTH-stimulated corticosterone production from endogenous cholesterol (Huijmans et al. 1981). Cholesterol needs the complete side-chain cleaving enzyme system for its conversion.

2. 22S-OH has an inhibitory effect on the conversion of 25-OH (Figs. 1 and 2). 25-OH is not an intermediate in the side-chain cleavage reaction; probably all steps of the cholesterol side-chain cleavage are involved in its conversion (Falke et al. 1975b).

3. 22S-OH has no effect on the conversion of 22R-OH (Figs. 3 and 4). This sterol is the first intermediate in the side-chain cleavage, according to the classical scheme with 22R-OH, 20R,22R-dihydroxy-cholesterol as the main intermediates (Burstein et al. 1975; Burstein & Gut 1976). The same is true for the epoxy-diol scheme involving $\Delta^{20\text{-}22}$-cholesterol, 20,22-epoxy-cholesterol and probably 22R-OH as intermediates (Kraaipoel et al. 1975a,b: hypothesis B).

From these results one may conclude that 22S-OH inhibits a step in the cholesterol side-chain cleavage, prior to 22R-OH. This means the transport of cholesterol to and within the mitochondria, binding to the cytochrome P-450 complex and conversion into 22R-OH (classical scheme, epoxy-diol scheme: hypothesis B) or into $\Delta^{20\text{-}22}$-cholesterol (epoxy-diol scheme: hypothesis A).

The cytochrome $P\text{-}450_{scc}$-complex is located at the matrix side of the inner mitochondrial membrane (Simpson 1979). The substrates have to reach that site. Mason et al. (1978) studied transport phenomena in rat adrenal mitochondria and found that several sterols, hydroxylated in positions 20, 24, 25 and 26 rapidly reached the cytochrome P-450, whereas exogenous cholesterol did not. The metabolism of these hydroxylated sterols was independent of prior treatment of the rats with the protein synthesis inhibitor cycloheximide, Falke et al. (1976) showed a lack of effect of cycloheximide on the conversion of 25-OH in adrenal cells. The same is true for 22S-OH (Huijmans, unpublished observation). These results suggest, that for the transport of hydroxylated sterols to the cytochrome P-450 complex no rapidly induced protein factor is needed. Different transport mechanisms for cholesterol and hydroxylated sterols are likely. This is supported by the description of two cytosolic binding proteins in L-cells and foetal mouse liver cells (Kandutsch et al. 1977). One protein specifically bound cholesterol and the other one 25-OH, 7-keto-cholesterol and 24-keto-cholesterol. However, no such studies are known for the adrenal. One may conclude that an effect of 22S-OH on the transport of cholesterol becomes less probable.

Binding of cholesterol to the cytochrome P-450 was reported to be rate-limiting in steroidogenesis (Mahaffee et al. 1974; Williams-Smith et al. 1976). It is difficult to distinguish between the binding process and the intramitochondrial movement of cholesterol; the latter process was also reported to be a rate-limiting step (Crivello & Jefcoate 1980). Orme-Johnson et al. (1979) studied numbers of binding sites in beef adrenocortical preparations. They found one binding site per heme moiety. Sterols like 22S-OH, 22R-OH and 20R,22R-dihydroxy-cholesterol did bind to the cytochrome P-450.

Our experimental results (see also Huijmans et al. 1981) suggest a competition of cholesterol and 22S-OH for the binding site on the cytochrome P-450. The affinity of 22S-OH may be stronger compared to that of cholesterol. This may be illustrated by the fact that small concentrations of 22S-OH have great influence on the ACTH-stimulated corticosterone production. In addition, the presence of ACTH has almost no effect on the conversion of 22S-OH (Fig. 5). If 22S-OH binds to the cytochrome P-450, it still remains to be proven whether it will be converted. Morisaki et al. (1976) showed 22S-OH to be a better substrate than cholesterol, when incubated with a purified bovine

adrenocortical cytochrome P-450 preparation. Our results with damaged bovine adrenal mitochondria, presented in Fig. 6, show 22S-OH to be a good substrate. In 25 min almost 60% of the initial concentration has disappeared. Two products are formed in equimolar amounts, one of which has been gas chromatographically identified as pregnenolone. The other compound could be identified as 22-keto-cholesterol by gas chromatography – mass-spectrometry (Alsema et al., subm. for publ.).

When rat adrenal cells were incubated with 22S-OH both the appearance of corticosterone (Huijmans et al. 1981) and the disappearance of 22S-OH (Fig. 5) were linear with time. This suggests the existence of a relationship. 22-Keto-cholesterol could not be detected in these adrenal cell incubations. Although 22-keto-cholesterol inhibits the ACTH-stimulated corticosterone production (Huijmans et al. 1981) a possible mechanism of action of 22S-OH by way of 22-keto-cholesterol is not supported by the present results.

In conclusion it appears very likely that 22S-OH is a substrate for steroid production in the adrenal cell. It exerts its inhibitory action on the ACTH-stimulated corticosterone production via a step prior to 22R-OH. The most probable mechanism is a competition between 22S-OH and cholesterol for the binding sites at the cytochrome P-450 complex.

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CHAPTER 6

EFFECTS OF SOME HYDROXYLATED STEROLS ON THE STEROID PRODUCTION IN ISOLATED RAT ADRENAL GLOMERULOSA AND FASCICULATA/RETICULARIS CELLS IN THE PRESENCE OF POTASSIUM, ANGIOTENSIN II OR ACTH.

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Effects of some hydroxylated sterols on the steroid production in isolated rat adrenal glomerulosa and fasciculata/reticularis cells in the presence of potassium, angiotensin II or ACTH

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Abstract. 22S-OH-cholesterol and 25-OH-cholesterol are good aldosterone precursors in isolated rat adrenal glomerulosa cells. In the two concentrations tested (25 and 50 μM) 25-OH-cholesterol stimulated the aldosterone production in a (not-linear) dose-dependent way. An increase in the 22S-OH-cholesterol concentration from 25 μM to 50 μM led to a decrease in the aldosterone production.

The exogenous substrate deoxycorticosterone, entering the steroidogenic pathway after the cholesterol side-chain cleavage, is a much better substrate than the sterols mentioned. These results suggest that the cholesterol side-chain cleavage is the rate-limiting step in the aldosterone production from both sterols.

We found no effect of the sterols on the potassium-induced aldosterone synthesis. This might be explained by the existence of separate pools of steroid intermediates within the adrenal cell.

In vitro a difference in steroid production rates exists between glomerulosa and fasciculata/reticularis cells. This may arise from differences in availability of endogenous steroid precursors like cholesterol. However similar differences can be observed if exogenous substrates like 25-OH-cholesterol or 22S-OH-cholesterol are used. These results therefore suggest that enzymatic activities in the steroidogenic pathway are more important than the cholesterol concentration in regulating steroid production in isolated rat adrenal glomerulosa and fasciculata/reticularis cells.

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In previous reports (Huijmans et al. 1981, 1982) we showed 25-OH-cholesterol (25-OH) and 22S-OH-cholesterol (22S-OH) to have different effects on the ACTH-stimulated corticosterone production in isolated rat adrenal cells. 25-OH is a good substrate for steroid production; even with an optimally-stimulating ACTH-concentration additional corticosterone is produced in its presence. In contrast 22S-OH inhibits the ACTH-stimulated corticosterone production in a dose-dependent way. These results were obtained with cell suspensions from whole rat adrenals. The three main types of adrenal cells were present in these suspensions, all contributing to the corticosterone synthesis (Bell et al. 1979). Zona fasciculata + zona reticularis cells comprise about 85% of the adrenal cortex volume (Bloom & Fawcett 1968). In these cell types regulation of steroid production is mainly ACTH-dependent. In zona glomerulosa cells other stimuli, such as potassium (K^+) and angiotensin II (A II), are at least equally important.

It is not known, whether hydroxylated sterols can influence steroid production in the presence of these latter compounds. In this paper we wish to report the role of 25-OH and 22S-OH as substrates for aldosterone production in isolated rat glomerulosa cells, as well as effects of these sterols on the K^+- and A II-induced aldosterone production. A comparison will be made with the aldosterone production from another exogenous substrate,

deoxycorticosterone (DOC), entering the steroidogenic pathway after the cholesterol side-chain cleaving enzyme system. In addition the conversion of 25-OH and 22S-OH and their effects on the ACTH-stimulated corticosterone production in isolated fasciculata/reticularis cells will be described.

Materials and Methods

Materials

Bovine serum albumin (Cohn fraction V), collagenase (type I) and DNase were obtained from Sigma Chemical Company. The albumin was purified by extensive dialysis against 0.9% NaCl and finally against double-distilled water. Hepes was from Calbiochem. 22S-OH was obtained from Makor; all other steroids and sterols were from Steraloids. [Asp^{1}Ile^5]-A II was a product of Organon. Hypertensin ([Asn^{1}Val^5]A II) was from Ciba Geigy.

All glassware coming in contact with the adrenal cells was siliconized (Siliclad, Clay Adams). Incubation media were sterilized before use by filtration through a Millipore filter (HAWP 02500; pore size 0.45 μm).

Methods

Preparation of glomerulosa and fasciculata/reticularis cells. Adrenal glands from male Wistar rats, weighing 190–210 g, were removed and dissected free of adhering fat tissue. The glands were incised followed by manual compression of each gland to extrude the zonae fasciculata and reticularis and the medulla from the capsule + zona glomerulosa. For the preparation of glomerulosa cells the capsules were cut in small pieces and washed with a Krebs-Ringer-Hepes buffer, pH = 7.35, supplemented with 3% (w/v) albumin and 0.2% (w/v) glucose (KRHAG). Cells were prepared by digestion with collagenase/DNase and by sucking and expelling them several times in a Pasteur pipette, as described by Falke et al. (1975a,b). The cells were centrifuged at 150 × *g* for 10 min. Finally the cells were resuspended in KRHAG and counted. Average yields were 30 800 cells per adrenal. The purity of the glomerulosa cell suspension was checked by microscopic examination after staining with haematoxylin/eosin. Generally, > 95% of the cells was of the glomerulosa type. The remaining fasciculata/reticularis cells were prepared as previously described (Falke et al. 1975a,b).

Adrenal cell incubations. Cells were incubated for 2 h in KRHAG at 37°C in a Dubnoff incubator shaking at 100 r.p.m. Depending upon the experiment, the number of glomerulosa cells used varied from 25 000 to 40 000 per ml and 150 000 to 200 000 per ml for fasciculata/reticularis cells. A II and ACTH were added in KRHAG. All sterols were added in 10 μl of ethanol. To incubations not containing sterols vehicle only was added. The Na^+ and K^+ concentrations in KRHAG were 112.3 mM and 3.6 mM, respectively. If higher K^+ concentrations were required, Na^+ was replaced by K^+.

After the incubation period the tubes were rapidly chilled in ice, tightly capped and stored at −20°C until further analysis.

Analytical procedures. Aldosterone was measured by radioimmunoassay (CEA, France) after extraction with dichlo-

Table 1.
Conversion of 25-OH and 22S-OH into corticosterone and their effect on the ACTH-stimulated corticosterone production in isolated rat adrenal fasciculata/reticularis cells. Incubations were done in duplicate.

	Corticosterone production (ng/10^5 cells/2 h) (mean and range)	
	No ACTH	0.5 ng/ml ACTH
Control	23 (23–23)	716 (699–733)
25 μM 25-OH	286 (274–297)	834 (778–889)
50 μM 25-OH	425 (384–466)	957 (948–966)
25 μM 22S-OH	220 (215–225)	629 (607–651)
50 μM 22S-OH	194 (182–206)	516 (513–518)

romethane. The recovery for this procedure was 86 ± 13%. The cross-reactivity for some relevant sterols and steroids were:

11-deoxycorticosterone	0.0025%
18-OH-corticosterone	0.01%
18-OH-11-deoxycorticosterone	0.03%
corticosterone	0.0025%
25-OH-cholesterol	< 0.0005%
22S-OH-cholesterol	< 0.0005%

Corticosterone was measured by fluorometry, as previously described (Huijmans et al. 1981).

Statistical analysis. When indicated, the results of three experiments with duplicate incubations were statistically evaluated using a two-way analysis of variance (Snedecor & Cochran 1967).

Results

Fasciculata/reticularis cells

The results with an adrenal fasciculata/reticularis cell suspension are shown in Table 1. 25-OH was a good substrate. 22S-OH was a less efficient substrate, especially at a concentration of 50 μM. In the presence of a maximally-stimulating concentration of ACTH (0.5 ng/ml) incubation with 25-OH resulted in an additional corticosterone production. 22S-OH in both concentrations inhibited the ACTH-stimulated corticosterone production.

Glomerulosa cells

Rat adrenal glomerulosa cells were incubated in the presence of several concentrations of K^+ or [Asp[1]Ile[5]]A II. K^+ was a potent stimulator of aldosterone production with an optimally-stimulating concentration at 8.4 mM. In further experiments 3.6 mM K^+ and 8.4 mM K^+ were used as non-stimulatory respectively maximally-stimulatory concentrations. A II was a weaker stimulator of aldosterone production in these cells. A maximum was reached at 10^{-8} M (confirming the results of Aguilera et al. (1981), Tait & Tait (1976) and Tait et al. (1980a)).

Consequently the two hydroxylated sterols, 25-OH and 22S-OH, were incubated with rat glomerulosa cells. The combined results of three experiments are shown in Fig. 1. Aldosterone production in the presence of 25 μM 25-OH exceeded the K^+-stimulated aldosterone production by a factor 5, but K^+ still had an additional effect ($P < 0.05$). Aldosterone production in the presence of 50 μM 25-OH was significantly higher ($P < 0.05$), compared with the lower concentration of 25-OH. K^+ had no significant effect at this higher 25-OH

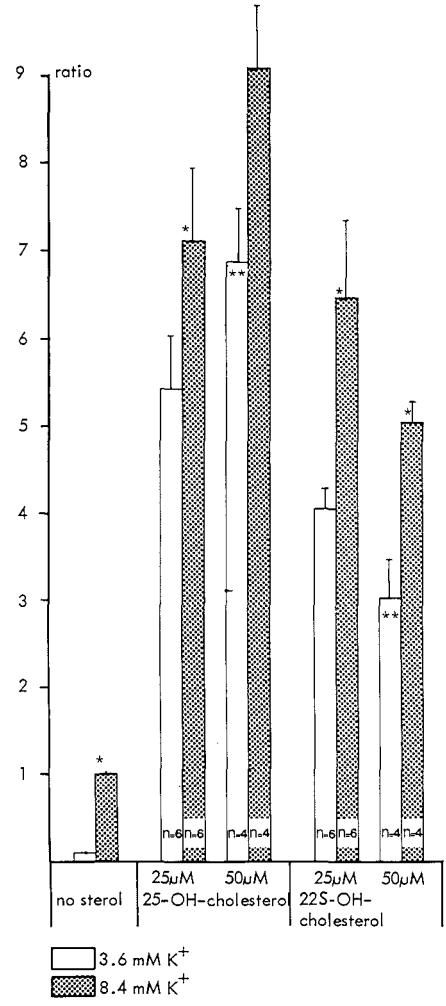

Fig. 1.
Ratio of aldosterone output in the presence of various stimuli and the aldosterone production at the maximally-stimulating concentration of K^+ in isolated rat glomerulosa cells. Results are given as the mean ± SEM of 2–3 experiments (in duplicate). Presence of 8.4 mM K^+ vs 3.6 mM K^+: $*P < 0.05$. Presence of 50 μM sterol vs 25 μM sterol: $**P < 0.05$. N: number of incubations.

concentration. The other sterol, 22S-OH, was a good substrate for aldosterone production too, although less efficient than 25-OH ($P < 0.05$). Incubation with the higher 22S-OH concentration resulted in a lower aldosterone production ($P < 0.05$). In both situations K^+ had an additional effect ($P < 0.05$).

[Asp^{1}Ile^5]A II had a small stimulatory effect on the aldosterone production by glomerulosa cells at the concentrations tested. A plateau was reached at 10^{-8} M; this level was maintained at higher concentrations up to 10^{-4} M (results not shown; see for comparison Tait et al. 1980a, Mendelsohn & Kachel 1981). Adrenal glomerulosa cells were incubated in the presence of 10^{-8} M [Asp^{1}Ile^5]A II or 10^{-8} M [Asn^{1}Val^5]A II and 25 μM 25-OH or 22S-OH. Results of a typical experiment are shown in Fig. 2.

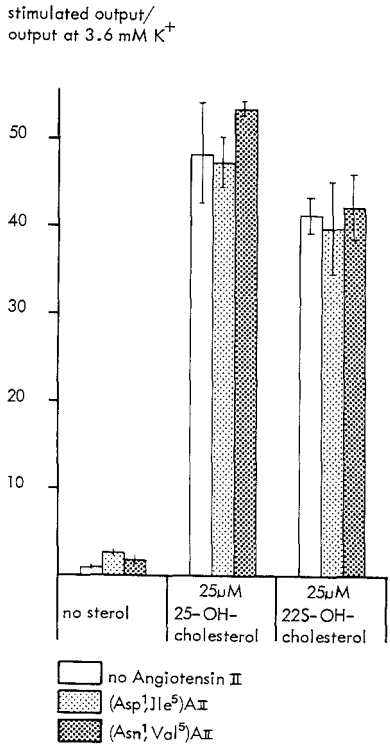

Fig. 2.
Effects of 25-OH and 22S-OH on the aldosterone production in isolated rat adrenal glomerulosa cells in the absence or presence of 10^{-8} M [Asp^{1}Ile^5]A II or 10^{-8} M [Asn^{1}Val^5]A II. Results are given as the mean and the range of duplicate incubations.

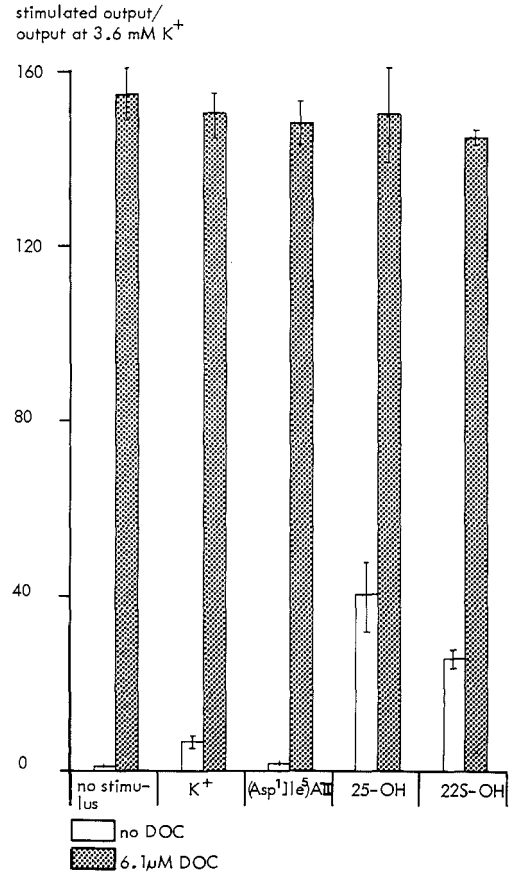

Fig. 3.
Effect of K^+ (8.4 mM), [Asp^{1}Ile^5]A II (10^{-4} M), 25-OH (25 μM) or 22S-OH (25 μM) on the aldosterone production in isolated rat glomerulosa cells in the absence or presence of 6.1 μM DOC. Results are given as the mean and the range of duplicate incubations.

We investigated the effect of 25-OH and 22S-OH, sterols which require the complete side-chain cleaving enzyme system for conversion into preg-

nenolone (Falke et al. 1975b; Alsema et al. 1982), on the conversion of DOC, a substrate entering the steroidogenic pathway after the side-chain cleavage. DOC was chosen, because it is known to be a good precursor for aldosterone production in rat adrenal capsules (Fattah et al. 1977). 6.1 μM DOC was rapidly converted into aldosterone in isolated rat glomerulosa cells (Fig. 3). Neither 8.4 mM K^+ or 10^{-4} M [Asp^{1}Ile^5]A II nor 25 μM 25-OH or 22S-OH had an effect on the aldosterone production in the presence of DOC.

Discussion

The aim of this study was to investigate possible effects of 25-OH and 22S-OH on rat adrenal glomerulosa cells. The method used in the preparation of these cells, however, does not preclude the presence of a small percentage of contaminating fasciculata cells. The question therefore arises, whether metabolites of fasciculata cells might be responsible for additional aldosterone production in the presence of the sterols. Taking into account the steroid production in fasciculata cells (as shown in Table 1) and literature data on steroid production profiles in isolated adrenal cells (Bell et al. 1979; Vinson et al. 1979; Tait et al. 1980a) an upper limit to the amount of aldosterone produced from fasciculata metabolites can be calculated. For 25-OH (50 μM) this does not exceed 5% of the total aldosterone production.

25-OH is a good substrate for aldosterone production in rat adrenal glomerulosa cells (Fig. 1). Using DOC, a substrate entering the steroidogenic pathway after pregnenolone, a much greater aldosterone production can be observed (Fig. 3). This could mean that the side-chain cleavage reaction is the rate-limiting step. 22S-OH is a good substrate too (Fig. 1). However an increase in the 22S-OH concentration leads to a decreased aldosterone production. As the conversion of DOC is not inhibited by 22S-OH (Fig. 3) this suggests that 22S-OH inhibits its own side-chain cleavage. Similar results are obtained for the corticosterone production in fasciculata/reticularis cells (see Table 1). In these cell types 22S-OH also has an inhibitory effect on the ACTH-stimulated corticosterone production, confirming earlier results with whole adrenal cell suspensions (Huijmans et al. 1981). We therefore conclude that 22S-OH has an effect on the cholesterol side-chain cleavage.

K^+ stimulates one of the first steps in aldosterone synthesis (probably the cholesterol side-chain cleavage) as well as one or more late steps (McKenna et al. 1978; Aguilera & Catt 1979). The apparent absence of an inhibitory effect of 22S-OH on the K^+ stimulation may be explained by the existence of separate pools of steroids within the cell, which are important in the control of steroidogenesis (Vinson et al. 1979). In particular the pool of 18-oxygenated steroids might play a key role in the stimulation by K^+ of the late steps in aldosterone formation.

In vitro there is a difference in steroidogenic capacity between glomerulosa and fasciculata cells. Estimated steroid production rates in fasciculata cells in the presence of ACTH or other stimuli exceed the production rates observed in glomerulosa cells by a factor 10 (Tait et al. 1980b). It could mean that the glomerulosa cell contains less endogenous steroid precursors (e.g. cholesterol). This is in agreement with histological findings; a smaller amount of lipid droplets has been found in the glomerulosa cells (Bloom & Fawcett 1968). However the availability of substrate can not be the only explanation as can be deduced from our results with the exogenous sterols 25-OH and 22S-OH. Corticosterone production from 25-OH in fasciculata/reticularis cells is 285 ng/10^5 cells/2 h (Table 1), whereas the mean aldosterone production from the same amount of 25-OH in glomerulosa cells is only 16 ng/10^5 cells/2 h (Fig. 1). The two additional steps in the production of aldosterone from corticosterone (18-hydroxylase and 18-dehydrogenase) might be responsible for this difference. However, as demonstrated with the exogenous substrate DOC, these steps are not rate-limiting. One may conclude that the cholesterol side-chain cleavage activity is responsible for the in vitro observed difference in steroidogenic capacity between glo merulosa and fasciculata/reticularis cells.

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CHAPTER 7

EFFECT OF 25-HYDROXY-CHOLESTEROL ON THE UPTAKE OF CORTICOSTERONE IN ISOLATED RAT LIVER CELLS.

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Effect of 25-Hydroxy-Cholesterol on the Uptake of Corticosterone in Isolated Rat Liver Cells

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Summary

Isolated rat liver cells are able to take up corticosterone. The Arrhenius plot of the uptake shows a biphasic course with a change in the slope around 25° C.

25-OH-cholesterol is also taken up; this phenomenon reaches a maximum at 10–15 minutes. After preincubating liver cells at 37° C in the presence of this sterol the phase transition is shifted to a higher temperature (32° C), as shown in the Arrhenius plot of the corticosterone uptake. At the same time the uptake of corticosterone is diminished. This cannot readily be explained by direct competition. The mechanism might involve an inhibition of an active uptake mechanism caused by a change in the plasma membrane.

Key-Words: *Hydroxylated Sterol – Steroids – Cell Membrane – Steroid Uptake*

Introduction

It is commonly assumed, that the first step of steroid hormone action is passive diffusion through the cell membrane. Some authors, however, reported mediated transport mechanisms (*Rao* 1981, *Milgrom, Atger* and *Baulieu* 1973; *Jonkers, Timmermans, de Jong, Lamberts* and *Birkenhäger* 1980). These observations are supported by the existence of stable, dexamethasone-resistent, murine thymoma cell lines, in which selective membrane permeability was a rate-limiting step in steroid hormone action (*Johnson, Newby* and *Bourgeois* 1984). The generalized steroid hormone resistance, observed in some species, also suggests a defect at the level of the cell membrane (*Lipsett, Chrousos, Tomita, Brandon* and *Loriaux* 1985).

The aim of the present study was to investigate possible effects of hydroxylated sterols on the uptake process. The rationale was, that in several types of cells adverse effects of hydroxylated sterols on membrane structure and function have been demonstrated. Human polymorphonuclear leukocyte chemotaxis is inhibited by oxygenated sterols (*Gordon, Bass* and *Yachnin* 1980), in human erythrocytes the calcium channel is modulated by oxygenated sterols (*Neyses, Locher, Stimpel, Streuli* and *Vetter* 1985), the osmotic fragility of human erythrocytes is diminished following the membrane insertion of oxygenated sterols (*Streuli, Kanofsky, Gunn* and *Yachnin* 1981).

Because the naturally occurring 25-OH-cholesterol (25-OH) is one of the more potent sterols in this regard (*Kandutsch, Chen* and *Heiniger* 1978), we used as a model system the effect of 25-OH on the uptake of corticosterone in isolated rat liver cells.

Materials and Methods

Materials

Bovine serum albumin (Cohn fraction V), collagenase (type I) and DNase were obtained from Sigma Chemical Company. The albumin was purified by extensive dialysis and lyophilization. Hepes was from Calbiochem. All sterols and steroids used were from Steraloids. [26-^{14}C]-25-OH (4.9 mCi/mmol) and [1,2-^{3}H]-corticosterone (45 Ci/mmol) were bought from Amersham. All glassware coming in contact with the cells was siliconized (Siliclad, Clay Adams). Incubation media were sterilized before use by filtration through a Millipore filter (HAWP 02500, pore size 0.45 μm).

Methods

For each experiment the liver of a male 200 gram Wistar rat was removed, cut into pieces and washed with 0.9% NaCl and with a Krebs-Ringer-Hepes buffer (pH = 7.35), supplemented with 3% (W/V) albumin and 0.2% (W/V) glucose (= KRHAG). Cells were prepared by digestion with collagenase/DNase using the method as described for rat adrenal cells (*Falke, Degenhart, Abeln, Visser* and *Croughs* 1975; *Huijmans, Degenhart* and *Kortleve* 1982), except that the liver cells were centrifuged at 100 xg for 5 minutes.

Preincubations and incubations of the liver cells were done at 37° C (except when stated otherwise) in a Dubnoff incubator, shaking at 100 rpm. After the incubation the cells were isolated on glassfibre filters (Whatman GF/C), using a Millipore filter manifold and washed twice with 4 ml KRHAG. The filters were dried at 50° C and solubilized at 37° C in Nuclear Chicago tissue solubilizer. After 12 hours 10 ml of toluene scintillation fluid, containing PPO and POPOP was added. Radioactivity was counted in a liquid scintillation counter (Mark III, Searle).

Statistics

Data treatment was performed as indicated in the legends to the figures and table. The slopes and intersection points of the straight lines in the Arrhenius plot (Fig. 2) were calculated by means of a numerical method for estimating the least sum of squares (*Draper* and *Smith* 1981).

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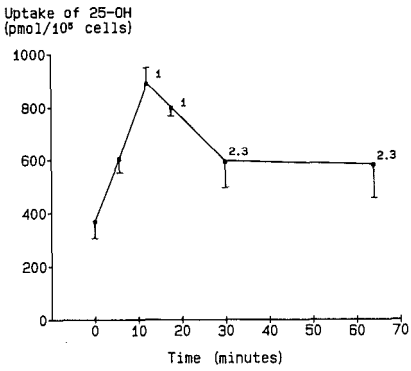

Fig. 1 Time course of the uptake of 21 μM [26-^{14}C]-25-OH in isolated rat liver cells at 37°C. Results, given as the mean±SEM of 3–5 incubations, are statistically evaluated with a test, described by Kruskal and Wallis 1952.
1: $P < 0.01$ versus t = 0 minutes
2: $P < 0.01$ versus t = 10 minutes
3: not significant versus t = 0 minutes

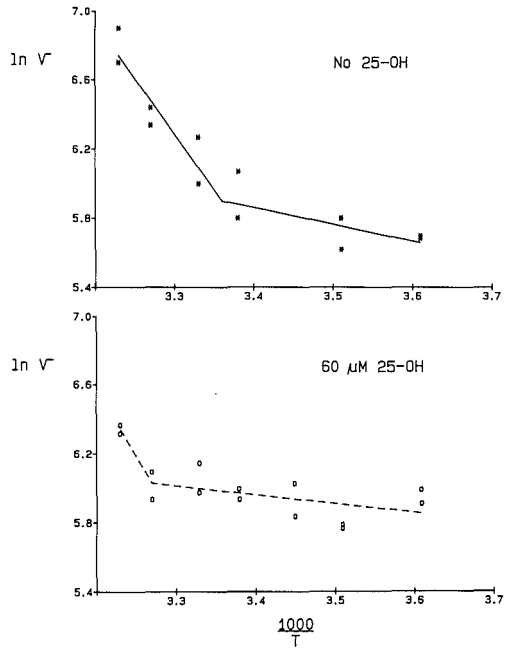

Fig. 2 Arrhenius plot of the uptake of 0.20 μM [^{3}H]-corticosterone in isolated rat liver cells at different incubation temperatures in the absence (upper) or presence (lower) of 60 μM 25-OH. Incubations were done in duplicate. The individual values, expressed as ln (pmol/min/10^5 cells), are plotted.

Table 1 Effect of 25-OH and several steroids on the uptake of corticosterone in isolated rat liver cells at 37°C. Cells were preincubated in the presence of the sterol/steroids for 15 minutes at 37°C. The incubation time was 10 minutes. The corticosterone concentration was 1.6×10^6 dpm/4 x 10^5 cells (16 nM). Results were statistically evaluated with a test described by Kurtz, Link, Tukey and Wallace 1965.

60 μM sterol/steroid	Percentage of [^{3}H]-corticosterone uptake (mean and range of duplicate incubations)	
Control	100	(99–101)
25-OH	60	(55–64)*
pregnenolone	26	(25–27)*
progesterone	28	(24–32)*
deoxycorticosterone	25	(24–26)*
corticosterone	26	(21–31)*
cortisol	26	(25–27)*

*$P < 0.01$

100% = 6.2×10^4 dpm/4 x 10^5 cells

Results

The time course of the uptake of 21 μM [26-^{14}C]-25-OH is given in Figure 1. A maximum was reached at 10 minutes, followed by a significant decline. The effect of 25-OH and several steroids on the uptake of corticosterone in rat liver cells is shown in Table 1. All substances tested inhibit the corticosterone uptake significantly.

We studied the effect of 25-OH on the uptake of corticosterone in rat liver cells at different incubation temperatures ranging from 4°C to 37°C. The cells were preincubated for 15 minutes in the absence or presence of 25-OH. The preincubation temperature was the same as the incubation temperature. The uptake of corticosterone over the first 50 seconds of the incubation was measured. The Arrhenius plot is shown in Figure 2. In the absence of 25-OH a change in the slope of the curve occurred at 25°C. The activation energy was 12.0 Kcal/mol from 37°C to 25°C and 2.3 Kcal/mol from 25°C to 4°C. In the presence of the sterol (60 μM) the changing point was 32°C with activation energies of 11.4 Kcal/mol and 0.8 Kcal/mol from 37°C to 32°C and 32°C to 4°C respectively.

Discussion

Membrane permeability can modulate steroid hormone action (*Johnson, Newby* and *Bourgeois* 1984). An important constituent of all cell membranes is cholesterol. Sterols hydroxylated in the side-chain occur in minor amounts only. Many reports describe the effects of insertion of hydroxylated sterols on cellular processes, e.g. the uptake of calcium (*Neyses* et al. 1985), platelet aggregation (*Shimida, Imada, Kikuchi, Inada, Morisaki, Ikekawa* and *Saito* 1984), diminished osmotic fragility of human erythrocytes (*Streuli* et al. 1981), the formation of echinocytes (*Hsu, Kanofski* and *Yachnin* 1980) or leukocyte chemotaxis (*Gordon, Bass* and *Yachnin* 1980). The latter effect could already be observed after 5 minutes of preincubation in the presence of the sterol. The sterols are inserted into the cellular mem-

brane, as shown e.g. for the insertion of 7β-OH-cholesterol into erythrocytes (*Hsu, Kanofsky* and *Yachnin* 1980). In hepatocytes the largest fraction of the unmetabolized 25-OH was also found in the plasma membrane (*Erickson, Matsui, Shrewsbury, Cooper* and *Gould* 1978).

Corticosterone is taken up by rat liver cells (see Fig. 2; upper part). With this amount of corticosterone the uptake is linear with the concentration. The Arrhenius plot of the uptake shows a biphasic course with a change in the slope around 25°C. This temperature lies well within the range (18°C to 31°C) reported for the critical transition temperatures of rat hepatocyte membranes (*Schachter* 1984). The activation energy of 12.0 Kcal/mol between 37°C and 25°C is in accordance with the value of 10–20 Kcal/mol, calculated for mediated transport processes (*Rao* 1981). From 25°C to 4°C the activation energy is 2.3 Kcal/mol, suggesting a diffusion process.

Some steroids can inhibit the corticosterone uptake (see Table 1), confirming Rao's observation (*Rao, Rao, Eckel* and *Breuer* 1977). These steroids were selected because of their structural resemblance to corticosterone. In addition most of these steroids are important intermediates in steroidogenesis in the rat. Without preincubation these steroids also inhibit the corticosterone uptake, though to a smaller extent. This inhibition may be due to competition at the level of the cellular membrane and/or intracellular corticosterone binding sites. Of the sterols/steroids tested only cholest-4-en-3-one was without effect. The results suggest that a certain structural resemblance of the sterol/steroid to corticosterone is correlated with its inhibitory action on the uptake. Quantitative statements are difficult to make, however.

In our study 25-OH is also taken up by isolated rat liver cells (see Figure 1). The initial uptake and, probably, insertion into the cellular membrane is maximal at 10 minutes and may be opposed by metabolism and excretion after that period (*Erickson* et al. 1978).

After preincubating rat liver cells in the presence of 25-OH the uptake of corticosterone is diminished (see Figure 2; lower part). It seems, that the inhibition by 25-OH is independent of the corticosterone concentration. The presence of 25-OH without preincubation has no direct effect on the corticosterone uptake, in spite of the fact that the 25-OH concentration exceeds the corticosterone concentration by a factor thousand (result not shown). A direct competition between 25-OH and corticosterone for entry into the liver cells is therefore less probable. Only with a preincubation time of 10–15 minutes, when the uptake of 25-OH is at its maximum, the corticosterone uptake is inhibited. The Arrhenius plot of the corticosterone uptake after preincubation with 25-OH also shows a biphasic course but with a change in the slope around 32°C. It is known from studies on artificial membranes, that an increase in the membrane sterol concentration is accompanied by a decrease in the membrane fluidity (*Cooper* 1977). The shift of the phase transition in the Arrhenius plot to a higher temperature (25°C to 32°C) may be a direct consequence of this lowered membrane fluidity.

The activation energy between 37°C and 32°C is not influenced by the presence of 25-OH, but the overall capacity of the uptake mechanism is decreased by 40% (at 37°C).

In conclusion our results show, that 25-OH has an inhibitory effect on the uptake of corticosterone in isolated rat liver cells. This is not readily explained by direct competition. The mechanism might involve an inhibition of an active uptake mechanism accompanied by a change in the plasma membrane.

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CHAPTER 8

EFFECT OF HYDROXYLATED STEROLS ON THE UPTAKE OF PREGNENOLONE IN ISOLATED RAT ADRENAL CELLS.

Introduction.

It is commonly thought that the uptake of steroids and sterols mainly proceeds via diffusion processes. However, the possibility has been discussed, that in rat liver cells a mediated transport mechanism is involved (for a review see Rao, 1981). It has been found that some steroids can inhibit the uptake of corticosterone in rat liver cells (Rao et al., 1977), while the sterol 25-OH-cholesterol also inhibits the uptake of corticosterone in rat liver cells (Huijmans et al., 1988; chapter 7: this thesis).

The aim of the present study was to analyse the effect of 22S-OH, 17S,20S-diOH and 25-OH on the uptake of labelled pregnenolone, the intracellular content of labelled steroids produced from pregnenolone and on the conversion of pregnenolone into metabolic products in isolated rat adrenal cells.

Methods.

Rat adrenal cells were prepared as described in Chapter 3. The cells were preincubated at 37°C for one hour in a Dubnoff incubator, shaking at 100 rpm in the presence or absence of the sterols tested (62 μM). After one hour [^{3}H]-pregnenolone (17.2 Ci/mmol; 165 nM) was added. The cells were incubated for 0, 0.5, 1.5, 5, 10, 20 and 40 minutes. Subsequently the cells were filtered on glass fibre filters (Whatman GF/C) using a Millipore filter manifold and washed twice with 4 ml KRHAG. The filtrate and the washing fluid were collected (see below).

The filters were dried at 50°C and solubilized at 37°C in Nuclear Chicago tissue solubilizer. After 12 hours 10 ml of toluene scintillating fluid, containing PPO and POPOP was added. Radioactivity was counted in a liqiud scintillation counter (Mark III, Searle).

The combined filtrate and washing fluid was extracted with chloroform/

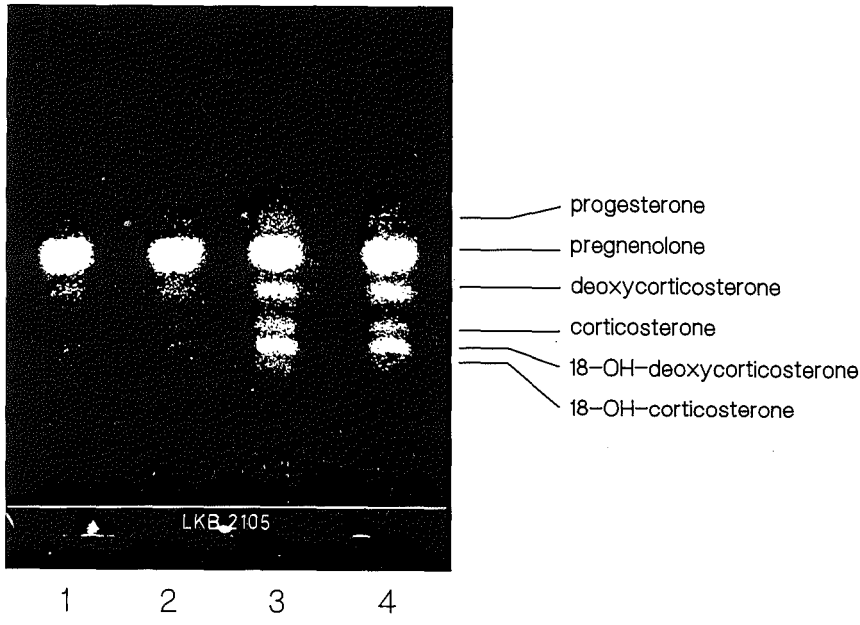

Figure 8.1: Example of a thin-layer chromatogram of the labelled steroids present in the incubation filtrates. Visualisation occurred with a radiochromatogram camera.
lane 1: control incubation after 90 seconds.
lane 2: 17S,20S-diOH incubation after 90 seconds.
lane 3: control incubation after 20 minutes.
lane 4: 17S,20S-diOH incubation after 20 minutes.

methanol and the steroids separated by thin-layer chromatography on silicagel plates (Merck, Darmstadt), using ethylacetate/acetone (99/1; V/V) as a developing solvent. Radioactivity was detected with a radiochromatogram camera (LKB, Sweden). Labelled material was extracted from the thin-layer plate with methanol and counted. An example of a thin-layer chromatogram is presented in Figure 8.1. Reference compounds were used for the identification.

Table 8.1: Mean of the radioactivity recovered in isolated rat adrenal cells after incubation with 165 nM [^{3}H]-pregnenolone at 37°C. The cells were preincubated for one hour in the absence or presence of the sterols. Incubations were done in duplicate; results are expressed as a percentage of the corresponding control incubation.

Incubation time (minutes)	17S,20S-diOH 62 μM	22S-OH 62 μM	25-OH 62 μM
0	71	104	109
0.5	93	122	113
1.5	92	170	107
5	85	129	116
10	75	110	99
20	85	112	102
40	86	112	95

Results.

The time course of the uptake of pregnenolone in isolated rat adrenal cells in the absence or presence of the sterols tested is shown in Table 8.1. In each experiment one sterol was tested against a control without sterol (in duplicate). The results were statistically evaluated with a test for the comparison of two curves, described by Gebelein and Ruhenstroth (1952). In the presence of 22S-OH an initial significant increase could be observed in the amount of intracellular radioactivity ($P<0.01$). After 10 minutes this uptake was back to control values. 25-OH was without effect. The presence of 17S,20S-diOH caused a significant decrease in the intracellular radioactivity from 5 minutes onwards ($P<0.05$).

The analysis of the radioactive compounds in the incubation media allowed an estimate of the conversion of pregnenolone. For the sake of simplicity the sum of progesterone, DOC, 18-OH-DOC and corticosterone was used to

measure the pregnenolone conversion. The results are presented in Figure 8.2. 17S,20S-diOH had a significant inhibitory effect on the pregnenolone conversion and product formation (in particular 18-OH-DOC and progesterone) ($P<0.005$). In the presence of 22S-OH, neither the pregnenolone conversion nor the total sum of products were changed. However, the concentration of 18-OH-DOC was significantly decreased ($P<0.05$). 25-OH had no effects on the pregnenolone conversion.

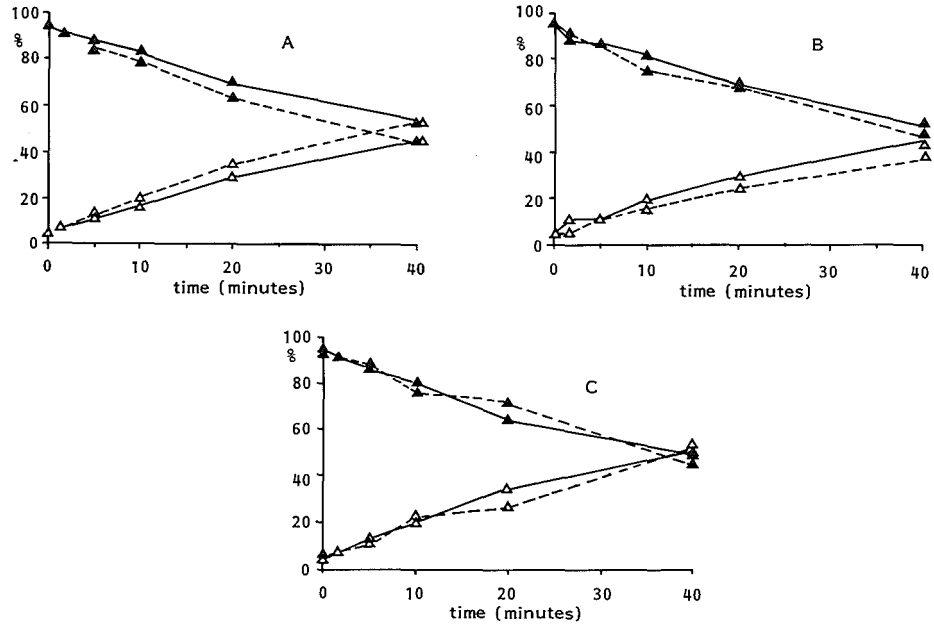

Figure 8.2: Time course of the conversion of pregnenolone (▲) into products (△) in isolated rat adrenal cells in the absence (– –) or presence (——) of 62 μM 17S,20S-diOH (A), 22S-OH (B) or 25-OH (C). Each point represents the mean of duplicate incubations. The results were statistically evaluated as described by Gebelein and Ruhenstroth (1952).

Discussion.

The intracellular adrenal steroid concentration is the result of uptake, synthesis, conversion and release. The uptake and release of steroids are commonly considered as passive diffusion processes. However mediated uptake mechanisms in rat liver cells have been discussed (see introduction). In rat adrenal cells some authors described the existence of macromolecules (probably proteins), which play a part in the control of secretion or synthesis of steroids (Sibley et al., 1980; Goddard et al., 1980). In particular the secretion of 18-OH-DOC, formed from endogenous precursors, is controlled by a mechanism involving a ouabain-sensitive process in the cell membrane (Sibley et al., 1980).

Pregnenolone is a good precursor for corticosterone production in isolated rat adrenal cells (Huijmans et al., 1981). The present results show that the three hydroxylated sterols tested act differently on the uptake and processing of pregnenolone by rat adrenal cells. 22S-OH increased the amount of intracellular radioactivity upon incubation with labelled pregnenolone. This can not merely be an increase in the permeability of the cellular membrane for pregnenolone, as 22S-OH has no effect on the total conversion of pregnenolone, nor on the sum of products. As mentioned before, especially the secretion of 18-OH-DOC formed from endogenous precursors is controlled by a ouabain-sensitive mechanism in the cellular membrane. It is also the concentration of 18-OH-DOC in the incubation medium, which is significantly decreased. One might speculate that 22S-OH has an inhibitory effect on the secretion of 18-OH-DOC, thus increasing its intracellular concentration. Feed-back inhibition of the 18-hydroxylase (corticosterone methyloxidase I) may direct steroid metabolism towards the production of corticosterone, thus not changing the overall sum of products (see figure 8.2).

In the presence of 17S,20S-diOH, the cellular radioactivity is decreased; less pregnenolone is converted and fewer products are formed in its presence, in particular less progesterone and 18-OH-DOC. This sterol may have an effect on the secretion of 18-OH-DOC too. In addition, an inhibitory effect on the uptake of pregnenolone seems probable.

25-OH has no effect on the intracellular radioactivity nor does it influence the conversion of pregnenolone into products. One explanation is that 25-OH is a much better substrate in rat adrenal cells than 22S-OH and 17S,20S-diOH and is rapidly cleared from the cell. The fact that 25-OH has

an inhibitory effect on the corticosterone uptake in rat liver cells (Huijmans et al., 1988), but no effect on the uptake of pregnenolone in rat adrenal cells may also reflect a difference in the clearance of 25-OH from the two types of cells. However, the existence of a mediated uptake mechanism in the liver cells and not in the adrenal cells may also explain the different effects of 25-OH.

In conclusion, the effects of these hydroxylated sterols on the uptake and conversion of pregnenolone in isolated rat adrenal cells are caused by changes in the cellular membrane, probably as a consequence of insertion of these sterols into the membrane.

CHAPTER 9

THE HUMAN FETAL ADRENAL AND HYDROXYLATED STEROLS

Introduction

The human fetal adrenal gland is unique in its steroidogenic properties, in that the 3β-HSD activity is low. An important function of the fetal adrenals is the production of precursors for estrogen synthesis with cholesterol as the main precursor. Some 30% of the cholesterol is derived from de novo synthesis in the fetal adrenal gland (Carr and Simpson, 1981b). The other part is derived from plasma LDL. This means that in the fetal adrenal gland, the cholesterol production and influx is high. On the other hand the regular steroidogenic pathway is functionally blocked by a low 3β-HSD activity. Our hypothesis was, that the human fetal adrenal might be used as a model system, in which the production of unusual hydroxylated sterols might occur.

As discussed in Section 3B.5, the isolation technique used for rat adrenal cells is also suitable for the isolation of human fetal adrenal cells. The cells retain their steroidogenic properties in comparison with those described in the literature. A separation of cells of the fetal and definitive zones can be achieved by a manual dissection method and by density gradient centrifugation through Percoll.

These cells have been used to study steroidogenesis from cholesterol. However, cholesterol is a very poor substrate, because it does not enter the cell at an acceptable rate. As shown in rat adrenal cells, hydroxylated sterols are much better substrates for steroid production (Huijmans et al., 1981, 1982). The aim of this study was to answere two questions:

1. are hydroxylated sterols converted in human fetal adrenal cells?
2. If so, what are the products of their conversion?

Methods

A survey of the fetal material used in this study is given in Section 3B.5. Part of the methods has already been described in Chapter 3A (isolation of cells; gaschromatography; estimation of cortisol and corticosterone) and Chapter 3B (cell separation).

Conversion of pregnenolone and conjugation.

Cells from the separated fetal and definitive zones of the human fetal adrenal in case nr 3 (18 weeks of gestation) were incubated with ^{3}H-labelled pregnenolone for 2 hours. The extraction procedure with acetone/dichloromethane, as described in Section 3A.5, was used. Radioactivity in the water layers was counted. DHEA-sulfate was retained in the water layers, as was confirmed with a DHEAS standard solution.

Steroids in the organic layer were separated by monodimensional thin-layer chromatography, using successively ethylacetate/acetone (6:4 V/V) and chloroform/ether (9:1 V/V) as the developing solvents. Radioactivity was visualized with a radiochromatogram camera (LKB, Sweden). The chromatography lanes were divided into segments with R_f-boundaries, as described in table 9.5. The location in this TLC system of several relevant steroids was tested with reference compounds.

Results and Discussion

In the human fetal adrenal the 3β-HSD activity is low. An important pathway of steroid metabolism is the conversion of cholesterol into DHEA via pregnenolone and 17α-OH-pregnenolone (Yanaihara and Arai, 1981). As stated in the introduction, we studied the metabolic fate of hydroxylated sterols. The results of incubations of human fetal adrenal cells in the presence of 22R-OH and 25-OH are presented in Table 9.1. The free sterols and steroids were analyzed. Control values in the absence of sterols are given between brackets.

22R-OH may serve as a precursor for the production of pregnenolone, 17α-OH-pregnenolone and DHEA. A striking phenomenon is, that the amount of sterol converted greatly exceeds the amount of products measured with this technique. As far as steroid metabolites are detectable by gas-chromatography, this technique does not show unknown peaks. Reference steroids tested in this system are e.g. 16α-OH-pregnenolone, testosterone, androstenediol, androstenedione and 16α-OH-DHEA.

In the presence of 25-OH, the production of pregnenolone, 17α-OH-pregnenolone and DHEA did not increase. However, some 10% of this sterol disappeared.

Both 22R-OH and 25-OH are good substrates for steroid synthesis in isolated rat adrenal cells. 22R-OH is a normal intermediate in the cholesterol side-chain cleavage. Several sterols, which are not

Table 9.1: Conversion of 22R-OH (20 μg/ml) and 25-OH (20 μg/ml) into pregnenolone, 17α-OH-pregnenolone and DHEA in isolated human fetal adrenal cells. Values found in control incubations without added sterols are given between brackets.

	22R-OH		25-OH
Weeks of gestation [case number]	15[1]	20[5]	15[1]
Number of cells/ml	$1.6x10^5$	$1.7x10^5$	$1.6x10^5$
Sterol converted (ng/10^5 cells/2 hrs)	6380	5090	1250
Production of:			
- Pregnenolone "	1180(nd)	520(10)	nd(nd)
- 17α-OH-pregnenolone "	1020(160)	1190(nd)	170(160)
- DHEA "	90(30)	540(10)	60(30)
Cholesterol (μg/10^5 cells)	10.2(9.8)	8.7(8.5)	10.3(9.8)

nd = not detectable

intermediates in cholesterol metabolism, but do have effects on steroid production or steroid uptake in rat adrenal cells, were tested in human fetal adrenal cells. Results are shown in table 9.2. Both 22S-OH and 17S,20S-diOH are precursors for steroid synthesis; more sterol is converted than can be accounted for by the formation of the measured products. Desmosterol is a very poor substrate in these cells, probably because this sterol does not enter the cell.

The conversion of 22R-OH and 25-OH was also investigated with cells of the separated zones of the human fetal adrenal (according to separation method 1). The sum of free and sulfate-conjugated sterols and steroids was measured by using a solvolysis procedure before the gaschromatographic

Table 9.2: Conversion of 22S-OH (40 μg/ml), 17S,20S-diOH (20 μg/ml) and desmosterol (15 μg/ml) by isolated human fetal adrenal cells (case number 10; 24 weeks of gestation). The number of cells was $3.5x10^5$ /ml).

	22S-OH	17S,20S-diOH	desmo-sterol	blank
Sterol converted (ng/10^5cells/2 hrs)	3290	1190	0	
Production of:				
- pregnenolone "	30	nd	50	40
- 17α-OH-pregnenolone "	250	165	nd	10
- DHEA "	90	20	80	40
Cholesterol (μg/10^5 cells)	8.8	10.3	10.0	9.5

nd = not detectable

determination. Results are presented in table 9.3. 22R-OH was a good substrate for steroid production in the cells of both zones. It is striking, that much of the sterol disappeared without a concomitant rise in any steroid, measured with this analytical procedure.
In contrast with the results in Table 9.1, some 50% of the 25-OH concentration disappeared.

These results show a deficit in the amount of substrate converted and the amount of products formed. This deficit may have several causes:

1. formation of cortisol and corticosterone which are not detectable by this gaschromatographic technique,
2. formation of conjugates, especially sulfate conjugates, although the main route with exogenous substrates proceeds via the unconjugated pathway (Cooke et al., 1970; Cooke and Taylor, 1971).

Table 9.3: Conversion of 22R-OH (20 μg/ml) and 25-OH (20 μg/ml) into pregnenolone, 17α-OH-pregnenolone and DHEA in the cells of the separated zones of the human fetal adrenal. Fetal age was 18 weeks (case number 3).

	Fetal zone			Definitive zone		
Number of cells	2.7×10^5/ml			1.1×10^5/ml		
	22R-OH	25-OH	Blank	22R-OH	25-OH	Blank
Sterol converted (ng/10^5cells/2 hrs)	7100	6930		10940	9670	
Production of:						
- pregnenolone "	350	nd	nd	320	nd	nd
- 17α-OH-pregnenolone "	470	nd	nd	490	nd	nd
- DHEA "	1410	540	520	720	300	nd
Cholesterol (μg/10^5 cells)	12.4	9.9	7.2	5.1	5.3	4.9

nd = not detectable

Table 9.4: Corticosterone and cortisol production by isolated human fetal adrenal cells (case number 6) in the presence of 22R-OH, 25-OH and progesterone.

Addition	Corticosterone (ng/10^5 cells/2 hrs)	Cortisol (ng/10^5 cells/2 hrs)
Blank	30	10
40 μg/ml 22R-OH	110	40
50 μg/ml 25-OH	140	nd
10 μg/ml progesterone	1340	1850

nd = not determined

Sub 1

The conversion of hydroxylated sterols into cortisol and corticosterone was investigated in the human fetal adrenal cells. For comparison, steroid production in the presence of progesterone is included. The results are shown in Table 9.4.

Table 9.5: Formation of radioactive products after incubation of fetal and definitive zone cells (case number 3) with labelled pregnenolone (20 μg/ml = 35,800 dpm/ml) for 2 hours. Results are expressed as dpm/10^5 cells and as a percentage of the added radioactivity.

	Fetal zone cells		Definitive zone cells		
# cells	2.7x10^5/ml		1.1x10^5/ml		
	dpm (total)	%	dpm (total)	%	
water layers	6610	18.5	530	1.5	
protein precipitate	1660	4.6	1650	4.6	
R_f-boundaries					reference compounds in resp. zones
	dpm/10^5cells	%	dpm/10^5cells	%	
0.69-1.00	310	2.4	320	1.0	progesterone androstenedione
0.60-0.69	4850	36.6	23,190	74.5	pregnenolone, DHEA 17α-OH-progesterone
0.52-0.60	2120	16.0	2,040	6.5	DOC, 17α-OH-pregn. androstenediol
0.40-0.52	760	5.8	2,160	6.9	cortisol, corticosterone, 16α-OH-preg
0.00-0.40	490	3.7	700	2.2	18-OH-DOC

22R-OH and 25-OH, which are good substrates for steroid production in rat adrenal cells, are not readily converted into cortisol and corticosterone in human fetal adrenal cells. One explanation, especially in the fetal zone cells, is the low 3β-HSD activity. Conversion of these sterols into other, non-detected metabolites appears the only remaining possibility.
These results show that cortisol and corticosterone production does not play an important role in the conversion of 22R-OH and 25-OH.

Sub 2

With cells of the separated zones of the human fetal adrenal, according to method 1 (see Chapter 3.13), steroidogenesis was investigated with labelled pregnenolone as a substrate. The analytical procedure is described in the Methods section. The results are presented in table 9.5. More radioactivity remains in the water layer of the fetal zone incubation, compared with the definitive zone cells, suggesting a higher sulfokinase activity in the fetal zone. These results also show, that the extent of sulfate conjugation by the fetal zone cells does not exceed 20% of the added pregnenolone. In the definitive zone sulfate conjugation is negligible.

Comparing the results in Tables 9.1 and 9.3, a solvolysis procedure before the gaschromatographic determination of the steroids does not increase the recovery of products to a considerable extent. This also suggests a low extent of sulfate conjugation in these experiments.

CONVERSION OF PREGNENOLONE AND 17α-OH-PREGNENOLONE

The conversion of sterols most probably proceeds via pregnenolone and 17α-OH-pregnenolone into DHEA (Yanaihara and Arai, 1981), as is also suggested by the results in Tables 9.1 and 9.3 for 22R-OH as a substrate. A comparison was made between the conversion of the sterols and the conversion of the proposed intermediates pregnenolone and 17α-OH-pregnenolone. Table 9.6 shows the results for a whole human fetal adrenal cell suspension. Only the free products were measured. The high 17α-OH-pregnenolone production in the presence of pregnenolone is in agreement with an active 17α-hydroxylase in the human fetal adrenal (Villee, 1969b). DHEA production is small. With 17α-OH-pregnenolone as a substrate, only a small part of the steroid converted can be explained by the production of DHEA.

Table 9.6: Conversion of pregnenolone (20 μg/ml) and 17α-OH-pregnenolone (20 μg/ml) in isolated human fetal adrenal cells. Control values are given between brackets.

	Pregnenolone		17α-OH-pregnenolone	
Weeks of gestation [case number]	15[1]	22[7]	20[5]	24[1]
Number of cells/ml	$1.6x10^5$	$3.8x10^5$	$1.7x10^5$	$1.6x10^5$
Steroid converted (ng/10^5 cells/2 hrs)	4550	4080	6740	7910
Production of:				
- 17α-OH-pregnenolone "	2680(160)	2960(nd)	-	-
- DHEA "	290(30)	360(nd)	680(10)	490(40)
Cholesterol (μg/10^5 cells)	8.8(9.8)	11.5(8.9)	8.0(8.5)	9.7(9.8)

nd = not detectable

We also studied the conversion of pregnenolone in cells of the separated fetal and definitive zones of the fetal adrenal (according to separation method 1). The steroids are measured after a solvolysis step to free possible sulfate conjugates. The results are presented in table 9.7. Pregnenolone is a good substrate. Expressed per 10^5 cells the production of 17α-OH-pregnenolone and DHEA is higher in the fetal zone than in the definitive zone cells. This is in agreement with the higher 17α-hydroxylase activity, found in the fetal zone (Voutilainen et al., 1979).
Although the production of 17α-OH-pregnenolone and DHEA is less in the definitive zone cells than in the fetal zone cells, more pregnenolone disappears. A difference in the low 3β-HSD activity (see Table 9.4) between both zones and the subsequent conversion of pegnenolone into 3-keto-Δ^4 steroids may partly explain this phenomenon.

The results in Table 9.5 also show that incubation of definitive zone

Table 9.7: Conversion of pregnenolone (20 μg/ml) in the isolated cells of the fetal and definitive zones of the human fetal adrenal. The fetal age was 18 weeks (case number 3). Control values are given between brackets.

	Pregnenolone	
	fetal	definitive
Number of cells/ml	2.7×10^5	1.1×10^5
Pregnenolone converted (ng/10^5 cells/2 hrs)	5580	8390
Production of:		
- 17α-OH-pregnenolone "	2410(nd)	1550(nd)
- DHEA "	1110(520)	390(nd)
Cholesterol (μg/10^5 cells)	7.8(7.2)	4.9(4.9)

nd = not detectable

cells with labelled pregnenolone yields more radioactivity in the area on the thin-layer chromatogram where cortisol and corticosterone are situated. This suggests a greater production of cortisol/corticosterone in the definitive zone, in agreement with what is known about steroidogenesis in the respective zones of the human fetal adrenal (Serón-Ferré et al., 1978a; Branchaud et al., 1978). It should be noted, however, that the presence of radioactivity in a zone of the thin-layer chromatogram is by no means an identification of steroids appearing in this zone.

The higher production of DHEA from pregnenolone in fetal cells (with solvolysis) compared with a whole fetal adrenal cell suspension (no solvolysis) may suggest sulfate conjugation of DHEA in the fetal zone. This is also confirmed by the results in Table 9.5.

The cholesterol content of the cells, expressed per 10^5 cells, as shown in tables 9.3 and 9.7, is higher in the fetal zone cells than in the definitive zone cells. This may be in accordance with the size of the

cells (Bloom and Fawcett, 1968): the fetal zone cells are bigger in size with a greater membrane surface.

In conclusion, the hydroxylated sterols 22R-OH and 25-OH are converted in isolated human fetal adrenal cells. The increase in the pregnenolone, 17α-OH-pregnenolone and DHEA concentration suggests that, at least 22R-OH, is converted via the normal metabolic route. The production of cortisol and corticosterone and of sulfate conjugates cannot account for the deficit in the sterol conversion.

A possible explanation is the conversion into conjugates other than sulfates, or the conversion into more polar products, which are not extracted from the incubation medium or not detectable with this capillary column. The availability of labelled sterols would be of great help in solving this problem.

As stated in the introduction to this chapter, the high steroid flux and the low 3β-HSD activity in the human fetal adrenal make the production of unusual hydroxylated sterols possible. Our results, presented in this chapter, confirm this possibility. This needs further study.

CHAPTER 10

GENERAL DISCUSSION

In several diseases abnormal sterol metabolism occurs; this may be a factor in the etiology of the disease, due to effects of hydroxylated sterols on cellular or subcellular processes. The aims of this study were to investigate:

1. short-term effects of hydroxylated sterols on basal and stimulated steroid production in rat adrenal cells and on steroid uptake in rat adrenal and liver cells,
2. the conversion of sterols in human fetal adrenal cells, as a physiological model of a 3β-HSD deficient organ.

STEROID PRODUCTION

Several sterols were found to be good substrates for corticosterone synthesis in isolated rat adrenal cells, e.g. 25-OH, 22R-OH and 20S-OH-cholesterol. 22S-OH was a poor substrate. This is in agreement with the findings of Lambeth (1986), that the structural requirements for binding to the cytochrome P-450$_{scc}$ and conversion into pregnenolone are more favourable for the intermediate 22R-OH than for 22S-OH. The 22R hydrogen of cholesterol is more close to the heme iron than the 22S hydrogen.

For 25-OH and 22S-OH no acute adverse effects on the functioning of the isolated rat adrenal cells were found. This conclusion is based on the linearity of the corticosterone production for up to 3 hours in the presence of 22S-OH (Chapter 4) or 25-OH.

In a pilot experiment, 22S-OH inhibited the ACTH-stimulated corticosterone production, whereas steroid production in the presence of 25-OH and ACTH was almost additive. For this reason the effect of 22S-OH was studied in more detail. The results in Chapters 4 and 5 allowed to locate the effect of this sterol at the level of the cholesterol side-chain cleaving enzyme system as the most probable site of action. The arguments in favour of this site are:

1. there was no effect of 22S-OH on the cAMP concentration in rat adrenal cells after stimulation with ACTH,

2. free cholesterol in the adrenal cells accumulated in the presence of 22S-OH plus ACTH, compared with the cholesterol concentration after stimulation with 22S-OH or ACTH,
3. there was no effect of 22S-OH on the conversion of exogenous pregnenolone (Chapters 4 and 8),
4. 22S-OH had no effect on the conversion of 22R-OH but slightly inhibited the conversion of 25-OH.

The most probable action mechanism of 22S-OH is an effect on the binding of cholesterol and 25-OH to the cytochrome P-450$_{scc}$ complex.

In damaged bovine adrenal mitochondria too, 22R-OH is a much better substrate for pregnenolone production than 22S-OH. When 22S-OH was used as a substrate, 22-keto-cholesterol was formed in addition to pregnenolone. 22-Keto-cholesterol was not detectable when a mitochondrial preparation was incubated in the presence of 22R-OH (see also: Alsema et al., 1982b). Possibly the slower conversion rate of 22S-OH allows normally minor pathways to become active; a nice example of unusual sterol formation. The conversion of 22S-OH into 22-keto-cholesterol might explain the inhibitory effect of 22S-OH on the ACTH-stimulated corticosterone production in isolated rat adrenal cells, as 22-keto-cholesterol is an inhibitor of this process (see Chapter 4). However, when rat adrenal cells were incubated in the presence of 22S-OH, 22-keto-cholesterol was not detectable. This difference in 22-keto-cholesterol production may be explained by:

1. the use of whole adrenal cells versus damaged mitochondria,
2. a species difference (rat versus cow).

The inhibitory effect of 22S-OH on the ACTH-stimulated corticosterone production, as seen in whole rat adrenal cell suspensions, could also be demonstrated in isolated rat adrenal fasciculata/reticularis cells. Apparently the presence of glomerulosa cells or steroid metabolism in these cells does not influence the overall inhibitory mechanism. One obvious reason is, that the zona glomerulosa is much smaller than the zonae fasciculata and reticularis. In addition, the steroidogenic capacity of glomerulosa cells may be different from that of fasciculata/reticularis cells. The combined aldosterone and corticosterone production in glomerulosa cells in the presence of ACTH is 10 times lower than the ACTH-stimulated corticosterone production in fasciculata cells (Tait et al., 1980b). This might be due to the lower cholesterol content of the glomerulosa cells. However from the results obtained with the exogenous

substrate 25-OH and from the fact that the pathway:

DOC ——> aldosterone

is not rate-limiting (Chapter 6), the most likely explanation is, that the cholesterol side-chain cleaving enzyme activity is responsible for the difference in the steroidogenic capacity, observed in vitro, between glomerulosa cells and fasciculata/reticularis cells. The use of hydroxylated sterols thus seem to be a good expedient in elucidating differences in the steroidogenic capacities, existing between several types of steroid producing cells.

STEROID UPTAKE

Effects of hydroxylated sterols on the uptake of steroids were studied in rat adrenal and liver cells. In liver cells 25-OH had an inhibitory effect on the corticosterone uptake, probably caused by a change in the cellular membrane. However, the same sterol had no effect on the uptake of pregnenolone in isolated rat adrenal cells. One possible explanation might be the difference, existing between liver and adrenal cells as to the metabolism of 25-OH, 25-OH being a good substrate in adrenal cells. A similar effect was seen in rat hepatocytes, where the HMG-CoA reductase activity was inhibited in the presence of 25-OH. The recovery of the activity was dependent on the clearance of 25-OH from the cells; with low concentrations of 25-OH the HMG-CoA reductase activity rose to 90% of the initial value, whereas with high concentrations of 25-OH activity remained low (Erickson et al., 1978).

Another sterol, 22S-OH had a similar inhibitory effect on the corticosterone uptake in rat liver cells after 15 minutes preincubation in the presence of the sterol. Like 25-OH, 22S-OH did not inhibit the uptake of pregnenolone in rat adrenal cells, although this sterol is a poor substrate compared with 25-OH. For this reason another explanation for the different effects of the sterols tested on the uptake of steroids in rat adrenal or liver cells might be a difference in the cellular membrane itself, with liver cells having a different uptake mechanism for corticosterone compared with the uptake of pregnenolone in rat adrenal cells. The results of our study show that hydroxylated sterols are able to influence the cellular membrane.

It has become apparent that the composition and fluidity of membrane lipids is critically regulated for an optimal cell growth and function. A

change in the fluidity of cell membranes can be an important factor in the pathogenesis of disease. Under conditions of an increased cholesterol to phospholipid ratio in plasma lipoproteins, the cholesterol content of red cell membranes is also increased, causing altered shape and properties (Cooper, 1977). This kind of effects are not restricted to red cell membranes, as discussed in Section 2.5. Summarizing, our study has shown effects of hydroxylated sterols on steroid uptake processes. In those diseases where an accumulation of cholesterol or other sterols has been described, these effects on uptake processes should be further evaluated. This can be the subject of future research.

HUMAN FETAL ADRENAL CELLS

The third aim of this study was the investigation of the effect of hydroxylated sterols on the basal steroid production in human fetal adrenal cells and the possible formation of unusual compounds. These cells were obtained using the isolation procedure for rat adrenal cells. This method yields viable cells with steroidogenic characteristics, in accordance with the literature. A procedure for separating the two types of human fetal adrenal cells by density gradient centrifugation on Percoll was proven to be effective; the characteristics of the cells in the separated bands are in agreement with those reported for fetal and definitive cells.
Several sterols were found to be converted in these cells. Part of the conversion products are intermediates in the main metabolic pathway of cholesterol to DHEA via pregnenolone and 17α-OH-pregnenolone. However, the conversion of the sterols greatly exceeded the formation of measurable products. One may speculate about the nature of these products. Cortisol and corticosterone were not found in measurable amounts. The formation of sulfate conjugates could also not account for the deficit. The more common steroids, like androstenediol, 16α-OH-DHEA, etc. could be ruled out by their absence in the gaschromatogram. The formation of very polar or acidic sterols or sterol/steroid conjugates other than sulfates is a possibility. This should be investigated further; the availability of labelled sterols would be of great help in solving this problem.

CLOSING REMARKS

This study has shown that in vitro hydroxylated sterols have several acute effects:

1. on steroid production by a (competitive) effect on the cholesterol side-chain cleavage,
2. on steroid uptake by inducing a change in the cellular membrane.

It should be stressed, that the presence of unusual sterols not necessarily leads to dramatic changes at short term, but may have long-term effects. One example is the presence of cholestanol in cerebrotendinous xanthomatosis. The contribution of accumulated sterols to the mortality in e.g. the congenital lipoid adrenal hyperplasia or Wolman's disease is not known with certainty. However, any profound change in membrane composition, either with effects on uptake of steroids or other metabolites, or with a more general disfunction of the cells, is very likely to result in deleterious effects on the whole organism.

In this thesis only a limited number of sterols were studied. Other sterols should be evaluated for their short-term and long-term effects on isolated cells and whole organisms as a subject for future research.

CHAPTER 11

SUMMARY

Several effects of sterols, oxygenated in the steroid nucleus or the side-chain, on cellular and subcellular processes have been described. The investigation of possible short-term effects of these sterols on basal and stimulated steroid production and on steroid uptake is the subject of this thesis.

Chapter 1 gives a general introduction and a statement of the problem. The accumulation of sterols in body fluids and tissues may play a role in the etiology of several diseases. This might be of importance, because in vivo and in vitro many adverse effects of sterols have been described. For this study the isolated rat adrenal and liver cells were used as model systems. In addition the properties of hydroxylated sterols as steroid precursors were tested in isolated human fetal adrenal cells as a model of a 3β-HSD deficient organ. The use of isolated human fetal adrenal cells is rather uncommon, as most authors use cell or tissue cultures. For this purpose, the applicability of the method for the isolation of rat adrenal cells to the human fetal adrenal and the separation of cells from the two zones in the human fetal adrenal were investigated.

Chapter 2 gives a review of the literature. Steroidogenesis and its regulatory factors in the rat adrenal (both zona glomerulosa and zonae fasciculata/ reticularis) and in the human fetal adrenal are discussed. The occurrence of unusual sterols and their effects on cellular and subcellular processes are described.

Chapter 3 describes the experimental methods, including isolation of cells, incubation conditions and analytical techniques. In addition, some characteristics of rat adrenal cells and an evaluation of several incubation parameters are presented, e.g. a dose-response curve for the stimulation of cells with ACTH and the relationship between steroid production on one hand and (1) concentration of cells and (2) the composition of the incubation buffer on the other hand.

A survey of the human fetal adrenals, obtained in this study, is given. Two parameters for the cell viability, the ATP content of the cells and the cortisol plus corticosterone production from exogenous progesterone, are

evaluated. Results are presented showing the low 3β-HSD activity in these cells. The separation by density gradient centrifugation of cells from the two zones of the human fetal adrenal is compared with a manual dissection method.

Chapter 4 deals with the conversion of hydroxylated sterols and the effects of sterols on the ACTH-stimulated corticosterone production in isolated rat adrenal cells. Several sterols were tested as substrates for steroid synthesis. 22R-OH, 25-OH and 20S-OH-cholesterol were found to be good substrates for corticosterone synthesis. 22S-OH was a minor substrate. This sterol inhibited the ACTH-stimulated corticosterone production. 22S-OH had no effect on the ACTH-stimulated cAMP concentration. In the presence of ACTH, this sterol increased the free cholesterol concentration. It had no effect on the conversion of exogenous pregnenolone. These results suggest that the inhibitory effect of 22S-OH on the ACTH-stimulated corticosterone production is situated at the level of the cholesterol side-chain cleavage. Acute adverse effects of 22S-OH on the functioning of adrenal cells have not been found. This is concluded from the linearity with time of the corticosterone production up to 3 hours in the presence of this sterol and ACTH.

Chapter 5 provides more evidence that the inhibitory effect of 22S-OH on the ACTH-stimulated corticosterone production is located at one of the first steps in the cholesterol side-chain cleavage by comparing the effect of 22S-OH on the conversion of 22R-OH (the first intermediate in the cholesterol side-chain cleavage) and of 25-OH (which needs the complete side-chain cleaving enzyme system). 22S-OH had no effect on the conversion of 22R-OH but inhibited the conversion of 25-OH.
The results obtained from the experiments with rat adrenal cells and with bovine adrenal mitochondria allow the hypothesis, that a direct relationship exists between the conversion of 22S-OH and the production of corticosterone by the adrenal cells and of pregnenolone by the adrenal mitochondria. In bovine adrenal mitochondria 22S-OH was also converted into 22-keto-cholesterol.

In Chapter 6 the conversion of hydroxylated sterols and their effect on stimulated steroid production in isolated fasciculata/reticularis and glomerulosa cells is described.
The inhibitory effect of 22S-OH on the ACTH-stimulated corticosterone production, as seen in a whole adrenal cell suspension, could also be

demonstrated in isolated fasciculata/reticularis cells.
In isolated glomerulosa cells 25-OH and 22S-OH were substrates for aldosterone production. Aldosterone production was proportional to the 25-OH concentration, but inversely proportional to the 22S-OH concentration, probably due to an effect of 22S-OH on the cholesterol side-chain cleavage. 22S-OH did not have an adverse effect on the potassium-stimulated aldosterone synthesis, probably due to an additional site of action of potassium, namely on the late steps in aldosterone synthesis.
The difference in in vitro steroid production rates from endogenous substrates between glomerulosa and fasciculata/reticularis cells could also be observed using exogenous substrates like 25-OH and 22S-OH. This suggests that the intracellular substrate concentration is less important than the steroidogenic enzyme activities in regulating steroid production in isolated adrenal cells.

Chapter 7 deals with the uptake of corticosterone in isolated rat liver cells. 25-OH inhibited the corticosterone uptake. This inhibition was temperature dependent, but independent of the corticosterone concentration within the concentration range measured. The inhibition was also dependent on the preincubation time of the liver cells in the presence of the sterol: a maximal effect was reached at the time of the maximal uptake of the sterol. The action mechanism of 25-OH might involve an inhibition of active uptake by inducing a change in the cellular membrane.

In chapter 8 the effect of several sterols on the uptake of pregnenolone in isolated rat adrenal cells is described. Pregnenolone was an efficient precursor for steroid synthesis. 25-OH had no effect on the uptake or the conversion of exogenous pregnenolone. In the presence of 22S-OH the amount of intracellular radioactivity at first increased, but returned to control levels later on. This can be explained by an effect of this sterol on the secretion of corticosterone precursors, like DOC.
17S,20S-diOH had an inhibitory effect on the uptake of pregnenolone, probably also via a structural change in the cellular membrane. The diminished uptake resulted in a lower pregnenolone conversion.

Chapter 9 describes the results with the human fetal adrenal cells. Several sterols were converted in these cells. Much more of the sterols disappeared than can be accounted for by the usual products of steroid biosynthesis, including cortisol, corticosterone and the production of sulfate conjugates. Gaschromatography did not reveal any unknown steroid in

significant amounts. The possibility of production of abnormal sterols/ steroids in this model system, as proposed in Chapter 1, is confirmed by these results.

In Chapter 10 the results obtained are discussed with respect to the aims of this study. Acute effects of the hydroxylated sterols tested seem to be restricted to a competitive effect on the cholesterol side-chain cleavage and/or to an induced change in the cellular membrane, probably caused by insertion of the sterol. The results obtained are put in a broader biological perspective.

CHAPTER 12

SAMENVATTING

Verschillende effecten van sterolen op cellulaire of subcellulaire processen zijn beschreven. Sterolen kunnen omschreven worden als verbindingen, afgeleid van cholesterol, die bijvoorbeeld extra keto- of hydroxylgroepen bevatten, gesubstitueerd in de kern of de zijketen van het cholesterol molecuul. Het onderwerp van dit proefschrift is het bestuderen van korte termijn effecten van een aantal sterolen op de basale en gestimuleerde steroid productie en op de steroid opname.

Hoofdstuk 1 geeft een algemene introductie en de probleemstelling. Ophoping van sterolen in lichaamsvloeistoffen en weefsels komt voor bij verschillende ziekten. Dit kan een rol spelen in het beloop van dergelijke ziekten, omdat in vivo en in vitro schadelijke effecten van sterolen beschreven zijn. Voor deze studie zijn de geïsoleerde bijnier- en levercel als modelsystemen gebruikt. Ook is de omzetting van gehydroxyleerde sterolen bestudeerd in geïsoleerde humane foetale bijniercellen, als model van een 3β-HSD deficiënt organisme. Het gebruik van geïsoleerde humane foetale bijniercellen is niet algemeen gangbaar; de meeste auteurs passen cel- of weefsel-cultures toe. Daarom werd nagegaan, in hoeverre de procedure, die gebruikt wordt voor de isolatie van rattebijniercellen, ook toepasbaar is op de humane foetale bijnier. Ook een scheidingsprocedure voor de twee typen cellen in de foetale bijnierschors werd bestudeerd.

Hoofdstuk 2 geeft een overzicht van de literatuur. De steroid synthese en de regulatie daarvan in de rattebijnier (zowel de zona glomerulosa als de zona fasciculata/reticularis), als ook in de humane foetale bijnier worden besproken. Het vóórkomen van een aantal sterolen wordt beschreven, alsmede hun effecten op cellulaire en subcellulaire processen.

Hoofdstuk 3 beschrijft de experimentele methoden, zoals de isolatie van cellen, incubatie omstandigheden en analytische technieken. Ook worden enkele karacteristieken van de rattebijniercellen, alsmede een evaluatie van de belangrijkste incubatie parameters gepresenteerd. Deze zijn: een dosis-respons curve voor de stimulatie van cellen met ACTH en de relatie tussen de steroid productie enerzijds en (1) het aantal cellen en (2) de samenstelling van de incubatie buffer anderzijds.

De belangrijkste gegevens van de in deze studie gebruikte humane foetale bijnieren worden vermeld. Twee parameters voor de vitaliteit van de cellen, te weten de ATP concentratie en de cortisol plus corticosteron productie uit exogeen progesteron, worden geëvalueerd. Ook kon de, in de literatuur beschreven, lage 3β-HSD activiteit in deze cellen worden bevestigd. Twee procedures om de cellen van de twee zones in de humane foetale bijnier te scheiden worden met elkaar vergeleken: centrifugatie door een dichtheids-gradiënt en een mechanische scheidingsmethodiek.

Hoofdstuk 4 behandelt de omzetting van gehydroxyleerde sterolen en het effect van sterolen op de ACTH-gestimuleerde corticosteron productie in geïsoleerde bijniercellen van de rat. Een aantal sterolen werd getest als substraat voor steroid synthese. 22R-OH, 25-OH en 20S-OH-cholesterol bleken goede substraten voor corticosteron synthese te zijn. 22S-OH werd minder goed omgezet. Dit sterol remde de ACTH-gestimuleerde corticosteron productie. 22S-OH had geen effect op de ACTH-gestimuleerde cAMP concentratie. In aanwezigheid van ACTH en 22S-OH hoopte vrij cholesterol zich op. 22S-OH had geen effect op de omzetting van exogeen pregnenolon. Deze resultaten suggereren, dat het remmend effect van 22S-OH op de ACTH-gestimuleerde corticosteron synthese gelocaliseerd is op het niveau van de cholesterol zijketen splitsing.
Tijdens het experiment werden geen verdere negatieve effecten van 22S-OH op het functioneren van bijniercellen waargenomen: de corticosteron productie als functie van de tijd bleef lineair.

Hoofdstuk 5 draagt aanvullend bewijs aan, dat het remmend effect van 22S-OH op de ACTH-gestimuleerde corticosteron productie een van de eeste stappen van de cholesterol zijketen splitsing treft. Hiertoe werd het effect van 22S-OH op de omzetting van 22R-OH (het eerste tussenproduct in de cholesterol zijketen splitsing) en op de omzetting van 25-OH (waarvoor het gehele zijketen splitsende systeem nodig is) met elkaar vergeleken: 22S-OH had geen effect op de omzetting van 22R-OH maar remde de omzetting van 25-OH.
De resultaten verkregen uit experimenten met bijniercellen van de rat en runderbijnierschors mitochondriën suggereren een directe relatie tussen de omzetting van 22S-OH enerzijds en (1) de productie van corticosteron in de bijnier-cellen en (2) de pregnenolon productie in de mitochondriën anderzijds. In de mitochondriën werd 22S-OH ook omgezet in 22-keto-cholesterol.

In hoofdstuk 6 wordt de omzetting van gehydroxyleerde sterolen en hun effect op de basale en gestimuleerde steroid productie in geïsoleerde fasciculata/reticularis en glomerulosa cellen beschreven. Het remmend effect van 22S-OH op de ACTH-gestimuleerde corticosterone synthese, zoals beschreven voor een celsuspensie van de bijnierschors (Hoofdstuk 4), kon ook worden aangetoond in geïsoleerde fasciculata/reticularis cellen.
In geïsoleerde glomerulosa cellen waren 25-OH en 22S-OH substraten voor de aldosteron synthese. De aldosteron productie was evenredig met de concentratie 25-OH, maar omgekeerd evenredig met de concentratie 22S-OH, waarschijnlijk ten gevolge van een effect van 22S-OH op de cholesterol zijketen splitsing. 22S-OH had geen remmend effect op de kalium-gestimuleerde aldosteron synthese, mogelijk omdat kalium ook op de laatste stappen in de aldosteron productie een stimulerende werking heeft.
In vitro bestaat er een verschil tussen de steroid productie-snelheden uit endogene substraten in glomerulosa en in fasciculata/reticularis cellen. Dit verschil is ook met exogene substraten, zoals 25-OH en 22S-OH, aantoonbaar. Hieruit blijkt, dat bij de regulatie van de steroid synthese in geïsoleerde bijniercellen de endogene substraat concentratie minder belangrijk is dan de enzymatische activiteit van het steroid-synthetiserende apparaat.

Hoofdstuk 7 handelt over de opname van corticosteron in geïsoleerde levercellen van de rat. 25-OH remde deze corticosteron opname. De remming was afhankelijk van de temperatuur, maar onafhankelijk van de gebruikte corticosteron concentratie. Deze remming was ook afhankelijk van de preïncubatie tijd van de levercellen met het sterol; het sterkste effect werd bereikt, wanneer de opname van het sterol maximaal was. Het werkingsmechanisme van 25-OH loopt waarschijnlijk via de remming van een actief opname-mechanisme door een verandering in het celmembraan.

In hoofdstuk 8 wordt het effect beschreven van verschillende sterolen op de opname van pregnenolon in geïsoleerde bijniercellen van de rat. Pregnenolon was een efficiënte precursor voor de steroid synthese. 25-OH had geen effect, noch op de opname noch op de omzetting van exogeen pregnenolon. In aanwezigheid van 22S-OH nam de intracellulaire radioactiviteit eerst toe, maar keerde later terug op het controle niveau. Dit kan verklaard worden door een effect van dit laatste sterol op de uitscheiding van corticosteron precursors, zoals DOC.
17S,20S-diOH had een remmend effect op de opname van pregnenolon, mogelijk

eveneens via een structurele verandering in het celmembraan. De verminderde opname resulteerde in een lagere pregnenolon omzetting.

Hoofdstuk 9 beschrijft de experimenten met de humane foetale bijniercellen. Een aantal sterolen werd omgezet in deze cellen. Een veel grotere hoeveelheid sterol verdween, dan verklaard kan worden op grond van de normale producten van de steroid synthese, inclusief cortisol, corticosteron en sulfaat conjugaten. Bij gaschromatografische analyse kon geen enkel onbekend steroid in een grotere hoeveelheid aangetoond worden. De, in dit modelsysteem geopperde, mogelijkheid voor productie van abnormale sterolen/steroiden wordt door deze resultaten bevestigd. Verder onderzoek op dit terrein is noodzakelijk.

In hoofdstuk 10 worden de verkregen resultaten besproken in het licht van de doelstellingen van deze studie. Acute effecten van de geteste sterolen lijken beperkt te blijven tot een competitieve remming van de cholesterol zijketen splitsing en/of tot een structurele verandering in het celmembraan, hoogstwaarschijnlijk veroorzaakt door inbouw van het sterol. De resultaten worden in een breder biologisch perspectief geplaatst.

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Curriculum vitae

De schrijver van dit proefschrift werd in 1950 geboren te Amsterdam. In 1966 behaalde hij het getuigschrift ULO-B aan de St. Dominicus ULO te Amsterdam. In 1968 werd het HBS-B diploma behaald aan het St. Ignatius college te Amsterdam.

In datzelfde jaar werd de scheikunde studie aangevangen aan de Vrije Universiteit te Amsterdam. Het doctoraal examen met als hoofdvak Biochemie (Prof. Dr. R.J. Planta) en als bijvakken Analytische Chemie (Prof. Dr. K. Gerritsma) en Histologie (Prof. Dr. H.L. Langevoort) werd afgelegd in december 1974.

Van begin 1975 tot half 1979 was hij als wetenschappelijk medewerker verbonden aan de afdeling Kindergeneeskunde (Erasmus Universiteit, Rotterdam. /Academisch Ziekenhuis Rotterdam/Sophia Kinderziekenhuis) (Hoofd: Prof. Dr. H.K.A. Visser), werkzaam op het laboratorium Kindergeneeskunde (Hoofd: Prof. Dr. H.J. Degenhart). Hij werd hiertoe in staat gesteld door een subsidie van de Sophia Stichting Wetenschappelijk Onderzoek.

Momenteel is hij waarnemend hoofd van het Stofwisselingslaboratorium van het Sophia Kinderziekenhuis in dienst van de Stichting Klinische Genetica, Regio Rotterdam.

Hij is gehuwd met Nel Boots en heeft drie kinderen: Saskia, Remco en Judith.