STUDIES ON ISOLATED RAT ADRENAL CELLS

PROEFSCHRIFT

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CONTENTS

LIST OF TRIVIAL NAMES AND ENZYMES

Adenylate cyclase	- ATP-pyrophosphate lyase (cyclizing) E.C. 4.6.1.1.
Aldosterone	- 11β,21-dihydroxy-18-oxo-4-pregnenolone-3,20-dione
Androstenedione	- 4-androstene-3,20-dione
Cholesterol	- 4-cholesten-3β-ol
Δ^{20-22}Cholesterol	- 5,20(22)-cholestadien-3β-ol
Cholesterol esterase	- Sterol-ester hydrolase, E.C. 3.1.1.13
Chymotrypsin	- E.C. 3.4.21.1
Collagenase	- E.C. 3.4.24.3
Corticosterone	- 11β,21-dihydroxy-4-pregnene-3,20-dione
Cortisol	- 11β,17α,21-trihydroxy-4-pregnene-3,20-dione
Dehydroepiandrosterone	- 3β-hydroxy-5-androsten-17-one
11-Deoxycorticosterone	- 21-hydroxy-4-pregnene-3,20-dione
11-Deoxycortisol	- 17α,21-dihydroxy-4-pregnene-3,20-dione
20R,22R-dihydroxycholesterol	- (20R,22R)-5-cholestene-3β,20,22-triol
DNAse	- Deoxyribonucleate 5'-oligonucleotidohydrolase, E.C. 3.1.4.5.
Elastase	- Pancreopeptidase E, E.C. 3.4.21.11
20,22-Epoxycholesterol	- 20ξ,22ξ-epoxy-5-cholesten-3β-ol
Hyaluronidase	- Hyaluronate 4-glycanohydrolase, E.C. 3.2.1.35 or Hyaluronate 3-glycanohydrolase, E.C. 3.2.1.36 or Hyaluronate lyase, E.C. 4.2.2.1
20S-Hydroxycholesterol	- (20S)-5-cholestene-3β,20-diol
22R-Hydroxycholesterol	- (22R)-5-cholestene-3β,22-diol
18-Hydroxycorticosterone	- 11β,18,21-trihydroxy-4-pregnene-3,20-dione
17α-Hydroxypregnenolone	- 3β,17α-dihydroxy-5-pregnene-3-one
17α-Hydroxyprogesterone	- 17α-hydroxy-4-pregnene-3,20-dione

3β-Hydroxysteroid dehydrogenase-isomerase	- 3β-hydroxy-5-steroid:NAD^+ 3-oxidoreductase E.C. 1.1.1.145 and 3-oxosteroid-Δ^4-Δ^5 isomerase, E.C. 5.3.3.1
17β-Hydroxysteroid dehydrogenase	- 3 (or 17)β-hydroxysteroid: $NAD(P)^+$ oxidoreductase, E.C. 1.1.1.51
Papaine	- Papainase, E.C. 3.4.22.2
Phosphodieterase	- 3',5'-cyclic-AMP 5'-nucleotidohydrolase, E.C. 3.1.4.17
Pregnenolone	- 3β-hydroxy-5-pregnene-20-one
Progesterone	- 4-pregnene-3,20-dione
RNAse	- Ribonucleate 3'-pyrimidino-oligonucleotidohydrolase, E.C. 3.1.4.22
Steroid-11β-hydroxylase	- Steroid, reduced-adrenal-ferredoxin: oxygen oxidoreductase (11β-hydroxylating), E.C. 1.14.15.4
Steroid-17α-hydroxylase	- Steroid, hydrogen-donor: oxygen oxidoreductase (17α-hydroxylating), E.C. 1.14.99.9
Steroid-18-hydroxylase	- Corticosterone, reduced-adrenal-ferredoxin: oxygen oxidoreductase (18-hydroxylating), E.C. 1.14.15.5
Steroid-21-hydroxylase	- Steroid, hydrogen-donor: oxygen oxidoreductase (21-hydroxylating), E.C. 1.14.99.10
Testosterone	- 17β-hydroxy-4-androsten-3-one
Trypsin	- E.C. 3.4.21.4

LIST OF ABBREVIATIONS

ACTH	- Adrenocorticotrophic hormone
AGI	- Aminoglutethimide-phosphate
ATP	- Adenosine triphosphate
c-AMP	- Adenosine 3',5'-cyclic monophosphate
5'-AMP	- Adenosine 5'-monophosphate
c-GMP	- Guanosine 3',5'-cyclic monophosphate
CLAH	- Congenital lipoid adrenal hyperplasia
DMSO	- Dimethyl sulfoxide
EDTA	- Ethylenediaminetetra-acetate
HEPES	- 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid
KRBAG	- Krebs-Ringer bicarbonate buffer, with albumin and glucose
KRHAG	- Krebs-Ringer Hepes buffer, with albumin and glucose
NADPH	- Nicotinamide-adenine dinucleotide phosphate (reduced)
NPS-ACTH	- Ortho-nitrophenyl sulfenyl derivative of ACTH

GENERAL INTRODUCTION

Research during the past few decades has resulted in detailed knowledge on the production of steroid hormones in the adrenal gland, but the picture is still far from complete. Hardly anything is known about the function of the human fetal adrenal gland which contains a specific fetal zone present only in man and some higher primates. The biochemistry of the inborn errors occurring in adrenal steroidogenesis is also poorly understood.

A very intriguing step in the biosynthesis of steroids is the conversion of cholesterol into pregnenolone. This cholesterol side-chain cleaving process is common to the biosynthetic pathways for all steroid hormones. Furthermore, hormonal regulation of steroid production almost exclusively takes place at this site. The exact mechanism of the side-chain cleaving process is still uncertain.

The experiments described in this thesis were performed to select and apply suitable methods which could extend the knowledge on some of the aspects of adrenal steroid biosynthesis, with emphasis on the function of cholesterol side-chain cleavage in the intact cell.

The isolated adrenal cell suspension was chosen as an experimental model. Mainly due to optimal contact between cells and medium, this incubation procedure offers some important advantages over other techniques. The high sensitivity of the cells to ACTH is a good illustration of this aspect. Rat adrenal tissue was chosen as it is much more easily available than fresh human adrenal tissue. The process of steroidogenesis in the rat adrenal is relatively well known, which makes this organ very well suited for the development of techniques that at a later stage might be applied directly to human adrenal tissue.

Several methods for the isolation of adrenal cells have been published, based on the lytic action of either collagenase or trypsin preparations. A variant of the existing

collagenase methods was developed. Cells can be incubated in a fixed volume of medium (batch incubations) or in a continuous medium flow (continuous flow incubations). A description of the methods used and the motivation for the selection of the technical procedures is given in Chapter III.

Batch and continuous flow systems each have different properties. In order to select the method most suited for the investigation of adrenal steroidogenesis, some properties of adrenal cells in both incubation systems were studied (Chapter IV).

Free cholesterol added to the incubation medium is not converted into corticosterone by isolated rat adrenal cells, most probably because the cell does not take up cholesterol in the free form. For this reason an alternative substrate was looked for. Such a substrate should be able to enter the cell in significant amounts directly from the medium. Furthermore, the substrate should not be an intermediate of the cholesterol side-chain cleaving process as, in these studies, this process was considered as a single step. The cholesterol derivative 25-hydroxycholesterol was found to meet these requirements (Chapter V).

As can be expected, a congenital deficiency of cholesterol side-chain cleavage causes more serious problems than deficiencies in other parts of the steroidogenic pathways. In fact, children born with such a defect, known as congenital lipoid adrenal hyperplasia (CLAH), in spite of treatment with steroids usually die soon after birth, contrasting with the clinical picture seen in the case of other inborn errors in steroidogenesis. A condition biochemically comparable to CLAH can be established in the rat as well as in the isolated rat adrenal cell. The corticosterone production was studied with adrenal cells in which a CLAH-like condition was induced (Chapter VI).

Inhibitors of protein synthesis have a negative effect on the stimulation of corticosterone production by ACTH. Although it is known that protein synthesis is an essential step in the

mechanism of action of ACTH, direct effects of inhibitors of protein synthesis on the steroidogenic pathways cannot be excluded. Furthermore, the contribution of mitochondrial protein synthesis to the mechanism of action of ACTH is uncertain. For these reasons the effects of two inhibitors of protein synthesis, cycloheximide and chloramphenicol, on corticosterone production were investigated (Chapter VII).

CHAPTER I

REVIEW OF THE LITERATURE ON ADRENAL STEROIDOGENESIS

1. Introduction

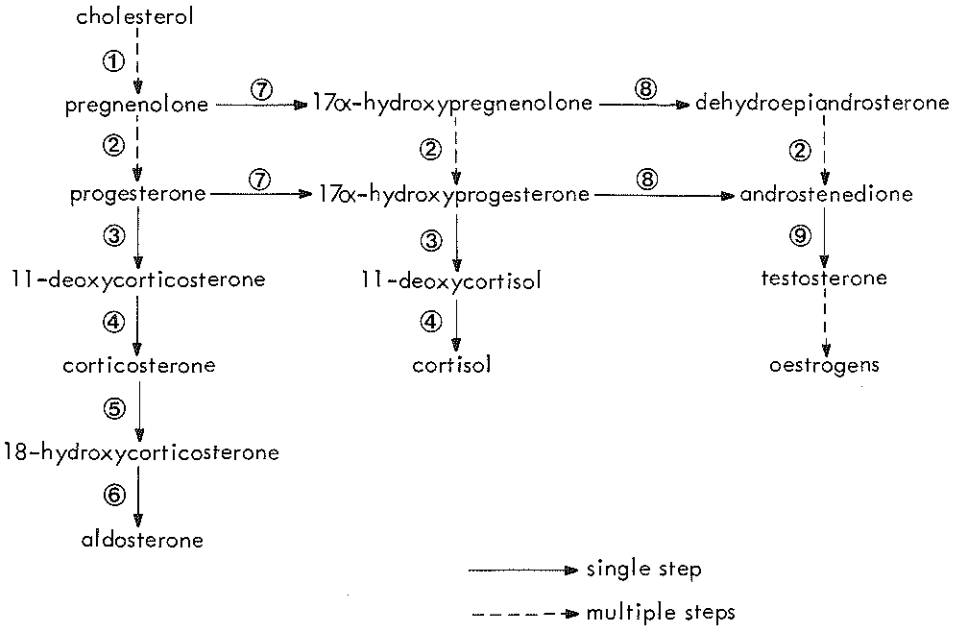
In higher vertebrates the adrenals are two relatively small organs situated above the kidneys. In these glands two functionally different tissues can be distinguished. The outer part, the adrenal cortex, produces steroid hormones and, judged by the weight, is the major part of the gland. The adrenal medulla, the central part of the adrenal gland, produces catecholamines.

Histologically three zones are found in the adrenal cortex. Starting from the outside these are the zona glomerulosa, the zona fasciculata and the zona reticularis. In man and some higher primates during intra-uterine life the larger part of the adrenal is occupied by a so-called fetal zone, which disappears soon after birth.

In this Chapter some aspects of steroid synthesis in the adrenal cortex will be discussed, with special emphasis on the role of cholesterol side-chain cleavage.

2. Pathways of adrenal steroid biosynthesis

The mammalian adrenal cortex has the ability to synthesize a variety of steroid hormones. The main products of the normal adrenal cortex are the C-21-steroids. Some of these, such as cortisol, have glucocorticoid activity, others, such as aldosterone, have mineralocorticoid activity, while yet others, such as corticosterone, have a combination of both. Under normal conditions the adrenal cortex may also produce small amounts of androgens (C-19-steroids) and oestrogens (C-18-steroids). The main pathways of adrenal steroidogenesis are shown in Fig. 1. Detailed descriptions of these pathways can be found in several recent reviews (Samuels and Uchikawa, 1967; Griffiths and Cameron, 1970; Schulster et al., 1976).



① cholesterol side-chain cleaving system

② 3β-hydroxysteroid-dehydrogenase-isomerase

③ steroid-21-hydroxylase

④ steroid-11β-hydroxylase

⑤ steroid-18-hydroxylase

⑥ 18-hydroxysteroid-dehydrogenase

⑦ steroid-17α-hydroxylase

⑧ steroid-17α,20-lyase

⑨ 17β-hydroxysteroid-dehydrogenase

Fig. 1

Outline of adrenal steroid biosynthesis.

In vivo cholesterol is the main substrate for the synthesis of all steroids. In vitro conversion of other sterols into steroids has been described (Solomon et al., 1956; Raggat and Whitehouse, 1966; Young and Hall, 1968) and it is possible that in vivo some of these sterols can act as substrates of minor importance. Cholesterol can be synthesized from acetate by the normal adrenal. However, there is good evidence that at least in some species, such as man and rat, plasma cholesterol is the main substrate for adrenal steroidogenesis (Vinson and Whitehouse, 1970; Borkowski et al., 1972). In the adrenal most of the cholesterol is stored as cholesterol esters in lipid droplets (Moses et al., 1969).

Steroid synthesis starts with the conversion of cholesterol into pregnenolone, a process called cholesterol side-chain cleavage. This is a multi step reaction. The central position of this step in steroidogenesis pathways and its relation to the regulatory mechanisms for adrenal steroidogenesis (Schulster et al., 1976) justify a more detailed discussion of this process in the next section. As can be seen in Fig. 1 the different pathways for the synthesis of the various steroids have several enzyme steps in common. As the zona glomerulosa is the single zone where a 18-hydroxy-steroid dehydrogenase is found, synthesis of aldosterone is limited to this zone. The synthesis of other corticosteroids is not restricted to a single zone.

In some rodents, such as the rat, the adrenal cortex possesses virtually no 17α-hydrolase activity. Consequently the main adrenal steroid in these animals is corticosterone and not cortisol (Slaga and Krum, 1976).

3. Cholesterol side-chain cleavage

The first step in the synthesis of steroids from cholesterol is the removal of part of the side-chain of the sterol. The products of this process are pregnenolone and most probably isocaproic aldehyde, but the exact nature of the reaction is not yet fully understood.

The cholesterol side-chain cleaving enzyme system is located in the mitochondria (Halkerston et al., 1961; Constantopoulos and Chen, 1961). The conversion of cholesterol into pregnenolone requires oxygen and NADPH. A cytochrome P-450 functions as the terminal oxidase in association with a flavoprotein and a non-haeme iron protein called adrenodoxin (Gower, 1975b). The side-chain cleaving enzyme system requires three molecules each of oxygen and NADPH (Shikita and Hall, 1974). This suggests the existence of intermediate products. Several authors have proposed mechanisms involving hydroxylation of cholesterol at positions 20 and 22, followed by a cleavage of the 20-22 bond (Sulmovici and Boyd, 1968; Burstein and Gut, 1971; Schulster et al., 1976). Early kinetic studies by Burstein and Gut (1971) critisized the so-called classical model in which cholesterol is first hydroxylated at C-22 and then at C-20 (Fig. 2). They found that the route via 20S-hydroxycholesterol is a very minor pathway and that most of the pregnenolone seemed to be generated via a route not including one of the two hydroxylated sterols.

Recent results obtained by Kraaipoel et al. (1975 a, b, c) led to the proposal of another model for the side-chain cleavage of cholesterol (Fig. 3). In this model a Δ^{20-22} unsaturated cholesterol and a 20,22 cholesterol epoxide were introduced as intermediates. The results of Kraaipoel et al. (1975 c) combined with those of Takemoto et al. (1968) and Burstein et al. (1974) definitely exclude any essential role of 20S-hydroxycholesterol. Whether 22R-hydroxycholesterol is an essential intermediate or a byproduct in this process remains to be established. At present, 20R,22R-dihydroxy-cholesterol is the only intermediate that is generally accepted. The pathway between 22R-hydroxycholesterol and 20R, 22R-dihydroxycholesterol is not entirely clear. As water seems to be involved in this part of the pathway, a conventional hydroxylation step is unlikely (Kraaipoel et al., 1975 b, c). The unsaturated Δ^{20-22} cholesterol is a very good substrate for pregnenolone synthesis in vitro (Kraaipoel et al., 1975 a)

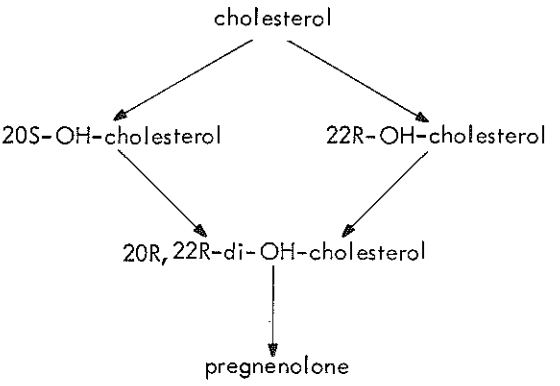


Fig. 2

Cholesterol side-chain cleavage according to the "classical model"

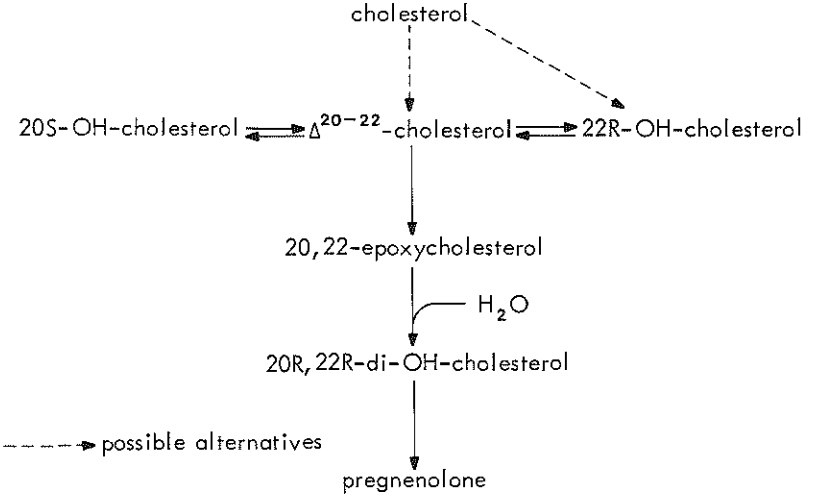


Fig. 3

Cholesterol side-chain cleavage according to the model proposed by Kraaipoel et al. (1975 a, b, c).

but to our knowledge this compound and the epoxide have never been isolated from adrenal tissue. At present the role of the epoxide is under discussion (Morisaki et al., 1976). However, the results of Kraaipoel et al. (1975 b, c) seem to indicate that there is an intermediate with the characteristics of an epoxide between the unsaturated cholesterol and 20R,22R-dihydroxycholesterol. The exact mechanism of the final step, the cleaving of the C-20, C-22 bond remains to be established.

4. On the mechanism of action of ACTH

The synthesis of glucocorticoids by the adrenal cortex is regulated almost exclusively by ACTH. The mechanism of action of ACTH is very complex and in this section an outline only of the stimulation of steroid production by ACTH will be presented. Comprehensive reviews of the literature can be found elsewhere (Garren et al., 1971; Gill, 1972; Sayers et al., 1974; Schulster, 1974 a; Schulster et al., 1976).

ACTH is produced by the adenohypophysis (Daughaday, 1974). It is a single peptide chain of 36 amino acids. In mammalian ACTHs the interspecies variation is found in the C-terminal part of the molecule (Jöhl et al., 1974). The biological activity resides in the N-terminal part (Schulster et al., 1976) and the frequently used synthetic 1-24 ACTH shows full biological activity.

ACTH has several effects on the adrenal. Its main short term effect is the stimulation of corticosteroid production. In vivo ACTH also increases adrenal blood flow (Stark and Varga, 1968). In addition to these short term effects ACTH also influences the growth of the adrenal cortex.

From a variety of experiments it is clear that ACTH does not enter the cell but exerts its effect from the outside (Taunton et al., 1967; Schimmer et al., 1968; Richardson and Schulster, 1972). ACTH is bound to receptor sites on the outer surfaces of the cellular membrane. At least two different receptor-systems for ACTH have been found, one with an apparent

dissociation constant of approximately 10^{-11}-10^{-12}M and another with an apparent K'd of approximately 10^{-8}M. The latter receptors are present in much larger numbers than the former (Lefkowitz et al., 1971; McIlhinney and Schulster, 1975), but the exact function of each of these receptors is unknown.

Inside the cell the rate-limiting step in ACTH-stimulated steroid production is situated somewhere between cholesterol and pregnenolone. This was first suggested by Stone and Hechter (1954) and confirmed by Davis and Garren (1968). It now seems that a more exact location for the effect of ACTH is the transport of cholesterol from extra-mitochondrial storage sites of cholesterol to the cholesterol side-chain cleaving system (Garren et al., 1971; Mahaffee et al., 1974; see also the discussion in Chapters V and VII).

Between the binding of ACTH to the cell and the stimulation of pregnenolone synthesis a number of processes are involved, which are only partly known. The first intracellular effect of ACTH is the formation of cyclic adenosyl 3',5'monophosphate (c-AMP) as so-called second messenger (Grahame-Smith et al., 1967; Robinson et al., 1971). In the case of ACTH the role of c-AMP as a second messenger seems well established, but the existence of other second messengers cannot be excluded. The experiments of Sharma et al. (1974) for example suggest that c-GMP may act as a second messenger at low ACTH concentrations Some investigators (Sayers et al., 1972; Moyle et al., 1973) have described stimulation of steroid production in isolated rat adrenal cells with ACTH concentrations that do not result in a measurable increase in c-AMP. A slightly better correlation was found by Mackie et al. (1972). Furthermore, the ortho-nitrophenyl-sulfenyl derivative of ACTH stimulated corticosterone production by isolated rat adrenal cells in a similar way as ACTH, but a small increase in c-AMP was only observed at relatively high concentrations of NPS-ACTH (Moyle et al., 1973). These results may indicate an ACTH-influenced compartmentation of c-AMP. ACTH stimulates production of c-AMP by activation of adenylate cyclase, a membrane-bound enzyme

which converts ATP to c-AMP (Lefkowitz et al., 1970; Kelly and Kortiz, 1971). Activation of adenylate cyclase requires Ca^{++} (Lefkowitz et al., 1971) and seems to be modulated in a still unknown way by several prostaglandins (Honn and Chavin, 1976). A generally applicable model for the activation of adenylate cyclase by hormones acting extracellulary is given by Cuatrecasas (1974). In this model the receptor and adenylate cyclase are distinct and separate structures moving independently in the fluid phase of the cellular membrane. Binding of a hormone to the receptor induces a change in the conformation of the receptor which enables the latter to combine with and to activate the adenylate cyclase. The resulting complex is probably stabilized by GTP (Rendell et al., 1975; Lin et al., 1975; Salomon et al., 1975).

The c-AMP formed can be inactivated by a phosphodiesterase, resulting in the formation of 5'-AMP (Schulster et al., 1976). Activation of steroid production by c-AMP starts with binding to a c-AMP-receptor-inactive protein kinase complex. This process and the next steps have been thouroughly studied by Garren and co-workers (Garren et al., 1971). After binding of cyclic AMP, the complex dissociates into a (c-AMP-receptor)-c-AMP complex and an active protein kinase, which may form dimers. The active protein kinase can incorporate phosphate from ATP into several proteins, including histones. So far the only substrate for the protein kinase identified as part of the regulatory mechanism of ACTH is cholesterol esterase (Garren et al., 1971; Trzeciak and Boyd, 1974), which stimulates hydrolysis of cholesterol esters after treatment of adrenal tissue with ACTH.

The free cholesterol formed has to be transported to the cholesterol side-chain cleaving system residing in the mitochondria. As cholesterol itself is very hydrophobic, it seems likely that some transport factor, probably a protein, is needed. At present it is generally accepted that protein synthesis is an essential part of the mechanism of action of ACTH (Schulster, 1974a; Schulster et al., 1976). This

knowledge was obtained largely by the use of inhibitors of protein synthesis (cycloheximide, puromycin, chloramphenicol). When the effect of ACTH on steroid production is blocked by cycloheximide, free cholesterol accumulates in the so-called lipid droplets (Garren et al., 1971). Therefore, the protein involved in the acute effect of ACTH seems to play a part in the transport of free cholesterol from the lipid droplets to the mitochondria. This is in contrast with the observation made by Mahaffee et al. (1974) that ACTH in the presence of cycloheximide stimulates accumulation of free cholesterol in the mitochondria. This indicates a role for the ACTH-dependent protein in the translocation of cholesterol in the mitochondria in order to make this sterol available to the cholesterol side-chain cleaving system. However, Mahaffee et al. isolated mitochondria under conditions which facilitate leakage of free cholesterol from the lipid droplets (Garren et al., 1971) and the observed accumulation of cholesterol in the mitochondria which was observed could be an artifact. Brownie et al. (1973), using electron paramagnetic resonance and optical difference spectra, observed in ACTH-treated, hypophysectomized rats an increased association of cholesterol with the cholesterol side-chain cleaving system. The effect was reversed by subsequent treatment with cycloheximide. This indicates that an ACTH-dependent protein may be involved in the binding of cholesterol to the cholesterol side-chain cleaving system.

Little is known about the protein involved in the acute effect of ACTH. Studies with actinomycin D (Garren et al., 1965; Schulster, 1974 b) provide evidence that for the acute effect of ACTH no newly-synthesized RNA is required. According to Schulster (1974 b) the RNA involved in the synthesis of the ACTH-dependent protein has a half-life of at least 70 minutes, indicating the presence of a relatively stable messenger RNA. The protein itself is labile, having a half-life of the order of several minutes (Garren et al., 1965; Rubin et al., 1973; Schulster et al., 1974). As stated above, the labile protein

may have a role in the transport of free cholesterol to the mitochondria. Schulster and Jenner (1975) have suggested that ACTH does not influence the synthesis proper of the labile protein, but its transformation from an inactive to an active form. In this model there is a continuous synthesis of the labile protein. If this is correct, the activation could involve phosphorylation by the activated protein kinase as mentioned earlier.

Several other factors may play a part in the acute stimulation of steroidogenesis by ACTH. Calcium in addition to its role in the activation of adenylate cyclase, is involved in many other processes in the cell and ACTH is known to influence the uptake of calcium by the adrenal cell and may also influence its intracellular distribution (Schulster et al., 1976). There is also evidence for a role of microfilaments in the mechanism of action of ACTH (Mrotek and Hall, 1975). Finally, ACTH probably does not have an acute stimulating effect on the cholesterol side-chain cleaving system itself (Chapter V, 3), hence all the cholesterol made available to this enzyme system can be immediately converted into steroids.

Fig. 4 presents a picture of a model including the main steps of the acute mechanism of action of ACTH on steroidogenesis which have been discussed in this section.

5. On congenital lipoid adrenal hyperplasia

Some of the experiments described in this thesis were performed in order to obtain more information about the biochemical aspects of congenital lipoid adrenal hyperplasia (CLAH). CLAH is a rare and almost always fatal inborn error in steroid biosynthesis. A location of the defect somewhere in the cholesterol side-chain cleaving system was proposed by Prader and Anders (1962) and has been proven for one case by Degenhart et al. (1972). CLAH is one of a large group of congenital defects in adrenal steroidogenesis (Degenhart et

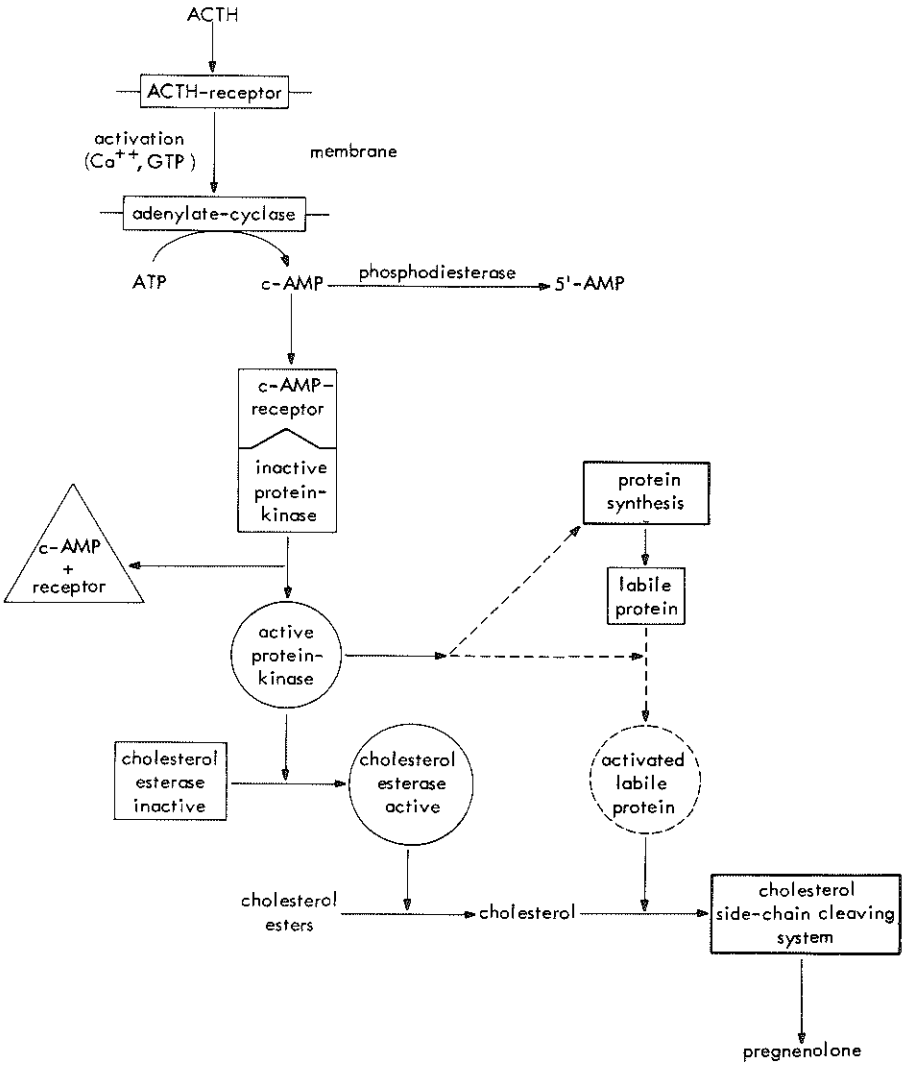


Fig. 4

Model of the mechanism of action of ACTH.

al., 1971) and by no means the most common (Bongiovanni, 1972), but it is of particular interest because the location of the defect of steroid synthesis results in most cases in an almost total absence of any steroid production, depending on the severity of the defect. There is a remarkably high mortality rate. This cannot be explained by the absence of steroid production, as children with congenital absence of the adrenals can be treated relatively easily (Sperling et al., 1973; Pakravan et al., 1974). Most of the studies on CLAH were performed post mortem and very little information on sterol and steroid metabolism is available. Tsutsui et al. (1970) found accumulation of cholesterol (both free and esterified) in the adrenal glands. In a very mild case of CLAH, Kirkland et al. (1973) observed some production of androgens which could be produced via an alternative pathway as proposed by Jungman (1968).

It is not possible to obtain fresh human tissue with this defect, but a CLAH-like situation can be induced in rats with aminoglutethimide (AGI), an inhibitor of cholesterol side-chain cleavage (Goldman, 1970; Gower, 1974). In this thesis an experiment with isolated rat adrenal cells in which the defect was immitated in a similar way will be presented (Chapter VI).

6. Epilogue

Cholesterol side-chain cleavage is a crucial step in adrenal steroidogenesis. It is the entrance to the pathways of steroid synthesis and although the process itself seems not to be influenced by ACTH, it is of great importance for the regulation of adrenal steroid production as ACTH controls the availability of the main substrate, cholesterol. Furthermore, a genetic defect in this process causes severe clinical problems.

Cholesterol side-chain cleavage has been studied using a wide variety of in vitro methods. These methods vary from studies with purified enzyme preparations, via intact

mitochondria and isolated cells to the intact organ. In this thesis cholesterol side-chain cleavage is studied in the intact cell. Although it includes several reactions the process was considered as a single step.

CHAPTER II

REVIEW OF THE LITERATURE ON ISOLATED ADRENAL CELLS

1. Introduction

The introduction, several years ago, of isolated adrenal cell suspensions (Kloppenborg et al. 1968; Halkerston and Feinstein, 1968; Swallow and Sayers, 1969) added an important new technique to the arsenal of in vitro methods available for the study of adrenal steroidogenesis. Although intact cells can now be isolated from almost all tissues, this Chapter will mainly deal with adrenal cells. The methods used for the isolation of the cells, the advantages of cell suspensions over other in vitro systems and several other aspects of isolated cells will be discussed.

2. Motivation of the use of isolated rat adrenal cells

As stated in the general introduction, little is known about the biochemistry of the human fetal adrenal gland and on the adrenal gland with inborn errors in steroid synthesis. Fresh human fetal adrenal tissue, especially tissue from the last trimester of intra-uterine life, is only scarcely available for in vitro research. The same is true of adrenals with inborn errors of steroid metabolism. Therefore the rat adrenal was chosen as a model system. Research during the past few decades has produced extensive knowledge on this gland. For this reason the rat adrenal is very well suited for the development of methods, which can be applied directly when human tissues become available. Steroidogenesis in the rat adrenal can be influenced by the use of specific inhibitors in order to imitate the situation in the human fetal adrenal gland, where 3β-hydroxysteroiddehydrogenase activity is low, or congenital lipoid adrenal hyperplasia where cholesterol side-chain cleavage activity is deficient (Degenhart et al., 1972).

The use of isolated adrenal cells was based on the definite advantages of this system over other incubation methods. With the exception of adrenal tissue cultures, the older in vitro methods mainly used tissue blocks (adrenal quaters, -slices etc..) for the study of steroidogenesis in intact adrenal cells. In these incubations only the outer tissue layers are in more or less direct contact with the surrounding medium. The inner layers have to communicate with the medium via diffusion of substrates and products through the outer layers. As a result they may degenerate to some extent and become an unaccountable source of nutrients and catabolic products.

In isolated cell suspensions the whole surface of each cell is in direct contact with the incubation medium (Fig. 5), thus allowing optimum exchange of information between cell and medium. Furthermore, different components in the medium can reach the cell in a more uniform way because differences

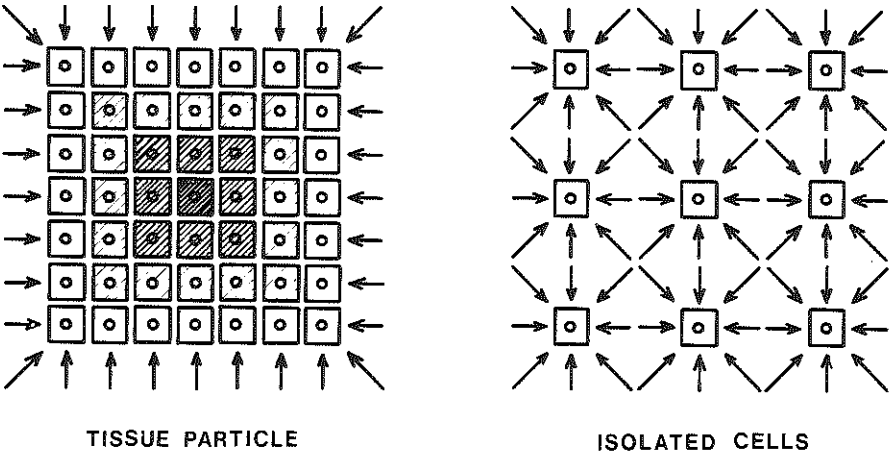


Fig. 5

Accesibility of cells in a tissue particle in comparison with the accesibility of isolated cells.

in diffusion rates through outer layers of tissue blocks have no effect.

These advantages of isolated cells are clearly illustrated by the high sensitivity of isolated rat adrenal cells to ACTH when compared with incubated rat adrenal quarters (Kloppenborg et al., 1968; Sayers et al., 1971). Another interesting experiment was published by Kuo and Chen (1973). They found severe mitochondrial damage in rat adrenal quarters after 1 hour continuous flow incubation, possibly due to anoxia. This did not occur in isolated rat adrenal cells after a comparable (batch) incubation. The better contact of all the incubated cells with the surrounding medium may also be responsible for the approximately tenfold higher steroid production per cell when isolated rat adrenal cells are compared with rat adrenal quarters (Kloppenborg et al., 1968; Richardson and Schulster, 1972).

Another advantage of isolated cells is the lack of inter-animal variation within one experiment, as the cells of tissues from several animals can be pooled before distribution over the incubation vessels.

Functionally different cells can be separated by physical methods (Miller and Phillips, 1969; Shortman, 1972), or by selective enzymatic destruction of specific cells (van Berkel et al., 1975). This allows the study of functionally homogeneous cell suspensions. A good example is the separation of cells from different adrenocortical zones by unit gravity sedimentation (Tait et al., 1974).

The advantages of freshly isolated cells are also obtained to some extent by using cultured adrenal cells. However, only part of the surface of these cells is in contact with the medium, the other part adhering to other cells or to the wall of the culture vessel. Furthermore, cultured cells may undergo transformation during long-term experiments (Rinaldini, 1958), while cell death may also occur. Normal rat adrenal cells in monolayer cultures may lose their response to ACTH (O'Hara and Neville, 1970).

Summarizing one may conclude that isolated rat adrenal cell suspensions offer several advantages for the study of steroidogenesis in short-term experiments.

3. The preparation of isolated adrenal cells

Methods for the isolation of intact cells from animal tissues were known long before the introduction of the adrenal cell suspension in 1968. Earlier methods were published for the isolation of embryonic cells (Moscona, 1952), hepatoma cells (Essner et al., 1954), kidney cells (Dulbeco, 1952; Younger, 1954; Melnick et al., 1955) and liver cells (Anderson, 1953). Cells obtained by these methods were used to grow cell cultures. According to Rinaldini (1958) cell cultures provide a "cleaner" system as compared with explant cultures, where the core of the explant may degenerate to some extent and become an unaccountable source of nutrients and catabolic products.

For reasons discussed in the previous section it is often preferable to perform metabolic studies on cells immediately after their isolation. In order to study isolated cells methods for their isolation have to meet the following conditions: (1) They should be fast enough to allow studies being performed immediately after isolation of the cells and (2) contrary to the situation with cell cultures the yield of intact cells has to be high, as fresh cells have almost no opportunity to repair damage caused by the isolation method and no time for multiplication.

The first method to fulfill the conditions mentioned above was published by Rodbell in 1964, who used collagenase to isolate fat cells. Nowadays, incubation of freshly-isolated cells has become a major in vitro technique and methods are available for almost all mammalian tissues, including liver (Berry and Friend, 1969; van Berkel et al., 1975), kidney (Michelakis, 1969), testis (Moyle and Ramachandran, 1973; Dufeau et al., 1974), hypophysis (Portanova et al., 1970) and of course adrenals (Kloppenborg et al., 1968; Halkerston and

Feinstein, 1968; Swallow and Sayers, 1969).

Isolation of intact cells from solid tissues requires destruction of the intercellular structures under conditions that do not interfere with intracellular functions. Current methods combine enzymatic digestion of intercellular material with some kind of gentle mechanical action and/or removal of bivalent cations (mainly Ca^{++}). For enzymatic digestion one has always used proteolytic enzymes. Collagenase and trypsin have been used in the vast majority of all cases. Other proteolytic preparations that have been used occasionally are elastase, pancreatine, papaine (Rinaldini, 1959), chymotrypsin (Kono, 1969a) and pronase (Gwatkin, 1963). Hyaluronidase, RNAse or DNAse are sometimes added to improve tissue dissociation (Rinaldini, 1959; Howard et al., 1967; Barowsky et al., 1973). Removal of Ca^{++} is of great importance in the isolation of liver cells (Berry, 1976). This is usually achieved by perfusion with Ca^{++}-free media sometimes combined with addition of chelating agents such as EDTA.

The methods that have been used for the preparation of isolated adrenal cells can be divided into two main groups, those using collagenase and those using trypsin. Preparation of isolated rat adrenal cells by incubation with collagenase was introduced by Kloppenborg et al. (1968). Other collagenase-based methods were published by Haning et al. (1970), Rivkin and Chasin (1971), Richardson and Schulster (1972), Moyle et al. (1973) and Mulder (1975). Collagenase is isolated from clostridium histolyticum (Mandl, 1953). Most methods apply crude preparations. Kloppenbrog et al. (1968) and Richardson and Schulster (1972) also used somewhat more purified preparations. Collagenase concentrations vary from 0.2 - 0.5 mg/ml for the partly purified preparations to 3 - 5 mg/ml for the crude preparations. In two cases, DNAse (0,05mg/ml) was added to the collagenase solutions. Collagenase has also been used for the isolation of cells from rabbit (Peach and Chui, 1974) and canine adrenals (Fredlund et al. 1975).

Swallow and Sayers (1969) introduced a method for the isolation of rat adrenal cells with trypsin. Modifications of this method were published by Sayers et al. (1971), Kitabchi and Sharma (1971), Nakamura and Tanaka (1971) and Lowry et al. (1973). In all cases the trypsin concentration was 0.25%. Cells from the adrenals of guinea pigs (Kolanowski et al., 1974), cats (Rubin and Warner, 1975) and humans (Kolanowski and Crabbé, 1976) have also been isolated with trypsin.

The proteolytic action is always combined with some kind of mechanical disruption of the tissue. With trypsin, carefully controlled stirring during incubation is always applied. When collagenase is used, dissociation of the tissue is completed after the incubation by repeated pippetting through the opening of a pipette or a silicon tube. A Potter-Elvehjem type of homogenator with a loose-fitting rubber pestle was used by Richardson and Schulster (1972). Non-dissociated tissue fragments are removed by filtration or precipitation. The cells are collected by centrifugation, varying from 100xg for 10 minutes (Haning et al., 1970) to 480xg for 30 minutes (Rivkin and Chasin, 1971).

After collection of the cells, residual proteolytic activity is removed either by extensive washing (2 - 3 times) in the case of collagenase or, when trypsin is used, by the addition of lima bean trypsin inhibitor. The yield of intact cells varies from 3×10^5 cells to 10^6 cells per adrenal gland. According to the DNA estimations of Richardson and Schulster (1972), this accounts for 5 - 10% of the cells in the intact gland. The steroid production responds to ACTH with a more or less sigmoid log-dose response curve. There are, however, differences in the sensitivity to ACTH of the various cell suspensions. For most cell suspensions steroidogenic response to ACTH begins at ACTH-concentrations somewhere between 1 and 50 µU/ml, while maximum steroidogenic response is usually found between 1 and 10 mU ACTH/ml. The most sensitive cell suspension was obtained by the group of Sayers (Sayers et al., 1971; Giordano and Sayers, 1971).

This cell suspension was sensitive enough to be used for ACTH bio-assays in rat plasma.

What causes the dissociation of adrenal tissue upon incubation with proteolytic enzymes? From the success of both collagenase and trypsin in producing cell suspensions one might conclude that removal of extracellular proteins, especially collagen, is the main reason. Pauly (1957), using histochemical methods, identified collagen fibers in rat adrenal capsules, along the blood vessels penetrating into the adrenal cortex and between cortex and medulla. However, very few collagen fibers were present in the area between the parenchymal cells of the adrenal cortex. These cells are surrounded by a network of reticular fibers. Histochemically these fibers are very similar to the reticulin in basement membranes, which can be digested with collagenase (Barowski et al., 1973). Consequently the action of collagenase (and part of the action of trypsin?) could be directed against these fibers. From the work of Kono (1969a) it is clear that the removal of collagen or collagen-like material alone does not cause dissociation of tissue. Highly-purified collagenase preparations, although capable of removing almost all collagen from rat tail tendon, only causes dissociation of adipose tissue or cardiac muscle when used in combination with trypsin. The success of collagenase is commonly attributed to trypsin-like activity present in the generally used crude or partially-purified collagenase preparations. It is not certain what the substrates for this non-collagenase proteolytic activity are but several possible candidates can be found in the literature. It is known that desmosomes in several mammalian tissues can be cleaved by trypsin (Borysenko and Revel, 1973). Desmosomes, however, are relatively rare in the adrenal cortex, especially in the zona fasciculata (Friend and Gilula, 1972). Another possibility is the septate like junction found in rat adrenal cortex and other steroidogenic tissues (Friend and Gilula, 1972). This kind of cell contact consists of the membranes of two adjacent cells separated to

a distance of 210-300Å by extracellular particles of a more or less spherical shape. Between these particles, which show a very tight adherence to the cell membranes, lies a network of very thin channels permeable by relatively large particles such as horse radish peroxidase and lanthanium gels. The particles are resistant to a short perfusion of the adrenal with crude collagenase, but can be digested with protease. No certainty exists as to the involvement of these structures in the tissue-dissociation process.

From the fact that all methods used for the isolation of adrenal cells need some kind of mechanical action it can be concluded that proteolytic activity alone is not sufficient. Apparently, the incubations are too short or intercellular materials are present which are not digested by the enzyme preparations used. As hyalonuridase does not improve adrenal dissociation (Barowsky et al., 1973) it is unlikely that these materials are mainly composed of hyaluronic acid.

The use of DNAse by Haning et al. (1970), Barowsky et al. (1973) and ourselves (Chapter III) is justified by its prevention of cellular aggregation by DNA, released from cells damaged during the isolation procedure. This kind of aggregation was described by Steinberg (1963) for embryonic tissues.

CHAPTER III

MATERIALS AND METHODS

1. Some aspects of the methods used

In this Section some general aspects of our method for the isolation of rat adrenal cells will be discussed. A detailed description of the procedure can be found in Section III.2.

Adrenal cell suspensions can be prepared successfully with both collagenase and trypsin. The literature on adrenal cells does not provide definitive arguments in favour of either of these enzymes. It is claimed that trypsin yields more reliable results and cells with a higher sensitivity to ACTH (Swallow and Sayers, 1969; Sayers et al., 1971). Due to differences in the incubation techniques, however, comparison between the results of various groups is difficult. Mulder (1975), who tried both the trypsin method of Sayers et al. (1971) and a collagenase method, prefered collagenase. Our preference for collagenase is based on the philosophy that even the action of crude preparations of this enzyme is more specific for extracellular material and therefore may cause less damage to the cells as compared with trypsin.

This preference is supported by reports concerned with the effects of trypsin and collagenase on non-adrenal mammalian cells. Surface charge of mammalian cells, for example, is much more influenced by trypsin than collagenase (Yamada and Ambrose, 1966) and an inactivation of effector systems (receptors etc.) for several peptide hormones in mammalian cells has been described (Kono, 1969b, c; Fain and Loken, 1969; Czech and Fain, 1970; Lissitzky et al., 1973). Crude collagenase did not have these effects (Kono, 1969b; Fain and Loken, 1969). Penetration of intact cells by trypsin has been found (Hodges et al., 1973) and also activation of adenylate cyclase (Ryan et al., 1975). Although to our knowledge none of these phenomena has been investigated in

adrenal cells, the occurrence in other cell types provides arguments in favour of collagenase.

In our procedure, collection and washing of the cells were performed at room temperature. In this our method differs from most of the other published methods, where these steps are performed at 0 - 4°C. While developing the techniques, we got the impression that working at room temperature resulted in a slightly higher steroidogenic response to ACTH. This was confirmed by Morita et al. (1975), who found an increase in both the sensitivity to ACTH and in the rate of corticosterone production when cells were isolated at 25°C instead of 0°C. This effect could also be seen in rat adrenal quarters, which were pre-incubated at different temperatures. Similar effects have also been described for lipolysis in adipose tissue (Okuda et al., 1971).

Another aspect in which our procedure differs from other methods is the use of HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) buffers. Usually, cell suspensions are prepared and incubated in bicarbonate-carbondioxide buffers. These buffers require the continuous presence of a CO_2-enriched atmosphere to achieve a stable pH. Another disadvantage is the pKa of bicarbonate (6,1) at 37°C, which results in sub-optimum buffering at physiological pHs. In our experience, the pH of bicarbonate buffers rises very fast as soon as the additional carbondioxide is removed. As this is often unavoidable (centrifugation, pipetting etc.), values higher than pH 8.0 were sometimes reached within 10 - 20 minutes. We therefore tried HEPES, one of the hydrogen-ion buffers described by Good et al. (1966). HEPES has a pKa of 7,31 at 37°C, resulting in optimal buffering at physiological pHs. Cells prepared and incubated in either bicarbonate or HEPES buffers responded to ACTH with a sigmoid log-dose response curve (see Chapter IV). In both buffers, 25-hydroxy-cholesterol stimulated corticosterone production.

Viability of cells is often evaluated by dye exclusion tests, which are based on the fact that viable cells do not

take up certain dyes, whereas non-viable cells do. Trypan blue is commonly used, although other suitable dyes exist. We have initially used this dye in concentrations of 0.2 - 1.0 mg/ml and found that almost all of our cells excluded the dye. Trypan blue tests are, however, highly unreliable (Tenant, 1964), the results depending strongly on the concentration of the dye. Furthermore, the composition of the medium in which the cells are suspended has a marked influence (Phillips, 1973). In our experiments the results may have been influenced by the fact that cells were always in contact with solutions containing 3% bovine serum albumin. Trypan blue has a greater affinity for protein in solution than for non-viable cells (Phillips, 1973). Another variable is the (almost uncontrollable) composition of the particular batch of dye used. This led Scott (1972) to exclaim: "Lies, damned lies and biological stains". We therefore discontinued the use of this test. Other, more reliable, tests for viability, such as the capacity of the cells to attach and grow in culture (Hilfer, 1973), are difficult to combine with routine incubations of freshly-isolated cells.

In our experiments only those cells showing well-defined boundaries on observation under a light microscope (magnification 400x) were counted. The response of the cells to ACTH was used as a rough indication of the quality of the cell suspension as a whole. Stimulation of adrenal steroid production by ACTH involves a number of processes in different parts of the cell (Garren et al., 1971; Schulster et al., 1976). It thus gives some information about the integrity of the cell. In fact, it has been found that ACTH does not stimulate steroidogenesis in adrenal homogenates (Kloppenborg et al., 1968), which indicates that cellular integrity is necessary for the effect of ACTH on steroidogenesis.

2. Materials

ACTH was a purified preparation of porcine ACTH obtained from Organon, Oss (Cortrophine®). The following materials were purchased from Sigma Chemical Company: albumin (bovine serum albumin, Cohn fraction V; chloramphenicol (D-(-)-threo-chloramphenicol), cholesterol, 20S-hydroxycholesterol, collagenase (type I) and DNAse (DN-100). AGI was a generous gift from Ciba Co., Arnhem. HEPES was obtained from Calbiochem, cycloheximide from Boehringer, Siliclad from Clay Adams, 22R-hydroxycholesterol from Ikapharm and 25-hydroxycholesterol from Steraloids. Other chemicals were p.a. quality and purchased from Merck or Baker.

5-Cholene-3β,24-diol was synthesized in our laboratory by $LiAlH_4$-reduction of the methyl ester of 3β-hydroxy-5-cholenic acid (Schwarz-Mann).

3. Detailed description of the methods

Isolated adrenal cells were initially prepared from the adrenal glands of normal male Spraque-Dawley rats (200 - 250g). At a later stage of this project, due to difficulties with the Spraque-Dawley line, we changed to male Wistar-R rats.

Glass surfaces in contact with the cells were siliconized (Siliclad) and sterilized. All incubations were carried out in Krebs-Ringer buffers at pH 7.4. The buffers contained glucose and bovine serum albumin. Depending on the main buffering component the buffers are called KRBAG or KRHAG. The composition of the buffers is presented in Table I. KRBAG was used in the first experiments, KRHAG in the more recent incubations. The KRBAG was equilibrated before use, against an oxygen-carbondioxide mixture (95% O_2 - 5% CO_2). The incubations in this buffer were performed in an atmosphere of the same gas mixture. Incubations in KRHAG were done in air. Before use the buffers were sterilized by filtration through a Millipore filter (HAWP 02500; pore size 0.45 μm).

Table I

Composition of the Krebs-Ringer buffers used (in mM).

	KRBAG	KRHAG
NaCl	110	110
KCl	4.75	4.75
$CaCl_2$	2.54	2.54
$MgSO_4$	1.18	1.18
NaH_2PO_4	1.18	1.18
$NaHCO_3$	25	--
HEPES	--	25
Glucose	11	11
Bovine serum albumin*	3% (w/v)	3% (w/v)

*Dialyzed against twice-distilled water and lyophilized.

Usually, ten rats were decapitated at approximately 9.00 am. The adrenals were removed and collected in saline at 0°C. This whole procedure took ca. 15 min. After removel of adhering fat tissue, the adrenals were cut in 6 - 8 pieces per gland. The pieces were washed twice with buffer (ca. 1 ml per adrenal equivalent). The washed tissue was incubated in a fresh solution of collagenase (5 mg/ml) and DNAse (0.05 mg/ml). The incubations were carried out in 25 ml Erlenmeyer flasks. Each flask contained the pieces of ten adrenals and 5 ml enzyme solution. The flasks were placed in a Dubnoff incubator shaking at 100 rpm at a temperature of 37°C. Every 20 min. the medium with the free cells was removed with a Pasteur pipette and replaced by 5 ml fresh enzyme solution. The removed cells were originally stored at 0°C, in later experiments this was done at room temperature. After the third 20 min. peroid the incubation was stopped. The remaining particles were dissociated by pipetting and expelling them 5 - 10 times in a Pasteur pipette. All 20 min. fractions were combined. Coarse particles were removed by letting them sediment during 5 min. The supernatant with the cells was centrifuged at room temperature at 80 - 100xg for 10 min.

The cell pellets were re-suspended in the original volume of buffer and washed 3 times by centrifugation at 100xg for 10 min. and re-suspension in buffer. After the last centrifugation the cells were suspended in the volume of buffer needed for the experiment. The cells were counted in a haemocytometer under a light microscope (magnification 400x). Yields were $3\text{-}10\text{x}10^5$ cells per adrenal. The cells were used immediately after isolation.

Adrenal cells were incubated in siliconized round-bottom glass tubes at 37^0C in a Dubnoff incubator shaking at 100 rpm. The total incubation volume was always 1.0 ml. Approximately $2\text{x}10^5$ cells were incubated in each tube. ACTH, aminoglutethimide, cycloheximide and chloramphenicol were added in 0.1 ml buffer. The sterols were added in 10 µl ethanol. This low amount of ethanol did not influence steroid production, nor did clouding occur after addition of the sterols. After 2 hrs incubation the tubes were tightly capped, frozen rapidly and stored at -20^0C.

For continuous flow studies the cells were incubated in a flow cell (Fig. 6). The medium was pumped through the cell with a Harvard infusion pump (Harvard Apparatus type 2202). The direction of the medium flow was from top to bottom. This way, the magnetic stirrer kept the cells in a fast movement just above the outflow filter and thus prevented them from being pressed into filter. Loss of cells was further minimized op optimizing the flow of the medium through the cell and the speed of the magnetic stirrer. In our experiments the medium flow was 0.19 ml/min and the speed of the stirrer 400-500 rpm. Usually no more than about $2\text{x}10^6$ cells were at a time incubated; use of more cells resulted in obstruction of the outflow filter. The outflowing medium was collected on ice and stored at -20^0C.

Corticosterone is the main product of isolated rat adrenal cells (Sayers et al., 1971; Kitabchi and Sharma, 1971; Schulster, 1973). Corticosterone was extracted from the samples with the procedure of Clark and Rubin (1969).

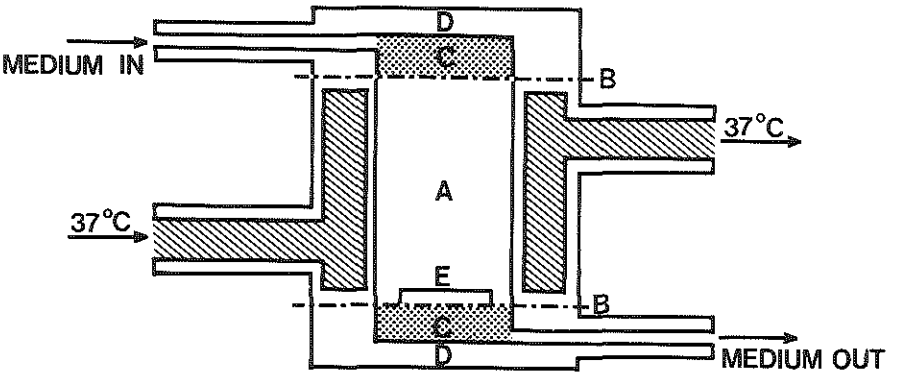


Fig. 6

Cross-section of the flow cell used for continuous flow incubations. The cell consists of a double-walled glass cylinder (A) with a capacity of 2 ml. The cylinder is closed at both ends by a Millipore filter (B) (HAWP 02500, pore size 0.45 µm), supported by disks of fritted glass (C) in the two glass end-pieces (D). Inside the cylinder is a small glass-coated magnetic stirrer bar (E). The temperature is kept at 37 ^{0}C by water flowing between the two walls of the cylinder.

After addition of 0.5 ml 1.0 M K_2CO_3 to 0.5 ml medium (plus cells), corticosterone was extracted with 5 ml dichloromethane (purified by acid washing, drying over solid Na_2SO_4 and destillation). From this extract 4 ml were mixed with 1.0 ml H_2SO_4: ethanol (3 : 1) and after 1 hr fluorescence was measured in the acid layer (excitation 468 nm, fluorescence 520 nm) with a Baird Atomic fluorimeter. Corticosterone concentrations were calculated from known amounts of corticosterone which were added to the buffer and extracted in the same assay. Table II shows the mean values and coefficients of variation after addition of known amounts of corticosterone to KRBAG. Fluorescence increased linearly with corticosterone concentrations up to 2 µg/assay. At higher concentrations the increase was smaller (Fig. 7). Table III shows the fluorescence of some sterols and steroids as percentages of the fluorescence

of corticosterone. Aminoglutethimide, cycloheximide and chloramphenicol did not give any fluorescence in this assay.

Whenever high concentrations of sterols were present in the incubate, the values were corrected to the fluorescence given by these sterols. The extent of the correction was based on the assumption that only a small fraction of the sterols present was converted into steroids. The validity of this assumption was supported by measurement of the steroid by means of gas chromatography. The method described by Kraaipoel et al. (1975a) was used with epicholesterol as an internal standard. Overall recovery of the sterols after a 2 hrs incubation with isolated rat adrenal cells was 85-90%.

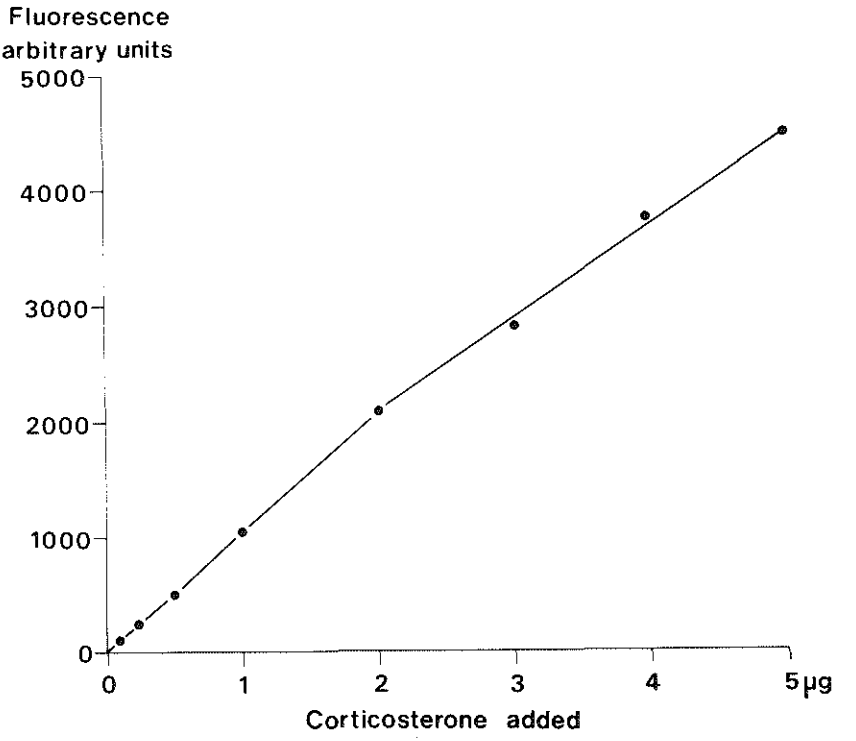


Fig. 7

Relation between amount of corticosterone added and fluorescence in the fluorimetric assay of corticosterone. Means of duplicates are plotted.

ACTH was estimated by radio-immuno-assay (Croughs et al., 1973), using Cortrophine® as a standard.

Table II

Mean fluorescence (arbitrary units) and coeffecients of variation (C.V.) obtained with known amounts of corticosterone.

Corticosterone added (ng)	number of estimations	fluorescence	standard deviation	C.V. (%)
0	6	6	1	17
200	6	126	6	5
400	6	232	19	8
1000	6	606	29	5

Table III

Fluorescence given by some sterols and steroids as percentage of the fluorescence of corticosterone.

cholesterol	0.20
20S-hydroxycholesterol	0.55
22R-hydroxycholesterol	0.55
25-hydroxycholesterol	0.55
pregnenolone	0.20
progesterone	0.10
11-deoxycorticosterone	0.50

4. Storage of isolated rat adrenal cells in liquid nitrogen

Isolated cells can be stored in liquid nitrogen after programmed freezing in the presence of cryoprotective agents, such as glycerol or dimethylsulfoxide (DMSO) (Shannon and Macy, 1973). Although it was not intended to use this method with isolated rat adrenal cells, a pilot experiment was

performed with these cells, as storage of cells could be of value in future experiments with human adrenals. If human adrenals are available they are seldom presented at a time of day which allows the immediate performance of an experiment. Furthermore, these adrenals are large enough to provide cells for more than one experiment, hence the surplus of cells from adrenals with e.g. a rare biosynthetic defect could be stored for later experiments.

Cells were frozen by two procedures in two commonly used concentrations of DMSO. After parts of a cell suspension were taken up in a medium containing either 4% or 10% DMSO, 1 ml portions of the suspension were sealed in a siliconized glass ampoule. One half of the ampoules was placed in the neck of a large liquid nitrogen container. The other half was frozen in a programmable freezing apparatus (Cryoson). In this apparatus the temperature was gradually lowered to the approximate temperature where ice formation starts. Then a rapid drop in temperature occurred to compensate for the heat generated by the crystallization process, followed by a gradual further decrease in temperature. The frozen capsules were stored overnight in liquid nitrogen. After rapid thawing at 37^{0}C and removal of DMSO by repeated washing and centrifugation (3x), the cells were counted to estimate the losses caused by this procedure. The response of the cells to ACTH (1 mU/ml) was compared with the response of freshly-isolated cells from the same suspension, part of which had undergone the whole procedure of addition and removal of DMSO, but without freezing. The results are summarized in Table IV. Although the response to ACTH was smaller than usually observed with rat adrenal cell suspensions some conclusions can be drawn from this experiment.

A. It is possible to store isolated adrenal cells for some time in liquid nitrogen with conservation of at least part of their response to ACTH.
B. Sufficient cells survive the procedure to enable further experimentation.
C. Treatment of cells with DMSO does not appear to have an

adverse influence on their steroidogenic response to ACTH.

D. Programmed freezing gives somewhat better results than direct freezing in a liquid nitrogen container.

E. The positive effect of adding DMSO immediately prior to freezing is counterbalanced by the far greater loss of cells.

Table IV

Effect of storage in liquid nitrogen on the response of adrenal cells to ACTH. Means of duplicates are given[1]

	Corticosterone ng/2hr/2x10^5 cells		
	control	ACTH 1.0 mU/ml	loss of cells %
fresh cells	60	340	-
cells treated with 4% DMSO	70	550	5
4% DMSO[2] container neck	35	45	25
10% DMSO[2] container neck	100	200	24
4% DMSO[2] programmed freezing	50	65	23
10% DMSO[2] programmed freezing	70	250	30
10% DMSO[3] programmed freezing	70	340	60

[1]Differences between duplicates were always less than 10%

[2]DMSO added approximately 1 hour before freezing

[3]DMSO added immediately before freezing

In its present form the technique is not perfect. Further evaluation may result in a smaller loss of cells with full preservation of the response to ACTH.

5. Conclusion

In Chapter II and III several aspects of the preparation and use of freshly-isolated cells with special reference to adrenal cells have been discussed. The isolation of cells from intact tissue has a long history, but suitable methods for the use of freshly-isolated cells have become available only recently. It is clear that incubation of freshly-isolated cells offers several major advantages over other in vitro techniques. Isolation of cells is usually performed by means of proteolytic action. Although both crude collagenase and trypsin are successfully used for the isolation of adrenal cells, there are arguments in favour of using the former.

CHAPTER IV

PROPERTIES OF ISOLATED RAT ADRENAL CELLS IN BATCH AND CONTINUOUS FLOW INCUBATIONS *

1. Introduction

This Chapter deals with the corticosterone production by isolated rat adrenal cells in two different incubation systems: batch and continuous flow incubations. In a continuous flow incubation system the secretion of products by the incubated tissue can be studied continuously without changes in the incubation volume and virtually without limitations to the number of samples that can be taken. Continuous flow incubations also offer other advantages over batch incubations. Accumulation of products in the incubation system, which may influence the metabolism in the incubated tissue by mass action or product inhibition (Birmingham and Kurlents, 1958; Péron et al., 1960; Clayman et al., 1970) is prevented. Re-entry of products in the tissue, which alters the ratios between the end products (Laplante and Stachenko, 1966), is also prevented. The concentration of every component in the medium can be changed at any time without altering the incubation volume. Free enzymes, entering the medium from the incubated tissue during the incubation procedure (Tsang and Carballeira, 1966), and differing in their metabolic properties from those shown by whole tissue, are removed and cannot metabolize the products secreted by the incubated tissue. There is no need for high initial substrate concentrations which might influence the metabolism (Ayres et al., 1960). If a steady state can be reached in the incubation system, the mathematics of Gurpide and Welch (1969) can be applied to study transport and enzyme reactions in the intact cell. Comparison of the properties of

* The results presented in this Chapter have been published: Acta Endocrinol. (Kbh) (1975) 78, 110., with the exception of Fig. 9.

adrenal cells in both, batch and continuous flow incubations makes it possible to select the system best suited for further experiments. For the methods used, see III.3.

2. Results

2.a Batch incubations

Fig. 8 shows the corticosterone production by our cell preparation as a function of increasing ACTH concentrations. A sigmoid log-dose response curve was found which is in agreement with the results of other investigators using collagenase for cell isolation (Kloppenborg et al., 1968;

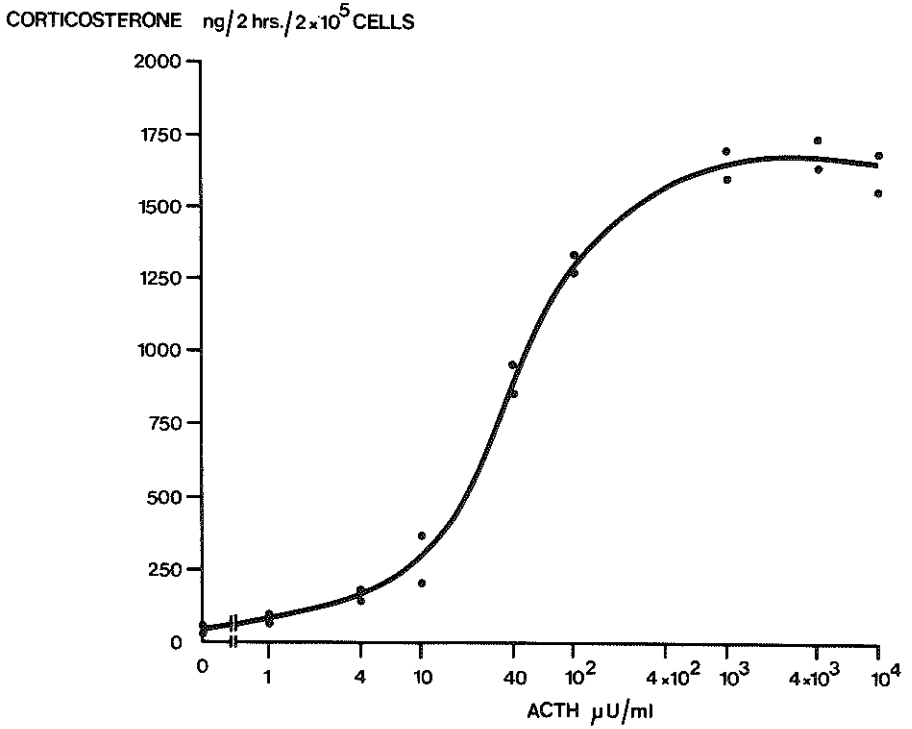


Fig. 8

Response to ACTH of isolated rat adrenal cells incubated in KRBAG. Duplicates are plotted.

Richardson and Schulster, 1972). A similar curve was obtained in KRHAG (Fig. 9). There was a linear relationship between the number of incubated cells and the amount of corticosterone produced (Fig. 10). This made it possible to correct all results for the number of incubated cells for comparison of results from different experiments. The relationship between corticosteroidogenesis and time in the presence of 1 mU ACTH/ml is shown in Fig. 11. There was a linear relationship up to 240 min. Pre-incubation of the cells during 3 h at 37°C in a KRBAG medium resulted in an increased corticosterone production at all ACTH concentrations tested (Fig. 12).

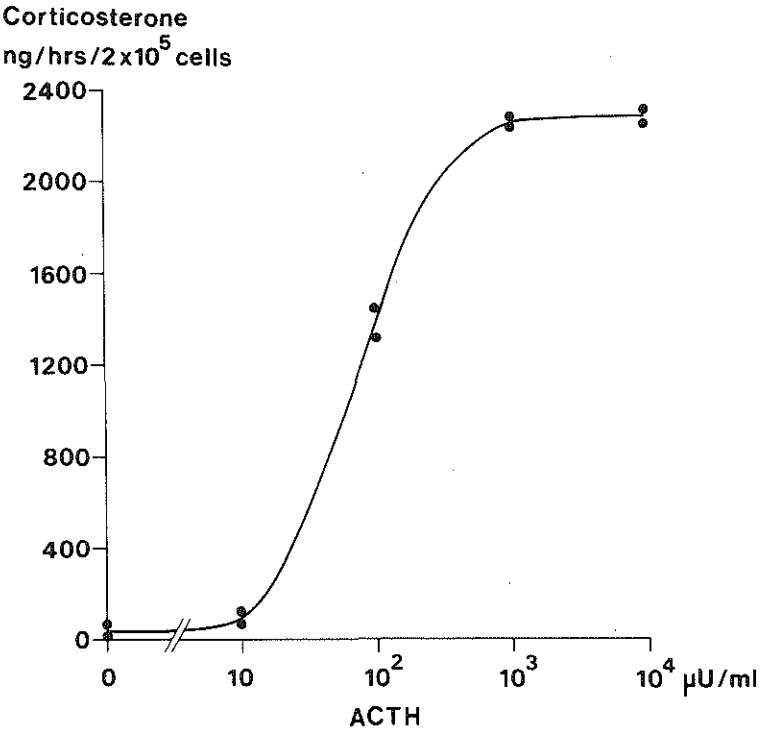


Fig. 9

Response to ACTH of isolated rat adrenal cells incubated in KRHAG. Duplicates are plotted.

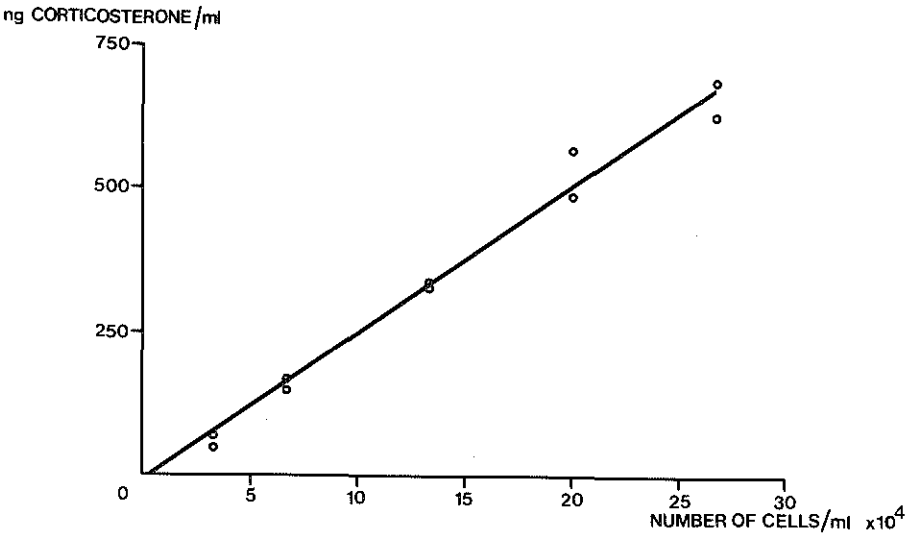


Fig. 10

Relation between the number of incubated cells and corticosterone production in the presence of 1 mU ACTH/ml. Total volume of each batch incubation was 1 ml. Incubation time was 2 hrs. Duplicates are plotted (KRBAG)

2.b Continuous flow incubations

Fig. 13 shows the results of two continuous flow incubations. Without ACTH the corticosterone production was very low ($\leq$ 4 ng/ml effluent). With 100 µU ACTH/ml medium the corticosterone production increased after a short lag, until after about 60-75 min. a maximum was reached, followed by a slow decrease. The response of the cells to ACTH was slightly altered during continuous flow incubations, as shown by Fig. 14. ACTH (100 µU/ml) was added to the medium after 0 and 90 min., respectively. In each experiment the increase in steroid production started after a short lag and after 60-90 min. maximum steroid production was reached. Compared with the first curve, the response to ACTH added after 90 min. was somewhat slower and maximum corticosterone production was slightly less.

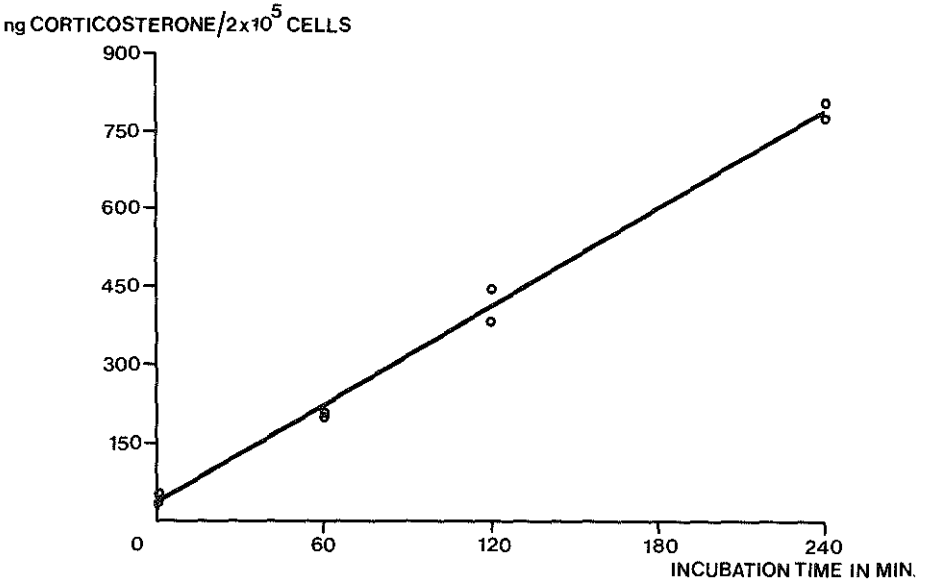


Fig. 11

Time course of corticosterone production in batch incubations in the presence of 1 mU ACTH/ml, added at time zero. Duplicates are plotted.

Cells still responded to ACTH after 3 hrs continuous flow incubation in KRBAG (Fig. 15), but corticosterone production at all ACTH concentrations tested was less than before continuous flow incubation. This decrease was most marked at the higher ACTH concentrations.

In all experiments the continuous flow incubation was started without ACTH in the flow cell. After the start there was an increase in ACTH in the flow cell until a constant concentration was reached.

In the experiment shown in Fig. 16, ACTH was estimated in the effluent from the flow cell. The increase of the corticosterone production started approximately 10 min. after the start of ACTH addition to the medium. The ACTH concentration reached its plateau about 10 min. before the maximum

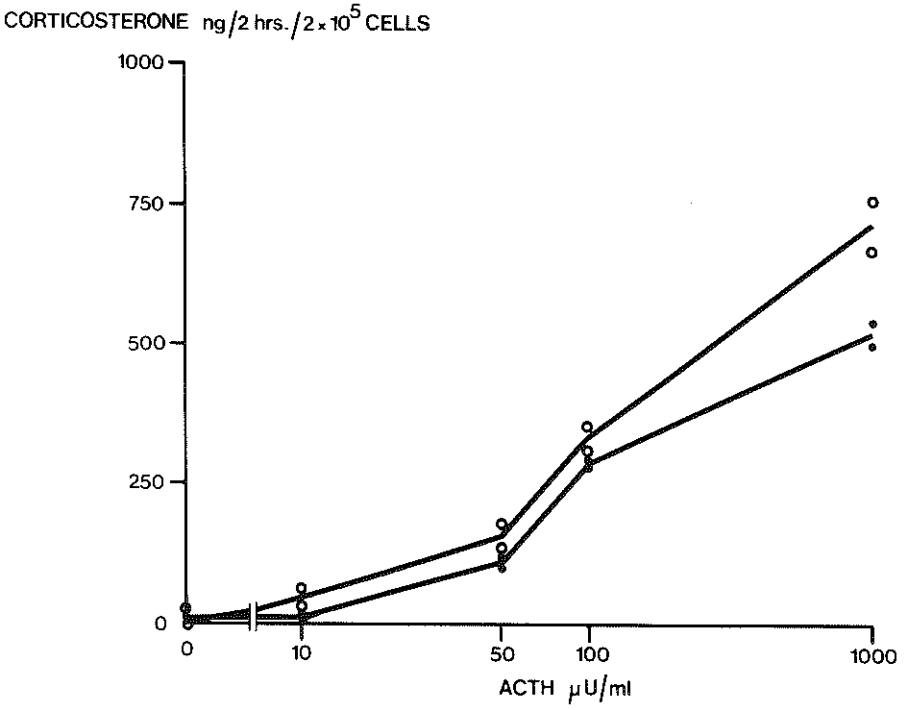


Fig. 12

Effect of 3 hrs pre-incubation on the response of isolated adrenal cells to ACTH in batch incubation. Pre-incubation was carried out in a Dubnoff incubator at 37°C in an O_2-CO_2 (95% - 5%)-atmosphere in KRBAG. Duplicates are plotted for the corticosterone production before (●——●) and after (O——O) pre-incubation.

corticosterone production.

3. Discussion

Isolated rat adrenal cells prepared by our method, when incubated with ACTH, show a sigmoid log-dose response curve similar to other isolated rat adrenal cell suspensions described in the literature (Kloppenborg et al., 1968; Swallow and Sayers, 1969; Richardson and Schulster, 1972).

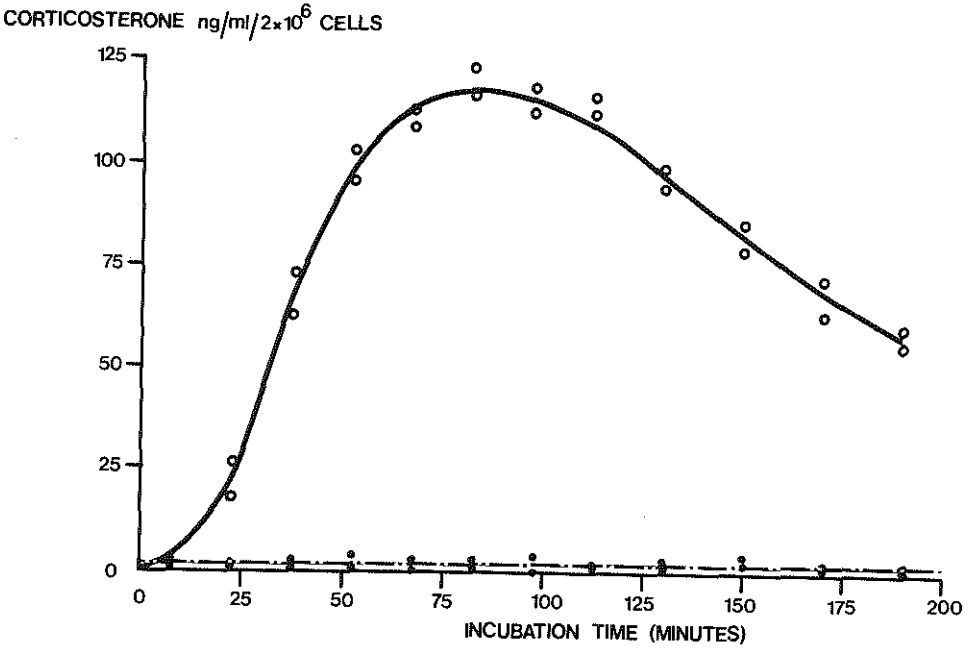


Fig. 13

Steroid production by isolated adrenal cells in continuous flow incubations without (●——●) and with 100 µU ACTH/ml (O——O). Addition of ACTH was started at t=0. The first 120 min. fractions were collected over periods of 15 min., after that over periods of 20 min. The flow rate was 0.19 ml/min. Values for duplicate estimations are plotted.

In the presence of 1 mU ACTH/ml a linear correlation exists between the number of incubated cells and the corticosterone production. Pre-incubation for 3 hrs slightly increased steroid production.

Incubation of our isolated rat adrenal cells in a permanent medium flow did not result in a continuous steroid production. ACTH stimulated the corticosterone production in this system. However, this production decreased after some time instead of being maintained at the maximum level reached. Steady state conditions were not reached either in other continuous flow systems for rats adrenal quarters (Saffran and Rowel, 1969;

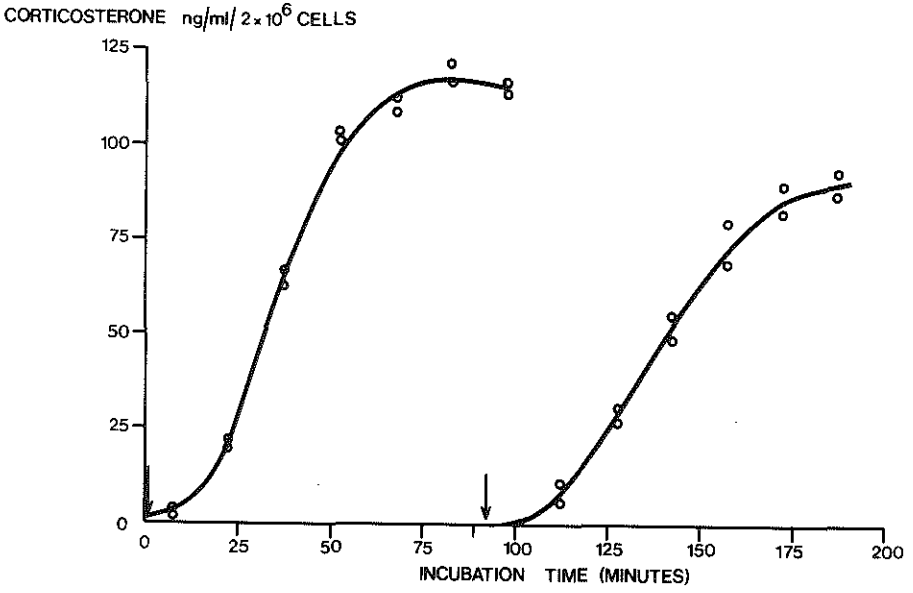


Fig. 14

Steroid production by isolated adrenal cells in continuous flow incubations with ACTH (100 µU/ml) added at t=0 min. and t=90 min. Arrows indicate start of ACTH addition to the medium. Values for duplicate estimations are plotted.

Schulster et al., 1970) or isolated rat adrenal cells (Schulster, 1973; Schulster and Jenner, 1975).

It is not clear why steroid production after prolonged incubation decreased. It is very likely that experimental imperfections rather than physiological properties of the cells are responsible for the failure to reach a steady state. Urquhart and Li (1968) perfused adrenals from acutely hypophysectomized dogs with whole blood. They found a continuous cortisol production in the presence of ACTH for at least 50 min. A continuous corticosterone production during 20 min. was observed by Pearlmutter et al. (1971) with superfused rat adrenal quarters.

Some time after we finished our continuous flow experiments,

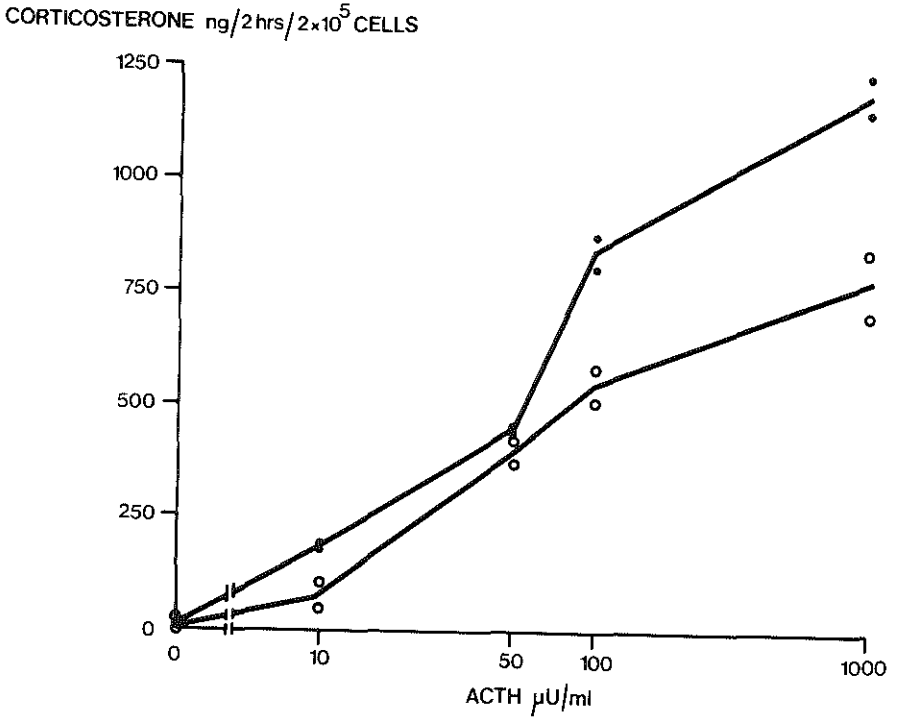


Fig. 15

Effect of 3 hrs continuous flow incubation of isolated adrenal cells in KRBAG (without ACTH) on the response of the cells to ACTH. One half of a cell suspension was used for direct batch incubations with different concentrations of ACTH (●——●) while the other half of the suspension was first incubated in the flow cell for 3 hrs. After this continuous flow incubation these cells too were incubated with ACTH (O——O).

Lowry et al. (1974) published a method in which adrenal cells are superfused while embedded in a Bio-Gel P-2 column. In this very elegant method ACTH produced a constant steroid production for more than 20 min. Similar methods have been applied by Mulder (1975) to adrenal and pituitary cells and by Cooke et al. (1975) to interstitial tissue and Leydig cells from rat testes. Neither of these authors reports constant steroid production.

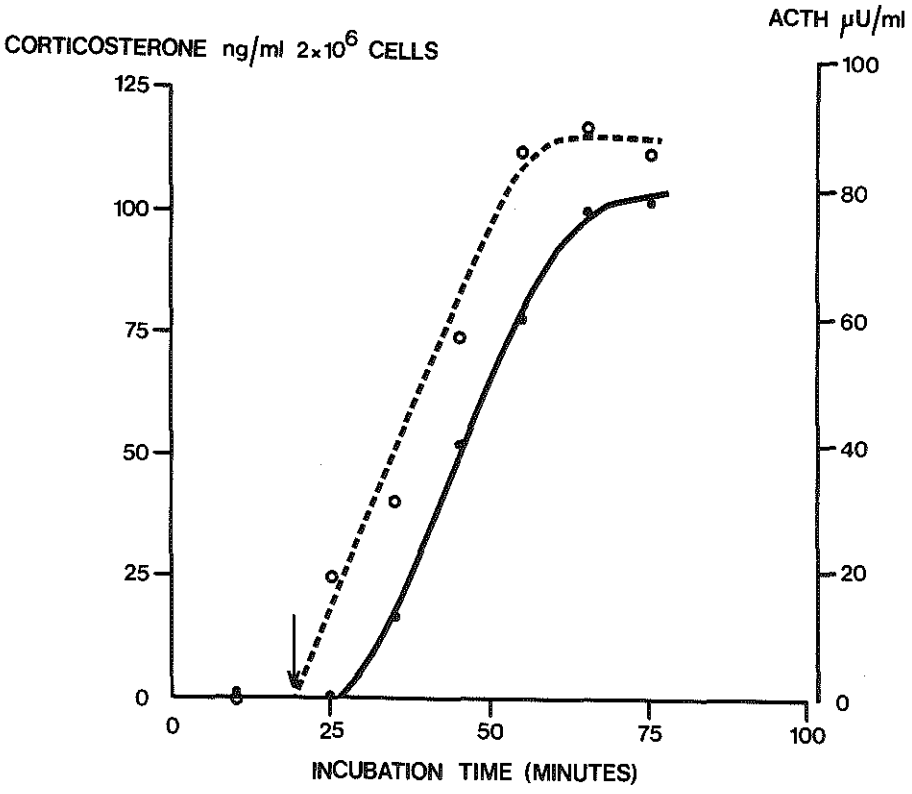


Fig. 16

Increase of ACTH concentration (O——O) and corticosterone production (O——O) during a continuous flow incubation of isolated adrenal cells. Arrow indicates the start of ACTH addition (100 µU/ml) to the incubation medium KRBAG after 20 min. The means of duplicate estimations are plotted.

Isolated rat adrenal cells can produce corticosterone continuously for at least 240 min. in a batch incubation in the presence of 1 mU ACTH (Fig. 11). Kitabchi and Sharma (1971) also found a continuous steroid production for 180 min. by a batch of rat adrenal cells in the presence of 50 µU ACTH/ml or 1 mM dibutyryl-cyclic AMP. Thus isolated rat adrenal cells are able to produce corticosterone continuously in the presence of ACTH.

Furthermore, in our experiments the corticosterone production per adrenal cell per 240 min. was of the same order of magnitude as the production per adrenal cell in a 200 min. continuous flow incubation. Ageing of the cells as a cause of the decreased production was excluded by the increased response of the cells to ACTH after 3 hrs pre-incubation in a batch (Fig. 12). This indicates that during the continuous flow incubation some factor necessary for the steroidogenic response to ACTH is lost, which is in agreement with the results shown in Fig. 14 and 15. The slower and lesser response to ACTH observed after 90 min. continuous flow incubation, while no significant loss of cells had occurred, indicates a decreased steroidogenic capacity of the cells. Fig. 15 shows that the steroidogenic response to ACTH decreases after 3 hrs continuous flow incubation. The A-50 (the ACTH-concentration giving maximal steroid production) (Schwyzer et al., 1971; Seelig and Sayers, 1973) is about 50 μU/ml, both before and after continuous flow incubation. This implies that the affinity of the cells to ACTH is not seriously affected. The decrease in corticosterone production is therefore probably caused by a slowdown of one or more processes occurring between the binding of ACTH to the cell membrane and the production of corticosterone.

There is a time lag between the start of the increase in ACTH concentration and the increase in steroid production (Fig.16). This can be explained by the fact that ACTH needs some time to reach the adrenal cells in the flow cell. Furthermore, it is known that adrenal cells do not immediately react to ACTH with increased steroid production (Richardson and Schulster, 1972). Finally, in this experiment the effluent was collected in 10 min. fractions.

Corticosterone production by isolated rat adrenal cells can be studied in batch or in continuous flow incubations. The latter system has some theoretical advantages. It is, however, rather laborious, which severely limits the number of experiments that can be performed. The potential of the

method is further reduced by the failure to establish a constant steroid production. For these reasons it was decided to use the batch incubation system in further experiments.

CHAPTER V

EFFECT OF 25-HYDROXYCHOLESTEROL AND OTHER STEROLS ON CORTICOSTERONE PRODUCTION BY ISOLATED RAT ADRENAL CELLS *

1. Introduction

The adrenal cholesterol side-chain cleaving system can convert a wide variety of sterols, including 20S-hydroxycholesterol, 22R-hydroxycholesterol and 25-hydroxycholesterol, into steroids (Burstein and Gut, 1971). Usually, the metabolism of these sterols has been studied in vitro, using isolated mitochondria or crude preparations of the cholesterol side-chain cleaving system (Burstein and Gut, 1971; Jefcoate et al., 1974) and little is known about the metabolism of these sterols in the intact adrenal cell. Hall and Young (1968) observed a conversion of {7α-^{3}H}20S-hydroxycholesterol into cortisol in bovine adrenal slices. Sharma (1973) found production of deoxycorticosterone and corticosterone from {7α-^{3}H}20S-hydroxycholesterol in isolated rat adrenal cells. In each case, steroid production from 20S-hydroxycholesterol occurred both in the absence and presence of ACTH.

This Chapter describes the corticosterone production by isolated rat adrenal cells incubated with 20S-hydroxycholesterol, 22R-hydroxycholesterol or 25-hydroxycholesterol and the effects of ACTH, aminoglutethimide-phosphate (AGI) and cycloheximide. Emphasis is put on 25-hydroxycholesterol, in view of the fact that side-chain cleavage of this sterol involves all steps required for cholesterol side-chain cleavage. In the existing models for cholesterol side-chain cleavage (Burstein and Gut, 1971; Kraaipoel et al., 1975a, b, c; Hochberg et al., 1976), metabolism of 20S-hydroxycholesterol or 22R-hydroxycholesterol requires only part of the side-chain cleaving system. For the methods used, see III.3.

* The results presented in this Chapter have been published: Mol. Cell. Endocrinol. (1975) 3, 375.

2. Results

In Fig. 17 the production of corticosterone by isolated rat adrenal cells under several conditions is presented. Without ACTH, addition of 5 µg/ml of 20S-hydroxycholesterol, 22R-hydroxycholesterol or 25-hydroxycholesterol caused a marked increase in corticosterone production (Fig. 17, left). 20S-hydroxycholesterol proved to be the best substrate, followed by 22R-hydroxycholesterol and 25-hydroxycholesterol, respectively. No increase was found after addition of cholesterol. AGI (70 µM) inhibited corticosterone production, both in the control incubation and in the presence of each of the sterols. Fig. 17 (right) also shows the effect of the sterols on corticosterone production in the presence of 1 mU ACTH/ml. Significant stimulation under these condition was found with 5 µg/ml of 20S-hydroxycholesterol and 22R-hydroxycholesterol. Very small effects were seen with cholesterol and 25-hydroxycholesterol. The effect of cholesterol is definitely not significant and was not found in other experiments, e.g. the one shown in Fig. 18. The effect of 25-hydroxycholesterol was not significant either. Nevertheless, a small effect of 5µg 25-hydroxycholesterol/ml was found in all our experiments, including the experiment in Fig. 18. Corticosterone production in the presence of ACTH and the sterols was inhibited by AGI (Fig. 17, right). This effect was most marked with 25-hydroxycholesterol.

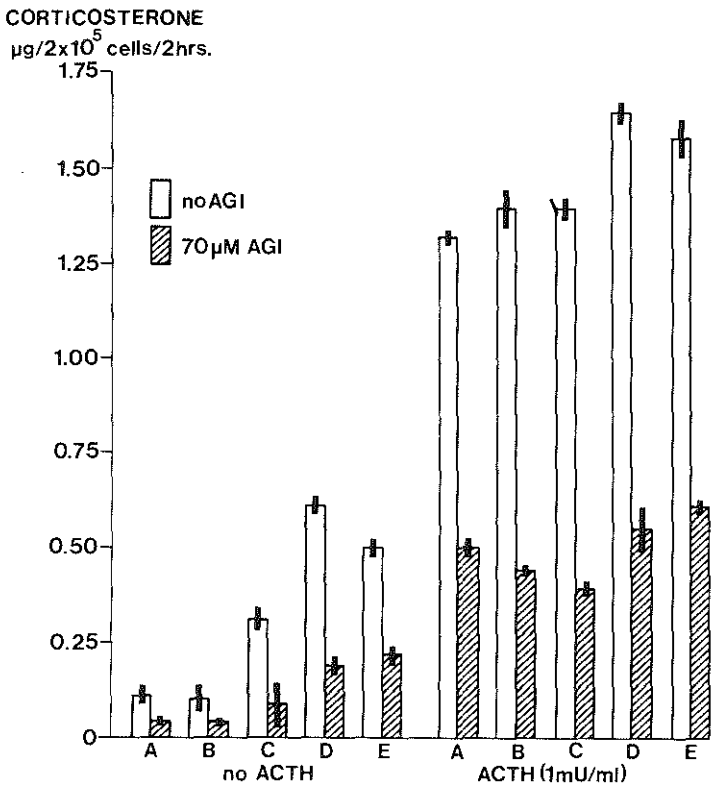


Fig. 17

Corticosterone production by isolated rat adrenal cells in the presence of cholesterol (B), 25-hydroxycholesterol (C), 20S-hydroxycholesterol (D) and 22R-hydroxycholesterol (E). Sterols were present at a concentration of 5 µg/ml. (A) is the control. Black bars indicate the differences between duplicate incubations.

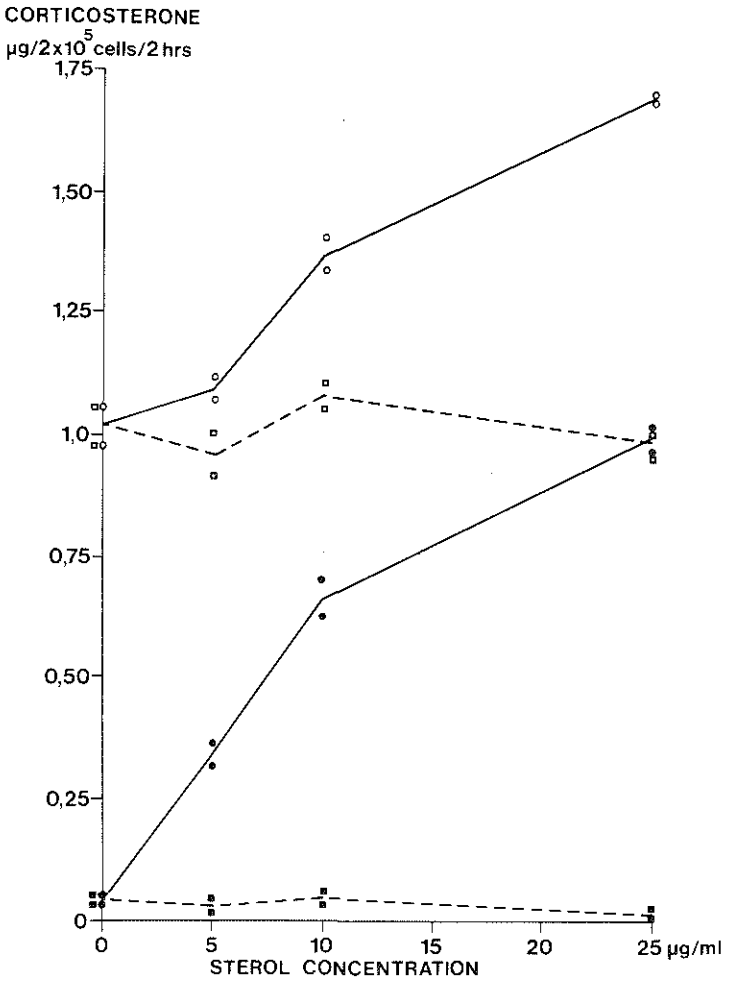


Fig. 18

Corticosterone production by isolated rat adrenal cells in the presence of 25-hydroxycholesterol (●——●), 25-hydroxy-cholesterol + 1 mU ACTH/ml (O——O), cholesterol (■——■) and cholesterol + 1 mU ACTH/ml (□——□). Duplicate incubations are plotted.

It should be mentioned that maximum stimulation of corticosterone production in this series of experiments was found with 10-100 mU ACTH/ml (see also Fig. 19). Therefore the experiment, shown in Fig. 17, was performed with a submaximal ACTH concentration.

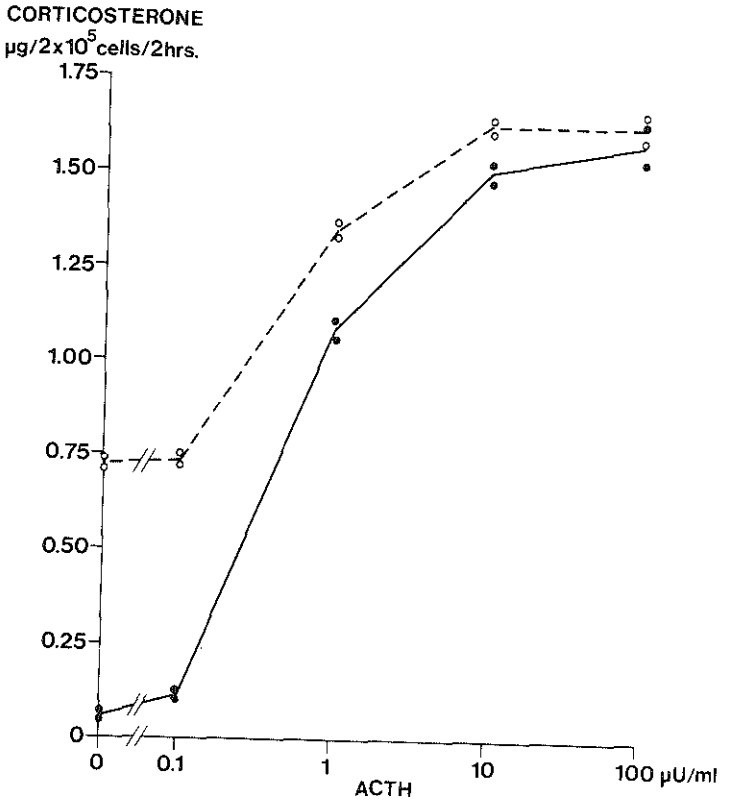


Fig. 19

Corticosterone production by isolated rat adrenal cells in the presence of increasing ACTH concentrations without 25-hydroxycholesterol (●——●) and with 25 µg 25-hydroxycholesterol/ml (O——O). Duplicate incubations are plotted.

The increase in steroid production caused by 25-hydroxycholesterol depends on the concentration of the sterol (Fig. 18). Higher concentrations of 25-hydroxycholesterol resulted in an increase in corticosterone production both in the absence and in the presence of ACTH (1 mU/ml). Cholesterol even at 25 µg/ml, had no effect on steroid production, whether ACTH was present or not.

Fig. 19 shows the effect of 25-hydroxycholesterol (25 µg/ml) in the presence of increasing ACTH concentrations.

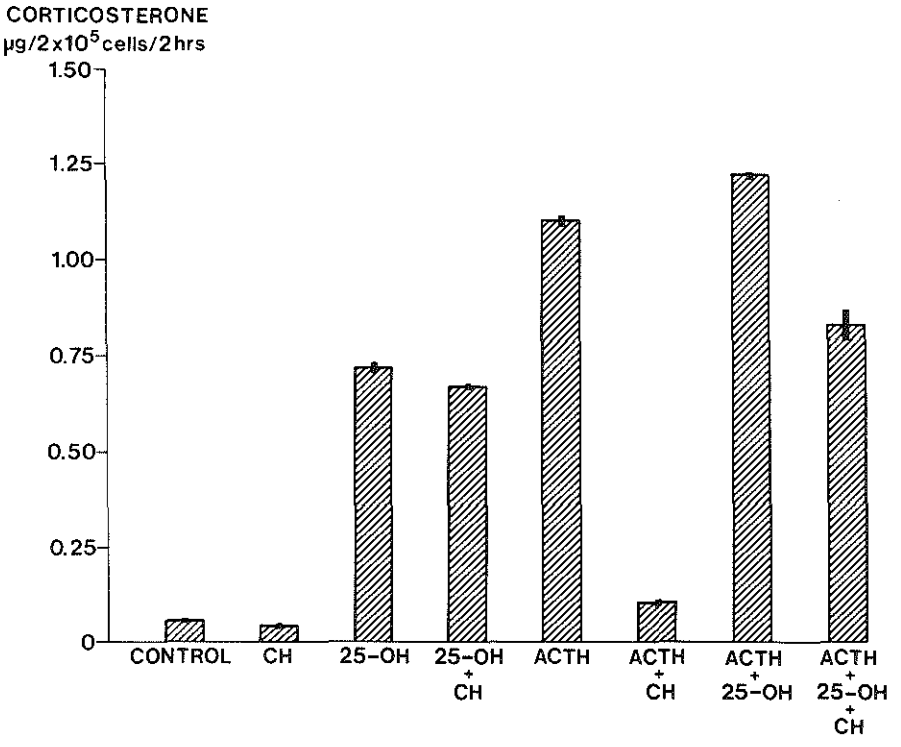


Fig. 20

Effect of 25 µM cycloheximide (CH), 1 mU ACTH/ml and 25 µg 25-hydroxycholesterol/ml (25-OH) on corticosterone production by isolated rat adrenal cells. Black bars indicate the differences between duplicate incubations.

The stimulation of corticosterone production is clearly seen without ACTH and also at sub-maximum ACTH concentrations, but disappears when the stimulation of steroid production by ACTH is highest.

The effect of cycloheximide (25 μM) on corticosterone production in the presence of ACTH 1 μM/ml and/or 25-hydroxycholesterol 25 μg/ml) is shown in Fig. 20. Cycloheximide inhibited approximately 95% of the effect of ACTH, but had no significant effect on the stimulation of corticosterone production by 25-hydroxycholesterol. In the presence of both ACTH and cycloheximide, 25-hydroxycholesterol caused the same stimulation of steroid production.

3. Discussion

Corticosterone production by isolated rat adrenal cells is stimulated by 20S-hydroxycholesterol, 22R-hydroxycholesterol and 25-hydroxycholesterol. In the presence of a sub-maximum ACTH concentration a similar stimulation was found. This indicates that these sterols are good substrates for the cholesterol side-chain cleaving system in the intact adrenal cell. Hall and Young (1968) and Sharma (1973), working with bovine adrenal slices and isolated rat adrenal cells, respectively, recovered ^{3}H-labeled C_{21}-steroids, starting with {7α-^{3}H}20S-hydroxycholesterol as a substrate. The synthesis of corticosterone from these sterols by isolated rat adrenal cells in the absence of ACTH is compatible with the current hypothesis on the mechanism of action of ACTH. According to this hypothesis, ACTH exerts its effect prior to the cholesterol side-chain cleaving system (Garren et al., 1971; Schulster, 1974). The reason for the absence of a stimulating effect of cholesterol, a phenomenon also reported by Sharma (1973), is not clear. Maybe, under our conditions exogenous cholesterol cannot reach the side-chain cleaving system fast enough to stimulate

steroid synthesis. Moreover Sharma (1973) found that cholesterol inhibited the effect of ACTH on corticosterone production. The absence of such an effect in our experiments may be due to the difference in the cholesterol concentration used. The highest cholesterol concentration in our experiment was 25 µg/ml (= 0.065 mM) while Sharma used 192 µg/ml (= 0.5 mM). Formation of monomolecular layers of cholesterol on the cell surface may have been responsible for the inhibition caused by the high cholesterol concentration used by Sharma.

In the classical scheme of cholesterol side-chain cleavage, 20S-hydroxycholesterol and 22R-hydroxycholesterol are considered to be intermediates. In the model proposed by Kraaipoel et al. (1975a, b, c) however, these two sterols are merely by-products. The calculations of Burstein and Gut (1971) also indicate that the larger part of the steroid production from cholesterol does not involve either sterol as an intermediate. In the models of Kraaipoel et al. (1975a, b, c), Burstein and Gut (1971) and Hochberg et al. (1976) the metabolism of these two sterols involves only part of the cholesterol side-chain cleaving system. For that reason they are of limited use only for the study of the cholesterol side-chain cleaving system. This is why most of our work was carried out with 25-hydroxycholesterol, a good substrate for the study of the cholesterol side-chain cleaving system.

The occurrence of 25-hydroxycholesterol in mammals has been repeatedly described. It is present among other sterols in human cord blood (Eberlein, 1965), human aorta (van Lier and Smith, 1967) and human brain (van Lier and Smith, 1969). Formation of 25-hydroxycholesterol from cholesterol by a 25-hydroxylase was found in rat liver (Björkhem and Gustafsson, 1974). A 25-hydroxylase for cholecalciferol is also present in mammalian liver (Norman and Henry, 1974). Conversion of 25-hydroxycholesterol to pregnenolone by the cholesterol side-chain cleaving system had already been found by Burstein and Gut (1971) in acetone powders of bovine adrenal mitochondria and by Degenhart et al. (1974) and

Jefcoate et al. (1974) in bovine and rat adrenal mitochondria.

Evidence exists that side-chain cleaving of cholesterol and 25-hydroxycholesterol takes place by the same enzyme system. Pregnenolone formation from 25-hydroxycholesterol is inhibited by AGI, an inhibitor of the first step of cholesterol side-chain cleavage (Degenhart et al., 1974; Kraaipoel et al., in prep.). This also indicates that all steps of cholesterol side-chain cleaving are involved in the metabolism of 25-hydroxycholesterol. The competition between cholesterol and 25-hydroxycholesterol (Jefcoate et al., 1974) is further evidence for conversion of both sterols by the same enzyme system. The inhibition of the metabolism of 25-hydroxy-cholesterol by AGI (Fig. 17) indicates that in the intact cell this sterol too, is converted by the cholesterol side-chain cleaving system. The absence of additive stimulation by 25-hydroxycholesterol at maximum ACTH concentrations points in the same direction. It seems that isolated rat adrenal cells at maximum ACTH concentrations the substrate flux to the side-chain cleaving system matches the full capacity of this system; as a result, no extra effect of 25-hydroxy-cholesterol can be seen. The results on which Jefcoate et al. (1974) base their assumption that the cytochrome P-450 for side-chain cleavage of cholesterol is present in three different forms may just reflect different binding properties of cholesterol and 25-hydroxycholesterol.

ACTH pre-treatment in vivo has no effect on the total cholesterol side-chain cleaving activity (Koritz and Kumar, 1970), nor on the K_m or V_{max} of the enzyme system (Bell and Harding, 1974) in rat adrenal mitochondria. ACTH thus appears to have no direct effect on the cholesterol side-chain cleaving system. However, Sharma (1973) found that ACTH stimulated the conversion of {7α-^{3}H}20S-hydroxycholesterol in isolated rat adrenal cells, while N^6,O^2-dibutyryladenosine 3',5'-monophosphate did not. This is in contrast with the results of Hall and Young (1968), who found that ACTH slightly inhibited the conversion of {7α-^{3}H}20S-hydroxy-cholesterol in bovine adrenal slices.

Neither Hall and Young (1968) nor Sharma (1973) measured specific activities of the compounds involved, which makes exact interpretation of their results difficult.

Our results are consistent with the absence of a direct effect of ACTH on the side-chain cleaving system. The metabolism of 25-hydroxycholesterol in the absence of ACTH indicates that the side-chain cleaving system is invariably present in an active form. At sub-maximum ACTH concentrations only part of the activity is needed for side-chain cleavage of endogenous cholesterol and an additive effect of 25-hydroxycholesterol can be observed. Only at maximum ACTH concentrations is the full capacity of the side-chain cleaving system used for endogenous cholesterol and 25-hydroxycholesterol cannot cause extra steroid production. Our results with cycloheximide indicate that ACTH has no direct effect on the side-chain cleaving system. Both in the absence and in the presence of ACTH, cycloheximide had no effect on the degree of stimulation caused by 25-hydroxycholesterol (Fig. 20). The failure of cycloheximide to influence the effect of 25-hydroxycholesterol is further proof of the localization of the effect of ACTH on events prior to the side-chain cleaving system.

CHAPTER VI

EFFECTS OF 25-HYDROXYCHOLESTEROL AND AMINOGLUTETHIMIDE ON CORTICOSTERONE PRODUCTION BY ISOLATED RAT ADRENAL CELLS. A MODEL FOR CONGENITAL LIPOID ADRENAL HYPERPLASIA? *

1. Introduction

Aminoglutethimide phosphate (AGI) is a well-known inhibitor of cholesterol side-chain cleavage (Gower, 1974), a process involving several steps. In the recent model of Kraaipoel et al. (1975a, b, c), the first step is the conversion of cholesterol into 5,20(22)-cholestadien-3β-ol. This is the main site of AGI action (Degenhart et al., 1974; Kraaipoel et al., in prep.). Inhibition of cholesterol side-chain cleavage by AGI is non-competitive (Bell and Harding, 1974).

The effect of AGI on the adrenal is comparable to congenital lipoid adrenal hyperplasia (CLAH) (Goldman, 1970), a rare and often fatal human inborn error due to a deficiency of one or more of the components of the cholesterol side-chain cleaving system (Prader and Siebenman, 1957; Degenhart et al., 1972; Kirkland et al., 1973). As can be concluded from the results in Chapter V, the analog of cholesterol, 25-hydroxycholesterol, is a good exogenous substrate for the cholesterol side-chain cleaving system in isolated rat adrenal cells. Both in isolated rat adrenal cells and in bovine adrenal mitochondria, conversion of 25-hydroxy-cholesterol into steroids is inhibited by AGI (Degenhart et al., 1974; this thesis, Chapter V). This Chapter describes the effect of AGI on the production of corticosterone by isolated rat adrenal cells incubated with ACTH and/or 25-hydroxycholesterol. The possibility exists that C-24 sterols

* The results presented in this Chapter have been published: Mol. Cell. Endocrinol. (1976) 4, 107.

can function as intermediates in the metabolism of 25-hydroxycholesterol (Degenhart et al., 1975). For that reason we also tested 5-cholene-3β,24-diol in our incubation system. For the methods used, see III.3.

2. Results

The conversion of 25-hydroxycholesterol (25 µg/ml) into corticosterone was completely inhibited by 70 µM AGI (Fig. 21, curve C). Complete inhibition of the effect of ACTH (1 mU/ml) required 280 µAGI (Fig. 21, curve A). 50% inhibition of the

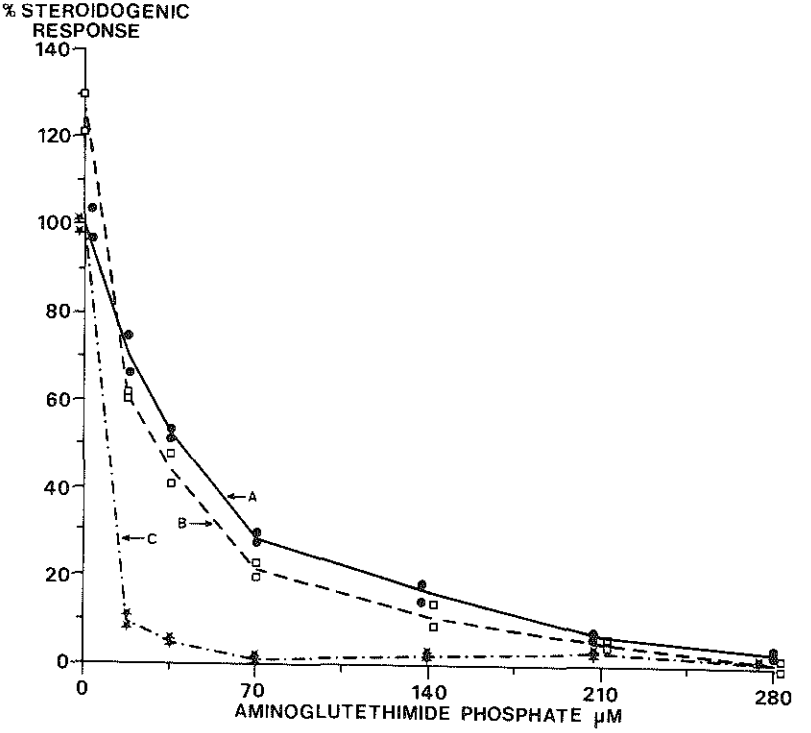


Fig. 21

Effect of AGI on corticosterone production by isolated rat adrenal cells. Cells were incubated with 1 mU ACTH/ml (curve A); 1 mU ACTH/ml + 5 µg 25-hydroxycholesterol/ml (curve B) and 25 µg 25-hydroxycholesterol/ml (curve C). Results are expressed as percentages of corticosterone production in the presence of 1 mU ACTH/ml without AGI (curves A and B) or 25 µg 25-hydroxycholesterol/ml without AGI (curve C). Duplicate incubations are plotted.

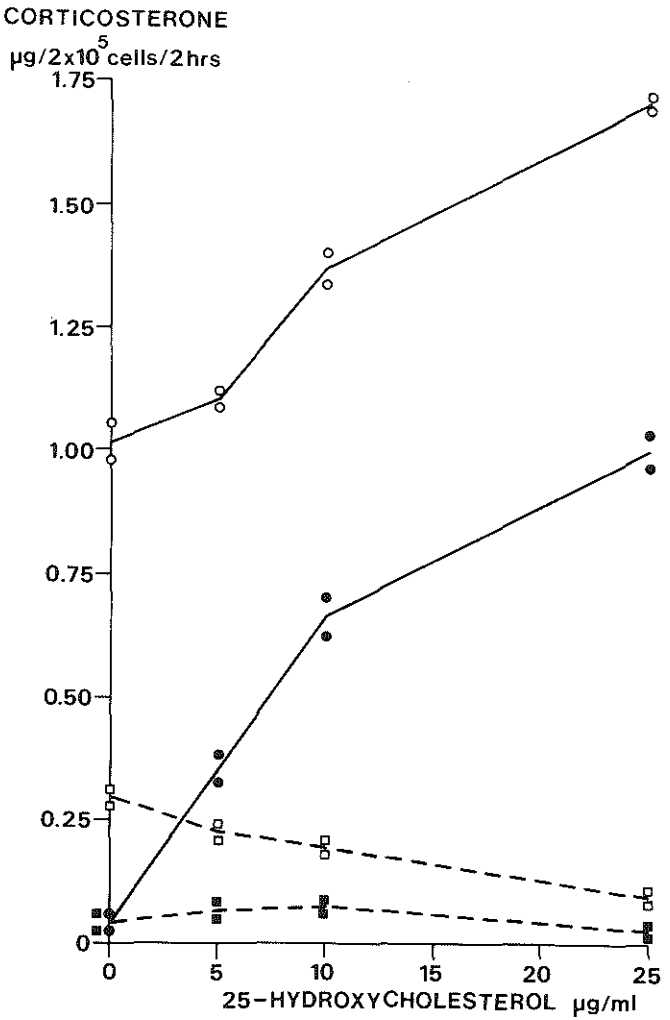


Fig. 22

Corticosterone production by isolated rat adrenal cells in the presence of 25-hydroxycholesterol (●——●), 25-hydroxycholesterol + ACTH (1 mU/ml) (O——O), 25-hydroxycholesterol + AGI (70 µM) (■---■) and 25-hydroxycholesterol + ACTH (1 mU/ml) + AGI (70 µM) (□---□). Duplicate incubations are plotted.

effects of 25-hydroxycholesterol and ACTH was obtained with approximately 10 and 40 μM AGI, respectively. Fig.21 also shows the effect of AGI in the presence of both ACTH (1 mU/ml) and 25-hydroxycholesterol (5 μg/ml) (curve B). In the presence of ACTH alone, 25-hydroxycholesterol induced a further increase in corticosterone production. With 5 μg 25-hydroxycholesterol/ml the increase varied from 10 to 25%, but was always present. At higher concentrations of sterol more pronounced differences were observed (Fig. 22). However, in the presence of both ACTH and AGI, addition of 25-hydroxycholesterol resulted in an additive decrease of corticosterone production. This effect of 25-hydroxycholesterol was observed at all AGI concentrations tested. The stimulation of corticosterone production by 25-hydroxycholesterol was dose-dependent, whether ACTH was present or not (Fig. 22). It should be mentioned that the ACTH concentration used (1 mU/ml) did not cause maximum stimulation of steroid production. Maximum stimulation in this series of experiments was usually found with 10-100 mU ACTH/ml. The extra stimulation of steroid production by 25-hydroxycholesterol did not occur with maximum stimulating ACTH concentrations (this thesis, Chapter V). AGI (70 μM) almost completely inhibited steroid production at all 25-hydroxycholesterol concentrations tested. The ACTH-stimulated corticosterone production from endogenous precursors was only partially inhibited by 70 μM AGI. Again, addition of 25-hydroxycholesterol resulted in a further inhibition of the corticosterone production. This inhibition increased at higher concentrations of the sterol.

Fig. 23 shows the effect of 5-cholene-3β,24-diol on the corticosterone production by isolated rat adrenal cells. This C-24 sterol had no effect on the basal steroid production but it partially inhibited the effect of ACTH.

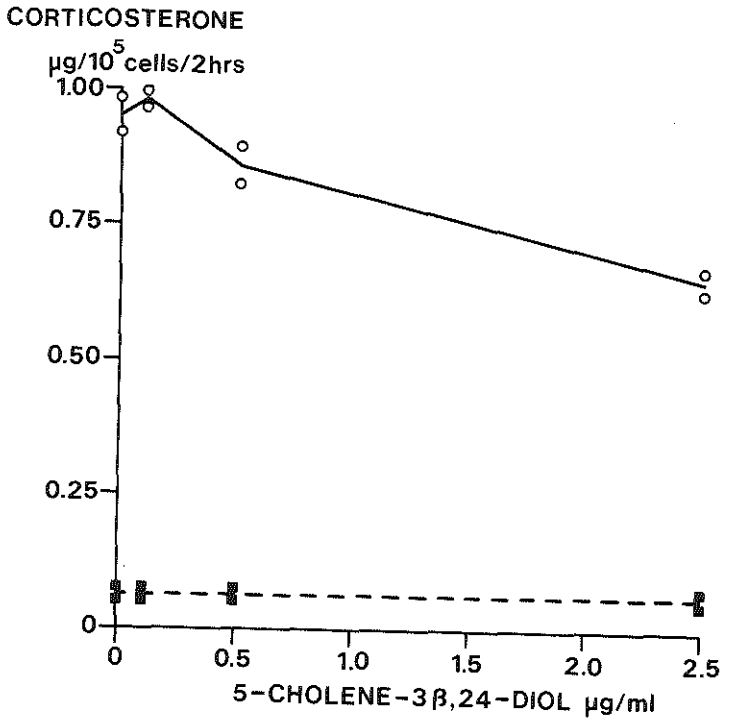


Fig. 23

Effect of 5-cholene-3β,24-diol on steroid production by isolated rat adrenal cells incubated without further additions (■——■) or with ACTH (1 mU/ml) (O——O). Duplicate incubations are plotted.

3. Discussion

Both ACTH and 25-hydroxycholesterol stimulate corticosterone production by isolated rat adrenal cells. In both cases corticosterone production is inhibited by AGI. Compared with its effect on the conversion of 25-hydroxy-cholesterol into corticosterone, AGI concentrations 4 times higher are required for a similar degree of inhibition of the ACTH-stimulated steroid production.

The effect of AGI on the ACTH-stimulated steroid production can be attributed to inhibition of the side-chain cleavage of

the endogenous substrate cholesterol. There is ample evidence that ACTH stimulates the steroid production by increasing the cholesterol supply to the cholesterol side-chain cleaving system (Garren et al., 1971; Brownie et al., 1973; Trzeciak and Boyd, 1973; Bell and Harding, 1974). AGI causes accumulation of free cholesterol in ACTH-stimulated rat adrenals (Dexter et al., 1967). For that reason it is improbable that inhibition of the ACTH-stimulated steroid production by AGI is due to inhibition of an ACTH-dependent process prior to cholesterol side-chain cleavage. On the other hand, as discussed in Chapter V ACTH appears to have no effect on the cholesterol side-chain cleaving system itself. In the presence of AGI, addition of 25-hydroxycholesterol causes additive inhibition of ACTH-stimulated corticosterone production. We offer two hypotheses to explain this effect, one based on competition between endogenous cholesterol and exogenous 25-hydroxycholesterol for side-chain cleavage, the other based upon production of inhibiting substances, probably from 25-hydroxycholesterol.

In bovine adrenal mitochondrial preparations, 25-hydroxycholesterol inhibits the side-chain cleavage of labeled cholesterol (Raggatt and Whitehouse, 1966; Simpson and Boyd, 1967). In rat adrenal mitochondria competition between cholesterol and 25-hydroxycholesterol was shown by Jefcoate et al. (1974). Our results indicate that the inhibition of side-chain cleavage by AGI is much greater for 25-hydroxycholesterol than for endogenous cholesterol. The dilution of the fraction of mitochondrial cholesterol available for side-chain cleavage, by 25-hydroxycholesterol, may explain the observed effect of 25-hydroxycholesterol in the presence of ACTH and AGI.

The following evidence of the existence of sterols with an adverse action on cellular metabolism is available:

1. With very few exceptions patients with CLAH die within a few months after birth (Prader and Siebenman, 1957; O'Doherty, 1964; Tsutsui et al., 1970; Kirkland et al.,

1973). This is surprizing, because patients with the more common forms of adrenal insufficiency and even patients with congenital adrenal absence can be treated relatively easily (Sperling et al., 1973; Pakravan et al., 1974; DiGeorge, 1975). One is tempted to conclude that in CLAH the defective adrenal produces one or more substances that are responsible for the severity of this disease. Such a production of toxic metabolites is not uncommon with inborn errors of metabolism (Bondy and Rosenberg, 1974). In CLAH these supposedly toxic metabolites ("adrenotoxins") could be produced from cholesterol which accumulates in the adrenal (Tsutsui et al., 1970). Perhaps similar processes can be induced by AGI. It should be noted, however, that no detailed studies on the sterol composition in CLAH adrenals are available.

2. 5-cholene-3β,24-diol (a C-24 sterol) inhibits the effect of ACTH on corticosterone production by isolated rat adrenal cells (Fig. 23).
3. 3β,20S-dihydroxy-5-cholenic acid (also a C-24 sterol) has strong anti-aldosterone properties (British patent, 1962).
4. Several sterols, including 25-hydroxycholesterol, inhibit growth of cultured cells (Chen et al., 1974).

Production of C-24 sterols from cholesterol is a common process in mammalian liver, an example is the production of bile acids (Danielsson and Sjovall, 1975). Small amounts of sterols with hydroxylated side-chains, which may be intermediates in the production of C-24 sterols, are normally present in human cord blood (Eberlein, 1965), human aorta and human brain (van Lier and Smith, 1967, 1969). Although this is less probable, one should consider the possibility that even in short-term experiments certain sterols inhibit one or more processes involved in corticosterone biosynthesis. Acute toxic effects of 25-hydroxycholesterol itself on our cells are unlikely, because this sterol is a good substrate for steroid production in the absence of AGI.

CHAPTER VII

DIFFERENT EFFECTS OF CYCLOHEXIMIDE AND CHLORAMPHENICOL ON CORTICOSTERONE PRODUCTION BY ISOLATED RAT ADRENAL CELLS*

1. Introduction

The available evidence for the involvement of protein synthesis in the mechanism whereby ACTH stimulates adrenal steroidogenesis comes mainly from experiments with inhibitors of protein synthesis (Ferguson, 1963, 1968; Farese, 1964; Garren et al., 1965; Schulster et al., 1970). Among the inhibitors of ACTH-stimulated steroid production in rat adrenals are cycloheximide and chloramphenicol (Farese, 1964; Garren et al., 1965; Ferguson, 1968; Schulster et al., 1970). In mammalian tissue, cycloheximide is a specific inhibitor of microsomal protein synthesis (Beattie et al., 1967) and short-term side effects that interfere with steroid production are unknown. Chloramphenicol is a specific inhibitor of mitochondrial protein synthesis in mammalian tissues (Kroon, 1965; Garren and Crocco, 1967). Chloramphenicol, however, has some side effects that may interfere with steroidogenesis. It inhibits mitochondrial oxidation of NADH and may also act as an uncoupler (Freeman and Haldar, 1967; Mackler and Haynes, 1970). Some direct effects of chloramphenicol on adrenal steroid production are also known (Marusic and Mulrow, 1972); Kortiz and Wiesner, 1975). We were interested in the effects of both antibiotics on steroid production independent from the mechanism of action of ACTH. As cholesterol cannot be used as an exogenous substrate for intact adrenal cells (Chapter V), we investigated the effects of cycloheximide and chloramphenicol on the conversion of 25-hydroxycholesterol into pregnenolone. This conversion involves the entire cholesterol side-chain

* The results presented in this Chapter have been published: Mol. Cell. Endocrinol. (1976) 4, 331.

cleaving system (Chapter V), which is considered as the rate limiting step in steroidogenesis. With the exception of some of our earlier experiments (Chapter V) this is the first report on the effects of both antibiotics on the conversion of 25-hydroxycholesterol in intact adrenal cells. For the methods used, see III.3.

2. Results

As shown in Fig. 24 cycloheximide inhibited the stimulating effect of 10 mU ACTH/ml on the corticosterone production by isolated rat adrenal cells. Inhibition was 50% with approximately 4.5 µM cycloheximide. Chloramphenicol also inhibited the effect of 10 mU ACTH/ml but only at much higher concentrations (Fig. 25). Approximately 0.65 mM chloramphenicol were required for 50% inhibition. Fig. 26 shows the effects of cycloheximide and chloramphenicol on the conversion by isolated rat adrenal cells of 25-hydroxy-cholesterol (25 µg/ml into corticosterone. While cycloheximide at concentrations that inhibited the effect of ACTH had no effect on the conversion of 25-hydroxycholesterol into corticosterone, 1.0 mM chloramphenicol caused approximately 50% inhibition of this process. The low solubility of chloramphenicol in KRHAG prevented the use of higher concentrations.

The different effects of cycloheximide and chloramphenicol were also seen in the presence of both ACTH and 25-hydroxy-cholesterol (Fig. 27 and 28). 25-Hydroxycholesterol (25 µg/ml) stimulated steroid production in the absence of ACTH and caused a small additive stimulation in the presence of 1 or 10 mU ACTH/ml. ACTH-stimulated corticosterone production was also inhibited by cycloheximide and chloramphenicol (25 µM and 1.0 mM, respectively). Inhibition was not complete, especially not at the higher ACTH concentration. The amount of stimulation caused by 25-hydroxycholesterol was not decreased in the presence of cycloheximide or cycloheximide + ACTH

(Fig. 27). On the contrary, chloramphenicol alone or in the presence of ACTH reduced the amount of stimulation caused by 25-hydroxycholesterol (Fig. 28). This effect was most marked at the higher ACTH concentration.

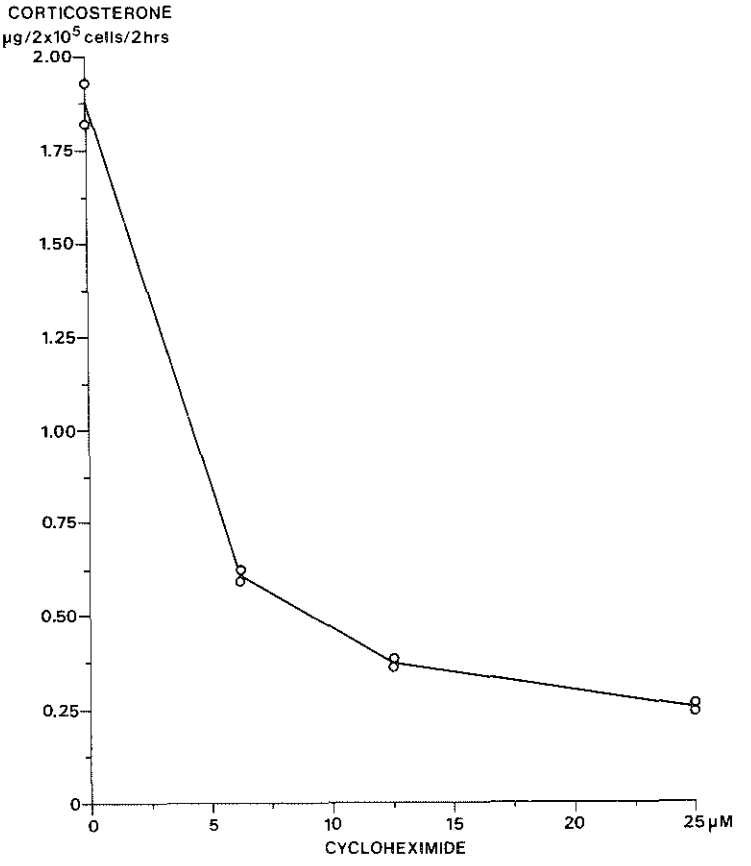


Fig. 24

Effect of cycloheximide on ACTH-stimulated corticosterone production by isolated rat adrenal cells. ACTH concentration 10 mU/ml. Duplicate incubations are plotted.

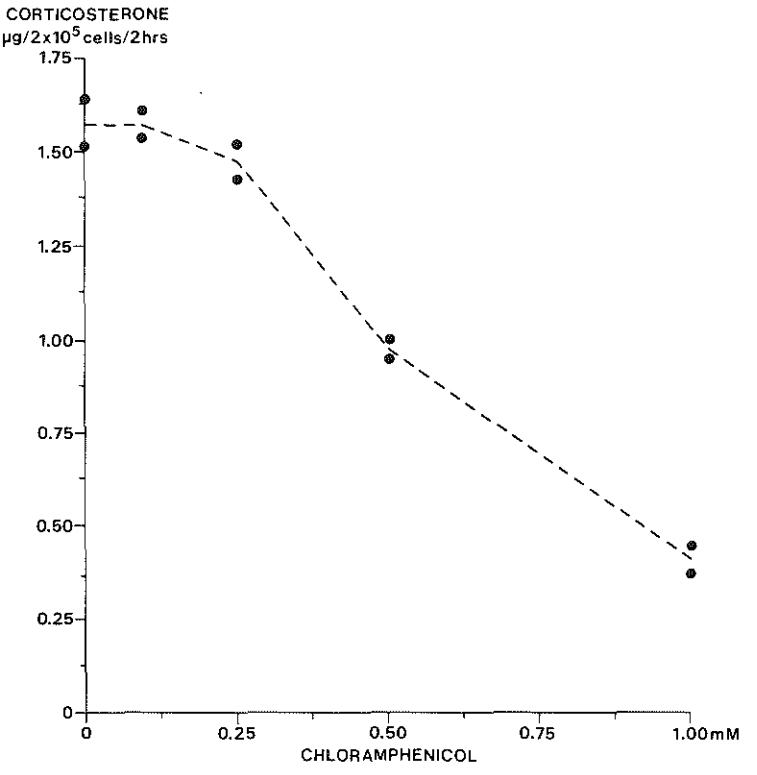


Fig. 25

Effect of chloramphenicol on ACTH-stimulated corticosterone production by isolated rat adrenal cells. ACTH concentration 10 mU/ml. Duplicate incubations are plotted.

3. Discussion

The stimulation of steroid production in isolated rat adrenal cells by ACTH was inhibited by cycloheximide and chloramphenicol in accordance with the results of other investigators (Farese, 1964; Garren et al., 1965; Ferguson, 1968; Schulster et al. 1970). Relatively high concentrations

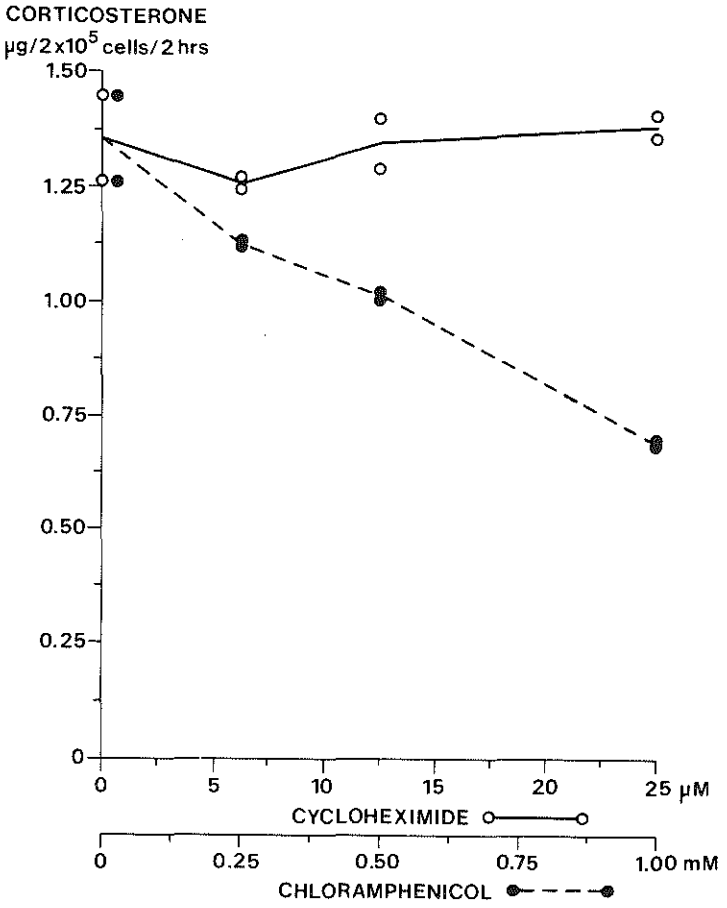


Fig. 26

Effect of cycloheximide and chloramphenicol on corticosterone production from 25-hydroxycholesterol by isolated rat adrenal cells. 25-Hydroxycholesterol concentration 25 µg/ml. Duplicate incubations are plotted.

of chloramphenicol were required. This was also found by Ferguson (1968) working with rat adrenal quarters.

Cycloheximide is a specific inhibitor of microsomal protein synthesis in mammalian tissue (Beattie et a., 1967). It has no effect on adrenal cholesterol synthesis from acetate or on the conversion of pregnenolone or progesterone into corticosterone by rat adrenals (Davis and Garren, 1968;

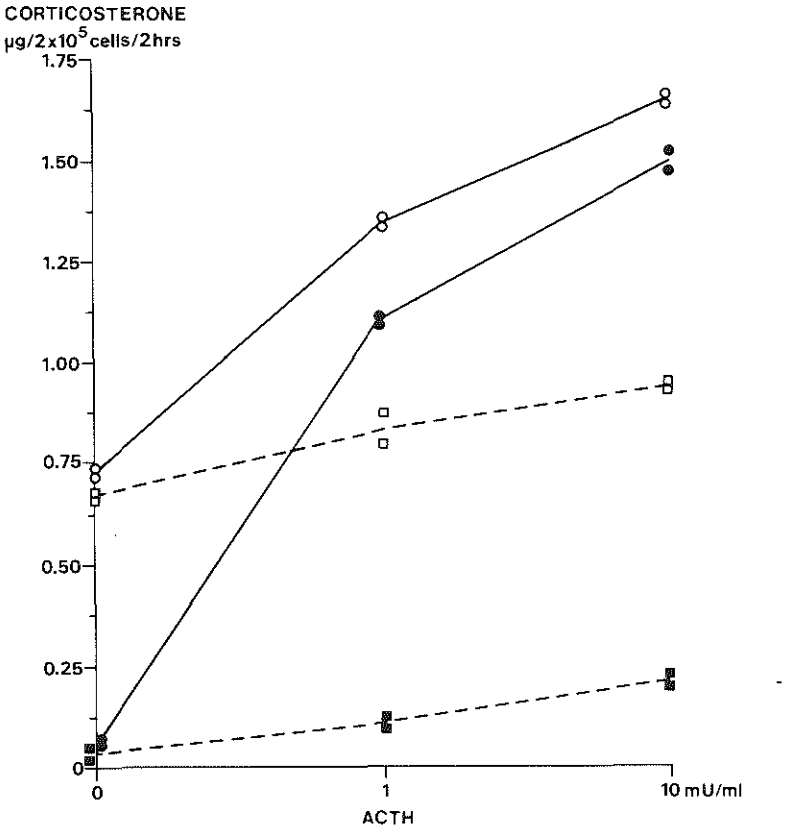


Fig. 27

Effect of cycloheximide on corticosterone production by isolated rat adrenal cells under several conditions. Cells were incubated with ACTH (●——●), ACTH + 25-hydroxy-cholesterol (25 µg/ml) (O——O), ACTH + cycloheximide (25 µM) (■----■) and ACTH + 25-hydroxycholesterol (25 µg/ml) + cycloheximide (25 µM) (□----□). Duplicate incubations are plotted.

Schulster et al., 1970). Possible effects of cycloheximide on the cholesterol side-chain cleaving system could not be investigated in the intact cell as exogenous cholesterol is not converted into steroids by intact adrenal cells (Chapter V). Measurement of pregnenolone production from endogenous cholesterol in the presence of cyanoketone cannot be used,

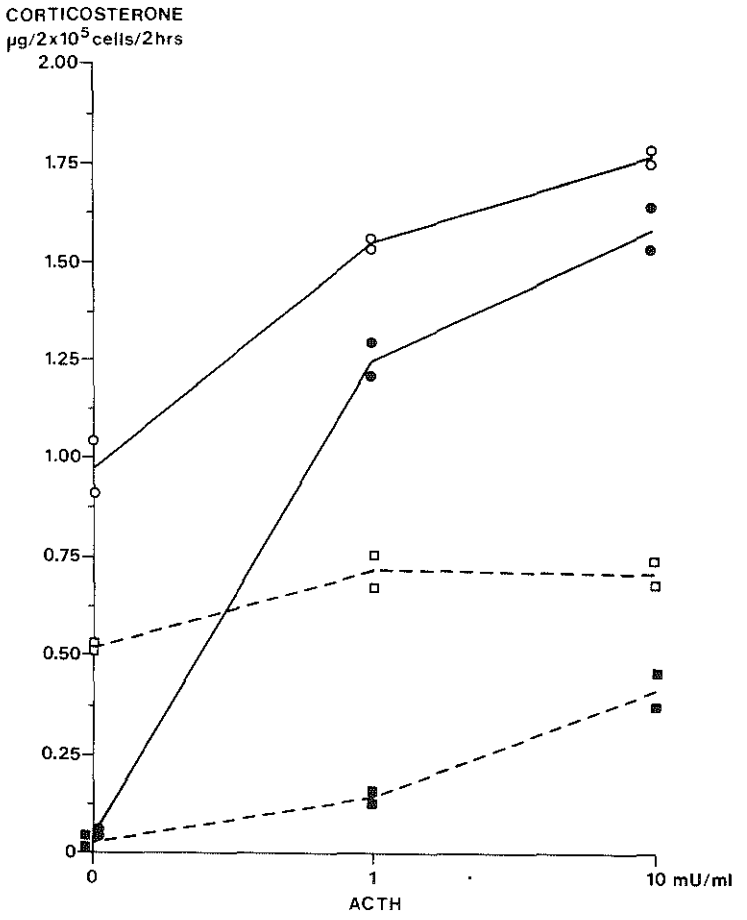


Fig. 28

Effect of chloramphenicol on corticosterone production by isolated rat adrenal cells under several conditions. Cells were incubated with ACTH (●——●), ACTH + 25-hydroxycholesterol (25 µg/ml) (O——O), ACTH + chloramphenicol (1.0 mM) (■----■) and ACTH + 25-hydroxycholesterol (25 µg/ml) + chloramphenicol (1.0 mM) (□----□). Duplicate incubations are plotted.

as endogenous cholesterol is stored mainly outside the mitochondria (Moses et al., 1969) and the ACTH-dependent labile protein is generally considered to play a role in the process which makes this cholesterol available to the side-

chain cleaving system (Garren et al., 1971). For that reason any effect of cycloheximide on the synthesis of this labile protein would obscure an effect of this inhibitor on the side-chain cleaving system itself. However, 25-hydroxycholesterol is a good exogenous substrate and its conversion into corticosterone requires the same steps as the conversion of endogenous cholesterol (Chapter V). Furthermore, the results of Jefcoate (1975) indicate that 25-hydroxycholesterol binds to the same site on the cholesterol side-chain cleaving system as the cholesterol transported to this system under the influence of ether stress in vivo. Jefcoate et al., (1974) studied the effect of in vivo pre-treatment of rats with cycloheximide on the conversion of 25-hydroxycholesterol in isolated adrenal mitochondria. This pre-treatment had no effect on the conversion of the sterol in pregnenolone. However, cycloheximide was not present in the mitochondrial incubations. In addition it should be kept in mind that mitochondria are a poor substitute for intact cells and that extramitochondrial effects of cycloheximide may have consequences for intra-mitochondrial processes. The results presented in this Chapter are an extension of our earlier results with cycloheximide (Chapter V), indicating that this antibiotic has no effect on the cholesterol side-chain cleaving system in the rat adrenal. Since no other side-effects of cycloheximide that might interfere with the mechanism of action of ACTH are known, its effect on ACTH-stimulated steroid production must be ascribed to inhibition of microsomal protein synthesis. Cycloheximide has no effect on the conversion of 25-hydroxycholesterol either in the absence or in the presence of ACTH. This is consistent with the conclusion of other investigators that ACTH has no direct effect on the cholesterol side-chain cleaving system (Koritz and Kumar, 1970; Bell and Harding, 1974). This conclusion was based on experiments with mitochondria of pre-treated rats.

Chloramphenicol is a specific inhibitor of mitochondrial protein synthesis in mammalian tissues including the adrenal

(Kroon, 1965; Garren and Crocco, 1967). This could imply that mitochondrial protein synthesis is also an essential part of the mechanism of action of ACTH. Our results and the results of other investigators do not support this theory. As shown in this Chapter chloramphenicol directly interferes with the conversion of 25-hydroxycholesterol into corticosterone. This effect of chloramphenicol is of the same order of magnitude as its effect on ACTH-stimulated steroid production. In bovine adrenal mitochondria chloramphenicol inhibited the conversion of deoxycorticosterone into corticosterone and aldosterone (Marusic and Mulrow, 1972). This inhibition was completely overcome by NADPH. The isomer of chloramphenicol, L-(+)-threo-chloramphenicol, which has no effect on protein synthesis by isolated rat liver mitochondria (Freeman, 1970b) but shares the effect of chloramphenicol on mitochondrial respiration (Freeman, 1970a), is almost as effective as chloramphenicol in inhibiting the effect of adenosine 3',5'-cyclic monophosphate on isolated rat adrenal cells (Kortiz and Wiesner, 1975). Chloramphenicol inhibits the NADH oxidation in rat liver mitochondria and may also act as an uncoupler (Freeman and Haldar, 1967; Mackler and Haynes, 1970). The results of Marusic and Mulrow (1972) indicate that chloramphenicol interferes with the NADPH production in adrenal mitochondria. The effect of chloramphenicol on mitochondrial energy production may explain both the diminished NADPH production and the inhibition of leucine incorporation in non-mitochondrial proteins. This inhibition of leucine incorporation was observed by Koritz and Wiesner (1975). For that reason the effect of chloramphenicol on ACTH-stimulated steroid production is at least partly due to inhibition of one or more of the processes involved in the conversion of 25-hydroxycholesterol into corticosterone by isolated rat adrenal cells, probably the NADPH production. Inhibition of ACTH-dependent microsomal protein synthesis may also play a role.

FINAL DISCUSSION

The aim of the studies described in this thesis was the selection and application of suitable in vitro methods for the investigation of steroid production by intact adrenal cells. Within this general framework our attention was mainly focussed on the first step in steroid biosynthesis, i.e. the side-chain cleavage of cholesterol, which is closely connected with the regulation of adrenal steroid production by ACTH. Cholesterol side-chain cleavage was considered as a single step, because it was not intended to study the nature of this complex and partly unknown process.

Incubation of freshly-isolated rat adrenal cells proved to be a very useful in vitro method. It offers some important advantages over more conventional incubation techniques. These advantages are best illustrated by the high sensitivity of isolated cells to ACTH reported by all laboratories applying this technique. The sensitivity of our cell suspensions to ACTH varied somewhat from batch to batch, but was always comparable with sensitivities reported in the literature. Another advantage, the absence of interanimal variation, is illustrated by the small differences in duplicate incubations in all our experiments.

Continuous flow incubation of adrenal cells offers some additional advantages over batch incubations of adrenal cells. This incubation system, however, in the way it was developed in this study, proved to be very laborious. For this reason, the batch incubation system was chosen as the experimental model in later studies.

The conversion of several sterols into corticosterone was studied, in order to find a suitable exogenous substrate. The natural substrate for adrenal steroidogenesis, cholesterol, proved to be a poor exogenous substrate. It is virtually not converted into corticosterone by isolated adrenal cells. Stimulation of corticosterone production was obtained with some cholesterol derivatives with hydroxyl groups in the side-

chain. From these sterols 25-hydroxycholesterol was chosen, as its conversion into corticosterone involves the entire cholesterol side-chain cleaving process. The other sterols tested had to pass through only some of the steps between cholesterol and pregnenolone. Conversion of 25-hydroxy-cholesterol was concentration-dependent and there is very good evidence that this compound is converted by the same enzyme system as that responsible for the side-chain cleavage of cholesterol.

The combination of isolated rat adrenal cells with 25-hydroxycholesterol as exogenous substrate proved to be very useful for the study of several aspects of adrenal steroid biosynthesis. This combination opened up the possibility to study the steroidogenic pathway, including all the steps of cholesterol side-chain cleavage, in the intact adrenal cell. Previously this could only be studied with the cholesterol precursor acetate or with intermediates in cholesterol synthesis, or via long-term pre-treatment with labeled cholesterol. Apart from the fact that under normal conditions only a minor part of the adrenal cholesterol is derived from de novo cholesterol synthesis, one is faced with effects of the experimental conditions on the synthesis and intra-cellular distribution of cholesterol. Hardly anything is known about the distribution of 25-hydroxycholesterol in the adrenal cell.

The conversion of 25-hydroxycholesterol in the absence of ACTH opened the possibility to discriminate between the effect of experimental conditions on the mechanism of action of ACTH and the effects on the steroidogenic pathways proper. This is very clearly demonstrated in the experiments where the effect of inhibitors of protein synthesis on the stimulation of steroid production by ACTH was studied. In these experiments, it could be shown that cycloheximide inhibited ACTH-stimulated steroid production only by its effect on the mechanism of action of ACTH, while the effect of chloramphenicol was at least partly due to other effects, resulting in inhibition of processes related to the

conversion of 25-hydroxycholesterol into corticosterone.

Very intriguing results were obtained when the congenital defect known as congenital lipoid adrenal hyperplasia was imitated in our system. although several explanations can be given for the phenomena that were observed, the results indicate that under these conditions (which are due to the inhibition of cholesterol side-chain cleavage) part of the large amount of available substrate can be converted into abnormal metabolites via alternative pathways. The existence of such abnormal metabolites is still highly speculative, but there is reason to believe that they are responsible for at least some of the problems encountered in the treatment of congenital lipoid adrenal hyperplasia.

SUMMARY

Adrenal steroid synthesis can be studied in vitro by a wide variety of techniques. The work described in this thesis was aimed at the study of the regulation of adrenal steroid production in normal and pathological conditions. Chapter I gives a general description of adrenal steroid synthesis with emphasis on the process of cholesterol side-chain cleavage and the mechanism of action of ACTH. This Chapter also includes a short summary of current knowledge about congenital lipoid adrenal hyperplasia.

Chapters II and III deal with isolated adrenal cells. After a short review of the historical aspects of the use of isolated cells, arguments are presented for our idea to use an incubation system for isolated rat adrenal cells. The methods used by several other groups for the isolation of adrenal cells are reviewed, and a detailed description is given of our own method. Adrenal cells are generally isolated with the aid of either trypsine or partly purified collagenase preparations. Although both enzyme preparations produce good adrenal cell suspensions, there are several arguments in favour of the use of collagenase. A HEPES-based buffer system is introduced as an alternative to the classical bicarbonate buffers. A preliminary test on the storage of isolated adrenal cells in liquid nitrogen is discussed.

Chapter IV describes several properties of isolated rat adrenal cell suspensions in both batch and continuous flow incubations. Isolated rat adrenal cells showed a sigmoid log-dose response to ACTH. The response to ACTH was slightly increased by pre-incubation during 3 hrs. In the presence of 1 mU ACTH/ml a linear correlation existed between the amount of corticosterone produced and the number of cells incubated. At this ACTH concentration corticosterone production was constant for at least 4 hours. In continuous flow incubations steroid production is stimulated by ACTH. Maximum production was obtained after approximately 1 hour, followed by a slow

decrease. During continuous flow incubations the response to ACTH decreased. The continuous flow method was very laborious, which was the main reason to discontinue the use of this technique.

The effect of several sterols on corticosterone production by isolated rat adrenal cells is described in Chapter V. Cholesterol had no effect on corticosterone production whether ACTH was present or not. Hydroxylated sterols stimulate steroid production, both in the presence and in the absence of ACTH. From these hydroxylated sterols 25-hydroxycholesterol was chosen for further experiments. Evidence for its conversion into corticosterone via the cholesterol side-chain cleaving system is presented.

Chapter VI deals with the effect of aminoglutethimide phosphate on the corticosterone production by isolated rat adrenal cells. Aminoglutethimide is an inhibitor of cholesterol side-chain cleavage and its effects resemble the situation found in congenital lipoid adrenal hyperplasia. AGI inhibited the effects of both 25-hydroxycholesterol and ACTH on steroid production by isolated adrenal cells. In the presence of ACTH and AGI, 25-hydroxycholesterol produced an additional inhibition. This effect may either be caused by substrate competition at the site of the side-chain cleaving system or by production of a yet unknown inhibiting substance. This inhibiting substance could be a C-24 sterol such as 5-cholene-3β,24-diol which partly inhibited the effect of ACTH.

In Chapter VII the effects of cycloheximide and chloramphenicol on the stimulation of steroid production by ACTH and the conversion of 25-hydroxycholesterol are presented. Cycloheximide is an inhibitor of microsomal protein synthesis and is also known as an inhibitor of ACTH stimulated steroid synthesis. Concentrations of cycloheximide which almost completely inhibited the effects of ACTH on corticosterone production, have no effect on the conversion of 25-hydroxycholesterol. This indicates that the effect of

cycloheximide on steroid production is related to the mechanism of action of ACTH, without an additional effect on any rate-limiting step in the steroidogenic pathways. Chloramphenicol, an inhibitor of mitochondrial protein synthesis but with several other effects, reduced the effects of both ACTH and 25-hydroxycholesterol on corticosterone production. Therefore the effect of this inhibitor is at least partly due to effects on steroidogenic pathways and results obtained with chloramphenicol cannot be used as arguments for the involvement of mitochondrial protein synthesis in the mechanism of action of ACTH.

SAMENVATTING

De steroid synthese in de bijnier kan met verschillende technieken in vitro worden bestudeerd. Het in dit proefschrift beschreven werk was gericht op het bestuderen van de regulatie van de steroid productie door de bijnier in normale en pathologische omstandigheden.

Hoofdstuk I bevat een algemene beschrijving van de steroid synthese in de bijnier met enige nadruk op de cholesterol-splitsing en het werkingsmechanisme van ACTH. Dit hoofdstuk geeft tevens een beknopt overzicht van de bestaande kennis omtrent de congenitale lipoid hyperplasie van de bijnier.

Hoofdstuk II en III handelen over geïsoleerde bijnier-cellen. Na een korte samenvatting van de geschiedenis van het gebruik van geïsoleerde cellen worden de argumenten gepresenteerd die ons geleid hebben tot het gebruik van geïsoleerde bijniercellen. De bestaande methoden voor het isoleren van bijniercellen worden besproken, en een gedetailleerde beschrijving van onze eigen methode wordt gegeven. Bijniercellen worden doorgaans geïsoleerd met behulp van trypsine of gedeeltelijk gezuiverde collagenase preparaten. Beide enzym-preparaten leveren goede bijniercel suspensies, maar er zijn argumenten die een voorkeur voor collagenase rechtvaardigen. Als alternatief voor de klassieke bicarbonaat buffers wordt een op HEPES gebaseerde buffer geïntroduceerd. Voorts worden enige voorlopige resultaten omtrent het bewaren van geïsoleerde bijniercellen in vloeibare stikstof gepresenteerd.

In hoofdstuk IV worden een aantal eigenschappen beschreven van geïsoleerde rattebijniercel suspensies in "batch" en "continuous flow" incubaties. Geïsoleerde rattebijniercellen reageerden op ACTH met een sigmoidale log-dosis respons. Pre-incubatie gedurende 3 uur versterkte de reactie op ACTH enigszins. In aanwezigheid van 1 mU ACTH/ml bestond er een lineaire correlatie tussen de corticosteron productie en het aantal geïncubeerde cellen. Bij deze ACTH concentratie vond gedurende 4 uur een constante corticosteron productie

plaats. Tijdens "continuous flow" incubaties stimuleerde ACTH de steroid productie eveneens. Na ongeveer een uur was de productie maximaal, waarna een langzame daling volgde. Daar de "continuous flow" incubatie vrij bewerkelijk bleek te zijn, werd deze methode niet gebruikt bij latere experimenten.

Het effect van enkele sterolen op de corticosteron productie door onze bijniercellen wordt in hoofdstuk V beschreven. Cholesterol had zowel in aan- als in afwezigheid van ACTH geen effect op de corticosteron productie. Gehydroxyleerde sterolen stimuleerden in beide gevallen de steroid productie. Van deze sterolen werd 25-hydroxycholesterol gekozen voor latere experimenten. De verkregen gegevens wijzen op een omzetting van dit sterol in corticosteron via het cholesterol splitsend systeem.

Hoofdstuk VI behandelt het effect van aminoglutethimidefosfaat op de corticosteron productie door rattebijniercellen. AGI remt de cholesterol splitsing en schept zo een situatie die overeenkomt met die bij congenitale lipoïd hyperplasie van de bijnier. AGI remde zowel de effecten van ACTH als die van 25-hydroxycholesterol op de steroid productie door geïsoleerde bijniercellen. In aanwezigheid van zowel ACTH als AGI veroorzaakte 25-hydroxycholesterol een extra remming. Dit effect zou kunnen worden veroorzaakt door substraat competitie op het cholesterol splitsend systeem of door productie van een tot nu toe onbekende remmende stof. Dit zou een C-24 sterol kunnen zijn zoals 5-choleen-3β, 24-diol, dat het effect van ACTH gedeeltelijk remde. De effecten van cycloheximide en chloramphenicol op de stimulatie van de steroid productie door ACTH en de omzetting van 25-hydroxycholesterol worden in hoofdstuk VII gepresenteerd. Cycloheximide, een remmer van de microsomale eiwitsynthese, remde de stimulatie van de steroid productie door ACTH. In een concentratie waarbij het ACTH-effect nagenoeg was verdwenen, had deze remmer geen effect op de omzetting van 25-hydroxycholesterol. Dit wijst erop dat het effect van cycloheximide in verband staat met het werkingsmechanisme van ACTH, zonder dat het enig effect

heeft op andere snelheidsbeperkende stappen in de synthese van corticosteron. Chloramphenicol, een remmer van de mitochondriële eiwitsynthese, maar met diverse bekende bijwerkingen, remt zowel de stimulatie van de steroid productie door ACTH als de omzetting van 25-hydroxycholesterol. De remmende werking van deze stof is derhalve voor een deel te wijten aan beïnvloeding van de biosynthese van de steroiden. Resultaten verkregen met chloramphenicol kunnen dan ook niet worden gebruikt als argument voor een rol van de mitochondriële eiwitsynthese in het werkingsmechanisme van ACTH.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd in 1946 te Groningen geboren. In 1963 behaalde hij het getuigschrift H.B.S.-B aan het Sint-Maartenscollege te Haren (Gr). Datzelfde jaar werd een begin gemaakt met de scheikundestudie aan de Rijksuniversiteit te Groningen. Het doctoraal examen scheikunde, met als hoofdvak biochemie en als bijvak experimentele endocrinologie, werd in 1969 afgelegd. Vervolgens diende hij enige tijd het landsbelang in het laboratorium van het Militair Hospitaal "Dr. A. Mathijsen" te Utrecht. Van 1970 tot eind 1974 was hij als wetenschappelijk medewerker verbonden aan het Academisch Ziekenhuis Rotterdam/Sophia Kinderziekenhuis en Zuigelingenkliniek, hiertoe in staat gesteld door een subsidie van de Stichting Wetenschappelijk Onderzoek Sophia Kinderziekenhuis. Momenteel is hij in een vergelijkbare, door TNO gesubsidieerde functie verbonden aan de afdeling Interne Geneeskunde van het Zuiderziekenhuis te Rotterdam.

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