STEROID BIOSYNTHESIS

AND

THE BRAIN-TESTIS AXIS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE

AAN DE ERASMUS UNIVERSITEIT TE ROTTERDAM,

OP GEZAG VAN DE RECTOR MAGNIFICUS

PROF.DR.C.J.VAN DER WEIJDEN

EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.

DE OPENBARE VERDEDIGING ZAL PLAATS VINDEN OP

WOENSDAG 27 JUNI 1973,

DES NAMIDDAGS TE 4.45 UUR

DOOR

FOCKO FREDERIK GEERT ROMMERTS

GEBOREN TE EDE

1973

bronder—offset b.v.—rotterdam

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Dr. J.K. Grant

Ter nagedachtenis aan
Neeltje van Slooten

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VOORWOORD

Het vermelden van één auteur op de omslag van dit proefschrift betekent niet dat het proefschrift een produkt van een éénling is. Het tegendeel is waar; velen hebben bijgedragen tot de totstandkoming van dit proefschrift.

Allereerst bedank ik mijn ouders voor de mogelijkheden die zij mij hebben geboden tijdens mijn studie.

Prof.Dr. H.J. van der Molen, beste Henk, veel dank ben ik je verschuldigd voor de goede begeleiding bij het in dit proefschrift beschreven werk. Ik heb nog meer geleerd van je inzet en inzichten in vele zaken die met onderzoek en onderwijs te maken hebben, zoals brandpreventie, volleybal, demokratie, organisatie en fakulteitsproblematiek.

Prof.Dr. W.C. Hülsmann, beste Wim, hartelijk dank voor het kritisch doorlezen van het manuskript. Ik heb veel genoten van je biochemische kennis en het vermogen om deze kennis zeer praktisch te gebruiken.

Dr. J.K. Grant, I appreciate very much your critical reading of the manuscript. This is beneficial for the cooperation between Great Britain and The Netherlands in the Common Market.

Dr. B.A. Cooke, beste Brian, bedankt voor de prettige en vruchtbare samenwerking en de vele leerzame diskussies (eerst in het Engels en daarna in het Nederlands). In het ons zo vertrouwde BALLS CAMP hebben we samen heel wat genoeglijke uurtjes doorgebracht onder het genot van het dissekteren van testisweefsel.

Mej. J.W.C.M. van der Kemp, beste Annemiete, het aantal voorletters voor jouw naam geeft het aantal jaren aan dat we hebben samengewerkt. In deze periode heb jij het leeuwendeel van onze experimenten voor je rekening genomen. Ik ben je zeer dankbaar voor deze volharding en voor

de prettige samenwerking. Erkentelijk ben ik ook voor de bijdragen van Hiske Rockx, Els Cassa en Wietske Schiphorst.

Beste Marja Decae, jij hebt er voor gezorgd dat wat ik onder woorden heb gebracht leesbaar is geworden. Hiervoor en voor het besturen van de regiekamer van de afdeling Chemische Endocrinologie heb ik niet te verwoorden dank.

Beste Pim Clotscher, dank zij jouw hulpvaardigheid en technische kennis funktioneerden de verschillende instrumenten van spoelmachine tot komputer optimaal, waardoor het proefschrift nu en niet 3 maanden later klaar is gekomen.

Dr. I. Kraulis, beste Ilze, met jou heb ik anderhalf jaar in wijlen het BRAIN DEPARTMENT gepioneerd met rattehersenen. Dank voor je bijdrage en de eerste lessen in Engels.

Beste Rudy van Doorn, bedankt voor jouw bijdrage die met de dissektiemikroskoop zichtbaar en zonder meetbaar was.

Beste Peter Frederik, jij hebt de "brain-testis axis" op overtuigende wijze op de omslag kunnen brengen en ik ben je daarvoor zeer erkentelijk.

De vruchtbare samenwerking met F.H. de Jong, als kollega bij de fakulteitsbrandweer, maar ook als statistikus, als ethisch adviseur en als Frank stemt mij tot dankbaarheid.

Bijzonder erkentelijk ben ik ook voor de vele goede kontakten die er bestaan met anderen binnen de Biochemie afdeling. Door deze veelheid van goede zaken is de Biochemie afdeling voor mij als een tweede thuis.

Ik stel het op hoge prijs dat de waterpoloverenigingen Polar Bears en S.V.H. beslag op mijn tijd hebben gelegd.

Mijn vrouw dank ik tenslotte voor haar inbreng bij het bewaren van de harmonie tussen de verplichtingen in mijn eerste en tweede thuis.

LIST OF ABBREVIATIONS AND TRIVIAL NAMES

ACTH	- Adrenocorticotrophic hormone
3β-Androstanediol	- 5α-Androstane-3β,17β-diol
Androstenediol	- 5-Androstene-3β,17β-diol
ATP	- Adenosine-5'-triphosphate
cAMP	- Adenosine-3':5'-monophosphoric acid, cyclic
21-Corticosteroid acetyl transferase	- Acetyl-(CoA):21 corticosteroid acetyl transferase (E.C. 2.3.1.99)
Dehydroepiandrosterone	- 3β-Hydroxy-5-androsten-17-one
Dehydroepiandrosterone sulfokinase	- 3'-Phosphoadenylyl sulphate: 3β-hydroxysteroid sulpho transferase (E.C. 2.8.2.2.)
Dihydrotestosterone	- 17β-Hydroxy-5α-androstan-3-one
Esterase	- a-Specific esterase (E.C. 3.1.1.1.)
FSH	- Follicle stimulating hormone
HCG	- Human chorionic gonadotrophin
3α-Hydroxysteroid dehydrogenase	- 3α-Hydroxysteroid: NAD(P) oxidoreductase (E.C. 1.1.1.50)
3β-Hydroxysteroid dehydrogenase	- 3β-Hydroxysteroid: NAD(P) oxidoreductase (E.C. 1.1.1.51)
11β-Hydroxysteroid dehydrogenase	- 11β-Hydroxysteroid: NAD(P) oxidoreductase (E.C. 1.1.1.99)
17β-Hydroxysteroid dehydrogenase	- 17β-Hydroxysteroid: NAD(P) oxidoreductase (E.C. 1.1.1.64)
Δ^5-3β-Hydroxysteroid dehydrogenase	- Δ^5-3β-Hydroxysteroid: NAD(P) oxidoreductase (E.C. 1.1.1.51)
I.U.	- International Unit
Oestradiol	- 1,3,5 (10)-Oestratriene-3,17β-diol
Phosphodiesterase	- Orthophosphoric diester phosphohydrolase (E.C. 3.1.4.1.)
Pregnenolone	- 3β-Hydroxy-5-pregnen-20-one
Progesterone	- 4-Pregnene-3,20-dione

Prostaglandin E_1	- 11α,15(s)-Dihydroxy-9-oxo-13-trans-prostenoic acid
Q_{O_2}	- Rate of oxygen uptake (μl O_2/hr per mg protein)
RNA	- Ribonucleic acid
5α-Steroid reductase	- 5α-Steroid: NAD(P)Δ^4-oxido-reductase (E.C. 1.3.1.99)
7α-Steroid hydroxylase	- Steroid NAD(P)H oxygen oxido-reductase (7α hydroxylating) (E.C. 1.14.1.99)
Sterol-sulphate sulphohydrolase	- Sterol-sulphate sulphohydro-lase (E.C. 3.1.6.2.)
Steroid C_{17}-C_{20}-lyase	- 17α-Hydroxy-20-oxo-steroid: C_{17-20} acetate lyase (E.C. 4.1.99)
Testosterone	- 17β-Hydroxy-4-androsten-3-one

CHAPTER 1

INTRODUCTION

1.1 The brain-testis axis

The significance of testicular function was shown as early as 1849 by Berthold[1] when he observed atrophy of the comb in castrated cockerels, which could be restored by implantation of a testis in the abdomen. It was only in the beginning of the twentieth century, however, that Bouin and Ancel[2] postulated the formation of certain hormonal principles in distinct cell types of the testis. The identification of testosterone as the biologically active androgen of the bull testis in 1935[3] marked the beginning of biochemical studies of testis function. The measurement of testicular hormonal products has been difficult for a long time because only small quantities are produced. However, present techniques such as radioimmunoassay permit the measurement of picogram quantities of testosterone[4]. Parallel with the development of these techniques, insight has been gained into the endocrine function of the testis.

The importance of the brain in relation to endocrine functions is well documented. A proper understanding of the details of biochemical mechanisms which may play a role in this respect, is however lacking. A functional relationship between the testes and the brain was described for the first time by Moore and Price in 1932[5]. The presence of certain hormones in the pituitary, the so-called trophic hormones, which could stimulate other endocrine

organs was established in 1926 by Zondek and Smith[6]. In 1932 Hohlweg and Junkman[7] postulated a "sex center" in the hypothalamus. The importance of this brain area was firmly established by Green and Harris[8] who demonstrated the neurohumoral control of the adenohypophysis. Conclusive evidence for effects of steroid hormones in the hypothalamus was not reported until much later; Davidson and Sawyer in 1961[9] and Lisk in 1962[10] found that implants of crystalline androgens in the anterior hypothalamus caused gonadal atrophy. Although recently other sensitive areas for steroid hormones have been demonstrated in the limbic system[11], the precise site of biochemical action of steroid hormones in the brain is still unknown.

The many studies on the endocrine function of brain and testis have resulted in a generally accepted model for the relationship between the two organs (Fig. 1). The pituitary secretes different hormones and these hormones are transported in the circulation to their various peripheral target organs. Under influence of the gonadotrophic hormones FSH and LH, different processes in the testes are triggered off and the testes synthesize and secrete steroid hormones. These steroid hormones may in turn interact with the brain. Excess of a steroid hormone has generally an inhibiting effect on the secretion of trophic hormones by the pituitary. A deficiency of a steroid hormone frequently results in an increased secretion of trophic hormones (especially in male rats). Thus the level of the steroid hormones in the circulation and in the brain acts as a negative feedback signal to adjust the secretion of trophic hormones by the pituitary. This simple model can be considered as a self-regulating system and illustrates one of the most important interactions between the testis and the brain. It must be kept in mind, however, that in the total endocrine system several other interactions occur and much more complicated systems can operate. In this respect many other stimuli (catecholamines, epiphyseal hormones, light and stress) that origi-

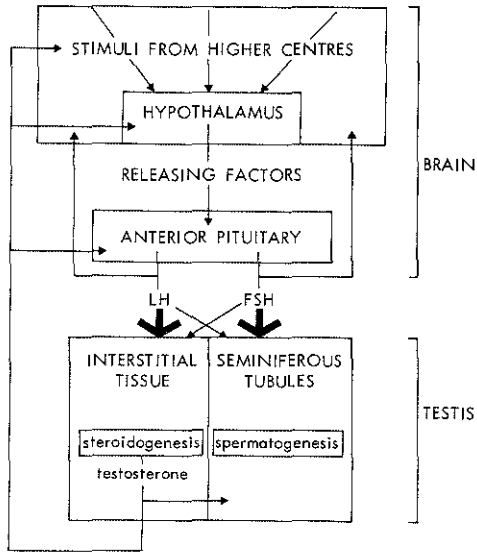

FIG. 1. Interactions and hormonal links in the brain-testis axis.

nate in the brain and influence testis function could be considered[12]. For the present study it was tried to investigate relevant biochemical aspects of both organs independently. The function of the brain and the testis in the endocrine system of the rat will therefore be described separately and in more detail.

1.2 The brain and the control of gonadotrophin secretion

It appears from many experiments using lesion and implantation techniques that most elements for the control of pituitary function are present in the hypothalamus[13]. It has been found, however, that also parts of the limbic system such as the hippocampus and amygdala, contain

elements which are of importance for the control of gonadotrophin secretion[11]. Direct effects of androgens on the pituitary have also been reported[14]. The contradictory results in many publications on the localization of certain so-called hypophysiotrophic areas illustrate the complexity of structures in the brain. Due to this complex structure it has been very difficult to investigate the relationship between various brain areas and the pituitary. Nervous connections between various brain areas and the hypothalamus have, however, been demonstrated. It has been shown that different types of nerves (monoaminergic, cholinergic and serotonergic) all play an important role in the endocrine function of the hypothalamus[15]. In the hypothalamus the afferent nerves are connected with special neurons which secrete neurohormones, the so-called releasing factors. The process of neurosecretion takes place close to a conglomerate of blood vessels, the portal system. In the portal system a blood flow exists from the hypothalamus to the pituitary. This portal system is used to transfer the releasing factors from the hypothalamus to the hypophysis and thus establishes a connection between the two compartments. In the anterior part of the pituitary the different releasing factors interact with specific cell types and stimulate production and release of trophic hormones into the systemic circulation. The posterior part of the pituitary or neurohypophysis is innervated with neurons originating from the hypothalamus. There are no connections with the portal vessels. The secretion of the neurohypophysial hormones oxytocin and vasopressin is therefore exclusively controlled by nerves. For the adenohypophysis it is thought that different groups of neurons from the hypothalamus, secreting different releasing factors, control the secretion of FSH and LH via the portal system. Recently, however, the existence of a FSH releasing factor has been disputed because in experiments it has been shown that synthetic LH Releasing Factor caused release of both LH and FSH <u>in vitro</u> and <u>in vivo</u>[16]. Much

work has been done, especially in female rats,to identify groups of neurons with comparable properties and thus make an anatomical description of certain centres possible. Centres for tonic and cyclic release mechanisms for FSH and LH have now been shown for female rats[17,18]. Stimuli from higher brain centres may also influence the endocrine function of the hypothalamus because of the many nervous connections. For example, effects of stress, light conditions and smell on the endocrine systems have been shown[19].

Apart from the influences of higher brain regions we can distinguish two hormonal feedback mechanisms: a short loop feedback in which anterior pituitary hormones act on the central nervous system and a long loop feedback in which gonadal hormones act on the nervous system of the hypothalamus[20].

The male rat does not show cyclic variations of gonadal function such as occur in the female rat, although there are seasonal variations. It has been demonstrated by Barraclough[21] that after postnatal injections of androgens (also called neonatal androgenization) female rats become acyclic and anovulatory. Therefore it has been concluded that under these conditions androgens desensitize or destroy the nerve cells responsible for the cyclic secretion of hormones. This effect of steroid hormones is an example of an irreversible effect. The role of gonadal hormones in maintaining the normal endocrine balance is however a reversible effect.

General accepted models for the biochemical mechanisms of steroid hormone action in the brain do not exist. Only in the last two years there have been a few attempts to study the uptake and metabolism of steroid hormones in brain tissue (chapter 2).

1.3 Control of steroid production in the testis

In the testis there are two functionally different compartments: the seminiferous tubules and the interstitial tissue (Fig. 2). In the seminiferous tubules spermatogenesis takes place. Primary spermatogonia are attached to the basement membrane, and cells at successive stages of the spermatogenic cycle (spermatocytes, spermatids, spermatozoa) are pushed towards the lumen of the tubule. The spermatozoa are then transported through the tubules via the rete testis to the epididymis for further transport. The

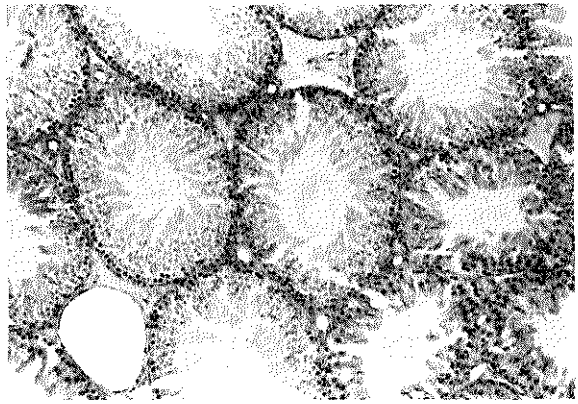

FIG. 2. Photomicrograph of rat testis tissue. The testis was fixed by perfusion with glutaraldehyde 2.5% in 0.1 M phosphate buffer pH 7.4 and stained with periodic acid Schiff and hematoxylin. This section (x 130 magnification) shows circular shaped seminiferous tubules which contain Sertoli cells and germ cells in various stages of spermatogenesis and in between the seminiferous tubules the interstitial tissue (stained structure) and blood vessels (unstained and circular).

seminiferous tubules are embedded in connective tissue which contains interstitial cells (Leydig cells) and many blood vessels. In intercellular spaces in between the tubules and the interstitium there is also a system of lymph vessels[22]. The endocrine function of the testis is predominantly determined by the interstitial tissue. Androgens produced in this compartment are secreted into the blood and possibly into the lymph and transported to peripheral organs to exert their actions. Normal testis function is dependent on the trophic hormones FSH and LH. Experimental evidence[23,24] led to the hypothesis that FSH acts directly on the germinal epithelium and that LH acts on the interstitial cells. Recent binding data for FSH and LH in the testis support this general hypothesis[25,26]. The biochemical specificity of the action of these hormones, however, has not yet been elucidated. It is known that androgens are required for maintenance of spermatogenesis[27] and thus a close relationship must exist between the endocrine function of the interstitial cells and the germinal function of the tubules. It has, however, been postulated that within the tubules also androgens can be produced in Sertoli cells[28].

Investigations with whole testis tissue will always give information which is the result of the presence of the two different tissue compartments. The introduction of a dissection technique by Christensen and Mason[29] made it possible to isolate specific tissues for the investigation of the isolated compartments.

The main steroid secreted by the testis of the rat is testosterone[30]. The precursor for this steroid is cholesterol and secretion of products is regulated mainly by the regulation of production in the tissue. In steroid producing tissues no real storage of hormones has been shown. This is in contrast to organs which produce protein hormones such as the pituitary where hormone containing granules are present[31]. The stimulation of testicular steroid biosynthesis by LH has been shown <u>in vivo</u> and <u>in vitro</u>[32]. It

is thought that LH has a direct effect on the Leydig cells in the interstitial tissue. Different effects of other endocrine organs such as the adrenal and thyroid on testicular steroid production have been shown[33]. The actions of hormones secreted by these organs may be described as permissive or modulating actions. The biochemical mechanisms for the control of steroid production have been investigated in great detail for the adrenal gland. From the results obtained for the adrenal a model has been proposed for the trophic regulation of steroid biosynthesis[34]. According to Garren et al.[34], ACTH activates adenyl cyclase in the cell membrane, which results in the formation of cAMP. Subsequently this nucleotide binds to a receptor and activates a protein kinase which catalyses the phosphorylation of a ribosomal moiety, thereby modulating the translation of mRNA(s). This results in the production of a "regulator-protein" which facilitates translocation of cholesterol to the mitochondrion where pregnenolone formation (the rate limiting step in steroidogenesis) takes place. Also the hydrolysis of cholesterol esters to free cholesterol is activated by a cAMP dependent protein kinase. The testis has been investigated in less detail, possibly because the steroid producing cells in the testis are closely connected with the sperm producing cells thus making it difficult to work with a homogeneous population of cells which produce steroids. The importance of steroid production in the testis for the maintenance of spermatogenesis made it attractive to investigate if the concept proposed for the adrenal can be applied as a suitable model for describing the regulation of steroid production in the testis.

1.4 Scope of this thesis

In the hypothalamus and testis steroids play an important role. Knowledge of the biochemical control mechanisms which include steroids and which operate in testis and brain is lacking. It was decided to investigate with biochemical techniques: the metabolism of steroids by brain tissues and the biochemical mechanism of action of trophic hormones on the endocrine function of the testis.

The first part of this thesis (chapter 2) deals with the interaction of steroids with brain tissue. Results from experimental work on metabolism of steroids in brain will be discussed in relation to results from the literature on biochemical processes in brain which are of importance for the regulation of trophic hormone secretion.

In the second part of this thesis (chapter 3) results on the regulation of steroidogenesis in testis tissue will be discussed. Special attention has been given to the role of cAMP in the control of steroidogenesis.

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INTERACTIONS OF STEROIDS WITH BRAIN TISSUE

The importance of steroids in the regulation of the gonadotrophin secretion is well established[1], however, the biochemical mechanism is poorly understood. Three stages may be of importance for the biochemical action of steroids on the brain: (i) uptake* of steroids by specific cell types, (ii) metabolism of the steroid molecules, (iii) metabolic effects caused by the steroids. These various steps can be incorporated in a hypothetical model for the biochemical interaction of steroids with brain tissue (Fig.3). The

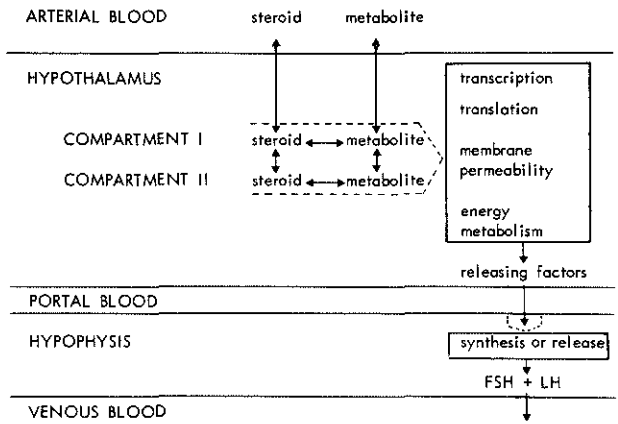

FIG. 3. Interactions of steroids with brain tissue.

* "Uptake" has been used to describe a process of entry of steroids into tissues or cells.

interactions of steroids with brain, which influence the production and/or release of hypothalamic and hypophysial hormones may be characterized by: a) the effect of the brain on the steroids (uptake, metabolism of steroids) and b) possible biochemical effects of steroids on processes in the brain (transcription, translation, energy metabolism). In this chapter particular emphasis will be paid to the effects of the brain on the steroids.

2.1 Uptake of steroids

The general concept concerning the mechanism of steroid hormone action includes the uptake of hormones by cells[2]. In many cases the uptake involves specific binding proteins. In brain tissue uptake of radioactive steroids *in vivo* and *in vitro* has also been demonstrated. The uptake of oestradiol in male and female rat brain has been investigated most extensively[1-9]. A receptor* for oestradiol has been isolated from the soluble fraction of hypothalamic tissue[10]. Stumpf[8] observed that radioactive oestradiol was concentrated in areas which have also been accepted as hormonal feedback areas.

The uptake of androgens in brain has also been demonstrated[5,6,11,12,13]. An androgen receptor in hypothalamic tissue and pituitary has been found by Samperez *et al.*[11]. However, it has been reported by Scherrat *et al.*[14], that no uptake of testosterone could be measured. Many similarities have been observed for the localization of the hor-

* "Receptor" has been used in this chapter to define macromolecules which bind steroids with a high affinity and a certain specificity without knowing the implications in the mechanism of action of steroids.

mones in the brain after uptake of testosterone and oestradiol in male and female rats. The quantitative uptake of testosterone and oestradiol, however, was found to be different; in the hypothalamus and the limbic system a preferential retention for oestradiol over testosterone was observed[6]. It appears that testosterone and oestradiol have comparable actions on the inhibition of trophic hormone release[15] and on establishing a non-cyclic gonadal function after neonatal administration to female rats[16] (also called neonatal androgenization). The potency of oestradiol in these experiments was found to be higher than testosterone.

The comparable inhibitory effects of testosterone and oestradiol in brain oppose the completely different peripheral stimulatory effects of both steroids. It should be kept in mind, however, that in all studies on uptake of steroids radioactively labelled steroids have been used and in most cases only the behaviour of the radioactivity is described without knowing the nature of the steroid. No conclusion can therefore be drawn on the precise interaction of a particular hormone and a receptor unless the structure of the bound steroid is known.

2.2 Metabolism of steroids

For a proper understanding of hormonal action in a certain cell type one has to consider metabolic transformations of steroids in these cells. Catabolites of steroids have long been considered as biologically inactive compounds. This opinion has changed since it was demonstrated that dihydrotestosterone, a catabolite of testosterone, could act as a physiologically active substance[17]. Metabolic reactions of steroids may therefore be an integral part of the mechanism of action of steroids. This was an

important motivation for us to investigate the metabolism of steroids in brain tissue.

It was found that in cerebral tissue of the male rat 5α-steroid reductase and 17β-hydroxysteroid dehydrogenase were present (appendix paper I). These enzyme activities have also been reported by other investigators in rat brain tissues[18-25], in human foetal brain tissue[18,26] as well as in dog brain tissue[27]. 3α-Hydroxysteroid dehydrogenase has also been detected in brain[24,25,26]. Other steroid converting enzymes in cerebral tissues that have been reported are: 11β-hydroxysteroid dehydrogenase[28-31], 21-corticosteroid acetyltransferase[32,33], dehydroepiandrosterone sulphokinase[34] and sterol-sulphate sulpho hydrolase[35]. Recently an aromatizing system for conversion of testosterone to oestradiol has been found in rat and human brain tissue[36-39].

A better understanding of the functions of these enzymes can possibly be obtained from a description of their specificity, cellular and subcellular distribution. In this respect it is important to realize that different workers have used different techniques for isolation of cellular and subcellular fractions and for measurement of the rate of steroid metabolism.

The specificity of the enzymes has not been investigated. The distribution of enzymes catalysing the formation of oestrogenic compounds (the aromatizing system), over various brain regions has been investigated using broken cell preparations of the tissues. Relatively high enzyme activities were detected in the hypothalamus and the limbic system[38]. As has been indicated before, oestradiol receptors have been shown in these areas and also important centres for the regulation of the gonadotrophin secretion have been found to be present. For the study of the 5α-reductase in various brain areas several authors have used minces[18,21,23]. It was found that more radioactive testosterone was converted to dihydrotestosterone in the hypophysis than in the hypothalamus. Conclusions

about enzyme activities in the various brain areas can however not be obtained from these studies, because in minces co-factor levels and permeability factors may determine the steroid conversion. In our studies (appendix paper I) we have used homogenates and we have investigated proper conditions for the measurement of enzyme activities. We have observed that enzyme activities, expressed as nmole of metabolite formed per hour, in the pituitary are lower than in the hypothalamus, thus contrasting the results with minces.

The subcellular distribution of enzymes was investigated with ultracentrifugation techniques for separation of the different subcellular organelles. However, the various subcellular preparations are derived from different cellular populations and are therefore inhomogeneous. Arguments for the localization of the 3β- and 17β-hydroxysteroid dehydrogenases in the soluble fraction and the presence of the 5α-steroid reductase in microsomes are described in appendix paper I. Other reports on subcellular localizations of these enzyme activities in brain tissue have not been published.

The possible role of steroid metabolism in brain under *in vivo* conditions is not clear. It is very difficult, if not impossible, to study cerebral metabolism under *in vivo* conditions with exclusion of all possibilities for peripheral conversions. Sholiton *et al.*[25] have reported conversions of steroids in hepatectomized and totally eviscerated male rats but one may wonder if these conditions give a good impression of the *in vivo* situation. Perfusion experiments with cerebral tissue have been described by Knapstein *et al.*[34], who reported sulpho-conjugation of dehydroepiandrosterone. We ourselves have tried to study metabolism of implanted crystalline [^{14}C]-progesterone in the hypothalamus, but it was not possible to detect any metabolites in the brain. The regional localization of oestrogen metabolites in brain after injections of oestradiol, as found by Luttge and Whalen[40] may indicate

a regional cerebral metabolism. Specific uptake after peripheral conversions cannot be excluded however.

2.3 Actions of steroids

To elucidate a possible function of steroid metabolism in the regulation of the gonadotrophin secretion the metabolic reactions of steroids in brain have to be investigated in relation to effects of specific metabolites on trophic hormone secretion. This will be difficult to accomplish because in the experimental model peripheral conversions must be absent whereas the regulation of the gonadotrophin secretion must be undisturbed.

Direct evidence for a function of steroid metabolites in brain is therefore lacking, although observations from various experiments may indicate a physiological role for steroids or steroid metabolites. In studies on the physiological effects of steroids it was observed that dihydrotestosterone injections in female rats had no effect on sexual behaviour and did not cause neonatal androgenization[41,42]. It appears therefore that no role exists for the 5α-reduced compound in this respect. Injections of steroids such as testosterone or androstenedione which may be converted to oestrogenic steroids but not of dihydrotestosterone induced changes in the reproductive behaviour and caused neonatal androgenization of the brain[39,41]. Oestradiol injection also caused neonatal androgenization in neonatal female rats[43]. From these experiments evidence may be derived for an obligatory aromatizing step in the action of androgens on behaviour. On the other hand it was found that both testosterone and dihydrotestosterone could suppress the serum levels of FSH and LH in male rats[44]. Also inhibiting actions of oestrogens on LH and FSH secretion are well known in male and female rats[15,16]. These

Observations indicate that the aromatizing step for the regulation of trophic hormone secretion is not obligatory, otherwise dihydrotestosterone could not suppress levels of FSH and LH. For cerebral control of behaviour and secretion of trophic hormones it thus seems likely that different metabolites may be required. These observations seem to be supported by results from experiments with prostatic tissue. In these experiments it has been found that dihydrotestosterone induces hyperplasia and 3β-androstanediol induces hypertrophy of cells[45]. Both steroid metabolites can originate in prostatic tissue from testosterone, thus active compounds have been formed from the precursor testosterone. Metabolic reactions controlling the quantities of various metabolites may be considered as an intracellular device for controlling the level of physiologically active steroid hormones in the cell. The findings on prostatic and brain tissue may illustrate the possible importance of metabolism in the action of steroid hormones on target tissues.

2.4 Effects of steroid hormones on metabolic processes

The next step in the biochemical mechanism of action of steroid hormones may be a regulation of metabolic processes in brain (Fig. 3). Theoretically it may be considered that steroids can interfere in all steps necessary for the secretion of hormones. Some of the possibilities have been investigated.

The best investigated biochemical parameter is the oxygen uptake of tissues of various brain areas. Moguilevsky et al.[46,47] have demonstrated that the Q_{O_2} in the hypothalamus and the limbic structures of female rats shows fluctuations which correlate with the stage of the sexual cycle. The Q_{O_2} of the anterior hypothalamus of pre-

puberal rats was found relatively high in tissues from rats with a male pattern of gonadotrophin secretion and low in tissue from rats with a female pattern[48]. The depression of the Q_{O_2} in anterior hypothalamic tissue from prepuberal rats which were neonatally androgenized with testosterone, gives evidence for a direct action of testosterone *in vivo*[48]. *In vitro* effects of testosterone on the Q_{O_2} of hypothalamic tissue could, however, not be demonstrated[49]. Although the fluctuations in the oxygen uptake illustrate changes in biochemical processes they give no information about possible effects on specific biochemical reactions.

Effects of steroids on protein synthesis *in vivo* in various brain areas have been investigated by measuring incorporation of radioactive aminoacids[50-53]. Conflicting results have been observed by Litteria and Timiras[50] who found inhibition of [^{14}C]-lysine incorporation into hypothalamic nuclei after administration of oestradiol and by Seiki *et al.*[53] who found a positive effect of a combination of progesterone and oestradiol on [^{3}H]-leucine incorporation. In these studies pharmacological doses of steroids were used. Moguilevsky *et al.*[51] have investigated protein synthesis in different hypothalamic areas during the sexual cycle of female rats and have found differences in different areas and fluctuations during the cycle.

Protein catabolism in the hypothalamus has also been investigated[54,55]. Non-specific peptidase activity was found to increase after injections of oestrogens into ovariectomized animals. All studies on protein metabolism, however, were not specific with respect to the quality of the proteins and therefore the observed effects may have no relation to the regulation of gonadotrophin secretion.

In analogy with hormonal effects on RNA metabolism in peripheral organs, the action of testosterone[56] and cortisol[57] on brain RNA metabolism have been investigated. It was found that hypothalamic tissue behaved differently from other brain areas. Finally, possibilities for actions

of steroid hormones on nerve-transmission processes have been investigated. Steroids in vivo appear to influence catecholamine metabolism[58,59].

Interpretation of the effects of steroids on metabolic processes for the in vivo situation is almost impossible, because of the many different experimental conditions and the different complex structures of brain tissue which have been used. Obviously more detailed investigations are therefore necessary to obtain better insight into the role of biochemical mechanisms in the control of gonadotrophin secretion.

In conclusion, it appears that for the biochemical mechanism of steroid hormone action on the brain, evidence exists for: (i) uptake and binding of steroids in various brain areas which are involved in the regulation of gonadotrophin secretion; (ii) metabolism of steroids; (iii) requirement of different metabolites for cerebral control of behaviour and secretion of trophic hormones. For the effects of steroids on metabolic processes in the brain, however, no general concept can be proposed.

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REGULATION OF STEROID BIOSYNTHESIS IN TESTIS TISSUE WITH SPECIAL REFERENCE TO THE ROLE OF CAMP

For the regulation of steroidogenesis by trophic hormones in testes a working hypothesis has been used which is derived from the model of Garren et al.[1] for the effects of ACTH on adrenal steroid production (Fig. 4). In this model it is considered that the following consecutive stages are involved in the action of ACTH on the adrenal: (I) binding of hormone to the cell, (II) activation of adenylcyclase, (III) enhanced production of cAMP, (IV) activation of protein kinase and action on protein synthesis, (V) effects of specific proteins or activated protein kinases on substrate availability for the cholesterol side-chain cleavage enzyme. Based on various experimental fin-

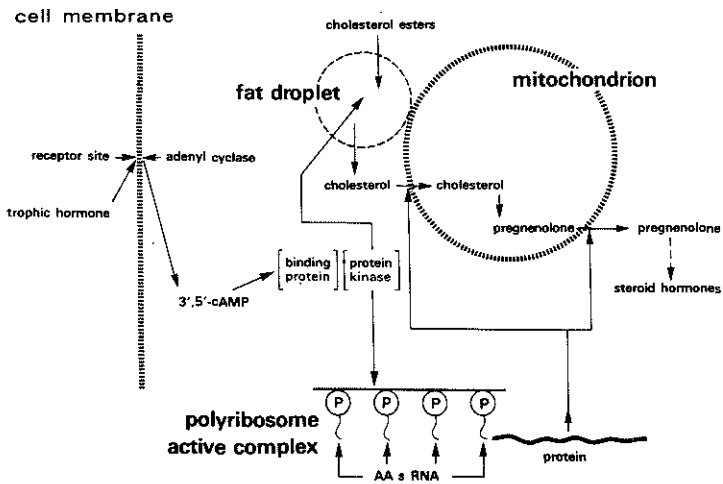

FIG. 4. Scheme for control of steroidogenesis

dings for other endocrine organs other explanations have also been proposed for the action of trophic hormones on steroid production in the testis[2]: a) increased production of intracellular NADPH through activation of phosphorylase, b) increased blood flow through the testis and c) stimulated removal of pregnenolone from mitochondria, thus abolishing the inhibition of the side-chain cleavage activity. The many proposed models partially reflect the lack of information on the biochemical regulation of endocrine testis function. For a better understanding of the operating biochemical mechanisms more detailed information is necessary. We have therefore investigated the role of cAMP in the gonadotrophic stimulation of steroidogenesis in testis.

It is essential when investigating the biochemical mechanism of hormone action, to work with specific methods of analysis and also where possible with specific cell or tissue types. A great deal of work on the testis has been carried out on the whole gland. Results from these investigations in relation to trophic hormone action are difficult to interpret, however, because in the testis many different cell types are present. In order to investigate in which cell types steroid biosynthesis occurs and which biochemical mechanisms regulate the production, isolated tissue compartments should be investigated first. This type of investigation must not of course exclude the possibilities of interaction between the different tissue compartments and therefore the whole gland and combinations of different tissue compartments must also be investigated.

3.1 Isolation of tissue compartments

The dissection technique as described by Christensen and Mason[3] for the isolation of interstitial tissue and seminiferous tubules from the rat testis, has been used in

the present study. The homogeneity of the fractions could not be fully characterized by examination of histological preparations, so that other techniques were examined. It was found that interstitial tissue has a high esterase activity which could be used as a marker for isolated interstitial tissue and which was applied to characterize the purity of tissue fractions (appendix paper II). It has been shown that esterase activity can be isolated in microsomal fractions[4] and is dependent on pituitary hormones (appendix paper II). The steroid converting enzymes, steroid $C_{17,20}$-lyase, 3β-hydroxysteroid dehydrogenase and 5α-steroid reductase are also localized in the endoplasmic reticulum and are equally dependent on gonadotrophic stimulation[5,6]. This correlation between activities of esterase and the steroid converting enzymes may therefore support the usefulness of esterase as a marker of testicular steroid producing cells.

The selective localization of esterase in interstitial tissue has also been used to calculate the % amount of interstitial tissue in testis (appendix paper II). The % amount of interstitial tissue in testis was found to be between 13 and 23%. Christensen and Mason[3] reported that interstitial tissue occupied 6% of the testis. This lower value can possibly be explained by rat strain differences or losses during the dissection for which no corrections were applied. Histological methods for quantification of Leydig cells in testis have been reported. Heller et al.[7] estimated in human testis preparations the ratio of the number of Sertoli and Leydig cells but they did not relate the number of Leydig cells to the amount of testis tissue. Kothari et al.[8] observed that in dog testis 15% of the total volume was occupied by Leydig cells.

3.2 Receptors for trophic hormones and adenylcyclase in testis

A first event in the action of trophic hormones on target organs is in many cases an interaction of the hormone with a receptor. For testis it has been reported that radioactive FSH is preferentially bound to seminiferous tubules[9,10] and that radioactive LH and HCG are predominantly bound to interstitial tissue[11,12]. There is little information on the subcellular localization of the receptors in testis. A few studies have demonstrated, however, an association with membranes fractions[13,14]. It has been found that binding of glucagon and insulin both occurred in liver plasma membranes. In addition, a correspondence was shown between the binding of glucagon and the adenylcyclase activity[15]. Also hormone sensitive adenylcyclase activity has been identified in cytoplasmic membranes of fat cells[16]. It has been postulated therefore that the receptor and the adenylcyclase form together a specific hormone sensitive system. The localization of this system in plasma membranes fits with the concept that hormones on the outside of the cytoplasmic membrane affect the conformation - and thus the activity - of the adenylcyclase on the inside of the cell membrane. It has also been reported that adenylcyclase may be associated with the microsomal fractions of fat cells[17] and with mitochondrial fractions of dog testis[18] and rat testis[19] and with nuclei of the prostate[20]. The purity and characterization of the subcellular fractions used in these studies were not beyond criticism. The exact localization and the hormone specificity of these adenylcyclases should therefore be reinvestigated, because the presence of an intracellular localized trophic hormone dependent adenylcyclase seems unlikely. With the presence of intracellular hormone dependent adenylcyclases the function of cAMP as intracellular messenger of hormone action would become superfluous and

trophic hormones should be transported through the cell membrane to interact with intracellular adenylcyclase. It has been shown that FSH and LH stimulate cAMP production in testis tissues[18,21-25]. It appears that LH or HCG stimulate cAMP production in interstitial tissue[24] and FSH in seminiferous tubules[23]. Increased formation of cAMP following addition of prostaglandin E_1 to total testis tissue *in vitro* has been reported by Butcher and Baird[26]. Kuehl *et al.*[27] have presented evidence for an intermediate function of prostaglandins in the action of LH on the production of cAMP and steroidogenesis in the mouse ovary. Whether the trophic hormone would only influence cAMP formation through the intermediate formation of prostaglandins can only be concluded after more information is available.

3.3 cAMP and steroid production

The ability of cAMP to stimulate testicular steroid production was first shown by Sandler and Hall[28] in 1966 and by Connell and Eik-Nes[29] in 1968. Hence at that time independent effects of trophic hormones on cAMP production and effects of cAMP on steroid production were known, but there was no evidence for a causal relation between cAMP and steroid production. We have therefore investigated the effect of trophic hormones on the endogenous production of cAMP and testosterone in different testis tissues of the rat. An *in vitro* incubation technique has been chosen for the investigation of total testis or tissue fractions. Testosterone was used as a parameter for steroid production because it is the quantitatively most important steroid secreted by the rat testis[30] and because its production can be stimulated by small doses of HCG in *vivo*[31].

In many investigations conversions of radioactively

labelled substrates have been studied to characterize stimulation of steroid production or cAMP production. Sometimes labelled precursors are used for labelling of the substrate pool[32]. Theoretically, however, such studies may at best give an impression about the enzyme activities present. If radioactive compounds can penetrate into the cell, they must reach an equilibrium state with the substrate pool. Sometimes this is not possible during the incubation period and in such cases one does not study the rate of conversion of the substrate.

The actual endogenous production depends, in addition to the presence of enzyme activities, on the endogenous amounts of available substrate. In the ovary it has been demonstrated that the active cholesterol pool for steroidogenesis is regulated by the cholesterol uptake from plasma, synthesis from acetate and synthesis from cholesterol esters[33]. In many studies on steroidogenesis in testis tritiated cholesterol has been used as a substrate for the production of testosterone[28,34-38]. Also [^{14}C]-acetate has been used to label the cholesterol substrate pool[29,38,39]. Effects of LH or HCG on incorporation of radioactivity from cholesterol into testosterone could be measured, although the percentage conversions were very low (in the order of 0.1%).

In our experiments endogenous production of cAMP and testosterone during the incubation period has been calculated from the change in the levels in tissue and incubation medium together. In this way the release of hormones by the tissue cannot be measured. On the other hand by measuring the sum of the levels in the tissue and the medium release phenomena cannot complicate the interpretation of the results. Production, calculated from a change in level, is the net result of formation and degradation of products. Thus production can be stimulated by stimulation of synthesis or inhibition of catabolic reactions. Hall[34] has presented evidence for the stimulation by LH of the conversion of cholesterol to testosterone whereas no

other effects of LH on the pathway to testosterone could be demonstrated. Also increased production of cAMP by hormonal activation of adenylcyclase in testis has been shown[22,25]. No evidence has been found for inhibiting effects of trophic hormones on catabolism of cAMP and testosterone. Catabolism of cAMP and testosterone, however, does occur in testis tissue.

3.4 Catabolism of steroids and cAMP in testis

Conversions of testosterone and androstenedione to androstanediols have been demonstrated and from the structure of the main metabolites it can be concluded that in rat testis tissue the following enzymes are present: 17β-hydroxysteroid dehydrogenase[40,42,43], 5α-steroid reductase[41-43], 3α-hydroxysteroid dehydrogenase[42], 3β-hydroxysteroid dehydrogenase[44] and 7α-steroid hydroxylase[45]. In isolated interstitial tissue 5α-steroid reductase and 17β-hydroxysteroid dehydrogenase are present whereas in isolated tubules 3α- and 17β-hydroxysteroid dehydrogenases and 5α-steroid reductase have been detected[43]. The extent of metabolism under our experimental conditions is unknown. It is clear, however, that due to metabolism, the calculated production is an underestimation of the real production.

After the formation of pregnenolone from cholesterol two pathways may result in the biosynthesis of androgens[46]. Based on the steroid structure of the intermediates in the two pathways a "Δ^4-route" and a "Δ^5-route" have been distinguished. It may be possible therefore that pregnenolone is converted via Δ^5-compounds to androstanediols and may not be measured in the testosterone pool. Bell et al.[40] have presented evidence, however, that the main route for biosynthesis of testosterone in the rat is via Δ^4-compounds. This preference for the Δ^4-route may be explained by a very

active Δ^5-3β-hydroxysteroid dehydrogenase and Δ^5-Δ^4 isomerase in rat testis when compared with other testis tissues from other species[47].

cAMP, the other parameter used to study the effect of gonadotrophins, is metabolized by cyclic nucleotide phosphodiesterase which is present in many tissues[48]. In testis tissue this enzyme has also been demonstrated[49,50] and thus the degradation of cAMP in testis tissue will influence the net production of cAMP.

3.5 Gonadotrophic stimulation of testicular testosterone production in vitro

When we started our experiments on the in vitro stimulation of the endogenous testosterone production in rat testis tissue some reports on the in vitro testosterone production in testicular tissue or tumours had been published. Stimulation of testosterone production was reported for mouse interstitial cell cultures with cAMP[51], mouse Leydig cell tumours with LH[52], testis tissue from 20 day old rats with cAMP and LH[28,35] and for rabbit testis slices with cAMP and LH[29]. In the first series of our experiments on the relationship between cAMP and testosterone production no reproducible effects of HCG could be obtained on testosterone production. In many experiments testicular testosterone production could not even be stimulated with high doses of HCG (10 I.U.). Hypophysectomy, pretreatment of rats with HCG, or the addition of albumin to the incubation medium did not improve these results. When testes from 20 day old rats were used, testosterone production could be stimulated with HCG which was in agreement with results published by Sandler and Hall[35]. Hall also reported that the rate of incorporation of radioactivity from cholesterol into testosterone in 20 day old rats was most sensitive to stimulation with HCG[36]. Testes of

these rats, however, were so small that they could not be used for dissection, which was necessary for a more detailed investigation of the regulation of steroidogenesis. Further work with tissue from normal adult rats showed that after preincubating the tissue for 1 h before the addition of HCG and fresh medium, a consistent stimulation of cAMP and testosterone production could be obtained. Similar observations have been reported for corpus luteum tissue[53] and for quartered adrenals[54]. These effects of preincubation may theoretically be explained by removal of an endogenous effector. In rat adipocytes the release of a hormone antagonist has been shown[55]. For testis, however, there is no evidence for such an antagonist.

3.6 cAMP as second messenger for trophic hormone action on total testis tissue

In the first series of experiments criteria for cAMP as a second messenger in trophic hormone action on testosterone production by total testis tissue were investigated. The following evidence (appendix paper III) supported the possible function of cAMP as a second messenger:

- HCG increases the levels of testosterone and cAMP in total testis tissue.
- The increase in cAMP levels precedes the increase in testosterone levels.
- Dibutyryl-cAMP increases testosterone levels in total testis tissue.

In the course of our experiments Dufau et al. also reported stimulation of testosterone production in testis with HCG, LH and dibutyryl cAMP[56-59], but no effects were observed with FSH alone or together with LH[58]. These authors incubated total unteased testis and were able to stimulate testosterone production with 0.5 ng LH/ml. Moyle

et al.[60] also reported stimulation of testosterone production with LH in Leydig cell tumours. It thus appears that in total testis tissue cAMP is an intermediate in the action of LH or HCG on steroidogenesis. However, in testis many different cell types are present and from experiments with whole testis tissue no conclusions can be drawn on the specificity of cAMP and steroid production. Therefore it was decided to investigate this relationship in a homogeneous cell system and to study the in vitro steroid production in isolated interstitial tissue and seminiferous tubules.

3.7 Testosterone production in isolated interstitial tissue and seminiferous tubules

During incubations with interstitial tissue testosterone levels increased, whereas with tubules an apparent decrease in testosterone levels was observed[61]. The localization of steroid producing cells in the interstitial tissue is thus clear. The absence of changes in testosterone levels during the incubation of tubules suggest the absence of steroid production in tubules. Production in tubules might have been present, however, if the synthesis were balanced by degradation of the products, so that the net production was zero. De novo steroid production can also be studied with isolated mitochondria which has the advantage that catabolic enzymes which are mainly microsomally bound or present in the cytoplasm[6] are absent. When the endogenous steroid production (expressed as the production of Δ^5-pregnenolone and testosterone) in isolated mitochondria from interstitial tissue, tubules and total testis tissue are compared, it appears that 92-97% of the steroid production in testis is produced by mitochondria from the interstitial tissue[4]. We have concluded that these results

strongly suggest the absence of de novo testosterone synthesis in tubules which is in agreement with observations made by Hall et al.[37]. This is in contradiction, however, with the conclusions of Lacy et al.[62] and Irusta et al.[63] that steroid biosynthesis from cholesterol may take place in the seminiferous tubules.

3.8 Stimulation of cAMP and testosterone in interstitial tissue with different doses of LH

As stated above it has been shown that endogenous testosterone production occurs in the interstitial tissue and therefore this tissue was also used to study the effects of LH on cAMP and steroid production (appendix paper IV).

cAMP and testosterone production could be stimulated by LH. The effects of LH on steroid production were different, however, in experiments with tissues from different rats. In some experiments a stimulation of the testosterone production could be shown with 2 ng LH/ml whereas in other experiments 20 ng LH/ml was necessary. Although stimulation of steroid production could be obtained with 20 ng LH/ml, stimulation of cAMP formation in interstitial tissue required minimally 200 ng LH/ml (appendix paper IV). Comparable observations for the amounts of ACTH required to stimulate cAMP and steroid production have been made with adrenal tissue[64,65]. The stimulation of testicular steroidogenesis with 20 ng LH/ml without a simultaneous effect on cAMP production could be interpreted as a direct hormonal effect on steroidogenesis without the involvement of cAMP, although it may also reflect the inadequacy of the analytical method for cAMP to detect small differences. Significant effects on cAMP may occur in a particular intracellular compartment, which cannot be measured if the total system is analysed. On the other hand if may be pos-

sible that all measurable effects on cAMP levels are caused by overstimulation of the adenylcyclase by unphysiological doses of trophic hormone.

3.9 Effects of isolation of testicular tissues on testosterone production

Of the total protein in testis tissue 17% is estimated to be interstitial tissue protein (appendix paper II). Thus in isolated interstitium the number of Leydig cells per unit of protein is 6 times higher compared to the total testis tissue. It may be expected therefore that the steroid production in isolated interstitial tissue is 6 times higher than in total testis tissue. In practice, however, lower values were observed in interstitial tissue (appendix paper IV). In contrast, the cAMP production in interstitium was about 6 times higher than in total testis[24]. Hence effects of the tissue isolation are apparently not detectable at the level of the adenylcyclase system, but are reflected somewhere else in the sequence of reactions, which regulate steroidogenesis. This can also be concluded from observations made by Dufau et al.[57] who found that the stimulation of testosterone production with dibutyryl-cAMP was less in teased testis tissue than in unteased tissue. These authors also reported that teasing of the testis tissue resulted in a diminished steroid production in the presence of LH. They were not able to stimulate testosterone production in interstitial tissue. Other testicular preparations have been used under different experimental conditions for the measurement of steroid production in vitro (Table I). Interpretation of the results is difficult because different incubation conditions have been used. It is striking, however, that the testosterone production in isolated interstitial tissue is relatively low compared to the production in whole testis. This may be explained by

a limitation of nutritional factors in isolated interstitium. The addition of glucose stimulated the steroid production in the presence of LH (appendix paper IV). Glucose may possibly act as a substrate for interstitial tissue.

TABLE I

Testosterone production *in vitro* in different testicular preparations from normal adult rats using various additions. Production is expressed per 1.5 g wet weight of whole testis tissue during an incubation period of 4 hours.

testicular preparation	additions	glucose 0.2%	testosterone production in ng	reference
unteased testis	LH or HCG	+	1100	58
unteased testis	LH	-	1400	paper IV
teased testis	HCG or dibutyryl-cAMP	+	100	57
teased testis	LH, HCG or dibutyryl-cAMP	-	900 - 1400	paper III and IV
teased testis	LH	+	3100	paper IV
interstitial tissue	HCG	+	production (not stimulated)	57
interstitial tissue	LH	-	80 - 260	paper IV
interstitial tissue	LH	+	1400	paper IV

Effects of glucose on oxygen uptake of interstitial tissue have however not been found[66]. In contrast, glucose can be shown to be a substrate for oxidative processes in tubules[66] and for protein synthesis in total testis[67] as well as for maintaining ATP levels[68]. Other obligatory endogenous substrates for the interstitial tissue have been suggested: lipids have been mentioned[66,69], but no conclusive evidence was presented, and Hamberger *et al.*[70] reported an increase of the Q_{O_2} of interstitial tissue with

succinate. Effects of these substrates on steroid production have not been measured.

The time lag between the addition of the trophic hormone and the response of the steroid production also seems to be dependent on manipulation of the tissue. In our studies with total testis tissue and interstitial tissue a time lag (of between 30 and 60 min) was found before significant increases in testosterone production could be observed. Dufau et al.[58] observed a marked stimulation of the testosterone production after 15 min (the earliest time of sampling). Clear stimulation of the in vivo testosterone production in the rat has been reported after 15 min (earliest time)[31]. In perfusion experiments with dog testis a time lag of less than 10 min was reported[71]. A comparison between our observations and those by others is however not completely valid, because we have measured under conditions which do not reflect possible release mechanisms. This is in contrast with the experiments in the cited publications where release of steroids may be dependent on trophic hormones. Regulation of release has been postulated by Eik-Nes for steroids in the testis[71] and has been demonstrated for the release of free fatty acids from adipocytes under the influence of cAMP[72].

3.10 Interactions between interstitial tissue and seminiferous tubules

When levels of cAMP during a four hour incubation period of interstitial tissue and total testis tissue are compared, it appears that in total testis cAMP levels reach a maximum at 30 min (appendix paper III), whereas in interstitial tissue the cAMP levels continue to increase throughout the whole incubation period up to 4 hours (appendix paper IV). The decrease of cAMP levels in total tes-

tis after 30 min may be caused by degradation of cAMP by phosphodiesterase. The absence of this effect in interstitial tissue may indicate a low phosphodiesterase activity in this tissue. It is possible, therefore, that cAMP produced in the interstitial tissue is transported to the tubules and metabolized. Transport of cAMP out of testis tissue has been reported by Catt et al.[59] and Dufau et al.[25]. Tubular degradation of cAMP produced in interstitial tissue may be considered as an example of an interaction between these two tissues.

Other evidence for interactions between the two compartments by steroids has also been published. After infusion of labelled Δ^5-pregnenolone into blood vessels of the interstitial compartment of rabbit testis, labelled steroids could be identified in the tubules[73]. Also the comparatively high levels of testosterone in tubules only after incubation of total testis tissue[61] support the occurrence of transport of steroids between the two compartments.

The low testosterone production in interstitial tissue in comparison with total testis tissue (Table I) may possibly be caused by a more readily removal of essential factors from isolated interstitial tissue than from intact tissue, where leakage may be smaller and supply by the lymphatic surrounding of the cells may still be present. The dependency of isolated interstitial tissue on added glucose is larger than in total testis tissue.

3.11 Biochemical mechanism of action of cAMP in testis tissue

Several mechanisms have been proposed for the next steps in the biochemical action of trophic hormones after the production of cAMP. In the hypothetical model presented

in figure 4 effects on protein kinase, protein synthesis and on the availability of cholesterol have been indicated. In testis tissue a cAMP dependent protein kinase has been detected[74]. After administration of LH or dibutyryl-cAMP *in vivo* stimulation of protein synthesis has been shown to occur in Leydig cells *in vitro*[75]. In various total testis preparations comparable effects were observed with FSH[76-78]. Also stimulation of RNA synthesis by FSH in total testis has been described[79]. Effects of trophic hormones *in vivo* on different cholesterol pools in testis have been described by various investigators[80-82]. Trophic hormones have also been reported to stimulate enzyme activities, such as lactate dehydrogenase[83] and alcohol dehydrogenase[84] which are not directly related to steroidogenic processes. It is difficult to establish the significance of these results because many results have been obtained from experiments with total testis tissue and also many effects have not been related with the steroid production. Also some effects may be a consequence of regulation of growth under influence of the trophic hormone. This may be an effect distinct from effects on steroidogenesis. At least for the adrenal different mechanisms have been proposed for the effect of ACTH on steroidogenesis and on growth[85].

In summary, it has been shown that testicular steroidogenesis occurs in interstitial cells with cAMP as the intracellular mediator of LH and HCG action. Isolated interstitial tissue can be used to study effects of trophic hormones on cAMP and testosterone production and also possibly on intermediate steps. Interactions between seminiferous tubules and interstitial tissue, however, may influence the testicular production of cAMP and testosterone.

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SUMMARY

The interaction between the two compartments of the brain-testis axis is established by hormones. Steroid hormones regulate gonadotrophin secretion via processes in the brain and gonadotrophic hormones regulate the endocrine and spermatogenic function of the testis (chapter 1). Available information of this feedback system in the rat is derived mainly from investigations using biological and anatomical techniques. Information on the biochemical mechanisms of both processes is not well documented. An investigation was therefore carried out into the:

A. Metabolism of steroids in brain tissues as a possible factor in the regulation of the gonadotrophin secretion (chapter 2).
B. Regulation of steroidogenesis in testis with special reference to the role of cAMP as "second messenger" for trophic hormone action (chapter 3).

A. From experiments on the metabolism of steroids by brain tissue it has been concluded (appendix paper I) that:
   1. 3α- and 17β-Hydroxysteroid dehydrogenase and 5α-steroid reductase activities are present in brain tissue.
   2. Using ultracentrifugation techniques 3α- and 17β-hydroxysteroid dehydrogenase activities could be demonstrated in the cytosol, whereas 5α-steroid reductase was isolated in microsomes.
   3. Relative activities of 5α-steroid reductase are low in the pituitary and cortex when compared with the activity in total brain tissue. Relative 17β-hydroxy-steroid dehydrogenase activities were not different in hypothalamus, pituitary, cerebellum and cortex.

In chapter 2 the following results from the literature have been reviewed:

1. The uptake of steroids by brain tissue
2. The possible significance of steroid metabolism for the mechanism of action of steroids in the brain
3. The effects of steroids on metabolic processes in brain.

B. From experiments on the role of cAMP in the mechanism of action of trophic hormones on testosterone production in different testicular tissues it has been observed (appendix papers II, III and IV) that:

1. Consistent stimulation of cAMP and testosterone production in testis tissue in vitro by LH and HCG could only be obtained after preincubation of the testis tissue.
2. cAMP may be considered as "second messenger" for trophic hormone action in testis because:
   a. with HCG and LH an increase of the cAMP production was observed which preceded the increase of the testosterone production in testis tissue.
   b. dibutyryl-cAMP stimulated the testosterone production in total testis.
3. The purity of isolated seminiferous tubules can be characterized by the activity of non-specific esterase which is mainly localized in interstitial tissue. From the distribution of esterase over tubules and interstitial tissue the relative amount of interstitial tissue was calculated to be between 13 and 23%.
4. The minimum amounts of LH required to stimulate measurable cAMP and testosterone production in isolated interstitial tissue (5 mg wet weight) were 200 ng LH/ml and 20 ng LH/ml respectively.
5. The production of testosterone expressed per amount of total testis tissue during incubations in the presence of LH was lower in interstitial tissue compared to total testis tissue.

6. Testosterone production in interstitial tissue in the presence of LH can be stimulated with glucose.

In chapter 3 results from the literature and from experiments described in the appendix papers II, III and IV are reviewed. For the role of cAMP in the regulation of steroidogenesis it has been concluded that:

1. For detailed investigation of the biochemical mechanisms of trophic hormone action, tissue fractionation is required for obtaining homogeneous tissue or cell preparations.
2. It is unlikely that endogenous steroid production occurs in seminiferous tubules.
3. The presence of adenylcyclase activity in nuclear and mitochondrial fractions may indicate actions of trophic hormones without cAMP as intracellular second messenger.
4. The measured production of cAMP and testosterone in the testis may be an underestimation of the real production because of the presence of metabolizing enzymes for the two compounds.
5. Gonadotrophic stimulation of endogenous cAMP and testosterone production in unteased testis, teased testis and interstitial tissue indicates that interactions between seminiferous tubules and interstitial tissue may influence the production of cAMP and testosterone.

SAMENVATTING

Hormonen spelen een belangrijke rol bij de interaktie tussen de hersenen en de testis. De sekretie van gonadotrofinen in de hersenen wordt o.a. gereguleerd door steroiden geproduceerd in de testis; deze gonadotrofinen hebben vervolgens een invloed op de steroidproduktie en de spermatogenese in de testis (hoofdstuk 1). De momenteel bestaande opvattingen over dit systeem met inwendige terugkoppeling in de rat zijn in hoofdzaak verkregen uit biologische en anatomische onderzoekingen. Er is weinig bekend over de biochemische mechanismen die optreden bij de interaktie tussen de hormonen in de hersenen en in de testis. Daarom is onderzoek verricht over:

A. Het metabolisme van steroiden in hersenweefsels als mogelijke faktor in de regulatie van de gonadotrofinesekretie (hoofdstuk 2).
B. De regulatie van de biosynthese van steroiden in de testis; in het bijzonder de betekenis van cyclisch adenosinemonofosfaat (cAMP) als "tweede boodschapper" bij de werking van gonadotrofe hormonen (hoofdstuk 3).

A. Onderzoekingen over het metabolisme van steroiden door hersenweefsels hebben tot de volgende konklusies geleid (artikel I van de appendix):
   1. In hersenweefsel is 3α- en 17β-hydroxysteroid dehydrogenase en 5α-steroid reductase aktiviteit aanwezig.
   2. Met behulp van ultracentrifugatie bleek dat de 3α- en 17β-hydroxysteroid dehydrogenase aktiviteiten zijn gelokaliseerd in de cytosol van hersenweefsel homogenaten, terwijl 5α-steroid reductase geïsoleerd kan worden in de microsomen.

3. De relatieve aktiviteit van 5α-steroid reductase in hypofyse en in cortex is laag in vergelijking met de aktiviteit in totaal hersenweefsel. De relatieve 17β-hydroxysteroid dehydrogenase aktiviteiten in hypothalamus, hypofyse, cerebellum en cortex zijn onderling niet verschillend.

Tevens bevat hoofdstuk 2 een samenvatting van gegevens over:

1. de opname van steroidhormonen door hersenweefsel
2. de mogelijke betekenis van steroid metabolisme in de hersenen voor het werkingsmechanisme van steroiden
3. de effekten van steroiden op metabole processen in de hersenen.

B. Uit de resultaten van experimenten over de funktie van cAMP bij het biochemisch werkingsmechanisme van trofe hormonen op de biosynthese van testosteron in de testis (artikelen II, III en IV van de appendix) zijn de volgende konklusies getrokken:

1. Stimulatie van cAMP en testosteron produktie in testisweefsel in vitro door LH of HCG kon alleen worden waargenomen na preinkubatie van het weefsel.
2. cAMP kan worden beschouwd als "tweede boodschapper" bij de werking van trofe hormonen op de testis omdat:
   a. in aanwezigheid van LH of HCG een stimulatie van de cAMP produktie is waargenomen voorafgaande aan de stimulatie van de testosteron produktie.
   b. dibutyryl-cAMP de testosteron produktie in totaal testisweefsel stimuleert.
3. De zuiverheid van geïsoleerde seminifere tubuli kan gekarakteriseerd worden op grond van de aktiviteit van niet-specifieke esterase in interstitieel weefsel. Uit de verdeling van esterase aktiviteit over seminifere tubuli en interstitium is berekend dat de hoeveelheid interstitieel weefsel 12 tot 23% van de totale testismassa bedraagt.

4. De kleinste hoeveelheden LH nodig voor de meetbare stimulatie van de cAMP en testosteron produktie in circa 5 mg (nat gewicht) interstitieel weefsel waren respektievelijk 200 ng LH/ml en 20 ng LH/ml.
5. De produktie van testosteron uitgedrukt per gewichtshoeveelheid totaal testisweefsel tijdens inkubaties in aanwezigheid van LH was voor interstitieel weefsel lager dan voor totaal testisweefsel.
6. De testosteron produktie in interstitieel weefsel in aanwezigheid van LH werd gestimuleerd door glucose in het inkubatie medium.

In hoofdstuk 3 is een samenvatting gegeven van resultaten uit de literatuur en van de in de artikelen II, III en IV van de appendix beschreven experimenten over de mogelijke funktie van cAMP bij de produktie van steroiden in de testis. Hieruit zijn de volgende konklusies getrokken:

1. Voor het onderzoek naar het verband tussen de produktie van cAMP en testosteron moet bij voorkeur een zo homogeen mogelijk weefsel- of celpreparaat worden gebruikt.
2. Het is onwaarschijnlijk dat in de seminifere tubuli endogene testosteron produktie plaats vindt.
3. De aanwezigheid van adenylcyclase aktiviteit in kern- en mitochondriale frakties kan een aanwijzing zijn dat de werking van trofe hormonen niet uitsluitend plaats vindt via cAMP als intracellulaire "tweede boodschapper".
4. Door de aanwezigheid van metaboliserende enzymen voor cAMP en testosteron in de testis, is het waarschijnlijk dat de experimenteel bepaalde produktie van deze verbindingen kleiner is dan de werkelijke.
5. Uit het effekt van gonadotrofinen op de produkties van cAMP en testosteron in verschillende testispreparaten kunnen aanwijzingen verkregen worden dat een interaktie tussen het interstitieel weefsel en de seminifere tubuli een effekt op de testosteron en cAMP produktie kan hebben.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren in 1943 te Ede en behaalde in 1960 het diploma H.B.S.-B aan het Marnix College te Ede. In hetzelfde jaar werd met de scheikundestudie begonnen aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen in de wiskunde en natuurwetenschappen (letter g) werd afgelegd in 1964. Het doctoraal diploma scheikunde (hoofdvak: analytische chemie, bijvak: farmakologie) werd in juli 1968 behaald. Gedurende de periode augustus 1963 tot augustus 1966 was hij als studentassistent verbonden aan het Analytisch-Chemisch Laboratorium van de Rijksuniversiteit Utrecht en vanaf augustus 1966 tot augustus 1968 aan de Medische Faculteit Rotterdam te Rotterdam. Sinds augustus 1968 is hij als wetenschappelijk medewerker verbonden aan de afdeling Biochemie II van de Medische Faculteit Rotterdam waar het hier beschreven onderzoek werd verricht. In oktober 1970 werd het diploma behaald voor Brandwacht 2e klasse (beschikking Ministerie van Binnenlandse Zaken 15-1-1970 nr. EB 70/U 23).

APPENDIX PAPERS

Reprinted from

Biochimica et Biophysica Acta
Elsevier Publishing Company, Amsterdam - Printed in The Netherlands
BBA 55950

OCCURRENCE AND LOCALIZATION OF 5α-STEROID REDUCTASE, 3α- AND 17β-HYDROXYSTEROID DEHYDROGENASES IN HYPOTHALAMUS AND OTHER BRAIN TISSUES OF THE MALE RAT*

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(Received July 1st, 1971)

SUMMARY

1. The presence of steroid-converting enzymes in different brain areas as well as the subcellular distribution of these enzymes have been studied.

2. Identification of metabolites following incubations of various steroids with brain tissue indicated that 5α-steroid reductase (EC 1.3.1.99) and 3α- and 17β-hydroxysteroid dehydrogenases (E.C. 1.1.1.50 and EC 1.1.1.51) are present.

3. The subcellular localizations of these steroid-converting enzymes were studied with ultracentrifugation techniques. From the comparison of the specific activities of steroid-converting enzymes with marker enzymes (NADH-cytochrome *c* reductase and lactate dehydrogenase) and other characteristic parameters (RNA, DNA and protein content) it was concluded that hydroxysteroid dehydrogenases are present in the soluble fraction and the 5α-steroid reductase in the microsomes.

4. Ratios of the specific activities of 5α-steroid reductase in different brain tissues relative to the specific activity in total brain were: hypophysis, 0.3; hypothalamus, 1.0; cerebellum, 1.6; cortex, 0.3. No significant differences were found between the specific activities of 17β-hydroxysteroid dehydrogenase in the different brain tissues.

INTRODUCTION

Certain steroid hormones influence the secretion of gonadotrophins from the pituitary[1-8]. The mechanism by which steroids regulate this secretion is unknown. It

Abbreviations: The following trivial names have been used in this paper: Progesterone, 4-pregnene-3,20-dione; androstenedione, 4-androstene-3,17-dione; testosterone, 17β-hydroxy-4-androsten-3-one; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; oestradiol, 1,3,5(10)-oestratriene-3,-17β-diol; androsterone, 3α-hydroxy-5α-androstan-17-one; oestrone, 3-hydroxy-1,3,5(10)-oestratrien-17-one; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; 5α-pregnanedione, 5α-pregnane-3,20-dione; 5α-androstanedione, 5α-androstane-3,17-dione; 5α-steroid reductase, 5α-steroid: NAD(P) Δ^4-oxidoreductase; 3α-hydroxysteroid dehydrogenase, 3α-hydroxysteroid: NAD(P) oxidoreductase; 17β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid: NAD(P) oxidoreductase.

* Presented in part at the third International Congress on Hormonal Steroids, Hamburg, September 7–12, 1970 (abstr. 488).

has been shown that after administration of radioactively labelled oestradiol and testosterone, the radioactivity was found to be taken up selectively in specific brain areas such as the hypothalamus and hypophysis[4–7]. However, in most of these studies the nature of the radioactivity was not identified. Catabolites of steroids were until recently considered biologically inactive compounds. Since the demonstration that dihydrotestosterone as a catabolite from testosterone can act as a physiologically active substance *in vivo*[8], the possibility should be considered that catabolites of the hormonal steroids can also influence the secretion of gonadotrophins. We have studied the occurrence and distribution of different steroid converting enzyme activities in different brain areas, as well as in subcellular fractions of brain tissue. While this study was in progress the *in vitro* metabolism of testosterone by brain tissue has been reported[9–15].

MATERIALS AND METHODS

Solvents used for extraction, crystallization and chromatography were analytical grade and redistilled before use. Unlabelled steroids were obtained from Steraloids and recrystallized before use.

Labelled steroids. [4-^{14}C]progesterone (60 mC/mmole), [1,2-^{3}H]progesterone (50 C/mmole), [4-^{14}C]androstenedione (60 mC/mmole), [1,2-^{3}H]androstenedione (50 C/mmole), [4-^{14}C]testosterone (60 mC/mmole), [1,2-^{3}H]dihydrotestosterone (44 C/mmole) obtained from New England Nuclear Corporation or the Radiochemical Centre, were purified by paper chromatography before use. Compounds were accepted as pure when less than 0.1% impurities were present.

Substrates and co-factors for enzyme assays were obtained from Boehringer.

For incubations of steroids with brain tissue two different incubation media were used: (1) For incubations of whole brain tissue approximately 10 mg tissue protein was suspended in 1 ml Krebs–Ringer buffer pH 7.4 containing 121 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 16.5 mM Na_2HPO_4 and 10 mM glucose. (2) For incubations with subcellular fractions, approximately 10 mg tissue protein was suspended in 1 ml of a phosphate buffer pH 6.5 (ref. 16) containing 1 mM KH_2PO_4, 1 mM $MgCl_2$ and 0.32 M sucrose.

Brain tissues were obtained from 3–6-month-old white male Wistar rats, weighing 200–300 g. The rats were killed by decapitation and the total brain was removed within 1 min, and immediately cooled in cold buffer solution (0°). When necessary, brain tissue of different rats was pooled.

The hypophysis was isolated in total. For isolation of hypothalamic tissue, the brain was placed on its dorsal surface and the following cuts were made: (see ref. 17) (1) Transversely through the optic chiasma. (2) Transversely near the mammillary bodies. (3) Bilaterally sagitally at a 3-mm distance from the midline. (4) For the ventral part, horizontally 1 mm under the basal surface. (5) For the dorsal part, horizontally 2 mm under the basal surface.

For isolation of cortical tissue thin sections of about 1 mm thick were sliced from the dorsal surface. The cerebellum was isolated in total. Different brain tissues were homogenized in Krebs–Ringer solution or in the buffered 0.32 M sucrose solution. When subcellular fractions were prepared homogenization conditions were: tissue

concentration 10% (w/v), speed of teflon pestle 800–1000 rev./min, ten up and down strokes, clearance between teflon pestle and glass tube wall 0.20–0.25 mm.

For isolation of subcellular fractions[18] (see also Fig. 1) the homogenate of whole brains was centrifuged at $900 \times g$ for 10 min and the supernatant was decanted. The sediment was washed once by resuspending the pellet in half the original volume

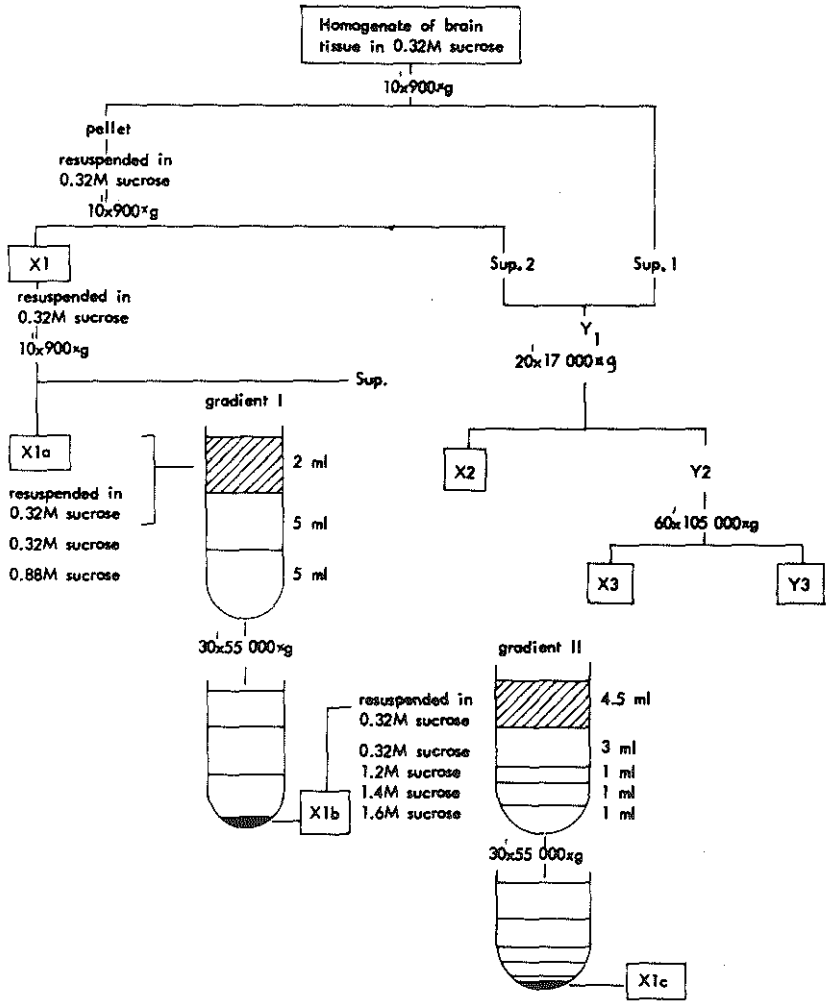

Fig. 1. Flow sheet for isolation of various subcellular fractions from brain tissue homogenates. For details see MATERIALS AND METHODS.

sucrose solution followed by centrifugation at $900 \times g$ for 10 min. The washed pellet was used as fraction X_1. The combined supernatant fractions Y_1 were centrifuged at $17\,000 \times g$ for 20 min and a supernatant fraction Y_2 was decanted. The pellet was used as fraction X_2. Subsequently the supernatant fraction Y_2 was centrifuged at $105\,000 \times g$ for 60 min and a supernatant fraction Y_3 was decanted. The pellet was used as fraction X_3. The precipitates (X) were resuspended in sucrose solution to give a protein

concentration of about 10 mg protein/ml. The Y_3 fraction always contained in the order of 3 mg protein/ml. In some cases fraction X_1 was further purified to enrich the nuclear content. Therefore fraction X_1 was washed again by resuspending the pellet to half the original volume of sucrose solution, and centrifuged at $900 \times g$ for 10 min, which gave a precipitate X_{1a}. For further purification fraction X_{1a} was resuspended in one tenth of the original volume sucrose solution and 2-ml portions were layered over a discontinuous gradient prepared by filling tubes with 5 ml 0.88 M sucrose and 5 ml 0.32 M sucrose. After centrifugation at $55000 \times g$ for 30 min in a Beckmann SW 40 Ti rotor, a pellet X_{1b} could be obtained. For a last purification, X_{1b} was centrifuged again for 30-min at $55000 \times g$ on a gradient prepared by filling tubes with respectively 1 ml 1.6 M sucrose and 4.5 ml suspension of X_{1b}. The pellet after centrifugation was designated X_{1c}. The different X fractions were resuspended in 0.32 M sucrose solution at a protein concentration of about 10 mg protein/ml.

Incubations

Unlabelled and ^{14}C- or ^{3}H-labelled steroid substrates dissolved in benzene–methanol, (9:1, by vol.) were pipetted into 5-ml incubation flasks.

Following the addition of 4 drops propyleneglycol–methanol, (1:10, by vol.) the solvents were evaporated at 40° under a nitrogen stream. To the residue was added 0.3 ml aqueous solution containing 3.3 mM $NADP^+$, 33 mM glucose 6-phosphate, 460 mU/ml of glucose-6-phosphate dehydrogenase. Subsequently the incubation was started by addition of 1 ml of the tissue suspensions (2–10 mg protein). For each steroid substrate a blank incubation was performed with a tissue fraction that was boiled for 5 min. Incubations were carried out at 37° in air.

To study the qualitative aspects of steroid converting enzymes 2 μg ^{14}C-labelled steroids containing 10^6 disint./min were incubated for 3 h. To study the quantitative aspects of the steroid converting enzymes, 15 μg ^{14}C- or ^{3}H-labelled steroids contaning 10^6 disint./min were incubated for 1 h. The incubations were terminated by addition of 4 drops glacial acetic acid and 30 μg of non-radioactive progesterone, testosterone and androstenedione were added to the media as carrier steroids.

Extraction, purification and isolation of steroids

The incubation media were extracted 3 times with 2 ml of diethylether each time. The combined ether extracts were evaporated under nitrogen at 40° and the residue was dissolved in 2 ml methanol–water, (1:1, v/v). To freeze out fatty material the methanol solutions were kept overnight at −20°. After centrifugation at $1000 \times g$ for 10 min at −10° the supernatant was transferred to a clean centrifuge tube, evaporated and subjected to paper chromatography.

The latter was carried out at 22° on Whatman No. 20 paper strips, 2 cm wide and 50 cm long, in the system Bush A-2 containing ligroin–methanol–water, (50:35:15, by vol.) or the modified system Bush B-1 containing ligroin–benzene–methanol–water (25:25:35:15, by vol.). Detection of radioactive compounds was carried out with a Packard radiochromatogram scanner. Steroid fractions were eluted from paper with methanol.

Radioactivity was measured with a liquid scintillation counter. Crystalline steroid fractions were counted in 15 ml of a toluene solution containing 4 g diphenyl-

oxazole (PPO) and 40 mg 1,4-*bis*-2-(5-phenyl oxazolyl) benzene (POPOP) per l. Water-containing fractions were counted in 15 ml of a dioxane solution containing 60 g naphthalene, 4 g PPO, 200 mg POPOP, 100 ml methanol and 20 ml ethylene glycol per l. Fractions containing tissue residues were counted in 10 ml Insta-Gel (Packard Instruments Co.). Quench corrections were applied with a channels ratio counting procedure or with an external standard counting procedure. Steroid fractions were characterized by the chromatographic behaviour of their derivatives and by crystallization to constant specific activities after addition of pure reference steroids.

Reductions of steroids were performed with sodium borohydride. 0.1 mg $NaBH_4$ was dissolved in 1 ml cold methanol and added to the dry steroid extract. The mixture was kept at 0° for 30 min and, following addition of 2 ml water, extracted twice with 1 ml ethyl acetate.

Oxidations of steroids were performed with chromium trioxide. 0.2 ml of a solution containing 1 mg CrO_3 in 90% acetic acid was added to the dried steroid extract. The mixture was kept at room temperature for 1 h. After adding 2 ml distilled water extraction was carried out with two times 1 ml ethyl acetate. Subsequently the extract was washed twice with 1 ml 8% $NaHCO_3$ solution and once with 1 ml distilled water.

Acetylations were performed by incubating the dried steroid extracts with 0.2 ml pyridine and 0.2 ml acetic anhydride for 2 h in a desiccator, followed by evaporation to dryness.

Crystallization to constant specific activity. After addition of 100 mg of appropriate carrier steroid to the radioactive steroid containing about 10^5 disint./min, crystallizations were carried out in several solvent mixtures (see RESULTS). Samples from various crystal and liquor fractions containing about 5–10 mg steroid were taken and the mass was determined by weighing in a counting vial. The corresponding radioactivity was measured after adding counting fluid.

Subcellular tissue fractions were characterized by analysing the distribution patterns of marker enzymes or other specific parameters for cell constituents.

Lactate dehydrogenase (EC 1.1.1.27) activity in various fractions was assayed by the oxidation of NADH according to the method of JOHNSON[19].

NADH cytochrome c reductase (EC 1.6.2.2) activity measurements were measured as described by SOTTOCASA *et al.*[20], except that antimycine A (1 μg/ml) was used instead of rotenone.

Cytochrome c oxidase (EC 1.9.3.1) was assayed polarographically by measuring oxygen consumption with a Clark electrode at 37° in 1.7 ml solution containing 75 mM potassium phosphate buffer, pH 7.4, 60 μM cytochrome *c* and 1.7 mM sodium ascorbate and cytochrome *c* oxidase. Mitochondria were preincubated for 1 h at 0° in 0.2% Lubrol-WX (I.C.I. Comp.) in order to release latent cytochrome *c* oxidase activity.

Carboxyl esterase (EC 3.1.1.1) activity was assayed spectrophotometrically by measuring the rate of hydrolysis of *p*-nitrophenylacetate at 400 nm. The incubation medium contained 0.1 M Tris–HCl buffer pH 8 0.3 μM eserine, 1 mM *p*-nitrophenylacetate. The incubation was started by adding the enzyme solution.

Protein content was estimated according to the method of LOWRY *et al.*[21]. Solutions for standard curves were prepared in the same media as the unknown samples.

DNA content was estimated as described by BURTON[22].

RNA was isolated from fractions according to the method of FLECK AND BEGG[22]. The RNA content was calculated from ultraviolet absorption values at 260 and 233 nm, according to BALAZS AND COCKS[24]; μg RNA/ml = $13.4 \times (3.13\ A_{260} - 0.80\ A_{233})$.

Electron microscopic observations of sedimentable fractions were carried out after fixing the various pellets with glutaraldehyde and staining with osmium tetraoxide according to the method of DEL CERRO *et al.*[25].

Expression of results. Activities of 5α-steroid reductase (EC 1.3.1.99) and 3α- and 17β-hydroxysteroid dehydrogenases (EC 1.1.1.50 and EC 1.1.1.51) were expressed as nmole specific metabolite formed per h. Quantitation was carried out by estimating the radioactivity in the metabolite fraction in relation to the total radioactivity after elution from paper. The amount of formed metabolite was estimated by multiplication of the percentage conversion as found after paper chromatography with the total amount of substrate present at the beginning of the incubation.

RESULTS

The characterization of metabolites

After incubations of ^{14}C-labelled progesterone, androstenedione and testosterone with total brain homogenates, paper chromatographic analysis of the extracts of the incubation media gave patterns of radioactivity on the paper strips as represented schematically in Fig. 2.

The pattern of radioactivity represented approximately 90% activity in the region of the original substrate and a few percentages of activity in regions different

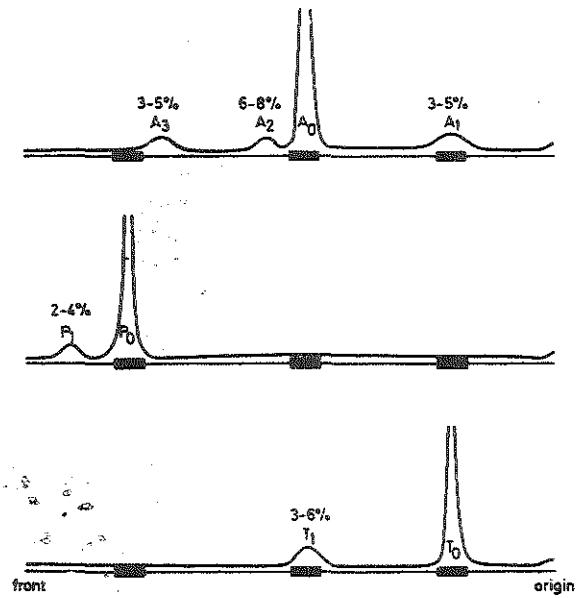

Fig. 2. Distribution of radioactivity after paper chromatography of the steroid extract in Bush A-2 system. The curves represent the amount of detected radioactivity. The bars indicate ultraviolet absorbing areas of reference steroids androstenedione (A), progesterone (P) and testosterone (T). The percentages indicated give the fraction of the total radioactivity that is present in the metabolite fractions. A_0, P_0 and T_0 are the unmetabolized steroid substrates.

from the original compound. Blanks contained only a single radioactive peak. Incubations with progesterone and testosterone gave rise to metabolites P_1 and T_1 respectively. The R_F value of T_1 was comparable with that of androstenedione. Incubations with A gave three metabolites A_1, A_2 and A_3. The R_F value of A_1 was comparable with that of testosterone and the R_F value of A_2 and A_3 was between those of P and A. Steroid fractions with comparable R_F values were eluted, combined and rechromatographed to check homogeneity. It was found that all the fractions indicated in Fig. 2 behaved as single compounds. To determine the identity of these steroids, chromatographic data of different derivatives were collected. Information concerning the presence or absence of hydroxyl groups or oxo groups was obtained through oxidation, reduction and acetylation of all the isolated steroids (see Table I). In all steroid fractions oxo groups were present. Hydroxyl groups could be detected in

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF DERIVATIVES OF DIFFERENT STEROID FRACTIONS ISOLATED DURING PAPER CHROMATOGRAPHY

The steroid fractions A originated from androstenedione, P from progesterone, and T from testosterone. Fractions indicated as A_0, P_0 and T_0 represent the unconverted substrates. Derivative formation has been carried out as described under MATERIALS AND METHODS. +, increase in R_F value; −, decrease in R_F value; o, no change in R_F value.

Steroid	*Change in R_F value through derivative formation*			*Indications for presence of:*	
	Oxidation	*Reduction*	*Acetylation*	*–OH group*	*=O group*
A_0	o	−	o	no	yes
A_1	+	−	+	yes	yes
A_2	+	−	+	yes	yes
A_3	+	−	o	no	yes
P_0	o	−	o	no	yes
P_1	o	−	o	no	yes
T_0	+	−	+	yes	yes
T_1	+	−	+	yes	yes

the steroids A_1, A_2, T_0 and T_1. In A_0, P_0, A_3 and P_1 no hydroxyl groups could be shown. The high R_F value of P_1 and A_3 compared to respectively P_0 and A_0 might reflect the absence of the double bond at C_4 in these catabolites. Steroids formed were identified by crystallization to constant specific activity (see Table II). When constant specific activities were obtained after crystallization of any of the various fractions with a particular carrier steroid, this was accepted as proof of the identity of the fraction. The identification of the various fractions was as follows: A_3 as 5α-androstanedione, P_1 as 5α-pregnanedione, T_1 as dihydrotestosterone, A_2 as androsterone and A_1 as testosterone. With other crystallization experiments (not in the table) A_0, P_0 and T_0 were identified as original substrates, respectively androstenedione, progesterone and testosterone.

Investigation of localizations of steroid converting enzymes

A study of the reaction velocity of the steroid converting enzymes as a function of time, tissue and substrate concentration showed that: (1) product formation was linear with time to about 75 min, (2) total tissue homogenates containing 3–20 mg protein gave reaction velocities that were linear with protein concentration and (3)

TABLE II

CRYSTALLIZATION TO CONSTANT SPECIFIC ACTIVITY (DISINT./MIN PER mg) OF ^{14}C-LABELLED CATABOLITES A_3, P_1, T_1, A_2 AND A_1 TOGETHER WITH 100 mg UNLABELLED STEROIDS

The catabolites were isolated from incubations of [^{14}C]androstenedione (A), [^{14}C]progesterone (P) and [^{14}C]testosterone (T) with brain tissue homogenates (see MATERIALS AND METHODS).

Samples	*Subfraction*	*Specific activities (disint./min per mg)*			
		Starting material	*Crystallization from*		
			Aqueous methanol	*Aqueous ethanol*	*Aqueous acetone*
A_3+5α-androstanedione	Crystals	335	337	334	333
	Mother liquor	—	347	333	337
P_1+5α-pregnanedione	Crystals	540	538	538	535
	Mother liquor	—	536	550	538
T_1+dihydrotestosterone	Crystals	432	347	334	330
	Mother liquor	—	526	402	338
A_2+androsterone	Crystals	354	349	350	347
	Mother liquor	—	362	350	350
A_1+testosterone	Crystals	245	252	251	243
	Mother liquor	—	270	257	251

reaction velocities were linear with substrate concentrations up to 30 μg steroid/ml. On the basis of these observations the following incubation conditions were chosen for studying the quantitative amounts of enzyme present: incubation time 1 h, protein concentration 5–10 mg, steroid concentration 15 μg/ml. Although this substrate concentration was not high enough to saturate the enzyme, the conversion of the substrate was so low (less than 10%) that the reaction velocity was found to be constant during 1 h. Most measurements of the 5α-steroid reductase activities were performed with testosterone as substrate. When progesterone or androstenedione were used as substrate, 5α-reductase activities could also be measured, although they were less accurate because it was more difficult to separate products from the incubated substrate and the conversion rates were lower. When activities in various subcellular fractions were compared and ratios calculated, no differences were observed when P, T or A were used. This may indicate that no different 5α-steroid reductases are present in brain tissue. 17β-Hydroxysteroid dehydrogenase activities were measured by incubating androstenedione. 3α-Hydroxysteroid dehydrogenase was measured by incubating dihydrotestosterone. Results of incubations of the steroid substrates with subcellular fractions X_1, X_2, X_3 and Y_3 are given in Fig. 3. Both the 3α- and 17β-hydroxysteroid dehydrogenases show the highest activity in the soluble fraction. For the 5α-reductase the relative specific activity is the highest in the microsomal fraction. This enzyme activity also appears to be located in the nuclear fraction, although quantitatively less than in the microsomes. Distributions of enzymes and other specific parameters in the 4 fractions were used for characterization of the subcellular fractions[26] (Fig. 4). The concentration of nuclei in the X_1 fractions is shown by the high relative specific activity value of DNA. In all other fractions the DNA content was very low. The highest activity of cytochrome *c* oxidase in the X_2 fraction is an indication for the concentration of mitochondria. Microsomes (fraction X_3) have been characterized with RNA, NADH–cytochrome *c* reductase and eserine insensitive carboxyl esterase. The first two parameters have been used as microsomal

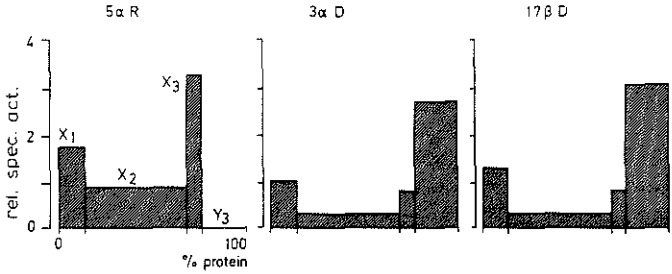

Fig. 3. Distribution of 17β-hydroxysteroid dehydrogenase (17βD), 3α-hydroxysteroid dehydrogenase (3αD) and 5α-steroid reductase (5αR) in various subcellular fractions X_1, X_2, X_3 and Y_3 of brain tissue. X_1 is nuclear fraction; X_2 is mitochondrial fraction; X_3 is microsomal fraction; Y_3 is supernatant fraction. The characterization of the fractions is given in Fig. 4. On the ordinate enzyme concentrations have been expressed as relative enzyme activity (rel. spec. act.) as the ratio of percent recovered activity to percent recovered protein. On the abcissa the percentages of recovered protein in the subcellular fractions have been indicated.

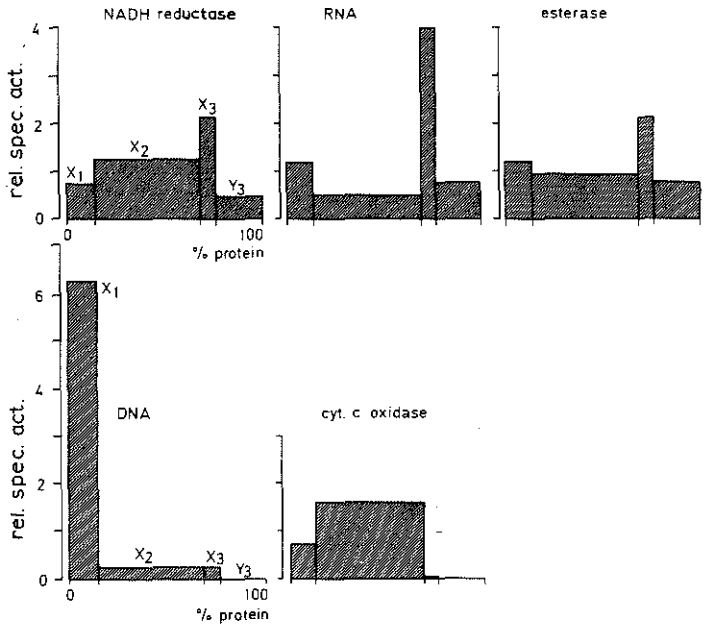

Fig. 4. Distribution of marker enzymes: NADH cytochrome *c* reductase (NADH reductase), carboxyl esterase (esterase), cytochrome *c* oxidase (cyt. *c* oxidase) and RNA and DNA in subcellular fractions of brain tissue X_1, X_2, X_3, Y_3. For further explanations see Fig. 3.

markers in brain[27,28] whereas the esterase has been used as a microsomal marker in liver[29]. The distribution patterns for the two microsomal enzymes and RNA were comparable. The relative specific activity values were the highest in the microsomal pellet. Lactate dehydrogenase was used as marker enzyme for the soluble fraction Y_3[30]. This enzyme activity was highest in the soluble fraction. Sedimentable fractions were also characterized by electron microscopy. Fraction X_3 appeared to contain the largest amounts of microsomes, although microsomes were also present in all other sedimentable fractions. For the distribution of the 5α-reductase it was found that

besides a high activity concentration in the microsomal fraction, the nuclear fraction also possessed a high activity. The X_1 fraction was further investigated. Fractions X_{1a}, X_{1b} and X_{1c} were prepared from X_1 for obtaining purified nuclear fractions with increasing content of DNA. With these fractions, incubations were carried out and the specific activities of the steroid reductase were expressed relative to DNA content (Table III). The results clearly demonstrate that the 5α-steroid reductase activity relative to DNA decreases during purification. This means that DNA and the steroid reductase behave differently.

TABLE III

SPECIFIC ACTIVITIES OF 5α-STEROID REDUCTASE IN VARIOUS NUCLEAR FRACTIONS OF BRAIN TISSUE

The 5α-steroid reductase activities are expressed as the amount of nmole steroid (dihydrotestosterone) formed per mg protein or μg DNA. Total homogenate and a washed 900 × *g* pellet (X_{1a}) were used as two nuclear fractions. Further purified fractions (X_{1b}, X_{1c}) were obtained by density gradient centrifugation with different sucrose gradients. The DNA to protein ratio has been used as a characteristic parameter for the purity of the fractions. For further details see MATERIALS AND METHODS.

Fraction	A $\left(\frac{\mu g\ DNA}{mg\ protein}\right)$	$B = 5\alpha\text{-}reductase$ $\left(\frac{nmole\ steroid}{mg\ protein}\right)$	$B/A = \frac{5\alpha\ reductase}{DNA}$ $\left(\frac{nmole\ steroid}{\mu g\ DNA}\right)$
Total homogenate	15	3.0	0.2
X_{1a} (900 × *g* pellet)	111	7.0	0.06
X_{1b} (gradient I)	123	5.0	0.041
X_{1c} (gradient II)	239	9.0	0.038

To study the enzyme distribution in various brain tissues, homogenates were made of two types of hypothalamic tissue, hypophysis, cortex, cerebellum and total brain tissue. Incubations with these homogenates were performed under the same conditions as for the study of the subcellular localization of enzymes. The specific activities of 17β-hydroxysteroid dehydrogenase and 5α-steroid reductase in the particular brain tissues have been expressed relative to the specific activity in total brain homogenates (Fig. 5). The hypophysis homogenates showed the characteristic ab-

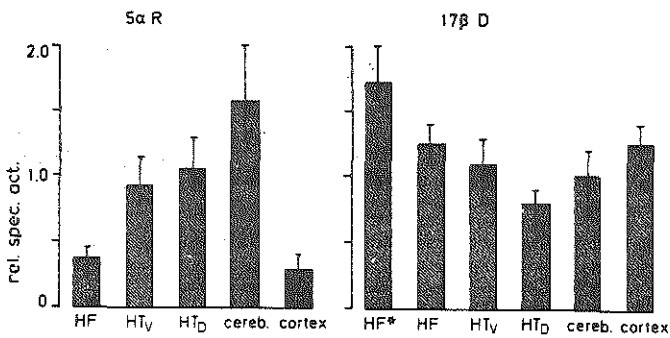

Fig. 5. Distribution of 5α-steroid reductase (5αR) and 17β-hydroxysteroid dehydrogenase (17βD) in hypophysis (HF), ventral and dorsal part of hypothalamus (HT_V and HT_D, respectively) cerebellum (cereb) and cortex. The enzyme concentrations in the different tissues have been expressed as relative specific activity (rel. spec. act. = the ratio of specific activity in a special tissue and the specific activity in total brain homogenate). Values are given as mean values of three experiments. Experimental errors have been indicated by the range.
* Not corrected for contaminating erythrocytes (see text).

sorption bands of haemoglobin at 540 and 570 nm, thus indicating the presence of haemoglobin from haemolyzed erythrocytes. Rat erythrocytes contain a high 17β-hydroxysteroid dehydrogenase activity[31,32]. Corrections have therefore been made for contamination of the homogenates by blood by measuring the dehydrogenase activity in an amount of diluted blood that was equal to the amount of blood present in the hypophysis homogenates. In the other brain tissues no haemoglobin could be detected by measurements at 540 and 570 nm. The relative specific activities of 5α-steroid reductase in hypophysis and cortex were lower than in hypothalamus and cerebellum. No other significant differences were found. For the 17β-hydroxysteroid dehydrogenase the measured relative specific activity in the hypophysis appeared to be high in comparison with hypothalamus, cerebellum and cortex. When corrected for the contamination with blood the 17β-dehydrogenase activity in hypophysis was comparable with activities in the other investigated tissues.

DISCUSSION

On the basis of the steroids isolated after incubations with various labelled precursors we conclude that brain tissue of the male rat contains 5α-steroid reductase, 17β-hydroxysteroid dehydrogenase and 3α-hydroxysteroid dehydrogenase. This confirms previous reports for the existence of 17β-hydroxysteroid dehydrogenase and 5α-steroid reductase in rat brain tissue[10–15]. The occurrence of the 3α-hydroxysteroid dehydrogenase activity in brain tissue has not been reported previously. Other enzymes or reactions in brain tissues that have been reported are 11β-hydroxysteroid dehydrogenase[33–36], 21-corticosteroid acetyltransferase[37,38] as well as the formation of sulfo conjugated dehydroepiandrosterone[39].

The significance of these *in vitro* investigations in relation to the metabolism of steroids by brain tissue *in vivo* is not known. LUTTGE AND WHALEN[40] found regional localization of oestrogen metabolites in rat brain tissue after intravenous injections of labelled oestradiol. They indicated a possible specific localization of 17β-hydroxysteroid dehydrogenase in brain. Some evidence for the *in vivo* activity of 5α-reductase also could be derived from experiments carried out in this laboratory (in collaboration with I. Kraulis) to study *in vivo* uptake of [^{3}H]testosterone by brain tissue of castrated male rats. After 15 min, brain tissue appeared to contain more labelled dihydrotestosterone than was present in plasma. However 5α-reductases outside the brain can also cause formation of dihydrotestosterone. Brain perfusion could give a better impression of the functions and capacity of the existing enzymes *in vivo*. We tried to study metabolism of implanted crystalline [^{14}C]progesterone in the mediobasal hypothalamus, but no radioactive metabolites could be detected in brain tissue. After one day the radioactivity present in the brain was still progesterone and part of the radioactivity had leaked into the circulation. A finding which does not support a physiological function for 5α-reduced metabolites was the observation of BEYER *et al.*[41], who found that *in vivo* dihydrotestosterone injections did not alter oestrous behaviour in ovariectomized rabbits. However, the fact that injections are not comparable with endogenously produced steroids cannot be ignored.

The subcellular distributions of the steroid-reducing enzymes have been studied with 4 subcellular fractions. Conclusions have been made by comparing distribution patterns of DNA, RNA and marker enzymes with the steroid-reducing enzymes. The

3α- and 17β-hydroxysteroid dehydrogenases were found to be soluble because the enzyme distribution pattern was comparable with lactate dehydrogenase, which has been used many times as cytoplasmic marker enzyme[30]. The finding that distribution patterns of sedimentable subcellular fractions were not comparable with the two steroid dehydrogenases also indicates the presence of 3α- and 17β-hydroxysteroid dehydrogenases in the soluble fraction. The distribution pattern of 5α-steroid reductase was found to be comparable with microsomal markers RNA, antimycine-insensitive NADH–cytochrome *c* reductase and eserine insensitive carboxyl esterase[27–29]. Particularly the highest relative specific activity value in the microsomal fraction was clear. Distribution patterns from markers of nuclei, mitochondria and the cytoplasma were different from the 5α-steroid reductase. We conclude from these findings that the 5α-steroid reductase is predominantly microsomal bound. However, there was a small concentration of 5α-steroid reductase activity in the nuclear fraction, in contrast to the three microsomal markers. Although quantitatively this localization may be less significant, qualitatively it can be of importance. It has been suggested that the specific metabolism of testosterone to the physiologically very active dihydrotesterone in the nuclei of the prostate might have wide implications[42]. When 5α-steroid reductase is present in nuclei, brain tissue could be compared with the prostate[43]. The present study of the 5α-steroid reductase activity in different purified nuclear fractions showed a different behaviour of DNA and 5α-steroid reductase activity. The 5α-steroid reductase activity relative to DNA decreased during purification. We therefore concluded that the reductase activity in the nuclear fraction could be explained by a microsomal contamination or a very loosely nuclear bound 5α-reductase activity. The presence and localization of 5α-reductase in microsomes of brain tissue together with the soluble 3α- and 17β-hydroxysteroid dehydrogenases can be compared with the situation occurring in liver[44]. However, the localization study has been done with total brain tissue and because brain is composed of many different cell types, different subcellular distributions may exist in different cell types.

The distribution of steroid-reducing enzymes in different brain areas can possibly give indications to specific properties or functions. 5α-Reductase activities in pituitary and cortex homogenates were found to be low in comparison with hypothalamus and cerebellum. Both JAFFE[10] and KNIEWALD *et al.*[13] found after incubations of minces more dihydrotestosterone in pituitary than in the hypothalamus. The discrepancy between these results may be caused by differences in the preparation of the tissue.

The 17β-hydroxysteroid dehydrogenase activity in hypophysis homogenates was high in relation to total brain homogenates. High activities of 17β-hydroxysteroid dehydrogenase in pituitary tissues were also observed by JAFFE[10]. These observations must be interpreted carefully, because the pituitary is highly vascularized and therefore contains much blood with a relatively high 17β-dehydrogenase activity[31,32]. After correcting for this contamination, no differences in 17β-dehydrogenase activity in the different tissues could be observed in our experiments.

Studies that have been done on steroid metabolism in brain tissue so far have in common that they are not specific for cells responsible for the regulation of gonadotrophins. At this moment it is known that the neurons responsible for regulation of the secretion of gonadotrophins are localized in particular areas but even there they are always mixed with other cell types[3]. All preparations that do not contain isolated

neurons therefore give "diluted information". Histochemical techniques could possibly give more selective information than the incubation techniques described, but we could not show any dehydrogenase activity in brain tissue with histochemical techniques. To study specific interactions of steroids with specified cells in brain tissue, other techniques, for example microdissection, will have to be applied.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to Miss A. van der Kemp, Mrs. H. Rockx, Mr. P. Wordsworth and Mr. W. van Ewijk for their expert assistance and to Dr. I. Kraulis, Dr. J. Moll and Dr. G. P. van Rees for helpful discussions.

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DISSECTION OF WET TISSUE AND OF FREEZE-DRIED SECTIONS IN THE INVESTIGATION OF SEMINIFEROUS TUBULES AND INTERSTITIAL TISSUE FROM RAT TESTIS

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Received for publication August 3, 1972

Summary

Seminiferous tubules and interstitial tissue were dissected out from freeze-dried sections and from wet tissues of the rat testis. The results of these preparation procedures were compared in regard to the distribution of a nonspecific esterase activity and of radioactive labeled steroids. Nonspecific esterase activity was found 50 times higher in interstitial tissue than in the seminiferous tubules, when samples dissected from wet tissue were analyzed. When specimens from freeze-dried sections were used for assays, this ratio was somewhat lower. In normal rat testes, the amount of interstitial tissue varied from 13 to 23%. The percentage of interstitium increased to about 50% in rats fed a diet lacking essential fatty acids. In seminiferous tubules isolated by the wet dissection technique there was no indication of the presence of interstitial tissue. Both fractionation procedures are useful in the analysis of enzyme activities, but the dry dissection method is preferable for studying the distribution of diffusible compounds, like steroids, because

during wet dissection some redistribution of labeled steroids did occur.

Introduction

In the testis there are at least two functionally different tissue compartments: the interstitial tissue which contains the steroid-producing Leydig cells, and the seminiferous tubules which contain the germ cells in various stages of spermatogenesis and the Sertoli cells. The physiology of these compartments and their interrelationship cannot be adequately understood without a separate analysis of each type of tissue. To accomplish this purpose, Christensen and Mason introduced a procedure by which the tubules and the interstitium can be separated by the dissection of the wet tissue (5). This technique has been used in studies on steroid production in both tissue fractions (5, 6, 11). Galjaard et al. (10) employed Lowry's dissection technique (12) using freeze-dried cryostat sections from which they isolated, under the microscope, seminiferous tubules and interstitium. They applied this procedure to the study of steroid transport between the two tissue compartments.

The purpose of the present paper is to compare the two separation procedures in regard to the purity of the tissue types that were isolated. The purity and cross-contamination of the isolated fractions were checked by analyses of enzyme activities and of radioactive labeled steroids.

Materials and methods

Testicular tissue was obtained from normal adult male rats (Wistar strain), 3-4 months old, weighing 200-250 g, or from "essential fatty acid (EFA)-deficient rats" that

were fed a diet lacking essential fatty acids. Hypophysectomized rats were used 1 week after hypophysectomy. The rats were killed by decapitation. The testes were removed and the testicular tissue was obtained by the removal of the tunica albuginea.

Wet dissection of the testicular tissue as described by Christensen and Mason (5), was performed on a petri dish maintained at $0^{\circ}C$. Tubules were removed from the tissue by the use of jeweler's forceps, and these tubules will henceforth be referred to as the "unwashed tubules fraction." The interstitial tissue fraction was examined under a dissection microscope in order to remove any small fragments of tubules. Remaining minute tissue fragments, cells and condensed water on the wet dissection Petri dish were collected by rinsing with a buffer solution (35 mM Na_2HPO_4 and 15 mM KH_2PO_4); this material is referred to as the "residue fraction." In another Petri dish, other seminiferous tubules were dispersed into a phosphate buffer and were washed three times, each time with a fresh buffer solution. The tubules were collected with forceps from the solutions after each of the washings, but small pieces of tubules were left in the washing fluids. The tubules washed in the phosphate buffer are specified in this paper as the "washed tubules fraction."

Dry dissection was carried out according to Lowry's method (12). Testicular tissue was frozen in isopentane cooled with liquid nitrogen ($-155^{\circ}C$); cryostat sections (6-20 μ) were cut at $-15^{\circ}C$ and freeze-dried in glass tubes ($-45^{\circ}C$ and 10^{-3} mm Hg) overnight. The lyophilized sections were used for microscopic dissection at $20^{\circ}C$ and 40% relative humidity. The seminiferous tubules and interstitial tissue were isolated by using razor blade fragments and hair points. The dry weights of the isolated tissue specimens (0.1-1 μg) were determined by weighing them on quartz fiber balances (4).

Nonspecific esterase activity was measured using either phenylacetate or α-naphthylacetate as substrate.

Phenyl esterase activity was assayed in the testicular tissue compartments isolated by wet dissection. The enzyme solution was prepared by sonication for 15 sec at 20 kHZ with an amplitude of 5 μm at 0°C of 10-50 mg tissue in 0.5 ml phosphate buffer. This solution could be stored at -20°C without loss of enzyme activity for at least 1 week. The enzyme solution was added to 0.1 M Tris-HCl buffer (pH 8) containing about 1 mM p-nitrophenylacetate, and the rate of hydrolysis of p-nitrophenylacetate at 23°C was measured at 400 nm.

Naphthyl esterase activity was determined using the method of Galjaard et al. (9).

Protein was measured according to Lowry et al. (13).

Histochemical staining for nonspecific esterase activity was done according to Pearse (22). Cryostat sections (6-10 μ) of testicular tissue and washed tubules were fixed in neutral formalin for 10 min. After rinsing with distilled water, the sections were incubated at 20°C for 2 min in a solution containing 20 ml 0.15 M Na_2HPO_4, 0.5 ml 1% α-naphthylacetate in 50% acetone and 1.6 ml hexoazonium salt of pararosanilin (0.8 ml 4% $NaNO_2$ and 0.8 ml 4% pararosanilin in 2 M HCl). Incubation of the sections was terminated by rinsing with distilled water.

Electrophoresis: The isoenzyme pattern of esterases was obtained by electrophoresis using a 7.5% polyacrylamide gel (15). Protein solutions were applied in 10-μl fractions; the concentrations (milligrams of protein per milliliter) used in the different fractions were 0.4-2 for interstitial tissue, 0.4-2 for testis tissue and 40-80 for seminiferous tubules. A current of 2 ma/tube was applied for 5 min and was then increased to 4 ma (150 volts). The electrophoresis was terminated 20 min after the indicator bromophenolblue had moved through the lower (anodal) surface of the gel. The gels were then incubated at 37°C for 10 min in accordance with the procedure employed by Markert and Hunter (14); α-naphthylacetate was used as the substrate and fast blue BB, as a coupling agent.

Infusion of radioactive steroids: Testis tissue was labeled with radioactive steroids by the infusion of [^{3}H]-pregnenolone, (10-40 µC; 0.2-0.8 µg) in 2 ml solution containing 1% Tween 20 and 0.9% NaCl, into rats under ether anesthesia (8). Important side branches of the aorta were ligated, and the aorta itself was clamped off just above its junction with the internal spermatic artery. After ligating the arteries, the injection was made for a 2-min period retrograde in the aorta immediately below the spermatic artery. After the radioactive infusion, blood was flushed through the testis for 2 min, and the testis was then removed.

Radioactivity in tissue was measured with a liquid scintillation counter. The tissue samples were digested in 1 ml Soluene (Packard Instruments Company) at 20°C followed by the addition of 15 ml of a toluene scintillation solution containing 4 g diphenyloxazole and 40 mg 1,4-bis-2-(5-phenyloxazolyl)benzene/liter.

In protein-containing solutions, radioactivity was measured after the addition of 10 ml Instagel (Packard Instruments Company). Quench corrections were applied with an external standard ratio procedure.

Results

Specific activity of esterase as a marker for interstitial tissue: In the normal rat testis a high naphthyl esterase activity has been demonstrated histochemically in the interstitial tissue, whereas in the seminiferous tubules hardly any activity was detected (3, 19). These findings were confirmed by the quantitative determination of the phenyl esterase activity in testicular tissue, and in washed seminiferous tubules and interstitial tissue that were isolated by a wet dissection (Table I). The specific activity of phenyl esterase was always much higher in the interstitium than in the tubules; in testes from

TABLE I

Specific Activities of Phenyl Esterase (Micromoles of Nitrophenol per Minute per Milligram of Protein; Mean Value ± S.D. and Number of Observations) in Preparations of Rat Testis Tissue

Preparations	Normal	EFA-Deficient*	1 Wk. after Hypophysectomy
Intact testis	0.39 ± 0.11(17)	4.45 ± 0.9(4)	0.07 ± 0.005(4)
Washed tubules (a)	0.045 ± 0.013(17)	0.17(2)	0.039 ± 0.005(4)
Interstitial tissue (b)	2.35 ± 1.2(17)	9.3(2)	0.26 ± 0.03(4)
b/a	52	55	7

* EFA-deficient rats were fed a diet lacking essential fatty acids.

normal and EFA-deficient rats this difference was about a factor of 50. In EFA-deficient rats phenyl esterase activity of the interstitial tissue was much higher than in normal rats. The specific activity of esterase in the interstitial tissue from hypophysectomized animals was much lower than in the two other groups of animals; the same was true of the ratio of enzyme activities in the interstitial tissue and seminiferous tubules.

Based on these results, the specific activity of esterase was used as a marker for the interstitial tissue in comparative studies of the two dissection techniques. After wet dissection of the testis, three fractions were obtained: interstitial tissue, unwashed tubules and a residue. The results of analyses of these fractions (Table II) showed that the mean specific esterase activity and the protein content of the residue were in the same order of magnitude as that of the interstitial tissue.

The residue fraction consisted of germinal cells from the tubules, broken cells and fragments of connective tissue. During the wet dissection process, contamination of the seminiferous tubules with material from the residue

TABLE II

Protein Content and Phenyl Esterase Activity (Mean Value ± S.D.; Number of Observations) in Testis Tissue Fractions Isolated with the Wet Dissection Technique

Isolated Fraction	% Protein*	Phenyl Esterase (µmole Nitrophenol/ Min/mg Protein)
Interstitial tissue	8 - 12(4)	2.4 ± 1.7(7)
Unwashed tubules	70 - 78(4)	0.20 ± 0.16(4)
Residue	10 - 20(4)	1.1 ± 1.3(7)

* Protein content is expressed relative to the sum of the fractions and presented as the range of four determinations.

TABLE III

Specific Activity of Phenyl Esterase (Micromoles of Nitrophenol per Minute per Milligram of Protein) in Tubular Fractions* and in Washing Fluids during Washing of Isolated Seminiferous Tubules

Washing	Exp. 1		Exp. 2	
	Tubular fraction	Washing fluid	Tubular fraction	Washing fluid
0	0.45		0.086	
1	0.32	1.4	0.071	0.22
2	0.13	0.45	0.052	0.17
3	0.09	0.35	0.052	0.11
4	0.09	0.15	0.045	0.065
5				0.047

* The tubular fraction, isolated with the wet dissection technique, was washed with phosphate buffer.

is likely to occur. The decrease of specific activity of esterase in tubules after repeated washings (Table III) indicated that when wet dissection was used, the tubules required additional processing for the removal of contaminating interstitial components. The specific activities of esterases in both the tubular fraction and in the washing fluids became constant after three to five washings, so that further purification could not apparently be achieved by additional washings.

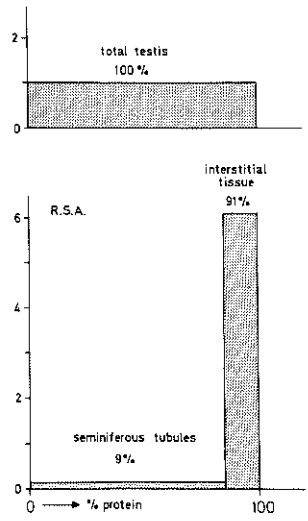

FIG. 1. Distribution of phenyl esterase activity between isolated seminiferous tubules and isolated interstitial tissue. On the ordinate enzyme concentrations have been plotted as relative specific enzyme activity (R.S.A.) as the ratio of percentage of recovered activity to percentage of recovered protein. On the abcissa the percentages of recovered protein in the fractions have been indicated. The percentages indicated in the figure give the fractions of the total testis esterase activity present in the isolated tissue fractions.

Characterization of tubular esterase: Esterase activity could be demonstrated histochemically in the seminiferous tubules, but to a much lower extent than in the interstitial tissue.

The tubular fraction, as obtained by wet dissection, accounted for about 10% of the total esterase activity of the testis (Fig. 1). In order to ascertain whether this determination reflected accurately the tubular esterase activity or whether it was inaccurate as a result of contamination from interstitial tissue, we have studied the electrophoretic characteristics of the esterase activity of both tissue fractions. The results of polyacrylamide gel electrophoresis on wet dissected testis tissue (Fig. 2) showed that the isoenzyme pattern of the interstitium was different from that of the tubular fraction. The two tissue fractions had three bands A, B and C, in common; the interstitium contained two additio-

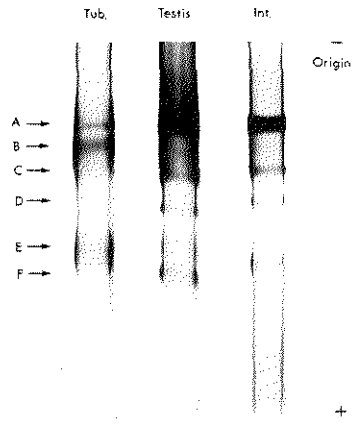

FIG. 2. Isoenzyme patterns of esterases obtained by polyacrylamide gel electrophoresis of different testis tissues. The tubular fraction contains bands A, B, C and E; the interstitial tissue contains bands A, B, C, D and F; and whole testis contains the bands A, B, C, D, E and F.

nal bands, D and F; and the tubules an additional band E. If the tubules were contaminated by interstitial tissue it was not reflected in their specific electrophoretic pattern. The isoenzyme pattern of the whole testis did show all isoenzymes of both tissue compartments (Fig. 2).

To analyze further the purity of the tubules, esterase activities were measured in testicular tissue from hypophysectomized rats (Table I). The specific esterase activity in the interstitial tissue from the hypophysectomized rats was about a factor 10 lower than that occurring in the interstitial tissue from normal rats. However, the specific esterase activity in washed tubules from normal and hypophysectomized rats did not differ one from the other. If the dissected tubules had been contaminated with interstitial tissue, one would have expected a significantly lower specific esterase activity in the tubules of hypophysectomized rats because of the correlation with the much lower specific activity of esterase in the interstitium of these animals.

Comparison of the dry and wet dissection technique using nonspecific esterase: To compare the results of tissue fractionation by wet and dry dissection methods for nonspecific esterase activity, rat testis tissue was divided into two parts. One part was used for wet dissection and the other, for dry dissection. The two fractions obtained by wet dissection were also sectioned in the cryostat and lyophilized. The specific activity of esterase was

TABLE IV

Naphthyl Esterase Activities (Micromoles of Naphthol per 20 Minutes per Milligram of Dry Weight; Mean Values ± S.D. and Number of Observations) in Testis Tissue Fractions Prepared by the Wet and Dry Dissection Techniques

Exp.	Dry Dissection			Wet Dissection		
	Tubules (a)	Interstitial tissue (b)	b/a	Tubules (c)	Interstitial tissue (d)	d/c
1	4.8 ± 1.9(5)	53 ± 20.8(5)	11	2.3 ± 0.4(8)	55 ± 12(7)	23
2	3.7 ± 1.8(10)			1.5 ± 0.2(10)		
3	5.3 ± 1.8(3)	67 ± 7(2)	12	1.7 ± 0.5(4)	52 ± 20(4)	24

determined in all fractions, using naphthylacetate as a substrate (Table IV). The specific activity of esterase in the interstitial tissue was about the same for both preparation methods, and thus indicated a relatively high purity of these fractions. The esterase activity in the seminiferous tubules differed according to the method used and was always somewhat higher in dry dissection specimens. Also the standard error of the analyses after dry dissection was greater than after wet dissection.

The amount of interstitial tissue in the rat testis: Information about the amount of interstitial tissue in the whole testis was obtained from protein and esterase analyses of dissected fractions, the assumption being that the specific activity of esterase (S.A.) in the interstitium and the washed tubules (Table I) represented the true esterase activity of the pure tissue compartments. The percentage of interstitial tissue was calculated as follows: 100 S.A.·testis = (100 - X) · S.A. tubules + X · S.A. interstitium, where X represents the percentage of protein present in interstitial tissue.

In testes from normal rats the percentage of interstitial tissue was 17.4 ± 6.3 (11) (mean value ± s.d.; number

of observations). In testes from EFA-deficient rats, a much higher amount (in the order of 50%) was found.

Comparison of the dry and wet dissection techniques using [^{3}H]-pregnenolone: Both dissection techniques have been applied in the study of the nature and distribution of steroids in the testis. To test the usefulness of the two isolation procedures for the analyses of soluble compounds, the distribution of infused [^{3}H]-pregnenolone over the different testicular tissue compartments was studied. After an *in vivo* infusion with [^{3}H]-pregnenolone the testis was divided in two parts. One part was processed using the wet dissection method, and from the other part, interstitium and tubules were isolated using freeze-dried cryostat sections. From these tissue specimens, specific radioactivities were determined. In two experiments, the

TABLE V

Radioactivity (Disintegration per Minute per Milligram of Protein) in Rat Testis Tissue Fractions after *in Situ* Infusion of Testis with [^{3}H]-Pregnenolone

Fraction	Dissection Exp. A		Dissection Exp. B	
	Dry	Wet	Dry	Wet
Interstitial tissue	3.6×10^5	2.2×10^5	6.5×10^5	3.4×10^5
Washed tubules	1.6×10^5	1.2×10^5	1.7×10^5	1.6×10^5
Testis		1.6×10^5	2.5×10^5	2.3×10^5
Residue		5.7×10^5		2.1×10^5
Washing fluid 1		2.9×10^5		3.2×10^5
Washing fluid 2		6.5×10^5		7.5×10^5
Washing fluid 3		11.4×10^5		8.7×10^5

* Important side branches of the aorta were ligated and 10-40 µC radioactivity was infused retrogradically in the aorta just below the spermatic artery of an anesthesized rat. One testis was divided. One part was dissected with the dry technique, the other part with the wet technique. Tissues were pooled and about 5 µg dry weight was counted per vial.

specific radioactivity in interstitial tissue was much higher when the dry dissection method was used, while the values of radioactivity for tubular tissue were about the same regardless of the technique employed (Table V). The variable amounts of radioactivity in the residue fraction after wet dissection indicated possible redistribution of the steroids during this isolation process.

Because of the increase in specific radioactivity in the subsequent washing fractions, relatively more radioactivity than protein was lost during the washing of the tubules (Table V). For the purpose of analyzing steroids in the different testis tissue compartments, freeze-dried cryostat sections were superior to those obtained from wet dissection.

Discussion

Two dissection techniques, described for the isolation of interstitial tissue and seminiferous tubules from the rat testis (5, 10, 11), have been compared. Both the dissection of wet tissue and of freeze-dried cryostat sections resulted in a good separation of the two tissue compartments. The activity of nonspecific esterase was 50 times higher in interstitial tissue than in tubules. It has been shown that nonspecific esterase is bound to microsomes in testis tissue (26) as well as in brain (23) and in liver tissues (24). Therefore, we have concluded that this esterase can be used as a marker for the interstitial tissue or tissue fragments. The low enzyme activity in the washed tubules could have indicated the absence of interstitial tissue contamination. This premise could further be supported by the presence of different isoenzyme patterns for esterase activity in the tubules and in the interstitial tissue. Moreover, the presence of similar, very low esterase activity in the tubular fractions, isolated from normal rats and from hypophysectomized

rats, denoted the tubular esterase activity was apparently independent of that in the interstitium.

The wet dissection method for isolating the two testicular tissue compartments had the advantage of being an easier procedure than dry dissection, and therefore far greater quantities of tissue could be obtained within a specified time. During 1 hr of wet dissection, an individual was able to isolate about 75 mg of interstitial tissue, but when the dry dissection method was used for a comparable time, the tissue output was decreased nearly 1000 times. The easy separation of tissue compartments by wet dissection was probably due to the structural makeup of the rat testis; each tubule is surrounded by a firm layer of myoid cells which in most areas is separated from the connective tissue of the interstitium by extensive lymphatic vessels (7). A distinct advantage of tissue prepared by the wet dissection method is the potentiality it affords researchers for subsequent investigation on metabolic processes requiring intact cells or for differential centrifugation studies on subcellular structures.

The dry dissection method provides the benefit of a direct microscopic control during the isolation procedure which, in principle, also makes possible the isolation of specific parts within one tissue compartment. Particular cell types or tubular regions containing germ cells in various stages of spermatogenesis might be isolated from the tissue sections. The chief limitation of the dry dissection method is the difficulty in microscopic recognition of cell types in the unstained lyophilized sections. While it is possible to dissect out all of the seminiferous epithelium, the resulting tubular preparation might be contaminated because of its close contact with the interstitial tissue.

The esterase activity in tubules dissected by the dry method was higher than the activity in tubules isolated by wet dissection (Table IV). This differential could have been caused by the fact that during microscopic dissection

of the lyophilized sections, the more central part of the tubular wall was used in an effort to avoid contamination with the interstitial tissue. Histochemical staining revealed a higher esterase activity in the central region than in the peripheral parts of the tubules (3). The results of experimentation with a testis infused with [^{3}H]-pregnenolone showed that redistribution of radioactivity did occur during the dissection of wet tissue. The nature of the steroids had no bearing on the radioactive redistribution. From our results on the study of total radioactivity in these experiments, we cannot conclude that metabolism occurs during wet dissection, but the possibility should be further investigated. During dissection of freeze-dried tissue, metabolism is unlikely. We have therefore concluded that in studies on identification, localization and transport of steroids, the dry dissection technique is preferable. Analytical results on identification and localization of steroids in different tissue compartments as obtained from specimens prepared by wet dissection (20, 21) might not reflect the actual situation at the instant when the tissue was removed from the live animal. For a correct interpretation of the analyses made on isolated tissue compartments, we determined that it was necessary to check the purity of the fractions by the use of a marker. In our experience it has been very difficult to distinguish residues of tubular cells from interstitial cells with histologic techniques, especially when the original structure was disturbed. The findings presented from our experiments (Table IV and Fig. 1) imply that the quantitative determination of esterase activity could serve as a reliable marker. Another marker for interstitial tissue which has been used is the enzyme activity of 3β-hydroxysteroid dehydrogenase (2, p. 503). In the case of the rat testis it is, however, arduous to study the quantitative distribution of this enzyme over the tubules and the interstitial tissue. The quantitative data that we have obtained which showed much higher esterase activity

in the interstitium than in the seminiferous tubules is in accordance with qualitative histochemical analyses made by Niemi and Ikonen (19) and Baust, Goslar and Tonutti (3). Additional advantages of esterase activity as a marker are the stability of the enzyme (16), the microsomal localization (23, 24, 26) and the high dependability of the quantitative analysis. If performed in microvolumes, reliable enzyme determinations can be carried out in less than 0.2 µg dry weight of interstitial tissue and in approximately 2 µg of tubular tissue. The esterase activity in the interstitial tissue is mainly localized in the Leydig cells (18). In this respect the effect of hypophysectomy on the esterase activity (2, p. 497; 19) coupled with the suggestion of Myers et al. (17) that phenyl esterase activity might correlate with cholesterol esterase could indicate a possible functional role for the non-specific esterase.

By using esterase activity as a marker, we estimated the relative amount of interstitial tissue in normal adult male rats varied between 13 and 22%. These calculations were made on the assumption that the specific enzyme activities of the isolated fractions were actually those of the interstitial tissue and of the seminiferous tubules and that the quantitative results had not been influenced by contaminated specimens. The absence of a significant difference in the specific esterase activities in wet and dry dissected interstitial tissue gave support to the theory of the purity of the samples. Evidence for the purity of the isolated seminiferous tubules has been discussed in the Results section of this paper. The calculated percentage of interstitial tissue composing the rat testis seems high when compared with histologic sections examined visually, but the estimated area of interstitial tissue in some sections ranged from 9 to 17% of the total area. The reported values of 13 to 22% are much higher than the value of 6% arrived at by Christensen and Mason (5). This distinctive discrepancy could possibly be ascribed to differences

in the dissection techniques and to variations in the type of animal used in the two investigations.

Very high percentages of interstitial tissue (about 50%) occurred in testes from EFA-deficient rats; our findings are in agreement with a previous publication (1). The occurrence of this abnormal ratio between the amounts of the two testicular tissue compartments in EFA-deficient rats calls for a careful interpretation of analytical results obtained in the study of whole testis tissue from this type of animal (25).

Acknowledgments

The authors wish to express their gratitude to Mrs. H. Rockx and Dr. N.J. de Both for measuring esterase activities, and to Mr. T.M. van Os for the preparation of the photographs and Miss D. Hill for stylistic alterations to this paper.

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STIMULATION OF 3′,5′-CYCLIC AMP AND TESTOSTERONE PRODUCTION IN RAT TESTIS *IN VITRO*

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Received 15 June 1972

1. Introduction

It has been shown that ICSH (LH) and HCG can stimulate testicular steroidogenesis both *in vitro* and *in vivo* and it has been suggested that 3′,5′-cyclic AMP is the intracellular mediator of this process [1]. However, the necessary experimental evidence for cAMP being the "second messenger" [2] in the testis has not been obtained. This is in contrast to other steroid producing tissues e.g. the ovary and adrenal gland where there is good evidence for cAMP being a mediator of trophic hormone action on steroidogenesis [3, 4]. The present communication describes experiments carried out to examine three criteria for the role of cAMP as second messenger in testosterone production in the testis. It has been found that:

1) The increase in cAMP levels precedes the increase in testosterone production in HCG stimulated tissue.
2) Dibutyryl-cAMP stimulates testosterone production.
3) Theophylline (with and without HCG) has a variable effect on testosterone production.

2. Materials and methods

HCG was obtained from N.V. Organon (Oss, The Netherlands) (3500 I.U./mg. rat seminal vesicle weight test) and N^6-2′-*O*-dibutyryl-cAMP from N.V. Boehringer, Mannheim. These compounds were dissolved in Krebs-Ringer-Bicarbonate buffer (KRB) immediately before use.

[1,2-^{3}H] Testosterone (45 Ci/mmole) was obtained from the Radiochemical Centre, Amersham and purified by paper chromatography (Bush A-2 system containing ligroin, methanol, water, 50:35:15, by vol and Bush B-1 system containing ligroin, benzene, methanol, water, 25:25:35:15, by vol). [^{3}H] cAMP (Adenosine-^{3}H(G) 3′,5′-cyclic phosphate, ammonium salt, 24 Ci/mmole) was obtained from New England Nuclear and checked for purity by paper chromatography (isopropanol, ammonium hydroxide, H_2O, 70:10:30 by vol); no impurities were found.

Wistar strain rats, 10 weeks old, weighing 200–250 g were killed by decapitation. The testes were removed, decapsulated, slightly teased and separately preincubated for 1 hr at 32° in 6 ml Krebs-Ringer-Bicarbonate (KRB) in open 50 ml beakers with shaking in an atmosphere of 95% O_2 and 5% CO_2. Each testis was then removed with forceps from the medium and teased into 12–20 pieces. One piece (approx. 100 mg wet weight) from both the left and the right testis from one rat was added to 0.5 ml KRB or KRB containing 1.5 mM dibutyryl-cAMP, 10 mM theophylline or 10 I.U. HCG per 0.5 ml as indicated. Incubations were carried out for 5–240 min at 32° in an atmosphere of 95% O_2 and 5% CO_2 and were stopped by cooling the vessels in ice immediately followed by addition of the internal standards [^{3}H]cAMP and [^{3}H]testosterone. The samples were sonicated (20 KHz, amplitude 5 μm) at 0° for 30 sec and then extracted with acetone (2 × 2 ml).

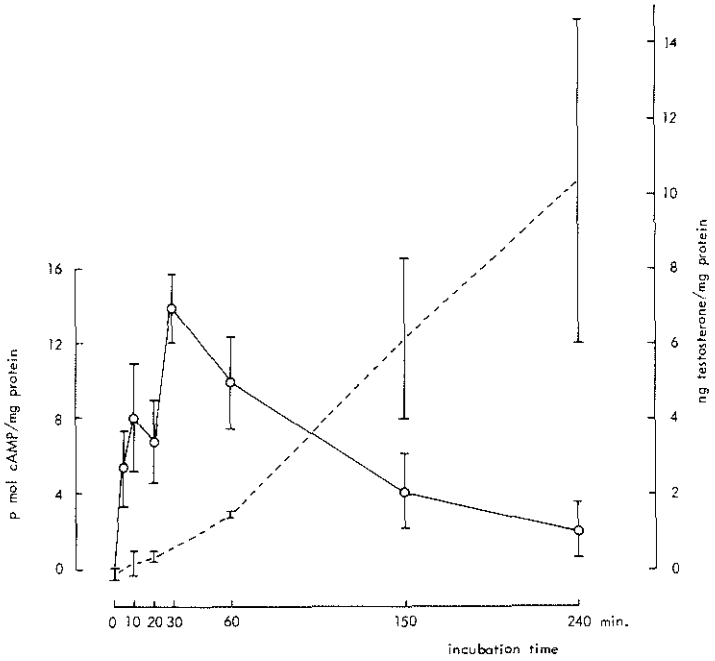

Fig. 1. Time course relationship of cAMP (——) and testosterone (- - - -) production in preincubated total rat testis tissue incubated with HCG (10 I.U.) *in vitro*. The values presented (means ± S.E.M., $n = 3$ to 6) are the difference between the levels of stimulated and unstimulated tissues at each time period. For incubation conditions see text.

Acetone was evaporated under N_2 at 45° and the remaining water phase was extracted with ether (3 × 1 ml). Testosterone was assayed in the combined ether phases by gas–liquid chromatography as described by Brownie et al. [5]. For cAMP measurements 20 μl 50% (w/v) trichloroacetic acid was added to the water phase (made up to 1 ml) to precipitate residual protein.

cAMP was isolated by chromatography of the trichloroacetic acid/water mixture over Dowex (50W × 8, 200–400 mesh) ion exchange resin columns [6]. Eluted cAMP was assayed by saturation analysis [7]. Tissue samples were dissolved in M NaOH for estimating protein according to Lowry et al. [8].

3. Results and discussion

A reproducible stimulation of testosterone and cAMP production *in vitro* by HCG was found only when total testis tissue was preincubated for 1 hr at 32°. When the tissue was not preincubated, testosterone production was high and it was difficult to stimulate further production. It is possible therefore that inhibitors are removed from the tissue by the preincubation procedure.

In control incubations over a period of 4 hr cAMP levels decreased from 12 to 3 pmole/mg protein and testosterone levels increased from 2.4 to 4 ng/mg protein. Addition of HCG caused an increase in cAMP levels that preceded the increase in testosterone levels (fig. 1). A significant increase in

Table 1
Correlation between change in cAMP and testosterone levels in total rat testis tissue during stimulation with HCG *in vitro*.

Experiment number	cAMP (pmole/mg protein/20 min incubation)			Testosterone (ng/mg protein/240 min incubation)			X
	No additions (a)	HCG (10 I.U.) (b)	b−a (X)	No additions (c)	HCG (10 I.U.) (d)	d−c (Y)	Y
1	5.6	16.2	10.6	2.8	12.1	9.3	1.1
2	9.8	16.6	6.8	4.2	22.1	17.9	0.4
3	6.4	9.2	2.8	1.7	5.3	3.6	0.8
4	7.2	7.7	0.5	2.9	4.0	1.1	0.5
5	10.4	17.1	6.7	7.8	22.4	14.6	0.5

Table 2
Effect of dibutyryl-cAMP on testosterone levels in total rat testis during incubation *in vitro*.

Experiment number	Testosterone (ng/mg protein)					
	180 min incubation			240 min incubation		
	No additions (a)	Dibutyryl-cAMP (1.5 mM) (b)	b−a	No additions (c)	Dibutyryl-cAMP (1.5 mM) (d)	d−c
1	3.1	5.8	2.7	3.2	16.3	13.1
2	3.7	4.8	1.1	4.1	7.6	3.5
3	2.5	9.2	6.7	3.2	25.3	22.1
4	3.4	16.8	13.4	2.8	13.3	10.5
5	2.4	10.3	7.9	2.5	16.1	13.6

Table 3
Effect of theophylline on testosterone levels in total rat testis tissue during incubation *in vitro*.

Experiment	Testosterone (ng/mg protein/240 min incubation)					
	No additions (a)	Theophylline (10 mM) (b)	b−a	HCG (10 I.U.) (c)	HCG (10 I.U) + theophylline (10 mM) (d)	d−c
1	3.1	2.7	−0.4	4.8	4.0	−0.8
2	4.1	7.6	3.5	12.4	6.2	−6.2
3	2.9	2.6	−0.3	4.0	7.3	3.3
4	7.8	7.8	0	22.5	16.0	−6.5

cAMP levels ($P < 0.025$) was found during 10 min incubation whereas testosterone levels were not significantly increased until 60 min ($P < 0.001$).

Although a stimulation of cAMP and testosterone production was always observed, the degree of stimulation varied. A correlation was found, however,

between the change in cAMP levels during 20 min and the change in testosterone levels during 240 min (table 1). For example, when the cAMP increase during 20 min was small, there was also a small increase in the production of testosterone. In this respect the results of Dufau et al. [9] are of interest. They found that HCG stimulated testosterone production in decapsulated total testis *in vitro*, but if the testis was teased apart a much lower stimulation occurred. Additions of dibutyryl-cAMP (1.5 mM) resulted in a stimulation of testosterone production especially during incubation periods of more than 180 min (table 2). These results are in agreement with data published by other investigators [9–11]. In an attempt to increase testosterone production by inhibiting the breakdown of cAMP, theophylline (10 mM) was added to inhibit phosphodiesterase activity. However, a consistent effect of this compound on testosterone production when added alone or with HCG, could not be demonstrated. In some experiments an inhibition of testosterone production by theophylline was observed (table 3). Comparable inhibiting effects on corticosteroid production have also been reported by other workers for the adrenal gland [12, 13]. Therefore 10 mM theophylline is apparently unsuitable for testing the participation of cAMP in hormone action on steroid producing tissues.

It may be concluded from the present observations on the time course of cAMP and testosterone production during HCG stimulation and from the effect of dibutyryl-cAMP, that cAMP could be a mediator of trophic hormone action on the testis. However, because of the inhomogeneous nature of total testis tissue only tentative conclusions can be drawn and this work is therefore being extended to testis interstitial tissue and seminiferous tubules. Results already obtained show that HCG specifically stimulates cAMP production in interstitial tissue [6] and that testosterone production can also be stimulated in this tissue by HCG.

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STIMULATION OF 3',5'-CYCLIC AMP AND TESTOSTERONE PRODUCTION IN RAT TESTICULAR INTERSTITIAL TISSUE IN VITRO BY LUTEINIZING HORMONE

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Received for publication April 6, 1973

Introduction

It has been shown that human chorionic gonadotrophin (HCG) and luteinizing hormone (LH) will stimulate steroidogenesis in testes *in vivo*[1]. The intracellular mediator of this trophic hormone action is thought to be 3',5'-cyclic AMP (cAMP) because *in vitro* experiments have shown that (i) HCG or LH stimulates cAMP and testosterone production in testis tissue[2-7], (ii) the increase in cAMP production precedes the increase in testosterone production[3] and (iii) dibutyryl-cAMP stimulates testicular testosterone synthesis *in vitro*[2,3,5] and *in vivo*[1]. However, because of the different cell types present in testes only tentative conclusions can be drawn. It is possible, for example, that cAMP production is stimulated in cell types that are not involved in steroidogenesis.

In vitro studies with separated testis tissues have shown that LH specifically stimulates cAMP production in the interstitial tissue[7] and that this tissue is the main site of testosterone biosynthesis[8]. It was therefore deci-

ded to investigate the effect of LH on the relationship between cAMP and testosterone synthesis in isolated interstitial tissue in vitro.

The results obtained are in accordance with cAMP being an intracellular mediator of LH action. Both cAMP and testosterone production in interstitial tissue were stimulated by LH and the increase in cAMP preceded the increase in testosterone production. The addition of glucose was found to increase the production of testosterone in LH stimulated interstitial tissue. The magnitude of the observed increased testosterone production in interstitial tissue was, however, lower than might be expected from the relatively higher number of Leydig cells in this tissue compared with the total testis.

Materials and methods

Ovine LH (NIH-LH-S18, 1 unit/mg) was a gift from the Endocrinology Study Section, National Institute of Health, Bethesda, Maryland. Testis tissue was obtained from 10-13 weeks old rats (Wistar strain). Some rats were used 11-15 days after hypophysectomy, starting on the day after hypophysectomy these rats received daily subcutaneous injections of 10 µg LH. The isolation of the tissues, incubation conditions and the extraction procedure were as published previously[3,7], except that in all experiments 50 µg γ globulin but no theophylline and in some experiments 0.2% glucose were present in the incubation medium. The following amounts of tissue (expressed as weight of protein per volume incubation medium) were used: unteased testis 70 mg/2 ml, teased testis 5-10 mg/0.5 ml and interstitial tissue 0.3-1.0 mg/0.5 ml.

cAMP was isolated as described previously[3] and assayed by saturation analysis[9]. Testosterone was measured by ra-

dioimmunoassay essentially as described by Furuyama et al.[10], except that the tissue extracts were not chromatographed. Samples were incubated with antiserum at 4°C for 16 hours and separation of free and bound testosterone was achieved with dextran coated charcoal (0.5 ml containing 250 mg charcoal and 25 mg dextran T250 per 100 ml borate buffer).

Evaluation of the procedure for testosterone estimation showed that the coefficient of variation of the within assay precision was approximately 13% for samples containing between 0.3 and 50 ng (n=114). The coefficient of variation of the between assay precision for mean values of duplicate determinations was approximately 14% for samples containing between 1 and 30 ng (n=32). The specificity and accuracy of the method under the experimental conditions used was evaluated by comparing the results of estimations by radioimmunoassay and gas-liquid chromatography[11]. The correlation coefficients between estimations by radioimmunoassay and gas-liquid chromatography of total testis tissue extracts (n=54) and interstitial tissue extracts (n=12) were 0.95 and 0.94 respectively.

Results and discussion

The time course relationship for cAMP and testosterone production during incubations of interstitial tissue in the presence of 200 ng LH/ml is given in Fig. 1. The first detectable increase in cAMP levels was 5 to 10 min after the addition of LH while stimulation of testosterone production was not noticeable until 30 to 60 min. These results are similar to observations with total testis tissue[3]. It is striking, however, that cAMP in interstitial tissue continuously increased during 4 hours incubation whereas in total testis tissue a decrease was found already after

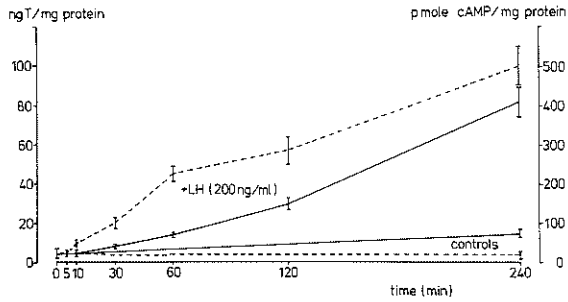

FIG. 1. Time course relationship for cAMP (---) and testosterone (——) production in interstitial tissue in the presence of 200 ng LH/ml. The values presented are means + S.E.M. from 3 different duplicate incubations with tissue from 3 different rats. Tissues were incubated in the presence of 0.2% glucose.

30 min incubation. The difference may be explained if cAMP is released in the intact gland from the interstitial cells and metabolized elsewhere in the testes e.g. in the seminiferous tubules. During incubation of whole testis in vitro, release of cAMP into the incubation medium has been shown by Dufau et al.[5] and phosphodiesterase activity has been detected in seminiferous tubules[12].

The dose-response relationship between LH and testosterone has been investigated with interstitial tissue and was compared with results from incubations with teased and unteased testes (Fig. 2). With interstitial tissue from some rats a stimulation of testosterone production was found with 0.002 µg LH/ml but stimulation was consistently obtained only with 0.02 µg LH/ml. The amount of testosterone formed, varied from one rat to another with higher doses of LH (0.2-2.0 µg/ml) especially in the teased testis tissue and interstitial tissue. In this series of experiments the testosterone production in stimulated interstitial tissue and in total testis tissue in the absence of glucose was between 4 and 12 ng testosterone/mg protein/4 hr. Glu-

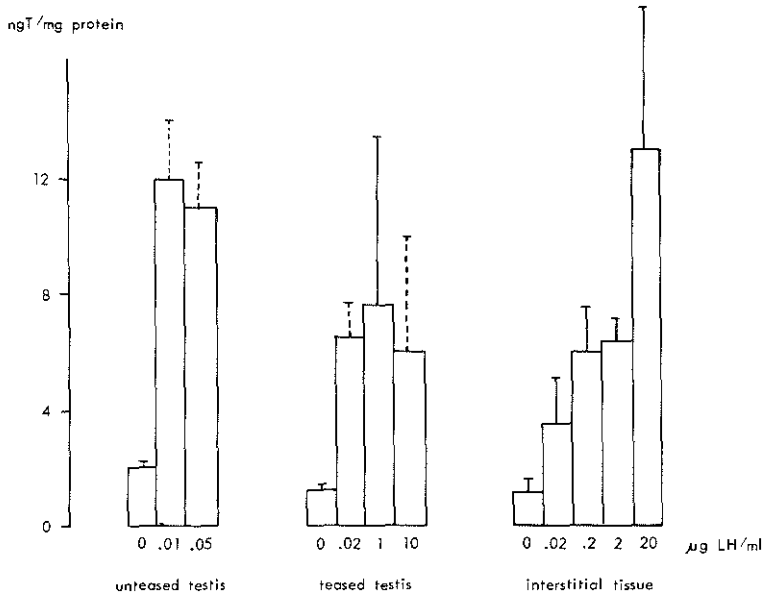

FIG. 2. Effects of various amounts of LH in vitro on testosterone production by unteased testis, teased testis and interstitial tissue. Tissues were incubated for 240 min at 32°C. Zero time values were subtracted. Mean values ± S.E.M. (——) for n=3 to 6 or mean value and the range (---) for n=2 to 3 are indicated, n is the number of observations with tissues from different rats. Tissues were incubated without glucose added to the incubation medium.

cose was added to the incubation medium when investigating the time course relationship for cAMP and testosterone production and it was found that the amount of testosterone produced in LH stimulated interstitial tissue was much higher (72.8 ± 20.8; mean value ± S.D. n=6) than the production by tissues in the absence of glucose. The amount of testosterone produced in total testis tissue in the presence of LH was also increased when glucose was added (26.6 ± 5.6; n=3). It may be concluded, therefore, that the testicular preparations used in the two series of experiments produced different amounts of steroids, presumably

because of the addition of glucose. The effect of glucose on steroid production was therefore investigated within one experiment (Table 1). It was confirmed that a higher testosterone production is obtained in the presence of glucose thus clearly indicating the necessity of this compound in

TABLE 1

EFFECT OF GLUCOSE ON PRODUCTION OF TESTOSTERONE DURING INCUBATIONS OF INTERSTITIAL TISSUE IN VITRO

Testosterone production (ng/mg protein/4 h)		
Rat	without glucose	with glucose (0.2%)
1	5.3	20.0
2	5.0	36.1
3	2.7	12.7

Interstitial tissue was obtained from 3 normal rats (1, 2 and 3). Zero time values (2.7-3.6 ng T/mg protein) have been subtracted. Each value is a mean from duplicate incubations. Incubations were carried out over a period of 4 hours in the presence of 200 ng LH/ml.

addition to LH for a high steroid production. This is somewhat surprising because Gomes[14] has reported that glucose had no effect on oxygen uptake by isolated interstitial tissue and he therefore concluded that glucose was not utilized by this tissue. From these observations it may be concluded therefore that oxygen uptake does not correlate with the effect of glucose on steroid production in interstitial tissue.

Approximately 17% of the total amount of protein in the testis is present in the interstitial tissue[13], therefore the isolated interstitial tissue should theoretically

produce approximately 6 times more testosterone per mg protein when compared to the total testis, stimulated with the same amount of LH. The absence of a proportionally higher production by isolated interstitial tissue (Fig. 2) may reflect a decreased steroid production in this isolated tissue. This low steroid production may be explained by destruction of the tissue during dissection. However, this is not reflected in the relatively high cAMP production in isolated interstitial tissue compared with total testis tissue[7]. Another explanation may be a lack of essential factors from the tubules which might be required for optimal steroid production.

When cAMP and testosterone production in interstitial tissue from hypophysectomized rats were studied (Table 2), it was found that with 20 ng LH/ml only testosterone production was stimulated, whereas with 200 ng LH/ml both cAMP and testosterone production were stimulated. Other studies with theophylline added to the incubation medium to inhibit metabolism of the cAMP, have shown that 100 ng LH/ml was required to detect a change in cAMP production in isolated

TABLE 2

PRODUCTION OF cAMP AND TESTOSTERONE DURING INCUBATIONS OF INTERSTITIAL TISSUE IN VITRO

Incubation time (min)	LH concentration (ng/ml)	Testosterone ng/mg protein			cAMP pmole/mg protein		
		4	5	6	4	5	6
0	0	0.3	0.5	1.8	4.0	4.0	7.0
120	0	0.8	1.8	3.0	5.2	3.9	14
120	20	1.3	3.6	5.5	4.8	5.0	17
120	200	3.2	3.5	17	22	21	158

Interstitial tissue was obtained from hypophysectomized rats which were injected daily for 11-15 days with 10 µg LH.
Each value is a mean from duplicate incubations with tissue from rat 4, 5 or 6 carried out in the presence of 0.2% glucose.

interstitial tissue from normal rats (reference 7 and unpublished observations). It has also been reported[15] that trophic stimulation of adrenal cortex may result in an increased corticosteroid production without effects on the cAMP production. The absence of an effect on cAMP production when steroid production is stimulated may reflect a non-obligatory role of cAMP in the control of steroidogenesis. It is possible, however, that with the analytical techniques used, small differences in cAMP levels which could have stimulated steroid production remain undetectable.

In conclusion, the results of the present study clearly demonstrate that the testosterone production by isolated interstitial tissue *in vitro* can be stimulated by LH. This is in contrast to the results of Dufau *et al.*[2] who reported that testosterone production of an interstitial cell fraction could not be stimulated *in vitro* and they therefore suggested that the intact testis was required for the synthesis of testosterone. Although our results do not support the latter suggestion, the reason for low production of testosterone in interstitial tissue remains to be elucidated.

Acknowledgments

The authors wish to thank the NIH Endocrinology Study Section for gifts of the LH.

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