Autoradiography of diffusible substances
Localization of steroids in the testis

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Appendix: Publications I-VI

Publication I
P.M. Frederik & L.G. van Doorn (1973). J. Reprod. Fert. **35**: 117-121.
A technique for perfusion of rat testes in situ through the internal spermatic arteries.

Publication II
P.M. Frederik & D. Klepper (1974). J. Microscopie **19**: 11a-12a.
Freeze Drying of unfixed tissue samples for the autoradiographic localization of steroid hormones in testes.

Publication III
P.M. Frederik & D. Klepper (1976). J. Microscopy **106**: 209-219.
The possibility of electron microscopic autoradiography of steroids after freeze drying of unfixed testes.

Publication IV
P.M. Frederik, D. Klepper, G.J. van der Vusse & H.J. van der Molen (1976) Mol. Cellul. Endocrinol. 5:123-136.
Dynamics of steroid uptake in rat testis studied by quantitative autoradiography.

Publication V
P.M. Frederik, H.J. van der Molen, D. Klepper & H. Galjaard. J. Cell Sci. accepted for publication.
Electron microscopic autoradiography of tritiated testosterone in rat testis.

Publication VI
P.M. Frederik, J.J.H. Fortuin, D. Klepper & H. Galjaard (1977). Histochem. J. **9**:89-96.
Autoradiographic detection of mucopolysaccharide accumulation in single fibroblasts.

Introduction

1.1 The testis

The testis contains two cell compartments respectively having an endocrine and an exocrine function. The seminiferous tubules contain a cell renewal system concerned with the production of spermatozoa, and are the exocrine part of the organ. In the other compartment, the interstitial tissue, Leydig cells are found having an endocrine function in the biosynthesis of androgenic steroids.
The studies presented in this thesis are mainly concerned with two questions regarding the role of steroids in the testis:
– Firstly, which (sub) cellular structures are involved in the synthesis and secretion of androgen in the Leydig cells.
– Secondly, which testicular cells or cell organelles are targets for androgen and estrogen action and what is the route by which these steroids reach their targets.
To answer these questions tritiated steroids have been administered to the rat testis under various experimental conditions. The incorporated radioactivity was subsequently localized in tissue sections by autoradiography both at the level of the light and of the electron microscope. Before discussing the merits and the technical problems of this experimental approach an outline of the role of steroids (among other factors) in testis in general and for spermatogenesis in particular will be given.

In the seminiferous tubules germinal cells are present intermingled with non dividing cells, the Sertoli cells. Spermatogenesis involves a highly ordered sequence of mitotic and meiotic divisions of the germinal cells (Leblond & Clermond 1952) which can be divided into three distinct phases (Clermont 1972). The first phase concerns the spermatogonia (stem cells) which proliferate to give rise to spermatocytes and simultaneously maintain their number by stem cell renewal (Clermont 1972, Moens & Hugenholz 1975, Huckins 1971, van Keulen & de Rooij 1974). A chalone, i.e. a tissue specific substance inhibiting mitoses (see Bullough 1975 for review) has been thought to regulate stemcell proliferation in the testis (Clermont & Mauger 1974, 1976). The second phase involves primary and secondary spermatocytes going through meiosis and mitosis which leads to the formation of haploid cells, the spermatids. For the prophase of the reduction division in the rat no hormone seems to be required, but for the completion of meiosis the presence of testosterone seems to be necessary (see Steinberger 1971). The third phase involves a number of transformation by which spermatids differentiate into spermatozoa. The spermatozoa are shed into the tubular lumen. Steinberger (1971) has argued that the pituitary hormone FSH (Follicle Stimulating Hormone) is involved in the final stages of spermatid differentiation. This effect may be mediated by Sertoli cells since recent evidence point towards the Sertoli cells as targets for FSH action (Means 1975).

Luteinizing hormone (LH) is the other pituitary hormone involved in testis function. LH regulates the testosterone production by the Leydig cells (for review see Hall 1970, Cooke 1976). Testosterone plays a key role in the regulation of spermatogenesis since it has been shown that administration of testosterone can maintain spermatogenesis in hypophysectomized animals (Woods & Simpson 1961, Ahmad et al 1973). As yet it is not known which cells and cellorganelles are targets for the possible effect of testosterone on spermatogenesis. Studying the distribution of testosterone in the testis may therefore contribute to our understanding of the regulation of spermatogenesis. It has been the aim of our studies to localize testosterone (and its percursors and metabolites) in the testis at the cellular and subcellular level. Such a localization study may provide data about the sites of synthesis and secretion of testosterone, about the intracellular and extracellular transport of testosterone and finally they may reveal the target cells or cell organelles for testosterone or for its metabolites. Autoradiography with the light- or electron microscope seems to be the method of choice for such a localization study and it enables in situ localization at the cellular and subcellular level. This method in principle avoids the disruption of cells and tissues which may cause redistribution of steroids.
Such disruptive procedures are commonly employed when biochemical methods are used for cellular and subcellular localization studies.

1.2 Autoradiography

When a compound labelled with a radioactive nuclide is incorporated in tissue the distribution of radioactivity can be visualized by autoradiography. In autoradiography a photographic emulsion is used to record the site of radioactivity in a tissue section or (part of) a cell. The principles of autoradiography are as old as the discovery of radioactivity itself (see Rogers 1973 for review) but the important contributions of autoradiography to biological problems came after the Second World War when a number of radioactive isotopes became available which were important in biological processes. Current methods for autoradiography are still based on techniques developed by Bélanger and Leblond (1946) and Pelc (1947) for the application of photographic emulsions to histological sections. Tissue sections containing a labelled compound are exposed to the photographic emulsion for an appropriate time, followed by development of the photographic emulsion. The sections with the developed emulsion still attached are then studied with the light microscope and these autoradiograms will reveal silver grains over labelled tissue components. The resolution of these autoradiograms may equal the optical resolution of the light microscope (see Rogers 1973). An even higher resolution of autoradiograms has been obtained by combining electron microscopy with autoradiographic techniques (Caro & Van Tubergen 1962, Salpeter et al. 1969, Blackett & Parry 1973), thereby enabling the localization of radioactivity in subcellular organelles.

The application of autoradiography to biological problems is restricted. In principle only those compounds which remain in tissue after treatments

with fixative solutions, dehydration solvents and embedding agents can be localized whereas diffusible substances (including steroid hormones) are washed out during these procedures (Roth 1969).
For the autoradiographic localization of diffusible substances at the light microscope level several reliable techniques (notably for steroids) are available. Extraction of labelled substances is completely prevented by exposing cryosections to a dry photographic emulsion (Appleton 1964, Stumpf 1964). Several groups have attempted to extend this method to the electron microscope level (Appleton 1969, Christensen 1969, Hodson & Marshall 1970). Although progress has been made recently in sectioning procedures, technical problems still prevent the practical realization of this method (see chapter 2). To retain diffusible substances in tissues modifications of the conventional embedding procedures have also been used. Wilkse & Ross (1965) used frozen died tissue fragments which were fixed in the vapour phase with osmiumtetroxide or paraformaldehyde and which were then vacuum embedded in Epon. This method was further developed by Stirling & Kinter (1967) and used for the electron microscopic localization of tritiated galactose (see chapter 2).
In chapter 2 of this thesis we will describe the techniques which we have developed and tested for electron microscopic autoradiography of tritium labelled steroids in the testis and the theoretical principles on which they are based.

1.3 Survey of experimental work

In order to link the potential capabilities of autoradiography to the study of the localization of steroids in testis we first had to find a suitable way to introduce sufficient tritiated steroids into the testis.
For this purpose a technique was developed for vascular perfusion of rat testis which is described in appendix paper 1; further improvements are described in appendix paper 3. Perfusion of the isolated organ has the advantage over injections in the peripheral circulation that labelled steroids enter the testis without being diluted in blood or being taken up and eventually metabolized by other organs.
By perfusing rat testis with tritiated steroids we found an uptake of tritium label in amounts that can be detected by electron microscopic autoradiography (ca. 1 μCi/gr fresh weight, Rogers 1973).
Techniques employed for autoradiography of steroids are described in detail in chapter 2 and the appendix papers 2 and 3. Several techniques have been applied. Initially we tried to localize precursors of testosterone biosynthesis by precipitating them with digitonin during the processing for microscopy (appendix paper 3). We found however no quantitative retention of those steroids for which a reaction with digitonin has been reported (Mizuhira et al 1970). Other approaches involve freezing of tissue to immobilize diffusible substances. The formation and growth of ice crystals during freezing influences both the ultrastructure and localization of diffusible substances and may limit the autoradiographic resolution (chapter 2). Using frozen testis as starting material we have made ultrathin frozen sections, but the low number of suitable sections obtained was a major problem in our hands (chapter 2). Better results were

obtained with ultrathin sections from testis tissue which had been freeze dried, fixed in osmium vapour and embedded in Epon (appendix paper 3). The effects of the procedures of the freeze drying methode on the preservation of the ultrastructure and the retention of steroids are discussed in chapter 2. Using the freeze drying method we have studied the localization of steroids in testis by autoradiography. Preliminary results obtained by light microscopic autoradiography were published in appendix paper 3 and more extensive quantitative data on the distribution of oestradiol, testosterone and pregnenolone and their metabolites in the rat testis are presented in appendix paper 4. Based on the innovations described in appendix paper 3 and chapter 2 the autoradiographic observations on the localization of testosterone in testis were further extended to the electron microscope level (appendix paper 5). In chapter 3 we have tried to relate the data obtained by autoradiography to the in vivo situation in the rat testis.
The techniques, developed for the autoradiography of steroids can also be applied to localization studies of other diffusible substances. An example is given in appendix paper 6, where the localization is studied of accumulated acid-mucopolysaccharides in cultured fibroblasts derived from patients with a genetic metabolic disease.

Techniques for autoradiography of diffusible substances

2.1 Introduction

The current concepts of the fine structure of cells and tissues are largely based on electron micrographs from sections of chemically fixed, dehydrated and embedded material. Cell constituents are chemically modified by these preparative procedures. In addition, ions and other non fixed cell constituents ('diffusible substances') will be extracted. The procedures of fixation, dehydration and embedding limit localization studies with the electron microscope. Reactions aimed at the demonstration of specific cell or tissue components are mostly carried out during the early steps of the preparative procedures; either by adding a specific reagent to the fixative solution (e.g. for the demonstration of inorganic ions, Pearse 1973) or after mild aldehyde fixation (frequently employed in enzyme cytochemistry and immunocytochemistry, Pearse 1973). Such procedures however do not permit the electron microscopic localization of small organic molecules like steroids (see section 2.2). Alternatives to chemical fixation, solvent dehydration and embedding have been described since the earliest application of electron microscopy to biological material in the early forties (Sjöstrand 1943) and mostly involve freezing of biological material (Bretschneider & Elbers 1952). However none of them has resulted in a generally accepted substitute for the continuously improving conventional procedures (see Mühlethaler 1973 for review). More recently, efforts have concentrated on cryobiological techniques enabling localization of diffusible cell constituents (Roth 1969). In this chapter the available techniques will be evaluated for their applicability to the localization of diffusible substances. The preparative steps will be considered especially with respect to their implications for the morphological preservation and the retention of steroids. Table 2.1 shows the microscopic techniques that have been used for the in situ localization of steroids. They are largely based on two principles: **freezing,** thereby transferring water from solution into the biological inert form of ice and/or **chemical reaction,** rendering steroids insoluble. Digitonin added to the fixative and dehydration solutions is supposed to form insoluble precipitates in situ with certain steroids (Mizuhira et al, 1970). The digitonin method for the autoradiographic localization of steroids is thus entirely based on chemical fixation. Immune precipitation of steroids has been carried out on frozen sections fixed in acetone (Bubenik et al 1975). This procedure involves both freezing and chemical reactions. Principles of freezing and chemical reactions are also both involved when freeze drying, vapour fixation and embedding are used to retain steroids in tissue for their subsequent localization by autoradiography. Freezing is the only 'fixation' procedure when frozen (thin) sections are used for autoradiography.

The next sections will deal with a more detailed description of these techniques and our own results concerning the application of these techniques to the autoradiography of steroids in testes.

Table 2.1

Method	Frozen sections	Freeze drying and vapour fixation	Steroid protein interaction	Digitonin precipitation
Light microscopy	Fitzgerald (1961) Appleton (1964) Stumpf (1964)	Wilske & Ross (1965) Attramadal (1969c) Nadler et al (1969) Fredrik & Klepper (app. papers 3, 4)	Bubenik et al (1975) Haferkamp et al (1968) Uriel (1975) Tchernitchin (1971, 1972)	
Electron microscopy	Appleton (1969) Christensen (1969)	Stirling & Kinter (1967) Eckert (1969) Frederik & Klepper (app. papers 3,5)		Mizuhira et al (1970) Frederik & Klepper (app. paper 3)
Extraction of triated steroids	0%	1-25%	0-20%	70%

2.2 Chemical fixation

2.2.1 Digitonin

Digitonin gives an insoluble precipitate with cholesterol and other steroids with a 3β-hydroxyl group and a double bond between carbon atoms 5 and 6. This reaction has been proposed for the histochemical demonstration of cholesterol in tissue. After fixation of adrenal tissue in a digitonin containing medium typical alterations of the membranes were observed, which were interpreted as a cholesterol-digitonin precipitate (Ökrös 1968, Szabó et al. 1974). Digitonin has also been used during processing of liver and nervous tissue for the autoradiographic localization of cholesterol and gave a retention of free cholesterol between 85% and 99% (Gautheron & Chevalier 1971, Scallen & Dietert 1969, Sterzing & Napolitano 1972, Stein & Stein 1971). More recent electron microscopic observations from a model study where digitonin was added to purified egg lecithin suggest that in addition to interactions between digitonin and cholesterol, interactions between digitonin and phospholipid occur as well (Frühling et al 1971) which have formerly been erroneously interpreted as cholesterol-digitonin complexes. Mizuhira et al (1970) have suggested to use digitonin precipitation for the autoradiographic localization of androgens in testes, but have not given data on the retention of steroids. We have repeated these experiments (appendix paper 3) using the rabbit as experimental animal because in rabbits androgen synthesis occurs preferentially via the Δ^5-pathway*. Thus the number of digitonin reactive intermediates is expected to be greater than in the rat, where androgen synthesis follows mainly the Δ^4-route*. From the experiments reported in appendix paper 3, it appears that treatment with fixatives containing digitonin will not retain tritiated testosterone and its precursors in testis tissue or in agar blocks. In addition membrane rearrangements were observed (see appendix paper 3) probably due to the surface activity of digitonin. The extraction of 3 β hydroxy-Δ^5 steroid precursors of testosterone might have been caused by an incomplete reaction between these 3 β hydroxy-Δ^5 steroids with digitonin or by a small solubility of digitonides in the dehydration media employed.

Because of the low retention of steroids and the alterations in the ultrastructure, the digitonin method for the autoradiographic localization of steroids in testis was not further investigated.

2.2.2 Steroid-protein interactions

Steroid binding proteins have been localized by autoradiography after incubating cryostat sections of tissues with tritiated steroid (Tchernitchin et al 1971, Tchernitchin 1972, Uriel 1975), eventually followed by

Footnote:
The Δ^5-pathway of testosterone biosynthesis from pregnenolone involves as intermediates: 17α-hydroxypregnenolone, dehydroepiandrosterone and Δ^5 androstenediol.
The Δ^4-pathway of testosterone biosynthesis from pregnenolone involves as intermediates: progesterone, 17α-hydroxyprogesterone and androstenedione.

extraction of non specifically bound steroids (Tchernitchin & Chandross 1973, Tchernitchin et al 1973). With this method binding sites of steroids (if not transferred during incubation) are demonstrated in situ but no information will be provided about the occupation of such binding sites in vivo.
The localization of endogenous steroids has been studied by incubating cryostat sections with solutions of antibodies labelled with fluorescein or peroxidase (Haferkamp et al 1968, Bubenik et al.1974, 1975). The method involves a brief fixation of the cryotome sections in cold acetone followed by rinsing in buffer before adding the antibody solution. No difference was observed between the steroid localization of acetone fixed and non-fixed sections. The use of steroid-antibodies for the localization of steroids appears unattractive since it involves incubations with aquous solutions which may induce changes in localization of steroids before the reaction with antibody.

2.3 Physical fixation

2.3.1 Introduction

Freezing can be considered as the physical fixation of water. Frozen tissue has been used as starting material for a number of ultrastructural studies, some involving modified forms of fixation, dehydration and embedding, others involving none of these procedures. The most direct ways for looking at frozen tissues are offered by cryosectioning procedures or by making replicas from fracture planes of frozen tissue (freeze-etch preparations). Chemical fixation in aquous solutions is sometimes employed prior to freezing in cryosectioning and freeze etch procedures. Chemical fixation can be carried out in the vapour phase or with non aquous solutions when ice has been removed from the tissue by dissolving ice in organic solvents (freeze substitution, Bullivant 1965) or by sublimation (freeze drying).
From these methods freeze drying (Stirling & Kinter 1967) and cryosectioning (Appleton 1969, Christensen 1969, Hodson & Marshall 1970) have been used for the autoradiographic localization of diffusible substances at the electron microscope level. Since frozen tissue is the starting material for these autoradiographic procedures, the limitations set by the freezing process deserve careful analysis.

The key event during freezing is the arrangement of water molecules in ice crystals. During formation of ice crystals in cells solutes are separated from ice. This segregation causes translocation of all solutes (including diffusible substances) and may be accompanied by alterations of the ultrastructure due to the disruptive growth of ice crystals. The process of freezing should therefore be carried out in such a way that the size of ice crystals remains as small as possible. This is certainly true for autoradiographic studies at the electron microscope level where a high resolution is required.

Another prerequisite is that the spatial relationship between ice and solutes is maintained during procedures for autoradiography so that the 'true' localization is reflected in the final micrograph. Also during melting of frozen tissue or rehydration of freeze dried tissue uncontrolled translocation of solutes and morphological changes may occur. It is clear that such artefacts hamper a correct interpretation of the relationship between autoradiographic grains and the location of the radioactive source and this may create uncertainity when autoradiographic results are related to the in vivo situation. In the following paragraphs the cryobiological aspects of freezing and freeze drying will be discussed. Subsequently the application of these principles in the preparation of autoradiographs for the localization of diffusible substances, i.e. steroids in testis tissues, will be discussed.

2.3.2 Cryobiological aspects of autoradiography

2.3.2.1 Review of the principles of freezing

The ultimate size of ice crystals in a frozen object is dependent on crystal nucleation rate and crystal growth rate, which are temperature and pressure dependant. Control of these rates is mainly a problem of heat exchange which depends on the specimen and coolant characteristics. The impact of crystal nucleation rate and crystal growth rate on freezing of dilute solutions and biological material has been discussed by Stephenson (1960a), Meryman (1956, 1960a, 1966) and Riehle & Hoechli (1973). Ice crystal formation may start from inclusions (heterogenous nuclei) or from aggregates of water molecules (homogenous nuclei). The formation of homogenous nuclei of critical size (having an equal probability of growing or vanishing) depends greatly on the temperature during crystallization. The occurrence of critical nuclei increases between -30 and -40 °C with a factor 10^{10} whereas their size decreases from 30 Å to 20 Å in the same temperature range. A cristal nucleus exceeding the critical size will grow and this growth is also temperature dependent with a maximum at -6 °C (Riehle & Hoechli 1973, misquoted by Moor 1973) and decreases towards 0 °C and towards -120 °C (the minimum temperature at which ice crystal growth has been observed, Meryman 1966).
The occurrence of small ice crystals in rapidly frozen cells has been explained by assuming that crystallization is initiated below the freezing point (supercooling). When crystallization starts in a supercooled solution it will be initiated by many homogenous nuclei of small size with hardly any subsequent growth. Under conditions of extreme heat removal (cooling rate $> 10^4$ °C/sec) only part of the water will be trapped in crystal lattices whereas most of the water will solidify as an amorphous glass (vitrification). Riehle & Hoechli (1973) postulated that a high pressure (2.100 bar) decreased the instability of the supercooled state thereby enabling vitrification of biological specimen at lower cooling velocities ($10^4 \rightarrow 10^2$ °C/sec). Is has not been possible to employ pressure freezing in our experiments because the required complicated apparatus is not commercially available. Van Venrooij et al (1975) have demonstrated that during freezing at atmospheric pressure supercooling will in fact decrease the freezing velocity (= the cooling velocity immediately after

solidification). In dilute glycerol solutions crystallization starts between the freezing point (-1°C for a 5% solution) and -15°C (see also Mazur, 1970). Van Venrooij et al (1975) also measured the cooling velocity at different locations within a sample (Ø 1 mm) and observed the highest freezing velocity in the centre and at the periphery of a sphere or cylinder frozen by immersion in Freon 22. The intermediate part of the sample freezes at a much slower rate and displays larger ice crystals in a freeze fracture replica, whereas small ice crystals are found around the centre and at the periphery of the frozen sample. Despite the prediction by Meryman in 1956, from theoretical considerations, the occurrence of small ice crystals in the centre of a frozen spherical sample is commonly neglected (p. 54 Riehle & Hoechli 1973, see also Moor 1973, Christensen 1971, Hodson & Marshall 1970). The freezing velocity within a (biological) specimen is higher for smaller samples. This increase in freezing velocities at corresponding areas, will occur at a rate roughly proportional to the square of the reduction of the size of the sample (Van Venrooy et al 1975). With a sample diameter less than 0.3 mm frozen in Freon 22 the freezing velocity exceeds 10,000°C/sec. at every location, resulting in vitrification of the whole sample. Samples of such a size can conviniently be obtained by spraying a cell suspension into a cooling medium (Williams 1953, Bachmann & Schmitt 1971, Vervegaert et al 1975). Spraying cannot be used when tissue relationships have to remain intact, as in the case of autoradiography of steroids in testis (see discussion appendix paper 3). Moreover most mammalian cells are disrupted when they are sprayed through a small spraying nozzle. We have confirmed this with a fibroblast suspension prepared as described in appendix paper 6.

The use of cryoprotectiva like glycerol and dimethyl sulfoxide which penetrate into cells has been very useful for the improvement of the morphological preservation of cells frozen at intermediate cooling velocities (10°C/sec, Moor 1964). These cryoprotectiva probably act on binding of intracellular water thereby reducing the amount of water available for crystal formation (Meryman 1968). Cells protected by glycerol behave during freezing as partially dehydrated cells, occurring in nature as frost hardened cells (Moor 1973). This behaviour is also comparable to solutions with a high dissolved solid content (e.g. 20% gelatine W/V, Mac Kenzie & Luyet 1962) which show vitrification when cooled at intermediate cooling velocities. We did not use glycerol in our studies because its effect on the localization of soluble compounds is not known and it considerably lowers the water vapour pressure thereby increasing the drying times for freeze drying. In addition glycerol causes ultrastructural alterations of cells (Moor 1971, Moor & Hoechli 1971, Plattner et al 1973).
Where freezing is used for the autoradiographic localization of diffusible substances, we have taken the following considerations into account:
1. Cryoprotective agents cannot be used.
2. Pressure freezing can as yet not be used.
3 Samples of the smallest practical size have to be frozen.
4. By the choice of suitable quenching medium freezing should be as rapid as possible.

2.3.2.2 Experimental determination of cooling velocities

Where histological integrity is the goal, freezing should be as rapid as possible. For the preparation of cryosections and frozen dried tissue we have compared several quenching liquids on the basis of cooling velocities in testis tissue samples (table 2.2). Measurements were made with a copper /constantan thermocouple (Φ 100μ). The EMF of the thermocouple was recorded as a function of time with an U.V. recorder. The highest cooling velocities were observed in Freon 22 and propane whereas much lower cooling velocities were obtained with isopentane, liquid nitrogen and a mixture of liquid and solid nitrogen ('solid nitrogen'). Freezing by sandwiching the tissue sample between brass blocks cooled to the temperature of liquid nitrogen ('freeze clamping') and freezing with solid nitrogen sometimes resulted in cooling velocities comparable to those obtained with Freon 22. It was however difficult to obtain reproducable results with freeze clamping and freezing in solid nitrogen. As a consequence of the results presented in table 2.2 we have mainly used Freon 22 in our autoradiographic studies on the localization of diffusible substances.

Table 2-2

Cooling velocity of testis tissue samples (ca. 10 mg) in several quenching media (cooled with liquid nitrogen)

Quenching medium	Temperature interval (-°C)	Cooling velocity (°C/sec.)
Isopentane	10-110	222
Liquid nitrogen	0- 65	80
Solid/liquid nitrogen	0- 65	250
Propane	0- 20	833
	20- 80	1540
Freon 22	0- 30	1540
	20- 80	1430

2.3.2.3 Review of the principles of freeze drying

Tissues can be dehydrated bij placing frozen tissue in a vacuum. Ice has a relatively high vapour pressure even at -70°C (1×10^{-3} mm Hg, Wheast & Selby 1966) and the ice will sublimate if it is kept at this temperature and in close proximity to a cold trap at a lower temperature. Freeze drying for dehydration of cells has generally been used for the preservation of labile substances which might otherwise be extracted or decomposed when liquid fixatives and dehydration solvents are used (see Pearse 1968). Since there was no freeze-dry equipment commercially available that

could meet the requirements of electron microscopic autoradiography we developed the freeze dryer with ancillary equipment described in appendix paper 3. The physical aspects of freeze drying forming the basis of this design can be largely derived from treatments of Malmström (1951) for histological work and from King (1971) for commercial drying of foods. The physical principles discussed in the next paragraphs formed the basis of our design of a freeze drying module.

The rates of freeze drying are determined by
1. The introduction of heat to supply the energy required for sublimation.
2. The transfer of water vapour from the subliming ice front through the already dried shell.
3. The removal of water vapour that reaches the surface of the specimen.

Energy required for sublimation
The heat of sublimation must be supplied from a heat source from which it is transferred, under the influence of a temperature difference, to the site where sublimation occurs. In the freeze dryers developed by Meryman (1960b), Boyde & Echlin (1973) and Dormans (1976) the heat approaches the sublimation front through the already frozen dried shell, which has poor heat conducting properties. By conduction through ice better heat transfer is found to the sublimation front (Meryman 1960b, King 1971). As a consequence the rate of freeze drying is now dependent on the removal of water vapour from the sublimation site ('mass transfer controlled' King 1971). Heat conduction through ice is employed in freeze-drying of liquids (coffee, orange juice) and may be advantageous in histological freeze drying. The maximum rate of sublimation (Qn) of ice can be derived from the kinetic theory of gasses (Malmström 1951):

$$Qn = 0.25\,(P_T - Pa)\ \frac{1}{\sqrt{T}} \quad (1)$$

Qn is expressed in $gr/cm^2/sec$. P_a is the pressure (in mm Hg) in the vacuum system, P_T is the saturated vapour pressure of water at a given temperature T. From equation (1) it follows that the rate of evaporation of water from the sample depends on two parameters, the vacuum Pa and the specimen temperature T. Since P_T is a function of T it is not an independent variable. When working under sufficient vacuum ($Pa < 10^{-2}\ P_T$) only the specimen temperature determines the sublimation rate.

Vapour transfer through the dried shell
The coefficient of diffusion, which is a parameter of the obstruction introduced by the dry shell is dependent on the size of the 'holes' formerly occupied by ice (King 1971). Estimates of the diffusion coefficient have been made for slowly frozen large specimen (King 1971) but not for rapidly frozen tissue. With the much smaller holes found after freeze drying of rapidly frozen tissues vapour flow may be influenced by vascular pathways and cracks which provide intermittant low resistance to vapour flow (Meryman 1960b). As a rough estimate of the resistance to vapour flow in frozen dried tissue Stephenson (1960b) predicted that a 1 mm frozen dried layer lowers the drying rate with a factor 10^3 relative to the maximum sublimation rate.

Removal of water vapour

The rate of removal of the evaporated water (Qr) can be calculated from Knudsens equation (Malmström 1951):

$$Qr = \frac{4}{3}\frac{2\pi M}{RT_S} \times \frac{r^3}{L}(P_a - P_V) \quad (2)$$

where r = radius of the tubing through which the gas flows, L = length of path from sample to trap, T_S = specimen temperature. Qr is given in gm/cm²/sec. R is the gas constant, M is the molecular weight and P_V = partial pressure of water at the trap, P_a = pressure in the vacuum system. As can be seen from this equation the radius of the tubing and the distance to the vapour trap are important factors in the design of a freeze dryer. The factor $\frac{r^3}{L}(P_a - P_V)$ is a measure of the impediment of water molecules leaving the specimen towards the trap (Malmström 1951). The rate of removal can be optimized by placing a cold trap in close proximity to the specimen. With the cold trap at liquid nitrogen temperature the partial water vapour pressure will be 10^{-24} mm Hg (Malmström 1951). With a liquid nitrogen trap and specimen temperature between -50 and -100°C the trapping efficiency will only depend on the solid angle over which the trap covers the specimen.

We have constructed a freeze drying module based on these physical considerations with the following specifications

1. the specimen temperature is thermostatically controlled and kept below -70°C to avoid crystallization during freeze drying;
2. a high vacuum (10^{-6} mm Hg) is used to optimize the rate of evaporation;
3. a liquid nitrogen trap is placed close to the specimens which are covered over a large solid angle in order to collect directly the water molecules emerging from the specimen.

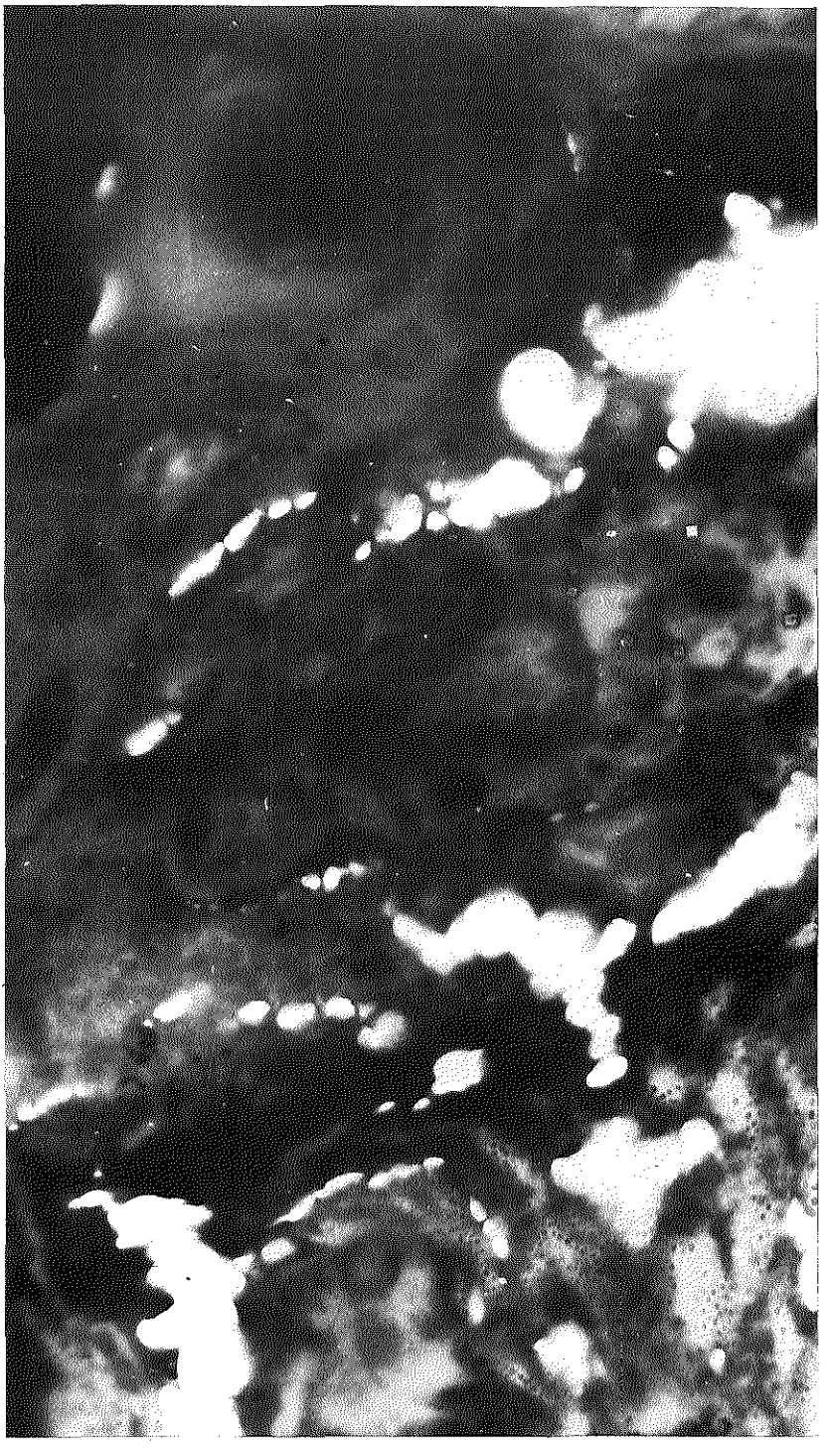
The design (described in appendix paper 3) is essentially based on the freeze-etch apparatus of Steere (1969), who suggested that after some modifications this type of apparatus would become an excellent freeze dryer (Steere 1973).

2.3.3 Review of cryo-techniques for autoradiography of diffusible substances

2.3.3.1 Use of cryosections

Light microscopy

A photographic emulsion can be brought in contact with a frozen or frozen dried cryotome section for the autoradiographic localization of radioactive diffusible substances. The first successful applications of this technique were reported by Fitzgerald (1961). Considerable improvements of the originally proposed procedures have been described by Appleton (1964) for frozen sections and by Stumpf (1964) for frozen dried sections. These improvements involve the lowering of the cutting temperature and a better attachment of the sections to the photographic emulsion. In comparative studies both methods have revealed the same localization of radioactivity (Rogers & Brown-Grant 1971, John & Rogers,



autoradiography of steroids, a technique which was expected to give a more reproducible yield of large numbers of thin sections. Initial experiments with frozen dried testis were performed with commercially available tissue dryers. The ultrastructure in these preparations was damaged but the tissue relationships were preserved enabling the identification of cell types. Upon improving the freeze-drying procedure (sections 2.3.2.3) we expected a better preservation of the ultrastructure (Stirling & Kinter 1967). This was confirmed with the developments described in appendix paper 3. A good preservation of the ultrastructure was achieved in conjunction with a small (less than 1%) extraction of tritiated steroids during embedding (appendix paper 3). Material obtained according to these procedures was used for light microscopic studies concerning the localization of androgens and oestradiol in rat testis (appendix papers 3 and 4) and the localization of mucopolysaccharides in fibroblasts (appendix paper 6). A source of extraction of steroids (and other diffusible substances) was eliminated by cutting ultra thin sections on a ultracryotome without using floatation liquids. Encouraging results were obtained with a Reichter FC 150 ultracryotome (appendix paper 2) but we were not able to reproduce these with consistency. In later studies we found that thin sections of frozen dried testis can be obtained reproducibly and in sufficient numbers by 'dry' cutting of Epon embedded tissue at low temperatures using a LKB-cryokit (appendix paper 3), allowing autoradiographic localization of steroids at the electron microscope level (appendix paper 5).

2.3.4.2 Discussion of the freeze-dry technique

Ultrastructure and localization diffusible substances

Upon freezing of spherical or tubular tissue fragments (Ø 1 mm) in Freon 22 small ice crystals are found at the periphery and around the centre with larger ice crystals in the intermediate part (section 2.3.2.1). Ice crystal formation is accompanied by segregation between water and its solutes. When (small) ice crystalls are visible in a micrograph this picture also reflects the degree to which translocation of water soluble substances has occurred during freezing and freeze drying. After freezing of small tissue samples every subsequent step must be carried out under conditions that prevent as much as possible further ice crystal growth, in order to achieve the maximal resolution in the final autoradiograph. During freeze drying the important parameter for growth of ice crystals (section 2.3.2.1) is the temperature of the frozen zone. At the start of drying in our freeze dryer the temperature of the frozen zone deviated less than 5°C from the temperature of the specimen support, which indicated a good thermal contact between these two. Theoretically (eq. 1 section 2.3.2.3) freeze drying will initially proceed at the maximum rate of 90μ/cm^2/hr ($\simeq$ 1 mm/cm^2/11 hr) under the employed conditions (specimen at -85°C, water vapour trap, high vacuum). Theoretically

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fig. 2.1 Frozen thin section, 7200x.
Mouse testis was frozen in liquid/solid nitrogen and sectioned on an ultracryotome (Sorvall FTS) with a dry glass knife. The section was freeze dried and covered with a thin layer of carbon.

therefore our specimen could be completely dehydrated within 11 hrs if the already frozen dried tissue does not impair water vapour flow. However such a dry shell certainly increases resistance to vapour flow especially after rapid freezing (section 2.3.2.2). Therefore it is difficult to predict to which level freeze drying has proceeded after overnight drying at -85°C. In principle information about the progress of drying can be gained by measuring weight loss as a function of drying time. However such measurements are complicated and we have confined ourselves to the freeze dry schedule outlined in appendix paper 3. At present we have to assume that during drying at -85 C only the outer rim of the sample is dehydrated and that the more interior parts are dehydrated upon slow warming from -85°C. This will probably occur at ice temperatures between -60°C and -40°C when the maximum rate of freeze drying is between 5 mm/cm²/hr and 600 mm/cm²/hr. After this freeze drying procedure free water has been sublimated but bound water may still be present. Part of this bound water is removed during secondary drying at 30°C at the end of the freeze dry run.
During the subsequent fixation with osmium tetroxide vapour contrast is obtained in the specimen. The fixing action of osmium tetroxide vapour involves the crosslinking of unsaturated compounds (especially phospholipids, Stoeckenius & Mahr 1965). Translocation or elimination of labelled steroid is not to be expected at this stage. During embedding two effects have to be considered: the passage of the surface of the embedding medium through the specimen with possible effects of surface tension (Hanzon & Hermodsson 1960) and diffusion along

Table 2.3

Translocation of diffusible substances during procedures for autoradiography. Possible sources of translocation and their prevention

Source of translocation	Treatment to prevent (minimize) translocation
Freezing	High freezing velocity
Crystal growth during freeze drying	Controlled freeze drying
Fixation in osmium tetroxide vapour	Only compounds reacting with osmium tetroxide vapour are fixed
Embedding in Epon 812	Embedding in an inert low viscosity resin
Section floating on bath liquids	Dry sectioning
Application of a photographic emulsion	Sections protected with a carbon layer

boundary layers, and the solvent action of the embedding medium. Embedding does not result in appreciable losses of tritiated steroids (appendix paper 3) and a number of other compounds (see appendix paper 5). When conventional procedures for fixation, dehydration and embedding are used, lipids (and lipid like substances) are extracted to a much greater extend (see Stein & Stein 1971 and Williams 1969 for reviews) than with the freeze drying method. Damage to the ultrastructure seems to be limited since a striking similarity can be observed between the ultrastructure found in frozen dried and embedded tissue and ultrastructure of cryosections of unfixed tissue (Hodson & Williams 1976, Spriggs & Wynne-Evans 1976) and freeze substituted tissue (Dempsey & Bullivant 1976). In those cryosections as well as in our frozen dried tissue a good preservation is found at the outer layer of the sample with a gradual increase in ice crystal size towards the interior (resulting in 'knitting', Hodson & Williams 1976). We have not been able to observe a central part with good structural preservation which was expected as explained in section 2.3.2.1 in serial sections (2.5 - 5 μ) around the midplane of a frozen dried and embedded testis tissue sample. This may be due to the irregular size of the sample, with an ill defined geometrical centre, or more probably, to freeze drying of the interior at elevated temperature ($>$ - 85°C).
Dry sectioning and dry mounting of the sections prevents the diffusion of label towards the photographic emulsion. Table 2.3 summarizes the possible sources of translocation during tissue processing for autoradiography of diffusible substances.

Ice crystals and autoradiographic resolution
In the final section freezing damage with its concommittant segregation of water and solutes should be considered as the major source of redistribution of diffusible substances. The optimal autoradiographic resolution (H.D. value; as defined by Salpeter, Bachmann & Salpeter 1969) can be estimated for 1000 Å thick sections as 1400 Å for Ilford L4 emulsion developed in Kodak D19 (Salpeter, Budd & Mattimoe 1974). A decrease in resolution to 1600 Å can be predicted by interpolation from graphs presented by Salpeter et al (1969) with ice crystal diameters of 700 Å. Such crystals are found at a depth of 7 μ from the surface of a freeze dried tissue sample (e.g. fig. 2 appendix paper 5). Accordingly a further decrease in resolution to 1700 Å corresponds to ice crystals having a diameter of 1400 Å which are found at a depth of 20 μ from the edge.
These figures imply that quantitation of autoradiograms is distinctly possible at a considerable depth in the frozen dried sample. It can however also be learned that optimal autoradiographic resolution can only be achieved at the two or three outermost cell layers. Optimal resolution in the autoradiographs can be expected in the whole specimen when ice crystals everywhere in the sample are smaller than 700 Å. This will only be achieved by freezing very small samples or a monolayer of cells such as the fibroblasts used in appendix paper 6. Such samples when freeze dried allow application of the more sophisticated methods of aquisition of autoradiographic data and their statistical treatment (Blackett & Parry 1973, Parry & Blackett 1976).

2.4 Summary and conclusions

In section 2.1 (and appendix paper 3) results were discussed concerning precipitation of steroids by addition of a specific reagent to the fixative. For an optimal retention of steroids (or other compounds under investigations) the fixative and the specific reagent should have the same diffusion velocity and the same solubility in cell and tissue components, whereas the precipitate should be completely insoluble in the liquids employed. Our experiments have demonstrated that these conditions are not fullfilled when digitonin is added to all solutions for tissue processing. Hence precipitation of precursors in testosterone biosynthesis by this method is not possible.

Freezing of tissue immobilizes all its components at approximately the same time (physical fixation, section 2.3). When frozen tissues are used deleterious effects of freezing and freeze-drying have to be taken into account (section 2.3.2). Frozen tissue may be sectioned directly in order to use the resulting sections for autoradiography (section 2.3.3.1). Alternatively, frozen tissue samples can be dried in vacuo, fixed in osmium vapour and embedded in order to enable sectioning for autoradiography (section 2.3.3.2). We have followed both approaches to investigate their applicability to the localization of steroids in testis with autoradiography at the electron microscope level (section 2.3.4). The yield of cryosections giving good morphological details was too small in our hands for reliable autoradiographic studies. A much higher yield of sections, with a well preserved ultrastructure was obtained after freeze drying of tissue samples (appendix paper 3, sections 2.3.4). In section 2.3.4.2 the factors have been discussed which contribute to the translocation of steroids and ultrastructural alterations during the freeze dry process. By relating these factors to the autoradiographic resolutions it has been argued (sections 2.3.4.2) that optimal resolution can only be achieved at the outer cell layers of the frozen dried sample whereas the autoradiographic resolution will gradually decrease towards the more interior parts.
Experimental evidence described in this chapter indicates that at the present time the freeze-dry method is the only method that appears applicable to the study of the localization of steroids by autoradiography at the electron microscope level.

Localization of diffusible substances by autoradiography

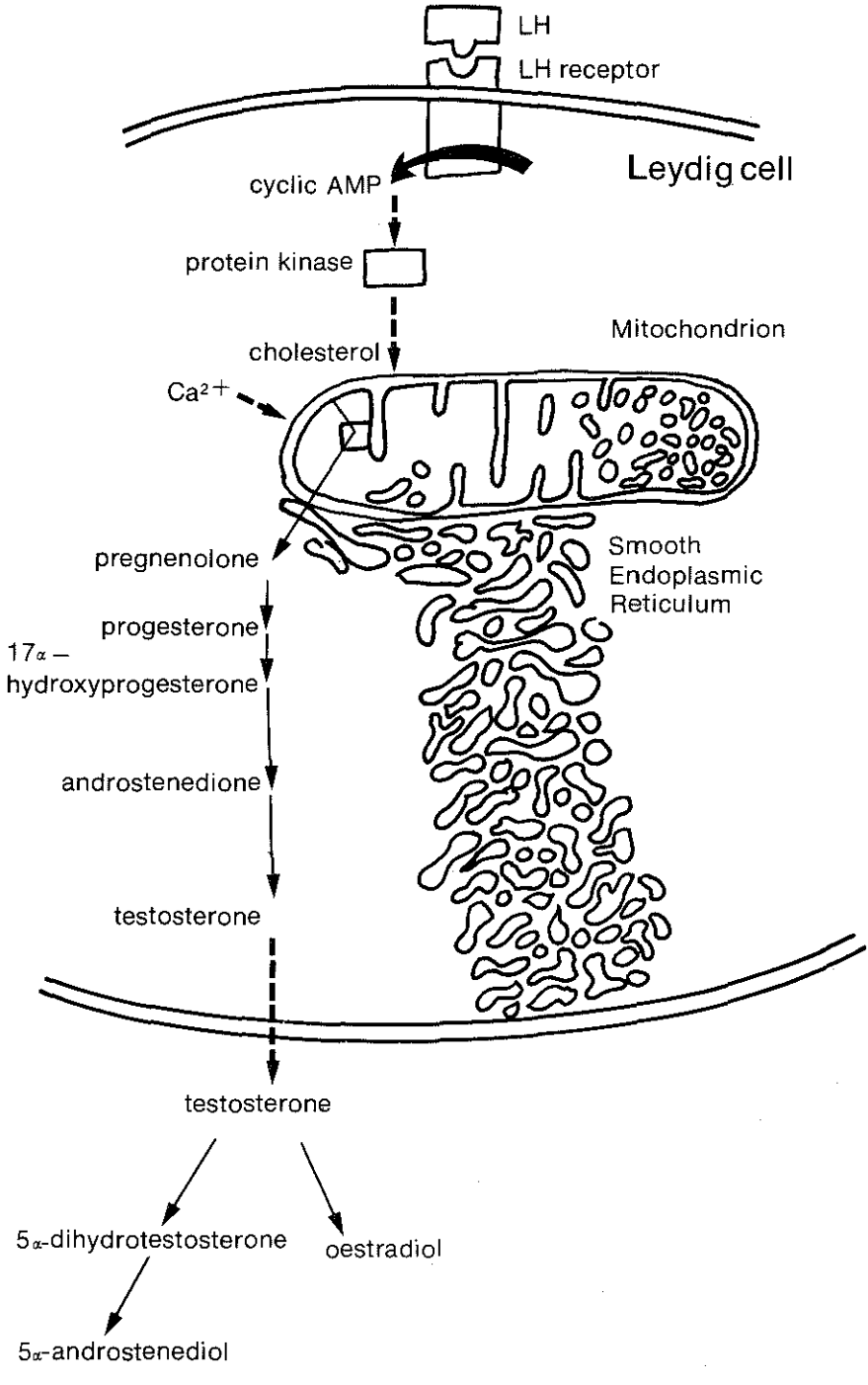
General aspects of steroid localization will be summarized in order to provide the background information for the integration of autoradiographic data in a more generalized picture of the dynamics of steroids in testis*.

3.1 Biosynthesis and transport of steroids in testis

The production of testosterone in the testis is restricted to the Leydig cells of the interstitial tissue (Cooke et al 1972a). A scheme of the biochemical pathways of testosterone biosynthesis is given in figure 3.1. The activity of the cholesterol-side-chain cleavage enzyme complex (SCE) responsible for the conversion of cholesterol to pregnenolone is present in mitochondria and is rate limiting in the overall testosterone production (Hall 1970). Pregnenolone is converted to testosterone by enzymes mainly located in the smooth endoplasmic reticulum of the Leydig cells (for reviews see Tamaoki 1973, 1975). The testosterone produced leaves the Leydig cells and can be found in testicular venous blood and in testicular lymph of the interstitial compartment (Cooper & Waites 1975, Setchel & Waites 1975). Previous experiments with whole body autoradiography seemed to indicate that injected testosterone did not enter the seminiferous tubules (Appelgren 1969). However, testostorene could be recovered from the seminiferous tubules both after dissection from fresh tissue and from freeze dried sections of testis (Galjaard et al 1970, Parvinen et al 1970, Rommerts et al 1973, van Doorn et al 1974). No data were available on the localization of steroids within the seminiferous tubules. The entrance of tritiated steroids in the testis has also been investigated by analysis of the tissue fluids. After injection of steroids a rapid equilibrium was always found between steroid concentrations in testicular venous blood and in testicular lymph whereas the rate of appearance of steroids in the rete testis fluid was found to decrease in the order or testosterone>progesterone>pregnenolone>5α reduced androgens>oestrogens>corticosteroids (Cooper & Waites 1975). With respect to the relatively free passage of these steroids as lipophylic substances the blood testis barrier appears to resemble the blood-brain barrier (Dhopeshwarker & Mead 1973). Testosterone produced in the Leydig cells may be further metabolized to 5α reduced metabolites (Ficher & Steinberger 1968 see also van der Molen et al 1975). In young rats the conversion of testosterone to oestradiol probably occurs in the Sertoli cell (Armstrong et al 1975). De Jong et al (1973) have demonstrated

* For extensive reviews the reader is referred to: Handbook of Physiology, section 7 vol. 5 (D.W. Hamilton & R.O. Greep eds.) Amer. Physiol.Soc., Washington D.C. (1975); Hormonal regulation of spermatogenesis (F.S. French, V. Hansson, E.M. Ritzen & S.N. Nayfeh, eds.) Plenum Press, New York & London (1975); The testis (A.D. Johnson, W.R. Gomes & N.L. Van Demark eds.) Acad. Press, New York (1970).

Fig. 3.1 Biosynthesis and metabolism of testosterone



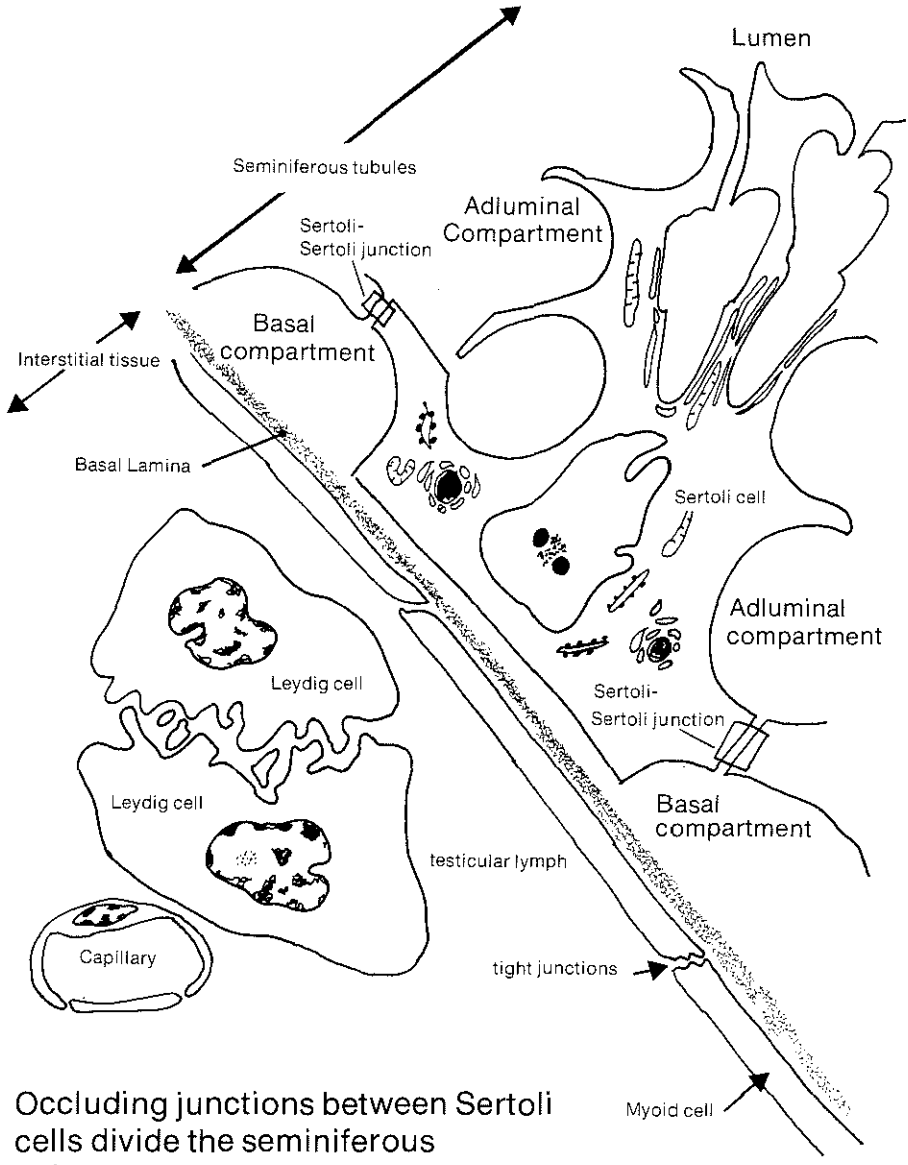
that testes of adult rats produce oestradiol which is secreted into the venous blood.

Oestradiol can also be bound by a receptor protein (Brinkmann et al 1972) in the nuclei of the Leydig cells (Mulder et al 1973). The interaction of a steroid with a cytoplasmic receptor protein is generally considered as the initial step in the effect of steroids on their target cells (for reviews see Chan & O'Malley 1976a, b, c). A transformed steroid-receptor complex enters the nucleus and may induce the synthesis of specific nucleic acids and proteins. An androgen receptor protein found in the seminiferous tubules is probably present in the highest concentration in Sertoli cells (Mulder et al 1975) although its presence in Leydig cells cannot be excluded. Apart from the receptor protein, an androgen binding protein (ABP) is found in the seminiferous tubules. ABP is secreted by Sertoli cells into the seminal fluid under the influence of FSH or testosterone (Ritzen et al 1975). The physiological role of ABP in testis is not known (Rommerts et al 1976).

3.2 Morphological aspects of steroid localization in the testis

The morphology of the Leydig cells in seasonal breeding animals shows cyclic changes (for review see Christensen 1975). These morphologic changes have been correlated with variations in testosterone production. In some non seasonal breeding animals (not in rats) morphological changes can also be observed after long term stimulation with LH (Massa & Aoki 1976). The testosterone produced by the Leydig cells under control of LH, is released by an as yet unknown mechanism (Chistensen 1975) into the surrounding testicular lymph. From the testicular lymph testosterone can either enter into the vascular bed or into the seminiferous tubules. In contrast to the relatively free access of steroids to the seminiferous tubules it has been reported that other compounds such as acridine dyes (Kormano 1968), extracellular tracers like horse-radish peroxidase and lanthanum nitrate (Dym & Fawcett 1970, Fawcett et al 1970) and sucrose (Setchell & Singleton 1971) do not enter the seminiferous tubules. The barrier for extracellular tracers in most instances seems to be the myoid cells. It has been observed that the horse-radish peroxidase and lanthanum nitrate may pass the myoid cell barrier at some sites but further penetration is than prevented by tight junctions between adjacent Sertoli cells (Dym & Fawcett 1970, Fawcett et al 1970, see fig. 3.2). The junctional complexes between Sertoli cells divides the tubules in two compartments (see fig. 3.2) with a different composition of the extracellular fluid. The compartments along the basement membrane have a composition similar to lymph whereas the adluminal compartment has a completely different composition characterized by a high potassium bicarbonate and inositol content and a low protein content (for review see Setchel & Waites 1975). The tight junctions between Sertoli cells are not permanent and may break down transiently to permit a cluster of preleptotene spermatocytes to pass from the basal to the adluminal compartment of the tubules. Available evidence

Fig. 3.2 Functional compartments of the testis



Occluding junctions between Sertoli cells divide the seminiferous epithelium into a basal compartment occupied by spermatogonia and preleptotene spermatocytes and an adluminal compartment containing spermatocytes and spermatids (after Fawcett 1975).

suggests that during this passage the barrier remains intact through the formation of a new junctional complex (Fawcett 1975).

3.3. Localization of steroids in the testis studied by autoradiography

3.3.1 Localization of oestradiol in the testis

The site of oestradiol synthesis cannot be established with autoradiography since the formation of oestradiol from tritiated precursors is hardly detectable is testis tissues (de Jong et al 1974). From the work of Stumpf (Stumpf 1969, Stumpf et al 1971) it is known that administered oestradiol can be demonstrated with autoradiography in the nuclei of the Leydig cells. Concentration of oestradiol in the nuclei of the interstitial Leydig cells has also been demonstrated after testis perfusion with tritiated oestradiol (appendix papers 3 and 4) as well as after incubation of dissected interstitial tissue with tritiated oestradiol (P.M. Frederik & W. de Boer, unpublished observation). The accumulation of oestradiol in the nuclei as demonstrated by autoradiography could be prevented by incubating dissected interstitial tissue with tritiated oestradiol together with a hundred fold excess of unlabelled oestradiol. Binding of tritiated oestradiol to the nuclear receptor protein is prevented under similar experimental conditions (Brinkmann et al 1972, Mulder et al 1973). Therefore it appears most likely that the nuclear accumulation of oestradiol in Leydig cells observed by autoradiography is the result of the interaction of oestradiol with a receptor protein.

3.3.2 Localization of testosterone in the testis

3.3.2.1 Biosynthesis and transport studied by autoradiography

For the autoradiographic study of the transfer of testosterone and related steroids from their site of production towards their targets, we have perfused rat testis with tritiated percursors of testosterone. In preliminary studies testes of intact rats were perfused with ^{3}H-cholesterol. The uptake of free cholesterol and cholesterol stearate from the vascular bed was low (0,5% and 0,06% respectively, see also appendix paper 4 and Parvinen et al. 1970). After isolation and separation of the steroids the conversion of the incorporated cholesterol to androgens was hardly detectable, so that it was not possible to obtain detailed biochemical or autoradiographic results on the initial stages of steroidogenesis. Autoradiographic results obtained after perfusion of intact rats with tritiated pregnenolone have demonstrated an even distribution of radioactivity over all cell types. Distinct differences in grain densities over cells were obtained by using rats 5-6 days after hypophysectomy. Endogenous testosterone production in these rats is low (due to a low activity of the cholesterol side-chain cleaving enzyme, Menon et al 1975) but could be stimulated by perfusion with LH (unpublished observation). Enzymes involved in the synthesis of testosterone from pregnenolone are still present in the testis

5-6 days after hypophysectomy since after perfusing with ^{3}H-pregnenolone the formation of physiological amounts of testosterone could be demonstrated (appendix paper 4).
Upon autoradiography of testis tissue, high grain densities were observed above the Leydig cell cytoplasm in testes which were removed either 5 or 10 min. after perfusion with ^{3}H-pregnenolone. This localization corresponds to the presumed site of conversion of pregnenolone to testosterone (Tamaoki et al 1975). A specific localization of autoradiographic grains above the cytoplasm of Leydig cells was also found after perfusion of testes with ^{3}H-testosterone followed by 5 min. perfusion with buffer. Beardsley and Hilton (1975), studying the mouse testis by autoradiography according to the "Stumpf-method" (for review see Stumpf 1975) also found preferential localization of testosterone in the cytoplasm of the Leydig cells. The localization of radioactivity after perfusion of labelled testosterone is thus quite similar to the localization of radioactivity after pregnenolone perfusion. This may be due in part to the conversion of pregnenolone to testosterone. A distinction between testosterone and pregnenolone localization in the Leydig cell can possibly be made in future experiments by blocking the pregnenolone conversion (e.g. with cyanoketone, van der Vusse et al 1974) or by studying the localization of these hormones at the EM level. The time course study with tritiated pregnenolone indicates that the concentration gradient over the seminiferous tubule, which occurs immediately after the introduction of radioactivity, will gradually disappear after longer perfusion periods. By extrapolation of these results and from our initial autoradiographic studies with intact rats we have concluded that androgens are evenly distributed over the seminiferous tubules of the intact adult rat.

3.3.2.2 Interaction with target structures

The perfusion experiments with ^{3}H-testosterone (appendix paper 4 and 5) followed by perfusion with a hundred fold excess of unlabelled testosterone were an attempt to localize testosterone target cells under (more or less) physiological conditions. Under the experimental conditions the unlabelled testosterone should rapidly exchange with non specifically bound ^{3}H-testosterone so that tritiated testosterone would only be bound to the receptor sites. No concentration of label in nuclei could be demonstrated. After long term hypophysectomy nuclear concentration of androgen was demonstrated in the Sertoli cells with autoradiography (Sar et al 1975) as well as with biochemical methods (Mulder et al 1975). This ability can apparently be demonstrated only after long term hypophysectomy and not in the intact animal.
A detailed picture of the distribution of androgen radioactivity in testis after perfusion with ^{3}H-testosterone was obtained after autoradiography at the electron microscope level (appendix paper 5). For these studies testes from rats (5-6 days after hypophysectomy) were perfused with tritiated testosterone for 3 min., followed by 15 min. perfusion with buffer containing a hundred fold excess of unlabelled testosterone. A high grain density was observed above lipid droplets and areas rich in smooth endoplasmic reticulum such as found in the basal part of the Sertoli cells

and in the Sertoli cell cytoplasm surrounding the head pieces of elongated spermatids. The observations made by electron microscopic autoradiography provide no information about the role of androgen binding by proteins. The results may be explained by assuming that androgens are slowly exchanged between (phospho-) lipid rich areas and the cytoplasm.

Summary

Steroids produced in the interstitial tissue of the testis appear to be involved in the control of spermatogenesis in the seminiferous tubules. Little is known, however, about the transport and possible site of action of these steroids in the testis. In order to follow the intercellular and intracellular fate of steroids and to identify the cell types and cell organelles involved in steroid action, autoradiography seems the method of choice since this method avoids the disruption of cells and enables in situ studies at the level of the light microscope and the electron microscope. Steroids (and other 'diffusible substances') are however extracted when normal procedures of tissue preparation for microscopy are use. Therefore we have compared and developed preparative methods with the aim to retain steroids during all steps of tissue processing and to preserve the ultrastructure.

Chapter 2 and the appendix papers 2 and 3 deal with methods for autoradiography of steroids at the electron microscope level.

In chapter 3 and in the appendix papers 3, 4 and 5 the localization of steroids in testes is described, as observed with autoradiography. Testes of rats (5-6 days after hypophysectomy) were perfused with either tritiated pregnenolone, testosterone or oestradiol. The administration of tritiated steroid by perfusion was followed by perfusion with buffer for varying time intervals and subsequent processing of testis tissue samples for autoradiography using the freeze dry method.
All celltypes in the tubular and interstitial compartment contained radioactivity. Differences in the labelling of tubular cell types could only be demonstrated after the determination of the grain density distribution in the autoradiographs (appendix paper 4).
After pregnenolone and testosterone perfusion the highest density of autoradiographic grains was found above the cytoplasm of the interstitial Leydig cells (appendix paper 4). The grain density of the seminiferous tubules was highest in the basal layers and decreased towards the tubular lumen. The ratio between grain densities of the basal layer of cells and the adluminal layer of cells decreased when perfusion with tritiated pregnenolone was followed by 10 min. instead of 5 min. perfusion with buffer. In the same animal model the tubular radioactivity was analysed by autoradiography after perfusion of testis with tritiated testosterone followed by 15 min. perfusion with buffer containing a hundred fold excess of unlabelled testosterone. It was attempted with this experiment to detect possible target nuclei for andorgen in thhe seminiferous tubules, but the autoradiographs showed instead the highest grain densities above the basal cytoplasm of Sertoli cells and above lipid droplets within these Sertoli cells (appendix paper 4). These light microscopic observations were tentatively confirmed by studying the tubular grain density after autoradiography at the electron microscopic level. In addition evidence was obtained for a preferential localization of androgen in membranes of the smooth endoplasmic reticulum of Sertoli cell cytoplasm. From these results it can be concluded that in the testis,

androgens produced in the Leydig cells of the interstitial tissue can freely enter the seminiferous tubules without any preference for certain cell types or cell organelles. It was not possible to detect any specific accumulation of androgen which could be involved in the control of spermatogenesis.
Concentration of oestradiol in the nuclei of the interstitial Leydig cells was demonstrated by autoradiography after perfusion of rat testes (5-6 days after hypophysectomy) with tritiated oestradiol. The observed concentration of oestradiol is most likely the result of the interaction with a receptor protein.

The results presented in this thesis are the first account of autoradiography of steroids at the electron microscope level. The methods described can however also be applied for the localization of other 'diffusible' organic components in cells and tissues. This has been illustrated by the autoradiographic localization of accumulated acid mucopolysaccharides in cultured fibroblasts derived from a patient with a genetic metabolic disease; mucopolysaccharidosis type II (Hunter's disease). After labelling with $^{35}SO_4$ and using a special preparation procedure it was possible to distinguish normal cultured cells and enzyme deficient cells showing accumulation of (partly degraded) mucopolysaccharide. Since such a distinction could also be made at the single cell level after mixing normal and genetically defective cells, this method might also be useful in carrier detection of this X-linked disease and in its prenatal diagnosis (appendix paper 6).

Samenvatting

Steroid hormonen welke in het interstitiële weefsel van de testis geproduceerd worden, lijken betrokken te zijn bij de spermatogenese in de seminifere tubuli. Er is echter weinig bekend over het transport en de mogelijke plaatsen in de testis waar deze steroiden hun werking uitoefenen. Om de celtypen te identificeren die bemiddelen of doelwit vormen bij de werking van steroid hormonen leek autoradiografie de aangewezen methode. Bij deze methode wordt het stuk maken van cellen vermeden, en kan de lokalisatie van steroiden **in situ** bestudeerd worden zowel op licht- als op elektronen-microscopisch niveau. Bij gebruik van de normale procedures voor microscopie worden steroiden (en andere 'oplosbare verbindingen') geëxtraheerd. Met het oogmerk om de steroiden in het weefsel te behouden en tevens een goede preservatie van de ultrastructuur te verkrijgen, hebben wij een aantal prepareertechnieken vergeleken en verder ontwikkeld.

In hoofdstuk 2 en de appendix publicaties 2 en 3 worden de methoden behandeld voor de autoradiografische lokalisatie van steroiden op elektronenmicroscopisch niveau.

In hoofdstuk 3 en in appendix publicaties 3, 4 en 5 wordt de lokalisatie van steroiden in de testis beschreven zoals deze afgeleid kan worden uit autoradiografische studies.
Testes van ratten (5-6 dagen na hypophysectomie) werden geperfuseerd met getritieerd pregnenolon, testosteron of oestradiol. Na toedienen van het getritiëerde steroid werd de perfusie vervolgd met buffer gedurende verschillende periodes en vervolgens werden monsters genomen die volgens de vriesdroog methode autoradiografisch geanalyseerd werden. Alle cellen in het interstitiële- en tubulaire compartiment bevatten radioactiviteit. Concentratie-verschillen konden slechts vastgesteld worden nadat de korreldichtheid in de autoradiogrammen bepaald was (appendix publicatie 4). Na pregnenolon en testosteron perfusie werd de hoogste dichtheid van de autoradiografische korrels waargenomen boven het cytoplasma van de Leydig cel (appendix publicatie 4) De korreldichtheid van de seminifere tubuli was het hoogste boven de basale cellaag en nam af in de richting van het lumen. De ratio tussen de korreldichtheden van de basale laag en de adluminale laag van cellen nam af, wanneer de perfusie met getritieerd pregnenolon gevolgd werd door 10 min. perfusie in plaats van 5 min. perfusie met buffer. In dezelfde proefdieropstelling werd met autoradiografie de tubulaire radioactiviteit geanalyseerd na testes perfusie met getritiëerd testosteron gevolgd door 15 min. perfusie met buffer welke een hondervoudige overmaat bevatte aan niet getritieerd testosteron. Hoewel dit experiment beoogde doelwitcelkernen van testosteron op te sporen in de seminifere tubuli, lieten de autoradiogrammen de hoogste korreldichtheden zien boven het basale cytoplasma van de Sertoli cellen en boven lipide druppels in deze Sertoli cellen (appendix publicatie 4). Deze licht-microscopische waarnemingen werden voorlopig bevestigd door de bepaling van de tubulaire korreldichtheden met elektronen-microscopische autoradiografie. Bovendien werden hier aanwijzingen verkregen dat androgenen voorkeur vertonen

voor de membranen van het gladde endoplamatische reticulum van de Sertoli cel. Uit de resultaten kan worden afgeleid dat de androgenen, geproduceerd in de Leydig cellen van het interstitiële weefsel van de testis, onbelemmerd de seminifere tubuli binnengaan zonder enige voorkeur voor een bepaald celtype of celorganel. Een specifieke lokalisatie van androgenen welke mogelijk betrokken waren bij spermatogenese kon niet worden opgespoord.
Met autoradiografie werd na perfusie van ratte testes (5-6 dagen na hypophysectomie) met getritiëerd oestradiol een concentratie van oestradiol in de kernen van de Leydig cellen waargenomen. Deze concentratie wordt zeer waarschijnlijk veroorzaakt door de interactie van oestradiol met een receptor eiwit.

In dit proefschrift worden voor het eerst resultaten gegeven van de autoradiografische lokalisatie van steroiden op elektronenmicroscopisch niveau. De aangegeven procedures voor licht- en elektronenmicroscopische autoradiografie kunnen ook toegepast worden op andere 'oplosbare' organische bestanddelen van cellen en weefsels. Een voorbeeld hiervan vormt de autoradiografische lokalisatie van gestapelde zure-mucopolysacchariden in gekweekte fibroblasten afkomstig van een patient met een genetische metabole ziekte; mucopolysaccharidosis II (ziekte van Hunter). Na opname van $^{35}SO^{4}$ en met gebruik van een speciale procedure bleek het mogelijk om normale gekweekte cellen en enzym deficiënte cellen welke (gedeeltelijk afgebroken) zure-mucopolysacchariden gestapeld hadden, van elkaar te onderscheiden. Daar dit onderscheid ook op het niveau van de enkele cel gemaakt kon worden na het mengen van normale en genetisch defecte cellen, is de methode wellicht bruikbaar voor zowel het drager onderzoek van deze aan het X-chromosoom gebonden ziekte als voor de prenatale diagnostiek.

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Curriculum vitae

Op 30 oktober 1944 werd P.M. Frederik te Utrecht geboren. Het eindexamen HBS-B werd in 1962 behaald aan het Thorbecke Lyceum te Utrecht. In hetzelfde jaar werd de studie in de biologie aangevangen aan de Utrechtse Universiteit. Deze studie werd tijdelijk onderbroken voor activiteiten in het studenten verenigingsleven (senaat, redacties, per huifkar tussederusse). Hierna werd in 1967 het kandidaatsexamen (k) behaald en in 1970 het doctoraal examen met als hoofdvak planten-fysiologie (hydrobiologie & plantenfysiologie) en als bijvakken elektronenmicroscopie en biochemie. In de periode 1965-1970 werd de functie van student-assistent bekleed in de plantensystematiek (R.U. Utrecht) en in de hydrobiologie (ZWO). In januari 1971 volgde de aanstelling als wetenschappelijk medewerker aan de afdeling Biochemie (Chemische Endocrinologie) van de Erasmus Universiteit Rotterdam, waar het in dit proefschrift beschreven onderzoek werd verricht. In december 1975 werd tenslotte een aanstelling verleend aan de Rijks Universiteit Limburg (capaciteitsgroep Algemene Pathologie) waar een elektronenmicroscopisch laboratorium werd opgezet.

Appendix

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A TECHNIQUE FOR PERFUSION OF RAT TESTES *IN SITU* THROUGH THE INTERNAL SPERMATIC ARTERIES

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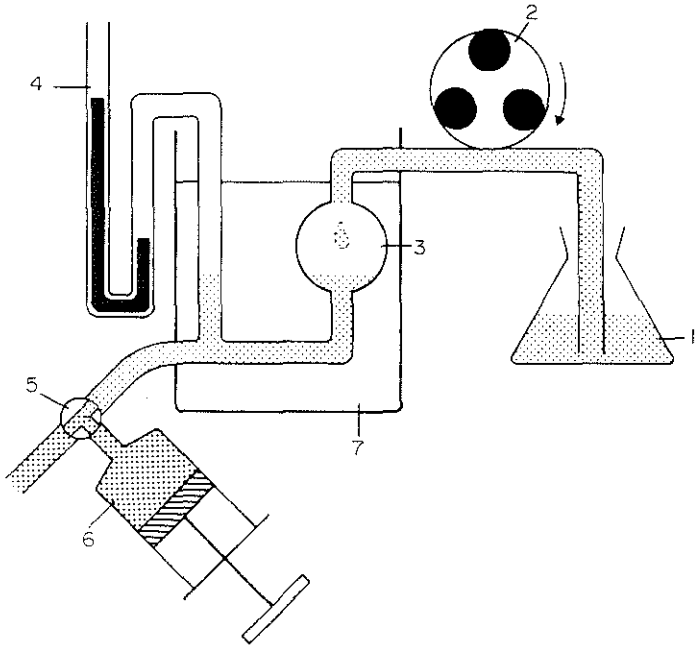
For studying the action of hormones on the testis, perfusion techniques have certain advantages (Eik-Nes & Hall, 1965), such as minimal peripheral conversion or dilution of a labelled compound before it reaches the testes. Techniques for the perfusion of rabbit testes (VanDemark & Ewing, 1963) and the testes of sheep and goats have been described. Unlike the rat testis (see Christensen & Mason, 1965; Cooke, de Jong, van der Molen & Rommerts, 1972; Rommerts, van Doorn, Galjaard, Cooke & van der Molen, 1973), however, the rabbit, sheep and goat testes have the disadvantage that after the perfusion, they cannot be dissected into seminiferous tubules and interstitial tissue. This paper describes a technique for the perfusion of rat testes *in situ* and the evidence derived by this procedure for an oestradiol-17β-binding macromolecule in isolated interstitial tissue.

A schematic outline of the perfusion apparatus is shown in Text-fig. 1. Providing a normal flow of 1·5 to 3 ml/min was maintained, the temperature of the testis tissue measured with an NTC thermistor probe was 31° C.

The perfusion medium used was a Krebs–Ringer bicarbonate buffer, pH 7·4, containing 1 mmol glucose and 10 i.u. heparin/ml. Labelled oestradiol ([2,4,6,7-$^3H_4$]oestradiol-17β, specific activity 100 Ci/mmol; Radiochemical Centre, Amersham, England) was checked for radiochemical purity by paper and thin-layer chromatography. Oestradiol-17β (10 μCi) was dissolved in 0·5 ml Krebs–Ringer bicarbonate buffer and perfused into the testes as described below.

The mature Wistar rats used were allowed free access to food and water up to the moment of ether anaesthesia. The abdomen was opened by a mid-line incision and the intestines were displaced to the right. The peritoneum was stripped from the aorta between the level of the left renal vein and the common iliac arteries (see Text-fig. 2), and 200 i.u. heparin were injected into the liver. The retrograde cannula (a hypodermic needle 40 mm long × 1·2 mm o.d., with a slightly blunted tip) was inserted at the bifurcation of the aorta. The tip of the cannula was moved cranially close to the origin of the left internal spermatic artery and then fixed with a ligature placed cranial to the origin of the ileolumbar arteries. By means of the roller pump, the pressure in the perfusion

system was kept at the level of the blood pressure in the aorta (about 80 mm Hg). The jaws of an arterial clamp were modified (Text-fig. 2) to allow clamping of the aorta in the small region between the branching of the left renal artery and the aortic crossing of the left renal vein. After clamping the aorta, the internal spermatic arteries were perfused with medium. When the testes were brought into the peritoneal cavity through the inguinal canals, they appeared pale, indicating the replacement of blood by buffer in the testis circulation.

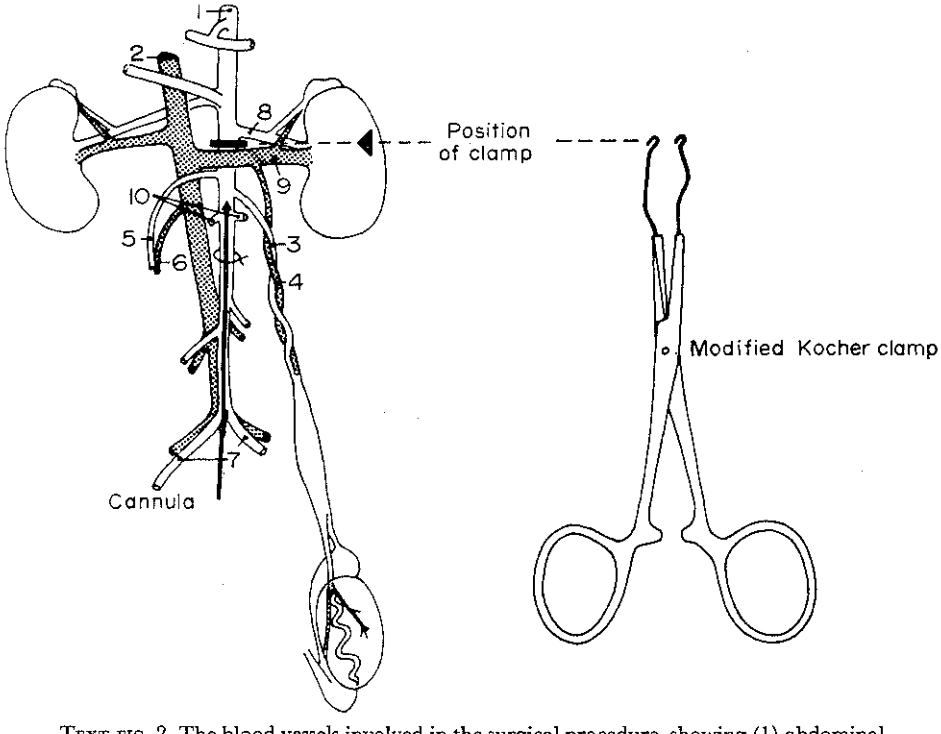


TEXT-FIG. 1. Perfusion apparatus. The perfusion medium was pumped from the storage flask (1) by a roller pump (2) to the bubble trap (3). The flow rate in the perfusion system was determined by counting the droplets falling into the bubble trap. The perfusion pressure was measured by a mercury-filled U-tube (4) connected to the infusion line coming from the bubble trap. A three-way stopcock (5) was interposed at a short distance from the end of the infusion line. Through this stopcock, the contents of a syringe (6) could be added to the perfusion medium under a controlled pressure. As many parts as possible of the perfusion apparatus were placed in a temperature-controlled water bath (7) kept at 40° C.

After perfusion of buffer for 5 min, the roller pump was stopped and ^{3}H-labelled oestradiol-17β was injected into the perfusion system by means of a syringe (6, Text-fig. 1) under controlled pressure. Perfusion by means of the roller pump was restored and continued for 1 min. The clamp on the aorta was then released and the testes were flushed with blood for less than 1 min, after which they were removed from the animal and kept at 0 to 4° C during the isolation of the oestradiol-17β receptor.

To prevent direct transfer of the infused compounds to the general circulation, perfusion has to take place only through the internal spermatic arteries. The outlet of the cannula was moved up the aorta close to the origin of the left internal spermatic artery (Text-fig. 2), thus excluding the lumbar arteries from

supply of blood or perfusion medium. The origin of the left ileo-lumbar artery is variable (Greene, 1963). The artery was excluded from the perfusion by fixing the cannula with a ligature cranial to its origin. With some animals, it was only possible to clamp the aorta cranial to the origin of the left renal artery. The left renal artery and vein were then also clamped near the hilus. With these precautions, perfusion will take place almost exclusively through the internal spermatic arteries. Before entering the general circulation, a small amount of the medium enters organs other than the testes through tiny branches of the



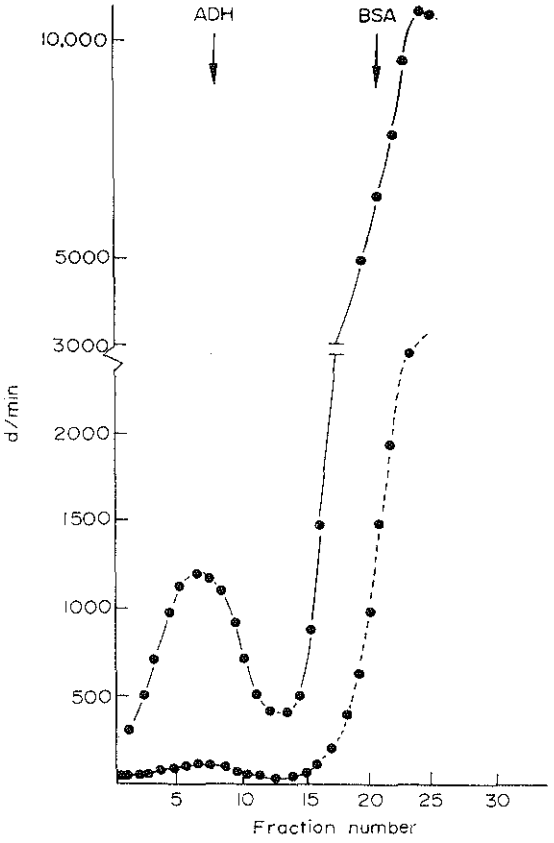
TEXT-FIG. 2. The blood vessels involved in the surgical procedure, showing (1) abdominal aorta, (2) vena cava, (3, 4) left internal spermatic artery and vein, (5, 6) right internal spermatic artery and vein, (7) common iliac arteries, (8, 9) left renal artery and vein, 2nd (10) third pair of lumbar arteries. Part of the jaws were removed from the arterial clamp. On the remaining parts, two stainless steel wires (0·8 mm o.d.) were attached with closely fitting semicircular blunt hooks.

internal spermatic artery which are difficult to clamp and which run to the epididymal fat and to the caput epididymidis. Anastomoses were also observed between the epididymal branch of the internal spermatic artery and the deferential artery which supplies the cauda epididymidis (Niemi & Kormano, 1965).

Electron microscope pictures of testis were obtained after glutaraldehyde (2·5%, in sodium cacodylate buffer at pH 7·4) perfusion according to the method described, followed by post-fixation in OsO_4 and routine dehydration, embedding and staining of thin sections. There was no ultrastructural evidence for hypoxia (Trump, Goldblatt & Stowell, 1965) or for tubular damage due to

the perfusion conditions (Linzell & Setchell, 1969), since the tubular structure appeared normal and Leydig cell mitochondria had matrix granules.

Perfusion *in situ* has greatly contributed to the characterization of oestradiol-17β-binding in the testis, because the radioactive oestradiol infused through the internal spermatic arteries was readily taken up by the cytosol in the testicular



TEXT-FIG. 3. Sucrose-gradient analysis of oestradiol-17β-binding by cytosols of interstitial tissue (——) and seminiferous tubules (– – – –). The amount of radioactive oestradiol (d/min) in each fraction is given as a function of the fraction number. Cytosol fractions (105,000-*g* supernatant) were labelled by infusion with 10^{-10} mol (10μCi) of oestradiol-17β into the testis and 200 μl (about 3 mg protein) of each fraction were layered over linear sucrose gradients (5 to 15% w/v). The gradients were centrifuged for 18 hr at 49,000 rev/min in a Beckman L-65 B ultracentrifuge equipped with an SW 65 rotor. After centrifugation, approximately thirty fractions were collected from each centrifuge tube. Radioactivity in the fractions was determined by liquid scintillation counting. Bovine serum albumin (BSA, $S_{20,w}$: 4·6S) and yeast alcohol dehydrogenase (ADH, $S_{20,w}$: 7·4S) were used as markers for calculation of the sedimentation value. The position of the markers is indicated by arrows. (From Brinkmann *et al.*, 1972.)

interstitial tissue. Far less radioactivity was found in the cytosol of the seminiferous tubules (Text-fig. 3).

The sedimentation profile of the interstitial cell cytosol showed a sharp peak of radioactivity in the 8S region. Only a small elevation in the corresponding region of the sedimentation profile was found for the tubular cytosol. The

specificity and affinity of this binding principle have been discussed by Brinkmann, Mulder, Lamers-Stahlhofen, Mechielsen & van der Molen (1972).

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FREDERIK * P.M. and KLEPPER D. — **Freeze-drying of unfixed tissue samples for the autoradiographic localization of steroid hormones in testes.**

In testis interstitial tissue, steroids are produced which influence the differentiation of spermatogenic cells in the seminiferous tubules. The cell types acting as mediators or targets for the action of these steroids are not known. Electron microscopic autoradiography of steroids can potentially be a technique to study cellular transport and the nature of the targets for steroid hormones. Since steroids are classified as "soluble compounds" special techniques have to be used for their autoradiographic localization.

Two different techniques have been proposed to cope with the requirements of soluble compound autoradiography at the EM level. Appleton (1) and Christensen (2, 3) suggest cutting ultrathin sections from unfixed unembedded tissue on an ultracryotome. The sections obtained are then freeze-dried and brought in contact with a nuclear emulsion. Eckert (4) and Stirling and Kinter (5) suggest rapidly freezing of unfixed tissue samples, freeze-drying and then fixing with osmium tetroxide vapor and embedding in epon. Thin sections are then obtained in the normal way. The latter authors found that labelled compounds are partly extracted during floating of the section on the knife trough. To prevent this extraction we have obtained ultrathin sections on a cryo-ultratome using dry glass knives. Best cutting conditions were found to be — 40 °C for the specimen and — 70 °C for the knife. Preliminary results suggest that these sections can be used for EM autoradiography of soluble compounds.

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The possibility of electron microscopic autoradiography of steroids after freeze drying of unfixed testes

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SUMMARY

A technique has been developed for the autoradiographic localization of steroids in testes at the light and possibly electron microscope level. During processing of the tissue there is no contact of tissue with water up to the moment of photographic development of the autoradiographs.

Tritium labelled steroids have been introduced into the testis through perfusion of the isolated organ. Small tissue samples were rapidly frozen, freeze dried and fixed with osmium vapour. The fixed tissue was embedded in Epon and thin sections could be cut from this material on an ultra-cryotome without the aid of floatation liquids. Results with light microscopic autoradiography indicate the potential of this technique. This procedure which avoids any contact of the tissue with water offers good prospects for autoradiography of steroids and other soluble compounds at the electron microscope level. A comparison is made with other proposed techniques for steroid autoradiography.

INTRODUCTION

Autoradiography of steroids might be a useful tool in the study of transport of steroids and the possible accumulation of these hormones in specific targets. Steroids belong to the group of so called 'soluble' compounds because they are extracted from tissues when the usual methods of fixation and dehydration for microscopy are used. Thus for steroid autoradiography special techniques have to be used. Several techniques have been proposed to cope with the requirements of steroid autoradiography at the electron microscopic level. Appleton (1969) and Christensen (1969) recommended the cutting of unfixed unembedded frozen tissue with a low temperature cryotome. The cutting temperature has to be lower than $-70°C$ to avoid destruction of subcellular structures through recrystallization of ice in the specimen. In order to exclude extraction of soluble materials during sectioning the use of a floatation liquid in the knife trough was avoided. Sections obtained in this way can be freeze dried and could be brought in contact with a photographic emul-

The following trivial names were used: 5-androstenediol, 5-androstene-3β,17β-diol; androstenedione, 4-androstene-3,17-dione; dehydroepiandrosterone (DHEA), 3β-hydroxy-5-androsten-17-one; pregnenolone, 3β-hydroxy-5-pregnen-20-one; oestradiol, 1,3,5(10)-oestratriene-3,17β-diol; testosterone, 17β-hydroxy-4-androsten-3-one.

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sion although the last part of this procedure has not been fully explored (Christensen & Paavola, 1972).

A different approach has been used by Stirling & Kinter (1967). They also used as starting material rapidly frozen unfixed tissue which was subsequently freeze dried, fixed with osmium tetroxide vapour and embedded in Epon. Sections were obtained in the normal way and were collected by floating them on water in a knife trough. The use of this method is restricted to labelled substances which are insoluble in Epon and in water after their reaction with osmium vapour.

A third approach to the autoradiographic localization of steroids has been proposed by Mizuhira *et al.* (1970), who suggested fixation of steroids by adding digitonin to the fixative and dehydration liquids. Digitonin reacts specifically with steroids having a 3β-hydroxy-5-ene structure yielding an insoluble steroid—digitonide. The merits of this method for the autoradiographic localization of cholesterol has been reviewed by Stein & Stein (1971). The results obtained by the digitonin fixation of cholesterol are equivocal (see Frühling *et al.*, 1971).

We have tried to compare the usefulness of the three above mentioned approaches for the autoradiography of steroids. In this paper we will report about the failure of the digitonin (Mizuhira *et al.*, 1970) method to accomplish our goal. We will further describe a freeze dry method which is essentially the same as that described by Stirling & Kinter (1967). We will pay special attention to the tissue morphology and to cutting of Epon embedded tissue without the use of floatation liquids.

MATERIALS AND METHODS

Animals

For testis perfusion with dehydroepiandrosterone (DHEA) New Zealand rabbits were used. For the other testis perfusions Wistar rats were used, 5–6 days after hypophysectomy. All the animals had free access to food and water up to the moment of anaesthesia with sodium pentobarbital (6 mg/100 g body weight) which was administered intraperitoneally.

Perfusion of testes

The surgical procedure used for perfusion of rabbit testes was essentially the same as described by Christensen (1965) for the guinea pig. For the perfusion of rat testes this technique was slightly modified. The testis was placed upon a support with a hypodermic needle (0·4 mm o.d. 10 mm long) in a fixed position. The testis artery running just below the testis capsule was cannulated under a dissecting microscope and was fixed to the needle with a ligature (Fig. 1). The perfusion medium used was a Krebs-Ringer bicarbonate buffer pH 7·4, saturated with 95% O_2/5% CO_2, containing 1 mmol glucose and 10 iu heparin/ml. The apparatus for perfusion under controlled pressure and flow has been described previously (Frederik & Van Doorn, 1973).

Steroids

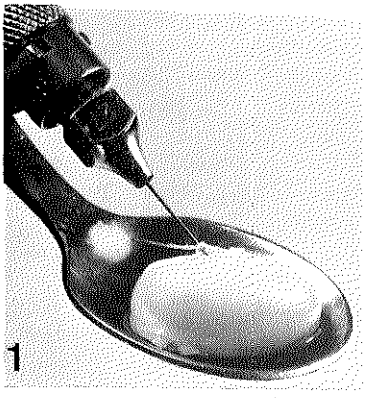
The radiochemical purity of labelled steroids was checked by paper and thin layer chromatography. For rabbit testis perfusion 7α-^{3}H-DHEA was used with a specific activity of 27 Ci/mmol. Rat testes were perfused with approx 30 μCi of either oestradiol (2,4,6,7-$^3H_4$, sp. act. 100 Ci/mmol) or testosterone (1,2,6,7-$^3H_4$, sp. act. 100 Ci/mmol).

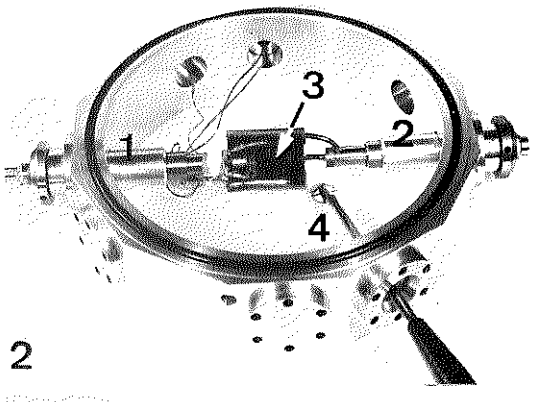
For a model study tritium labelled pregnenolone (7α-^{3}H, sp. act. 15 Ci/mmol), DHEA and testosterone were dissolved in 1·5% agarose (Difco-Bacto) in 0·1 M cacodylate buffer pH 7·3. Non-radioactive steroids were added to obtain the same

specific activity of each of the steroids. Of each steroid two concentrations in agar were prepared: 1 μg/ml (sp. act. 15 Ci/mmol) and 1 mg/ml (sp. act. 15 mCi/mmol.)

Fixation with digitonin

Rabbit testes were perfused for 2 min with buffer followed by perfusion for 15 min with 15 ml buffer containing $1{\cdot}03 \times 10^7$ dpm of labelled DHEA. The testes were then fixed by perfusion with 2·5% glutaraldehyde in 0·1 M cacodylate buffer (pH 7·3) containing 0·2% digitonin and 4% polyvinylpyrrolidone (PVP). After perfusion small tissue samples were cut from the testis which were stored overnight in 0·1 M cacodylate buffer containing 4% PVP and 0·2% digitonin. Post-fixation was carried out for 1 h in 1% OsO_4 in 0·1 M cacodylate buffer containing 0·2% digotinin. The tissues were dehydrated through graded acetone saturated with digitonin. Finally the tissue was embedded in Epon. Thin sections were cut on a Reichert OmU_2 microtome and the sections were post-stained with uranyl acetate and lead citrate.





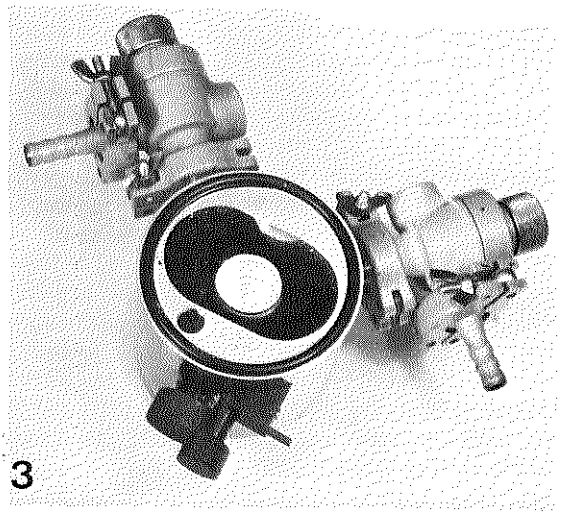


Fig. 1. Rat testis placed on a support for perfusion of the isolated organ.
Fig. 2. Freeze dry module. (1) specimen arm; (2) shield arm; (3) specimen holder; (4) manipulator with lid for specimen holder.
Fig. 3. Desiccator for osmium vapour fixation and Epon embedding.

The amounts of free and digitonin bound steroids in testis tissue were analysed directly after perfusion fixation. Testis tissues were extracted with chloroform/methanol (2/1, v/v). This extract was evaporated to dryness, non-labelled steroids were added, and the free steroid was extracted with ether.

The steroids in the ether extract were separated using paper chromatography essentially as described previously by De Bruijn (1973). After initial separation using a Bush AII system, the fraction containing testosterone and androstenediol was eluted and these steroids were separated by paper chromatography with a Bush BI system. Areas corresponding with testosterone and androstenediol were eluted and the amount of radioactivity in the eluates was estimated. The fraction containing DHEA and androstenedione was acetylated and further separated by paper chromatography with a Bush AII system. DHEA-acetate and androstenedione were separated in this way and following elution the amount of radioactivity associated with each of these steroids was estimated. The residue of the original extract was treated for 2 h with pyridine at 50°C to hydrolyse the steroid digitonides (Fieser & Fieser, 1959). The dissociated steroids were extracted with ether and were also analysed by paper chromatography. Directly after perfusion fixation the total amount of radioactivity in the testes was determined after solubilizing tissue samples in Soluene (Packard) and estimation of the amount of the radioactivity with liquid scintillation counting. In the same manner radioactivity was determined in the Epon embedded samples. In a parallel experiment agar blocks containing labelled steroids were fixed by immersion in the same fixative as used for rabbit testis perfusion. These agar blocks were further treated in the same manner as the testis tissue. The amount of radioactivity in the agar blocks at the beginning and at the end of the procedure was determined by liquid scintillation counting.

Freeze drying

After perfusion of rat testis several tissue samples (wet weight approx. 10 mg) were immersed in Freon 22 cooled with liquid nitrogen. For freeze drying the samples were transferred to the specimen holder of the freeze drying apparatus, a modified freeze etch module of the type developed by Steere (1969). The temperature of the specimen was thermostatically controlled. A rotatable shield could cover the specimen over a large solid angle (see Fig. 2). During freeze drying the shield was kept at −196°C through circulation of liquid nitrogen. Pressurized Dewars supplied the nitrogen to the specimen arm and to the shield reservoir by electronically controlled solenoid valves.

Before loading of the freeze dryer, the specimen stage and the shield were cooled to their working temperatures of −85°C and −196°C respectively. Pumping was started after screwing the specimen holder on the specimen-stage. The ultimate vacuum maintained was better than 10^{-5} torr during a 15 h freeze dry run. At the end of a freeze dry run specimen and shield were allowed to warm up slowly. Drying was completed by increasing the temperature of the specimen to 30°C by means of a resistance mounted in the specimen stage. During warming the shield was again cooled to −196°C. The specimen holder was covered with a lid by means of a manipulator and dry nitrogen was admitted. The specimen holder containing the dry specimen under vacuum was finally dismounted and transferred to a desiccator (compare with Müller, 1957).

In this desiccator (Fig. 3) an Epon container was placed in the specimen space. Osmium vapour can be admitted to the specimen space by a side port. The lid covering the specimen holder is attached to a spring. The lid is removed when proper vacuum has been established and osmium vapour can be admitted to the specimen. Vapour fixation was carried out overnight at 37°C. Fixation was termi-

nated by pumping away the osmium vapour and immersing the specimen in Epon by rotating the Epon container.

Further embedding and polymerization was carried out with an Epon mixture according to Luft (1961). The loss of radioactive label in Epon was determined by liquid scintillation counting.

Cutting of freeze-dried embedded tissues for autoradiography

For light microscopy 1–2 μm section were cut on a LKB III or Reichert OmU_2 microtome using dry glass knives.

Sections were adhered to microscopic slides with a thin layer of Entalan (Merck). Alternatively sections were floated upon a drop of water on the slide and the water was allowed to evaporate at room temperature.

Ultra-thin sections were cut on a LKB III microtome equipped with the 'cryokit' low temperature cutting attachment. Sections were obtained with a specimen temperature of $-70°C$ and a knife temperature of $-120°C$. Dry glass knives were used with a knife angle of 48° and a clearance angle of 5°. The tissue sections were transferred from the knife edge with a teflon tipped probe and flattened against a formvar coated copper grid. Sections were viewed in a Philips EM 300 electron microscope without additional staining.

Light microscopic autoradiography

Slides bearing 1–2 μm sections were coated with carbon. Ilford K5 emulsion was applied to the sections by the loop method (Caro & Van Tubergen, 1962). After an exposure time of about 10 weeks the emulsion was developed in Kodak D 76. After a brief wash in distilled water the autoradiographs were fixed for 5 min in 20% $Na_2S_2O_3$. They were photographed without additional staining under phase contrast.

RESULTS

Digitonin fixation of steroids

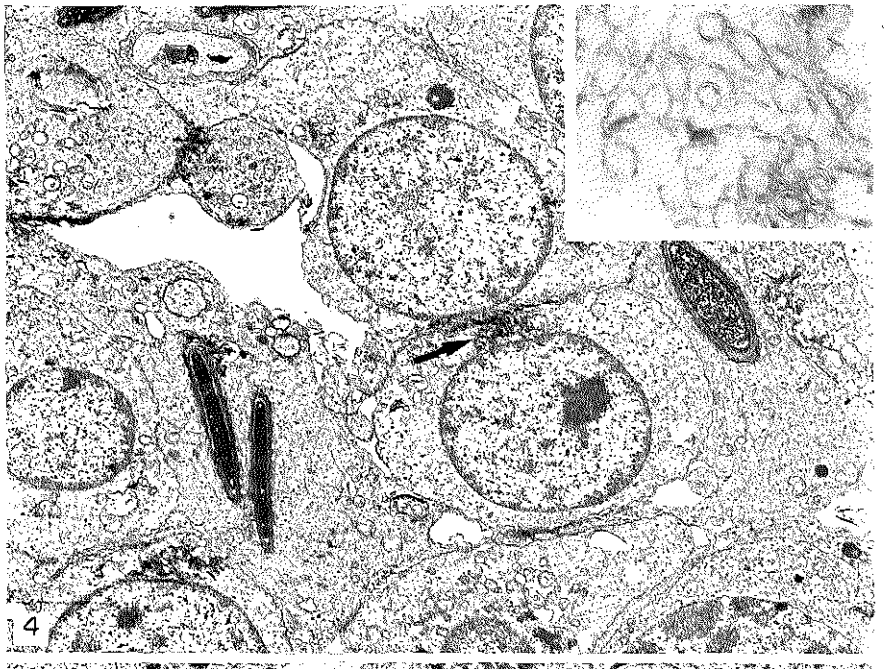
After post-fixation, dehydration and embedding the rabbit testes contained 35% of the initial amount of radioactivity. The analysis of free and bound steroid is given in Table 1. Only 10% of the steroids with the 3β-hydroxy-5-ene structure (DHEA and 5-androstenediol) was fixed as digitonide. The recovery of radioactivity of steroids dissolved in agar and subjected to the same fixation dehydration

Table 1. Fixation of steroids after rabbit testes perfusion with ^{3}H-DHEA, followed by perfusion with 2·5% glutaraldehyde containing 0·2% digitonin

	Percentage total amount of labelled steroids	Percentage of steroid bound in digitonide fraction
Testosterone	46	1·8
5-androstenediol	27	3·5
DHEA	21	2·1
Androstenedione	6	0·5

Table 2. Recovery of steroids dissolved in agar after fixation dehydration and embedding in digitonin containing media

Concentration of steroid in agar	Percentage radioactivity retained after fixation, dehydration and embedding		
	Testosterone	DHEA	Pregnenolone
1 μg/ml	n.d.	5	44
1 mg/ml	0·7	23	42



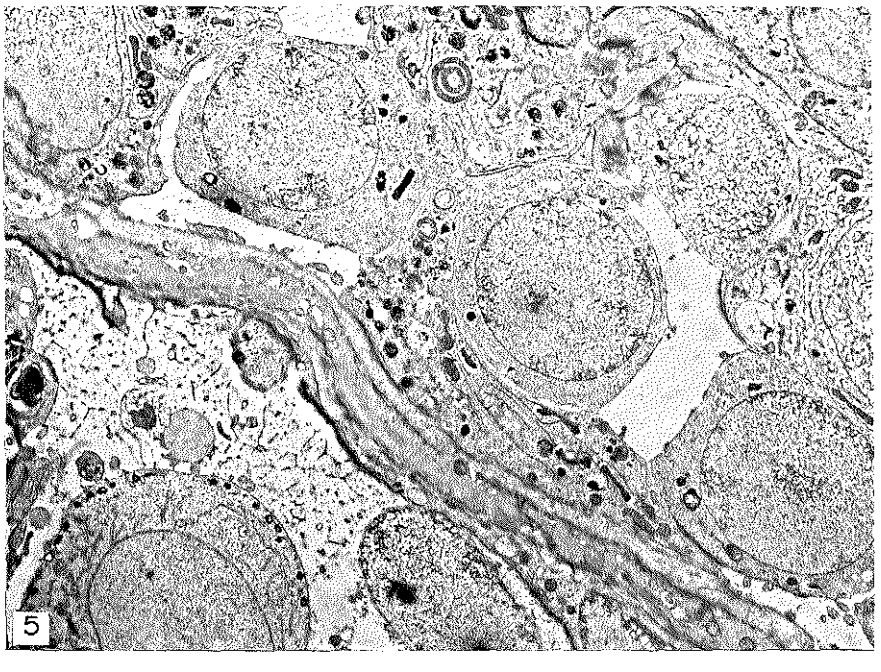


Fig. 4. Rabbit testis after treatment with digitonin. Fixatives, dehydration liquids and Epon contained digitonin. Arrow: membrane rearrangements shown at higher magnification in the inset. × 3150; inset × 32,400.

Fig. 5. Rat testis after freeze drying, osmium vapour fixation and Epon embedding, no further staining. At the lower left the best structural preservation is found. This area corresponds to the outer rim of the frozen tissue sample. The more interior parts exhibit a gradual decrease in the preservation of the fine structure.

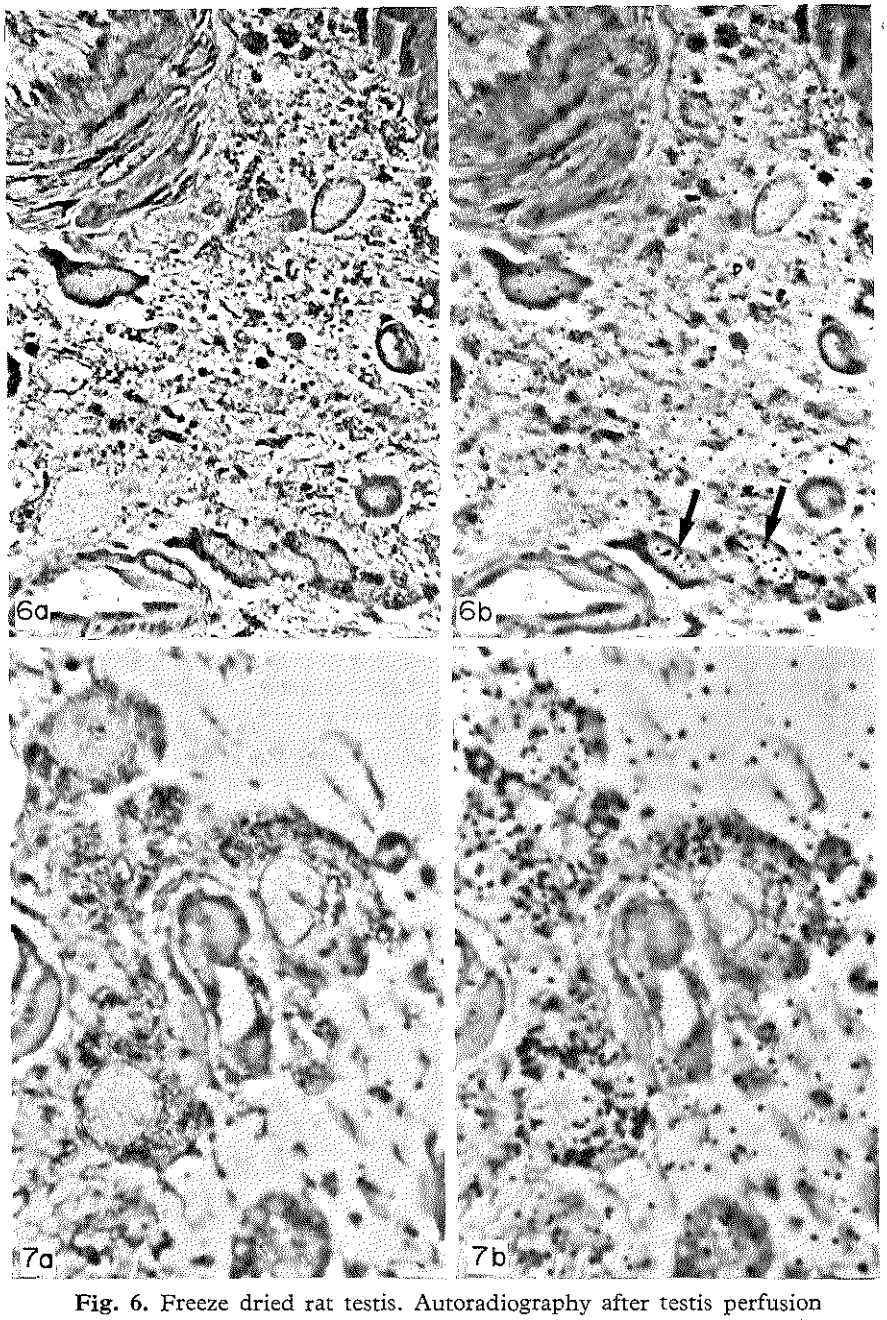


Fig. 6. Freeze dried rat testis. Autoradiography after testis perfusion with ^{3}H-estradiol. (a) is focused on the tissue, (b) is focused on the grains. The label is concentrated in the nuclei of some of the interstitial, cells (arrows). The interstitial compartment is contaminated with cell debris due to the breaking of seminiferous tubules during the sampling prior to freezing. ×788.

Fig. 7. Freeze dried rat testis. Autoradiography after testis perfusion with ^{3}H-testosterone. (a) is focused on the tissue, (b) is focused on the grains. The label is concentrated in the cytoplasm of the interstitial cells, whereas above the nuclei a distinct lower labelling is found. ×1710.

and embedding procedure as the tissues, is given in Table 2. The amount of retained steroid never exceeded 50%.

The preservation of the ultrastructure of the digitonin fixed testis was generally good (Fig. 4). However, in some parts of the tissue membrane rearrangements could be observed. These are shown at higher magnification in the inset of Fig. 4.

Freeze drying

An example of the ultrastructure of the frozen dried rat testis is given in Fig. 5. A reasonable preservation of the tissue was seen at the peripheral parts of the frozen block. The more central parts showed a course structure. Loss of labelled steroids in the embedding medium was lower than 1%.

Rat testes were perfused for 5 min with buffer, 5 min with buffer containing labelled estradiol followed by 15 min with buffer alone. With light microscopic autoradiography an accumulation of the steroid was observed in the nuclei of the interstitial cells (Fig. 6). Testes perfused with labelled testosterone revealed a completely different localization of the label. After a short labelling time (5 min buffer, 5 min buffer with labelled testosterone and 5 min buffer), the cytoplasm of the interstitial cells shows heavy labelling when compared with the labelling of the surrounding tissue (Fig. 7).

The application of the freeze dry technique to the autoradiography at the electron microscope level was further explored by the development of a dry cutting technique for Epon embedded material. An example of the electron microscopy of a thin section cut at temperatures below 0°C is given in Fig. 8. No results have yet been obtained with the autoradiography of such thin sections.

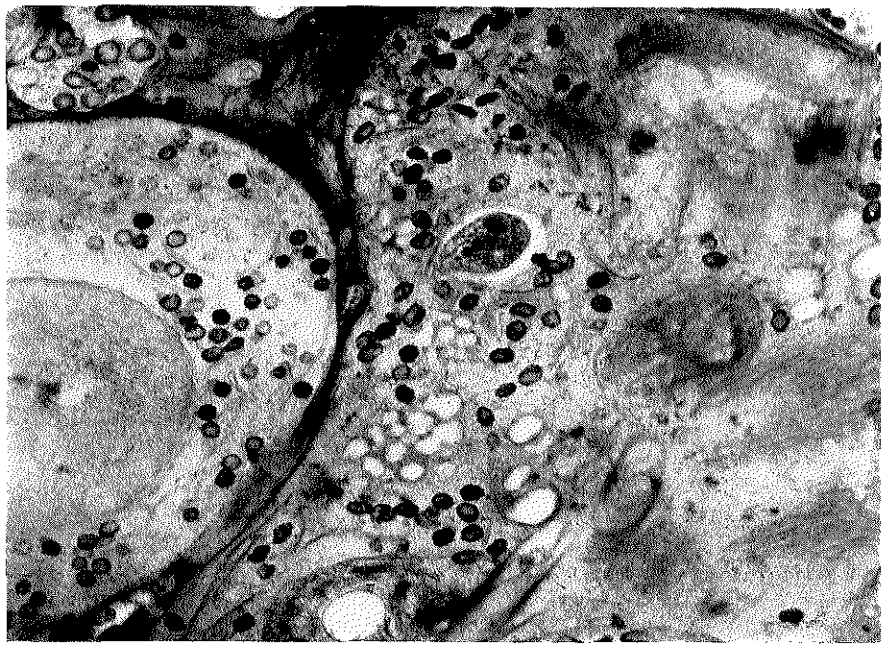


Fig. 8. Freeze dried rat testis. Thin section obtained by dry cutting of Epon embedded tissue at low temperatures on a cryo-ultratome. The picture was taken from an area representing the outer rim of the frozen tissue block. × 11,970.

DISCUSSION

The addition of digitonin to fixation and dehydration liquids did not prevent the loss of steroids. A slightly better retention of steroids could be observed with steroids having the favourable configuration for the formation of digitonides, but the retention never exceeded 50%. Complete recovery of the radioactive label is one of the prerequisites for autoradiography as was pointed out by Stumpf & Roth (1968). With the batch of digitonin used in the present experiments the described method certainly failed in this respect.

The solvent action of digitonin may have caused membrane rearrangements. Frühling *et al.* (1971) found similar rearrangement patterns in mixtures of digitonin with a cholesterol free lecithin preparation.

A good retention of label was observed with the freeze drying procedure. Floating of the sections in the knife boat gave a 25% loss of the label. Therefore the sections were glued to microscopic slides for light microscopic autoradiography. In other experiments it was found that flattening of these sections on a drop of water did not interfere with the autoradiographic resolution. Tissue free plastic areas at the border of the tissue or in blood vessel lumen, show background labelling only. Spreading of activity from highly labelled sources into nonlabelled areas was not observed.

The observed localization of estradiol in testis tissues was the same as reported by Stumpf (1969) and Stumpf *et al.* (1971). Our results on the localization of testosterone are in agreement with those of Beardsly & Hilton (1975), who used the same technique as Stumpf. From these data it can be concluded that the described freeze dry technique revealed the same steroid localization as the dry mount technique of Fitzgerald (1961) as improved by Stumpf & Roth (1964).

Most steroids found in tissues are not free, they are incorporated in lipid rich structures, loosely bound to proteins (as has been reported for serum albumins), or associated with specific binding proteins. During treatment of the freeze dried tissue with osmium vapour steroids are fixed, most probably by coupling of the (unsaturated) steroids to cellular structures. This coupling may eventually give rise to a translocation over a small distance. The extraction of less than 1% of the labelled steroid in the embedding medium might have resulted from an incomplete coupling action of the osmium tetroxide vapour.

We have to compare the above mentioned possible cause of translocation with the segregation between water and solutes inherent to the freezing of fresh tissue. The cooling velocity of a specimen is determinating the ice crystal size and thus the segregation between water and solutes. Van Venrooij *et al.* (1975) have demonstrated that the highest cooling velocities occur in the interior and at the outer rim of a frozen cylinder or sphere. In these areas small crystals could be observed in a freeze etch study. In comparable areas with a high cooling velocity a good ultrastructural preservation was expected in our freeze dry study. This is illustrated by Fig. 5, which displays the part of a section including outer cell layers of the frozen block together with more interior layers. The outermost cells of the frozen tissue block are also shown in Fig. 8 from a section which was cut 'dry' on an ultracryotome. From these observations it is clear that only the few outer cell layers of a frozen tissue block would be suitable for autoradiography at the electron microscopic level. The useful application of light microscopic autoradiography to the whole frozen tissue block depends on the desired limit of resolution.

Translocation of steroids due to the fixation reaction is probably negligible compared to the ice crystal formation. In order to obtain a high cooling velocity only small tissue samples (*c.* 10 mg) were frozen since the cooling velocity is inversely proportional to the square of the droplet diameter (Van Venrooij *et al.*,

1975). By taking such small samples the testis seminiferous tubules were undoubtedly disrupted, which resulted in the liberation of germinal cells. These cells, together with cell fragments were trapped in the interstitial mesh-work. As a result the interstitial compartment became contaminated with germinal elements and cell debris (see Figs. 6a and 7a). These contaminating elements can be distinguished morphologically from interstitial tissue. Therefore we have deliberately chosen for a high cooling velocity and optimal ultrastructural preservation, thus accepting a slight impairment of the tissue architecture.

From the Epon embedded tissue thin sections could be cut on a cryo-ultratome. We have also tried other means to obtain dry sections. These included siliconizing the glass knife, the use of silicone or fluorcarbon liquids in the knife trough, and the use of an embedding of the tissue in a harder Epon. Ultrathin sections could not be obtained using these approaches with the exception of hard Epon blocks. Dry cut sections from this material showed, however, an unacceptable degree of compression. A better yield of acceptable sections was obtained when these were cut from Epon embedded material with the ultra-cryotome rather than from fresh frozen unfixed tissue cut on the same instrument. An additional advantage of the described sectioning method is the higher stability and the ease of manipulation of Epon thin sections over the cryosections. Since all contact with aqueous solutions was avoided sections obtained from freeze dried, vapour fixed and Epon embedded material may be reconsidered as starting point for the electron microscopic autoradiography of soluble compounds. The application of this freeze dry technique now remains only restricted to compounds which are insoluble in Epon or rendered insoluble in Epon after their reaction with the anhydrous osmium tetroxide vapour.

ACKNOWLEDGMENTS

We wish to thank H. J. van der Molen for his support of this work. We also thank P. J. M. Hartwijk for his outstanding technical assistance in modifying the freeze dry module. H. W. A. de Bruijn has made helpful suggestions for rat testis perfusion and is thanked for steroid determinations. T. M. van Os and J. H. Fengler are thanked for preparing the photographs.

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DYNAMICS OF STEROID UPTAKE IN RAT TESTIS STUDIED BY QUANTITATIVE AUTORADIOGRAPHY

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Localization of radioactive steroids in rat testis was studied by autoradiography of tissue sections. For autoradiography, small tissue samples were frozen, freeze-dried under vacuum, fixed with osmium vapor and embedded in epon. The transfer of radioactive steroids was studied after in vitro perfusion of radioactive steroids into the testes isolated from hypophysectomized animals, thereby excluding the interference of endogenous steroids. Quantitative autoradiography on the basis of grain densities after perfusion of testes with tritiated pregnenolone or testosterone, revealed an accumulation of the label in the Leydig cell cytoplasm. After longer perfusion periods the amount of label in the seminiferous tubules increased and a preferential localization was observed in the basal cytoplasm of Sertoli cells and in lipid droplets. Perfusion of testes with estradiol-17β resulted in a distinctly different pattern of radioactivity in the autoradiographs. A high labeling of the Leydig cell nuclei was observed in combination with a low general labeling of all the other cell structures. The results suggest that different steroids are localized in different specific areas of the rat testes in vivo.

Keywords: steroids; autoradiography; testis; perfusion; Leydig cells; Sertoli cells; hypophysectomy.

Autoradiography and immunofluorescence have been used to study the localization of steroids in testes. With an autoradiographic technique for soluble compounds the distribution of steroids was studied after injection of tritiated steroid in the intact (Stumpf, 1971; Beardsly and Hilton, 1975) or long-term, hypophysectomized animal (Smith et al., 1975). However, by injecting steroids into the peripheral circulation one has to consider the possibility of metabolic conversion of the steroids before they reach the testes. Using an immunofluorescence technique for detection of immunoreactive androgens an attempt has been made to study the distribution of endogenously produced steroids (Bubenik et al., 1975). However, as this technique

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includes a brief wash of cryosections in cold acetone and an incubation in antibody solution, the possibility of steroid dislocation cannot be excluded, since androgens are soluble in the liquids employed.

In the present study we have tried to investigate the uptake and transport of steroids from their site of synthesis in the interstitial tissue to their possible target in the seminiferous tubules following perfusion of rat testes with tritiated steroids. Rats were used 5–6 days after hypophysectomy, because in these animals endogenous steroid production is reduced but all the developmental stages in the germinal epithelium are still present. We have studied the localization of steroids in testes using autoradiography at the light microscopic level with a modified Stirling and Kinter (1967) technique (Frederik and Klepper, 1976). This technique involves freezing of tissue samples, freeze-drying, osmium vapor fixation and embedding in epon. In a previous paper (Frederik and Klepper, 1976) we have discussed the applicability of this technique for the autoradiography of steroids in testes. It was found that less than 1% of the labeled steroids was extracted during the embedding procedure. A quantitative evaluation of the autoradiographic results will be given in this paper.

MATERIALS AND METHODS

Animals

Male rats of the Wistar-R strain were used 5–6 days after hypophysectomy. The animals had free access to food and water up to the moment of anaesthesia with sodium pentobarbital (6 mg/100 g body weight) which was administered intraperitoneally.

Testes perfusion

For perfusion of rat testes a surgical procedure was used, which was essentially a modification of the procedure described by Christensen (1965) for perfusion of guinea pig testes. The testis artery was canulated with a 27-gauge hypodermic needle and the organ was placed upon a support during perfusion (Frederik and Klepper, 1975). During perfusion, pressure and flow were controlled as described previously (Frederik and Van Doorn, 1973). The perfusion medium used was a Krebs–Ringer bicarbonate buffer (KRBG) containing 1 mg/ml glucose and 1 IU heparin/ml. The buffer was oxygenized with 95% O_2/5% CO_2. Further details concerning the duration of perfusion are given in the Results section.

Radiochemicals

The purity of all radiochemicals used was checked by paper and thin-layer chromatography. The steroids used were estradiol (2,4,6,7-$^3H_4$, spec. act. 85 Ci/mmol), testosterone (1,2,6,7-$^3H_4$, spec. act. 84 Ci/mmol) and pregnenolone (16-^3H, 21 Ci/mmol). For perfusion 30 μCi of each steroid was evaporated to dryness and subsequently dissolved in 1 ml KRBG containing 0.1% Tween.

Analysis of steroids

Rat testes were perfused for 3 min with 30 μCi [^{3}H]pregnenolone followed by 10 min perfusion with KRBG buffer and the testes were chilled in ice-cold 0.25 M sucrose. After removing the tunica albuginea the remaining testis tissue was homogenized in 10 ml 0.25 M sucrose using an automatic Potter homogenizer. To the homogenate 15 μg of each of the following steroids containing 2000 dpm ^{14}C were added as carrier steroid and as internal standard: pregnenolone, progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone. The homogenate was then extracted 3 times with 15 ml ethyl acetate. The collected extracts were evaporated under N_2 and were subjected to paper chromatography on Whatman no. 20 paper strips in a Bush A2 system (Neher, 1964). Detection of radioactive steroids on paper chromatograms was carried out with a Packard radiogramscanner (model 7200). The areas corresponding to pregnenolone plus progesterone (R_f: 0.50–0.60), to androstenedione (R_f: 0.40), to testosterone plus 17α-hydroxyprogesterone (R_f: 0.15) and to unknown compounds at the origin (R_f: 0.00), were eluted separately with 10 ml ethanol. After evaporation of the ethanol the residues were chromatographed on silicagel plates (Merck F254, 20 × 20 cm) using a toluene/ethyl acetate (9 : 1, v/v) solvent system. Areas corresponding to testosterone, androstendione, 17α-hydroxyprogesterone, progesterone, pregnenolone and to a region with unidentified compound(s) 'X' were eluted with ethanol. After evaporation of ethanol, the residues were dried in a vacuum dessicator for 1 h followed by acetylation with an acetic acid anhydride–pyridine (1 : 1, v/v) mixture for 30 min at 45°C. After evaporation of the reagents under N_2, the acetylated steroids were subjected to thin-layer chromatography in the toluene/ethyl acetate (9 : 1, v/v). The areas corresponding to testosterone acetate, 17α-hydroxyprogesterone, androstenedione, progesterone, pregnenolone acetate and the unknown compound(s) 'X' were eluted with ethanol into counting vials and the amount of radioactivity was subsequently determined by liquid scintillation counting.

Autoradiography

Rat testes were perfused with tritiated steroids for 3 min followed by perfusion for varying times with KRBG buffer (see Results). At the end of the perfusion the testes samples (wet weight approx. 10 mg) were frozen in freon 22. The frozen tissue samples were freeze-dried under vacuum (lower than 10^{-5} mm Hg), fixed in osmium tetroxide vapor and embedded in epon (Frederik and Klepper, 1975). From the epon-embedded blocks 2-μm sections were cut and fixed on microscopic slides by floating them on a drop of water which was then allowed to evaporate at room temperature. A thin layer of carbon was evaporated onto the sections to prevent chemography. Photographic emulsion (Ilford K5) was applied to the sections by the loop method (Caro and Van Tubergen, 1962). Some sections were exposed to daylight and were stored together with the other sections in order to estimate the incidence of negative chemography. After two months of exposure all the autoradiographs were developed (in Kodak D76, 3 min), rinsed in distilled water and

fixed in 20% $Na_2S_2O_3$ (5 min). Photographs of the sections were taken under phase-contrast microscopy without staining.

For analysis of the grain distribution in the sections a grid was placed in the eye-piece of the microscope. At the magnification used (1600X) each grid square represented an area of 27 μm^2 in the section. The observed grain densities were expressed as mean number of grains per grid square. The comparison of grain densities between consecutive experiments can be difficult. Therefore, all sections were cut by the same experimentator on the same microtome (LKB III) using a setting of 2 μm. Emulsion was applied over the sections with one batch of emulsion within the same week (except for the estradiol experiment). The same exposure time was used for all experiments.

By comparing the differences between grain densities observed for different sections within one experiment, it was found that the variance of the grain density of a certain structure within a section was, in most cases, greater than the variance of the mean grain densities of the same structure in different sections. The variation in thickness of the sections was thus apparently negligible as compared to the variation in the labeling of a specific structure. Based on these observations a comparison of the absolute figures for grain densities in different experiments seems to be justified, although we have also used ratios of grain densities of different areas in a single experiment.

RESULTS

Perfusions with pregnenolone and testosterone

Perfusion of testes for 3 min with 30 μCi [16-^{3}H]pregnenolone was followed by 5-min perfusion with KRBG buffer. Grain densities were determined from the autoradiographs (see fig. 1) in: cytoplasm of the interstitial Leydig cells (CL), nuclei of the Leydig cells (NL), peritubular cells consisting of Sertoli cells and germinal cells up to the stage of pre-leptotene spermatocytes (PT), a basal layer of round spermatids (SP_1) and round spermatids plus residual bodies close to the tubular lumen (SP_2). The observed grain densities are given in table 1 (Experiment 1). A micrograph from this experiment is shown in fig. 2. The results reflect a specific localization of the labeled steroid(s) in the cytoplasm of Leydig cells.

A different grain-density distribution was observed when perfusion with tritiated pregnenolone for 3 min was followed by perfusion with KRBG buffer for 10 min rather than 5 min. Under these conditions, labeling was observed mainly in the peritubular layer (Exp. 2 in table 1; fig. 3) and a decreasing grain density was observed towards the tubular lumen. In a parallel experiment the conversion of the perfused pregnenolone to androgens was determined (table 2). The main metabolite found was testosterone. Only 22.2% of the total amount of recovered tritiated steroids was as pregnenolone.

Exp. 3 in table 1 (see also fig. 4) shows the results obtained after perfusion of

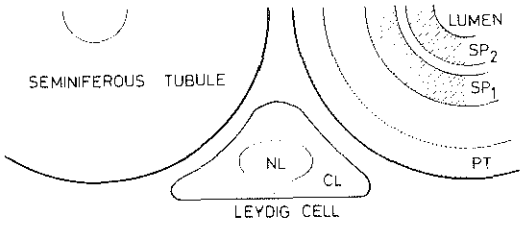

Fig. 1. Schematic representation of testis giving the areas studied for determination of grain densities. NL = nucleus Leydig cell; CL = cytoplasm Leydig cell; PT = basal layer of cells in the seminiferous tubules containing Sertoli cells and germinal cells up to the stage of pre-leptotene spermatocytes; SP_1 = basal layer of round spermatids; SP_2 = round spermatids and residual bodies adjoining the tubular lumen.

rat testis with tritiated testosterone followed by 5-min perfusion with KRBG buffer. The highest labeling was observed in the cytoplasm of the Leydig cell whereas the grain density in other structures studied was lower. These data are comparable to those obtained after pregnenolone perfusion (3 min – 5 min KRBG).

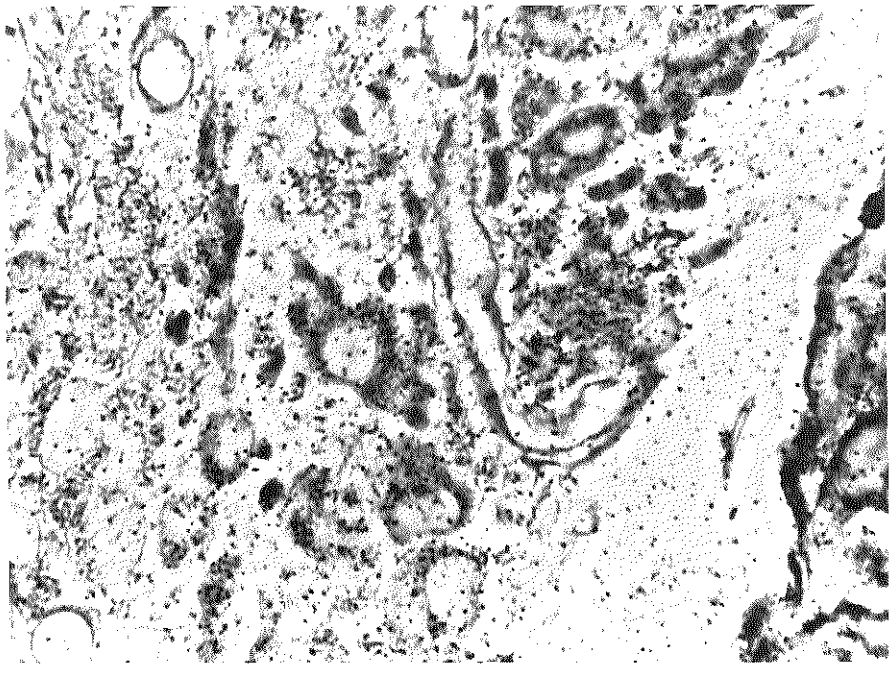

Fig. 2. Autoradiograph obtained after perfusion of rat testes for 3 min with [^{3}H]pregnenolone and 5 min with buffer. Prominent labeling of the cytoplasm of the Leydig cells can be observed (710×).

Table 1
Grain densities of autoradiographs. Perfusion of rat testes (5–6 days after hypophysectomy) for 3 min with tritiated steroid followed by a buffer perfusion for a period as indicated. Grain densities are given as mean number of grains per 27 μ^2 together with the standard error of the mean. The number of areas counted is given in parenthesis. BG refers to the background grain density. The other abbreviations are given in fig. 1.

Exp.	Steroid	Time (min)	BG	NL	CL	PT	SP_1	SP_2
1.	Pregnenolone	3– 5	1.04 ± 0.06 (169)	4.2 ± 0.5 (22)	18.7 ± 0.9 (35)	5.1 ± 0.5 (50)	1.5 ± 0.2 (50)	2.9 ± 0.5 (60)
2.	Pregnenolone	3–10	0.56 ± 0.04 (500)	5.4 ± 0.5 (48)	23.2 ± 0.6 (72)	12.9 ± 0.5 (91)	8.4 ± 0.7 (64)	1.9 ± 0.2 (171)
3.	Testosterone	3– 5	1.10 ± 0.07 (300)	6.0 ± 0.4 (45)	36.4 ± 1.1 (46)	7.8 ± 0.5 (56)	4.7 ± 0.3 (46)	4.8 ± 0.3 (46)
4.	Testosterone (+ 100-fold excess of cold testosterone)	3–15	0.35 ± 0.03 (500)	4.4 ± 0.3 (60)	22.9 ± 0.7 (60)	5.1 ± 0.4 (50)	2.7 ± 0.3 (50)	2.6 ± 0.3 (50)
5.	Estradiol	3–15	0.75 ± 0.04 (350)	13.8 ± 0.8 (28)	5.8 ± 0.6 (21)	1.9 ± 0.2 (30)	3.7 ± 0.4 (30)	2.1 ± 0.4 (20)

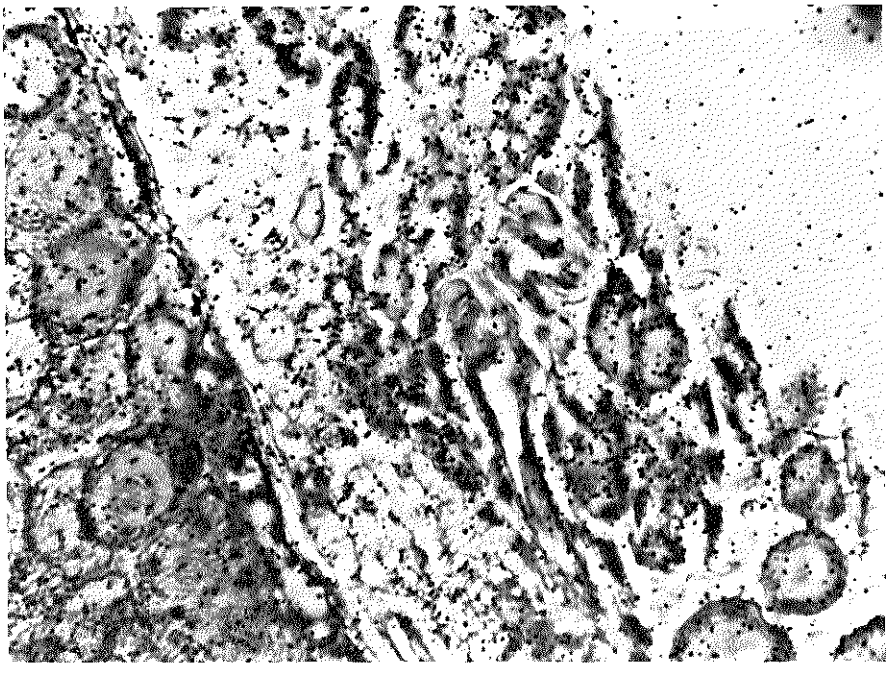

Fig. 3. Autoradiograph obtained after perfusion of rat testes for 3 min with [^{3}H]pregnenolone and 10 min with buffer. Leydig cell cytoplasm is labeled together with the basal cell layer of the seminiferous tubule.

An attempt was made to detect the possible presence of specific high-affinity low-capacity binding sites for testosterone using perfusion of testes with [$^3H_4$]testosterone followed by 15 min perfusion with KRBG buffer containing a 100-fold excess of unlabeled testosterone. The results of grain-density estimations are given

Table 2
Distribution of radioactivity between steroids in whole testis homogenate after perfusion with [^{3}H]pregnenolone (3 min) and 10 min with buffer. Results are expressed as percent of the total amount of labeled steroids recovered.

	Radioactivity recovered (%)
Pregnenolone	22.1
Progesterone	3.2
17α-OH-progesterone	3.4
Androstenedione	4.5
Testosterone	48.7
Compound (s) X	18.1

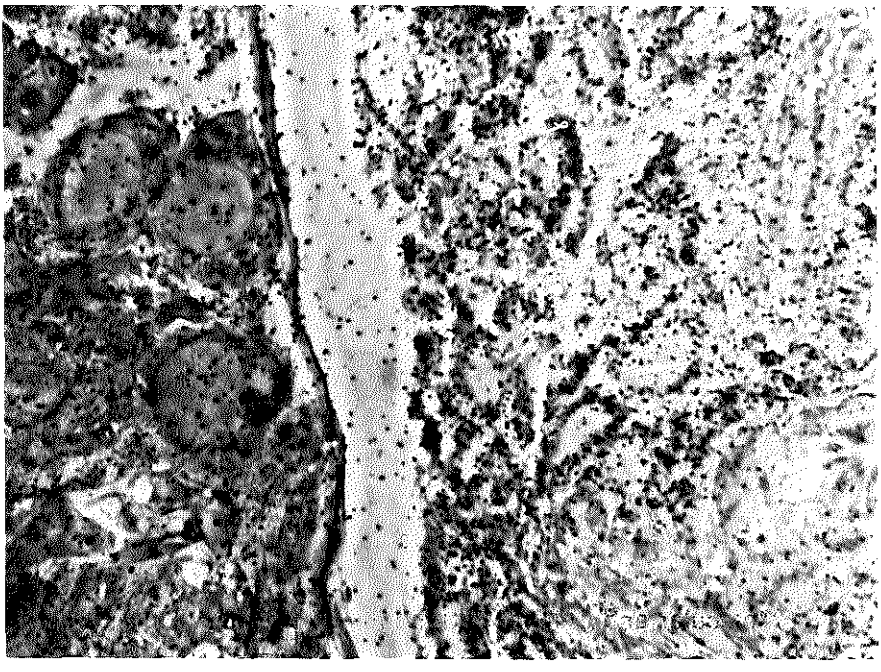

Fig. 4. Autoradiograph obtained after perfusion of rat testes for 3 min with [^{3}H]testosterone and 5 min with buffer. Note the heavy labeling of Leydig cell cytoplasm together with a low labeling of the Leydig cell nuclei and the cells from a blood vessel (lower right) (710×).

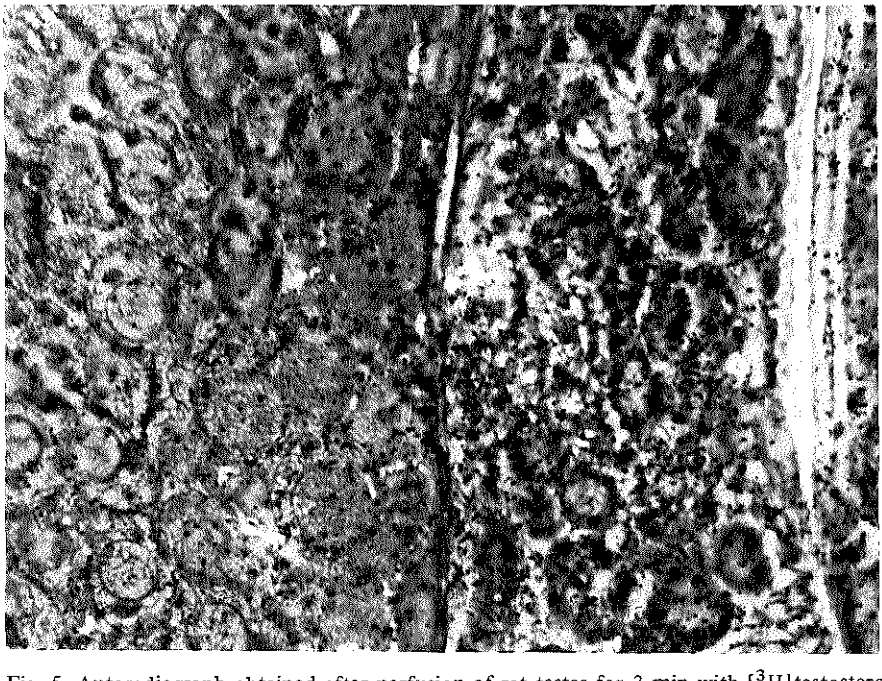

Fig. 5. Autoradiograph obtained after perfusion of rat testes for 3 min with [^{3}H]testosterone and 15 min with buffer containing 100-fold excess of unlabeled testosterone. Leydig cell cytoplasm is labeled and the basal cell layer of the seminiferous tubule (left) shows an inhomogenous labeling (710×).

Table 3
Tubular grain densities. Grain densities in the basal parts of Sertoli cell cytoplasm, lipid droplets and midpieces of spermatids. Units and abbreviations are the same as in table 1 and fig. 1. The values for the basal cell layer of the seminiferous tubule (PT, cf table 1) are included for comparison.

Exp.	Steroid	Time (min)	Sertoli cytoplasm	PT	Lipid droplets	Midpieces
4	Testosterone (+ 100-fold excess of cold testosterone)	3–15	10.4 ± 0.5 (36)	5.1 ± 0.4 (50)	21.0 ± 1.5 (20)	
2	Pregnenolone	3–10	12.5 ± 0.7 (30)	12.9 ± 0.5 (72)	12.4 ± 1.2 (8)	5.8 ± 0.5 (60)

in table 1 (Exp. 4) and in fig. 5. The tubular labeling in this experiment was also determined for comparison with the pregnenolone experiment (3 min – 10 min KRBG buffer). In both experiments, grain densities were also determined in Sertoli cell cytoplasm along the basement membrane, in lipid droplets and in midpieces of spermatids. A high labeling was observed in all these structures (table 3). For the testosterone experiment the labeling of Sertoli cell cytoplasm was significantly different ($P < 0.001$) from the labeling of the peritubular layer as a whole.

Perfusion with estradiol

Testes were perfused with [$^3H_4$]estradiol for 3 min followed by 15 min perfusion with KRBG buffer. In these studies the highest labeling was observed in the Leydig cell nuclei (table 1; figs. 6 and 7). In all the other areas studied a much lower grain density was observed.

Table 4
The ratios (± standard error) of the grain densities in either the basal cell layer of the seminiferous tubule (PT) or nuclei of Leydig cells (NL) compared to the grain densities in the cytoplasm of the Leydig cells (CL) have been calculated from the values in the same experiment. Grain densities are corrected for background labeling.

Exp.	Steroid	Time (min)	PT/CL	NL/CL
1	Pregnenolone	3–15	0.248 ± 0.033	0.196 ± 0.032
2	Pregnenolone	3–10	0.547 ± 0.027	0.213 ± 0.023
3	Testosterone	3– 5	0.189 ± 0.015	0.139 ± 0.014
4	Testosterone (+ 100-fold excess cold testosterone)	3–15	0.213 ± 0.019	0.178 ± 0.014
5	Estradiol	3–15	0.269 ± 0.057	2.524 ± 0.335

It was not possible to detect any preferential nuclear localization of the tritiated steroids within the seminiferous tubules. The reported data are summarized in a set of histograms in fig. 8. The ratios of grain densities of Leydig cell nuclei over Leydig cell cytoplasm and of the grain densities of the peritubular layer over Leydig cell cytoplasm are given in table 4.

DISCUSSION

Evaluation of the autoradiographic technique

Following perfusion with tritiated pregnenolone the label appeared initially in the cytoplasm of the Leydig cell. After a longer perfusion period the radioactivity was shifted towards the seminiferous tubules, which is reflected in the ratio of grain densities in peritubular over Leydig cell cytoplasm (table 4). At this time after perfusion most of the pregnenolone was converted to testosterone (table 2).

A similar picture was obtained after perfusion with testosterone. Perfusion with labeled testosterone followed by perfusion with 100-fold excess of cold testosterone resulted in a further shift of label towards the seminiferous tubules when compared with the results after perfusion with labeled pregnenolone (3 min – 10 min KRBG buffer). The results of this pulse-chase experiment reflect that labeling in the Sertoli cell cytoplasm remained high whereas labeling in the germinal elements was decreased. In all the experiments with either labeled pregnenolone or testosterone, the highest uptake of steroid was found in the cytoplasm of the interstitial Leydig cells. However, a different localization of radioactivity was found after testis perfusion with [$^3H_4$]estradiol. In these experiments the largest amount of radioactivity appeared in the nuclei of the Leydig cells whereas all the other structures showed an almost equal activity after 15-min perfusion with KRBG buffer.

Relation between grain densities and physiological situation

One of the aims was to study the transport of androgens from their site of synthesis in the Leydig cell to their possible site of action in the seminiferous tubules. Androgens are synthesized from cholesterol by a sequence of enzymes, initiated by the cholesterol side-chain cleavage enzyme (SCE). The activity of this enzyme is rate-limiting in testicular androgen biosynthesis. After perfusion of testes of (intact) normal rats with [^{3}H]cholesterol, the uptake of label was very low using our perfusion system, and the subsequent conversion of the labeled precursor to androgens was hardly detectable (P.M. Frederik, G.J. van der Vusse and H.J. van der Molen, unpublished observations). Hence, testes perfused with cholesterol were ruled out as a suitable model for the study of androgen transport by means of autoradiography. After 3-min perfusion of testes from intact rats with tritiated steroid followed by 15-min perfusion with buffer, a rather diffuse localization of the labeled steroid was found using autoradiography, especially with testosterone and pregnenolone and to a lesser extent with estradiol. This was in accordance with previously published results (Parvinen et al., 1970; Rommerts et al., 1973; Cooper and

Waites, 1975). The diffuse localization can be explained by the occupancy of most of the binding sites by endogenous unlabeled steroids. To prevent the interference of endogenous steroids we have only used rats 5–6 days after hypophysectomy. In these animals the activity of the cholesterol side-clain cleaving activity is reduced (Menon et al., 1975), whereas the activity of other enzymes involved in androgen biosynthesis is still sufficient to convert the perfused pregnenolone to testosterone (Samuels and Helmrich, 1956; Purvis et al., 1973; see also table 3). Therefore one may expect that after [^{3}H]pregnenolone perfusion the label follows the same pathways as endogenously produced pregnenolone. This is further supported by a comparison of the mass of [^{3}H]pregnenolone taken up during perfusion with the mass of pregnenolone that would have been produced during a similar time period in the intact animal. From the perfused pregnenolone, 10 μCi (150 ng) is taken up by the testis in 3 min. This mass is of the same order of magnitude as the production of pregnenolone from endogenous precursors in intact animals (Van der Vusse et al., 1975). These data together with the observation that 5–6 days after hypophysectomy all stages in the cycle of the seminiferous epithelium are still present, support our belief that the results obtained in these animals after perfusion of tritiated pregnenolone may mimic testosterone biosynthesis and transport in the intact animal.

The present observations from autoradiograms are apparently in contrast with the biochemical demonstration of a nuclear testosterone-binding protein in the seminiferous tubules (Mulder et al., 1974; Hansson et al., 1974). However, the number of free binding sites available from this binding protein are low in the intact rat and also in rats one week after hypophysectomy. Therefore the small amount of tritiated androgens that can be bound by this binding protein is not likely to result in a distinct increase of nuclear labeling above a level detectable with autoradiography.

The presence of a cytoplasmatic androgen-binding protein (ABP) has been described in the Sertoli cells of both intact rats and rats 8 days after hypophysectomy (Vernon et al., 1974; Hagenäs et al., 1975). After testis perfusion with tritiated pregnenolone or testosterone, ABP may bind tritiated testosterone. Labeling of the Sertoli cell cytoplasm remained high, even when perfusion of the labeled steroids was followed by perfusion with 100-fold excess of unlabeled testosterone, although the grain densities over the germ cells diminished (table 3). The remaining activity of the Sertoli cell cytoplasm may be explained if no equilibrium was obtained between the unlabeled and labeled steroid in our pulse-chase experiment thereby leaving tritiated testosterone bound to ABP. An alternative explanation for this observation may be that the observed steroid localization largely reflects a partition of steroids between a lipid (e.g., smooth endoplasmic reticulum, lipid droplets) and a water phase.

A specific binding of estradiol-17β in testis tissue has been demonstrated in biochemical studies (Brinkmann et al., 1972; Mulder et al., 1973) and has also been inferred from a previous autoradiographic study (Stumpf, 1969). The concentration

of estrogens used in the present study (approx. 30 ng [$^3H_4$]estradiol/g testis) was much higher than the physiological concentration (30 pg/g testis and 170 pg/g interstitial tissue; De Jong, 1974), but still a distinct uptake of radioactivity in the nuclei of the interstitial tissue was observed. Since the localization of estradiol radioactivity was completely different from the localizations of androgen radioactivity it appears reasonable that the retention of estradiol was the result of interactions with specific estradiol-binding proteins (Mulder et al., 1974).

The grain-density distribution in the autoradiograms (of fig. 7) reflect a larger capacity of the estradiol-concentrating mechanism in the nuclei of Leydig cells compared with the nuclear androgen-binding in seminiferous tubules.

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Electron microscopic autoradiography of tritiated testosterone in rat testis

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Summary

The feasibility of a technique for autoradiography of diffusible substances has been further tested by analysing the localization of steroids in rat testes with the light and electron microscope. Testes of rats were perfused with tritiated testosterone (3 min.) followed by 15 min. perfusion with buffer containing a 100 fold excess of unlabelled testosterone. Tissue samples were frozen, freeze dried, fixed in osmium vapour and embedded in epon. To exclude extraction of steroids contact with water and other solvents was prevented during cutting of thin sections on an ultracryotome and further treatment for autoradiography.
Light- and electron microscopic observations indicate that the highest concentration of labelled testosterone was present within the basal parts of the Sertoli cell cytoplasm and in lipid inclusions of Sertoli cells within the seminiferous tubules. The published micrographs are the first account of autoradiography of steroids at the electron microscope level.

Introduction

Androgenic hormones are involved in the initiation and maintenance of spermatogenesis (Steinberger 1971). The main sites of testicular androgen production are the Leydig cells of the interstitial tissue (Hall 1970) from which these hormones can readily be transported to the seminiferons tubules (Cooper & Waites 1975, Parvinen, Hurme & Niemi 1970, Rommerts, van Doorn, Galjaard, Cooke & van der Molen 1973). Whether androgens produced in the Leydig cells are involved in the regulation of spermatogenesis or this occurs through androgens which are produced within the seminiferons tubules by Sertoli cells (Vinson & Whitehouse 1973), remains to be elucidated. Another problem concerns the specific target cells or cellorganelles within the tubules for the androgens. If such targets accumulate androgens they could in principle be identified by autoradiographic procedures at the light and electron

microscope level. However, steroid hormones are diffusible substances which will be extracted when the usual methods for preparation of tissue sections for microscopy are used. Several methods have been described for the autoradiographic localization of diffusible substances with the electron microscope. By cryoultramicrotomy of unfixed frozen tissue (Appleton 1969, 1974, Christensen 1969, 1971) contact with solvents can be avoided but problems with the subsequent handling of the frozen sections for autoradiography have not yet been solved (Baker & Appleton 1974, Christensen & Paavola 1972). Alternatively frozen tissue may be freeze dried, fixed in osmium vapour and embedded in epon (Wilske & Ross 1965, Stirling & Kinter 1967). Quantitative retention in the epon blocks has been reported for steroids (Attramadal 1969, Frederik & Klepper 1976, Wilske & Ross 1965) and a number of other compounds (Frederik, Fortuin & Klepper 1977, Nadler, Bénard, Fitzsimons & Leblond 1969, Stirling & Kinter 1967, Stirling, Schneider, Wong & Kinter 1972, Wilske & Ross 1965) but considerable extraction was observed from epon sections when these were floating on the water of the knife trough. In previous publications (Frederik & Klepper 1974, 1976) we have described that this extraction can be circumvented by cutting epon embedded tissue on an ultracryotome without the use of flotation fluids. The validity of this method has been further tested in autoradiographic studies at the electron microscope level after perfusion of rat testis with tritiated testosterone. The first results of these studies and data on the autoradiographic localization of testosterone at the light microscope are presented in this paper.

Materials and methods

Mature Wistar rats (R-strain) were used 5-6 days after hypophysectomy. Perfusion of the isolated testes was performed as described previously (Frederik & Klepper 1976) under controlled temperature and pressure (Frederik & van Doorn 1973). The perfusion medium used was a Krebs Ringer bicarbonate buffer (KRBG) containing 1 mg/ml glucose an 1 I.U. herparin/ml. The buffer was saturated with 95% O_2/5% CO_2. After a short initial perfusion period with buffer, 30μ Ci of testosterone (1,2,6,7-^{3}H, S.A. 84 Ci/m mol) dissolved in 1 ml KRBG was infused into the testis. In order to eliminate non-specific binding of testosterone, perfusion was continued for 15 min. with buffer containing a 100 fold excess of unlabeled testosterone. At the end of the perfusion the testis was decapsulated and small tissue samples (wet weight ca. 10 mg) were frozen in Freon 22 cooled with liquid nitrogen.

The frozen tissue was freeze dried overnight at -85° C in vacuo, (less than 10^{-6} mm Hg), fixed in osmium tetroxide vapour at a temperature of 45° C and embedded in Epon (Luft 1961). Details of the equipment used have been described elsewhere (Frederik & Klepper 1976). Thin sections were cut from the embedded tissue on an LKB III microtome with a Cryokit attachment. Sectioning was performed on bare glass knives (knife angle 48°, clearance angle 5°) with a knife temperature of -120° C and a specimen temperature of -80° C (Frederik & Klepper 1976). The sections were removed from the knife edge with an eye-lash probe and were mounted on formvar coated copper grids. Good contact with the carrier film was achieved by pressing the sections onto the grids (Appleton 1974,

Christensen 1971). The sections were coated with a thin layer of carbon and a monolayer of Ilford L4 emulsion using the loop method (Caro & van Tubergen 1962). After 10 weeks of exposure at -18° C the autoradiographs were developed in Kodak D76 (3 min.), rinsed in distilled water and fixed in 20% $Na_2S_2O_3$ (5 min.). Photographs were taken with a Philips EM 300 electron microscope without any further staining of the sections. Autoradiograms were also studied at the light microscope and grain densities were determined.

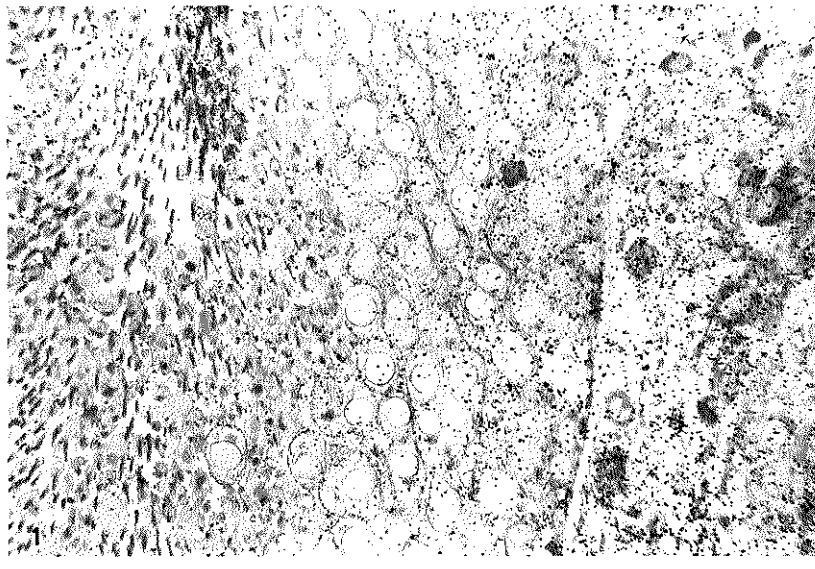
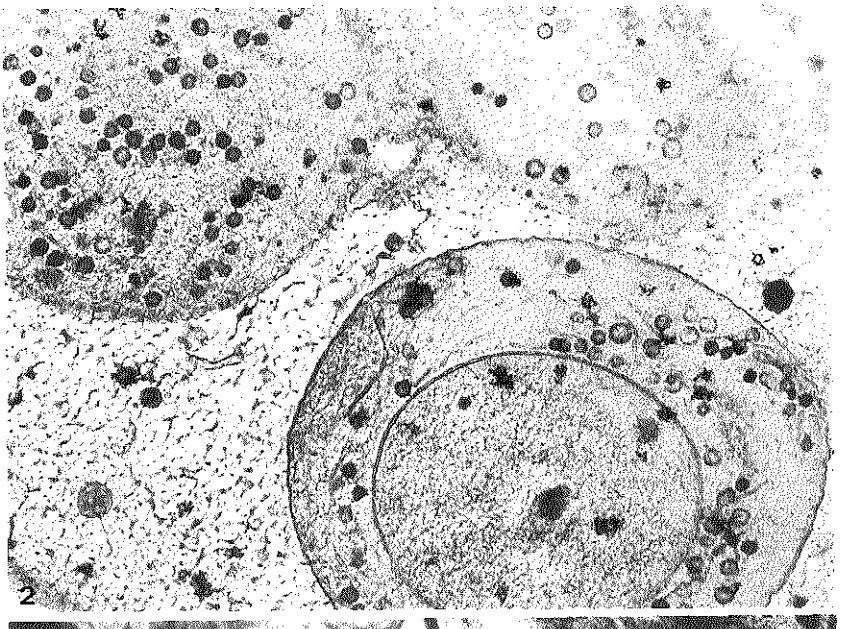


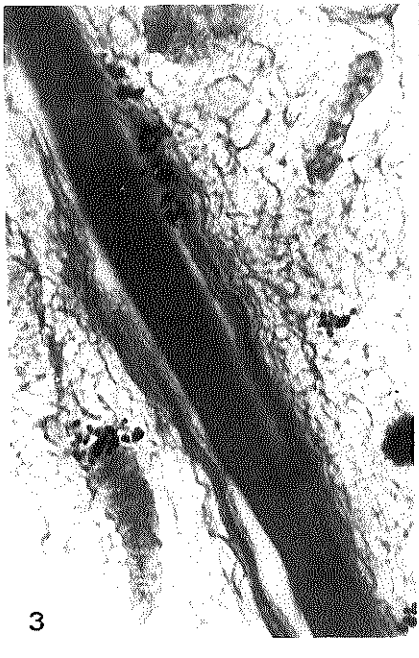
Fig. 1-6. Autoradiograms of rat testes perfused with testosterone. Testes were perfused for 3 min. with 30μCi tritiated testosterone, followed with 15 min. perfusion with buffer containing a hundred fold excess of unlabelled testosterone. Tissue samples were freeze dried, fixed in osmium tetroxide vapour and embedded in epon.
Fig. 1. Light micrograph of an autoradiogram (2μ section), unstained, phase contrast. A high grain density can be observed above the interstitial tissue. See text for the analysis of grain densities above the seminiferous tubules. Ilford K5 emulsion, 670 x.

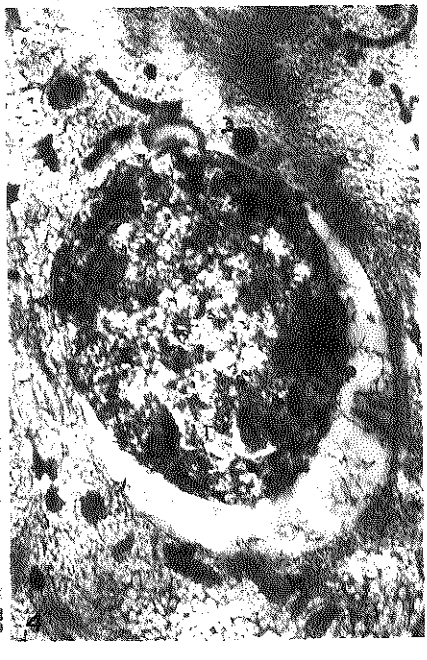
Results

A light microscopic picture of an autoradiogram from a testis perfused with ^{3}H-testosterone is shown in fig. 1. Estimation of the grain density distribution (expressed as number of grains/27$\mu^2 \pm$SEM, n = number of areas counted, 1-2 areas per cell) revealed that grain densities in the basal parts of the Sertoli cell cytoplasm (10.4 $\pm$ 0.5,n = 36) and in lipid droplets within the Sertoli cells (21.0 $\pm$ 1.5, n = 20) were higher than the grain density above the basal layer of round spermatids (2.7 $\pm$ 0.3,n = 50,

background 0.35 $\pm$ 0.03,n = 500) (Frederik, Klepper, van der Vusse & van der Molen 1976). Autoradiograms from the same animal experiment were prepared for electron microscopic studies (fig. 2-6). Fig. 2 shows an autoradiogram of spermatids. A satisfactory preservation of the morphology of these cells can be observed. The photographic grains are evenly distributed over the cellular structures with a density of about







20 grains/100μ^2. A high grain density can be found (59/100μ^2) above the lipid droplets (fig. 3). It should be noted that with the employed fixation procedure the contents of the lipid droplets are preserved, yielding an electron opaque structure. This complicates the identification of photographic grains above these structures. Fig. 4 shows the nucleus of an elongated spermatid with the surrounding Sertoli cell cytoplasm. Several autoradiographic grains are found in a row above the border line of these two cells. In fig. 5 and 6 the relatively high labelling above the basal parts of the Sertoli cell sytoplasm (39gr/100μ^2) is shown. Background grain density in this experiment was 1.5 gr/100μ^2 (total area counted 1315μ^2).

Discussion

The results obtained in the present study are the first account of the autoradiographic localization of tritiated steroids at the electron microscope level. The method used seems to fulfil the requirements for autoradiography (Stumpf & Roth 1968) with respect to: morphological integrity of the tissue sections, satisfactory high resolutio autoradiography and total label recovery. Preservation of the tissue morphology is achieved only for the superficial layers, an aspect inherrent to the freezing proces (Van Venrooij, Aertsen, Hax, Vervegaert & Verhoeven 1975). More interior layers are characterized by the same type of structural damage (knitting') as was observed in frozen thin sections obtained from a corresponding depth in the frozen tissue (Hodson & Williams 1976). High resolution autoradiography is achieved by using the well established loop technique with Ilford L4 emulsion (Caro & van Tubergen 1962, Rogers 1973). With respect to the total recovery of tritiated steroids (Attramadal 1969, Frederik & Klepper 1976, Wilske & Ross 1965) the only critical steps are the coating with photographic emulsion and the subsequent exposure of the autoradiograms. Precautions taken to restrict diffusion of steroid into the photographic emulsion include the use of a thin layer of carbon on the tissue section, the application of the emulsion in a gelled form and the storage of the sections at low temperature (-18° C) during exposure. The electron microscopic autoradiograms thus may give a faithful representation of the distribution of tritiated steroids in rat testis.

Fig. 2-6. Electron micrographs of autoradiograms. Contact with any liquid was avoided when sections were cut on an ultracryotome, mounted on carrier grids and covered with a thin layer of carbon. Ilford L4 emulsion, no post-staining. See text for analysis of grain densities.
Fig. 2. Round spermatids. A good preservation of the ultrastructure can be observed. 7450 x.
Fig. 3. Elongated spermatid. Several grains in a row are found at the spermatid-Sertoli cell border. 24.900 x (note: Some erosion of the grains has occured probably due to the high intensity of the elctron beam used during the study of the head-piece of the spermatid.)
Fig. 4. Lipid droplet within a Sertoli cell. A high grain density is found over the electron dense content. 8870 x.

With light microscopic autoradiography the highest concentrations of tritiated testosterone in the seminiferous tubules were found in the basal cytoplasm of Sertoli cells and in lipid droplets (Frederik et al. 1976). The evidence from the present EM autoradiography is in qualitative agreement with these observations because similar relationships between the grain densities were found. However more extensive studies followed by statistical analysis of the data obtained are required to support this evidence. In addition the present electron microscopic study suggests that the infused labelled testosterone could be found in the membranes which surround spermatids. From the light microscopic as well as the electron microscopic observations it appears that there is a preferential localization of the infused testosterone in lipid rich areas (membranes, lipid droplets).
Sar et al (1975) observed with autoradiography a nuclear concentration of testosterone in cells of the seminiferous tubules from rats hypophysectomized at 35 days of age. By determination of grain densities in light microscopic autoradiograms we were not able to demonstrate a concentration of testosterone above any nucleus in the seminiferous tubules. This difference may be explained by the apparent inability of nucleair androgen binding protein (Hansson, McLean, Smith, Tindall, Weddington, Nayfeh, French & Ritzen 1974, Mulder, van Beurden-Lamers, de Boer, Brinkmann & van der Molen 1974) to concentrate detectable amounts of tritiated testosterone after perfusion. In contrast nuclear oestradiol binding in the interstitial Leydig cells (Stumpf 1969) could be detected with autoradiography after comparable perfusions (Frederik & Klepper 1976, Frederik et al 1976) of testis with oestradiol.

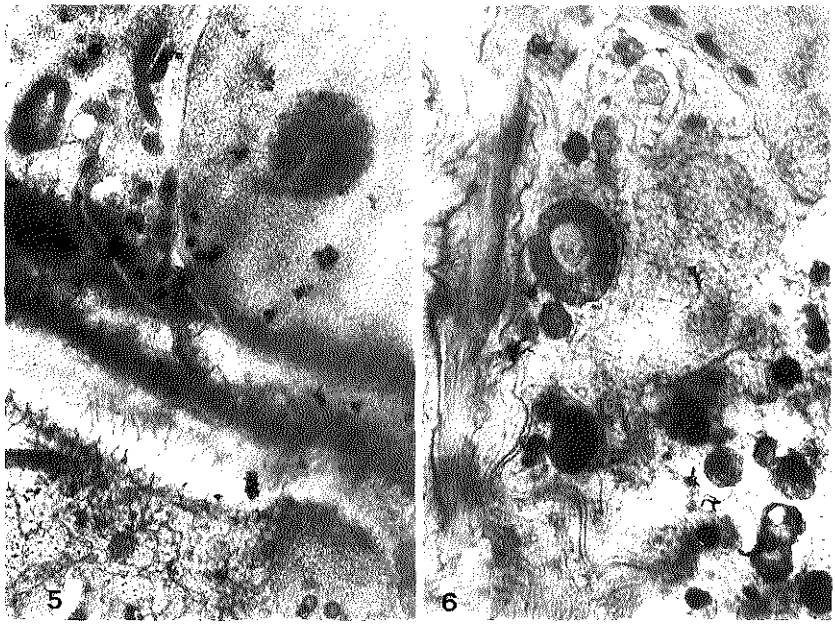


Fig. 5-6. Basal cytoplasm of Sertoli cells containing typical Sertoli cell mitochondria. 6890 x and 11.700 x.

We greatly appreciate the valuable advices of H. Boeré (LKB, The Netherlands) concerning the cryo-sectioning of plastic embedded tissues.

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Autoradiographic detection of mucopolysaccharide accumulation in single fibroblasts

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Synopsis. A method is described for localizing acid mucopolysaccharides autoradiographically in cultured cells. Normal fibroblasts and fibroblasts, from patients suffering from Mucopolysaccharidosis II disease (MPS II), were cultured for six days in the presence of $^{35}SO_4$ and one day in unlabelled medium. The cultured cells were transferred to a plastic film dish and, after settling, they were rapidly quenched, freeze-dried, fixed in osmium tetroxide vapour and embedded in Epon. Grain counting after autoradiography in 2 μm sections revealed a significant difference ($P > 0.001$) in $^{35}SO_4$ incorporation in the perinuclear cytoplasm of MPS II cells and control cells grown under the same conditions. Autoradiography was also performed after mixing MPS II cells and control fibroblasts in a ratio 1:1.8 prior to freezing and the same ratio was found between labelled and unlabelled fibroblasts. These results demonstrate the feasibility of the present autoradiographic technique for the detection of the acid mucopolysaccharide storage at the single cell level.

Introduction

The genetic metabolic defects in patients with Mucopolysaccharidoses (McKusick, 1972, 1975) are, in most cases, expressed among other things in the storage of acid mucopolysaccharides in cultured skin fibroblasts or amniotic fluid cells from these patients (Neufeld, 1974). The diagnosis of these diseases can be based either on the analysis of the responsible enzymic defect or on the demonstration of intracellular accumulations of acid mucopolysaccharides (MPS).

The impairment of normal degradation of MPS in cultured cells is usually demonstrated by labelling with $^{35}SO_4$ and the subsequent measurement of the incorporated radioactivity by scintillation counting (Fratantoni *et al.*, 1969). This procedure requires

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something of the order of 10^6 cells per assay. In the prenatal diagnosis of metabolic defects however, it is advantageous if biochemical analyses can be performed on small numbers of cells since this reduces the waiting period between amniocentesis and diagnosis (Galjaard *et al.*, 1974*a*, 1975*a*; Kleyer *et al.*, 1975).

It was one of the purposes of the present study to develop an autoradiographic technique that would enable the assay of MPS-accumulation in small numbers of cultured (amniotic fluid) cells. In principle, such a procedure would also give information at the single cell level, and might, therefore, contribute to more basic studies on enzyme-deficient cultured cells like those concerned with metabolic correction (Fratantoni *et al.*, 1968; von Figura & Kresse, 1974; Galjaard *et al.*, 1974*b*; Reuser *et al.*, 1976) and genetic complementation (de Weerd-Kastelein *et al.*, 1972; Galjaard *et al.*, 1975*b*).

Autoradiography of soluble substances such as acid mucopolysaccharides is complicated by the fact that serious losses will occur if conventional preparative methods are used (Lagunoff *et al.*, 1962; see also Pearse, 1968). However, Frederik & Klepper (1974, 1976) have developed a method that enables steroids to be studied autoradiographically both at the light microscope (Frederik *et al.*, 1976) and the electron microscope levels (Frederik & Klepper, 1976). The present study deals with the adaption of these methods to an analysis of the accumulation of acid mucopolysaccharides in single cultured cells.

Materials and methods

Cell cultivation and $^{35}SO_4$ incorporation

Normal human fibroblasts and fibroblasts from a patient with Mucopolysaccharidosis II disease (MPS II) are cultured in glass bottles on Eagle's medium (MME, Difco), supplemented with 15% foetal calf serum, 1 mM Na_2SO_4, 12 mM $NaHCO_3$, penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). For an experiment, cells are harvested by trypsinization and suspended in Eagle's medium supplemented as above but with 5 mM instead of 12 mM $NaHCO_3$ and additional organic buffers [HEPES, PIPES and BES, 10 mM of each (Eagle, 1971), pH 7.2]. Plastic petri dishes (35 mm overall diameter) are seeded with 2 to 3 × 10^5 cells/dish in 2 ml medium and incubated at 37°C in a fully humidified atmosphere of 5% CO_2 and 95% air. After 2 days the culture medium is changed with the same medium containing 150 μCi $^{35}SO_4$/ml in addition. (Carrier-free $Na_2{}^{35}SO_4$ was obtained from Amersham as a sterile solution in isotonic saline.) After 6 days culturing in medium containing $^{35}SO_4$ (medium change every two days), the cultures are rinsed three times with saline and incubated for another day in unlabelled medium. Then the cultures are rinsed again, trypsinized (for 15 min in 0.5 ml 0.25% trypsin per dish), counted, and split into two parts. One part is used for scintillation; counting of intracellular ^{35}S radioactivity. The other part is centrifuged, resuspended into medium, and 2 × 10^4 cells (in 100 μl medium) are seeded on a dish (Fig. 1) with a bottom of Teflon FEP film (gauge 25 μm Technomara A.G., Zürich). Before seeding, polylysine is pipetted on the bottom of the dish, left for 5 min at 20°C and subsequently the excess of polylysine is removed by rinsing several times with saline. This procedure facilitates the attachment of the cells (Mazia *et al.*, 1975). The cells are allowed to settle on the dish for 4 hr at 37°C, after which the medium is removed by rinsing with saline and finally with a volatile buffer (ammonium acetate, 0.15 M, pH 6.8).

Preparation for autoradiography

The whole dish is quickly frozen in liquid Freon 22 cooled with liquid nitrogen, and is then freeze-dried overnight at $-85°C$ *in vacuo* ($< 5 \times 10^{-6}$ mm Hg) using apparatus described previously (Frederik & Klepper, 1976). After lyophilization, the dish is transferred to a desiccator for subsequent fixation for 18 hr in osmium tetroxide vapour at 45°C. Embedding was carried out by immersing pure Epon 812 *in vacuo* into the plastic film dish, followed by several changes with a complete Epon mixture (Luft, 1961) at atmospheric pressure. After polymerization, the Epon disc containing the monolayer of fibroblasts is removed from the culture dish (Fig. 1; see also van Ewijk & Hösli, 1975) and

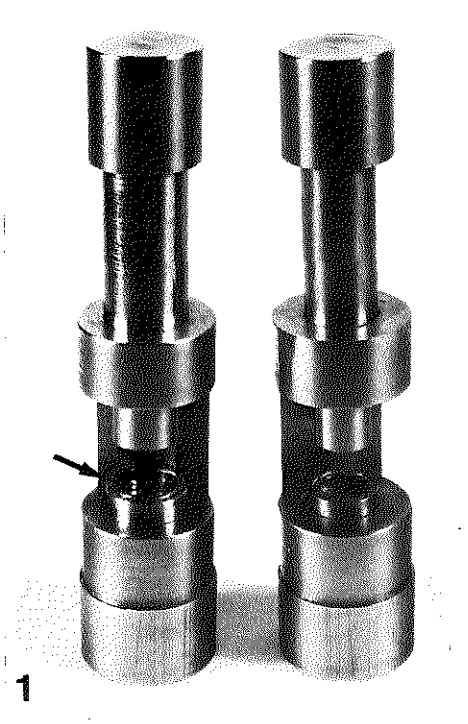
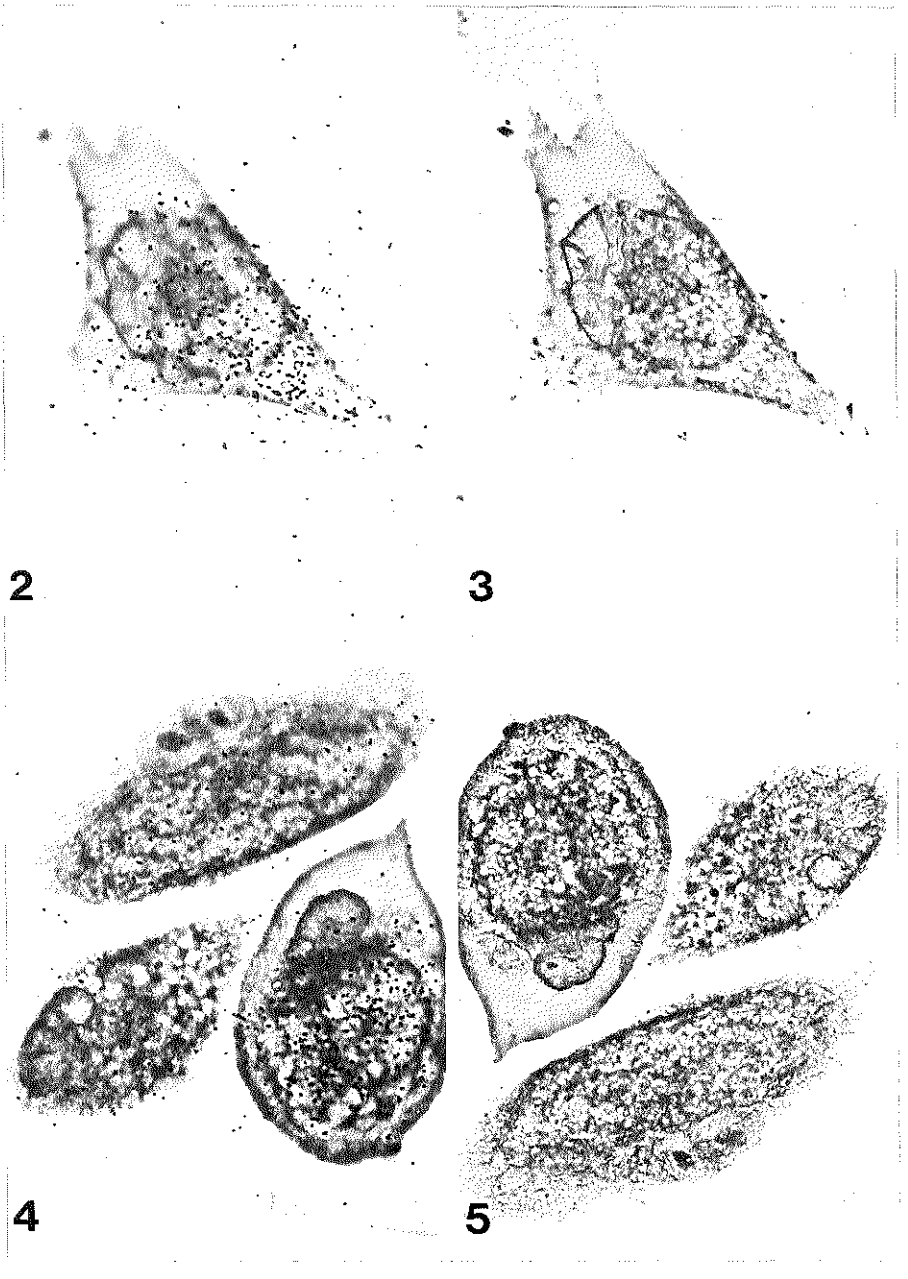


Figure 1. Removal of Epon-embedded cells from the plastic film dish. The plastic film dish (arrow) is made of two concentric rings with the substrate interposed. The freeze-dried, vapour-fixed and embedded cells in the well are liberated by punches, one for removal of the outer ring (left) and one for removal of the inner ring (right) of the dish, yielding a disc of epon with the embedded cells at the bottom. × 0.8

is sawn into four pieces. Each piece is cemented on top of a premoulded Epon block and sections (2 μm) are cut parallel to the substrate on a LKB III microtome. Sections are collected on glass slides and a thin layer of carbon is evaporated on the slides. Photographic emulsion (Ilford K5) is applied to the sections as a monolayer with a platinum loop (Caro & van Tubergen, 1962). The sections are exposed for 10 days at $+4°C$ in light-tight boxes containing silica gel. From each lot of sections, one section is exposed to daylight and stored together with the other sections to check the incidence of negative chemography (Rogers, 1973). The autoradiograms are developed in Kodak D76 (3 min 20°C), rinsed in distilled water, and fixed in 20% $Na_2S_2O_3$ (5 min, 20°C). Sections are examined without additional staining under a phase contrast microscope. Grain counting is carried out only for those cells where the nucleus is included in the section.

Results

The grain densities observed above fibroblasts from MPS II and control cells are given in Table 1. In the MPS II cell line the grain density above the nucleus is significantly different ($P > 0.001$) from the perinuclear cytoplasm. The grain densities above the perinuclear cytoplasm of the MPS II cells are significantly higher ($P > 0.001$) than



above the corresponding area in control cells. Micrographs from the autoradiograms are given in Figs. 2–5. These micrographs further demonstrate the differences in grain densities and also show the morphological preservation of the cells achieved with the employed technique. A clear difference between MPS II and control cells is also found for the intracellularly incorporated ^{35}S radioactivity as measured with liquid scintillation counting (Table 1). In order to study the discriminating ability of the autoradiographic technique, MPS II cells and control cells were mixed in a proportion of 1 : 1.8 prior to seeding them on the plastic film dish. After subsequent autoradiography a ratio of 1 : 1.7 was found between labelled and unlabelled cells for 100 cells observed (Figs. 4 & 5).

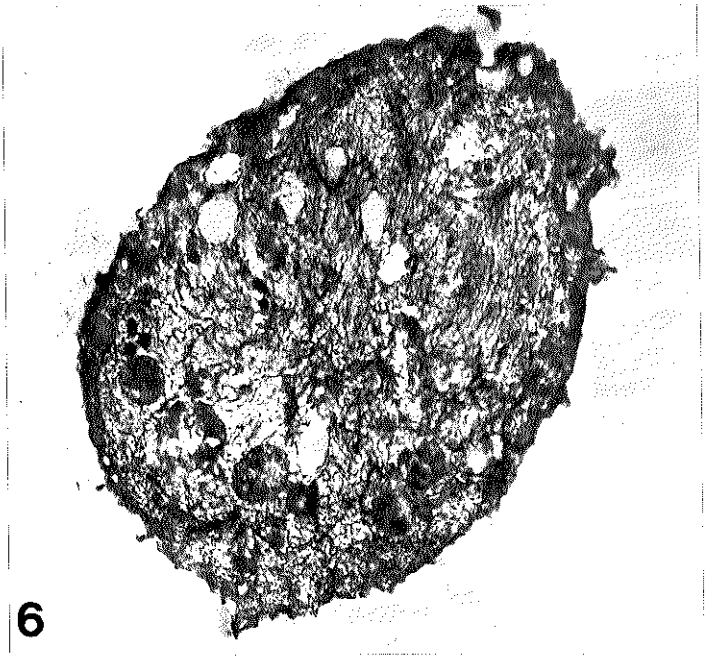


Figure 6. Electron microscopic picture of an MPS-II fibroblast (the same embedding as shown in Figs. 2 & 3). Several dense bodies can be observed indicating a retention of stored mucopolysaccharide in the preparative procedure. × 12800

Figures 2–4. Autoradiography of $^{35}SO_4$-labelled mucopolysaccharides in cultured fibroblasts. Cells were lyophilized, fixed in osmium tetroxide vapour and embedded in Epon. Sections (2 μm) were cut parallel to the substrate. Ilford K5 emulsion, phase contrast. × 1050

Figures 2 & 3. Fibroblast from a MPS II cell line grown in the presence of $^{35}SO_4$ (see text).

Figure 2. Focus on the autoradiographic grains. A high grain density can be observed above the perinuclear cytoplasm.

Figure 3. As Fig. 2, but focussed on the cell. A nucleus and nucleolus can be observed.

Figures 4 & 5. A mixed population of MPS II and control fibroblasts. Control and MPS II fibroblasts were cultured separately in the presence of $^{35}SO_4$ and mixed just prior to freeze-drying (see text).

Figure 4. Focus on the autoradiographic grains. Differences can be observed in the grain densities above the perinuclear cytoplasms.

Figure 5. As Fig. 4 but focussed on the cells.

Table 1. Grain densities are given as mean ± S.E.M. number of grains per area of 27 μm^2 and the number of areas counted is given in parentheses. The number of cells used for determination of the grain densities is given between parentheses in the left column. $^{35}SO_4$ incorporation in the cell is given as cpm ^{35}S-radioactivity/mg cell protein, found intracellularly after 80% ethanol-extraction of trypsinized cells from the same cultures as studied with autoradiography. See text for cell culture and labelling conditions.

	Grain densities			*Intracellular radioactivity*
	Cytoplasm	*Nucleus*	*Background*	
MPS II (n = 30)	14 ± 0.7 (82)	1.8 ± 0.3 (32)	0.08 ± 0.32 (300)	12.5×10^5
Control (n = 20)	3 ± 0.6 (61)	1.8 ± 0.3 (30)	0.11 ± 0.35 (200)	1.1×10^5

The same Epon blocks which were used for autoradiography were also used for making ultrathin sections. These sections were viewed without further staining in the electron microscope. An electron microscopic picture of a fibroblast from a MPS II cell line is shown in Fig. 6. Dense granules can be observed in the cytoplasm, probably indicating a retention of the lysosomal MPS in the preparative procedures.

Discussion

A significant difference in grain densities over the perinuclear cytoplasm was found between MPS II cells and control cells. The specific localization of high grain density above the perinuclear cytoplasm agrees with the localization of metachromatic staining in Toluidine Blue- or Alcian Blue-stained MPS II cell cultures (Danes & Bearn, 1966; Lagunoff *et al.*, 1962). The described method for freeze drying and autoradiography thus enables reliable detection of stored mucopolysaccharides at the single cell level. Working with 2 μm sections has the advantage over toto preparations in that a higher resolution can be obtained. Firstly, because a section is flat which excludes pressure artifacts caused by height differences, and secondly, because there is less overlap between organelles (e.g. nucleus, perinuclear cytoplasm and cell membrane). An additional advantage of the present method is the use of cells that had been trypsinized just before being freeze dried. Trypsinization removes the $^{35}SO_4$-mucopolysaccharide belonging to the cell coat and which does not contribute to the difference between normal and MPS II cells in cellular mucopolysaccharide content (Neufeld & Lautz, 1973; Fortuin & Kleyer, to be published).

By fixing cells in osmium tetroxide vapour, one has to consider the occurrence of negative chemography caused by oxidation of latent images. Therefore, all sections were coated with carbon before being covered with photographic emulsion and a few sections were exposed to daylight prior to storage with the other sections. Negative chemography could not be observed under the conditions employed.

The partly decomposed mucopolysaccharide stored in the lysosomes in MPS diseases are highly water soluble (Muir, 1973) which complicates their histochemical localization

(Danes *et al.*, 1970; Elleder, 1976). For the histochemical demonstration of mucopolysaccharides in sections, Pearse (1968) recommends the use of freeze-dried, vapour-fixed and embedded material. Osmium tetroxide vapour fixation seems to be advantageous in this procedure since such a fixation quantitatively prevents the extraction of sugars during embedding (Stirling & Kinter, 1967) and the quality of the fixation offers the possibility for electron microscopic autoradiography (Rogers, 1973; Frederik & Klepper, 1976; Stirling & Kinter, 1967).

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