Light and electron microscope localization of ^{3}H-dexamethasone 21-mesylate in adult rat testicular Leydig cells

by

Alison Stalker
Department of Anatomy,
McGill University,
Montreal, Quebec
Canada

March, 1990

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the Degree of Master of Science

To my parents,

and the rest of my family,

including Houdini

Acknowledgements

I would first like to thank Dr. Osmond, the Chairman of the Department of Anatomy, for having allowed me to work in this department.

I would also like to extend a very special thanks to Dr. Louis Hermo for his excellent guidance and support throughout this project as well as his constant, everyday enthusiasm.

I would also like to thank Dr. Tony Antakly for his guidance and support and the use of his computer for grain quantitation (at the LM level).

Further acknowledgements to:

- Dr. Nadler, for his invaluable advice, especially on the techniques of quantitative EM radioautography.

- Dr. Charlie Smith, for his instructive comments on the use of computer-assisted image analysis.

- the staff of the radioautographic lab, namely, Dr. B. Kopriwa, Fernando Evaristo, and Mohammed El-Alfy, for the processing and development of the LM and EM radioautographs.

- the staff (past and present) of the EM lab, in particular, Jeannie Mui, Pat Hales, and Matilda Cheung for the thin sections (RAGS).

- Dolores Raquidan, for her help in preparing the experiments.

- Dr. Cunxian Zhang for her help in the immunocytochemical experiments.

- Tony Graham, for his excellent photographic work (light and electron micrographs), and Ann Silkauskas, for her secretarial assistance.

- Dr. Clermont, for having translated the abstract.

This work was supported by grants to Drs. L. Hermo and T. Antakly from the Medical Research Council and a studentship to the author from the Fonds pour la Formation de Chercheurs et l'Aide a la Recherche (Fonds FCAR).

Abstract

The distribution of glucocorticoid receptors (GR) in the rat testis was examined in vivo using (3H)-dexamethasone 21-mesylate (DM) which binds covalently to GR. After injection with ^{3}H-DM, the testes of adrenalectomized, adult rats were processed for light microscope (LM) radioautographic (RAG) analysis. Control rats received simultaneously a 25-or 50- fold excess of cold dexamethasone. Quantitation of the label confirmed the presence of specific DM binding sites in both Leydig cells (86% of the label) and to a lesser degree, in the cellular layers of the seminiferous epithelium (14% of the label). These binding data were confirmed by LM immunocytochemistry. Interstitial macrophages were non-specifically labeled. In the Leydig cell, an electron microscope (EM) quantitative RAG analysis of 3H-DM binding sites showed that smooth endoplasmic reticulum (sER) and mitochondria were heavily labeled, with 53% and 31% of the total label, respectively. Cytosol (exclusive of all structures) and nucleus showed comparatively weak labeling, with 9% and 7% of the label, respectively. All other structures showed little or no labeling. While the presence of ^{3}H-DM in the cytosol and nucleus may represent the GR in its traditional compartments, the significance of the labeling of the sER and mitochondria remains to be clarified. It is possible that ^{3}H-DM was targeted to the mitochondria, perhaps to regulate transcription of some mitochondrial proteins. Furthermore, ^{3}H-DM may have been metabolized preferentially in the mitochondria and sER of testicular cells.

Name: Alison Stalker

Title: Light and electron microscope localization of ^{3}H-dexamethsone 21-mesylate in adult rat testicular Leydig cells.

Department: Anatomy

Degree: Master of science

RESUME

La distribution des récepteurs des glucocorticoides (GR) dans le testicule du rat, a été étudiée in vivo à l'aide du dexaméthasone 21-mésylate marqué au tritium (^{3}H-DM) qui se lie aux GR d'une façon covalente. Après injection de ^{3}H-DM, les testicules de rats adultes adrénalectomisés ont été prélevés et préparés pour la radioautographie en microscopie optique. Les rats contrôles ont reçus du DM non radioactif en doses 25 fois plus grandes. L'analyse quantitative de la réaction radioautographique confirme la présence d'une liaison spécifique du ^{3}H-DM aux cellules de Leydig (86% du marquage total) et aux cellules de l'épithélium séminifères (14%). Ces résultats ont été confirmés par immunocytochimie en microscopie optique. Les macrophages intertubulaires sont marqués non-spécifiquement. En microscopie électronique, la radioautographie montre que la liaison du ^{3}H-DM se retrouve au niveau de sER, 53% et des mitochondries, 31% du marquage total. Le cytosol (à l'exclusion de toute structure) et le noyau sont faiblement marqués i.e. 9% et 7% respectivement. Les autres organites cytoplasmiques ne montrent pas un marquage significatif. Tandis que la présence de ^{3}H-DM dans le noyau ou le cytosol correspond aux sites de liaison habituels du marqueur, les liaisons du ^{3}H-DM au sER et aux mitochondries sont inhabituelles et leur signification reste à être élucidée. Il est possible que le ^{3}H-DM associé contribue à la transcription des protéines mitochondriales. De plus le ^{3}H-DM peut être métabolisé d'une façon préférentielle dans les mitochondries et le sER des cellules testiculaires.

Auteur: Alison Stalker

Titre: Localization, en microscopie optique et électronique, du 3H-dexaméthasone 21-mésylate dans les cellules de Leydig de rats adultes.

Département: Anatomie

Diplôme: Maîtrise en science

Table of Contents

Introduction

Adrenal hormones

The adrenal gland is composed of two distinct organs, each of separate embryological origins, the medulla and the cortex.

The adrenal medulla forms the inner core of the gland and is responsible for secretion of two peptide hormones - epinephrine (adrenaline) and norepinephrine (noradrenaline) - both members of the catecholamine family. Removal of this organ does not impair life as most, if not all, of the hormonal effects of this tissue may be mediated elsewhere in the body.

The adrenal cortex is a steroidogenic organ which is essential for maintenance of life. It secretes three classes of steroid hormones: mineralocorticoids, which are involved in fluid and electrolyte balancing; glucocorticoids, important in metabolism, stress and immune responses; and the reproductive hormones - estrogens, progesterone, and androgens - which are secreted only in small amounts. This report will focus only on the glucocorticoid hormone.

Glucocorticoids

Glucocorticoids, C_{21} steroid hormones of adrenocortical origin, affect and regulate metabolic processes in a wide variety of tissues resulting in an enormous diversity of biological effects throughout the mammalian body (reviewed in Christy, 1971; Munck and Leung, 1977; Baxter and Rousseau, 1979). Amongst other effects, their actions ultimately raise hepatic glycogen deposition and blood glucose concentration (thus creating a diabetic condition) - particularly during periods of stress or anger - thereby creating stores of easily

obtainable energy.

In the liver, a major target tissue of glucocorticoids, the effects of these hormones are mainly anabolic and include increased RNA and protein synthesis (enzyme induction), increased glucose synthesis via increases in gluconeogenic enzymes, and ultimately, increased deposition of glycogen. Excess glucose from the liver is transferred to the blood (thereby raising blood sugar concentration) where it is made available for other tissues in times of increased energy needs.

In some peripheral or extra-hepatic tissues, muscle and adipose tissue in particular, the effects of glucocorticoids are mainly catabolic and therefore contrast heavily to those seen in the liver. Proteins and lipids are broken down to their individual components- amino acids and fatty acids (and glycerol), respectively. Glucose and protein synthesis is inhibited as is uptake of materials needed for their synthesis. Part of the breakdown products yielded by the catabolic activities in these tissues are mobilized to the liver to serve as precursors for glucose, and ultimately, glycogen synthesis.

Glucocorticoids also suppress the immune system, more specifically, lymphocyte proliferation, thereby causing involution of lymphoid tissues, i.e. thymus and lymph nodes. Their ability to reduce inflammations normally associated with infections and allergies has resulted in extensive clinical usage of these hormones as anti-inflammatory agents.

Regulation of glucocorticoid secretion

Glucocorticoids are under the positive control of the pituitary hormone, adrenocorticotrophic hormone (ACTH) - which is produced in the

corticotroph cells of the anterior lobe from the large precursor protein, pro-opiomelanocortin (POMC) (Chretien et al., 1978; Eipper and Mains, 1980). ACTH released into the bloodstream, in response to hypothalamic factors, including corticotropin releasing hormone (CRF) (Vale et al., 1981), stimulates synthesis of glucocorticoids in the adrenal cortex (fasciculata and reticularis zones) which then feedback to the anterior pituitary and inhibit ACTH (POMC) production in the corticotroph cells (Roberts et al., 1982) - a classic inhibitory feedback cycle.

Glucocorticoid receptor (GR)

Glucocorticoids exert their biological effects through a soluble, intracellular protein of approximately 94 kdal, the glucocorticoid receptor (GR).

Through the use of a number of techniques and tools, including limited proteolysis, deletion and insertional mutations, immunocytochemistry, and mutant cell lines, the structure of the general steroid receptor, GR included, has been elucidated and is known to consist of several functional domains intervened by two proteolytic sensitive hinge regions (Gehring, 1987; Hollenberg et al., 1987; Evans, 1988; Carlstedt-Duke and Gustafsson, 1988).

Receptor structure

The rat and human GR are proteins of 777-795 amino acid residues of which the first 403-406 form the amino terminus and include the immunogenic domain of the receptor (human GR, residues 77-262). Although little is known about the function of this terminus, deletion studies have shown that the presence of the entire region is necessary

for complete transcriptional activity. In the human GR, the severity of effects resulting from residue deletions range from 85% of normal (wild-type) transcriptional activity to as low as 10%, the latter occurring upon deletion of either the entire region or the small immunogenic domain (Hollenberg et al., 1987).

In the center of the receptor, adjoining the amino terminus, is the highly conserved DNA binding domain, rich in basic amino acid residues, particularly arginine, cysteine, and lysine, and without which any DNA binding and transcriptional enhancement can occur. This domain, approximately 87-104 amino acids long, contains a region of about 30 residues rich in cysteine and histidine residues which are thought to form finger-like structures which associate with the DNA (Berg, 1986).

A third domain, a region of about 245-278 amino acids (rat GR, residues 518-795) at the carboxy end, is responsible for binding the steroid. Since complete removal of this domain results in a constitutively active receptor, this region is thought to inhibit DNA binding in the absence of steroid. The GR affinity label, dexamethasone 21-mesylate, has been shown to bind covalently to cysteine 656 in this region of the rat hepatoma tissue culture (HTC) cell GR (Simons et al., 1987).

Receptor activation

In the absence of ligand (steroid), the GR is thought to exist within the cytoplasm as a heterodimeric complex with one or two 90 kdal heatshock proteins (hsp) associated with it (Housley et al., 1985; Bresnick et al., 1988, 1989). The presence of the hsp protein(s)

appears to stabilize the necessary conformation of the GR for ligand binding, since little or no steroid binding occurs in their absence (Bresnick et al., 1989).

Upon binding of the steroid, the receptor is "activated" to a state in which together with the steroid, it will translocate to the nucleus and associate with specific DNA sequences known as glucocorticoid responsive elements (GRE), whereby gene transcription will either be enhanced or inhibited. GREs are usually found upstream of the target gene, within the promoter regions (Yamamoto, 1985; Beato, 1989).

Although the detailed mechanism of receptor activation is still being elucidated, it is known that activation of the receptor-steroid complex can be achieved in vitro through increased temperature or ionic strength as well as cytosol dilution (Schmidt and Litwack, 1982). In the case of the human GR, this process is thought to occur in two distinct steps, the first of which is dissociation of the hsp protein(s) from the GR thereby yielding a partially activated 4-5S receptor-steroid complex, a step which is both temperature and molybdate sensitive (Harmon et al., 1988). The second step involves a yet unknown cytosolic peptide species of 72 kda whose presence appears to be necessary to confer full DNA binding activity to the receptor-steroid complex (Harmon et al., 1988).

Early Localizations of steroid receptors (SR)

In early studies, the steroid receptor (SR), including the GR, was detected primarily through radioreceptor assays, a method by which tissue homogenates and whole cells are incubated with a radiolabeled steroid thereby permitting quantitation of the SR (reviewed in Munck

and Leung, 1977; Baxter and Rousseau, 1979). The most widely used steroids in these studies were estrogens and progesterones. Such assays consistently localized the unactivated SR (from steroidectomized animals or cultured cells in the absence of steroid) in cytosolic fractions, with nuclear labelling increasing only after hormone administration, thereby prompting the formulation of the "two-step model" of steroid action. This model proposed that the steroid acts through an intermediary species, the cytoplasmic SR, to influence gene transcription as opposed to acting directly by itself (Jensen et al., 1968; Gorski et al., 1968).

However, with the emergence of improved radioassays (on different tissues and species) (Mester and Baulieu, 1972; Zava and McGuire, 1977; Callard and Mak, 1985), new immunocytochemical tools (King and Greene, 1984; Logeat et al., 1983; Radanyi et al., 1983), and cell enucleations (Welshons et al., 1984; 1985), it became apparent that the unoccupied ER and PR are found predominantly in the nucleus, and consequently, the early findings of these receptors in the cytoplasm were considered to be false, the result of artefactual translocation during the homogenization procedure. Nevertheless, the existence of a small population of cytoplasmic ER and PR, perhaps serving as a intracellular shuttle for the incoming steroid, was not ruled out completely.

Early localizations of the glucocorticoid receptor (GR)

As for ER and PR, the most widely used technique for GR detection was radioassays, usually with cortisol or dexamethasone, and again such assays consistently localized the unactivated GR (from adrenalectomized animals or cultured cells in the absence of glucocorticoid) in the

cytosol. However, the validity of these results has been recently questioned upon the discovery that both ER and PR appear to be exclusively nuclear, despite their previous cytosolic localizations.

Histological localizations of ^{3}H-glucocorticoid were performed by Stumpf (1971, 1988) by means of applying radiolabeled frozen tissue sections to precoated emulsion slides and processing them for light microscope (LM) radioautography.

The more recent development of immunocytochemical tools directed against the GR, including polyclonal antisera (Govindan and Sekeris, 1978; Eisen, 1980; Wilson et al., 1988) and monoclonal antibodies (Westphal et al., 1982; Grandics et al., 1982; Okret et al., 1984; Gametchu and Harrison, 1984; Eisen et al., 1985), has also allowed for the cellular localization of GR in a variety of tissues as well as provided an indispensable tool for its isolation and purification. These tools have also been used to study the subcellular distribution (at the histological level) of the unoccupied GR (in adrenalectomized animals), with a number of such studies confirming cytoplasmic immunostaining. Only upon treatment of animals with glucocorticoid was the GR localized in the nucleus (Antakly and Eisen, 1984; Antakly et al., 1985; Wikstrom et al., 1987). As of yet, however, the labile nature of the steroid has precluded any practical use of routine histological techniques for localization of the GR.

Covalent affinity labeling

One technique which can potentially overcome the problem of receptor-steroid dissociation is that of covalent affinity labeling, whereby an irreversible ligand-receptor complex is formed. This

technique makes use of a modified ligand (steroid), suitably equipped with an electrophilic functional group, which still retains both high affinity and specificity for its macromolecular (receptor) binding site (reviewed by Katzenellenbogen, 1977; Simons and Thompson, 1982). The formation of the irreversible receptor-steroid complex requires two steps, firstly, the routine noncovalent binding of the steroid to its receptor followed by the formation of a covalent bond between the electrophilic functional group of the ligand (steroid) and a specific amino acid residue found within the binding cavity of the receptor (Simons and Thompson, 1981, 1982).

Although there has not yet been any reported use of affinity labeling for examining the histological distribution of GR or other steroid receptors, the formation of a covalent receptor-steroid complex has prompted Antakly et al. (1984, unpublished data) to use affinity labeling in localizing, by histological methods, SR distribution within any given tissue. This thesis further elaborates this method and localizes the ^{3}H-dexamethasone 21-mesylate, a GR affinity label, in rat testis. Since the amount of SR within a cell affects the magnitude of response to the steroid (Miesfeld et al., 1986), knowledge of the distribution of GR within specific cell types of a given tissue is essential in understanding physiological actions of glucocorticoids.

Dexamethasone 21-mesylate: an affinity label for GR

The most suitable electrophilic affinity label for the GR appears to be dexamethasone 21-mesylate (DM) which has been shown to bind covalently to its steroid binding cavity, specifically labeling cysteine 656 through a thioether bond (Simons and Thompson, 1981;

Simons et al., 1987). It differs from dexamethasone only in that the carbon-21 hydroxyl group of the latter is replaced by a mesylate group (SO_3CH_3), thereby allowing it to react covalently with thiol groups (Simons et al., 1980). Although it has been shown to have some agonist ability, as demonstrated through induced tyrosine aminotransferase activity in HTC cells, DM is primarily an antiglucocorticoid (Simons and Thompson, 1981). Non-specific labeling of thiol groups in cellular proteins, including albumin, occurs only at relatively high concentrations of DM. However, specificity of the DM binding can be determined by competing an excess of cold (unlabeled) steroid (DM or dexamethasone) with the hot (labeled) steroid for the limited number of specific receptors.

Effects of glucocorticoids in the testis

Glucocorticoids, when administered in vivo and in vitro, are known to inhibit normal testicular steroidogenic function. Upon treatment with glucocorticoids, enriched cell populations, stimulated with gonadotropins, exhibited lowered androgen production and leutinizing hormone (LH) receptor content (Saez et al., 1977; Bambino and Hsueh, 1981). These inhibitory effects of glucocorticoids on normal Leydig cell function corroborate well with earlier binding studies of glucocorticoids in testicular cells. Although, specific binding sites for ^{3}H-dexamethasone in the testis were first demonstrated in cytosols of whole testis of juvenile rats (Ballard et al., 1974), an abundance of such binding sites was subsequently detected in the cytosolic and nuclear fractions of enriched interstitial cell populations as compared to the rest of the testis (Evain et al., 1976). However, the precise

localization of GR in the different cell types of the testis by using an in vivo approach remains to be determined.

Objectives of this study

In this report, LM quantitative radioautography was used to study the distribution of GR within testicular cells by using ^{3}H-DM as an affinity label. As of yet, the use of DM has previously been restricted to isolated whole cell (primarily HTC cells) or cell-free systems (Simons and Thompson, 1981, 1982; Eisen et al., 1981; Simons et al., 1983, 1987). Thus this study also signals the validation of DM as a GR affinity label in vivo and its ability to withstand routine histological techniques, as was shown by preliminary studies by Antakly et al. (1984, unpublished data). The limited biological activity of DM was not as much a concern of ours as was its ability to label the GR in intact animal systems.

The subcellular distribution of the DM binding sites in Leydig cells was also analysed through electron microscope (EM) quantitative radioautography with the main purpose of confirming a cytoplasmic or nuclear distribution of the GR in its unoccupied or unactivated state (as in adrenalectomized animals) and if cytoplasmic, to further analyze the distribution amongst the organelles. DM acts primarily as an antiglucocorticoid, possibly by preventing activation of the GR and its translocation to the nucleus. If such should be the case, then the majority of receptor-DM complexes should appear in the cytoplasm rather than the nucleus.

Objectives of the study - Summary

1. To examine the cellular distribution of GR in the testis using ^{3}H-DM as an affinity label and visualized through LM radioautography.

2. To study the subcellular distribution of the DM binding sites within the Leydig cell using EM quantitative radioautography.

Materials and Methods

Injection of ^{3}H-DM and tissue processing

In order to reduce competition between endogenous glucocorticoids and the injected ^{3}H-DM, rats were bilaterally adrenalectomized (Charles River Laboratory) 8-9 days prior to the experiment and maintained on 0.9% saline water and lab chow until use. Under general anesthesia, the left testes of 5 adult male Sprague-Dawley rats (250-300g) were exposed through an abdominal incision, and a single injection of 20 μCi ^{3}H-DM (specific activity 49.9 Ci/mmol) in phosphate buffered saline was administered into the interstitial space of each of the 5 testes. To test for specificity of ^{3}H-DM binding, the left testes of 3 control rats each received an interstitial injection of the same amount of labeled ^{3}H-DM as above in conjunction with either a 25- or a 50-fold excess of cold dexamethasone. The final injection volume for all animals was 100 μl.

Fifteen minutes after injection, the testes were fixed by perfusion through the abdominal aorta for 10 minutes with 2.5% glutaraldehyde buffered with 0.1M sodium cacodylate containing 0.05% $CaCl_2$ at pH 7.4. Prior to the introduction of fixative, lactated Ringer's solution was passed through the aorta and its branches for 2-3 minutes to clear the vessels of blood and unbound steroid. After removal of the testes, tissue close to the injection site was cut into small 1 mm^3 pieces, placed into the same fixative for 1-2 hours and washed overnight in 0.1M sodium cacodylate buffer at 4°C.

On the following day, the tissue was postfixed in potassium ferrocyanide-reduced osmium (Karnovsky, 1971) for 90 minutes

at 4°C, dehydrated in ethanol, propylene oxide and infiltrated and embedded in Epon 812.

Light Microscope (LM)

Radioautographic procedures

Semi-thin sections (1 μm), cut with glass knives, were stained with iron hematoxylin and processed for LM radioautography according to the procedure of Kopriwa and LeBlond (1962). Briefly, the slides were dipped in Kodak NTB2 emulsion and stored in dry air at 4°C for 21 days after which they were developed with Kodak D-170 and mounted under glass coverslips.

Radioautographic quantitation

A quantitative analysis of silver grains overlying the testicular tissue was conducted at the LM level with an oil immersion objective using computer-assisted image analysis (Smith et al., 1987). By using a Leitz LM equipped with a video camera, an image of the tissue was projected onto a television screen within the boundaries of a defined counting window (area -340,4348 um^2 or 100,224 pixels) The image was then digitized using a special card (Matrox Co., Montreal) installed within a microcomputer (IBM) which recorded the relative area (in pixels) occupied by the painted elements representing the silver grains.

To determine counts over cells of the seminiferous epithelium, five seminiferous tubules cut in cross section were chosen at random from each slide. Four fields per tubule were counted, one at each pole and at right angles to one another. A total of 20 tubular fields per slide was thus counted. Four slides per animal was counted thereby

representing a total of 80 tubular fields per animal (with the exception of two animals, one experimental and one control, for whom only two slides per animal were counted). Each field enclosed the various cell layers of the seminiferous epithelium extending from the basal plasma membrane of the cells of the basal compartment up to and including the maturing germ cells bordering the tubular lumen. The cells and connective tissue layers of the limiting membrane were excluded.

For the Leydig cells, cell counts were obtained from clusters of such cells found immediately adjacent to the respective tubular fields described above. A total of 20 fields of Leydig cells per slide were counted in this way. Four slides per animal was counted thereby representing a total of 80 tubular fields per animal (with the exception of two animals, one experimental and one control, for whom two slides per animal were counted). The area of the window was adjustable so as to exclude any non-Leydig cells such as macrophages, fibrocytes, and blood vessels also present in the interstitial space. Moreover, the counts were always standardized to the original area of 100,224 pixels. A statistical analysis of the results obtained from experimental and control animals was performed by using a Student's t-test.

Immunocytochemical localization of GR in the testis

In order to confirm the cellular distribution of GR in the testis, an immunocytochemical procedure developed earlier by Antakly and Eisen (1984) was applied. Briefly, the testes of ten adult Sprague Dawley rats were excised and fixed by immersion in Bouin's fluid for 24 hours

before being dehydrated in ethanol and finally embedded in paraffin. In order to increase the GR level, some rats were treated with dexamethasone (0.4 mg/day for 4 days). This treatment has been shown to result in an up-regulation of the GR level (Antakly et al., unpublished observations). Tissue sections (4 um) were cut, affixed to glass slides and processed for LM immunocytochemistry by using the avidin-biotin system (Vector Labs, Burlingham, CA). As a primary probe, a mouse monoclonal antibody (0.1 mg/ml of Ÿ-globulin) to the native GR was used (Gametchu and Harrison, 1984). Control tests were run in parallel and consisted of replacing the GR antibodies with non-immune hybridoma culture medium or non-immune IgG. Sections were counterstained with 0.1% methylene blue in order to distinguish cell morphology.

Electron Microscope (EM)

Radioautographic procedures

Thin sections of selected areas of the testicular interstitial space containing Leydig cells were cut (gold interference color) with a diamond knife and mounted onto celloidin-coated slides which were then coated with a thin monolayer of Ilford L4 photographic emulsion and placed in dry air at 4°C. After 3-4 months exposure under these conditions, the radioautographs were developed for 7 minutes in Agfa-Gevaert solution physical developer preceded by 1 minute gold latensification, a procedure that yielded small, compact silver grains (Kopriwa, 1975). Sections were then placed onto copper grids and poststained with uranyl acetate (5 mins) and lead citrate (2 mins) and examined with a Philips 400 EM.

Quantitative analysis of radioautographs

For each animal, portions of Leydig cells were selected in the EM, photographed at 15,200x and printed to a final magnification of 39,520x.

Definition of a true silver grain

In the solution physical development technique, a single radioactive source hitting a silver bromide crystal produces a silver grain usually entirely within the limits of the crystal (Nadler, 1979). However, the silver grain may not always be in the form of a single, compact deposit but rather may consist of a variable number of silver deposits clustered together (Nadler, 1979). Consequently, the number of silver deposits will not always be linear to the true number of silver grains, and more importantly, will not always be proportional to the radioactive content of the tissue or organelle. To overcome this problem, the diameter of each cluster of grains was measured on the EM micrographs in order to determine the number of silver bromide crystals responsible for each respective cluster. This was done using a transparency of a 5.5mm diameter circle which corresponded to the mean 140nm diameter of the silver bromide crystal in Ilford L4 emulsion at 39,520x magnification (Kopriwa et al., 1984). The circle was centered over a cluster and if all of the silver deposits were contained within the limits of the circle, they were all considered to be derived from one crystal only and therefore classified as a single silver grain. However, if the grain cluster exceeded the limits of the circle, two or more crystals were known to be responsible for the cluster and therefore the process was repeated until the number of crystals (i.e.

true grains) was determined.

Assignment of grains to organelle(s): exclusive and shared

For each experimental animal, an average of 557 silver grains from 34 micrographs was counted. For the controls, a mean of 90 silver grains from 27 micrographs was counted. To ascribe silver grains to organelles, grains were encircled with a resolution boundary circle of radius 76nm (magnified to 3.0mm) which represented the half-distance for tritium beta particles developed with a solution physical developer (Kopriwa et al., 1984). Therefore there existed a 50% probability that the radioactive source giving rise to the encircled silver grain was contained within the boundaries of the circle.

Grains were ascribed to organelle(s) as exclusive - if only one organelle was contained within the circle - or as shared if two or more were encompassed. Only those structures with 3% or more of the total exclusive grains were retained for further analysis. Grains belonging to those structures which did not meet this requirement were either omitted from the assessment (as for grains exclusively belonging to such structures) or were reassigned to one or more class(es) of structure(s) with which the grain was originally shared with and which met the requirement stated above.

Special consideration had to be given to silver grains which overlaid the tubules of the smooth endoplasmic reticulum (sER) or the cytosol immediately adjacent to them. In the Leydig cell, the average diameter of an individual sER tubule has been estimated to be 50-70nm (Russell and Burguet, 1977) , which is about the limit of resolving power of the radioautographic technique (Salpeter et al., 1969).

Surrounding each and every sER tubule is cytosol. Therefore, even in those cases where grains directly overlaid the lumen of a sER tubule, there still existed a possibility that such grains were the product of a cytosolic radioactive source. Consequently, grains which laid directly over sER tubules could not be ascribed to sER exclusively. Accordingly, in this study, grains were classified as "exclusive" to a category called cytosol (sER) only if 50% or more of the area within the resolution boundary circle was occupied by sER tubules (and the rest, cytosol). On the other hand, if a grain was situated within a circle consisting 50% or more of cytosol, it was classified as shared between the cytosol and sER.

Estimation of volume of a given organelle: Point hit study

For the point hit study, which is used to determine the relative volume of a given organelle in the Leydig cell, an overlay printed with equidistant points was placed over each EM micrograph and the structure underlying each point was recorded and expressed as percentages of total points used.

Circle hit study

In the circle hit study, a transparency printed with equidistant 50% probability resolution boundary circles (76nm radius, magnified to 3.0mm radius) was placed over each EM micrograph, and each circle was classified as either exclusive to or shared between the structure(s) contained within. The exclusive and shared counts for each organelle was combined into one total and expressed as a percent of the total circles used. Since circles could be counted more than once (if shared amongst two or more organelles), the total percentage exceeded 100.

Estimation of diffuseness of a given organelle: Circle/Point hit methods

For any given structure, the ratio of circle hits to point hits will always be greater than one, and will tend to unity the more the structure is compact. Therefore, structures which are spread throughout the cell, or, in other words, are diffuse, will have ratios greater than one. Accordingly, this ratio was used as a measure of diffuseness and was an important consideration in the assignment of shared grains to only one organelle (to be discussed).

For both circle and point hit studies, an average of 32 and 11 micrographs was used for each of the experimental and control animals, respectively.

Assignment of grains to only one organelle

In order to assign each grain shared between 2 or 3 organelles to the one organelle which was the most probable source of radioactivity for that grain, the shared grain counts were "corrected" according to the method of Nadler (1971). The resulting value ("corrected grain count") for a given organelle, obtained both by hand and with the aid of a computer program, took into account the exclusive grain count and the appropriate proportion of shared grains for that organelle as well as the correction for diffuseness for that organelle (discussed above). These values were expressed as absolute numbers and together yielded a total corrected grain count. The equations used to generate the "corrected grain counts" are discussed in detail by Nadler (1971).

The relative content and concentration of label for each organelle was also computed for each organelle, the former simply being the

percent of corrected grains for a given organelle and the latter, the ratio of the relative content of label over the percentage of relative organelle volume (point hit study) in the Leydig cell.

Statistics

To determine the significance in labeling of the four organelles. a statistical analysis of the values obtained for the relative content and concentration of label in Leydig cell was made using a Student's t-test. Only two organelles were examined at a time. All combinations of the four heavily labeled organelles were tested for both the relative content and concentration of ^{3}H-DM label. Probabilities less than 0.05 were considered significant.

Results

Light Microscope (LM)

Morphology of the rat testis

Under the LM (low power), the rat testis appears as an alternating series of cut seminiferous tubules - circular or elongated depending on the plane of section - and interstitial cell clusters. The clusters are suspended in a continuous lymphatic space, known as the interstitial space, which, in fact, surrounds each and every seminiferous tubule.

At higher magnification (40x), the wall of the tubules are seen to consist almost entirely of the seminiferous epithelium. Several cell layers thick, this epithelium is composed of germ cells, at all steps of development, and the somatic Sertoli cell, whose functions include nourishment and support of the germ cells (Figs.1,2). To the interior of the epithelium is a central lumen into which the maturing spermatozoa are released. Binding the entire tubule is an outer limiting membrane which appears under the LM as a single layer of squamous cells.

The predominant cell type of the interstitial clusters is the Leydig cell which can be identified by its long, polygonal shape, darkly stained cytoplasm and a nucleus which typically has 1-2 prominent nucleoli and condensed chromatin at its periphery (Figs.1,2). Another interstitial cell, the macrophage, is a large endocytic cell which is characterized by a pale-staining, foamy cytoplasm, and a nucleus which is often irregularly shaped (Fig.4). Other interstitial

cells include endothelial cells, lining the lumina of the blood vessels and occasional fibrocytes and blood-borne cells.

Radioautographic localization of ^{3}H-DM at the LM level

Fifteen minutes after injection of ^{3}H-DM into the interstitial space of the rat testis, an intensive radioautographic reaction was observed over the cells of the interstitial space - the Leydig cells in particular. Such cells displayed numerous silver grains over their cytoplasm and to a lesser degree, over their nucleus (Figs.1,2). In comparison, the radioautographic reaction overlying the cells of the seminiferous epithelium, regardless of the stage of the cycle of the seminiferous epithelium as identified by Leblond and Clermont (1952), was noticeably weaker than that observed over Leydig cells; the labeling was diffuse in nature with no one particular cell type, i.e. Sertoli or germ cell, showing more label than another (Figs.1,2).

In the presence of an excess of cold dexamethasone, the radioautographic reaction over Leydig cells and cells of the seminiferous epithelium was noticeably weaker than that observed for the experimental animals (Figs.3,4). However, the interstitial macrophages were still heavily labeled in control animals (Fig.4).

Quantitative data from LM radioautographs

For each animal (experimental and control), 20 fields were counted per slide and expressed as a single mean (Table 1). The means of each slide were then combined into an average value for each animal (Table 2). The average number of silver grains over Leydig cells obtained from five experimental animals and expressed as computer pixels ± S.D. was found to be 5181 ± 1794 (Table 2). An average value of only 1791 ±

733 was obtained in the case of the three control animals (Table 2). A Student's t-test revealed that the difference in number of silver grains between the experimental and control animals was significant ($p<0.01$), thus indicating a specificity of ^{3}H-DM binding to Leydig cells. The average number of silver grains over the cells of the seminiferous epithelium (Sertoli and germ cells) was 731 ± 72, again expressed as computer pixels, while that for the controls was 399 ± 72 (Table 2). A Student's t-test revealed a significant difference ($p<0.01$) between experimental and control values, thus indicating a specificity of ^{3}H-DM binding to the cells of the seminiferous epithelium. However, since no one seminiferous epithelial cell type was more heavily labeled than another, it can only be concluded tentatively at this time that binding is specific to these cells. An analysis of the quantitative data also revealed that only 14% of the total reaction counted was localized over the cells of the seminiferous epithelium. Background counts, measured using computer-assisted image analysis, were negligible.

Immunocytochemical localization of GR in the testis

To confirm the distribution of GR within specific cells of the testis, specific antibodies to the GR were reacted with histological sections of rat testis and subsequently visualized by peroxidase staining. Leydig cells were specifically stained, whereas cells of the seminiferous epithelium were also stained, but at a noticeably weaker level than Leydig cells (Figs. 5a,b). The immunocytochemical staining in Leydig cells was observed in the cytoplasm as well as in the nucleus, the cytoplasmic staining being predominant. This subcellular

distribution of GR in cytoplasm and nucleus is fully expected as demonstrated in previous immunocytochemical studies on several target cells (Antakly and Eisen, 1984; Picard and Yamamoto, 1987). Sections stained with non-immune IgG (Figs. 6a,b) or non-immune hybridoma culture medium showed absence of any specific staining.

Electron Microscope (EM)

Subcellular morphology of the adult rat Leydig cell

In adult rats, the Leydig cell cytoplasm is characterized by an extensive network of smooth endoplasmic reticulum (sER) and numerous mitochondria; the latter are often closely enveloped with sER. Other cytoplasmic structures include peroxisomes, a juxtanuclear Golgi apparatus, several cisternae of rough endoplasmic reticulum (rER), and a full complement of endocytic organelles, i.e. electron-lucent vesicles including endosomes, multivesicular bodies (MVB), and secondary lysosomes (Hermo and Lalli, 1988). Occasional lipid droplets and autophagosomes may be seen (Tang et al., 1988). The nucleus typically contains 1-2 nucleoli and a peripheral band of heterochromatin (reviewed in Christensen, 1975; Russell and Burguet, 1977).

Radioautographic localization of ^{3}H-DM at the EM level

Fifteen minutes after the injection of ^{3}H-dexamethasone 21-mesylate into the interstitial space of the rat testis, numerous silver grains were localized over the cytoplasm and to a lesser degree, over the nucleus. Over the cytoplasm, grains were distributed in a diffuse manner over the majority of organelles with the exception of a consistently strong radioautographic reaction close to or over the sER

and mitochondria (Figs.7-9).

Mitochondrial labeling (Figs.7,8) was found within the organelle (vertical arrows), towards the periphery (horizontal arrows), and over the outer mitochondrial membrane (arrowheads). Grains over the sER networks (Figs.8,9) were observed either directly over the lumen of the sER tubules (small arrows) or over the adjacent cytosol (circled) (Fig.9). Smooth ER enveloping mitochondria were also seen to be labeled (Figs.7-9, curved arrows). Interestingly, very few grains were found over sER associated with peroxisomes (Fig.8) and over cisternae of rER.

Relatively few grains were seen over the cytosol (Fig.7, circled), the Golgi apparatus (saccules, tubules, vesicles) (Fig.10), peroxisomes, and plasma membrane (including microvilli) (Fig.7, small arrows). All other cytoplasmic structures including endocytic vesicles (endosomes), MVB, and secondary lysosomes showed a very weak labeling- the latter two having no exclusive grains. Over the nucleus, grains were found both at the periphery, sometimes over the nuclear envelope, and centrally over the nucleoplasm (Fig.11).

To determine the specificity of binding of ^{3}H-DM in the Leydig cell, a 25- or a 50- fold excess of unlabeled dexamethasone was injected concurrently with the same dose of ^{3}H-DM as used in the experimental animals. Under these conditions, a similar distribution of silver grains as seen in the experimentals was observed. However, substantially fewer silver grains were seen in the control micrographs.

In the interstitial macrophages (Fig.12), grains were predominantly seen directly over vesicular endocytic elements

(endosomes) (horizontal arrows) or in the cytosol immediately adjacent to the vesicles (curved arrows). Few lysosomes were labeled at 15 minutes (arrowheads). Grains were not associated with mitochondria.

Assignment of grains to organelle(s): exclusive and shared

Using the 50% probability resolution boundary circle (76nm radius, magnified to 3.0mm radius) to ascribe silver grains to organelles, only four organelles were found to contain 3% or more of the total exclusive grain counts; cytosol (sER), mitochondria, cytosol, and nucleus. Tables 3 and 4 list the grain counts obtained for individual experimental and control animals, respectively. The pooled experimental and control results are displayed in Tables 5 and 6, respectively. Using the pooled experimental results (Table 5), together these four organelles accounted for 93% of the exclusive grains. The remaining 7% was associated with plasma membrane (including microvilli), Golgi apparatus, peroxisomes, and electron-lucent vesicles. Other structures, including lysosomes, MVB, and lipid bodies, had no exclusive grains of their own. Only 13% of the exclusive grains were nuclear. To simplify the study, only those four organelles containing 3% or more of the exclusive grain total, as listed above, were retained for further analysis. All other structures were disregarded and their grains were either omitted from the study, if exclusive to the structure, or assigned to the organelle(s) (cytosol (sER), mitochondria, cytosol, or nucleus) with which it was shared.

Circle hit study

The relative diffusivity of each organelle in the Leydig cell was determined by collecting random samples of the structures contained

within resolution boundary circles of the same diameter (6.0mm) used for ascribing grains to organelles. Tables 7 and 8 list the preliminary (crude) results of this study for each individual experimental and control animals, before any revisions were done. Tables 9 and 10 summarize the pooling of these preliminary results for both the experimental and control animals, respectively. The revised random sampling hits, expressed as percent hits, are listed in Tables 11 and 12. Focusing on the pooled experimental percent hits of the circle hit study (Table 12), sER showed the greatest diffusivity of all organelles in the Leydig cell with a value of 57.4. Cytosol was also relatively high at 33.8. Mitochondria and nucleus - both compact organelles - had similar values of 23.5 and 19.7, respectively.

Estimation of volume of a given organelle: Point hit study

The relative volumes of organelles in the adult rat Leydig cell (adrenalectomized) were assessed by collecting point samples from EM micrographs and each structure was expressed as percent of total points used. Table 11 lists the results for each individual experimental and control animal. Using the pooled hits of the experimental animals (Table 12), the extensive network of sER occupied the greatest proportion of the Leydig cell - 33% of the total cell volume. Cytosol and nucleus were similar at 25% and 21%, respectively. Mitochondria accounted for 14% of the total cell volume and the remaining 7% was occupied by the all other cellular structures -i.e. Golgi apparatus, rER, peroxisomes, microvilli, lysosomes, MVB, vesicles, autophagosomes, and lipid droplets.

Assignment of grains to only one organelle

Tables 13 and 14 summarize the individual results of the quantitative study for the experimental and control animals, respectively. Table 15 summarizes the pooled results of both the experimental and control animals. The corrected grain counts are shown in Row a of each of these tables and expressed in absolute numbers. The relative content of label (%) for each organelle is shown in Row b. In the experimental animals (pooled results, Table 15), cytosol (sER) was heaviest labeled with 1463 grains or 53% of the total corrected counts. Mitochondria accounted for 31% of the corrected grains with 855 grains. Cytosol and nucleus were similar in their labeling intensity at 253 and 210 grains or 9% and 7% of the total counts, respectively. When the relative volume of each organelle (point hit study, Table 12) was taken into consideration, the relative concentration of label could be determined (Row c). Since this parameter is a ratio of two percentages, it has no units. Mitochondria exhibited the highest relative concentration of label with a value of 2.15 - which was followed closely by cytosol (sER) at 1.63. Again cytosol and nucleus showed similarly low values - 0.36 and 0.33, respectively.

Specificity of ^{3}H-DM binding in the Leydig cell

The specificity of binding by ^{3}H-DM to the Leydig cell was confirmed at the EM level in the presence of a 25- or a 50- fold excess of cold dexamethasone. The data for each organelle was obtained in an identical manner as from experimental animals. In total, 293 grains from 81 micrographs were counted for the controls which averages to 3.6 grains per micrograph. In the experimentals, 2883 grains from 169

micrographs were counted giving rise to a mean of 17.1 grains per micrograph. Therefore, the labeling in the controls was only about 1/5th that seen in the experimentals.

Despite this reduction in grains, a similar pattern of labeling to that seen in the experimentals was observed in the controls. Cytosol (sER) showed the heaviest labeling with 154 grains or 57% of the total reaction followed by mitochondria with 61 grains or 23% of the total (Table 15, pooled results). Again, cytosol and nucleus showed fewer grains with only 29 and 26 grains or 11% and 9% of the total counts, respectively. For the relative concentration of label, mitochondria had the largest value at 1.83 - similar to the 1.67 obtained for sER. Again, both cytosol and nucleus were low at 0.43 and 0.55, respectively (Table 15).

Statistics

Of the four organelles studied in this analysis, cytosol (sER) was the heaviest labeled followed closely by mitochondria (Table 15). Both cytosol and nucleus showed weak labeling in comparison. When the difference of relative content of label was tested between cytosol (sER) and mitochondria using a Student's t-test, the former was shown to be significantly greater ($p<0.001$) than mitochondria (Row b, Table 16). However, when the relative volumes of these two organelles was taken into consideration, no significant difference was found in the relative concentration of label between these two organelles ($p>0.05$) (Row c, Table 16). However, both of these organelles showed significantly greater relative content and concentration of label than either cytosol or nucleus ($p<0.001$).

Discussion

Limitations

Before discussing the results, I would first like to present two limitations with our model which I feel should be an important consideration when interpreting the results.

Use of DM in vivo

Firstly, while the use of DM as a GR affinity label has been validated extensively in vitro, mainly through the combined use of immunoprecipitation and polyacrylamide gel electrophoresis (PAGE) (Eisen et al., 1981; Harmon, 1984), it must be emphasized that the administration of ^{3}H-DM in intact animals (in vivo) has thus far been very limited and therefore it was necessary to interpret the results with caution. Since identification of bona fide GR amongst the ^{3}H-DM labeled compound(s) would require the use of PAGE or immunoprecipitation, and such techniques obviously could not be used in a histological study such as this, we can only assume that ^{3}H-DM has labeled GR specific binding sites in this study. However, to substantiate this assumption, we have performed two control experiments. The first one was done to determine the specificity of DM binding in the testis and consisted of competing the labeled DM with an excess of cold dexamethasone. If the DM was binding specifically to the GR in such experiments, labeling would be reduced since the cold steroid would have a greater chance of saturating the GR than would the lesser amount of labeled steroid. These competition experiments were crucial since it has been shown that within any given cell, a variety of proteins exist, in addition to the GR, to which the DM may

covalently bind to, including small molecular weight compounds (particularly those with thiol anions, to which DM is attracted) and the low affinity albumin which is abundant in the interstitial space. However, only the GR is saturable. In the present work, competition experiments with unlabeled dexamethasone resulted in approximately a 65% reduction in overall ^{3}H-DM labeling, thus demonstrating specificity in DM binding within the testis. The remaining 35% may have been be due to non-specific binding of ^{3}H-DM to other cellular components and proteins, as will be discussed below.

Our second control consisted of LM immunocytochemical experiments, using the avidin-biotin peroxidase technique, both with a polyclonal antiserum and a monoclonal antibody to the GR. These experiments confirmed the presence of GR in the testis, the Leydig cells in particular.

In our discussion of the LM results, the assumption was made that ^{3}H-DM (at least 65% of it) has labeled the GR and the significance of our results was based on this assumption. In the EM, it became evident that some of the ^{3}H-DM labeling is due to mitochondrial labeling which may or may not represent bona fide GR.

Use of diffusible substances in radioautography

A second limitation of our model concerns the potential for artefactual subcellular ^{3}H-DM binding sites. As we are operating under the assumption that ^{3}H-DM is forming a covalent complex with GR, then we must consider the possibility that this complex may be a free entity within the cell, i.e. not attached to or incorporated into any membranous structures. Upon fixation of the cell, this free entity may

become fixed to an adjacent structure with which it is not normally associated. This phenomenon is referred to by Strumpf (1971) as translocation artifacts and is an important consideration when using diffusible substances in radioautography. However, if such is the case, then one would expect a equalized diffusion of ^{3}H-DM to all membraneous structures throughout the cytoplasm, thereby labeling all organelles with the same intensity.

Discussion of the results

Light microscope (LM)

^{3}H-glucocorticoid binding sites in the testis

Specific binding sites for the potent glucocorticoid, dexamethasone, within the testis were first demonstrated by Ballard et al. (1974), using juvenile, adrenalectomized rats. These findings contrasted with the previous work of Beato and Feigelson (1972) who had not detected any significant "receptor" activity within this tissue. However, it was only with the study by Evain et al. (1976) that an abundance of specific ^{3}H-dexamethasone binding sites (receptors) was detected in enriched interstitial (Leydig) cells of animals of different ages as compared to the testis as a whole.

Presence of ^{3}H-DM binding sites in rat testicular cells

Leydig cells

In the present work, computer-assisted quantitation of the LM radioautographs demonstrated specific glucocorticoid (^{3}H-DM) binding sites in adult rat testicular Leydig cells, as was revealed through a statistically significant reduction ($p<0.01$) in silver grains overlying these cells in control animals as compared to the experimentals. The

heavy labeling of macrophages still observed in the presence of an excess of cold dexamethasone indicated that these cells were taking up ^{3}H-DM non-specifically, possibly by fluid-phase endocytosis, a well-known function of these cells and secondly, that the area did in fact contain the labeled steroid.

Seminiferous tubules

Our data also showed specific ^{3}H-DM binding sites in the cells of the seminiferous epithelium. Compared to Leydig cells, however, the radioautographic reaction over these cells was less intense (14%). Similarly, the LM immunocytochemical reaction over the seminiferous epithelium was weaker than that over Leydig cells. Because no one particular cell type was heavily labeled, little can be said regarding the target cells of the ^{3}H-DM in the tubules. The presence of glucocorticoid binding sites in tubular cells is, however, compatible both with the study by Evain (1976) which revealed specific dexamethasone binding sites in isolated seminiferous tubules, though at 1/5th the level seen in Leydig cells, and recent studies demonstrating that GR, as assessed in rat seminiferous tubules in vitro, were found in cultured Sertoli cells, from both immature and mature testes, as well as within cultured myoid cells, and isolated germ cells, i.e. pachytene spermatocytes and round spermatids (Levy et al., 1987, 1989). Preliminary results also indicated the presence of an 8 kb mRNA species, the known length of GR transcripts, in Sertoli and peritubular cells as well as Leydig tumor cells (Levy et al., 1987, 1989).

Subcellular localization of ^{3}H-DM - LM level

In the LM, the majority of silver grains, representing ^{3}H-DM binding sites, was seen overlying the cytoplasm of the various cell types, the Leydig cell in particular. This is in agreement with the distribution of GR in other cell types (Antakly and Eisen, 1984; Antakly et al., 1985; Picard and Yamamoto, 1987). Since the GR is expected to translocate to the nucleus upon binding the steroid, its predominant localization in the cytoplasm following binding to ^{3}H-DM may be due to the decreased ability of the receptor-antagonist complex to translocate to the nucleus as noted above. Alternatively, the receptor-DM complex may require more than 15 minutes used in the present study to translocate to the nucleus, as opposed to the few minutes normally required for this event to occur (Schmidt and Litwack, 1982). However, this does not seem to be the case since no significant nuclear labeling was observed following a 60 minute injection of ^{3}H-DM in the testis (Stalker, unpublished results).

Functional implications of glucocorticoid in the testis

Previous studies have shown that the presence of GR is a prerequisite for glucocorticoid action (Munck and Leung, 1977; Baxter and Rousseau, 1979). Therefore, the present finding of an abundance of glucocorticoid binding sites in Leydig cells suggests that these cells may be targets for glucocorticoids in the testis. In fact, the most well-documented biological effect of glucocorticoid in the testis is the suppression of steroidogenesis (Saez et al., 1977; Bambino and Hsueh, 1981). This was first observed in patients with hyperadrenalism, clinically known as Cushing's syndrome (Gabrilove et al., 1974). In

hypophysectomized or intact rats, the administration of glucocorticoids to Leydig cells in vivo or in vitro decreased the stimulatory effects of gonadotropins on androgen production (Saez et al., 1977; Bambino and Hsueh, 1981). One mechanism by which glucocorticoids affect testosterone synthesis appears to be through inhibition of the activity of a steroidogenic enzyme, 17-hydroxylase (Welsh et al., 1982). Recently, it has been reported that glucocorticoids inhibit the cholesterol side-chain cleavage enzyme (P450scc) and its mRNA accumulation in normal Leydig cells, suggesting that this gene may be a glucocorticoid target (Hales and Payne, 1988a). Surprisingly, the same authors found an opposite glucocorticoid effect in a tumor Leydig cell line (Hales and Payne, 1988b). The observed reduction in hCG binding sites in glucocorticoid-treated cells (Saez et al., 1977) was not thought to be a causal factor in lowered androgen levels since full steroidogenic responses can be evoked even at reduced levels of gonadotropin binding (Mendelson et al., 1975).

Electron microscope (EM)

^{3}H-DM binding sites in the Leydig cell

In the Leydig cell, specific ^{3}H-DM binding sites were mainly observed over three organelles - cytosol (sER), mitochondria, and cytosol (exclusive of other membraneous organelles, filaments, and microtubules) thus confirming the predominance of cytoplasmic grains seen at the LM level. In comparison, relatively few grains were observed over the nucleus.

Cytoplasm

In the cytoplasm, in addition to the cytosol (sER), mitochondria, and cytosol, grains were also seen in association with other organelles including the Golgi apparatus and peroxisomes, though usually shared with one of the three aforementioned organelles. In fact, the exclusive grain counts for cytoplasmic organelles exclusive of the three listed above were too low to be considered as legitimate targets of ^{3}H-DM. Even cytosol (in the experimental animals) had only 4% of the total exclusive grains which is similar to the exclusive counts of both Golgi apparatus and plasma membrane (including microvilli), 2.7% and 3.0% of the total, respectively. But cytosol was retained in the analysis because it was a component in the majority of shared grains. Interestingly, few grains were associated with electron-lucent vesicles (including endosomes), MVBs, or secondary lysosomes, all of which are part of the endocytic pathway, one by which many peptide hormones enter a cell. Microvillar processes, the site of numerous hCG binding sites (Hermo and Lalli, 1988) only showed 2.9% of the total exclusive grains. Thus it appears that ^{3}H-DM entered the Leydig cell by diffusion through the plasma membrane.

Nucleus

Over the nucleus, grains were located both deep within the nucleus, over the euchromatin, as well as towards the periphery, and over the heterochromatin. Occasional grains were also seen over the nuclear envelope. Since 15 minutes is ample time for the steroid and receptor to translocate to the nucleus, it was not surprising to see nuclear labeling. However, as compared to the cytoplasm, the label over

the nucleus was very weak. Quantitation of grains in experimental animals revealed that of a total of 2781 corrected grains, only 210 or 7.6% of the total were nuclear. DM has been shown to act primarily as a glucocorticoid antagonist which undergoes only about a quarter of normal translocation levels (Simons et al., 1983), a property which may have contributed to the low numbers of nuclear grains seen.

Specificity of ^{3}H-DM binding

At the EM level, Leydig cells from animals injected simultaneously with ^{3}H-DM and an excess of cold steroid showed an 80% reduction of label which was an even greater displacement of label than seen at the LM where an excess of cold steroid resulted in a 65% reduction in label over both the seminiferous tubules and Leydig cells. Despite this reduction of grains over Leydig cells, the intracellular distribution of the grains was similar to that seen in the experimentals, most notably, cytosol (sER) and mitochondria showed heavy labeling with 57% and 23% of the total corrected grains, respectively. Thus it seems unlikely that the ^{3}H-DM, as a free entity within the cell (i.e. not attached or incorporated into any membraneous structure), underwent any random translocations during fixation thereby becoming fixed to an adjacent structure with which it is not normally associated.

Cytosol (sER): limited resolution of radioautographic technique

A substantial number of grains were observed over the extensive sER tubular networks which permeate the cell, both over the tubules and the adjacent cytosol. However, the task of classifying a grain within a sER network as exclusive to sER or shared between cytosol and sER was a

major problem since the limits of resolution for the radioautographic technique, about 100nm, were too large to be able to separate these two compartments. Grains found over dense sER networks in which little cytosol was present could not be classified as exclusive to sER since the surrounding cytosol may still have been the site of the radioactive source. Accordingly, a category called cytosol (sER) was developed for this study. Grains were classified as exclusive to this category only if 50% or more of the area of the resolution boundary circle was occupied by sER tubules and the rest, by cytosol. In cases where 50% or more of the circle was occupied by cytosol (and the rest, of sER tubules), grains were classified as shared between sER and cytosol. Thus the term cytosol (sER) considers the strong possibility that a grain over a sER network has arisen from a sER tubule without dismissing the presence of the adjacent cytosol. It is important to note that this classification scheme was used regardless of the structure (sER tubule or cytosol) directly underlying the grain itself.

Functional significance of the subcellular distribution of ^{3}H-DM

While the labeling of the cytosol and nucleus was not surprising since these are the two traditional compartments in which GR are thought to reside (the nucleus containing activated GR only), the labeling of the cytosol (sER) and mitochondria was unexpected. In the case of the cytosol (sER), one cannot dismiss the possibility of a cytosolic source giving rise to a sER grain, due to the small diameter of the tubules. If therefore these grains were assigned to the cytosol, it would mean that the total grain count for this organelle would be 62%, i.e. 53% + 9%. Such a rationale would thus increase the grain

counts over the cytosol. On the other hand, with the average diameter of a mitochondrion being 1000nm or 1 um, well above the limits of resolution for the radioautographic procedure, many grains are unquestionably arising from this organelle. It is possible that the ^{3}H-DM was targeted to the chromatin of the mitochondria, perhaps to regulate transcriptional activity of some mitochondrial proteins. Dexamethasone administration has been shown to stimulate mitochondrial protein synthesis thus suggesting some influence by the glucocorticoid on transcriptional activity in these organelles.

Steroidogenic metabolism

Alternatively, there exists a possibility that some of the injected ^{3}H-DM was targeted to the steroidogenic organelles of the Leydig cell, namely the mitochondria and the sER, for metabolism. This may have happened if the ^{3}H-DM saturated the GR in the cell thereby resulting in the excess ^{3}H-DM being diverted to the steroidogenic organelles. Recently, Picado-Leonard and Miller (1988) compiled a mini-database of sequence homologies amongst a number of steroid binding proteins, including several steroid receptors (SR), using residues 346-366 of the human P450c17 (human) steroidogenic enzyme as the standard. This sequence is thought to represent either the whole or a portion of the steroid binding site of this enzyme. As would be expected, sequence homology was very high between human steroidogenic P450 enzymes (P450c17, P450c21, P450scc, P450c11) (64/68 or 94% similarity). Although sequence similarity among human SR (progesterone, estrogen, glucocorticoid, androgen, and mineralocorticoid) was not as high as for the enzymes, it was still very conservative (61/85 or 72% similarity).

Between species, the same pattern was repeated (enzymes: 92/102 (90%); receptors: 74/102 (73%)) even with the inclusion of two avian SR. These results thus suggested little evolution of the steroid binding sequences in a variety of steroid binding proteins amongst different species. Thus it is highly probable that the ^{3}H-DM was recognized by the steroidogenic enzymes in the sER and mitochondria as metabolic agents.

^{3}H-DM subcellular distribution in other cell types

In support of this hypothesis, the distribution of ^{3}H-DM in three other cells of the male reproductive tract, namely the Sertoli cells of the seminiferous epithelium of the testis, the interstitial macrophage of the testis, and the epithelial principal cells of the epididymal initial segment, all of which are non-steroidogenic, do not show any significant labeling of mitochondria or endoplasmic reticulum with ^{3}H-DM. In the cases of the Sertoli and principal cells, fifteen minutes after injection of ^{3}H-DM into the interstitial space of the testis (for the Sertoli cell) and the underlying connective tissue space of the initial segment of the epididymis (for the principal cell), grains were located primarily over the cytosol in both of these cells. Very few grains were seen over or close to mitochondria. Interestingly, the nucleus again showed fewer grains than the cytosol. In the interstitial macrophage, ^{3}H-DM was found predominantly over endocytic organelles, in particular, small endocytic vesicles and endosomes (15 minutes) and lysosomes (1 hour). Again, few, if any, grains were seen over or adjacent to mitochondria or endoplasmic reticulum. In the LM, macrophages were heavily labeled with ^{3}H-DM even in the presence of a

25- or 50- fold excess of cold dexamethasone. Thus it appears that the ^{3}H-DM uptake in the macrophage was non-specific.

In conclusion, in the Leydig cells, ^{3}H-DM appeared to be targeted to at least one steroidogenic organelle, the mitochondria and perhaps to the sER as well. The special features of the DM which allow it to bind covalently to the steroid binding site of the GR might have allowed it to do such in the metabolic enzymes as well, thus allowing it to withstand the rigours of histological fixation. It is interesting to note that the unlabeled dexamethasone was able to compete with the ^{3}H-DM for the mitochondrial binding site. This may indicate some specificity in steroidogenic enzymes for glucocorticoid binding.

Further studies to be completed

To substantiate our results both at the LM and EM levels, and to provide further insight into the significance of our results, further studies should be completed. These include:

1. Use of a non-steroidogenic tissue. In this study, testis was chosen because of the apparent need to examine the cellular distribution of GR in this tissue, based on the functional effects of glucocorticoids previously examined in this tissue. However, since the Leydig cell which was the primary target of the ^{3}H-DM in the testis is also a steroidogenic cell, one has to consider the possibility that the DM was metabolized in the extensive steroidogenic organelles of this cell. Therefore a repeat of this study in a glucocorticoid responsive, non-steroidogenic, tissue would be an important control experiment. An ideal tissue for such a study would be the anterior pituitary, the corticotroph cells in particular, since the adjoining tissue, the

neurointermediate lobe, is devoid of GR (Antakly et al., 1985). We did in fact perform such experiments (although with much difficulty due to the dilution of the injected ^{3}H-DM in the bloodstream, local injections being difficult for this tissue), and attained success only with in vitro labeling, in which we found less labeling in the neurointermediate lobe as compared to the anterior pituitary (as counted with computer assisted image analysis). However, an EM study of these experiments was not pursued due to time constraints.

2. EM immunocytochemistry. Although the morphology is usually very poor in immunocytochemical experiments, due to the low level of fixation necessary to retain antigenicity, such experiments would allow for localization of the unoccupied GR. As well, the potential for translocation artifacts during the fixation process would be considerably decreased. The drawbacks to this technique are the poor morphology (when using lowicryl as the embedding medium) and the need for an extremely sensitive antibody (antiserum) to detect the relatively low levels of GR present in a given cell. We started to perform some immunocytochemical experiments at the EM level on Leydig cells both with polyclonal and monoclonal antibodies but did not meet with much success mainly due to time constraints which did not permit us to achieve ideal conditions.

Literature Cited

Antakly, T. and H.J. Eisen 1984 Immunocytochemical localization of glucocorticoid receptor in target cells. Endocrinology, 115: 1984-1989.

Antakly, T., A. Sasaki, D.T. Krieger, A.S. Liotta, and M. Palkovitz 1985 Induced expression of the glucocorticoid receptor in the rat intermediate pituitary lobe. Science 229: 277-279.

Ballard, P.L., J.D. Baxter, S.J. Higgins, G.G. Rousseau, and G.M. Tomkins 1974 General presence of glucocorticoid receptors in mammalian tissues. Endocrinology, 94: 998-1002.

Bambino, T.H., and A..W. Hsueh 1981 Direct inhibitory effects of glucocorticoids upon testicular leutinizing hormone receptor and steroidogenesis in vivo and in vitro. Endocr., 108: 2142-2148.

Baxter, J.D., and G.G. Rousseau 1979 Glucocorticoid hormone action. In: Monographs on Endocrinology. Baxter, J.D., and G.G. Rousseau, eds. Springer-Verlag, New York, Vol.12.

Beato, J.D. and P. Feigelson 1972 Glucocorticoid binding proteins of rat liver cytosol. 1: Separation and identification of the binding proteins. J. Biol. Chem. 247: 7890-7896.

Beato, M. 1989 Gene regulation by steroid hormones. Cell 56: 335-344.

Berg, J.M. 1986 Potential metal-binding domains in nucleic acid binding proteins. Science 232: 485-487.

Bresnick, E.H., E.R. Sanchez, and W.B. Pratt 1988 Relationship between glucocorticoid receptor steroid-binding capacity and association of the Mr 90,000 heat shock protein with the unliganded receptor. J. Steroid Biochem. 30: 267-269.

Bresnick, E.H., F.C. Dalman, E.R. Sanchez, and W.B. Pratt 1989 Evidence that the 90-KDa heat shock protein is necessary for the steroid binding conformation of the L-cell glucocorticoid receptor. J. Biol. Chem. 264: 4992-4997.

Carlstedt-Duke, J., and J.-A. Gustafsson 1988 Functional probing of glucocorticoid receptor structure. J. Steroid Biochem. 31: 593-597.

Callard, G.V., and P. Mak 1985 Exclusive nuclear location of estrogen receptors in Squalus testis. Proc. Natl. Acad. Sci. USA, 82: 1336.

Chretien, M., P. Crine, and N.G. Seidah 1978 Biosynthese de l'endorphine-beta. Bull. Schweiz. Akad. Med. Wiss., 34: 155-169 (Engl. Abstr.).

Christensen, A.K. 1975 Leydig cells. In: Handbook of physiology. Section 7. Vol.5. Astwood, E.B., R.O. Greep, eds. Washington, D.C. American Physiological Society. pp. 57-94.
Christy, N.D. 1971 The Human Adrenal Cortex. Harper and Row. New York.

Eipper, B.A., and R.E. Mains 1980 Structure and biosynthesis of pro-adrenocorticotropin/endorphin and related peptides. Endocr. Rev., 1: 1-27.

Eisen, H.J. 1980 An antiserum to the rat liver glucocorticoid receptor. Proc. Natl. Acad. Sci. USA, 77: 3893-3897.

Eisen, H.J., R.E. Schleenbaker, and S.S. Simons Jr. 1981 Affinity labeling of the rat liver glucocorticoid receptor with dexamethasone 21-mesylate Biol. Chem., 256: 12920-12925.

Eisen, L.P., M.E. Reichman, E.B. Thompson, B. Gametchu, R.W. Harrison, and H.J. Eisen 1985 Monoclonal antibody to the rat glucocorticoid receptor. J. Biol. Chem. 260: 11805-11810.

Evain, D., A.M. Morera, and M. Saez 1976 Glucocorticoid receptors in interstitial cells of the rat testis. J. Steroid Biochem., 7: 1135-1139.

Evans, R. 1988 The steroid and thyroid superfamily. Science, 240: 889-895.

Gabrilove, J.L., G.L. Nicolis, and A.R. Sohval 1974 The testis in Cushing's syndrome J. Urol., 112: 95-99.

Gametchu, B., and R.W. Harrison 1984 Characterization of a monoclonal antibody to the rat liver glucocorticoid receptor. Endocrinology, 114: 274-279.

Gehring, U. 1987 Steroid hormone receptors: biochemistry, genetics, and molecular biology. TIBS 12: 399-402.

Gorski, J., D.O. Toft, G. Shyamala, D. Smith, and A. Notides 1968 Hormone receptors: studies on the interaction of estrogen with the uterus. Recent Prog. Horm. Res. 24: 45-80.

Govindan, M.V. and C.E. Sekeris 1978 Purification of two dexamethasone-binding proteins from rat liver cytosol. Eur. J. Biochem. 89: 95-104.

Grandics, P., D.L. Gasser, and G. Litwack 1982 Monoclonal antibodies to the glucocorticoid receptor. Endocr., 111: 1731.

Hales, D.B., and A.H. Payne 1988a Regulation of cholesterol side-chain cleavage enzyme (P450scc) by glucocorticoids in Leydig cells: repression of DE NOVO synthesis and mRNA levels. Endocr. Soc., 70th Ann. Mtg., June 8-11, New Orleans, La., Abstr.537, p. 155.

Hales, D.B., and A.H. Payne 1988b Glucocorticoids stimulate P450scc gene expression in MA-10 tumor Leydig cells but have the opposite effect in normal Leydig cells. Regulation of Testicular Function: Signaling Molecules and Cell-Cell Communication. 10th Ann. testis Workshop, Dec. 8-11, Baltimore, Md. Abstr. 72, p.92.

Harmon, J.M., H.J. Eisen, S.T. Brower, S.S. Jr. Simons, C.L. Langley, and E.B. Thompson 1984 Identification of human leukemic glucocorticoid receptors using affinity labeling and anti-human glucocorticoid receptor antibodies. Cancer Res. 44: 4540-4547.

Harmon, J.M., M.S. Elsasser, L.A. Urda, and L.P. Eisen 1988 Activation of the human glucocorticoid receptor: evidence for a two-step model. J. Steroid Biochem., 31: 275-281.

Hermo, L., and M. Lalli 1988 Binding and internalization in vivo of [^{125}I]hCG in Leydig cells of the rat. J. Andrology, 9: 1-14.

Hollenberg, S.M., V. Giguere, P. Segui, and R.M. Evans 1987 Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. Cell, 49: 39-46.

Housley, P.R., E.R. Sanchez, H.M. Westphal, M. Beato, and W.B. Pratt 1985 the molybdate-stabilized L-cell glucocorticoid receptor isolated by affinity chromatography or with a monoclonal antibody is associated with a 90-92 KDa nonsteroid-binding phosphoprotein. J. Biol. Chem., 260: 13810-13817.

Jensen, E.V., T. Suzuki, T. Kawashima, W.E. Stumf, P.W. Jungblut, and E.R. DeSombre 1968 A two-step mechanism for the interaction of oestradiol with rat uterus. Proc. Natl. Acad. Sci. USA, 59: 632-638.

Karnovsky, M.J. 1971 Use of ferrocyanide-reduced osmium tetroxide in electron microscopy. Proc. 11th Am. Soc. Cell Biol., Nov. 17-20, New Orleans, La., Abstract 284, p. 146.

Katzenellenbogen, J.A. 1977 Affinity labeling as a technique in determining hormone mechanisms. In: Biochemical Actions of Hormones. G. Litwack, ed., Academic Press, New York, Vol. 4, pp. 1-84.

King, W.J., and G.L. Greene 1984 Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature, 307: 745-747.

Kopriwa, B., and C.P. LeBlond 1962 Improvements in the coating techniques of radioautography. J. Histochem. Cytochem., 10: 269-284.

Kopriwa, B.M. 1975 A comparison of various procedures for fine grain development in electron microscope radioautography. Histochemistry, 44: 201-224.

Kopriwa, B.M., G.M. Levine, and N.J. Nadler 1984 Assessment of resolution by half-distance values for tritium and radioiodine in electron microscopic radioautographs using Ilford L4 emulsion developed by "solution physical" or D-19b methods. Histochem., 80: 519-522.

LeBlond, C.P., and Y. Clermont 1952 Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann. N.Y. Acad. Sci., 55: 548-573.

Levy, F.O., L. Eikvar, B. Piotrowska, O. Oyen, T Jahnsen, and V. Hansson 1987 Characterization and cellular localization of glucocorticoid receptors in rat seminiferous tubules. Detection of glucocorticoid receptor mRNA. In: Cell Biology of the Testis and Epididymis. M.C. Orgebin-Crist and B.J. Danzo, eds. Ann. N.Y. Acad. Sci., 513: 487-489.

Levy, F.O., A.H. Ree, L. Eikvar, M.V. Govindan, T. Jahnsen, and V. Hansson 1989 Glucocorticoid receptors and glucocorticoid effects in rat Sertoli cells. Endocrinology, 124: 430-436.

Logeat, F., M.T. Vu Hai, A. Fournier, P. Legrain, P. Buttin, and E. Milgrom 1983 Monoclonal antibodies to rabbit progesterone receptor: Crossreaction with other progesterone receptors. Proc. Natl. Acad. Sci. USA, 80: 6456.

Mendelson, C., M. Dufau, and K. Catt 1975 Gonadotropin binding and stimulation of cyclic adenosine 3':5'-monophosphate and testosterone production in isolated Leydig cells. J. Biol. Chem. 250: 8818-8823.

Mester, J., and E.E. Baulieu 1972 Nuclear estrogen receptor of chick liver. Biochem. Biophys. Acta, 261: 236.

Miesfeld, R., S. Rusconi, P.J. Godowski, B.A. Maler, S. Okret, A.-C. Wikstrom, J.-A. Gustafsson, and K.R. Yamamoto 1986 Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. Cell, 46: 389-399.

Munck, A., and K. Leung 1977 Glucocorticoid receptors and mechanisms of action. In: Receptors and Mechanisms of Action of Steroid Hormones, part 2. J.R. Pasqualini, ed., Marcel Dekker, New York, p. 311.

Nadler, N.J. 1971 The interpretation of grain counts in electron microscope radioautography. J. Cell Biol. 49: 877-882.

Nadler, N.J. 1979 Quantitation and resolution in electron microscope radioautography. J. Histochem. Cytochem., 27: 1531-1533.

Okret, S., A.C. Wikstrom, O. Wrange, B. Andersson, and J.A. Gustaffson 1984
Monoclonal antibodies against the rat liver glucocorticoid receptor. Proc. Natl. Acad. Sci. USA, 81: 1609-1613.

Picado-Leonard, J., and W.L. Miller 1988 Homologous sequences in steroidogenic enzymes, steroid receptors, and a steroid binding suggest a consensus steroid-binding sequence. Mol. Endocr., 2: 1145-1150.

Picard, D., and K.R. Yamamoto 1987 Two signals mediate hormone-dependent localization of the glucocorticoid receptor. EMBO J., 6: 3333-3340.

Radanyi,C., I. Joab, J.M. Renoir, H. Richard-Foy, and E.E. Baulieu 1983 Monoclonal antibody to chicken oviduct progesterone receptor. Proc. Natl. Acad. Sci. USA, 80: 2854.

Roberts, J.L., C.C. Chen, J.H. Eberwine, M.J.Q Evinger, C. Gee, E. Herbert, and B.S. Schachter 1982 Glucocorticoid regulation of proopiomelanocortin gene expression in rodent pituitary. Rec. Prog. Horm. Res., 38: 227-256.

Russell, L., and S. Burguet 1977 Ultrastructure of Leydig cells as revealed by secondary tissue treatment with a ferrocyanide-osmium mixture. Tissue and Cell, 9:751-766.

Saez, J.M., A.M. Morera, F. Haour, and D. Evain 1977 Effects of in vivo administration of dexamethasone, corticotropin, and human chorionic gonadotropin on steroidogenesis and protein and DNA synthesis of testicular interstitial cells in prepubertal rats. Endocrinology, 101: 1256-1263.

Salpeter, M.M., L. Bachmann, and E.E. Salpeter 1969 Resolution in electron microscope radioautography. J. Cell Biol., 41: 1-20.

Schmidt, T.J., and G. Litwack 1982 Activation of the glucocorticoid-receptor complex. In: Physiological Reviews, 62: 1131-1192. S.G. Schultz, ed. American Physiological Society, Md.

Simons, S.S. Jr., M. Pons, and D.F. Johnson 1980 Alpha-Keto-mesylate: a reactive thiol-specific functional group. J. Org. Chem. 45: 3084-3088.

Simons, S.S. Jr., and E.B. Thompson 1981 Dexamethasone 21-mesylate: an affinity label of glucocorticoid receptors from rat hepatoma tissue culture cells. Proc. Natl. Acad. Sci. USA, 78: 3541-3545.

Simons, S.S. Jr., and E.B. Thompson 1982 Affinity labeling of glucocorticoid receptors: new methods in affinity labeling. In: Biochemical Actions of Hormones. G. Litwack, ed., Academic Press, New York, Vol. 9, pp. 221-254.

Simons, S.S. Jr., R.E. Schleenbaker, and H.J. Eisen 1983 Activation of covalent affinity labelled glucocorticoid receptor-steroid complexes. J. Biol. Chem., 258: 2229-2238.

Simons, S.S. Jr., J.G. Pumphrey, S. Rudikoff, and H.J. Eisen 1987 Identification of cysteine 656 as the amino acid of hepatoma tissue culture cell glucocorticoid receptors that is covalently labeled by dexamethasone 21-mesylate. J. Biol. Chem., 262: 9676-9680.

Smith, C.E., S. Williamson, D. Gauthier, and W.R.J. Funnell 1987 Low-cost automated particle counting board using an IBM-PC and the matrox PIP-1024 video digitizing board. McGill University. Technical Report No. 87-1.

Stumpf, W.E. 1971 Autoradiographic techniques and the localization of estrogen, androgen and glucocorticoid in pituitary and brain. Amer. Zoologist, 11: 725-739.

Stumpf, W.E. 1988 Hormone receptor autoradiography. In: Steroid Receptors and Disease: Cancer, Autoimmune, Bone, and Circulatory Disorders. Sheridan, P.J., K. Blum, M.C. Trachtenberg, eds., Marcel Dekker, New York, p.231.

Tang, X.M., Y. Clermont, and L. Hermo 1988 Origin and fate of autophagosomes in Leydig cells of normal adult rats. J. Androl., 9: 284-293.

Vale, W., C. Rivier, L. Yang, S. Minick, and J. Rivier 1981 Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and B-endorphin. Science, 213: 1394.

Welsh, T.H. Jr., T.H. Bambino, and A.W. Hsueh 1982 Mechanism of glucocorticoid-induced suppression of testicular androgen biosynthesis in vitro. Biol. Reprod., 27: 1138-1146.

Welshons, W.V., M.E. Lieberman, and J. Gorski 1984 Nuclear localization of unoccupied oestrogen receptors. Nature, 307: 747.

Welshons, W.V. B.M. Krummel, and J. Gorski 1985 Nuclear localization of unoccupied receptors for glucocorticoids, estrogens, and progesterones in GH_3 cells. Endocr., 117: 2140.

Westphal, H.M., G. Moldenhauer, and M. Beato 1982 Monoclonal antibodies to the rat liver glucocorticoid receptor. EMBO J., 1: 1467.

Wikstrom, A.-C., O. Bakke, S. Okret, M. Bronnegard, and J.-A. Gustaffson 1987 Intracellular localization of the glucocorticoid receptor: evidence for cytoplasmic and nuclear localization. Endocrinology, 120: 1232-1242.

Wilson, E.M., D.B. Lubahn, F.S. French, C.M. Jewell, and J.A. Cidlowski 1988 Antibodies to steroid receptor deoxyribonucleic acid binding domains and their reactivity with the human glucocorticoid receptor. Mol. Endocr., 2: 1018-1026.

Yamamoto, K.R. 1985 Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. 19: 209-252.

Zava, D.T., and W.L. McGuire 1977 Estrogen receptor. Unoccupied sites in nuclei of a breast tumor cell line. J. Biol. Chem., 252: 3703.

Table abbreviations

ER - endoplasmic reticulum (s - smooth, r - rough)
cyto - cytosol
mito - mitochondria
nucl - nucleus
pm - plasma membrane
MVB - multivesicular body

Table 1 - Quantitative radioautographic assessment of ^{3}H-DM labeling in testicular cells of experimental (A-E) and control (F-H) animals using computer-assisted image analysis. Each value represents the mean of 20 fields (± standard deviation) and is expressed in computer pixels.

	Animal	Leydig cells of Interstitium	Seminiferous Tubules
Experimental:	A	5476 ± 2309	764 ± 365
		6877 ± 4931	834 ± 438
		6485 ± 2853	553 ± 186
		5978 ± 3562	704 ± 310
	B	7669 ± 2884	1191 ± 337
		4987 ± 1603	664 ± 220
		3799 ± 1546	393 ± 196
		8695 ± 3220	996 ± 340
	C	2728 ± 1229	478 ± 278
		10606 ± 3468	815 ± 391
	D	2899 ± 1141	687 ± 290
		2767 ± 1276	634 ± 178
		2941 ± 953	667 ± 286
		3794 ± 1189	1095 ± 477
	E	2278 ± 1189	474 ± 204
		4500 ± 1272	765 ± 354
		1287 ± 567	233 ± 98
		1429 ± 1060	337 ± 137
Control:	F	3594 ± 1735	547 ± 285
		1576 ± 603	379 ± 169
		3384 ± 2059	516 ± 196
		1496 ± 698	285 ± 123
	G	1962 ± 939	426 ± 219
		1675 ± 626	433 ± 147
	H	2068 ± 920	482 ± 245
		2206 ± 735	582 ± 186
		1061 ± 477	284 ± 146
		1030 ± 531	350 ± 177

Table 2 - Quantitative radioautographic assessment of ^{3}H-DM labeling in testicular cells of experimental (A-E) and control (F-H) injected rats.

Experimental	Leydig cells	Cells of seminiferous epithelium
A	6926	738
B	6204	714
C	6288	811
D	3098	770
E	3390	620
Average value ± S.D.	5181 ± 1794*	731 ± 72

Control	Leydig cells	Cells of seminiferous epithelium
F	1816	449
G	2512	432
H	1046	317
Average value ± S.D.	1791 ± 733*	399 ± 72
	($p<0.01$)**	($p<0.01$)

* 80 light microscopic fields (20 fields/slide x 4 slides/animal) under oil immersion were counted for the two different groups of cells per animal (total 160 fields) (except for animals C and G where 40 light microscopic fields were counted for the two different groups of cells) and an average value then calculated for each animal. The average value obtained from the different animals of each group (experimental = 5; control = 3) was determined and expressed as computer pixels ± standard deviation (S.D.).

** Statistical significance between experimental and control animals for both groups of cells was assessed with a Student's t-test.

Table 3 - Crude grain counts over Leydig cells after injection of ^{3}H-dexamethasone 21-mesylate into the interstitial space of the rat testis - Individual experimentals.

	ANIMALS				
EXCLUSIVE	1	2	3	4	5
cytosol(sER)*	144 (34)**	214 (46)	128 (44)	57 (49)	71(42)
mitochondria	177 (42)	140 (30)	96 (33)	45 (39)	41(24)
nucleus	47 (5)	58 (12)	46 (16)	9 (8)	34(20)
cytosol***	23 (11)	22 (5)	8 (3)	3 (3)	6 (4)
pm/microvilli	20 (5)	13 (3)	8 (3)	-	4 (2)
Golgi apparatus	10 (2)	13 (3)	5 (2)	1 (<1)	10 (6)
peroxisomes	4 (<1)	5 (<1)	2 (<1)	-	2 (1)
vesicles (all sizes)	1 (<1)	1 (<1)	-	1 (<1)	-
	426	466	293	116	168
SHARED					
sER/other****	27	19	9	5	4
mito/other	2	-	1	-	-
cyto/other	13	13	9	2	11
sER/cyto	75	143	87	41	41
sER/cyto/other	18	21	7	3	7
sER/nucl	-	-	3	-	-
mito/sER	160	176	100	20	53
mito/sER/other	6	3	-	1	1
mito/cyto	44	36	17	4	17
mito/cyto/other	3	1	-	-	-
cyto/nucl	4	5	8	-	7
mito/sER/cyto	24	73	45	19	15
mito/sER/cyto/other	1	1	-	-	-
mito/sER/nucl	1	-	-	-	-
sER/cyto/nucl	-	2	3	-	1
	378	493	289	95	157

* cytosol(sER) = a specially developed category for grains over sER networks where the limited resolution of the radioautographic technique was not able to separate the sER tubules and the adjacent cytosol. Grains were classified as "exclusive" to this category only if 50% or more of the area of the resolution boundary circle in which they were situated was occupied by sER tubules (and the rest, cytosol).

** Percentage of exclusive grains.

*** Cytosol exclusive of all membrane-bound organelles, filaments, and tubules

**** Golgi apparatus, peroxisomes, electron-lucent vesicles, rER, autophagosomes, MVBs, lysosomes, and lipid droplets.

Table 4 - Crude grain counts over Leydig cells after injection of ^{3}H-dexamethasone 21-mesylate into the interstitial space of the rat testis - Individual controls.

	ANIMALS		
EXCLUSIVE	1	2	3
cytosol (sER)*	15 (43)**	21 (42)	16 (31)
mitochondria	13 (37)	16 (32)	8 (15)
nucleus	2 (6)	8 (16)	12 (23)
cytosol***	1 (3)	-	2 (4)
pm/microvilli	1 (3)	-	-
Golgi apparatus	2 (6)	3 (6)	8 (15)
vesicles (all sizes)	1 (3)	-	-
lysosome/MVB	-	2 (4)	6 (12)
	35	50	52
SHARED			
sER/other****	4	1	7
mito/other	-	1	-
cyto/other	4	1	5
sER/cyto	8	17	10
sER/cyto/other	2	4	5
sER/nucl	1	-	1
mito/sER	14	35	11
mito/sER/other	1	1	-
mito/cyto	2	-	1
cyto/nucl	-	2	3
mito/sER/cyto	-	8	3
sER/cyto/nucl	-	2	-
cyto/nucl/other	-	-	1
mito/sER/cyto/other	1	-	-
	37	72	47

* cytosol(sER) = a specially developed category for grains over sER networks where the limited resolution of the radioautographic technique was not able to separate the sER tubules and the adjacent cytosol. Grains were classified as "exclusive" to this category only if 50% or more of the area of the resolution boundary circle in which they were situated was occupied by sER tubules (and the rest, cytosol).
** Percentage of exclusive grains.
*** Cytosol exclusive of all membrane-bound organelles, filaments, and tubules.
**** Golgi apparatus, peroxisomes, electron-lucent vesicles, rER, autophagosomes, MVBs, lysosomes, and lipid droplets.

Table 5 - Pooled crude grain counts over Leydig cells after injection of ^{3}H-dexamethasone 21-mesylate into the interstitial space of the rat testis - Experimental.

GRAIN COUNTS

EXCLUSIVE			SHARED	
Organelle	Grains		Organelles	Grains
cytosol (sER)*	614	(42)**	sER/other****	64
mitochondria	499	(34)	mito/other	3
nucleus	194	(13)	cyto/other	46
cytosol***	62	(4)	sER/cyto	387
pm/microvilli	46	(3)	sER/cyto/other	56
Golgi apparatus	40	(3)	sER/nucl	3
peroxisomes	13	(<1)	mito/sER	509
vesicles (all sizes)	3	(<1)	mito/sER/other	11
			mito/cyto	118
	1471		mito/cyto/other	4
			cyto/nucl	24
			mito/sER/cyto	177
			mito/sER/cyto/other	1
			mito/sER/nucl	1
			sER/cyto/nucl	8
				1412

* cytosol(sER) = a specially developed category for grains over sER networks where the limited resolution of the radioautographic technique was not able to separate the sER tubules and the adjacent cytosol. Grains were classified as "exclusive" to this category only if 50% or more of the area of the resolution boundary circle in which they were situated was occupied by sER tubules (and the rest, cytosol).

** Percentage of exclusive grains.

*** Cytosol exclusive of all membrane-bound organelles, filaments, and tubules.

**** Golgi apparatus, peroxisomes, rER, microvilli, plasma membrane, lysosomes, MVB, electron-lucent vesicles, autophagosomes, and lipid droplets.

Table 6 - Pooled crude grain counts over Leydig cells after injection of ^{3}H-dexamethasone 21-mesylate into the interstitial space of the rat testis - Control.

EXCLUSIVE			SHARED	
Organelle	Grains		Organelles	Grains
cytosol (sER)*	52	(38)**	sER/other****	12
mitochondria	37	(27)	mito/other	1
nucleus	22	(16)	cyto/other	10
cytosol***	3	(2)	sER/cyto	35
Golgi	13	(10)	sER/cyto/other	11
lysosome	7	(5)	sER/nucl	2
microvilli	1	(<1)	mito/sER	60
multivesicular body	1	(<1)	mito/sER/other	2
vesicles (all sizes)	1	(<1)	mito/cyto	3
			cyto/nucl	5
	137		cyto/nucl/other	1
			mito/sER/cyto	11
			mito/sER/cyto/other	1
			sER/cyto/nucl	2
				156

* cytosol (sER)= a specially developed category for grains over sER networks where the limited resolution of the radioautographic technique was not able to separate the sER tubules and the adjacent cytosol. Grains were classified as "exclusive" to this category only if 50% or more of the area of the resolution boundary circle in which they were situated was occupied by sER tubules (and the rest, cytosol).

** Percentage of exclusive grains.

*** Cytosol exclusive of all membrane-bound organelles, filaments, and tubules.

**** Golgi apparatus, peroxisomes, rER, microvilli, plasma membrane, lysosomes, MVB, electron-lucent vesicles, autophagosomes, and lipid droplets.

Table 7 - Crude random sampling hits of Leydig cell structures obtained using the "circle hit" method (expressed as absolute numbers)- Individual experimentals.

	ANIMALS				
EXCLUSIVE	1	2	3	4	5
smooth ER	353	394	435	183	276
mitochondria	147	86	125	69	116
nucleus	345	286	359	97	291
cytosol	90	27	55	26	61
Golgi apparatus	56	28	27	5	30
pm/microvilli	116	53	69	16	40
peroxisomes	12	9	13	3	8
vesicles (all sizes)	-	5	8	2	2
lysosome/MVB	2	-	3	-	5
autophagosome	4	-	-	-	-
lipid	1	-	-	-	3
	1126	888	1094	401	832
SHARED					
sER/other*	33	52	62	31	51
mito/other	-	2	3	1	2
cyto/other	104	56	94	30	50
sER/cyto	308	287	313	131	259
sER/cyto/other	50	37	45	16	35
sER/nucl	1	5	3	-	3
mito/sER	161	204	200	98	162
mito/sER/other	3	1	2	1	8
mito/cyto	50	21	40	22	34
mito/cyto/other	1	2	4	1	-
cyto/nucl	30	20	27	5	29
cyto/nucl/other	-	2	-	-	-
mito/sER/cyto	19	67	79	30	67
mito/sER/cyto/other	-	4	2	-	1
mito/sER/nucl	-	1	2	-	-
sER/cyto/nucl	1	8	11	2	3
sER/cyto/nucl/other	-	1	-	-	-
mito/cyto/nucl	2	-	-	-	-
other	-	2	1	-	-
	763	772	888	368	704

* Golgi apparatus, peroxisomes, electron-lucent vesicles, rER, autophagosomes, MVBs, lysosomes, and lipid droplets.

Table 8 - Crude random sampling hits of Leydig cell structures obtained by using the "circle hit" method (expressed as absolute numbers)- Individual controls.

	ANIMALS		
EXCLUSIVE	1	2	3
smooth ER	128	109	84
mitochondria	46	18	28
nucleus	50	85	100
cytosol	5	13	11
Golgi apparatus	27	12	22
pm/microvilli	41	9	7
vesicles (all sizes)	3	1	-
peroxisomes	-	2	1
lysosome/MVB	2	3	1
autophagosomes	1	1	-
lipid	-	-	4
	303	253	258
SHARED			
sER/other*	29	19	29
cyto/other	17	18	13
sER/cyto	108	80	76
sER/cyto/other	22	11	7
sER/nucl	2	-	2
mito/sER	71	55	30
mito/sER/other	1	5	1
mito/cyto	6	3	6
cyto/nucl	4	9	4
mito/sER/cyto	23	12	11
mito/sER/cyto/other	-	-	2
sER/cyto/nucl	1	-	1
other	1	-	-
	285	212	182

* Golgi apparatus, peroxisomes, electron-lucent vesicles, rER, autophagosomes, MVBs, lysosomes, and lipid droplets.

Table 9 - Pooled crude random sampling hits obtained by using the "circle hit" method (expressed as absolute numbers) - Experimental.

EXCLUSIVE		SHARED	
Organelle	**Circles**	**Organelles**	**Circles**
smooth ER	1641	sER/other*	230
mitochondria	543	mito/other	8
nucleus	1386	cyto/other	334
cytosol	251	sER/cyto	1298
Golgi apparatus	146	sER/cyto/other	182
pm/microvilli	294	sER/nucl	12
peroxisomes	45	mito/sER	825
vesicles	17	mito/sER/other	15
lysosome/MVB	10	mito/cyto	167
autophagosome	4	mito/cyto/other	8
lipid droplets	4	cyto/nucl	111
		cyto/nucl/other	2
	4341	mito/sER/cyto	262
		mito/cyto/sER/other	7
		mito/sER/nucl	3
		sER/cyto/nucl	25
		sER/cyto/nucl/other	1
		mito/cyto/nucl	2
		other	3
			3495

* Golgi apparatus, plasma membrane, microvilli, peroxisomes, electron-lucent vesicles, lysosomes, MVB, autophagosomes, lipid droplets, rER.

Table 10 - Pooled crude random sampling hits obtained by using the "circle hit" method (expressed as absolute numbers) - Control.

EXCLUSIVE		SHARED	
Organelle	**Circles**	**Organelles**	**Circles**
smooth ER	321	sER/other*	70
mitochondria	92	cyto/other	49
nucleus	235	sER/cyto	264
cytosol	29	sER/cyto/other	48
Golgi apparatus	59	sER/nucl	4
pm/microvilli	57	mito/sER	156
peroxisomes	3	mito/sER/other	6
vesicles	4	mito/cyto	15
lysosome/MVB	6	cyto/nucl	17
autophagosome	2	mito/sER/cyto	46
lipid droplets	4	mito/sER/cyto/other	3
		sER/cyto/nucl	2
	812	other	1
			681

* Golgi apparatus, plasma membrane. microvilli, peroxisomes, electron-lucent vesicles, lysosomes, MVB, autophagosomes, lipid droplets, rER.

Table 11 - Percent hits on Leydig cell structures obtained by using the "Point hit" (PH) and "Circle hit" (CH) methods - Individual animals.

Experimental

	1		2		3		4		5	
Organelle	PH	CH	PH	CH	PH	CH	PH	CH	PH	CH
Smooth ER	29	49	40	64	29	58	32	64	37	56
Mitochondria	14	20	14	23	10	23	17	29	17	25
Cytosol	28	35	21	32	21	34	26	34	25	35
Nucleus	20	20	18	20	35	20	19	14	14	21
Other*	9	-	7	-	5	-	6	-	7	-

Control

	1		2		3	
Organelle	PH	CH	PH	CH	PH	CH
Smooth ER	37	65	33	63	31	55
Mitochondria	13	25	12	20	12	18
Cytosol	30	32	25	31	22	30
Nucleus	9	10	22	20	24	24
Other*	11	-	8	-	11	-

* Golgi apparatus, rER, peroxisomes, microvilli, lysosomes, MVB, electron-lucent vesicles, autophagosomes, and lipid droplets.

Table 12 - Percent hits on Leydig cell structures using the "Point hit" (PH) and "Circle hit" (CH) methods - Pooled results.

	Experimental		Control	
Organelle	PH	CH	PH	CH
Smooth ER	33	57	34	62
Mitochondria	14	24	12	21
Cytosol	25	34	26	32
Nucleus	21	20	18	17
Other*	7	-	10	-

* Golgi apparatus, rER, peroxisomes, microvilli, lysosomes, MVB, electron-lucent vesicles, autophagosomes, and lipid droplets.

Table 13 - Labeling of four organelles in the Leydig cell by ^{3}H-DM (after corrections) obtained from individual animals, expressed as absolute numbers (a) and percent of the total (b) - Experimental.

	CYTOSOL(SER)	MITOCHONDRIA	CYTOSOL	NUCLEUS
	a 330	a 319	a 71	a 49
1	b 43%	b 41%	b 9%	b 6%
	c 1.47	c 2.85	c 0.32	c 0.30
	a 550	a 242	a 74	a 61
2	b 59%	b 26%	b 8%	b 7%
	c 1.50	c 1.84	c 0.38	c 0.36
	a 324	a 149	a 40	a 54
3	b 57%	b 26%	b 7%	b 10%
	c 1.95	c 2.63	c 0.34	c 0.29
	a 123	a 62	a 15	a 9
4	b 59%	b 30%	b 7%	b 4%
	c 1.83	c 1.78	c 0.27	c 0.23
	a 163	a 71	a 39	a 36
5	b 53%	b 23%	b 12%	b 12%
	c 1.43	c 1.32	c 0.50	c 0.84

a = True silver grain count (absolute number). The sum of this row equals the total number of corrected grains.
b = Relative content of label (%) -
the ratio of the true grain count / corrected grain total
c = Relative concentration of label -
the ratio of relative content of label (%) / relative volume (%).

Table 14 - Labeling of four organelles in the Leydig cell by ^{3}H-DM (after corrections) obtained from individual animals, expressed as absolute numbers (a) and percent of the total (b) - Control.

	CYTOSOL(SER)	MITOCHONDRIA	CYTOSOL	NUCLEUS
	a	a	a	a
	40	17	9	2
	b	b	b	b
1	59%	25%	13%	3%
	c	c	c	c
	1.59	2.01	0.42	0.32
	a	a	a	a
	78	27	3	9
	b	b	b	b
2	66%	23%	3%	8%
	c	c	c	c
	2.02	1.91	0.11	0.36
	a	a	a	a
	45	11	15	14
	b	b	b	b
3	53%	12%	18%	17%
	c	c	c	c
	1.72	1.04	0.81	0.68

a = True silver grain count (absolute number). The sum of this row equals the total number of corrected grains.
b = Relative content of label (%) - the ratio of the true grain count / corrected grain total.
c = Relative concentration of label - the ratio of relative content of label (%) / relative volume (%).

Table 15 - Pooled results of the labeling of four organelles in the Leydig cell by ^{3}H-DM (after corrections), expressed as absolute numbers (a) and percent of the total (b).

Experimental (^{3}H-DM)

CYTOSOL(SER)	MITOCHONDRIA	CYTOSOL	NUCLEUS
a	a	a	a
1463	855	253	210
b	b	b	b
53%	31%	9%	7%
c	c	c	c
1.63	2.15	0.36	0.33

Control (^{3}H-DM + Dexa)

CYTOSOL(SER)	MITOCHONDRIA	CYTOSOL	NUCLEUS
a	a	a	a
154	61	29	26
b	b	b	b
57%	23%	11%	9%
c	c	c	c
1.67	1.83	0.43	0.55

a = True silver grain count (absolute number). The sum of this row equals the total number of corrected grains.
b = Relative content of label (%) - the ratio of true silver grain count / corrected silver grain total.
c = Relative concentration of label - the ratio of relative content of label (%) / relative volume (%).

Table 16 - Statistical analysis of the relative content (b) and concentration (c) of ^{3}H-DM in four Leydig cell organelles, tested using a Student's t-test.

			NUCLEUS
		CYTOSOL	b P > 0.05 c P > 0.05
	CYTOSOL (SER)	b P < 0.001 c P < 0.001	b P < 0.001 c P < 0.001
MITOCHONDRIA	b P < 0.001 c P > 0.05	b P < 0.001 c P < 0.001	b P < 0.001 c P < 0.001

Plate 1

Fig. 1. Low power photomicrograph of a portion of two seminiferous tubules and the intervening interstitial space (IS) of a testis from a rat injected with ^{3}H-DM. Note the abundance of silver grains overlying the Leydig cells (L) as compared to the weaker, more random grain distribution over the cells of the seminiferous epithelium (SE). bv, Blood vessel. X 600.

Fig. 2. High power photomicrograph of numerous Leydig cells (L) of the interstitial space (IS) of a rat testis injected with ^{3}H-DM. Such cells show many silver grains which are located mostly over the cytoplasm. bv, Blood vessel; seminiferous epithelium. X 1,200.

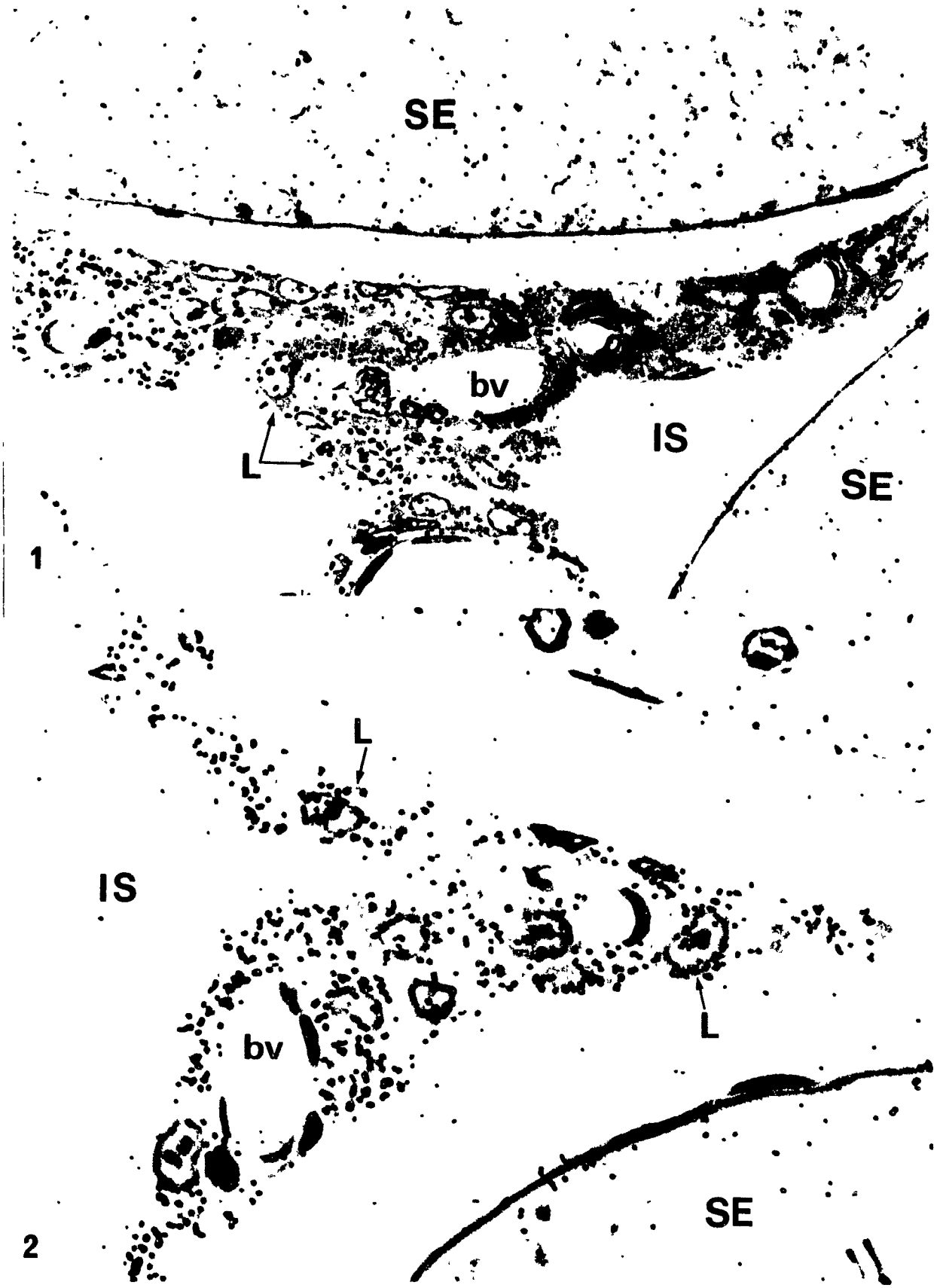

SE
bv
IS
L
SE
1
L
IS
L
bv
SE
2

Plate 2

Fig. 3. Light micrograph showing portions of two seminiferous tubules and many Leydig cells of the interstitial space (IS) of a rat testis injected with ^{3}H-DM in conjunction with a 25- fold excess of cold dexamethasone. Note that labeling over Leydig cells (L) and cells of the seminiferous epithelium (SE) is greatly reduced under such conditions. X 1,200.

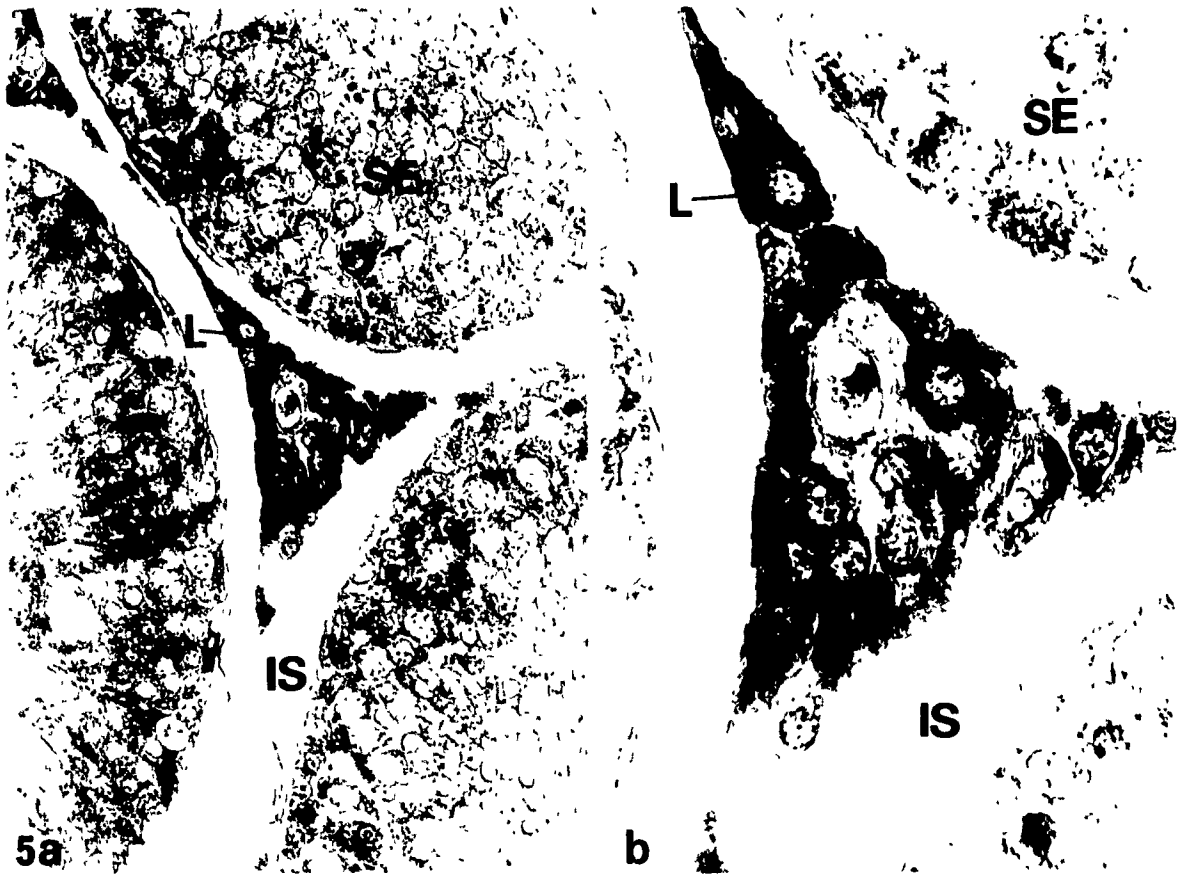
Fig. 4. High power photomicrograph of cells of the interstitial space (IS) of a rat testis injected with ^{3}H-DM in conjunction with a 50- fold excess of unlabeled dexamethasone. Leydig cells (L), identified by their large size and darkly stained cytoplasm, are weakly labeled. However, a few cells of smaller size and showing a paler cytoplasm, identified as macrophages (M), are well labeled reflecting non-specific endocytosis by these cells. bv, Blood vessel. X 1,500.


SE
IS
L
SE
3
IS
M
bv
L
M
4

Plate 3

Fig. 5. Immunocytochemical localization of glucocorticoid receptor (GR) in histological sections of the testis of rats treated with dexamethasone and reacted with GR antibody. Strong staining is seen over Leydig cells (L), while cells of the seminiferous epithelium (SE) are weakly stained. IS, interstitial space. Counterstained with methylene blue. **a**, X 400. **b**, X 1,000.

Fig. 6. Testicular tissue from a rat treated with non-immune rat IgG. The Leydig cells (L) and cells of the seminiferous epithelium (SE) show only background staining due to the methylene blue counterstain. IS, interstitial space. **a**, X 400. **b**, X 1,000.


SE
L
IS
5a
b

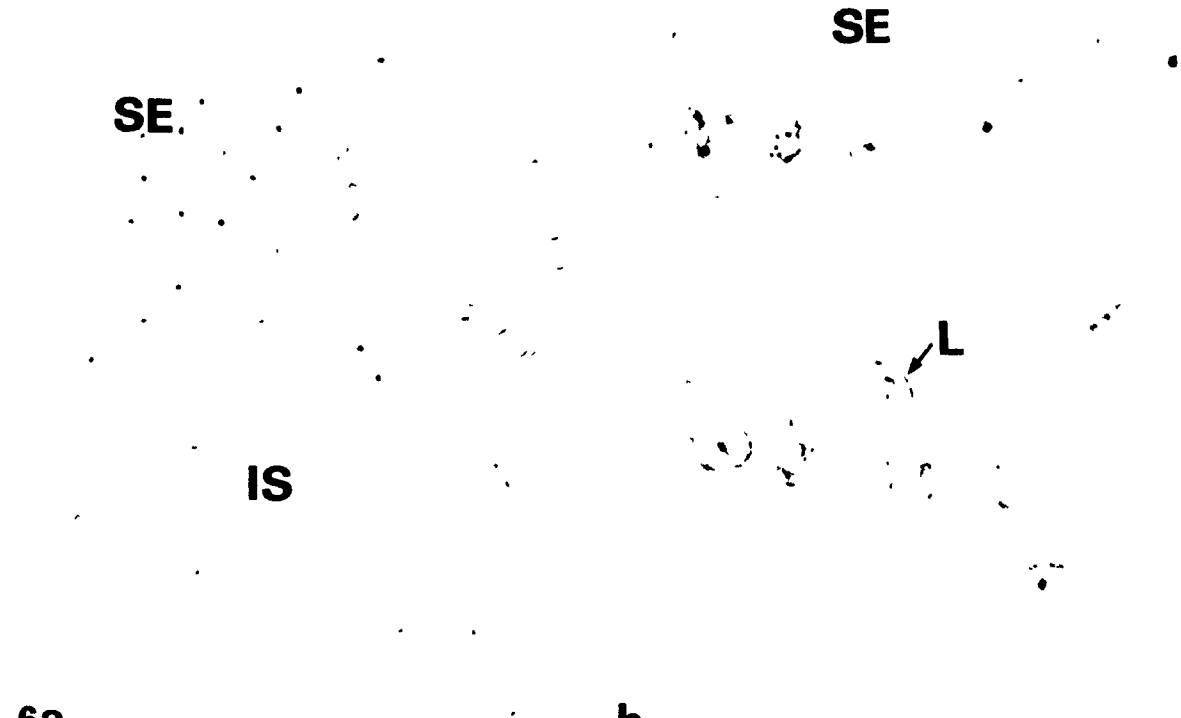

SE
SE.
L
IS
6a
b

Plate 4

Fig. 7. High power electron micrograph of portions of three Leydig cells 15-20 minutes after injection of ^{3}H-DM into the testicular interstitial space. Silver grains are predominantly associated with the mitochondria (m) - either deep to the interior (vertical arrows), towards the periphery (horizontal arrows), or over the outer mitochondrial membrane (arrowheads). Grains are also seen over the smooth endoplasmic reticulum (ser), including those tubules which are closely apposed to mitochondria (curved arrows), as well as cytosol (circled), and plasma membrane (small arrow). is: interstitial space. X 39,520.

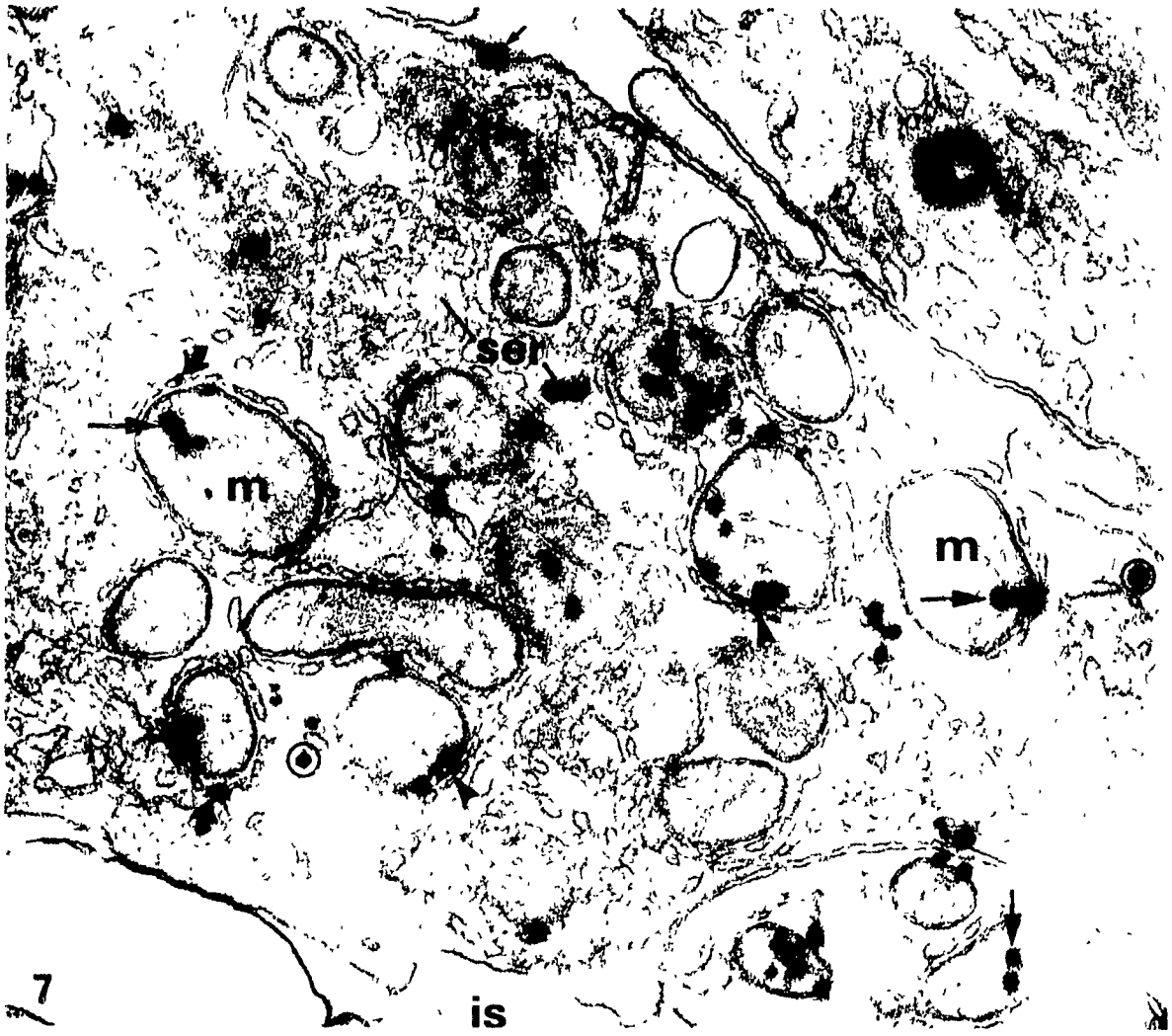

sar
m
m
7
is

Plate 5

Fig. 8. High power electron micrograph of a portion of a Leydig cell 15-20 minutes after injection of ^{3}H-DM into the testicular interstitial space. Grains are associated with mitochondria (m), smooth endoplasmic reticulum (ser), or shared between these two organelles (curved arrows). Over the mitochondria, grains are situated either deep to the interior (vertical arrows), or towards the periphery (horizontal arrows) - sometimes directly over the outer mitochondrial membrane (arrowheads). Note the absence of grains associated with peroxisomes. X 39,520.

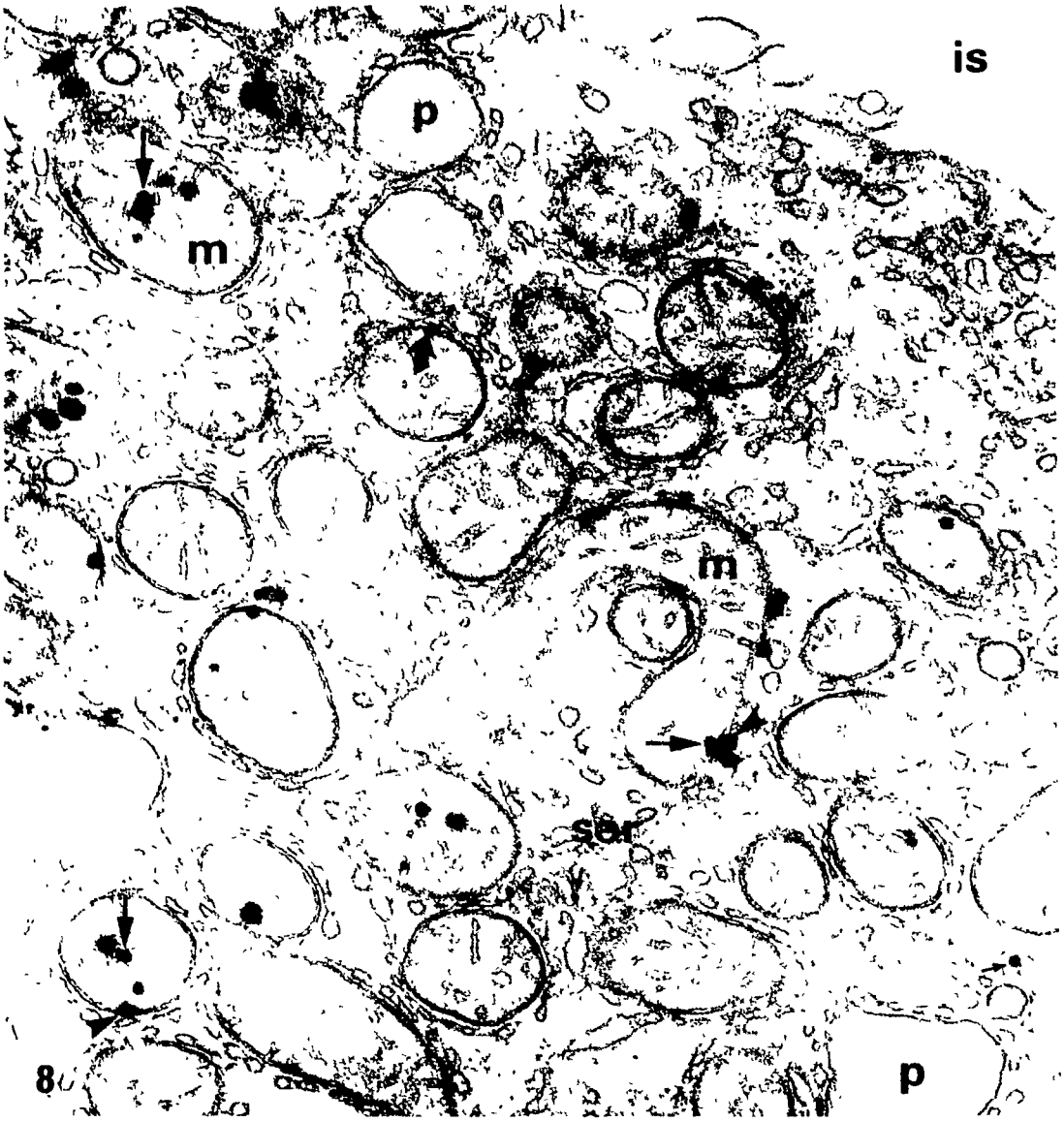
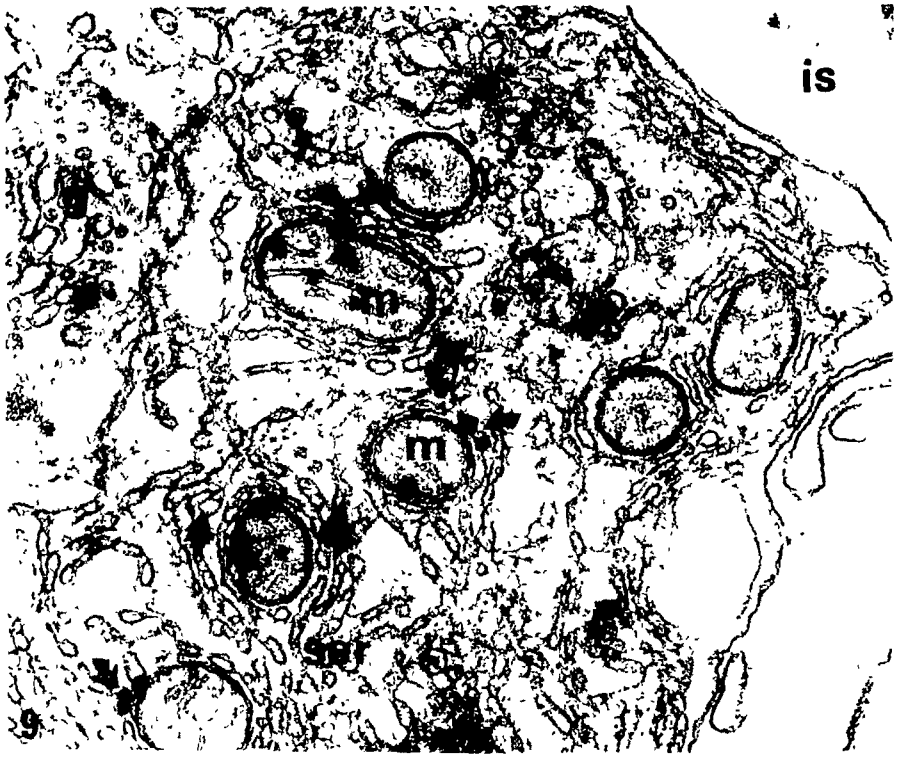

is
p
m
m
ser
p

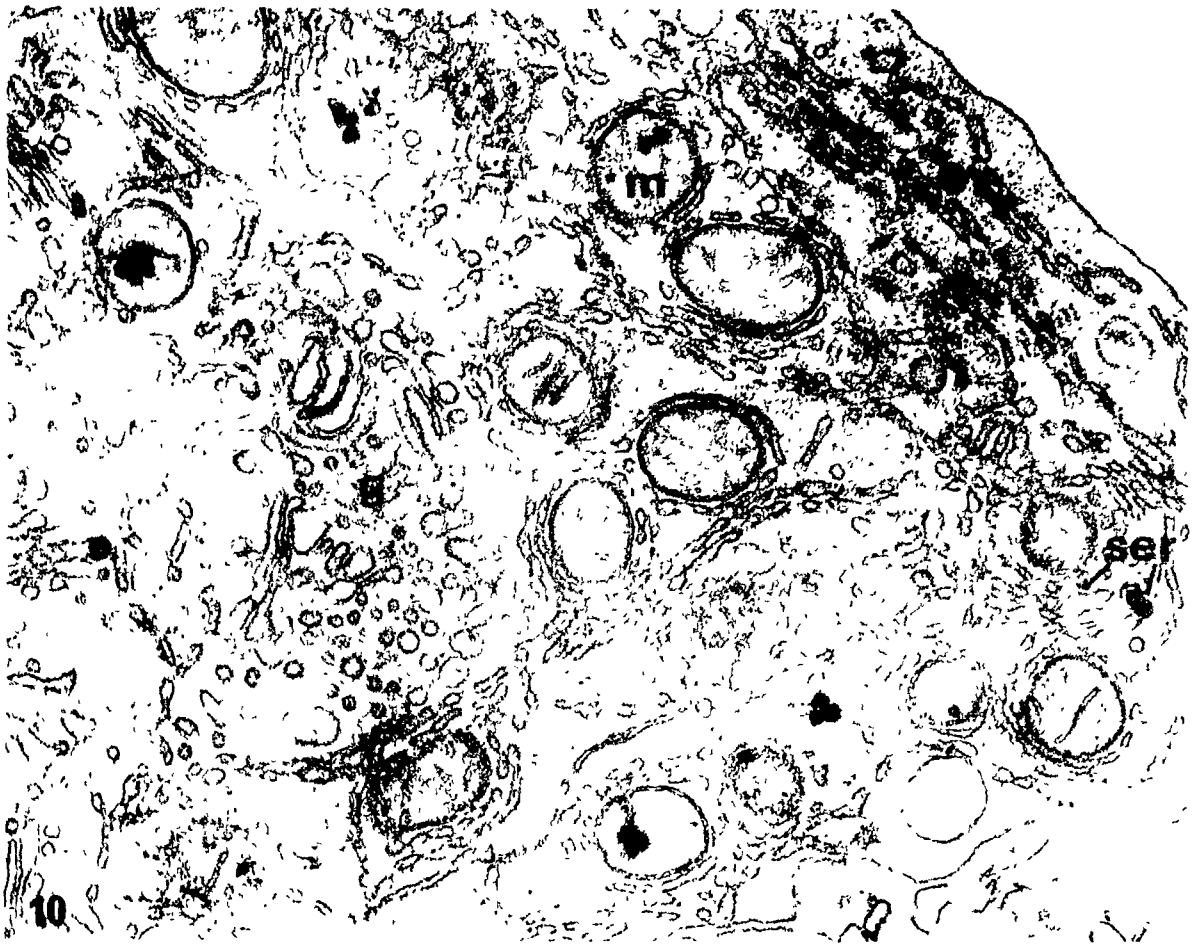
Plate 6

Fig. 9. High power electron micrograph of a portion of a Leydig cell 15-20 minutes after injection of ^{3}H-DM into the testicular interstitial space. Silver grains are associated predominantly with mitochondria (m) and smooth endoplasmic reticulum (ser). Grains over the sER are seen either directly overlying the lumen of the tubules (small arrows) or over the cytosol immediately adjacent to the tubules (circled). Many grains are seen over the tubules closely apposed to mitochondria (curved arrows). g: Golgi apparatus; is: interstitial space. X 39,520.

Fig. 10. High power electron micrograph of a portion of a Leydig cell 15-20 minutes after injection of ^{3}H-DM into the testicular interstitial space. Note the relative absence of silver grains from the Golgi apparatus (g). Grains are again seen in association with the mitochondria (m) and smooth endoplasmic reticulum (ser). X 39,520.



9



10

Plate 7

Fig. 11. High power electron micrograph showing portions of two Leydig cells 15-20 minutes after injection of ^{3}H-DM into the testicular interstitial space. Note the silver grains associated with the nucleus (n, arrows). Grains are also seen over mitochondria (m) and smooth endoplasmic reticulum (ser). X 39,520.

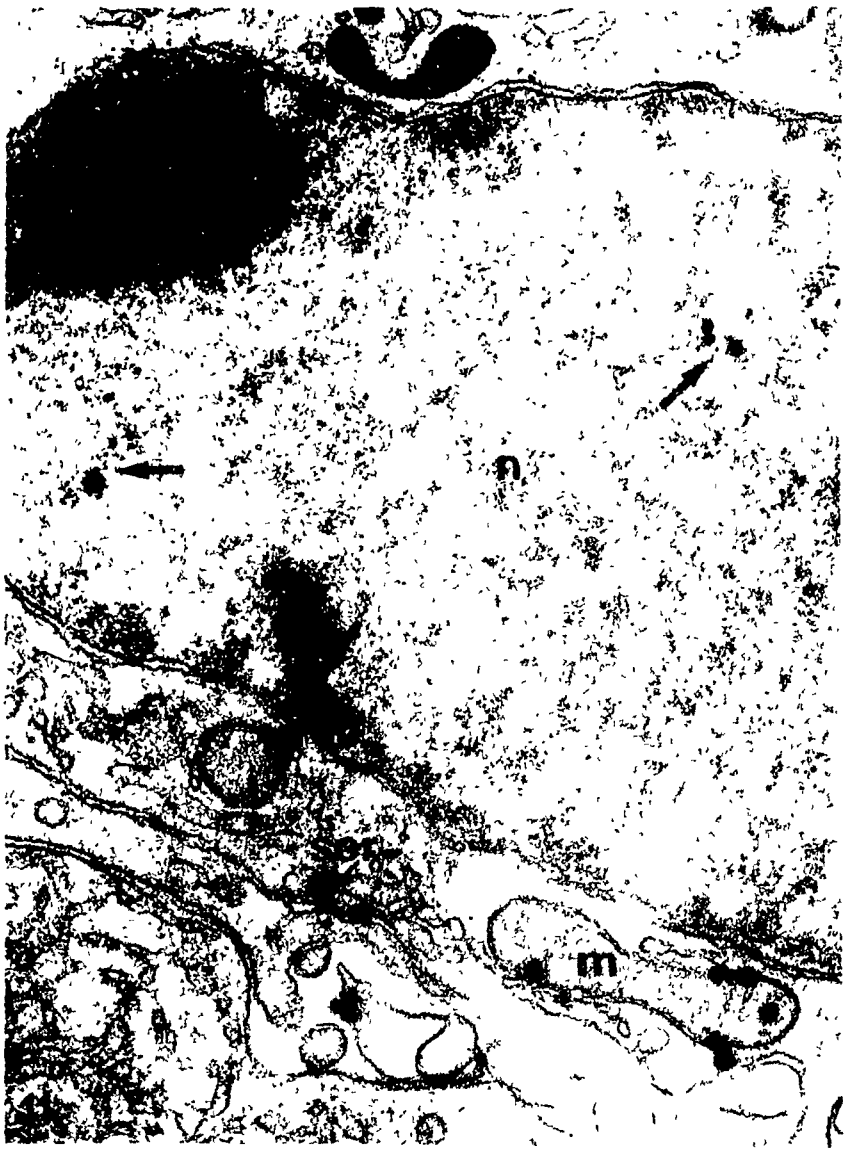
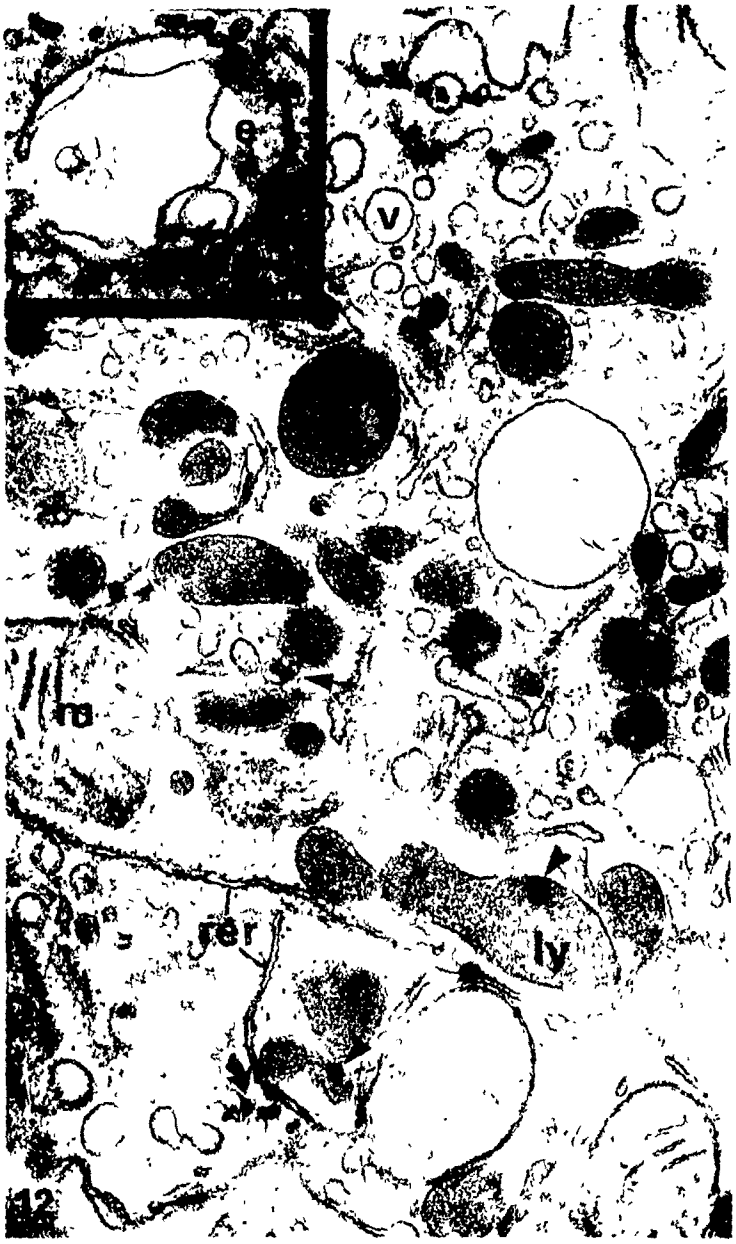

n
m

Plate 8

Fig. 12. High power electron micrograph of a portion of an interstitial macrophage 15-20 minutes after injection of ^{3}H-DM into the testicular interstitial space. Silver grains are predominantly associated with the extensive vesicular compartment (v) of this cell. Some are seen centered over the vesicles (arrows), including endosomes (e, inset) whereas others are situated over the cytosol immediately adjacent to the vesicles (curved arrows). Relatively few grains are associated with lysosomes (ly). Note the absence of mitochondrial (m) grains. X 39,520.


e
v
ro
rer
ly
12