MORPHOMETRIC, HISTOCHEMICAL, AND HORMONAL STUDIES OF THE TESTIS IN EXPERIMENTAL DIABETES MELLITUS IN THE RAT

by

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Great fleas have little fleas
upon their backs to bite 'em,
And little fleas have lesser fleas,
and so ad infinitum...
And the great fleas themselves, in turn
have greater fleas to go on;
While these in turn have greater still,
and greater still, and so on...

--Augustus De Morgan
A Budget of Paradoxes

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ABSTRACT

The hypothalamo-pituitary-testicular axis of diabetic rats was studied by morphometry, histochemistry, and hormone assays. Long Evans and Wistar strains were compared.

Using the streptozotocin model, there were 5 Long Evans groups: control, diabetic, drug-treated nondiabetic, insulin treated diabetic and semistarved rats. Wistar control and diabetic groups were added. After three months, testes were perfusion-fixed, incubated for Δ5,3β-hydroxysteroid dehydrogenase (HSD) activity, and processed for microscopy.

Analysis used ANOVAs, Tukey's and Chi-square tests, and orthogonal contrasts to separate effects of diabetes, streptozotocin, and starvation. Two-way ANOVAs compared strains.

The effect of diabetes (a)decreased seminiferous tubule diameter, (b)increased interstitial blood vessel density, (c)altered the frequency distribution of spermatogenic stages, (d)decreased HSD staining of Leydig cells, (e)increased Leydig cell lipid and smooth endoplasmic reticulum content, and (f)decreased testosterone and increased follicle stimulating hormone levels. Semistarvation had the latter two effects. Streptozotocin had no non-diabetogenic effect. Uremia and increased corticosterone levels were found only in Wistar diabetic rats.

The hypoandrogen state in diabetic Long Evans rats suggested primary Leydig cell compromise, not solely due to malnutrition, and preventable by insulin treatment. The streptozotocin model of diabetes was enhanced by the lack of uremia and stress in the strain.

INTRODUCTION

Definition

Diabetes is a term which refers to disorders characterized by excessive urine excretion (1), of which there are many. Diabetes mellitus is a metabolic disorder with decreased or absent ability to oxidize carbohydrates, usually due to faulty pancreatic beta cell activity in the islets of Langerhans. Consequent lack or deficiency of Insulin produces hyperglycemia with resulting glycosuria, and polyuria. Symptoms include thirst, hunger, weight loss, and weakness. Imperfect fat combustion leads to ketone body accumulation in the blood with metabolic acidosis and its symptomatology. Other complications of the metabolic derangement include pruritis, lowered resistance to pyogenic infections, angiopathy, arteriosclerosis, nephropathy, somatic and autonomic neuropathy, and proliferative retinopathy. These symptoms were recognized in ancient Chinese and Greek times (2), the hallmark sign of hyperglycemia being finally demonstrated by Bernard in 1859 (2). Since the discovery of insulin and its common usage in diabetic control programs, the study of diabetic complications has turned to that of their mechanism and prevention, rather than simple description of their pathology.

The precise etiology of diabetes mellitus (diabetes) is still in question, but autosomal genetic (307), metabolic (163) and environmental (13) factors are thought to play

important roles in susceptibility to the disease. Certain HLA genes have been linked (3,52,311) with a predisposition to an increased immunological response to some environmental event (possibly a viral infection), with the production of autoantibodies to islet beta cell-surface antigens (ICSA's) (7), or polyendocrine autoantibodies (14). While this remains theoretical, "in patients with hyperglycemia of recent onset the presence of ICSA's is a good predictor of a future need for insulin treatment."(3, 162, 167). Animal models exhibiting this feature (eg. BB Wistar rat) are currently being investigated as close parallels of human diabetes (8, 15, 18, 186, 230).

There are four types (2, 23, 306) of diabetes mellitus: (1) genetic (or primary) subdivided into Insulin-dependent and Insulin-independent, (2) pancreatic, (3) endocrine and (4) iatrogenic. The present study will concern itself with only the Insulin-dependent type of diabetes.

Prevalence

In North America, approximately 5% (3) of the population has been diagnosed as having diabetes. It is more common in relatives of diabetics than in the general population, suggesting the presence of a genetic factor in its occurrence (3). The prevalence varies in different countries, being nearly five times higher in the United States and Europe than in Japan (52).

Diagnosis

The diagnosis of diabetes (3) is suggested by a symptomatic history including polydipsia and polyuria, polyphagia and hunger, and weight loss. This clinical suggestion is confirmed by the finding of glycosuria and hyperglycemia. Glucose tolerance, the ability to respond to a glucose load, is also impaired, and is indicated by prolonged and abnormally increased blood glucose levels postprandially. Insulin treatment, now in a number of formulations and delivery systems combined with dietary control, seeks to correct this glucose intolerance, and to control the blood glucose levels within the normal fasting (3.9-6.0 mmol/L) and postprandial (less than 9.3 mmol/L at 1 hr) ranges (2).

Anatomy and Pathology of Diabetes

1.Pancreas

The islets of Langerhans are comprised of aggregated endocrine cells embedded in the exocrine (digestive) pancreas (59). They consist of alpha cells located peripherally and circumferentially, beta cells found centrally and most numerous, and diffusely scattered delta cells which produce glucagon, insulin, and somatostatin respectively (3, 4). This pattern is to some degree determined by age (24, 26), and by the precise location of a specific islet in the pancreas, with those most densely populated by alpha cells in the posterior, splenic part of the pancreas (21, 29). Islets are equally populated by beta

cells, but are of different sizes in anterior and posterior pancreatic regions (25).

The severity of diabetes is directly linked to the degree of reduction of total beta cell mass and degranulation of beta cells. These parameters correlate with extractable insulin, which is essentially zero in established insulin-dependent diabetics (3, 27). Rarely, in diabetes of recent onset (seen coincident with death due to severe symptoms at onset or by chance), the pancreas shows lymphocytic infiltration, called insulitis, in the islets of Langerhans (3, 28).

2.Blood Vessels

Both large and small vessels are affected earlier in the diabetic than in the healthy population by arteriosclerosis (22) and angiopathy respectively (2). The lesion, initially a thickening and duplication of the endothelial basement membranes, is seen in muscle, renal, skin, and retinal vessels (205). In the retina, vessels also exhibit microaneurysm formation, and there are new leaky vessels formed which allow vitreous hemorrhages, a process termed proliferative retinopathy (3).

3.Nephropathy

Nodular glomerulosclerosis (of Kimmelstiel-Wilson) in diabetes is an accumulation of glycoprotein material in the mesangium and in the basement membrane of glomerular capillaries. This lesion, termed diabetic nephropathy, is

thought to contribute to the increased permeability of the glomerular layers, and ultimately to become manifest as proteinuria, edema, and hypertension (3, 12).

4.Neuropathy

Neuropathy has been described in both the peripheral somatic and automonic nervous systems (11, 85, 165, 326, 216). Clinically it can present with loss of position or pain sensations in the feet (16), decreased deep tendon reflexes, decreased pupillary compensation to changes in light level (19), nocturnal diarrhea, a loss of beat-to-beat variability in resting heart rate (86), decreased blood pressure reflexes (20, 33), neurogenic bladder, or megacolon (5). Often, combinations of signs point to lesions of both somatic and autonomic pathways (17). Structural and functional studies of affected nerves showed decreased nerve conduction (88, 89, 99) and axonal transport (6), and endoneurial edema in biopsy specimens (10, 30). Experimentally (90), there has been reported abnormal myelination (98), with decreased myelin density, narrowed axons (161), mononuclear infiltrates and growth cones in regenerating distal axons.

5.Testicular Anatomy and Physiology

The testis, closely surrounded by the tunica albuginea (a dense layer of connective tissue) normally lies within the scrotum (31, 59).

Histologically (32, 35, 59, 229), the testis is

composed of many seminiferous tubules where the germinal epithelium (cells involved in spermatogenesis) as well as Sertoli cells are found. The tubules are surrounded by a smoothly contoured basement membrane delimiting the tubule compartment from the interstial compartment, which is comprised of Leydig cells (the site of testosterone synthesis), blood vessels and lymphatics. The Leydig cells are polygonal, and lie in groups or nests between the tubules. They have abundant acidophilic cytoplasm and large indented nuclei with coarse chromatin condensations and nucleoli. Assessment of human testicular histology uses parameters including mean tubule diameter, tunica propria thickness, and development of seminiferous epithelium and interstitial tissue (312, 313).

At the level of resolution provided by the electron microscope (EM), the Leydig cells lie loosely in the interstitial spaces, and have pseudopod-like microvillous projections from their cell surface. Their nuclei are indented, and pleomorphic, but generally central in the cell. The cytoplasm is largely occupied by smooth endoplasmic reticulum (SER), polyribosomes, and many elongated, dense mitochondria with tubular cristae. Occasional Golgi complexes and their associated vesicles, profiles of rough endoplasmic reticulum (RER), lysosomes, and lipid droplets occupy the remainder of the cytoplasm (36-39). In the normal cell, steroidogenic capacity has been found to be related to SER (34, 62, 145), vesicular mitochondria, and lipid droplets, which constitute an

organelle triad seen in all steroid-producing cells (318, 314).

Fertility in mammals is the endpoint of a complex system of endocrine (55, 56, 61, 67, 72, 74, 75, 77, 82, 87, 95, 131, 154, 204, 215, 221, 264, 293, 316, 317) and paracrine (41-45) regulatory mechanisms which ensure function of both spermatogenic and interstitial compartments. The hypothalamo- pituitary- testicular axis (summarized in Figure 1) stimulates Leydig cells by pituitary luteinizing hormone (LH) production to produce testosterone (61, 68, 69). Testosterone penetrates the seminiferous tubule basement membrane to act on the spermatogenic components (91, 92), and passes into the testicular lymphatics and capillaries to flow to distal target organs including sex accessory glands. The axis also acts directly upon Sertoli cells by pituitary follicle-stimulating hormone (FSH) production which in turn, facilitates spermatogenesis (3). Both interstitial and tubule compartments feed back to the pituitary, testosterone from Leydig cells, and 'inhibin' from Sertoli cells, inhibiting LH and FSH secretion, respectively (57, 58).

In addition, local regulation of the two compartments is thought to occur. Action of the Sertoli cell aromatase enzyme on testosterone produces estradiol, which can act on the cells undergoing spermatogenesis, and on Leydig cells (54, 76-79). As well, the proposed production of LHRH-like material by the rat Sertoli cell is thought to act upon Leydig cells in an initially stimulatory (short-term), then

in an inhibitory (long-term) manner (51) to locally modulate responsiveness to pituitary LH stimulation (46-50, 70, 104) or prolactin receptor numbers (53).

Steroidogenesis in the Leydig cell produces testosterone, primarily along the 5-ene pathway in man (1), but along the 4-ene pathway in rats (263), Testosterone is metabolized to 17-ketosteroids, androstenedione, and androsterone and to dihydrotestosterone (which is itself an essential androgen at sex accessory glands)(3). The enzyme 5alpha-reductase also degrades testosterone metabolites, which are conjugated by the liver and excreted in the urine. Sex steroid metabolism is further influenced by nutritional status of the animal (63).

Testis function is also modulated by influences on LH receptor turnover in the Leydig cell, including thyrotropin (63), prolactin (65, 66, 73, 80, 81, 83, 84, 95-97, 139, 164) and growth hormone (171). Adrenal function (105-107), stress (103, 135), nutrition (71, 63), and neurovascular parameters (59, 93, 94, 100, 101, 102, 299) also influence testicular function.

Diabetic Sexual Dysfunction

Impaired reproductive function in diabetic men has been recognized for over 100 years, and studied as a clinical entity by many authors, but despite this, it is often understated both in incidence and importance (109). Both insulin-dependent and non-insulin dependent diabetics report the occurrence of sexual dysfunction, including both

erectile impotence (169, 203) and infertility, at a younger age, and two to five times as often, as non-diabetic men (108-112, 120, 121).

The prevalence of these complications of diabetes, even despite insulin treatment is surprising: 29.4% of those under 30 years of age, and 72.6% of those over 60 years of age reported the gradual onset of erectile impotence (109). Other figures show up to 50% of all long-term diabetics will have sexual dysfunction of an organic (non-psychogenic) etiology. Clinically, impotence is more noticeable and thus more reportable than reduced fertility, and more studies have addressed this symptom (109).

The prevalence of impaired reproductive function does not correlate with the age of the patient at the onset of diabetes, but coincides with the duration of the disease (109), and with the appearance of other diabetic complications, notably angiopathy (115), and autonomic neuropathy (113, 116-118).

Clinical examination of patients exhibiting diabetic sexual dysfunction will frequently find decreased testicular size with loss of its normal firmness to palpation, a decrease in the size of the prostate, reduced seminal fluid volume (often to zero), and reduced fructose concentration in the ejaculate (109). A study of individuals complaining of infertility, which may preceed erectile impotence (111), showed abnormal sperm count, morphology and motility in one-third of patients examined (114).

Histological study of testicular biopsies (109, 117)

showed atrophy of seminiferous tubules with thickening of basement membranes, normal numbers of spermatogonia, decreased numbers of primary and secondary spermatocytes, frequent lack of mature sperm in tubule lumens, and decreased numbers of interstitial cells. There was also reportedly, a higher than normal frequency of spontaneous abortions in healthy wives of diabetic men without impotence, suggesting an increased proportion of genetically abnormal sperm (109, 122).

Measurement of immunoreactive insulin (IRI) levels in ejaculates of normal and diabetic men found no difference between the two groups. There was no increase in sperm motility, oxygen or glucose consumption, or lactate production when insulin was added directly to washed sperm. No improvement in semen quality or fertility followed 4 months of arginine treatment, which stimulates insulin secretion (119). This was substantiated in experimental work on drug-induced diabetes in rats (242). Thus neither acute metabolic influences on sperm behavior nor chronic changes in glandular or testicular function are directly correlated with decreased insulin levels in diabetes.

A number of clinical studies (summarized in Figure 2) have examined the endocrine parameters in reduced fertility as seen in diabetics (with or without impotence or neurogenic bladder), and in paraplegics who may have testicular histology similar to that in diabetes (57, 218-220). However, for various reasons, the conclusions are not certain. The diabetic subjects of these investigations

fell into many age groups, and the disease severity and duration was highly variable among them. The hormonal profiles often included only the serum testosterone level, which was consistently found to be normal (57, 101, 113, 117, 216, 217), even despite selection of symptomatic subjects. These patients were under insulin treatment, which may have confounded any deviation from normal values. Patients were also selected on the basis of diabetes, impotence or infertility, depending on the study, and this would further confound any trends in the data.

Biochemical studies on diabetic patients, regardless of histological appearance of the testes, have consistently indicated normal testosterone (109, 117-119, 123), prolactin (123), LH (109, 118, 119, 124, 135), and FSH (109, 118, 119, 123-125) in the peripheral circulation. Near-normal increases of LH and FSH in response to exogenous Gonadotropin-releasing hormone (GnRH) (118, 124-126) has also been reported. In light of abnormal spermatogenic capability, these hormonal values are paradoxically normal.

Animal Models of Diabetes Mellitus

To elucidate the details of reduced fertility in diabetes, a number of animal models have been used (138). There are hereditary models including the sand rat (159), genetically selected sucrose fed rats (156), the Chinese hamster (140), and the BB Wistar rat (138, 186). Until the development of the latter, the signs of disease occurring in

the animals did not closely parallel the human disease. The BB Wistar rat (230, 306), however, shows a high penetrance of juvenile-onset, ketosis-prone, insulin dependence. An autoimmune pathophysiology is involved in the disease process, in which the onset can be prevented with total lymphoid irradiation (237). There is also presentation of gonadal dysfunction (186). The monitoring of these animals is very costly of time and money, and unfortunately they were not available at the outset of the present project.

An older model for experimental diabetes is subtotal pancreatectomy (146, 147, 157, 158, 170, 277). It has been applied to dogs (141, 155), rats (146, 147), roosters (149), and cats (148). Rats subjected to this procedure are said to remain normoglycemic and free from disturbances of carbohydrate metabolism for two to three months. This "prediabetic" period is followed by a month of "incipient" diabetes, during which the symptoms of the disease develop. Finally a two to three month period of "manifest" diabetes ensues, with presentation of disease signs (147).

The papers available on the effects of pancreatectomy on the reproductive system report a reduction in fertility (146, 147) as a consequence of degenerated seminiferous tubules, atrophy of the accessory glands, diminished testosterone synthesis, and altered sexual behavior. Also present after the manifestation of diabetes were persistent hyperglycemia, total degranulation of the remaining pancreatic beta cells, and peri- and intra-insular fibrosis.

Drug induction is the most frequently applied tool to

model diabetes mellitus, with both alloxan (142) and streptozotocin (143, 144) acting as diabetogenic agents. Alloxan produces diabetes, but with a very narrow lethal:effective dose ratio in its administration, and its use is therefore becoming less common (172).

Streptozotocin, an antibiotic extracted from Streptomyces acromogenes, is diabetogenic due to its specific, rapid, and irreversible cytotoxic action upon pancreatic beta cells (153, 172). The drug is thought to act by binding to the beta-cell suface receptors for glucose, resulting in degranulation, and necrosis (152, 168). Its action can be blocked by prior administration of non-metabolizable 3-o-methyl-glucose (154), by NADH treatment (thought to rescue a blockage in energy production, 150, 151, 154, 172, 383), or by estradiol injection as little as 5 minutes prior to streptozotocin injection (187). This latter characteristic aids explanation of the higher susceptibility of male animals than female to the diabetogenic agent (185, 308).

There are also thought to be genetic and immune factors operative in determining susceptibility to the drug's action (160, 166, 201, 260, 334), including species sensitivity (192). Nutritional status (267) has also been shown to influence the diabetogenic action of the drug.

Rats treated with this compound, a 1-methyl-nitrosourea group linked to D-glucose (150, 172), exhibit persistent hyperglycemia, polyuria, polydipsia, and weight loss. These signs appear in a dose-related manner (191) after a single

intravenous injection, and are found to be correlated to decreased pancreatic insulin content after such treatment (172). The biological half life of streptozotocin, which is unstable to heat as a powder and to alkaline pH in solution (172), is estimated at 5 minutes in mice (190). Its lethal:effective dose range is much wider than alloxan, which it is now replacing (153, 172).

Despite this preference, several studies have demonstrated extrapancreatic cytotoxicity, causing epithelial pancreatic (175) and mesenchymal renal tumors in rats (182, 195), exocrine pancreas damage (194), and hepatocyte damage in mice (172, 183) and rats (184). The mouse hepatocyte damage occurred with or without successful induction of diabetes (183). The carcinogenic cytotoxicity is thought to result from nucleic acid alkylation (152), which makes the drug useful as a tumoricidal agent. Alloxan toxicity to the proximal renal tubules separate from the diabetic influence on glomerular epithelium and basement membrane has also been reported (188).

Recent work on the testis (189) suggested drug toxicity without diabetogenicity, as serum testosterone levels were reportedly decreased in nondiabetic streptozotocin-injected animals. It is therefore of crucial importance to control for the possibility of streptozotocin influences, apart from those of experimental diabetes, on reproductive function.

The Testis In Streptozotocin-Diabetic Rats

The structure of testes from streptozotocin-treated rats has been examined at both histological and ultrastructural magnifications. These studies have been made on Wistar or Sprague-Dawley male rats. Unlike the human testis, spermatogenesis in the rat occurs in "maturation waves" along seminiferous tubules (193, 196, 198-200, 251), which can be staged by microdissection under transillumination (238) and on histological sections. Fourteen such stages have been classically described, their relative frequency thought to be a marker for the duration of each stage in the meiotic division of spermatogenesis (196). The entire process of spermatogenesis in Long Evans rats takes 48 days, requiring 4 complete cycles through the fourteen stages (197).

Histological investigations of the testis in diabetic rats revealed sloughing (174, 178, 179) of the seminiferous tubule epithelium (primary spermatocytes and premature spermatids) and decreased numbers of Leydig cells (174, 178) accompanying reduced testis and seminal vesicle weight. The yield of Leydig cells in cell isolation procedures from testes of streptozotocin-diabetic males was decreased (224). Also noted were thickening of seminiferous tubule walls and occasionally, complete cessation of spermatogenesis, with those tubules containing only Sertoli cells and a few spermatogonia and primary spermatocytes. These changes were noted to be greater in severity and frequency with increased duration of diabetes, but because of large variation between

animals, they were not significantly correlated with parameters used to monitor body weight changes or hyperlycemia in diabetes. The animals were also found to have decreased fertility (181), but normal sexual behavior and penile reflexes (202).

Staging of spermatogenesis has been only qualitatively examined (178) with respect to decreased tubule cross-sectional area. Decreased numbers of sperm and spermatids were seen with mild reductions of tubule size, while spermatogenesis appeared to be blocked at spermiocyte stage I/II with severely reduced tubule size.

Animal models of diabetes mellitus have shown patterns of secondary or tertiary hypogonadism, or their combination, as summarized in Figure 3. Serum testosterone levels in these investigations showed a consistent decrease, marking gonadal hypofunction in the presence of reduced gonadotropins and lesions of the hypothalamic arcuate nucleus (143, 209).

In other work (127), the serum corticosterone level was increased in diabetic rats, implicating stress in the gonadal failure, although stress has been reported to have no effect on germ or interstitial cells of the testis (339). Stress of many types, including anesthesia and burns, has been shown to increase serum glucose levels in man (3). In many formats, stress has also been shown experimentally to affect blood hormone levels, most notably those of prolactin, LH, FSH, and testosterone (128-130, 132-135), apart from influences on epinephrine (136) and adrenal

steroids (135). These effects are thought to be coordinated at and above the level of the hypothalamus (131, 137).

Biochemical studies in streptozotocin-induced diabetes showed decreased LH (181, 208, 216, 225), FSH (176, 208, 221), serum testosterone (176, 181, 189, 208, 223), testicular testosterone (223), and testosterone synthesis *in vitro* (224). Pituitary LH content and hypothalamic GnRH content were similar to control levels (181). The binding of LH to its receptor on Leydig cells was found to be of normal (207), increased (226), or reduced (221) capacity. In the latter case, the receptor capacity was restored by insulin treatment (221). Administration of GnRH to diabetic animals stimulated normal LH levels, suggesting loss of sensitivity or responsiveness of the hypothalamus to the decreased gonadal function in untreated animals (207). This was confirmed by a study in which testosterone implants were found ineffective in suppressing LH in untreated diabetic animals, or in restoring accessory gland weights in insulin treated diabetic rats (208). Insulin treatment of diabetic animals did not increase testicular testosterone, nor did it improve fertility. Chorionic gonadotropin (hCG) combined with insulin treatment restored fertility to near-normal values, and testicular testosterone to greater than normal values in that study (206). In other investigations, testicular testosterone was restored by two weeks of insulin therapy, as was testicular androgen binding protein (ABP) content (223), although fertility was not tested.

At the EM level, Leydig cell alterations after 4 weeks (176, 177) and 12 months (209) showed many lipid droplets and a reduced amount of SER. In comparison (177), control animals were found to have abundant SER and no lipid inclusions. The cellular changes were interpreted as a loss of steroidogenic activity due to insulin deficiency, based on a re-establishment of normal cell profiles with insulin treatment. Serum gonadotropin and testosterone were not measured in this work, and no examination of cytotoxicity resulting from streptozotocin apart from the effect of diabetes was made.

To this end, a small study (189) examined serum testosterone and Leydig cell ultrastructure in both diabetic and nondiabetic Sprague-Dawley rats that received streptozotocin. Although hyperglycemia developed in only four out of six animals, all six showed a decline in serum testosterone from their pre-injection level. Morphometry by computerized planimetry of Leydig cell cytoplasm also showed alteration regardless of whether or not the drug-injected animals were diabetic. The alterations included a decrease in mitochondrial size and an increase in the size of lipid droplets, with no change in their numbers per micrograph. SER profiles tended to be less dilated than in those from control animals. Thus, the suggestion of streptozotocin toxicity to the Leydig cell has been encountered in steroidogenic organelles and in their product.

OBJECTIVES

In light of the above studies, the present investigation was undertaken with the following objectives.

1. To describe and quantify testicular histology, parameters including tubule diameter, blood vessel density, and the stages of the spermatogenic cycle.
2. To examine and quantify Leydig cell organelle structure, and relate it to measured metabolic and hormonal parameters.
3. To investigate the possible site of the reduction of testosterone synthesis in the steroidogenic pathway, by developing a method of labelling $\Delta 5,3\beta$-hydroxysteroid dehydrogenase (HSD) activity for examination by electron microscopy.
4. To measure serum corticosterone in order to elucidate a possible stress-induced change in testicular morphology and physiology.
5. To control for possible streptozotocin toxicity and separate it from the effects of diabetes on the various parameters studied.
6. To briefly investigate strain differences in the parameters of diabetic testicular function.

EXPERIMENTAL DESIGN

In order to investigate strain differences, the albino Wistar rat and the black, hooded Long Evans rat were used.

To enable algebraic separation of the influences of diabetes, streptozotocin, semistarvation (seen as protein-calorie malnutrition in untreated diabetes), and insulin treatment, five groups of Long Evans rats were incorporated. This was possible only because not all the animals who received streptozotocin injections (noted by Thliveris *et al*, 189) became diabetic. The nondiabetic but drug-treated animals thus constituted internal controls in the model.

Algebraic contrast expressions (L) for four such separate effects were derived using orthogonal transformations of group means (210),

$$L_{DM} = 1/4[\ C + S_{not} + S_{dmi} + STV\ ] - S_{dm}$$

$$L_{S} = 1/2[\ S_{dmi} + S_{not}\ ] - C$$

$$L_{STV} = 1/3[\ C + S_{dmi} + S_{not}\ ] - STV$$

$$L_{I} = S_{dmi} - S_{not},$$

where C = citrate-control

S_{dm} = streptozotocin-injected diabetic

S_{not} = streptozotocin-injected but non-diabetic

S_{dmi} = streptozotocin-injected diabetic treated with insulin

STV = semistarved

group means.

The standard error of each of these expressions was determined from the error mean square term in the analysis of variance (made on each parameter studied in the five groups), the root of the sum of coefficients in each contrast expression, and the harmonic mean of the group sizes.

MATERIALS AND METHODS

Animal Treatment and Care

Adult male Long Evans (black hooded) rats weighing 220 to 270 grams were allowed to acclimatize in separate mesh cages with food (Wayne F6 Rodent Blox) and water *ad libitum* for five days in standard laboratory conditions. They were then placed in metabolic cages for 24 hour collection of urine, and returned to their respective cages. After 24 hours, they were lightly anesthetized under ether, and a collection of blood by tail snip was made for determination of blood glucose levels, using the Ames glucometer, and baseline chemistry (including blood urea nitrogen, serum creatinine, and electrolytes).

Still anesthetized, rats were randomly assigned to one of five groups, as summarized schematically in Figure 4. Six animals received an injection of 0.5 ml of cold citrate buffer (pH 4.5, 0.1 M) via the tail vein. These animals constituted the citrate-control group, and were normoglycemic (blood glucose 6.82 $\pm$ 0.9 mmol/L). Eighteen animals received an injection of 40 mg/kg Streptozotocin (Sigma) dissolved immediately beforehand in the same amount of citrate buffer

vehicle, also via the tail vein. Three days later these drug-injected animals fell into two categories when blood was collected for glucose determination: rats either remained normoglycemic (7 animals), called streptozotocin-injected but not diabetic, or they were hyperglycemic (blood glucose > 22.3 mmol/L, 11 animals) . This latter group was subdivided randomly. Five animals were placed in an untreated group, called the streptozotocin-injected diabetic group. These three groups of animals were kept under standard laboratory conditions for three months with food and water *ad libitum*. The remaining six diabetic animals were placed in an insulin-treated group, referred to as the streptozotocin-injected, diabetic plus insulin treated animals. The insulin dosage (3 IU Ultralente Insulin, Connaught) was determined in a preliminary trial (appendix 1). These animals were given insulin injections daily at 0800 hours, and were kept with food and water *ad libitum* during the day (0800 - 1700 hours). During the night (1700 - 0800 hours), their food was restricted to four pellets (1.43 $\pm$ .15 grams per pellet), and water was *ad libitum*. Six additional animals, referred to as semistarved, were assigned to a food restriction program. Their food allowance was titrated weekly in order to match their weight changes to those of the untreated diabetic animals.

After one week, all animals were placed in metabolic cages for overnight collection of urine output. They were returned to their separate cages and food regimens for

twenty-four hours, and then weighed and bled under light ether anesthesia. These monitoring procedures were repeated every two weeks for the first month, and then every month for the next two months, with the exception of the semistarved group, as specified above. The experimental treatment period was three months. Thirty-six hours prior to sacrifice, animals were again placed in metabolic cages for overnight urine collection, and then returned to individual cages for twenty-four hours.

To verify that the insulin-treated diabetic animals were indeed insulin dependent at the end of three months, these animals were deprived of their injection of insulin on the day before sacrifice.

During the study, one diabetic insulin-treated animal died of pneumonia. One streptozotocin-injected nondiabetic animal was excluded from the study when a 72 gram renal tumor was noted at the time of sacrifice. All other animals were active and appeared healthy.

The number of Long Evans rats in each group was as follows: 6 citrate-control, 5 streptozotocin-injected diabetic, 6 nondiabetic streptozotocin-injected, 5 insulin-treated streptozotocin-diabetic, and 6 semistarved.

The subtotal pancreatectomy model of diabetes was initially incorporated as a non-drug comparison to streptozotocin-induced diabetes in this study. The following problems were encountered, pursuant to this aim.

Due to the unpredictable 6-7 month lag time between

surgery and manifest diabetes, the animals were pancreatectomized while young (approximately 80 grams body weight). This was necessary in order to avoid final examination of the testicular anatomy and physiology in senescent rats, which typically demonstrate gonadal failure and lack of pituitary response (342, 344-346, 348, 349). Use of such young animals was also needed in order to age and weight match the pancreatectomized rats to controls.

The procedure of pancreatectomy in the rat is difficult (158), both because the animals are small, and because of anatomical features specific to the rat (328). The rat pancreas is not retroperitoneal, as in man, but is dispersed in the mesentery of stomach and small intestine, occasionally as distal as the proximal jejunum. In addition, it is variably traversed by the common bile duct. Techniques to dissect pancreatic lobules away from mesentery wihout damaging duodenal and gastric vessels or bile duct (158, 146, 147, 157, 277) were practised, with noticeable improvement in yield of removed pancreatic tissue. However, only with splenectomy included in the procedure, was a large proportion of pancreas removed. Subsequently, there were very few post-surgical deaths, from bowel infarction with or without peritonitis, but only 4 of 23 became hyperglycemic, with lag times ranging from 2 weeks to 9 months.

Of animals who did not develop hyperglycemia, the pancreatic tissue at post mortem had apparently redeveloped from tissue remnants along gastric and duodenal vessels.

Histological examination of this tissue showed many islets of Langerhans, and the presence of functional beta cells was marked by positive staining for insulin with the acid aldehyde-fuchsin| method.

Interestingly, Wistar rats became hyperglycemic and polyuric much more often than Long Evans animals (4 out of 8, as compared with 4 out of 23). However, the Wistar rats also died more frequently without surgical or respiratory complications (3 out of 4 hyperglycemic Wistar rats within one month of pancreatectomy, as compared to zero out of 4 hyperglycemic Long Evans rats).

Although pancreatectomy is a non-toxic (that is, non-drug) induction of diabetes mellitus, the procedure involves removal of the exocrine pancreas as well as entire islets of Langerhans. Thus, digestive enzymes, glucagon, and somatostatin would also be deficient in this model, and a sham-operated group would be required to control for the effects of the surgical procedure on the animals. For all of the above reasons, this model of diabetes was excluded from the study.

Perfusion Procedure

Rats were sacrificed between 0800 and 1000 hours. They were weighed and lightly anesthetized under ether. Laparotomy was made by a midline incision to |expose the abdominal aorta which was ligated just caudal to the diaphragmatic incisura. The aorta was then cannulated between the point of fixation

and the celiac trunk with PE #90 tubing, which was connected by a three-way stopcock to 50 cc of phosphate buffer (pH 7.4, 0.1 M, 38°C.) at one port, and to 300 cc of 1% glutaraldehyde in phosphate buffer (pH 7.4, 0.1 M, 38°C.) at the other. Perfusion was immediately begun with buffer at the same time as the left renal vein was cut to allow perfusate outflow. This cleared the blood from the abdominal vessels, including those to the testes. The thorax was then rapidly opened, and blood was drawn from the left ventricle into a 5 cc syringe. The perfusate was then switched to the glutaraldehyde fixative, and allowed to flow such that the 300 cc would feed by gravity in 30-40 minutes. Immediately after sampling, a drop of the blood was placed on a dextrostix for use in the Ames glucometer, two capillary tubes were filled for hematocrit measurement, and the remainder was injected into a vacutainer tube for the separation of serum from blood cells during centrifugation.

Strain Differences

In order to compare the effects of streptozotocin diabetes on two different rat strains, a group of Wistar (Charles River, 250 - 300 grams) rats were obtained. A preliminary trial of streptozotocin injection using 40 mg/kg and 55 mg/kg on four and five animals respectively, produced only two hyperglycemic animals. Seven animals were then injected with a higher dose of streptozotocin (65 mg/kg) in cold citrate buffer (pH 4.5, 0.1 M), and when tested three

days later, all had been made hyperglycemic. Three more animals then received the drug in that dosage and all of those became hyperglycemic. The urine output of these animals rapidly became very high, and the animals were ill-looking and irritable. Two rats died within the first week in very poor condition. One died after each of three and four weeks following a few days of nasal discharge and inactivity, and post mortem examinations showed widespread lung consolidation. The Wistar rats were kept in a separate animal room, in an attempt to minimize further respiratory infection. After three months of monitoring, using the same schedules as with the Long Evans rats, three of the animals were irritable and cyanosed peripherally, and one had a respiratory infection. Seven animals received citrate buffer only (0.5 ml, 0.1 M, pH 4.5), and one died after 6 weeks, showing the same lung consolidation following nasal discharge. There were, therefore, at the end of the three month experimental period, six citrate injected control animals and three diabetic Wistar rats for subsequent assessment.

Chemistry

Routine chemistry on blood and urine samples determined the following parameters, used to monitor animal status: blood glucose, urine volume, hematocrit, blood urea nitrogen, serum and urine electrolytes (382), and serum and urine creatinine (382, 375). These studies were carried out in the Department of Pharmacology at the University of Manitoba, courtesy of Mr.

D. Jones.

Tissue selection and handling

Pancreas: After perfusion, a sample of tissue from the head of the pancreas was taken for light microscopy. Separate, sequential sections from this material were stained in Mallory-azan which stains alpha cell granules red, and in Aldehyde acid fuchsin which stains beta cell granules purple. A third section was stained with hematoxylin and eosin for routine histological examination.

Testis: After perfusion, the testes were dissected free from the epidydimus, and weighed. One was bisected longitudinally through the hilum, and decapsulated. Half the organ was taken for light microscopy, and blocked such that sections were made from the whole cut face. One millimeter slices were made of the remaining organ half, and blocks were diced from area-weighted regions of these slices (231, 232). Once selected, these blocks were combined, and randomly subdivided into three tubes. One tube was routinely processed for electron microscopy (see below), one tube was incubated for hydroxysteroid dehydrogenase activity and diastase action, and the third tube was incubated only for diastase action on the tissue. These histochemical incubations were made according to a predetermined process, established in a study on tissue from 15 animals (1).

Liver and Adrenal Cortex: These tissues were sampled for electron microscopy, and served as control tissues for the diastase and hydroxysteroid incubation respectively (1).

Tissue Preparation

For light microscopy, tissues were fixed in Bouin's fluid, dehydrated, and paraffin embedded. Sections were cut at a thickness of 5 microns, and stained with hematoxylin and eosin, or with the special stains for the pancreas (see above). Photography was done on a Nikon Optiphot microscope.

For electron microscopy, the routine procedure included a second period of fixation in the same proportions as the perfusate, for 2 hours. Portions of adrenal, liver, and testis tissues were diverted into the hydroxysteroid dehydrogenase and diastase procedures (see below). After this processing, those blocks as well as the unincubated remainder were postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol, methanol and propylene oxide, then infiltrated with and embedded in epon 812.

After block trimming, sections were cut on a Porter-Blum MT2-B Ultramicrotome at 1/2 micron thickness, and stained with toluidine blue for orientation. Blocks were retrimmed to precise tissue areas (for example, interstitium of testis) and cut for electron microscopic examination (341). These were collected on cleaned, uncoated copper 300 mesh grids, and stained with saturated aqueous uranyl acetate (45 minutes) and lead citrate (3-5 minutes). Grids were examined and

photographed using a Philips 201 electron microscope.

Histochemical Incubation

In order to label the site of activity of the enzyme $\Delta 5,3\beta$-hydroxysteroid dehydrogenase (HSD) for electron microscopic examination, the original light microscopy method (340) was adapted (1). Insoluble copper ferrocyanide was the reaction product of enzyme activity on dehydroisoandrosterone dissolved in dimethylsulfoxide, in the presence of nicotinamide adenine dinucleotide (NAD), potassium ferricyanide, copper sulfate and sodium citrate. The concentration of glutaraldehyde fixative was established at 1%, due to abolition of enzyme activity with higher concentrations. Negative controls for the reaction by modification of the incubation medium, included omission of substrate or cofactor, and addition of cyanoketone, an enzyme inhibitor.

Diastase (alpha-amylase) incubation was appended to the HSD incubation process, due to the similarity of particulate glycogen in control Leydig cells with the HSD reaction product.

Liver tissue was subjected to diastase incubation to determine the thoroughness of glycogen removal, and adrenal cortex was incubated for HSD activity as a positive control for the process.

The HSD and diastase incubations were carried out after glutaraldehyde fixation, and prior to postfixation with osmium

tetroxide.

Tissue Analysis

Coded slides and grids were examined to control for possible observer bias. Histology of the pancreas checked the status of beta cells in islets of Langerhans, by examination of islet architecture and staining.

Light microscopic study of the testis examined histopathology of germinal epithelium and interstitium, tubule diameter (Figure 4), blood vessel number (Figure 5), and stages of spermatogenesis. For determination of testis tubule diameter, slides were projected at 50 X magnification, and measurements were made from five preselected, area-weighted (232) regions of the organ section. In each area, the smallest diameter for each of 20 patent tubules was measured. To count blood vessels, slides were viewed at 100 X magnification, and five 1 mm^2 preselected, area-weighted (232) regions of cross- sectioned tubules were scanned through an ocular grid (Zeiss). All vessels in the outlined area were counted, respecting the "forbidden zone" (232), and this was adjusted to a number per tubule.

Electron microscopic study of adrenal tissue confirmed positive incubation for HSD activity. Liver sections allowed a check for completeness of glycogen removal.

Six blocks of testis from each animal were sectioned, two blocks selected randomly (232) from each of the three sets of testis blocks. Testicular tissue was primarily examined for

ultrastructural norms and departures therefrom, but adjacent cells of the germinal epithelium, including Sertoli cells were also viewed when they appeared in sections. Leydig cells from each section were randomly photographed at various magnifications, but especially at 1285X, such that photographic enlargement gave a final magnification of 10,000X for further study and morphometry. These prints were coded, and 10 prints from each animal were selected by computer-generated random number sequences for determination of: (1) average single, and total mitochondrial area, and number of mitochondria, (2) average single and total lipid droplet area, and number of droplets, and (3) total cytoplasmic area. These quantities were measured on an Apple II graphics tablet (384). Areal and numerical density of mitochondria and lipid droplets were determined by dividing, respectively, total mitochondrial area and mitochondrial number, by the total cytoplasmic area of the ten prints. Point counting of these same prints determined mitochondrial, smooth endoplasmic reticulum, rough endoplasmic reticulum, Golgi, and cytoplasmic matrix volume densities according to the principle of Delesse (231), where d = 1 cm, on 100 grid points per micrograph.

Leydig cells from blocks incubated for HSD activity were examined for frequency of electron dense reaction product deposit, and expressed as a proportion of cells stained.

Radioimmunoassay

Blood collected at the time of sacrifice was separated, and serum stored at -80°C. until thawing for assay. Radioimmunoassays of testosterone, estradiol and corticosterone were made in duplicate in single runs, by the Endocrinology laboratory of the Department of Medicine in the Health Sciences Centre, courtesy of Dr. C. Faiman.

Testosterone values were obtained using a Radioassay Systems Laboratories kit, modified to include an extraction procedure (233). The assay for serum corticosterone used charcoal-stripped serum standards (234). Estradiol assay was made according to EIR kit #155 (Radio Isotopen Service, Eidg. Institut fur Reaktofurschung, 5303 Wurenlingen, Schweiz).

Radioimmunoassays for rat LH (235) and rat FSH (236) in serum were carried out in single runs by Hazelton Laboratories, Diagnostic Assay Dept., 9200 Leesburg Turnpike, Vienna, Virginia, 22180. The FSH methodology used NIH FSH-RP1 as a reference preparation, with a minimum assay sensitivity of 114 ng/ml.

Statistical Analysis

Analysis of the data generated in this study included parametric analysis of variance (ANOVA) and Tukey's procedure for intercomparison of the 5 Long Evans group means. As well, orthogonal contrasts of means on the basis of single chosen factors (diabetes, streptozotocin, semistarvation, insulin treatment) were carried out (210).

For comparison of the 2 Wistar groups, non-parametric Mann-Whitney U tests were made.

A number of parametric correlation matrices and non-parametric Spearman rank order correlations were made between pairs of Long Evans and Wistar parameters, respectively. Multiple regression analyses were also made of the data, where appropriate.

The frequency distribution statistics of spermatogenic stages, tubule diameter histograms, morphometric point-counts and HSD staining were analysed using the Chi-square (X^2) routines.

Finally, two-way ANOVAs compared Wistar with Long Evans data.

The statistical computer programs in the "Stats Plus" (211) and "ANOVA II" (212) packages supplied by Human Systems Dynamics were used for some of these computations according to standard statistical procedures (210, 213, 214, 363).

RESULTS

LONG EVANS ANIMALS

Body Weight

Final body weights and total change in body weight are listed in Table 1. By Tukey's procedure, it was shown that the final weight of diabetic animals was significantly lower than that of insulin-treated diabetics ($p<.05$), and of non-diabetic but streptozotocin-injected animals ($p<.01$). Final mean weight of the semistarved group also was reduced compared to weight of those two latter groups, with the same level of significance.

Body weight change during the experimental period showed significant weight loss ($p<.01$) by the semistarved group compared to the other three non-diabetic groups, as well as a weight loss by the diabetic group compared to the same three groups. This indicated that the weight loss associated with this degree of food restriction did match diabetic weight loss, and that insulin treatment prevented that weight loss.

Orthogonal contrasts of both final weight and change in weight measures showed that diabetic and semistarvation effects both contributed to variance in body weight measures ($p<.01$). Streptozotocin treatment itself was found to contribute a significant positive influence to increase body weight ($p<.05$).

Blood Glucose

Mean pretreatment blood glucose for all Long Evans animals included at perfusion (n=26) was 6.8 ± 0.26 mmol/L (mean ± standard error). Final blood glucose group means are given in Table 1. Tukey's procedure indicates that the semistarved animals were able to maintain normoglycemia, and that insulin treatment of diabetic animals was able to maintain glycemia at levels not significantly higher than in control animals. Non-diabetic streptozotocin-injected animals also did not have significantly higher blood glucose levels than control animals.

Examination of orthogonal contrast expressions indicated that while diabetes had the greatest impact in raising normal blood sugar ($p<.001$), streptozotocin did itself raise glucose levels by this calculation ($p<.05$). As well, the overall effect of semistarvation decreased blood glucose ($p<.01$), likely due to the reduced variation within the food-restricted group of animals.

Non-parametric Kruskall-Wallis analysis of these values shows significant differences among them ($p<.001$), with rank sum ordering from greatest to least as follows: diabetic, insulin-treated diabetic, non-diabetic streptozotocin-injected, citrate-control, and semistarved groups.

Hematocrit

Comparison of group means (Table 1) using Tukey's procedure showed that diabetic rats had significantly lower

hematocrits than both control animals ($p<.05$), and insulin-treated diabetic animals ($p<.01$). There was no significant difference between control and any of the other group mean values.

Orthogonal contrasts showed that only diabetes had a significant effect to decrease hematocrit from control values ($p<.01$).

Urine Volume and Glucose

Mean urine output (Table 2) studied using Tukey's procedure showed significantly increased output ($p<.01$) in the diabetic group over the four other groups. There was no difference between any of the groups which were not diabetic. Orthogonal contrasts showed these changes were significant only for the effect of diabetes to increase urine output ($p<.001$).

Urine glucose measurements recorded trace or negative amounts of glucose in the urine of citrate-control, non-diabetic, insulin-treated diabetic, and semistarved animals. The untreated diabetic animals had levels of 3% or greater urine glucose, from day 3 after streptozotocin injection until sacrifice.

Creatinine Clearance and Blood Urea Nitrogen

The final creatinine clearance (CCr) and blood urea nitrogen (BUN) values are listed in Table 2. Tukey's multiple range analysis showed significant reduction in CCr

only in the diabetic group when compared to the non-diabetic streptozotocin-injected group ($p<.05$). Orthogonal contrasts revealed that the effect of diabetes was to significantly decrease ($p<.01$) creatinine clearance. The nondiabetogenic effects of streptozotocin itself and of semistarvation were not statistically significant.

Tukey's procedure applied to the BUN values showed that insulin-treated diabetic values were significantly lower than control ($p<.01$) and untreated diabetic ($p<.01$) values. Orthogonal contrast analysis revealed significant overall effects of both diabetes to increase ($p=.02$) and streptozotocin to decrease ($p<.01$) BUN in this study. Insulin itself was found to significantly decrease BUN from control values ($p<.02$). There was no effect of semistarvation on BUN demonstrated.

Testis Weight

Study of group means from single or combined testicle weight, and total testis weight per 100 grams of body weight (Table 3), and total testis weight as a fraction of body weight change showed no significant difference between any two means, despite the apparent trend to decreased weight among diabetic animals. Orthogonal contrasts also showed that neither diabetes, streptozotocin, nor semistarvation had a significant effect on testicular weight. Testis weight per 100 grams body weight was increased by the effect of semistarvation ($p<.05$), likely due to the decreased body

weight in the semistarved group.

Testicular Histology

Testicular histology of the citrate-injected control, the non-diabetic drug-treated group and the insulin-treated diabetic group were similar, and showed seminiferous tubules lined by germinal epithelium at various stages of spermatogenesis (Figure 6a-6d). A thin, regular basal lamina separated the tubule epithelium from the interstitum composed of Leydig cells and blood vessels in loose array between tubule profiles. In testes from diabetic animals (Figure 7a, 7c,and 7d), there was frequent sloughing of pachytene spermatocytes into the tubule lumen resulting in a thinned germinal epithelium. This was most apparent at stages IX-XII and II-IV where the heads of maturing spermatids were unusually close to the basal lamina, and the spermatocytes and early spermatids did not form regular 'columns' between their flagella. Some tubules at stages IV-VIII had very large round, vesicular bodies in their lumen. Testes of semistarved animals exhibited the pattern seen in control groups, but with occasional pachytene spermatocyte sloughing. The basal lamina in the diabetic group appeared irregular in thickness, and lacked the smoothly rounded profile seen in the control groups, but a more precise assessment of this component was made at the higher magnification of electron microscopy (see below).

Tubule diameter and blood vessel data are listed in

Table 3. For the five groups analysed by ANOVA and Tukey's procedure, there was a significant reduction in tubule diameter in comparisons between the diabetic and the citrate-injected control group ($p<.05$), and between the diabetic and non-diabetic streptozotocin-injected group ($p<.01$). Chi-square analysis of histograms constructed from all diameter measurements in each group (one hundred per animal) showed a significant ($X^2=438.9$, $df=36$, $p<.001$) shift to smaller tubule diameters in untreated diabetic animals. Blood vessel number per tubule showed no significant difference between any two groups by Tukey's procedure. There was, however, a significant negative correlation between tubule diameter and blood vessel number per tubule ($r=-.67$, $p<.01$, $v=24$), and a significant positive correlation of tubule diameter and testis weight as a fraction of proportional body weight change ($r=.48$, $p=.01$, $v=24$). Multiple regression analysis indicated significant prediction ($r=.49$, $F(2,23)=3.6$, $p=.04$) of testis weight as a fraction of body weight change by tubule diameter and blood vessel number criteria.

Diabetes had the only significant effect on either variable in examination of orthogonal contrasts, decreasing tubule diameter (mm x 50) by $1.52 \pm .8$ ($p<.001$), and increasing blood vessel number per tubule by 1.4 ± 1.1 ($p<.02$). These are approximately -10% and +30% changes respectively. Streptozotocin toxicity, as an influence on either parameter, was excluded.

The frequency distribution of seminiferous tubules at stages I, II-III, IV-VI, VII-VIII, IX-XI, XII-XIII, and XIV are given in Table 4a. Chi-square analysis of the raw frequencies shows significant (X^2=60.2, df=24, $p<.001$) dependence of the distribution on the animal treatment groups. Within each treatment group there were significant individual variations in stage frequency distributions only in the untreated diabetic ($p<.005$) and semistarved ($p<.01$) groups.

Analysis of the variance between frequencies of each stage for individual animals, using the nonparametric Kruskall-Wallis test, showed significant ($p=.008$) variation only at stage IX-XI. This may indicate the initial site of a block in spermatogenesis, and account for the sloughing of pachytene spermatocytes into the tubule lumen from these stages, and for the epithelial thinning seen in stages IX-XII and II-IV.

Testicular Ultrastructure

While the main emphasis of the present study focussed on the structure and function of the Leydig cell, observation of other features of the interstitium, the seminiferous tubule germinal epithelium, and the tubule basal lamina were made. These will be considered separately.

Leydig Cell: Qualitative examination of Leydig cells from citrate-control animals was summarized by the following description. Pleomorphic in shape, the perimeter of the cell was marked by many pseudopod-like microvillous projections, up to 3 microns long, in all directions from the cell body. These projected into the interstitial space and often contacted other Leydig cells or their projections (Figures 8 and 9). The space between adjoining Leydig cell membranes could occasionally be seen to contain dense material. Leydig cells were very rarely sheathed by a basal lamina.

The indented cell nucleus (often bilobed) had heterochromatin condensations along most of its internal nuclear membrane, and a prominent nucleolus appeared in many nuclei. The nucleus was usually central in the cell, and occupied approximately 1/4 to 1/3 of the cell area (Figures 8 and 9).

Cytoplasmic organelles in Leydig cells (Figure 10) included Golgi complexes, mitochondria, SER, and occasional RER profiles, centrioles, lysosome-like structures, glycogen, and multivesicular bodies. Very infrequently, a short cilium protruded from the cytoplasm. The Golgi complexes consisted of 4 to 6 stacks of elongated vesicles, and their associated round vesicles at either end. Often, two or more Golgi complexes appeared in an arc in the cytoplasm. Mitochondria were often dense in comparison to the cytoplasmic matrix. They were elongated or round in

sections, and dispersed throughout the cytoplasm. Mitochondrial cristae were usually tubular, although occasional cells had mitochondria with sparse plate-like cristae.

SER was very prominent in Leydig cells from control animals, and was not usually dilated by electron lucent material. Although composed of fine tubules or saccules, occasionally the SER appeared in whorls, with the tubules in longitudinal section seen in concentric arcs. The whorled pattern is characteristic of perfusion fixed Leydig cells (331). Polyribosomes and single ribosomes were seen in the cytoplasmic matrix.

Glycogen, demonstrated by its removal after diastase digestion, was often densely deposited in the cytoplasmic matrix. Occasional single or grouped RER profiles, lysosome-like bodies of variable density, and single or paired centrioles were seen.

Leydig cells from diabetic animals (Figures 11 and 12), appeared smaller in area, although this was not quantified. The SER was frequently seen in whorls, and often prominent in close juxtaposition to the outer mitochondrial membrane. Mitochondria were frequently bilobed and 'folded,' with an attenuated central region. Dense mitochondrial granules were observed more often than in mitochondria from control animals. Glycogen was dense in some Leydig cells, and lipid droplets appeared more numerous and larger than in Leydig cells from control animals.

Of note, the Leydig cells of one animal appeared patchy, in that the cytoplasm seemed negatively stained in contrast to a very dense mitochondrial matrix.

The Leydig cells from non-diabetic streptozotocin-injected animals were similar to those from control animals, with two exceptions: lipid droplets were more commonly seen, and SER appeared more sparse, with its tubules occasionally dilated. Glycogen was abundant in many cells.

In Leydig cells from insulin-treated streptozotocin-injected diabetic animals, a number of differences were observed from those in control animals (Figure 13). There appeared to be a decrease in the density of SER packing in the cytoplasm, with many cells having attenuated and widely-spaced stack-like arrays of SER. There also appeared to be much glycogen present, and in some cells mitochondria had a very dense matrix, which highlighted their tubular cristae. Lipid droplets were very infrequently seen.

In Leydig cells from semistarved animals (Figure 12), RER was more prominent than in other groups, often appearing in well-developed stack formations. Both mitochondria and lipid droplets seemed smaller than in Leydig cells from control animals.

Morphometry of the Leydig Cell: Morphometric analysis of Leydig cell cytoplasmic structures was carried out in two ways. First, computerized planimetry gave exact measures of mitochondrial features [single mitochondrial area (MA),

mitochondrial areal density in cytoplasm (MAT/CAT), and mitochondrial numerical density in the cytoplasm (MN/CAT)], and of lipid droplets [single lipid droplet size (LS), lipid areal density in cytoplasm (TL/CAT), and lipid numerical density in the cytoplasm (LN/CAT), where CAT=total cytoplasmic area]. These parameters are given in Table 5.

The reproducibility of measurements made on the graphics tablet (computerized planimetry) was tested by repeated tracing of a single area, designated as one square unit. The mean and standard error of 50 tracings was found to be 1.007 $\pm$ .006 square units.

Tukey's procedure revealed no significant difference between group means for any of the above mitochondrial parameters. By orthogonal transformation of these data, the effect of diabetes was seen to increase mitochondrial numerical density ($p<.05$).

With respect to lipid numerical density, Tukey's procedure showed that density was significantly increased over control levels in diabetic ($p<.05$) and semistarved ($p<.01$) groups. Lipid numerical density was also increased in Leydig cells from semistarved rats over levels measured in non-diabetic streptozotocin-injected ($p<.01$) and insulin treated diabetic ($p<.01$) animals. No significant differences were seen between groups for the lipid droplet size or lipid areal density measures. Orthogonal contrasts revealed an overall effect of diabetes to increase ($p<.05$) lipid numerical density.

The separated effects of semistarvation were seen to increase mitochondrial areal density ($p<.02$), and decrease single mitochondrion area ($p<.02$). Semistarvation effects also produced a significant increase ($p<.001$) in lipid numerical density. Streptozotocin had no significant effect on any of these parameters.

Also of note was a significant increase in the standard deviation of single mitochondrion area within the animals of the semistarved group (Tukey's procedure: $p<.05$, and also by orthogonal contrasts: $p<.01$).

Further analysis of these data in tests of correlation found that total lipid areal density was significantly correlated ($r=.76$, $p<.001$, $v=24$) with single lipid droplet size, and with lipid numerical density in the cytoplasm ($r=.66$, $p<.001$, $v=24$).

Two tests of mitochondrial data were not significant: mitochondrial areal density was not significantly correlated to single mitochondrion area ($r=.37$, $p=.06$, $v=24$) or to mitochondrial numerical density ($r=.19$, $p=.36$, $v=24$). However single mitochondrion area was significantly negatively correlated to mitochondrial numerical density in the cytoplasm ($r=-.81$, $p<.001$, $v=24$).

The lipid areal density measurements were found to be significantly negatively correlated to change in body weight ($r=-.42$, $p=.04$, $v=24$), and to the diameter of seminiferous tubules ($r=-.39$, $p=.05$, $v=24$).

The second morphometric approach made to Leydig cells was by point counting of cytoplasmic contents, including RER, SER, mitochondria, Golgi, and cytoplasmic matrix. This data is presented in Table 6.

Chi-square analysis of the subtotal frequencies showed highly significant (X^2=186.3, df=16, $p<.001$) treatment effects. Partitioned chi-square analysis of SER vs. 'other' subtotal frequencies also showed a significant contribution by diabetes ($p<.005$) to increase SER areal density, and of the insulin-treatment of diabetes ($p<.005$) to decrease SER areal density.

Comparison of group means for these data expressed as proportions of the cytoplasmic area was made using Tukey's procedure. The SER fraction in the insulin-treated diabetic group was significantly decreased from that seen in both diabetic ($p<.01$) and semistarved groups ($p<.01$). The combined SER and mitochondrial fraction was significantly lower in the insulin-treated diabetic group than in semistarved and control groups ($p<.05$), and from that fraction in the untreated diabetic and the non-diabetic streptozotocin-injected groups ($p<.01$).

The RER fractions in control and diabetic groups, and in diabetic and non-diabetic streptozotocin-injected groups were not significantly different. However, that fraction was significantly increased in the insulin-treated diabetic group ($p<.05$) and in the semistarved group ($p<.01$) when compared to the non-diabetic streptozotocin-injected group.

The Golgi fraction of cytoplasmic area in the non-diabetic streptozotocin-injected group was significantly greater than that in all other groups ($p<.01$).

Orthogonal transformation of these group means revealed an overall effect of diabetes to increase combined areal densities of SER and mitochondria ($p<.05$). The effect of semistarvation on areal densities was to increase RER ($p=.05$), to increase SER ($p<.01$) and to decrease Golgi fractions ($p<.001$). Insulin treatment itself had an overall effect to increase RER ($p<.001$), to decrease SER ($p<.01$), and to decrease combined SER and mitochondrial areal densities ($p<.001$).

To test the precision of the point counting approach to morphometry, this estimate of mitochondrial areal density was found to be significantly correlated ($r=.58$, $p<.01$, $v=24$) to the planimetric measurement of mitochondrial areal density. The point counts were also reproducible in time and within one animal.

Tests of correlation between morphometric parameters showed significant negative correlation of SER and mitochondrial cytoplasmic fractions ($r=-.38$. $p=.05$, $v=24$), and of Golgi and RER fractions ($r=-.48$, $p<.02$, $v=24$). In addition, the SER fractions in each animal were also significantly negatively correlated to changes in body weight ($r=-.53$, $p<.01$, $v=24$), to single mitochondrial area ($r=-.40$, $p<.05$, $v=24$), and to tubule diameter ($r=-.36$, $p=.05$, $v=24$).

Histochemistry of the Leydig Cell: Adrenocortical tissue blocks used as a control in each HSD incubation processing run were consistently positively stained, and substrate/cofactor omissions and reaction inhibition gave consistently negative controls. Reaction product was fine and dense, and was localized at or very near the external surfaces of SER membranes in the cytoplasm (Figure 15).

In liver tissue blocks used as a control for diastase action in each incubation process, glycogen removal was not always complete, leaving small amounts of glycogen in liver cells. Occasionally this was reflected by only partial digestion of glycogen from Leydig cells, and from vascular smooth muscle and endothelial cells in the testicular interstitium. However, the glycogen was distinct from the HSD reaction product, the latter being a single, dense deposit associated with the SER, and the former appearing as small particulate aggregations with irregular outline.

The HSD reaction product in non-diabetic streptozotocin-injected, untreated diabetic, and semistarved animals was distributed as in the Leydig cells from control animals, and in the HSD methodology study (1). Its localization was also identical, in that it exhibited the intracellular variability also reported previously (1). In insulin-treated diabetic animals, this feature was not seen, as the reaction product very heavily stained the SER and mitochondrial compartments. There were, however,

intercellular differences in staining intensity noted as in control animals.

As Leydig cells in sections from two doubly incubated blocks were photographed randomly, variable numbers of cells were studied per animal to determine a proportion of cells positively stained for HSD activity (Table 7). These frequencies were analyzed by a chi-square test and found to be very significantly dependent on treatment group (X^2=200.8, df=4, p<.001). Expressed as a proportion of cells stained, analysis of variance showed significant difference between groups (p<.001, F(4,20)=14.5). Tukey's procedure to compare group means showed significantly decreased (p<.01) staining proportion in Leydig cells of the diabetic group, compared to all four other group means.

Orthogonal contrast expressions showed that diabetes had the only overall effect, and decreased staining proportion (p<.001). Streptozotocin injection and semistarvation had no significant influence.

Tests of correlation revealed that Leydig cell HSD staining proportion was significantly correlated to serum testosterone (r=.39, p<.05, v=24), to seminiferous tubule diameter (r=.53, p<.02, v=24), and to change in body weight (r=.48, p=.02, v=24), and negatively correlated to interstitial blood vessel density (r=-.47, p<.02, v=24).

Multiple regression analysis showed HSD staining and the testis weight fraction of proportional body weight change to be significant criteria in the prediction of

seminiferous tubule diameter (r=.63, F(2.23)=7.6, p=.003).

Interstitium: In all cases, Leydig cells were seen in loose array within the interstitium, around blood vessels, adjacent to peritubular lymphatic cells and to the peritubular basal lamina. In control animals, the lymphatic channels were very seldom filled with debris which resembled large Leydig cell fragments devoid of plasma membrane, and including swollen mitochondria with tubular cristae, and dilated SER. A few macrophages were noted among the Leydig cells in control animals (Figures 8 and 9).

In interstitium seen in diabetic streptozotocin-injected animals, there appeared to be greater numbers of macrophages among the Leydig cells (Figure 11). These macrophages were large, with many filopodia projecting from the cell surface. Their cytoplasm was pale staining, and included many dense lysosome-like, multivesicular bodies, and some membrane-bounded crystalline structures, as in those seen in the interstitium of control animals. The interstitial macrophages were also noted in testes from insulin-treated diabetic and semistarved animals, although in lesser numbers than in the diabetic streptozotocin-injected animals.

The interstitium of diabetic animals appeared to contain more collagen fibers than seen in that of the testes from other groups.

Seminiferous Tubule Ultrastructure: Due to the presence of stages of the spermatogenic cycle seen in the rat seminiferous tubules, and to the complexity of their intercellular associations, a comprehensive survey of tubule ultrastructure was not within the scope of this study. However, a few observations were noted.

Sertoli-Sertoli cell junctions were vacuolated in some tubules of diabetic animals (Figures 11 and 18), while this was not a feature in control animals (Figures 9 and 17). As well, degeneration of pachytene spermatocytes and early spermatids were noted, and nuclear and cytoplasmic contents (for example, characteristic mitochondria) were often seen within Sertoli cell inclusions at the cell base (Figure 19).

In all animal groups, Sertoli cells were seen to contain lipid droplets of variable size, although their range exceeded the size of the cell nucleus only in streptozotocin-injected and semistarved animals. In addition, tubules from diabetic drug-treated animals exhibited giant residual bodies at and within the lumen (Figures 7c and 20). These membrane-bounded cell fragments appeared compartmented, and contained lipid droplets, occasional mitochondria, and particulate matter.

The tubule basal lamina complex in control animals (Figure 9) was composed of four layers. These were a thin basal lamina of the tubule itself (Figures 17-19) a second basal lamina between the first and the tubule pericyte, the peritubular myoid cell, and a third basal lamina distal to

the pericyte (Figures 9 and 21).

In diabetic and semistarved animals, this complex appeared thickened, with a wider space between the two adjacent basal laminae than in control animals. The lamina directly beneath the germinal epithelium also appeared thicker than that in control animals, although these impressions were not quantified. In occasional tubules from diabetic animals, there was a duplication of this layer.

Serum Hormones

Testosterone, corticosterone, estradiol, and FSH levels were determined by radioimmunoassay as given in the methods. The group means of these hormones are given in Table 7. The mean hormone levels of the 3 animals killed by decapitation were not significantly different from control animals (Mann-Whitney U test), but were not pooled with citrate-control group values for the following statistical analyses. However, two animals in the non-diabetic streptozotocin-injected group, which were not examined morphometrically, were included in the analysis of separate hormone values.

Testosterone: Tukey's procedure showed significantly reduced serum testosterone (Table 7) in diabetic and semistarved animals. Statistical differences ($p<.05$) were found between control and diabetic, control and semistarved, diabetic and insulin-treated diabetic, and semistarved and

insulin- treated diabetic group means.

Orthogonal contrast expressions showed that both diabetes (p=.03) and semistarvation (p=.01) had significant separate effects on measured testosterone.

In a correlation matrix, testosterone levels were significantly positively related to Leydig cell staining by HSD activity (r=.40, p<.05, v=24), and to changes in body weight (r=.45, p=.03, v=24). Negative correlation was found to lipid areal density (r=-.40, p<.05, v=24) and to cytoplasmic SER fraction (r=-.39, p=.05, v=24).

In a multiple regression analysis, serum testosterone and blood vessel number per tubule were found to be significant criteria in the prediction of tubule diameter (r=.69, F(2.23)=10.2, p<.001). Serum testosterone levels were significantly predicted by single mitochondrion area and lipid numerical density (r=.51, F(2,23)=4.0, p=.03), by SER volume density and lipid numerical density (r=.53, F(2,23)=4.57, p=.02), and by lipid numerical density and HSD staining (r=.55, F(2,23)=4.97, p<.02).

Corticosterone: Tukey's procedure to compare means (Table 7) showed no significant difference between groups. Orthogonal contrast expressions also showed no significant overall effect of diabetes, streptozotocin, or semistarvation on the concentration of this hormone.

Serum corticosterone levels were found to be significantly correlated with lipid droplet size (r=.52,

p<.01, v=24). Multiple regression analyses found corticosterone and blood vessel density (r=.67, F(2.23)=9.3, p=.001), and corticosterone and HSD staining of Leydig cells (r=.56, F(2,23)=5.2, p=.01) to be significant pairs of criteria in the prediction of tubule diameter.

Estradiol: In all animals studied, serum estradiol measures were below the level of sensitivity of the test, that is less than 30 pmol/L.

FSH: Tukey's procedure to compare group means (Table 7) showed increased serum FSH levels in diabetic and semistarved animals. Statistical differences were found between control and diabetic (p<.01) and between control and semistarved (p<.01) groups. There were also differences between untreated and insulin-treated diabetic groups (p<.01), between semistarved and insulin-treated diabetic groups (p<.01), between diabetic and non-diabetic streptozotocin-injected animals (p<.01), and between non-diabetic streptozotocin-injected and semistarved animals (p<.01).

Orthogonal contrast expressions revealed separate, significant overall effects of diabetes (p<.001) and semistarvation (p<.001) to increase serum FSH levels in this study.

The SER areal density of Leydig cells was found to be significantly correlated to serum FSH (r=.41, p<.05, v=22).

Serum FSH values were significantly negatively correlated to tubule diameter ($r=-.39$, $p=.05$, $v=22$), to lipid numerical density ($r=.70$, $p<.01$, $v=22$), and to the proportion of cells stained for HSD activity ($r=-.42$, $p<.05$, $v=22$). However, serum FSH was not significantly correlated to serum testosterone values ($r=-.35$, $p=.06$, $v=22$).

In multiple regression analyses to predict seminiferous tubule diameter, FSH and HSD staining ($r=.61$, $F(2,20)=5.8$, $p=.01$), FSH and blood vessel density ($r=.75$, $F(2,20)=12.8$, $p<.001$), and FSH and lipid areal density ($r=.52$, $F(2,20)=3.63$, $p=.04$) were found to have significant prediction value.

LH: Due to repeated difficulties encountered with quality control of the procedures used in this assay, there were insufficient serum samples to permit a report of the LH levels in the present study.

WISTAR ANIMALS

In all parameters, the citrate-control and streptozotocin-injected Wistar groups were studied parallel to Long Evans animals. The group means and the Mann-Whitney U test significance level of each parameter are presented in Tables 8-11. Due to the severe illness of the diabetic Wistar animals, and their susceptibility to respiratory illness, group sizes are small. In addition, testicular perfusion was not successful in five of these nine Wistar

rats, making fine morphometric distinctions between the two groups of questionable significance.

Final body weight and body weight change (Table 8) both showed significant difference between control and diabetic groups ($p=.02$, and $p=.002$ respectively). As well, blood glucose at perfusion ($p<.001$), urine output ($p<.02$), and blood urea nitrogen ($p=.008$) were significantly higher in the diabetic group (Table 8). Hematocrit and creatinine clearance were not different statistically between groups (Table 8).

Testicular Histology

Testicular weight (single, total, or total per 100 grams of body weight) was not statistically different in the two groups, despite a trend to decreased weight in diabetic animals (Table 8).

Histologic examination of testes from control animals showed smoothly contoured seminiferous tubules showing various stages of spermatogenesis, and surrounded by interstitium. The interstitium appeared to have a greater number of larger blood vessels with a well-developed muscular layer than in control Long Evans rats, although this was not quantified. As well, the interstitial cells (including Leydig cells) seemed smaller, though in greater numbers in each perivascular collection. There was occasionally marked sloughing of spermatocytes and early

spermatids into the tubule lumen. In two animals there were small areas of complete atrophy, where tubules were small, and populated only by Sertoli cells.

In diabetic animals, cross-sections of testis tubules were often indented, with an irregularly contoured basement membrane. There was frequent desquamation from thinned germinal epithelium, degeneration of spermatocytes within the epithelium, and several large areas of tubules with the "Sertoli cell only" pattern of atrophy (Figure 7b). In addition, there were many tubules at stages VI-VIII seen to contain large residual bodies, both at the lumenal surface of the epithelium, and in the lumen. These were round and multivesicular with both pale and darkly stained contents.

Measurement of tubule diameter (Table 8) showed no changes between group means or between histograms of diameter class distributions. Blood vessel density (Table 8) tended to be increased in diabetic animals, but this was not significant due to large standard errors.

Staging of spermatogenesis in seminiferous tubules (Table 4b) recorded the appearance of "Sertoli cell only" tubules, and when that category was included in a frequency distribution, there was a nearly significant dependence on treatment group (X^2=13.5, df=7, p=.06). Without the "Sertoli cell only" category, the distributions were quite independent of treatment effects. There were marked differences between stage frequency distributions within the animals of the diabetic group, although this was also not

significant (X^2=18.9, df=12, p=.09).

Testicular Ultrastructure

Leydig cell ultrastructure was of two patterns, and depended on tissue fixation. The first pattern, from perfusion fixed testes, was very similar to that seen in control Long Evans animals: Leydig cells were large and pleomorphic, with microvillous projections from the cell surface. A large indented nucleus, many dense mitochondria with tubular cristae, large amounts of SER, and occasional lipid droplets and RER profiles populated the cytoplasm. The second pattern, from testes fixed by immersion after unsuccessful perfusion, (Figure 21), was one of whole cells, and occasional areas of cytoplasmic contents lacking a continuous plasma membrane, lying between lymphatic cells of the interstitial space. Mitochondria with tubular cristae, and SER were present, although the SER was dilated with electron lucent material. The mitochondria were often swollen, with internal disruption of cristae and occasional rupture of their outer membranes.

Despite this disruption of some areas of Leydig cells in three control and two diabetic animals, certain changes could be appreciated in a comparison of the two treatment groups. Leydig cells from diabetic animals appeared to contain less glycogen, and more and larger lipid droplets than control animals. Interstitial macrophages were more frequently noted in diabetic than in control animals, and

their cytoplasm contained recognizable fragments of Leydig cell organelles.

Morphometry of the Leydig cells included the same parameters and methods of approach as examined in Long Evans rats. There were no significant differences between control and diabetic groups by planimetry (Table 9) of single mitochondrion size, mitochondrial areal density, or mitochondrial numerical density. Single mitochondrial area was, however, negatively correlated (rho=-.81, $p<.01$) with mitochondrial numerical density, as seen in Long Evans animals.

There were significant increases in lipid droplet size ($p=.007$), and in lipid areal density ($p=.018$), although not in lipid numerical density ($p=.06$). Lipid droplet size was positively correlated with lipid numerical density (rho=.84, $p<.01$) in these animals. Total lipid areal density was also correlated with lipid numerical density (rho=.83, $p<.01$), with single lipid droplet size (rho=.98, $p<.01$), and negatively correlated with body weight change (rho=-.95, $p<.01$) during the experimental course.

By point counting (Table 6) of SER, mitochondrial, RER, and Golgi areas in Leydig cell cytoplasm, there was an overall significant difference between control and diabetic groups ($X^2=13.06$, $df=4$, $p=.01$). The cytoplasmic fractions considered separately showed no statistical difference between groups. The areal fraction of mitochondria by point counting was significantly correlated

(rho=.76, p<.02) with the mitochondrial areal density as determined by computerized planimetry.

Histochemistry of HSD activity in Leydig cells on Wistar control animals showed an identical distribution to that seen in control Long Evans rats, with reaction product deposited at or very near the external SER surface. The inter- and intracellular variations in staining were also noted.

In Leydig cells from diabetic Wistar rats, there was no significant change from the control proportion of cells stained for HSD activity (Table 11), or in the pattern of that stain deposition. However, the proportion of Leydig cells positively stained was found to be significantly negatively correlated with tubule diameter in the Wistar animals (rho=-.68, p=.05).

Ultrastructure of Seminiferous Tubules

Germinal epithelium of control animals exhibited the degeneration of spermatocytes seen at the level of the light microscope. Also noted were many spermatocytes and immature spermatids within the tubule lumen, the latter often still attached to residual cytoplasmic fragments including lipid and characteristic condensed mitochondria.

In diabetic animals, the germinal epithelium was also disrupted, with frequent vacuolation of Sertoli cell cytoplasm, which separated maturing spermatids from supporting cytoplasm. Further, there were "Sertoli cell

only" tubules encountered, as well as those with complete desquamation of germinal and supporting elements. Sertoli cells were also notable by enormous lipid inclusions, occasionally equal in size to their large nuclei.

The basal lamina complex of the tubules in diabetic Wistar rats was irregular in contour, with apparent thickening of the tubule component. There was also widening of the space between layers of basal lamina, as in diabetic Long Evans animals.

Serum Hormones

Serum testosterone (Table 11) was significantly lower ($p=.045$) in diabetic animals compared to control. The level was positively correlated with the SER areal fraction in Leydig cell cytoplasm ($rho=.69$, $p<.05$). In addition, serum testosterone levels were significantly negatively correlated ($rho=-.67$, $p=.05$) with lipid droplet size.

Serum corticosterone concentrations (Table 11) were significantly higher ($p=.03$) in Wistar diabetics than in controls. Corticosterone levels were correlated negatively ($rho=-.71$, $p<.05$) with serum testosterone. Serum estradiol levels in both groups were below sensitivity of the assay.

Follicle-stimulating hormone levels were not measured in one control and in two diabetic animals due to insufficient serum samples (Table 11).

Luteinizing hormone levels were unavailable, due to repeated difficulties encountered in quality control of the

assay procedures.

STRAIN DIFFERENCES

Long Evans and Wistar control groups compared in chi-square tests, showed significant difference between strains in size-class histograms of tubule diameter (X^2=41.7, df=9, p<.001), and also in frequency distributions of spermatogenic stages (X^2=47.3, df=6, p<.001). The within-group variability of many parameters was greater in the Wistar than in the Long Evans diabetic animals. As well, the histology of testes from control Wistar rats included many features seen in testes from Long Evans diabetic animals.

In the ultrastructural study of Leydig cells, point counting of overall cytoplasmic organelle fractions between the two control groups were significantly different (X^2=16.4, df=4, p=.003). In addition, the proportion of Leydig cells stained by HSD reaction product was significantly lower in Wistar controls than in Long Evans rats (X^2=5.4, df=1, p=.02).

Two-way analyses of variance comparing treatment, strain, and treatment-strain interaction effects are summarized in table 12. Strain differences were found in urine output (increased over respective control levels more in diabetic Wistar than in diabetic Long Evans rats (p=.03), with significant (p=.01) interaction). Significant differences in morphometric parameters were also noted in

lipid size and total lipid areal density (increased more in diabetic Wistar than in diabetic Long Evans animals ($p=.004$, and $p=.008$ respectively), with significant ($p=.003$, and $p<.01$ respectively) interaction). Mitochondrial areal density was also significantly different between strains (greater ($p=.02$) in Wistar than in Long Evans animals). Mitochondrial numerical density tended to be greater in Wistar than in Long Evans rats, but the difference was not significant.

Point counting of cytoplasmic contents also showed significant strain differences in SER fractions (greater ($p<.001$) in diabetic Long Evans than in diabetic Wistar rats without significant ($p=.06$) interaction), and in Golgi fractions (greater ($p=.01$) in Long Evans than in Wistar rats).

There was significant interaction (interference, $p=.001$) between strain and treatment effects seen in corticosterone levels (diabetic Wistar greater than control Wistar levels, but diabetic and control Long Evans levels not different), although by analysis of variance, neither treatment or strain effect was significant in itself.

Serum FSH levels were also significantly different between strains (Wistar control greater than Long Evans control levels, $p=.01$), although there were no statistically significant treatment or interaction effects by this analysis.

Tables 1-12 follow.

For Tables 1-3 and 5-7, entries to the right of each group are mean ± standard error for each parameter. In the lower section of these tables are listed the probability of the F value from the analysis of variance on each parameter (p(F) ANOVA), and the probabilities of the orthogonal contrast expressions for diabetes (tDM), streptozotocin (tS) and semistarvation (tSTV). Arrows indicate an increase or decrease in each of these effects. Probability of ".000" indicates a value less than .00001.

For Table 4, entries are the sum of the frequency of each stage (or group of stages) within each group.

For Tables 8-11, entries are the mean ± standard error of each parameter in control and diabetic Wistar groups, and the probability of the value U from the nonparametric Mann Whitney U test of significance between groups.

Table 12 lists the probability of strain and treatment effects, and of strain-treatment interactions from two-way analyses of variance tests for each parameter.

Table 1: Body weight, blood glucose and hematocrit in Long Evans diabetic and control groups

Group	Body Weight (Final) (g)	Body Weight Change (g)	Blood Glucose (Final) (mmol/L)	Hematocrit (%)
Control (6)	426.5 ± 21.0	+94.6 ± 6.1	7.49 ± 0.8	45.7 ± 1.45
Diabetic (5)	371.4 ± 19.9	-1.0 ± 12.4	>22.2	40.8 ± .73
Not Diabetic (6)	485.0 ± 15.5	+132 ± 25	9.2 ± 0.8	45.3 ± .95
Diabetic & Insulin (5)	452.8 ± 11.0	+138 ± 6.0	8.97 ± 0.5	47.0 ± .63
Semistarved (6)	377.5 ± 6.1	-19 ± 8.1	6.57 ± 0.3	43.5 ± 1.15
p (F) ANOVA	.0003	.000	<.001	.008
t_{DM}	<.01 ↓	<.001↓	<.001↑	<.01↓
t_{S}	<.03↑	<.01 ↑	<.05↑	—
t_{STV}	<.01↓	<.001↓	<.01↓	—

Table 2: Final urine volume, creatinine clearance and blood urea nitrogen measures in Long Evans diabetic and control groups

Group	Urine Volume (ml/hr)	Creatinine Clearance (μl/min)	Blood Urea Nitrogen (mg/dl)
Control (6)	0.26 ± .02	1920 ± 275	27.6 ± .88
Diabetic (5)	1.96 ± .27	1241 ± 106	28.3 ± 2.3
Not Diabetic (6)	.41 ± .11	1731 ± 145	24.8 ± 3.5
Diabetic & Insulin (5)	.27 ± .02	2064 ± 217	17.2 ± 1.6
Semistarved (6)	.38 ± .02	1710 ± 45	22.9 ± 1.2
p (F) ANOVA	.000	.03	.002
t_{DM}	<.001 ↑	<.01 ↓	<.02 ↑
t_{S}	--	--	<.01 ↓
t_{STV}	--	--	--

Table 3: Testis weight, seminiferous tubule diameter and blood vessel density of testes from Long Evans diabetic and control groups

Group	Testis Wt per 100g Body Weight	Tubule Diameter (mm x 50)	Blood Vessel Density (number per tubule)
Control (6)	.81 ± .06	11.48 ± .20	3.44 ± .40
Diabetic (5)	.89 ± .04	9.85 ± .36	4.91 ± .74
Not Diabetic (4)	.72 ± .05	11.8 ± .70	3.55 ± .74
Diabetic & Insulin (5)	.73 ± .01	11.2 ± .19	3.62 ± .36
Semistarved (6)	.89 ± .08	11.0 ± .19	3.24 ± .10
p (F) ANOVA	NS	.006	NS
t_{DM}	—	<.001↓	<.02↑
t_S	—	--	--
t_{STV}	<.05↑	--	--

Table 4: Frequency distribution of the stages of spermatogenesis in seminiferous tubules from diabetic and control groups of (a) Long Evans and (b) Wistar strains

Group	I	II-III	IV-VI	VII-VIII	IX-XI	XII-XIII	XIV
Long Evans							
Control (6)	22	66	191	185	54	64	18
Diabetic (5)	24	81	117	159	53	45	21
Not Diabetic (4)	17	39	83	132	69	39	21
Diabetic & Insulin (5)	22	71	128	149	64	43	22
Semistarved (6)	37	92	142	152	100	47	30
Wistar							
Control	34	111	121	155	102	61	16
Diabetic	14	54	58	71	58	24	15

Table 5: Leydig cell proportions of mitochondria and lipid droplets measured by computerized planimetry of micrographs from Long Evans diabetic and control groups

Group	MA	MAT/CAT	MN/CAT	LS	TL/CAT	LN/CAT
Control (6)	.179 ± .016	.141 ± .006	.814 ± .07	.28 ± .10	.21 ± .10	.59 ± .19
Diabetic (5)	.169 ± .009	.140 ± .003	.842 ± .04	.57 ± .11	1.05 ± .18	1.98 ± .31
Not Diabetic (4)	.198 ± .03	.140 ± .01	.735 ± .06	.46 ± .20	.43 ± .24	.63 ± .30
Diabetic & Insulin (5)	.182 ± .01	.138 ± .006	.770 ± .13	.66 ± .32	.71 ± .42	.93 ± .10
Semistarved (6)	.137 ± .01	.119 ± .009	.926 ± .12	.36 ± .05	.92 ± .21	2.60 ± .45
p (F) ANOVA	NS	NS	NS	NS	NS	.000
t_{DM}	—	—	<.05↑	—	—	<.05↑
t_{S}	—	—	—	—	—	—
t_{STV}	<.02↓	<.02↓	—	—	—	<.001↑

MA= single mitochondrion area
MAT/CAT= mitochondrial areal/density
MN/CAT= mitochondrial numerical density

LS= single lipid droplet size
TL/CAT= lipid areal density
LN/CAT= lipid droplet numerical density

Table 6: Volume density of SER, RER, Golgi and mitochondria in Leydig cell cytoplasm from Long Evans diabetic and control groups

Group	SER	RER	Golgi	Mitochondria
Control (6)	.35 ± .008	.037 ± .007	.035 ± .005	.21 ± .01
Diabetic (5)	.37 ± .016	.042 ± .007	.038 ± .009	.20 ± .008
Not Diabetic (6)	.35 ± .015	.020 ± .003	.071 ± .006	.22 ± .005
Diabetic & Insulin (5)	.27 ± .018	.065 ± .009	.019 ± .004	.19 ± .007
Semistarved (6)	.38 ± .028	.068 ± .01	.028 ± .005	.17 ± .01
p (F)	.003	.003	.0001	NS
t_{DM}	--	--	--	--
t_S	--	--	<.01↑	--
t_{STV}	<.01↑	.05↑	<.001↓	<.01↓

Table 7: Leydig cell HSD staining and serum hormone concentrations from Long Evans diabetic and control groups

Group	HSD stain (proportion of positive cells)	Serum Testosterone (ng/ml)	Serum Corticosterone (ng/ml)	Serum FSH (ng/ml)
Control (6)	.89 ± .02	3.81 ± .61	1423 ± 165	147.2 ± 14.7
Diabetic (5)	.39 ± .08	1.02 ± .20	940 ± 116	280 ± 19.1
Not Diabetic (6)	.89 ± .04	1.78 ± .51	1148 ± 216	137.7 ± 6.6
Diabetic & Insulin (5)	.98 ± .02	4.38 ± 1.23	1138 ± 175	148.2 ± 14.8
Semistarved (6)	.88 ± .11	1.17 ± .49	995 ± 93	285.7 ± 76.6
p (F)	.000	.003	NS	.0002
t_{DM}	<.001↓	<.03↓	--	<.004↑
t_{S}	--	--	--	--
t_{STV}	--	<.01↓	--	<.001↑

Table 8: Body and testis weight, blood and urine chemistry, seminiferous tubule diameter and interstitial blood vessel density from Wistar control and diabetic groups.

Parameter	Wistar Control (n=6)	Wistar Diabetic (n=3)	p(Mann Whitney U)
Final body weight (g)	411.7 ± 19.2	297 ± 57	.019
Body weight change (g)	+103.5 ± 18.3	-23.3 ± 61.3	.002
Final blood glucose (mmol/L)	7.5 ± 1.1	>22.2	<.001
Hematocrit (%)	42.3 ± 3.4	43 ± 5.6	--
Urine volume (ml/hr)	.34 ± .12	1.18 ± .21	<.02
Creatinine clearance (µl/min)	1993 ± 538	1325 ± 275	--
Blood urea nitrogen (mg/dl)	22.5 ± 2.3	33.2 ± 6.9	.008
Testis weight/100g body weight	.806 ± .16	.907 ± .18	--
Tubule diameter (mm x 50)	10.84 ± .58	10.86 ± .24	--
Blood vessel density (no./tubule)	3.72 ± 1.3	4.19 ± .76	--

Table 9: Morphometry of mitochondria and lipid droplets by computerized planimetry of Leydig cells from Wistar control and diabetic groups

Parameter	Wistar Control (n=6)	Wistar Diabetic (n=3)	p(Mann Whitney U)
MA	.182 ± .03	.146 ± .02	--
MAT/CAT	.162 ± .02	.154 ± .015	--
MN/CAT	.09 ± .16	.109 ± .31	--
LS	.23 ± .25	2.4 ± 1.5	.007
TL/CAT	.003 ± .004	.055 ± .044	<.02
LN/CAT	.006 ± .008	.019 ± .009	--

MA= single mitochondrion area
MAT/CAT= mitochondrial areal/density
MN/CAT= mitochondrial numerical density
LS= single lipid droplet size
TL/CAT= lipid areal density
LN/CAT= lipid droplet numerical density

Table 10: Cytoplasmic volume density of SER, RER, Golgi, and mitochondrial fractions of Leydig cells from Wistar control and diabetic groups.

Parameter	Wistar Control (n=6)	Wistar Diabetic (n=3)	p(Mann Whitney U)
SER fraction	.31 ± .02	.28 ± .027	--
RER fraction	.046 ± .018	.051 ± .015	--
Golgi fraction	.022 ± .014	.012 ± .004	--
Mitochondrial fraction	.23 ± .04	.21 ± .02	--

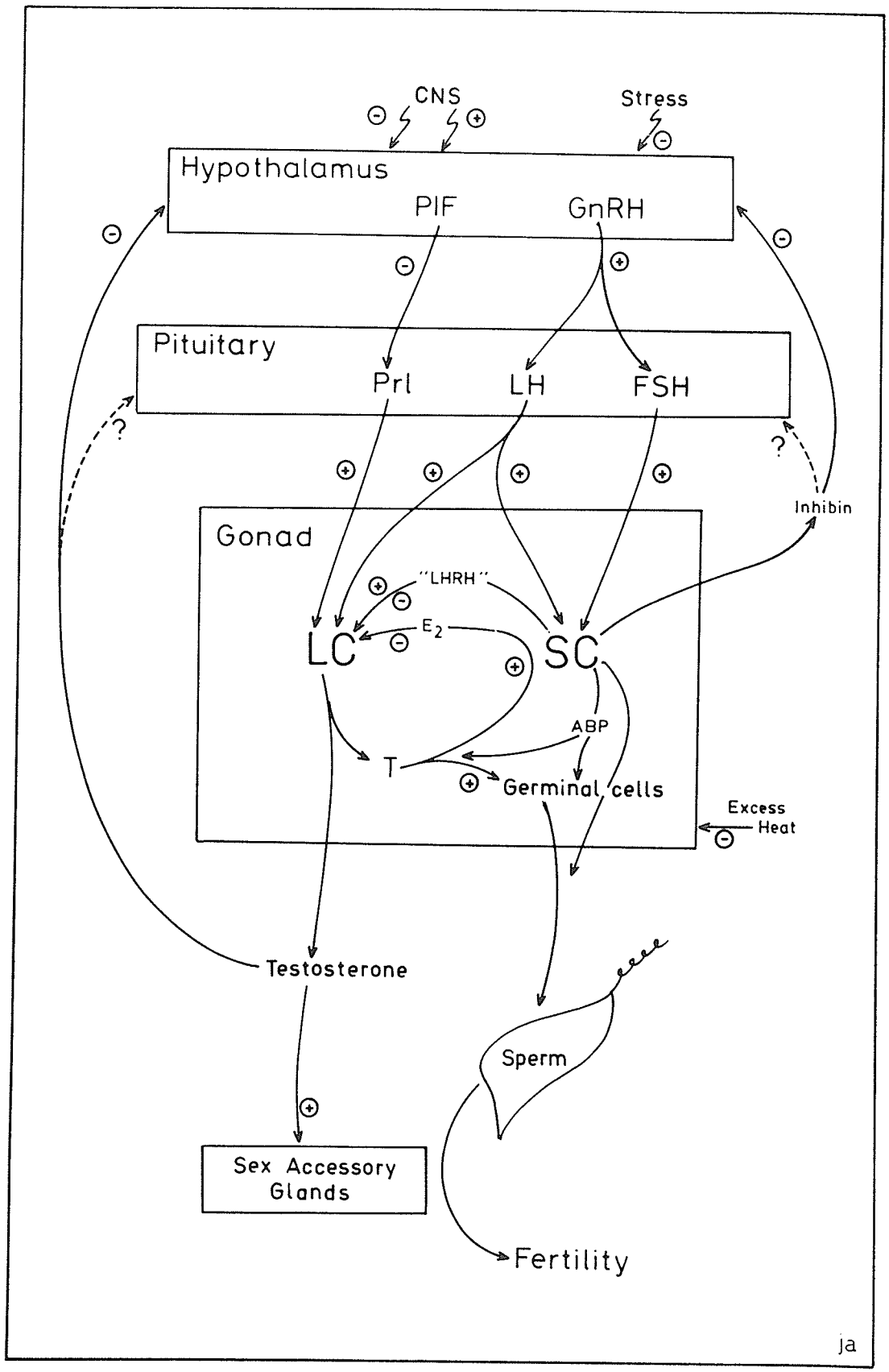
Table 11: Leydig cell HSD staining and serum hormones from Wistar control and diabetic groups.

Parameter	Wistar Control (n=6)	Wistar Diabetic (n=3)	p(Mann Whitney U)
HSD staining	.76 ± .12	.75 ± .28	--
Serum testosterone (ng/ml)	6.1 ± 3.4	1.1 ± .75	.045
Serum corticosterone (ng/ml)	703 ± 204	1323 ± 341	.03
Serum FSH (ng/ml)	334 ± 97	--	--

Table 12: Significance level of treatment, strain, and strain-treatment interaction effects from 2-way analyses of variance between Long Evans and Wistar control and diabetic groups.

	Strain	Treatment	Strain-Treatment Interaction
Bodyweight change	--	<.001	--
Urine volume	.03	<.001	.01
Blood glucose (final)	--	<.001	--
Blood urea nitrogen	--	.007	.01
Hematocrit	--	--	--
Testis weight/100g body weight	--	--	--
Tubule diameter	--	.006	<.02
Blood vessel density	--	--	--
MA	--	--	--
MAT/CAT	.02	--	--
MN/CAT	.06	--	--
LS	.004	<.001	.003
TL/CAT	.008	.001	.009
LN/CAT	--	.001	--
SER fraction	<.001	--	--
RER fraction	--	--	--
Golgi fraction	.01	--	--
Mitochondria fraction	--	--	--
HSD staining	--	.004	.01
Serum testosterone	--	.002	--
Serum corticosterone	--	--	.001
Serum FSH	.01	--	--

Figure 1: Schematic summary of the hypothalamic- pituitary-gonadal axis, as referenced in the introduction.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Fertility
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Figure 2: a. A table summarizing theoretical endocrine profiles of primary, secondary and tertiary hypogonadism. Abbreviations used in column headings are: sT: serum testosterone, sLH: serum luteinizing hormone, sFSH: serum follicle-stimulating hormone, LHRH: luteinizing hormone releasing hormone, 17KS: 17-ketosteroids, uE: urinary estrogens, uGnH: urinary gonadotropins, sPrl: serum prolactin, T with HCG: serum testosterone after stimulation with exogenous HCG. Arrows indicate increase or decrease from normal levels. Primary hypogonadism includes syndromes of LH receptor defect or deficiency, as well as defects of steroidogenesis. Secondary hypogonadism includes syndromes of reduced gonadotrope function and increased mammotrope function.

b. A table summarizing, by author, biochemical investigations of gonadal function in diabetic men.

	sT	sLH	sFSH	LHRH	17KS	uE	uGnH	sPrl	T with HCG
a. THEORETICAL									
Primary Hypogonadism	↓	↑	?↑	↑	?↑	?↑	↑	?	↓
Secondary Hypogonadism	↓	↓	↓	↑	↓	↓	↑	?↑	↑
Tertiary Hypogonadism	↓	↓	↓	↓	↓	↓	↓	?	↑
b. HUMAN STUDIES									
Kolodny	N								
Schoffling	↓								
Ellenberg	N								
Faerman	N								
Kent	N								
Kim	↓								
Brazales									
Bors	↓	↓	↓		↑	↑			
Young								↑	

Figure 2. (a) A summary of theoretical endocrinological parameters in primary, secondary, and tertiary hypogonadism.
(b) A summary of studies on the effects of Diabetes Mellitus on parameters of reproductive endocrinology in humans.

Figure 3: A table summarizing, by author, biochemical investigations of gonadal function in animals with experimentally-induced diabetes. Column headings are abbreviation as given in Figure 2. Other symbols include: LC: Leydig cell, N: normal and #: number.

	sT	sLH	sFSH	LHRH	17KS	uE	uGnH	sPrl	T with HCG
RAT STUDIES									
Rossi	↓	↓		N					
Schoffling	↓/↓LC								
Hunt	LC								
Bestetti				abN					
Paz		N		N					
Charreau		N	N						↓ # receptors
Oksanen			N						
Lui			↑						
Foglia	↓	↓	↓						
Paz	↓/↓LC	↓							↑
Orth		N	↓						
Murray	↓	N	↓						↓
Tesone	↓								↓ # receptors
Howland	↓	↑/↓	N						
Thliveris	↓								
Kuhn-Velten	↓								

Figure 3. A summary of studies on the effects of experimental diabetes on parameters of reproductive endocrinology in rats.

Figure 4: Schematic outline of the animal treatment groups of Long Evans rats, as described in the experimental protocol.

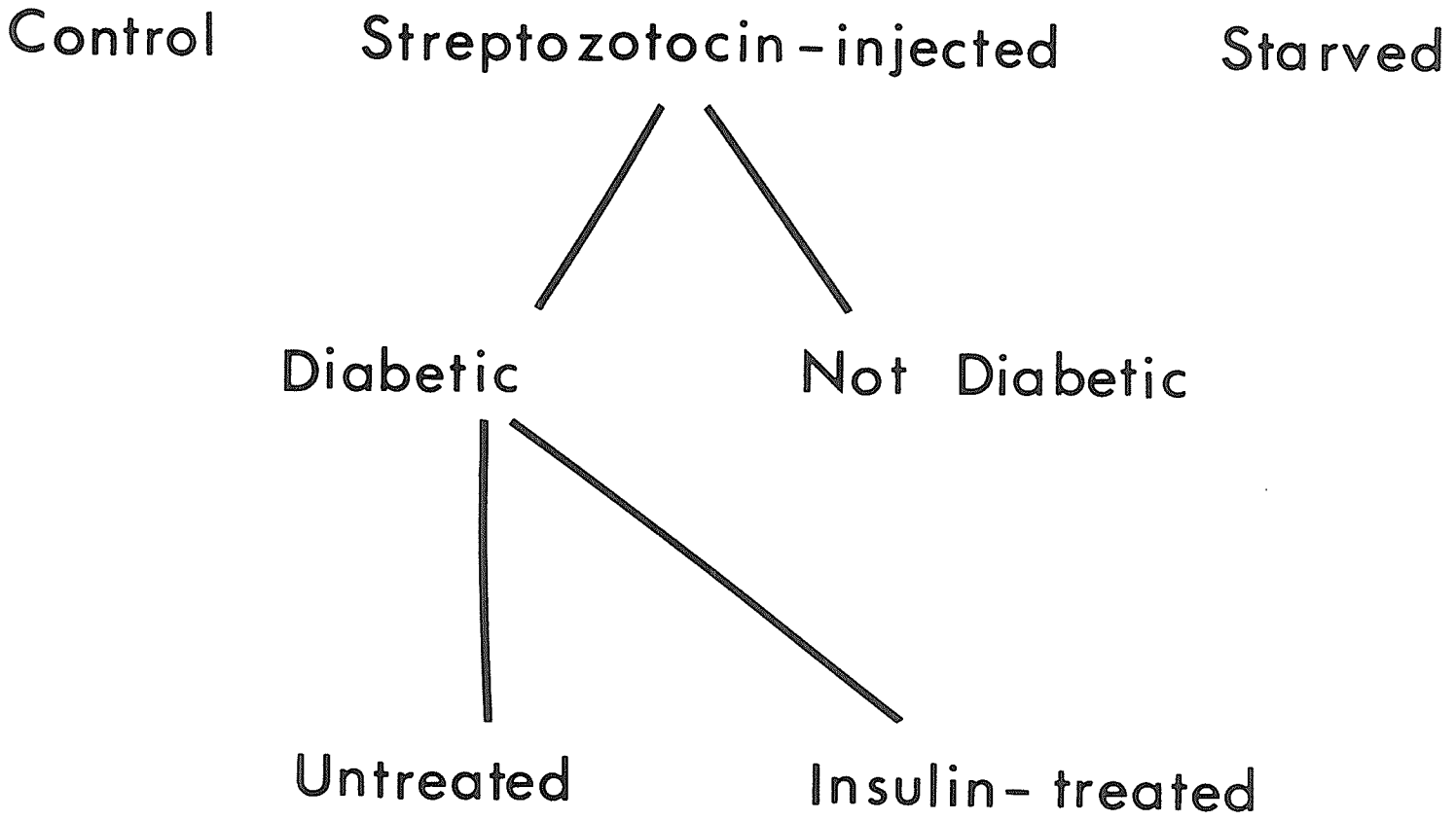

Control
Streptozotocin-injected
Starved
Diabetic
Not Diabetic
Untreated
Insulin-treated

Figure 5: An outline of the analysis of testicular histology showing the area-weighting plan (centre), the epidiascope projection (X50) of slides (right) for measurement of seminiferous tubule diameter, and the ocular grid projection (X100) of slides (left) for counts of interstitial blood vessels.

Analysis of Testicular Histology

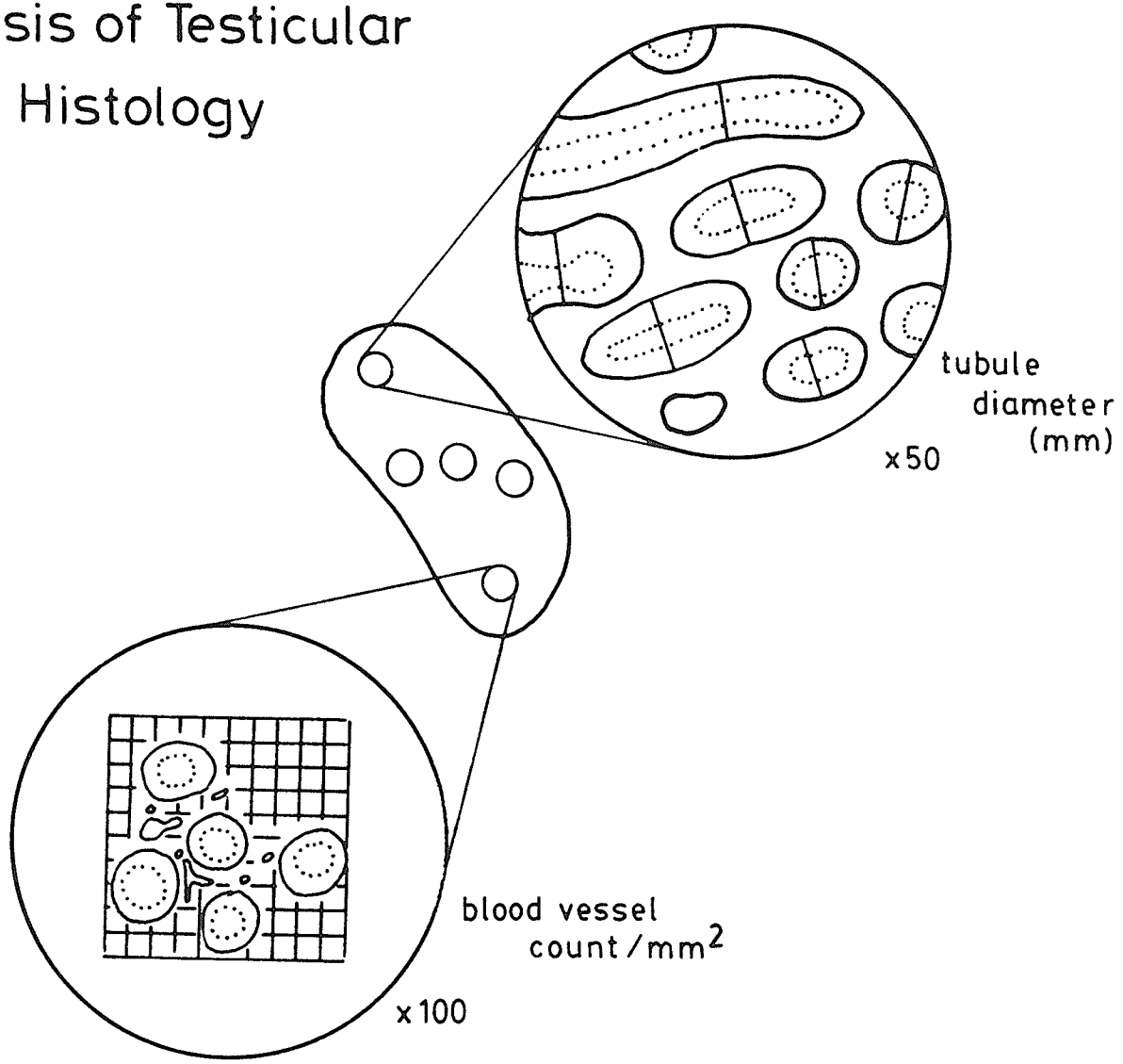


Figure 6:(H&E) (a) Light micrograph of testis from Long Evans control animal showing seminiferous tubules (st) surrounded by interstitial tissue (i) and blood vessels (bv). The tubule epithelium exhibits many stages of spermatogenesis. Original magnification: X90.

(b) Light micrograph of portions of three seminiferous tubules from the testis of a Long Evans control animal showing stages II and IX of spermatogenesis. Original magnification: X350.

(c) Light micrograph of portions of three seminiferous tubules from the testis of a Long Evans control animal showing germinal epithelium at stages II, IV, and XII of spermatogenesis. Also note cells of the interstitium (i) and blood vessels (bv). Original magnification: X350.

(d) Light micrograph of portions of two seminiferous tubules from the testis of a Long Evans control animal showing epithelium at stages VII and XIV of spermatogenesis. Original magnification: X350.

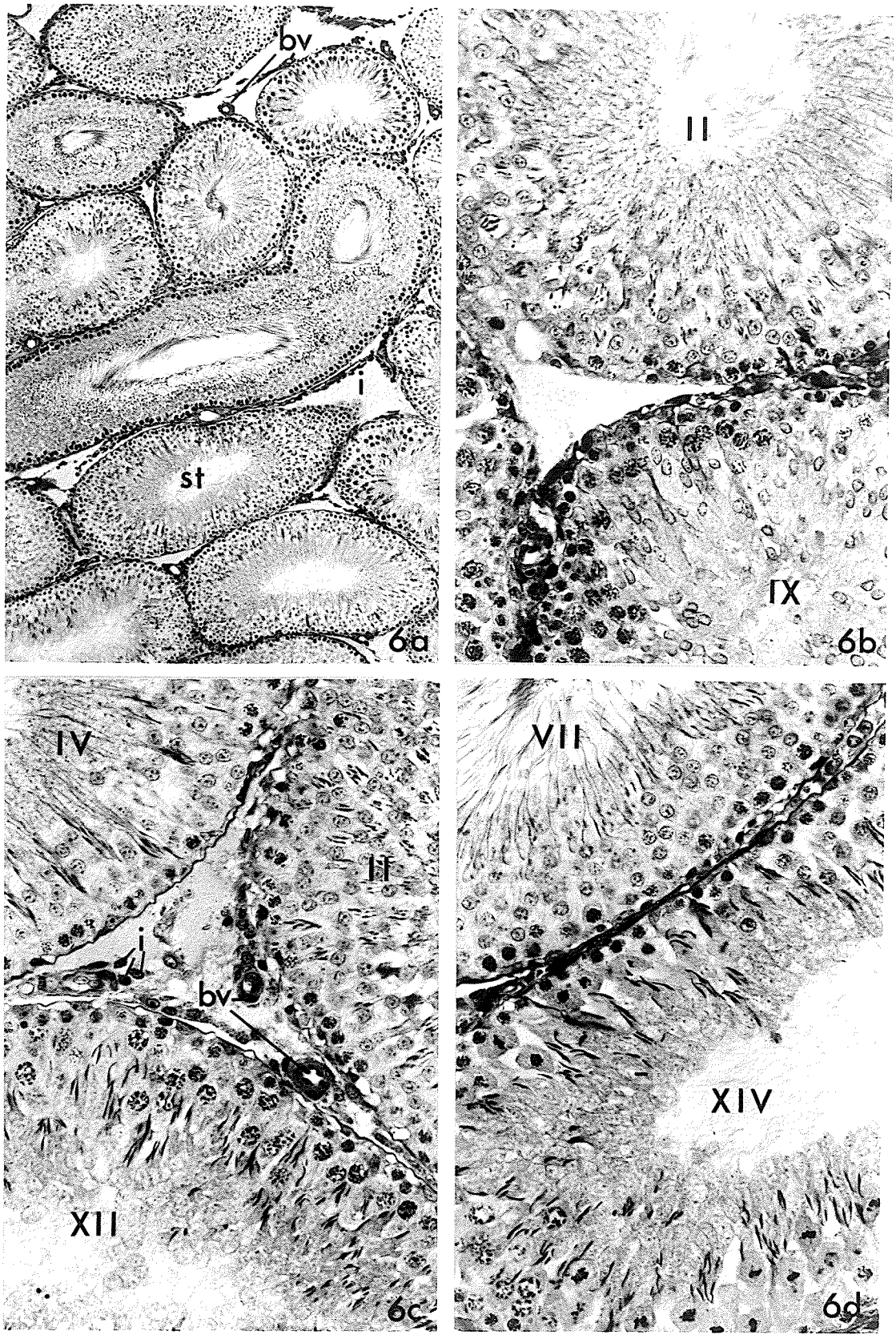
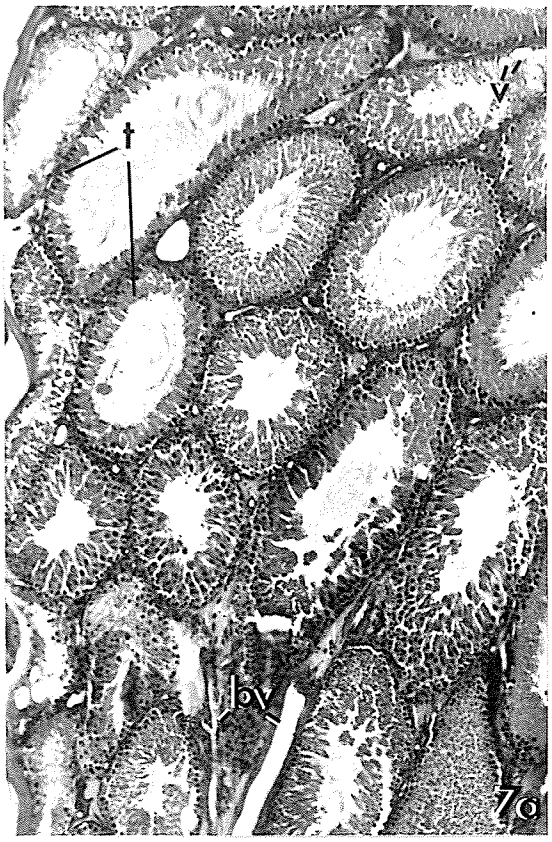

bv
i
st
6a
II
IX
6b
IV
II
i
bv
XII
6c
VII
XIV
6d

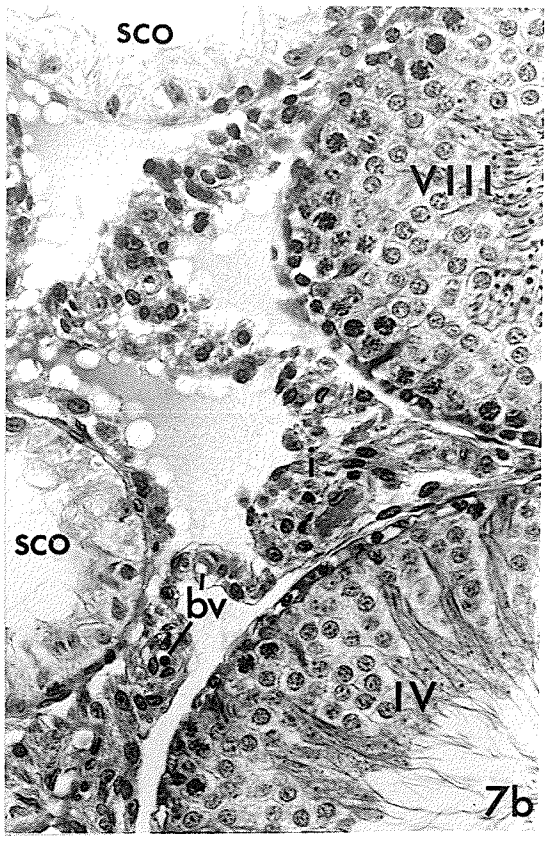
Figure 7:(H&E) (a) Light micrograph of testis of a diabetic Long Evans animal showing thinned (t) and vacuolated (v) epithelium within seminiferous tubules, which are surrounded by interstitium and blood vessels (bv). Original magnification: X90.

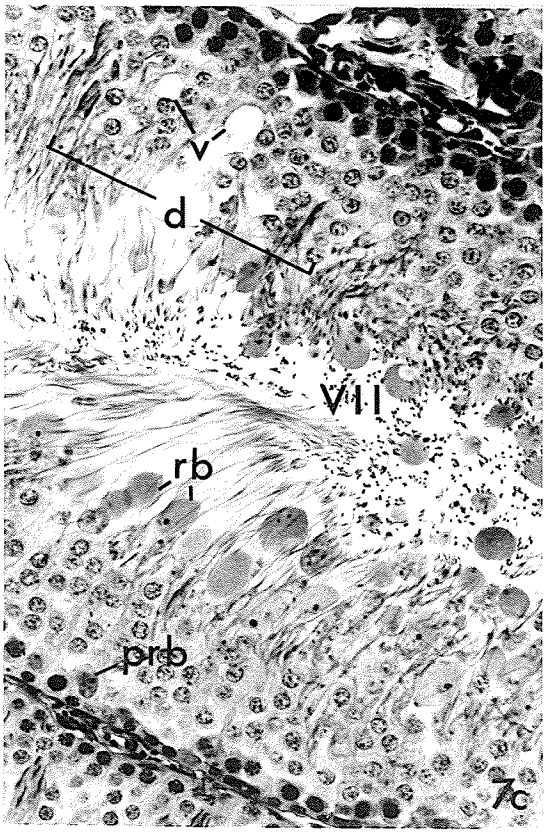
(b) Light micrograph of testis from a Wistar diabetic rat showing tubules at stages IV and VIII of spermatogenesis, and two tubules with Sertoli cells only (sco). Note the loose array of cells in the interstitium (i) and blood vessels (bv) surrounding the tubules. Original magnification: X350.

(c) Light micrograph of testis from Long Evans diabetic animal of tubules at stage VII of spermatogenesis showing vacuolation (v) and disruption of the epithelial pattern (d). Note large multivesicular residual bodies at the lumen (rb) and phagocytozed residual material (prb) at the base of the tubule. Original magnification: X350.

(d) Light micrograph of testis from Long Evans diabetic animal of tubules at stages IX and XIII of spermatogenesis. Note desquamated early spermatids (s), pachytene spermatocytes (ps), and pyknotic nuclei (p) within the lumen and the germinal epithelium. Also note the close proximity of spermatid heads to the basal lamina. Original magnification: X350.


v
t
bv
7a


sco
VIII
sco
bv
IV
7b


v
d
VII
rb
prb
7c

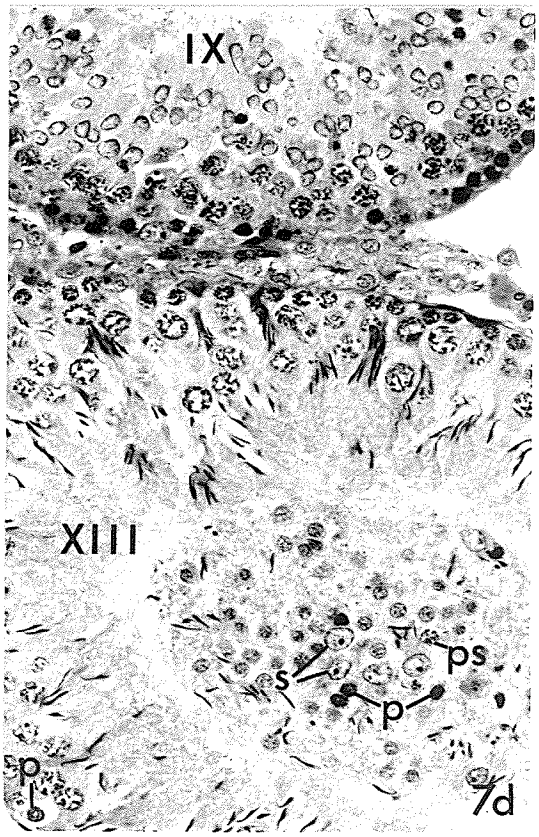

IX
XIII
s
ps
p
p
b
7d

Figure 8: Low power electron micrograph of testis from control Long Evans animal showing perfused capillaries (bv) within the interstitial space, and portions of two macrophages (mp). Parts of three seminiferous tubules (st) and numerous Leydig cells (LC) are also visible. Original magnification: X5,300.

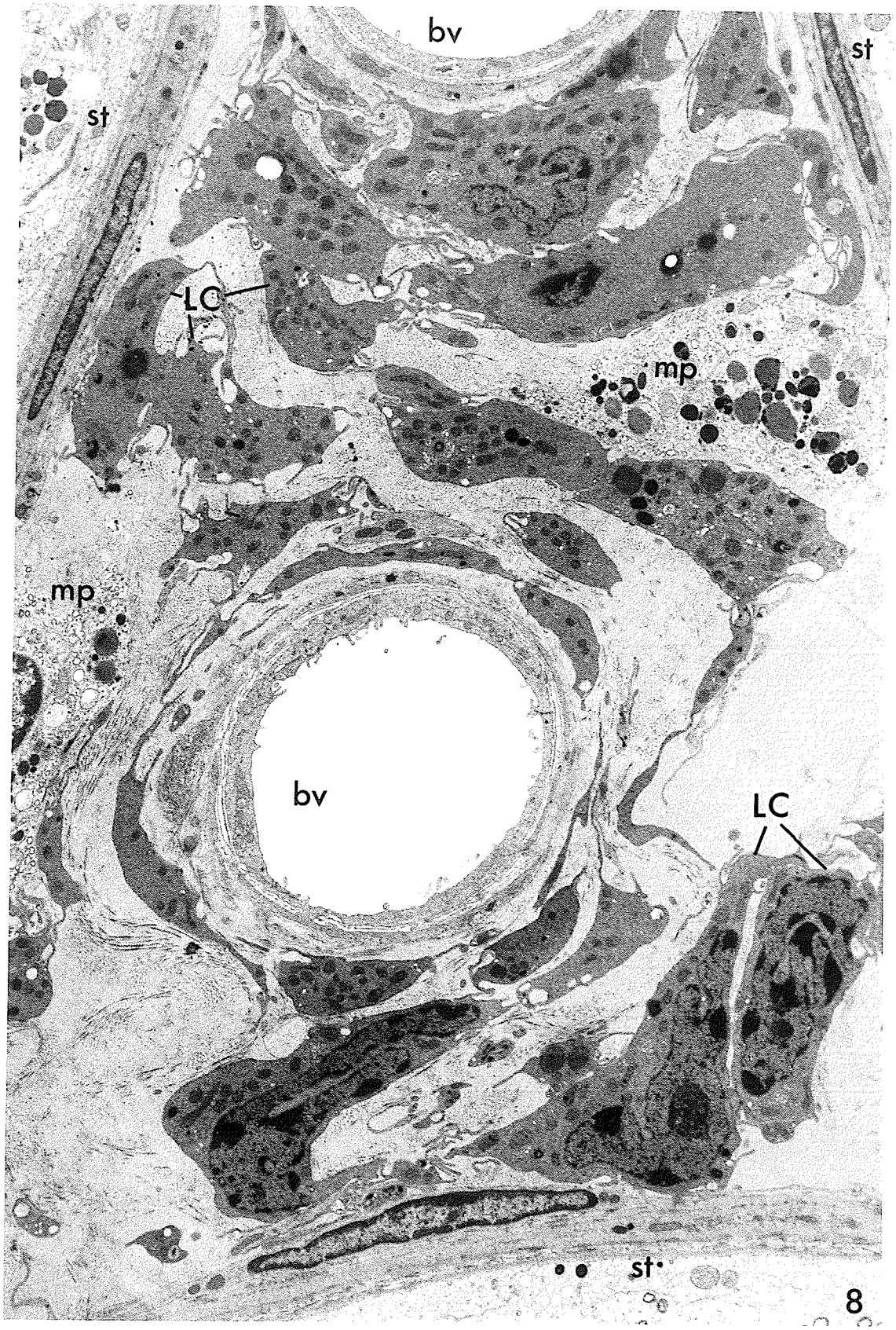
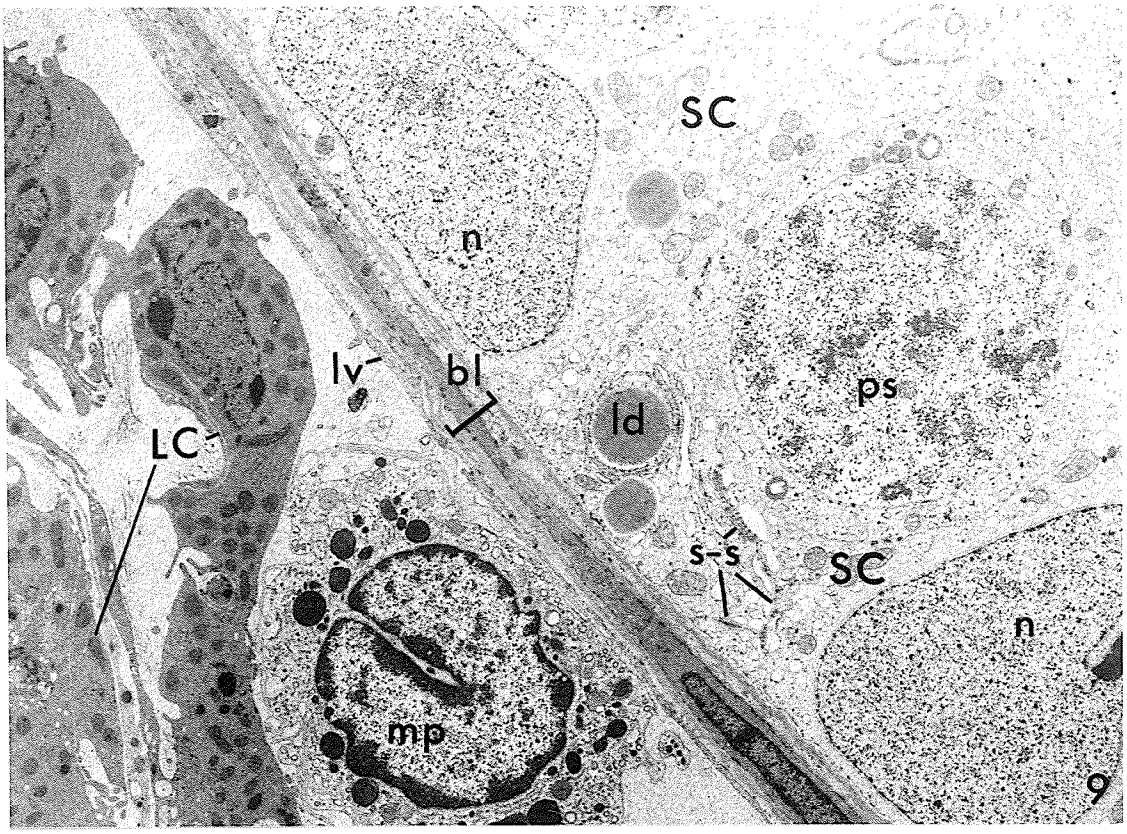

bv
st
st
LC
mp
mp
bv
LC
st
8

Figure 9: Low power micrograph of testis from control Long Evans animal showing proximity of Leydig cells (LC) and interstitial macrophages (mp) to lymphatic vessel (lv) and tubule basal lamina complex (bl). The complex is composed of four layers from tubule to interstitial space: tubule basal lamina, inner basal lamina of the peritubular myoid cell, myoid cell, and outer basal lamina of the myoid cell. Note nuclei (n) and lipid droplets (ld) within the Sertoli cells (SC), and Sertoli-Sertoli junctional complexes (s-s). Also note pachytene spermatocyte nucleus (ps). Original magnification: X5,500.

Figure 10: Higher power micrograph of Leydig cell from testis of control Long Evans animal showing dense mitochondria (m) with tubular cristae, Golgi complexes (G), and rough (rer) and smooth (ser) endoplasmic reticulum. Note the irregular cell outline exhibiting filopodia (f) and a cilium (c). Original magnification: X12,600.


SC
n
lv
bl
ps
ld
LC
s-s
SC
n
mp
9

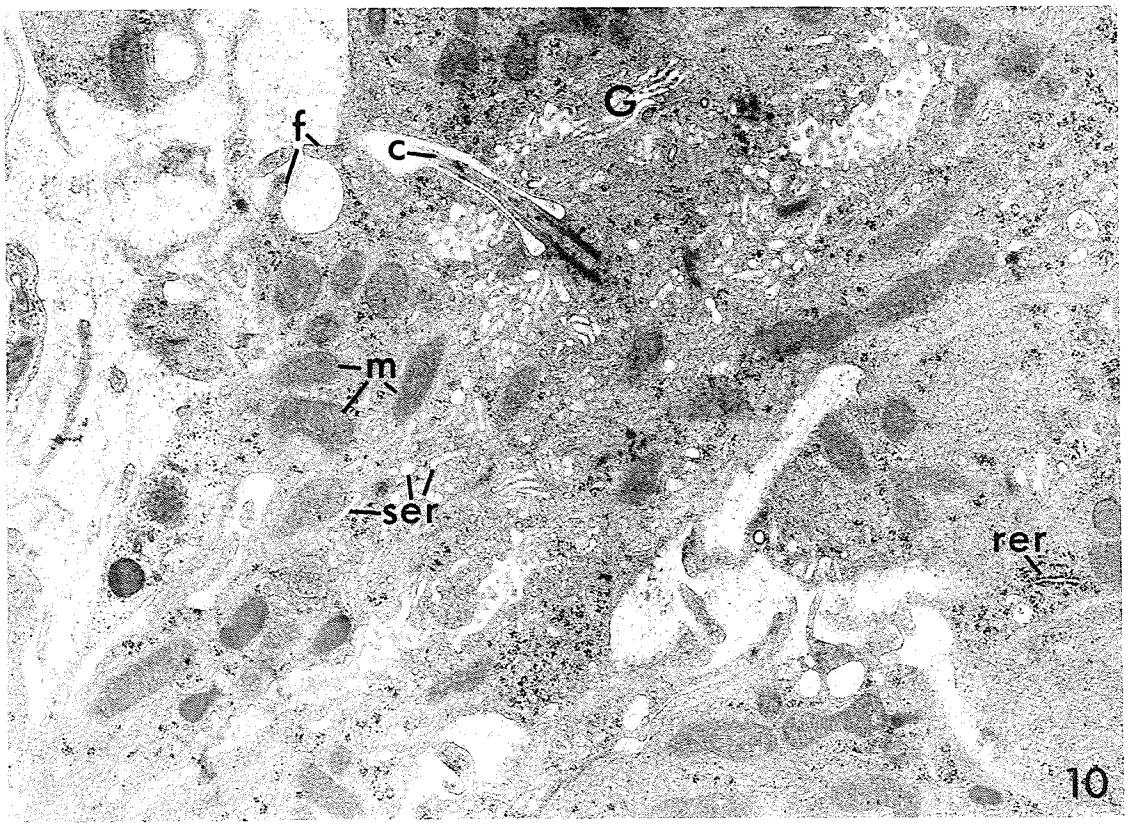
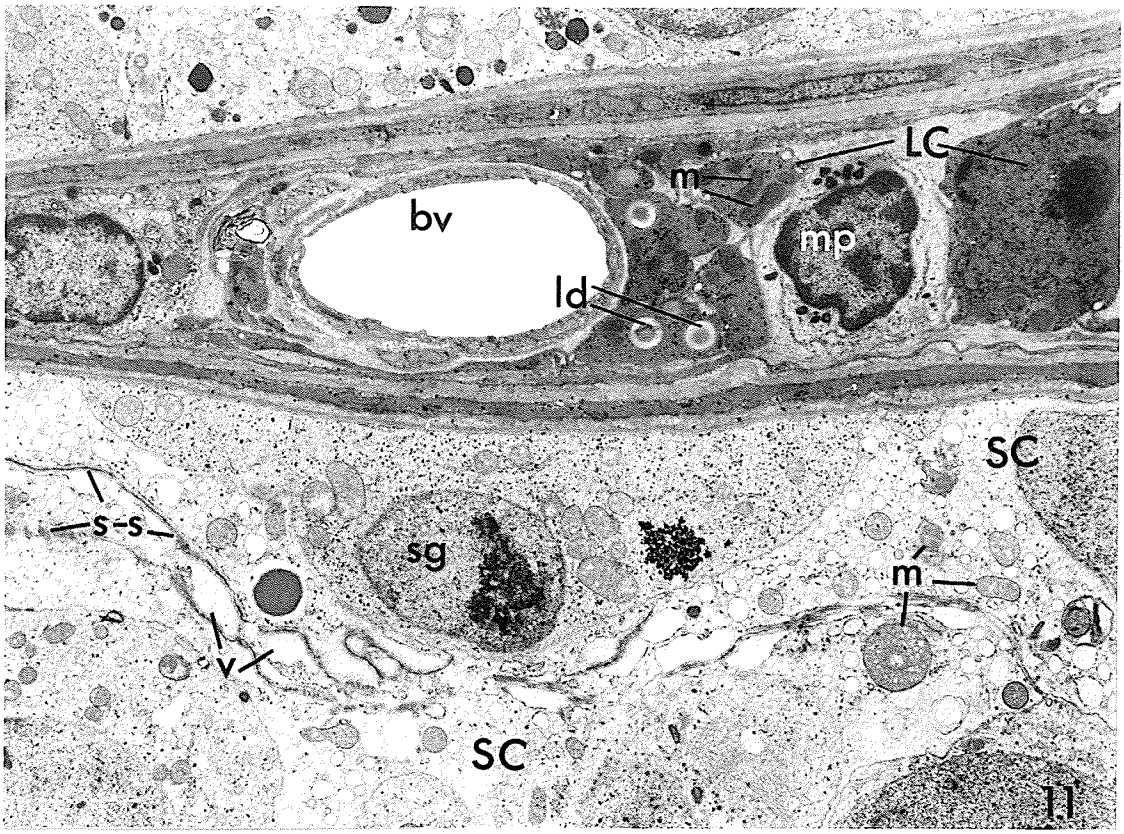
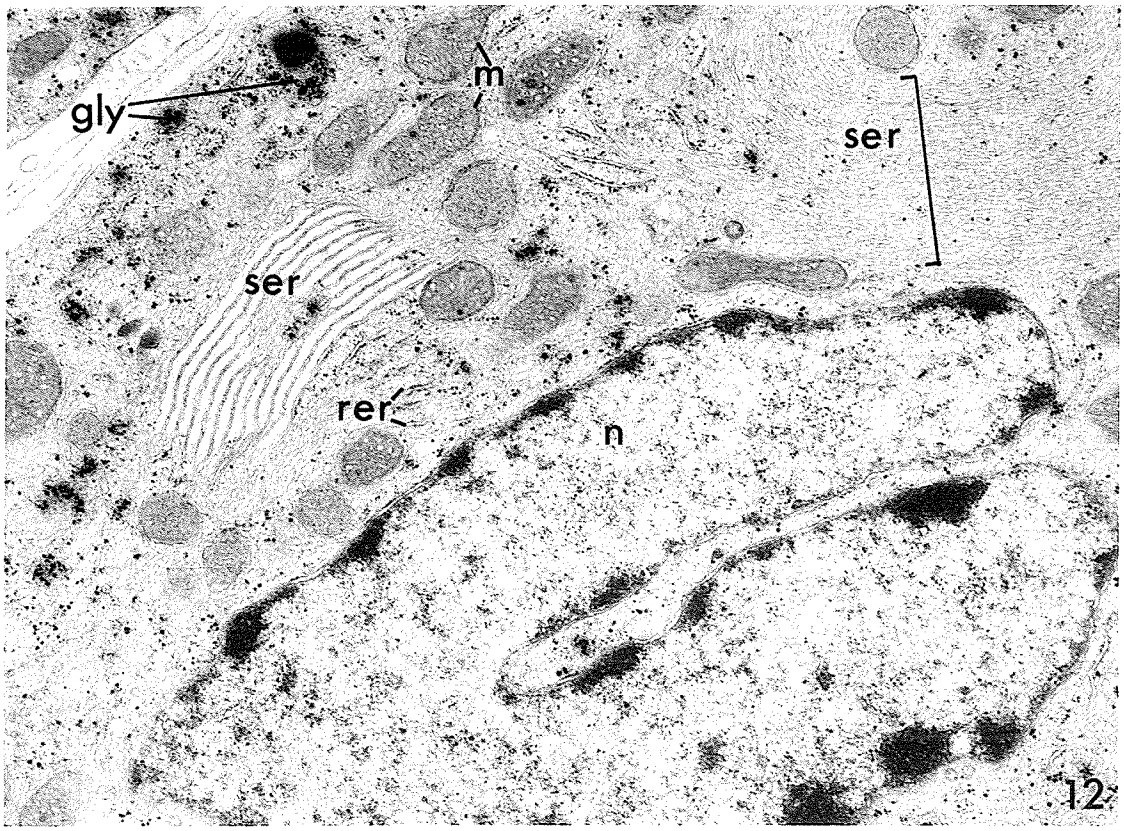

G
f
c
m
ser
rer
10

Figure 11: Low power electron micrograph of testis from a Long Evans streptozotocin-diabetic animal showing Leydig cells (LC) and interstitial macrophages (mp) in close proximity to a perfused blood vessel (bv) and portions of two seminiferous tubules (st). Note lipid droplets (ld) and dense mitochondria (m) in the Leydig cells. Also seen are a spermatogonium (sg) and Sertoli cells (SC) within the germinal epithelium. Note that the Sertoli cell contains mitochondria with tubular cristae (m), and that the Sertoli-Sertoli junctional complexes are vacuolated. Original magnification: X5,200.

Figure 12: High power electron micrograph of a Leydig cell from the testis of a diabetic Long Evans animal. Note the presence of large stacks and whorls of smooth endoplasmic reticulum (ser), mitochondria (m) with tubular cristae, glycogen (gly), rough endoplasmic reticulum (rer), and a prominent indented nucleus (n). Original magnification: X16,400.



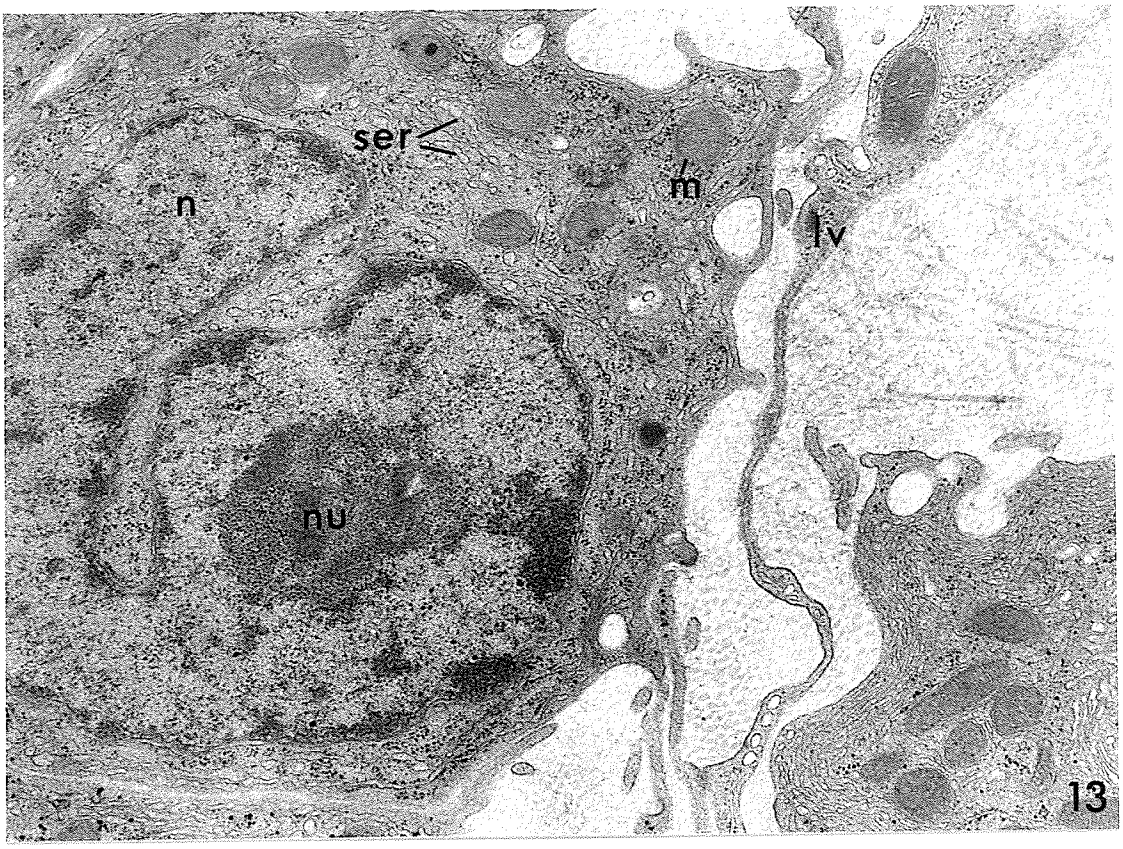
11



12

Figure 13: Electron micrograph of Leydig cells and lymphatic vessel wall (lv) from testis of an insulin-treated diabetic Long Evans animal. Note the indented nucleus (n) with prominent nucleolus (nu), mitochondria (m) with tubular cristae, and attenuation of widely spaced tubules of smooth endoplasmic reticulum (ser). Original magnification: X18,700.

Figure 14: Electron micrograph of Leydig cells from testis of a semistarved Long Evans animal. Note the nucleus (n) with nucleoli (nu), lipid droplet (ld), smooth endoplasmic reticulum (ser), mitochondria (m) with tubular cristae, and numerous stacks of rough endoplasmic reticulum (rer). Original magnification: X18,700.


ser
n
m
lv
nu
13

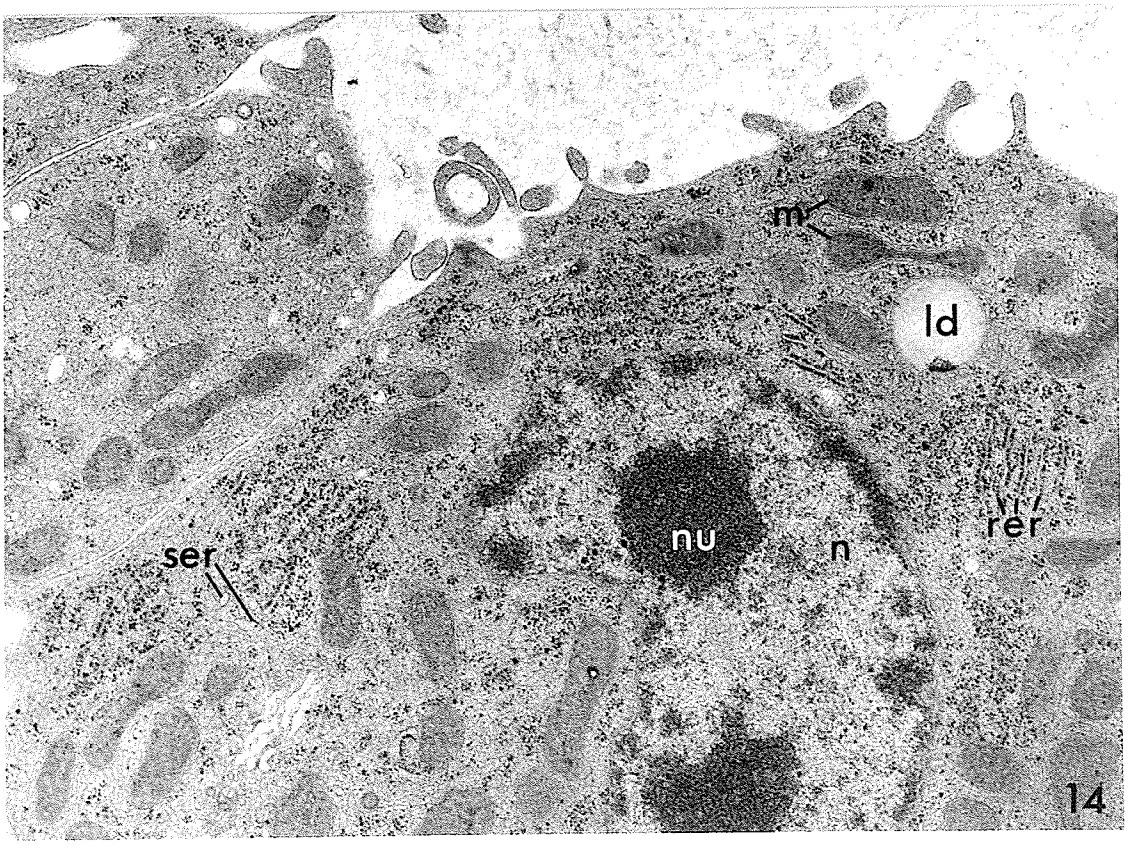
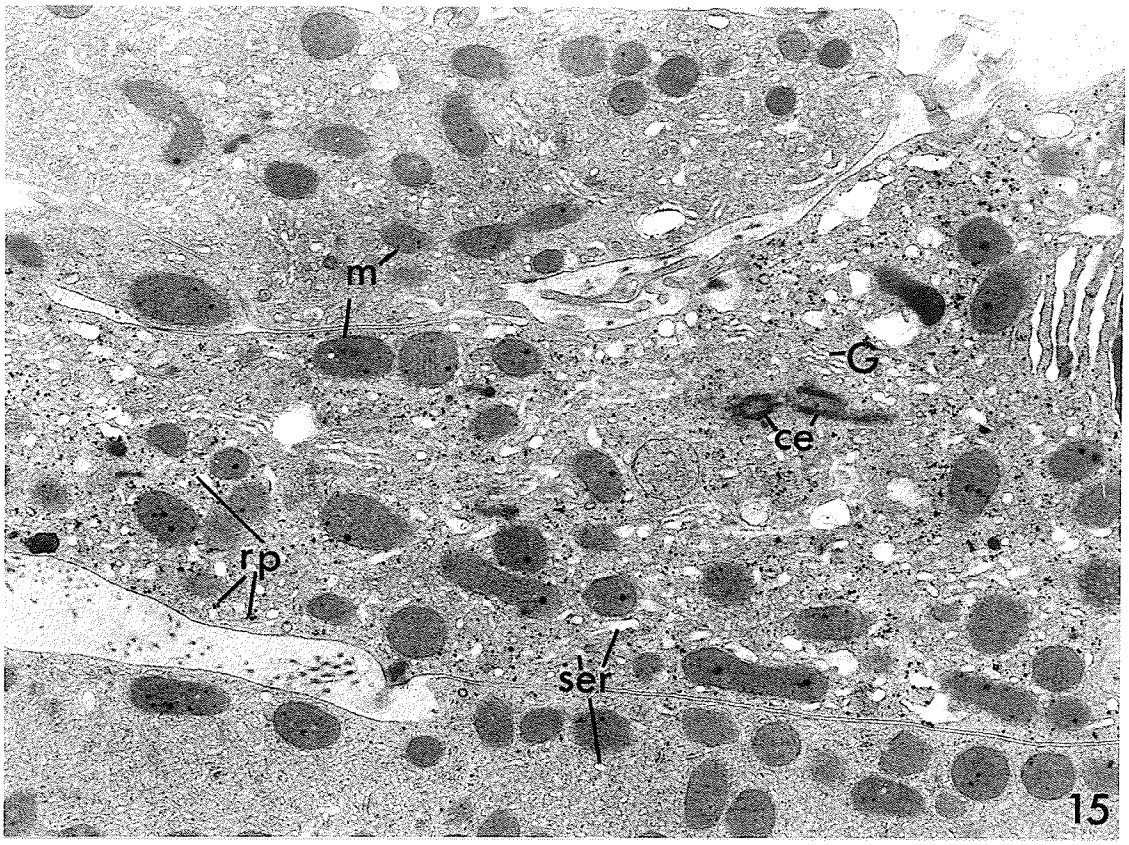

m
ld
rer
nu
n
ser
14

Figure 15: Electron micrograph of Leydig cells from testis of a control Long Evans animal. The tissue was incubated for HSD staining, and with diastase to digest glycogen. Note the presence of dense reaction product (rp) at or near smooth endoplasmic reticulum profiles (ser) within one cell, and its absence from two other Leydig cells. Also note mitochondria (m), a Golgi complex (G), and paired centrioles (ce). Original magnification: X12,800.

Figure 16: Electron micrograph of portions of three Leydig cells from the testis of an insulin-treated diabetic Long Evans animal. The tissue was incubated for HSD staining, and with diastase to remove glycogen. Note the deposits of reaction product (rp) in cells at different densities, as seen in the control situation. Original magnification: X20,700.


m
G
ce
rp
ser
15

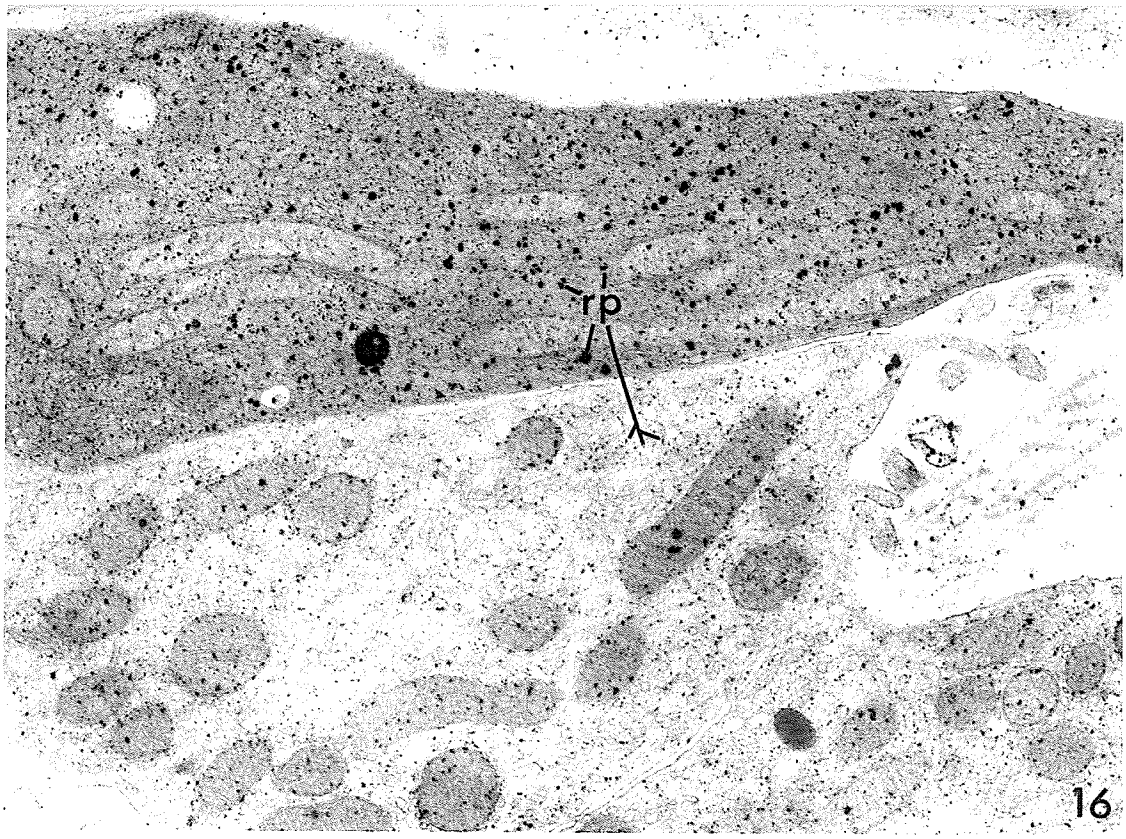
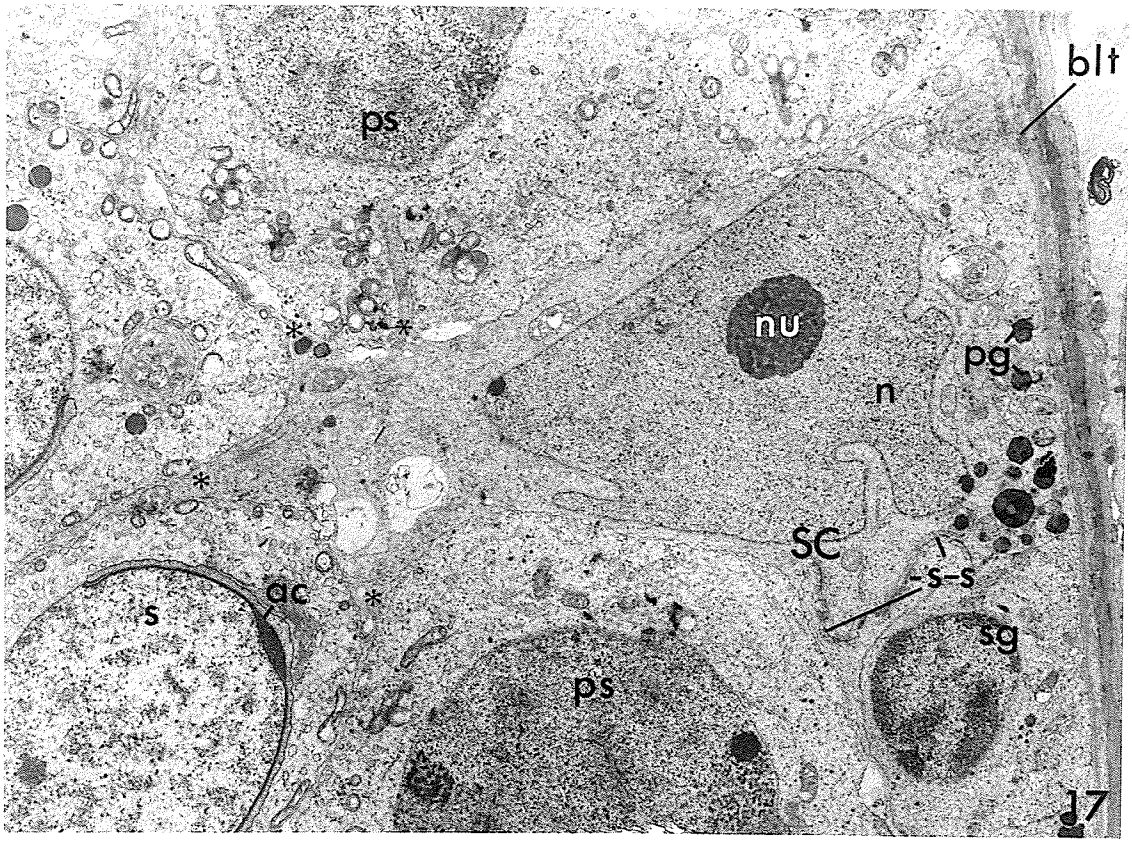

rp
16

Figure 17: Electron micrograph of a seminiferous tubule from a control Long Evans animal. Note the basal lamina of the tubule (blt), and a Sertoli cell (SC) with a large nucleus (n) and prominent nucleolus (nu). Also seen are small, dense pigment-like granules (pg) at the base of the cell, Sertoli-Sertoli cell junctions (s-s), and apical projections (*) between germinal elements. Note spermatids with early acrosomal caps (ac), pachytene spermatocytes (ps), and a spermatogonium (sg). Original magnification: X16,700.

Figure 18: Electron micrograph of a seminiferous tubule from a diabetic Long Evans animal showing a Sertoli cell (SC) on the basal lamina of the tubule (blt), and vacuolation of the mitochondria (m) and the cytoplasm (v) surrounding spermatid heads (s). Original magnification: X7,400.


blt
ps
nu
pg
n
SC
s-s
s
ac
sg
ps
17

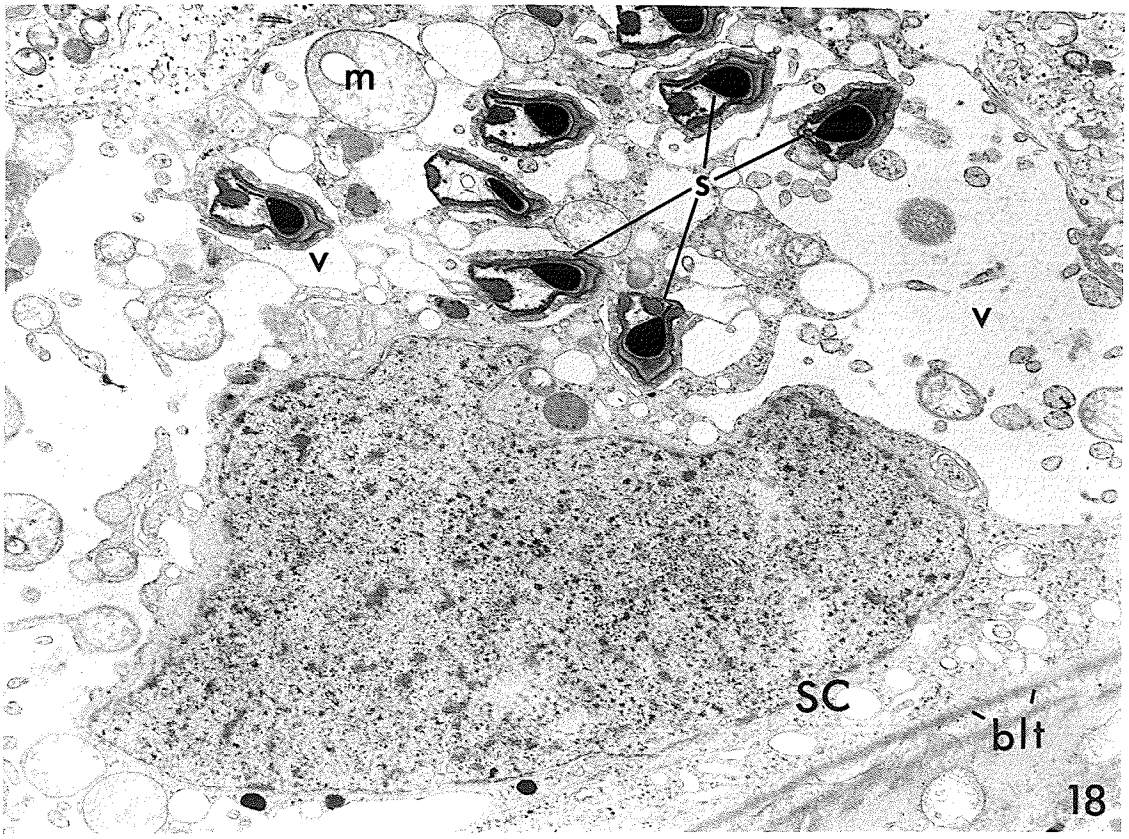
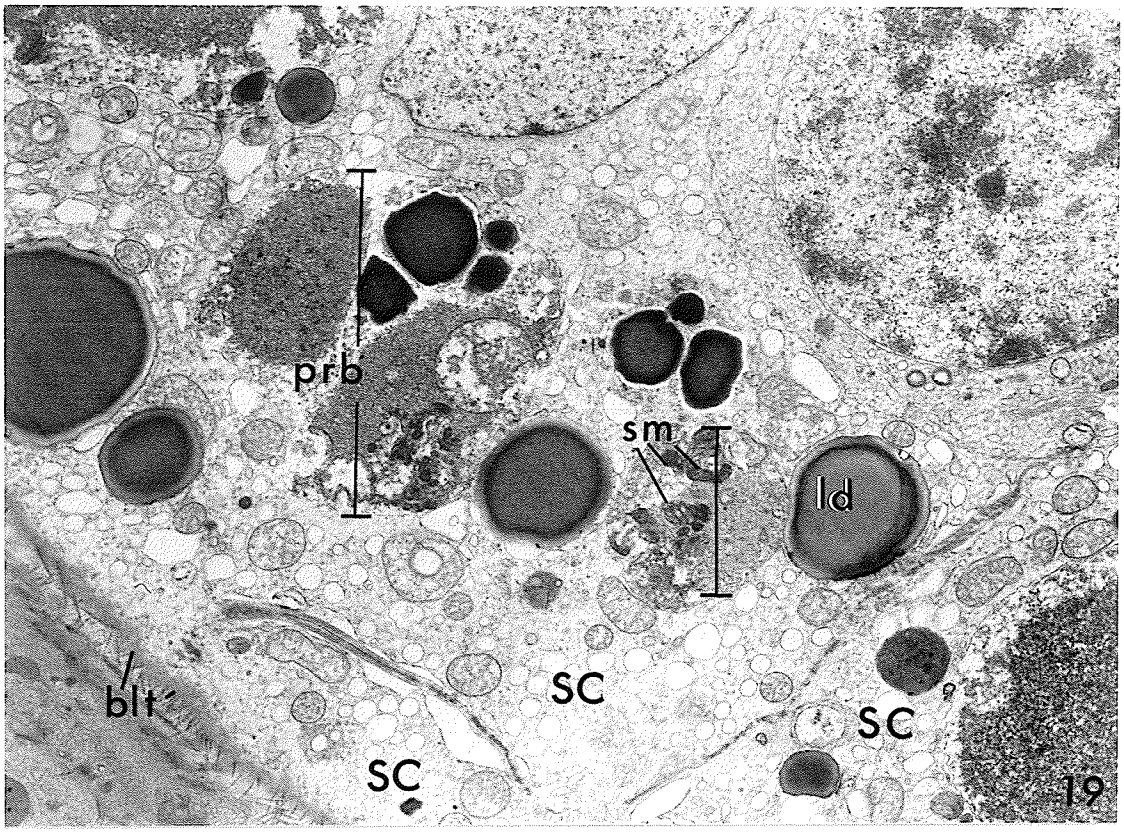

m
s
v
v
SC
blt
18

Figure 19: Electron micrograph of a seminiferous tubule from a diabetic Long Evans animal showing the base of a more than one Sertoli cell (SC) on the tubule's basal lamina (blt). Note the many lipid droplets (ld), and phagocytozed residual material (prb) which occasionally contains spermatid mitochondria (sm). Original magnification: X8,000.

Figure 20: Electron micrograph of the lumenal border (L) of a seminiferous tubule from a diabetic Long Evans animal showing giant multivesicular residual bodies (rb), Sertoli cell projections (sc), and an early spermatid (S) with characteristic condensed mitochondria (sm). Note the presence of spermatid tail cross sections (st) and spermatid heads in longitudinal section (sh). Original magnification: X8,000.


prb
sm
ld
blt
SC
SC
SC
19

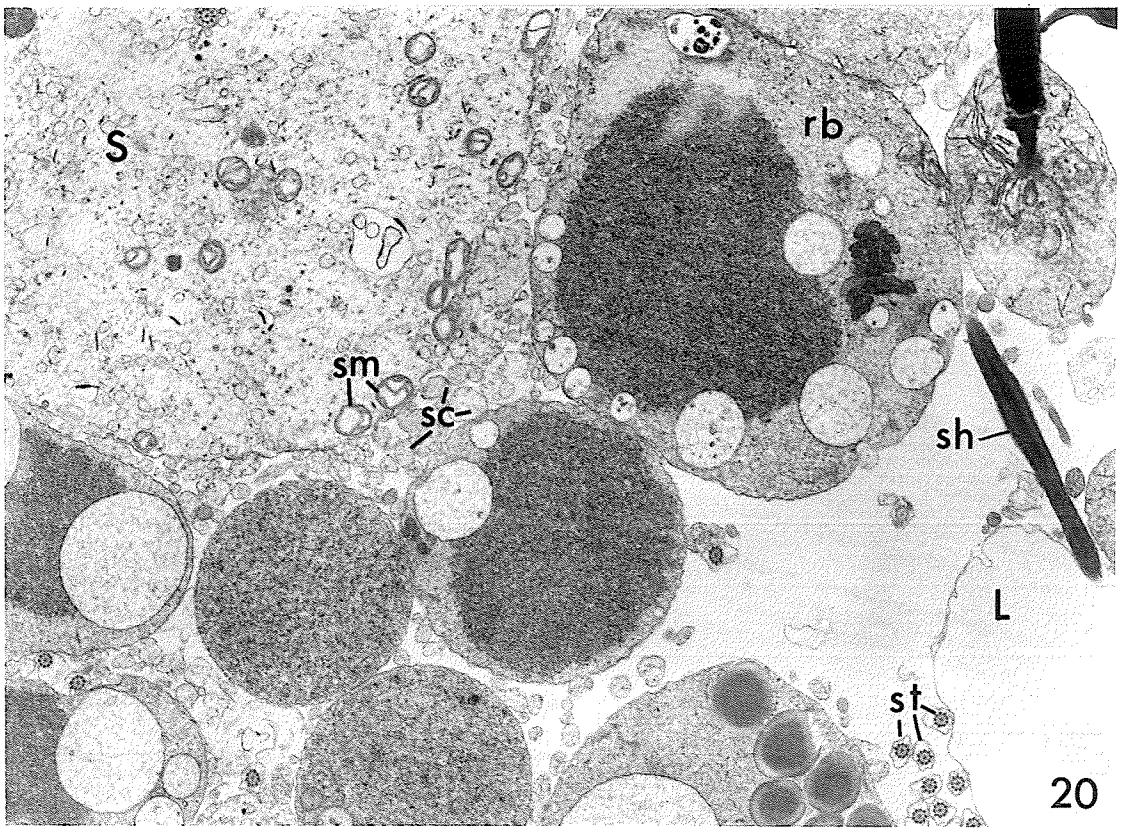
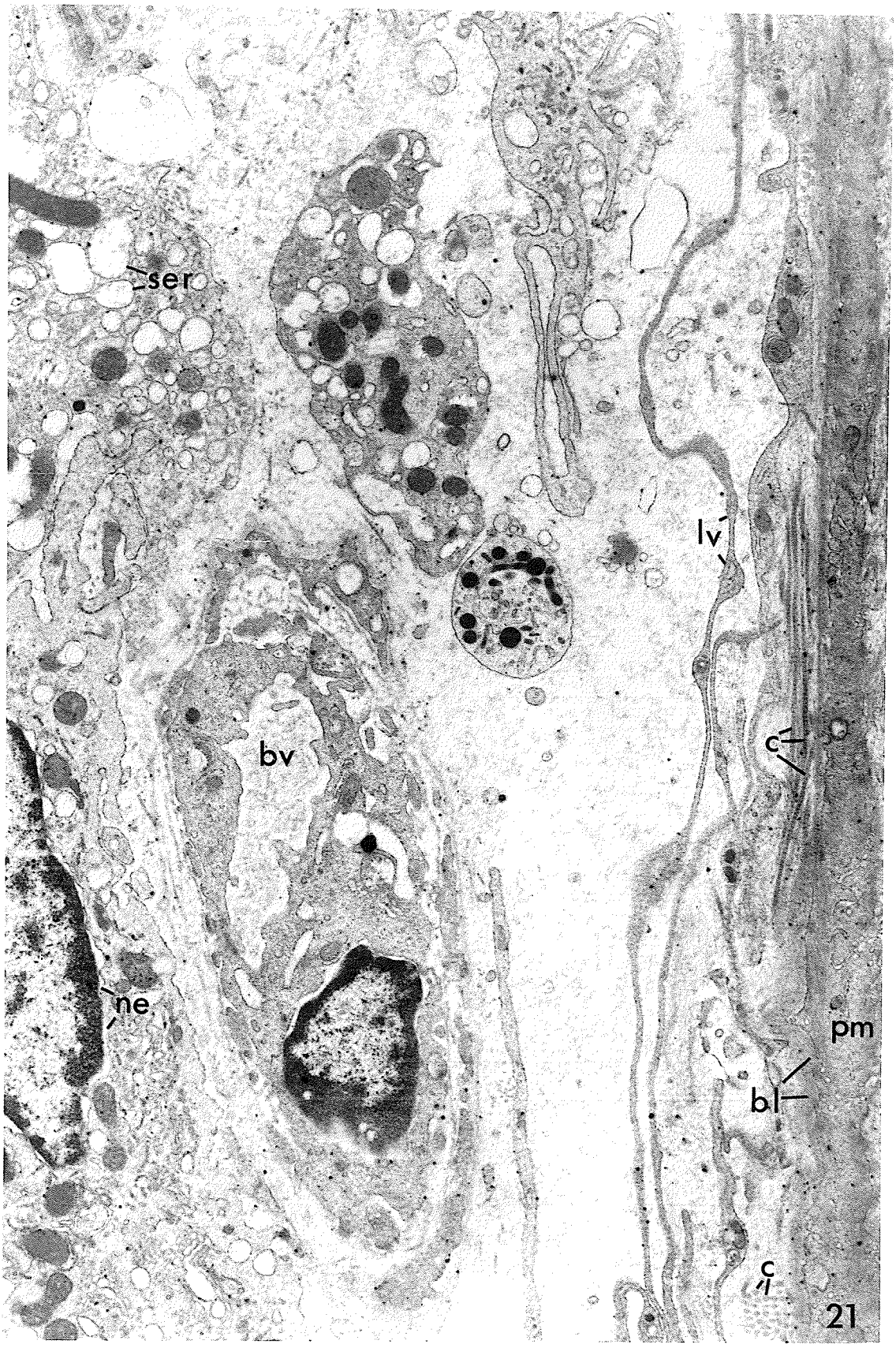

S
rb
sm
sc
sh
L
st
20

Figure 21: Electron micrograph of an interstitial area from an immersion-fixed testis of a diabetic Wistar animal. Note the swollen profiles of smooth endoplasmic reticulum (ser), and a vesiculated nuclear envelope (ne) in the Leydig cells. Also note a non-perfused blood vessel (bv), lymphatic endothelial cells (lv), collagen fibers (c), and a peritubular myoid cell (pm) and its basal lamina (bl). Original magnification: X11,500.


ser
lv
bv
c
ne
pm
bl
c
21

DISCUSSION

A number of questions arose from the results of the present study.

1. Were the animals hyperglycemic, or diabetic?
2. Was the insulin treatment adequate to control blood glucose?
3. Were the animals monitored in sufficient detail?
4. How is alteration of testicular interstitial blood vessel density to be interpreted?
5. Is staging of spermatogenesis a useful parameter to examine, and how can it be interpreted?
6. Was Leydig cell morphometry sufficient to determine cellular alterations, and how did the two methods differ in this ability? How did the results differ from those of previous studies?
7. Was the electron-microscopic HSD staining methodology found useful in examination of steroidogenesis, and how could it be improved?
8. Were the serum hormone levels consistent with testicular morphology in the control and experimental groups?
9. Were there significant strain differences between Long Evans and Wistar animals, and how did this influence interpretation of results?
10. Was the model effective in revealing the effects of diabetes, separable from streptozotocin toxicity, in investigation of the hypothalamo- pituitary- gonadal axis in streptozotocin-induced diabetes?

1. Diabetic or Hyperglycemic?

The cardinal signs of diabetes mellitus are hyperglycemia, polyuria, polydipsia, glycosuria and weight loss with high food intake in the presence of pancreatic beta-cell destruction. In animals injected with streptozotocin who became hyperglycemic, the blood glucose was very significantly elevated above the control range, as were urine glucose and urine output measures. The increased urine volume in diabetes was a reflection of the osmotic diuretic influence of hyperglycemic hyperosmolarity. Free water clearance was also much more negative in hyperglycemic animals than in the other four groups, confirming the hyperosmotic diuresis (304). Decreased hematocrit in the hyperglycemic group was another index of hypervolemia.

Creatinine clearance, a measure of glomerular filtration rate (304), was significantly decreased in the hyperglycemic group only when compared to the non-diabetic streptozotocin-injected animals. This implies that the large range of normal values seen in citrate-control rats was narrowed by treatment with the drug. BUN values were not significantly different between group means, although the orthogonal expression for the overall effect of diabetes showed it to statistically increase BUN and decrease creatinine clearance. While these are the usual findings in chronic diabetic renal disease, the mild elevation of BUN in these animals, especially in the context of normal plasma creatinine values (not shown) does not qualify as uremia.

It was important to exclude this as an influence on testicular function, as uremic men requiring peritoneal dialysis may be infertile or impotent (352, 354, 355, 359) as discussed below (section 9).

In summary, the persistently hyperglycemic animals in this study showed the cardinal signs of diabetes, and these signs were prevented by insulin treatment. This functional definition does not account for etiologic factors, and parallels to consequences of human diabetes must be made with caution.

2. Insulin Treatment

After trials of single and split doses of Lente insulin (Appendix 1), 3 IU per day of Ultralente was established to attempt glycemic control in the diabetic animals, combined with food restriction overnight. Blood glucose levels during the day continued to exhibit decreased glucose tolerance, but showed great moderation from the hyperglycemia in untreated diabetic animals (Appendix 1).

To document long-term trends in blood glucose, two comparisons were made between insulin-treated diabetic and non-diabetic drug-injected groups by two-way analyses of variance. First the serial blood glucose values during the entire insulin treatment interval were compared to those in the non-diabetic animals and no significant group, time, or interaction effects were seen. Second, the baseline glucose prior to streptozotocin injection, the 4-week glucose

reading, and the final glucose values at sacrifice were compared between the two groups. As the insulin-treated group did not receive insulin for 24 hours prior to perfusion, their insulin dependence was demonstrated by an increase in glucose at sacrifice. In this second analysis, therefore, significant group, time, and group:time interaction effects ($p<.001$) were demonstrated. Thus, while morning glucose tended to be above the normal range in the insulin-treated diabetic animals, it was not significant by either Tukey's procedure, orthogonal contrasts, or analysis of variance through the treatment interval, indicating adequate glucose control for the present study.

The adequacy of blood glucose control achieved in this study may be questioned in light of the neural, metabolic and endocrine modulation of insulin release in intact responses to fine changes in glycemia (3). In an experimental situation however, without more than one insulin injection per day, and insulin doses titrated to daily glucose measurements, truly normal glycemia would not be realistically achieved. For this reason, some researchers now use a minipump (272) adapted for rats to give close control of glucose with adjustable insulin delivery. Although desireable, that level of glucose control was not considered to be within the scope of the present study.

3. Monitoring and Protocol

The three main items monitored were blood sugar control, food and water intake, and duration of the treatment period. As discussed above, blood glucose was monitored frequently enough to determine statistically adequate insulin control, and to demonstrate significant and persistent hyperglycemia in diabetic animals. Blood glucose levels were measured in non-fasting rats, as it has been reported to more accurately represent experimental diabetic status than fasting levels (144).

Glucose perturbations within the 24 hour time frame would have contributed more detail to the status of insulin control, or its lack of regulation in untreated diabetic animals. However, frequent use of ether anesthesia, and technical requirements for regular, repeated glucose curves were felt to outweigh the advantages of such additional detail.

Food and water intake were difficult to quantify precisely in animal care, due to two factors. The food, in pellet form, was eaten by gnawing, and only part of a pellet was ingested. The remainder fell through the wire mesh of the cage floor. For this reason semistarved animals were weight-matched to diabetic animals as a group, rather than being pair-fed with individual animals.

Water supply bottles were prone to drip when moved or after drinking by the animals, causing overestimation of

consumption. The change in water level within the bottles was therefore only a rough marker of consumption, though it was apparent that diabetic animals drank much more than those of the other four groups.

Thus, food and water intake, and blood glucose control were monitored to the extent that they did not compromise technical and pragmatic concerns. They were not found to significantly interfere with interpretation of the data.

The three month duration of the experimental period was chosen in light of previous findings of reduced fertility after six weeks (181), and histological and ultrastructural alteration of the testis after 7 weeks of experimental diabetes (146, 147, 174, 177, 178, 181, 189). Such findings would be expected to be greater after longer duration of the diabetic state, if they were secondary to it. The treatment period was therefore lengthened to three months to enable changes to be well-established. This was not found to be complicated by age-induced alteration of testicular structure or function (342, 344-346, 348, 349), despite the animals being mature adults at the start of the experiment.

4. Testicular Blood Vessel Density: Real or Artifact?

Two observations gave rise to this question. First, the group means from blood vessel density measures were not significant by Tukey's procedure while in the face of a significant overall effect of diabetes to increase the density. As the orthogonal contrasts were defined a

priori, and tested an effect on the basis of within-group variance and between-group comparisons, their ability to determine significant specified influences was more rigorous than the former procedure, which tested "raw" group data on a multiple range basis.

Second, testis weight had a tendency (not significant) to decrease in diabetes. The decreased tubule diameter and germinal epithelial thinning could theoretically account for the weight decrease by shrinkage of tubules in the interstitial array.

However, the blood vessel counts were adjusted to a number per tubule as viewed through the ocular grid, with both counts and adjustment made with respect to the "forbidden zone" in stereological practices (231, 232). Therefore, a shrinkage artifact would be compensated for in these measurements.

Many authors have reported streptozotocin- or alloxan-diabetic rats to have decreased testicular weight (181, 209, 223, 225, 274, 275). However, there are also reports of experiments with no significant decrease in this parameter (227, 242, 276, 278, 326). This discrepancy may be a reflection of strain susceptibility to the dose of diabetogenic agent, route of induction of diabetes, of the age of the animal at induction, or of the severity and duration of the disease. In the present study, animals were mature adults, which conforms with a report of decreased susceptibility to testicular weight loss with increasing

animal age (242).

The removal of one testis at the beginning of the experiment, in order to compare its weight (and histology) with that of the remaining testis at sacrifice may have found a significant weight change. As the right and left testes were not significantly different in weight at sacrifice in this study, such a protocol may seen reasonable. However, this comparison could be complicated by the previously reported hypertrophy of the second testis after unilateral orchidectomy (62).

While previous authors have noted structural blood vessel alterations in diabetes, including basement membrane thickening and reduplication (334), or neovascularization (3), and functional changes including increased permeability (205), there are no reports of an increase in blood vessel density in the testicular interstitium. The increased number of vessels found in this study could be accounted for by increased coiling or tortuosity of capillaries, although this was not particularly noted in sections from tissue of diabetic animals.

Focal, diffuse seminiferous epithelial shedding, and Sertoli cell and spermatogonial degeneration resembling present histological findings, were reported in testicular ischemia, due to experimental clamping of the testicular artery (301, 302). Resolution of early ischemic damage occurred with fibrosis and neoformation of blood vessels (301), while tubules with significant ischemic damage could

not recuperate even after the return of arterial flow by collateral development (302). The tubule lesions reported in the contralateral (unclamped) testis also resembled the histology seen in the current study. They included lipid accumulation and vacuolation of spermatogonia, spermatocytes, Sertoli cells, and notably, spermatids. Such contralateral lesions were found to resolve if not severe. It is not known whether testicular ischemia in particular, occurs in diabetes, possibly due to focal microangiopathic disease, but the increase in vascularity noted in this study could be a clue to collateral development. Blood flow monitoring and silicone rubber perfusion casts of testicular vessels would clarify this question.

The implications of increased vascular density in testes from diabetic animals may also be important with respect to the heat transferred by blood. Body temperature is adjusted and regulated under the control of the sympathetic nervous system (281, 282) by changes in blood vessel diameter or flow (218), with peripheral constriction in cold and dilatation with heat. Density and anatomical placement of vessels also contribute to temperature regulation, as exemplified by the heat conservation provided by venae committantes (282). In the case of the human testis, temperature is also controlled by the cremaster reflex (282). However, this reflex is lacking in the rat (303), in which the scrotum is an outpouching of the peritoneum, covered by a thin layer of skin, with retention

of an open connection to the peritoneal cavity (303, 328). This simple scrotum allows a much larger gradient between testicular and body temperature in rats than in rabbits, which do not have a scrotum (303). Indeed, rat scrotal blood flow increases to twice normal values with an increase in external temperature from 33 to 37 degrees C., and no other tissue responds to this extent (310). Thus the lack of a cremasteric reflex is overcome largely by blood flow modifications, making anatomical vascular parameters even more crucial to temperature regulation.

It is also well-known that Sertoli (284, 287, 288, 290) and Leydig cell functions (284-286, 289, 309), as well as lysosomal membranes in early pachytene spermatocytes and early spermatids (305) are very sensitive to increases in ambient temperature. This has largely been studied in experimental cryptorchidism, which is accompanied by increases in testicular blood flow (517), and by external application of heat to the scrotum (285). Therefore, involvement of the autonomic nervous system in diabetic neuropathy, allows the possibility of compromised control of blood vessel diameter to combine with an increased vascular density (not an artifact of testicular shrinkage in this study), and increased vascular permeability. The sum of these alterations could significantly contribute to increased testicular temperature in the rat, to the detriment of Sertoli and Leydig function, and spermatogenesis.

5. The Stages of Spermatogenesis

The significant alteration of the frequency distribution of stages in the spermatogenic cycle (tested by Chi-square), and the site of that difference at stage IX-XI (by non-parametric analysis of variance) were interesting findings. Further analysis using control values as expected in a one-variable contingency table (213), also suggested that streptozotocin-induced diabetes may alter the spermatogenic process, and that insulin correction of diabetes to the level achieved in this study, did not entirely prevent that alteration. In as much as the frequency distribution is the outward marker of the relative duration of each stage in the cycle (193), a change in that distribution could imply that the dynamics of meiosis in sperm production were altered in diabetes.

This hypothesis has been tested previously in a study of tubule stages in hypophysectomized rats, with the conclusion that the stages were unchanged in their frequency (324). However, the conclusion was reached without statistical application to the frequency data. Indeed, re-examination of the data presented by those authors using a Chi-square analysis, does show significant variation between stage frequency distributions in their control and hypophysectomized rats. Furthermore, comparison of the control frequency distributions from that study, and control distributions from an earlier paper by the same laboratory

(193), revealed a significant difference by Chi-square analysis. The statistical difference was even larger with control distributions from the present study included.

Two factors may explain these retrospective findings. First, the ability to stage the seminiferous tubules may be consistent only within one study, or in one strain of rat, or the stage frequency distributions may change with time. While the latter possibility may occur after great inbreeding, the first two are more reasonable. A strain difference may be a factor in these comparisons. In the present study, a significant strain-dependent difference between Long Evans and Wistar control groups in the frequency distributions was shown. Indeed, the entire spermatogenic cycle time has also been found to vary with strain, being 48 days in Long Evans rats (198) and 51.6 days in Sprague-Dawley rats (294).

The two earlier studies (193, 324) were both on Sprague-Dawley rats, and it is conceivable that a strain may change over a span of years if it is highly inbred, as have age and weight parameters (355). These considerations would not account for the alteration seen in the present study, as the within-group Chi-square analyses were significantly dependent on individual animals only in the diabetic and semistarved groups.

Second, it is also possible that the significant difference is an artifact of the statistical method. As Chi-square analysis is based on proportional contributions

to marginal totals, each total of equal probability, it may not be a precise determination of deviation from biologically expected values, but only from mathematically expected values. In consideration of spermatogenesis, where the stages are not of equal frequency, their frequency is the prime index of the relative duration of each stage (193). Therefore, the stages are not expected with equal probability, and such a statistical measure indicates only that a difference exists, not its source.

Partitioning of Chi-square tables (240, 241, 256, 257, 259) may also not be entirely applicable to this situation, as the stages are arbitrary divisions of a continuous process, rather than distinct categories (that is, they are not binary choices). This further serves to emphasize the complexity of statistical applications to the spermatogenic process.

This complexity is not surprising, in view of the many cell types and kinetic processes in which they participate at even one stage of the cycle. Furthermore, each of the fourteen stages outlined by Leblond and Clermont (193, 194, 200) are traversed four times by each cell as it becomes a mature spermatid. This increases the difficulty of assessing the static representation of a dynamic process, composed of multiple inter-cellular associations.

However, it remains to explain the significant difference in tubule stage frequency at stages IX-XI. The period of spermatogenesis with the greatest relative

dependence on androgens occurs at stages VII-VIII, as judged by paracrine stimulation of testosterone production (356), by degeneration after hypophysectomy (243), and by correlation with LH level (243). Nuclear receptor density studies showed biphasic testosterone binding, with maxima at stages IX-XII and XIII-I (357). FSH dependence by Sertoli cells is greatest at stages VII-VIII (244) when there is also the highest concentration of testosterone (238), although LH and testosterone support must be present to permit FSH action (243). The highest androgen binding protein (ABP) production and release occurs at stages VIII-XI (248, 245, 238, 266), and maximal phagocytosis of residual body material from maturing spermatids is at stage IX, marked by a maximum lipid content (246). It is possible that this time period contains the most hormone-dependent cells of the entire spermatogenesis cycle, and therefore is the first to be altered in relative frequency with disruption of the hormonal milieu.

In this study, giant residual bodies in tubules at stages before and during spermiation, vacuolation of Sertoli cell cytoplasm, and greatly increased Sertoli cell lipid inclusions were noted. Previous studies have shown that cell remnants from spermatocyte-to-spermatid maturation are normally phagocytozed by Sertoli cells (246). Testicular ABP content was reported to be decreased in streptozotocin-induced diabetes (247, 259) as a result of defective testicular secretion or transport to the

epididymis. Lipid inclusions have been noted to be larger and more widely distributed across stages during germ cell and spermatocyte degeration (249, 261, 279, 280, 295, 296). All of these changes signify compromised Sertoli cell function.

The appearance of large residual bodies has been noted in many other experimental and clinical studies, such as testicular ischemic damage (270), experimental allergic orchitis (265), experimental cryptorchidism (271), vitamin A or E deficiency (273), heat exposure (296, 297), and in testicular feminization in mice (253). In all of these conditions, there was decreased Leydig cell responsiveness to LH or hCG stimulation (254, 255), thought to be secondary to disruption of spermatogenesis (255). The pattern of decreased response to testosterone feedback has been noted in untreated streptozotocin-diabetes (208, 268). This suggests an insulin facilitation of Leydig cell LH receptor function, separate from decreased responsiveness of the hypothalamo-pituitary unit to testosterone feedback.

The large residual bodies have been described as "multinucleate giant cells" in light microscope studies (270, 271, 273, 279, 280), but under the electron microscope they are not multinucleate. Instead, they are multivesicular, and contain large granular aggregations that are not membrane-bounded, homogeneous lipid inclusions and vacuoles, and mitochondrial remnants from spermatocytes, as seen in Figures 19 and 20 (252). They are often described

as aggregations of degenerating spermatocytes and spermatids (271, 273), although they do not contain flagellar material. Their presence may be the most obvious marker of greatly compromised Sertoli cell phagocytic function, appearing after the large lipid droplet accumulation at the cell base.

In summary, statistical analyses revealed a significant alteration of the spermatogenic cycle. This disruption was most notable at the stage known to be highly dependent on testosterone and FSH concentrations. The stage frequency changes, likely relative in nature, were not entirely prevented by insulin treatment in this study. The possibility that the intrinsic time sequence of the spermatogenic process may be altered in a non-relative manner affecting stage duration is intriguing. In view of the emphasis of modern research into male fertility control, the idea may be worth pursuing in greater structural and statistical detail.

6. Morphometry of Leydig Cells

The main question at hand is whether quantification of Leydig cell ultrastructure is a useful marker of endocrine and metabolic status. This involves consideration of error, methodology, and interpretation.

Reproducibility of measurements made by computerized planimetry was confirmed by determining a small standard error in use of the graphics tablet. This permitted many

measurements to be made consistently and rapidly. Ideally, more cell compartments would have been quantified by this method, but the area outline of SER, for example, was not practical. Therefore, point counts were made of organelle compartments. This also determined the mitochondrial areal fraction, and the two measures by different methods, were significantly correlated.

There may be concerns that the graphics tablet planimetry method has sampling (232) and interpretation drawbacks. It may be argued, for example, that the cross-sectional area of a mitochondrion with an elongated cigar-shape, may not be an accurate measure of its size. However, as demonstrated in the present study, the large numbers of mitochondria measured from 10 random micrographs per animal (250-450), did compute very similar average mitochondrial sizes with a unimodal distribution.

Semistarvation had the effect of decreasing mitochondrial size and increasing their numbers, while the effect of diabetes was to increase mitochondrial areal density in the Leydig cell cytoplasm, with no significant effect on their numerical density. This may be interpreted to show that compensation for metabolic compromise can occur by significant changes in either number or size, or by small changes in total area with only slight changes in size or number. This corresponds to the negative correlation between number and size, with neither parameter alone significantly correlated to mitochondrial areal density.

This suggests that total mitochondrial area in a Leydig cell tends to remain relatively stable under many conditions. That the mean mitochondrial areal density in Leydig cells from diabetic animals was not significantly different from those of control animals by Tukey's procedure, and was seen to be increased only in orthogonal contrast study, again shows the strength of the latter test.

The increased standard deviation of single mitochondrial size in starvation (where mean size tended to be decreased, although not significantly), may indicate the possibility of graded responsiveness within a population of mitochondria. The change in standard deviation would have been statistically expected in the diabetic group, with "bilobed" mitochondrial profiles, due to variations in sectioning angle across the profile. It may, however, reflect a true change in shape with treatment. An elongated tubular organelle in a control situation, may become smaller and flattened to a biconcave disc (which would have both bilobed and doughnut-shaped cross sectional profiles) to increase inner and outer membrane surface areas before a significant size decrease is noted. To confirm the presence of these fine adjustments would need more detailed morphometric investigation, including shape quantification by form-factor analysis (384).

The possibility of shape modification in response to metabolic insult is not unreasonable, considering the well-known separation of the mitochondrion itself into inner

and outer membranes and matrix compartments (58, 61, 314, 315). Demands put on energy production, or lipid substrate conversion, for example, in interaction with intra- and extra-cellular environs may provoke up- or down-regulation of specific enzyme pathways, and thus alter fine anatomical features. Such localized responses have been reported within one cell population (43, 44, 362) and within a single cell (1, 318, 320).

A pattern different from that seen with mitochondria, appeared in the lipid compartment of the Leydig cells in animals of this study. Lipid areal density was positively correlated to both single lipid droplet size, and to lipid numerical density. This suggests that size and number of lipid droplets may be modulated up or down together in a compensatory response to physiological changes. This would also allow for more gradual changes in droplet size or number in a response, and could account for the lack of significant difference between group means and effects in their measure.

The diabetes and starvation effects both increased lipid numerical density, in negative correlation with serum testosterone levels. Lipid areal density was also negatively correlated with testosterone. These findings support the concept of lipid droplet storage of steroid hormone precursors (58).

In point-counting quantification of Leydig cell cytoplasmic compartments, a more general representation of

intracellular activity was produced. This generality was primarily due to the compromise reached between desired detail in the micrograph magnification, resolution of the coherent grid test systems (231), and the time allotted to its achievement. In this case, medium magnification and grids to detect mitochondrial and SER dimensions were used. The test grid was randomly placed on the micrographs and thus did not include the total cytoplasmic area measured by planimetry. This would account for the relatively low, although statistically significant correlation between mitochondrial areal density measurements by the two morphometric methods.

While there were significant alterations in the size of organelle compartments, the overall changes portrayed a decreased area of the cytoplasmic matrix not occupied by organelles in Leydig cells from diabetic and semistarved animals. The reverse was true of the insulin-treated diabetic animals: their Leydig cells showed increased matrix area and decreased mitochondrial, SER, RER, and Golgi areas. Leydig cells from control animals showed a pattern intermediate between these two trends.

Insulin has been reported to have positive effects independent of ambient glucose concentrations, on renal function (188, 304, 334, 350), testosterone synthesis (251), and gonadotropin release (324), as well as stimulation of HSD activity, as noted in the present study. The organelle portrait of Leydig cell cytoplasm after insulin treatment

would seem to support the idea of increased efficiency in intracellular physiology in these animals, and an important direct influence of insulin on Leydig cell function. That suboptimal insulin treatment of diabetes could increase cellular capacity to supranormal levels, suggests also that normal physiological patterns are less than optimal.

The normal endocrine target cell functions with minimal receptor-hormone binding compared to total binding capacity (318). Indeed, the capacity to meet insult with compensatory hormonal and physiological responses would seem to insist on supranormal capacity and submaximal metabolic norms. This concept is supported by the apparently failed compensation for Leydig cell hypofunction in diabetic Long Evans animals with primary insulin deficiency, rather than deficient gonadotropin stimulation.

Reports of maximal testosterone production correlating with SER volume density (319, 324) and with HSD activity with substrates supplied in excess (321) substantiate these ideas. Although this seemingly conflicts with the present finding of increased SER during decreased testosterone secretion, the testosterone levels were basal rather than exogenously stimulated. Also the negative correlation between mitochondrial and SER compartments may be taken as an indication of attempted and failed compensation of the SER compartment to increase testosterone production. Interestingly, the serum testosterone level was better predicted by SER and lipid droplet size, number, or areal

density, and by lipid droplet number and HSD staining, which perhaps take into account the failed component of steroidogenesis.

To summarize, Leydig cell morphometry revealed significantly decreased mitochondrial size, and increased mitochondrial numerical density, lipid areal density, and SER volume density in semistarvation. Similar but nonsignificant trends in these Leydig cell organelle compartments were found in diabetes, as well as significantly increased lipid numerical density and increased mitochondrial numerical density. The lipid compartment was found to adjust capacity by parallel changes in number and size, while the mitochondrial compartment was found to be relatively fixed in area, with inverse changes in number and size. Point-counting was not as accurate as planimetry, but it was sufficient to define similar direct insulin-dependent trends in Leydig cell organellar constituents in Long Evans animals.

A number of contradictions have been found in this study, in comparison with previous reports. In two previous qualitative studies by Orth *et al* (177) and Rossi and Bestetti (209), Leydig cells in streptozotocin-diabetic rats were noted to contain much less SER than those in control animals and to have lipid droplets not present in the control situation. However, these conclusions were contradicted by a morphometric study by Thliveris *et al* (189) of many Leydig cells from each animal, which found no

change in SER or in mitochondrial or lipid droplet numbers per micrograph, but instead decreased mitochondrial size and increased lipid droplet size in diabetics. The contradictions were attributed to the possibility of strain differences between Wistar (177, 209) and Sprague-Dawley (189) rats, as well as to the precision of morphometric analysis compared to qualitative description.

In the current study, further contradictions to the report by Thliveris *et al* (189) are evident. First, in the present investigation, mitochondrial areal density and lipid numerical density were increased by the effects of diabetes, where they were determined per unit area of cytoplasm. These parameters were determined per micrograph by Thliveris *et al* (189). Second, no significant change in size of mitochondria or lipid droplets were found. This may result from differences in statistical design between the two studies. In the current investigation, smaller samples of Leydig cell organelles from only 10 micrographs per rat were taken from five different groups. This removed one possible bias to the results by not reducing standard errors with large samples from each animal. More stringent tests of significance using ANOVA, Tukey's test or orthogonal contrast analysis were made possible using five groups, than was made by a student's-t test between two groups (189). The decreased mitochondrial size and increased lipid droplet size revealed in the previous study by Thliveris *et al* (189) is suggested, however, by a

tendency among the group means in the present investigation.

Third, SER volume density was found increased by the effect of diabetes in Long Evans rats in the present investigation. In contrast, a virtual absence of SER in Leydig cells of diabetic Wistar rats (177), but abundant SER in Leydig cells of diabetic Sprague-Dawley animals (189) have been reported. The question of strain differences in this regard was unfortunately not answered by the present investigation, due to the poor perfusion of Wistar tissues, and the lack of LH data.

Fourth, in the current study, lipid droplets were present in control animals of both Long Evans and Wistar strains. They were also noted in control Sprague-Dawley animals by Thliveris *et al* (189). These findings contradict the previous work by Orth *et al* (177) and Rossi and Bestetti (209) on Leydig cells in control Wistar animals, which were found to lack lipid droplets. While this discrepancy is difficult to resolve, the existence of storage capacity for testosterone precursors in lipid droplets under control circumstances supports the concept of submaximal production in intact animals.

The cells labelled as Leydig cells of diabetic animals by Rossi and Bestetti (209) resemble interstitial macrophages seen in the present study (Figures 8, 9, and 10), which possess pale cytoplasm and pseudopodia, and contain dense granules and lysosomes. It is possible that long term diabetes in a mature rat may cause a transition

from steroidogenic to phagocytic cell types, and that the previous authors have mislabelled the cells. However, it is more likely that a large increase in the interstitial macrophage population occurred in combination with the aging process.

The differences between control ultrastructure noted by Orth *et al* (177) from that of the present study and of the report by Thliveris *et al* (189) are difficult to explain, and may result from differences in sampling technique, small sample size (n=2), or in the particular line of inbred Wistar animals studied by Orth and coworkers (177).

In summary, the current investigation of Leydig cell ultrastructure and morphometry contradicts previous findings in four main areas, likely due to a combination of differences in sampling, methodology and statistical design. Strain differences may be involved in measures of SER volume density and lipid content, though this remains to be confirmed.

7. Histochemistry

In examination of Leydig cell steroidogenesis, results of the HSD and diastase incubations were found to be consistent among tissue blocks from each animal, and with the preliminary methodology study, as previously published (1). The testicular HSD activity was found to be decreased in only the diabetic animals, confirming previous

biochemical work (327, 330).

There were, however, a number of problems encountered in application of this technique to the present project. First and foremost, the positively stained proportion of Leydig cells did not correlate very strongly with testosterone concentrations in the serum. This may not be surprising. A small number of Leydig cells, chosen with a systematically random method, were counted relative to the total gonadal population. There was subjective assessment of each cell's staining, and to maintain the consistency of this judgement, cells were called positively stained only if more than one quarter of their cytoplasm (as photographed) contained reaction product. This necessarily produced some sampling error, as not all cytoplasmic areas viewed were the same size. There was also staining variability within control cells noted in the preliminary study (1), placing further limitations on reliability of the judgement.

Although these methodological constraints are important, the HSD activity measured *in vitro*, with substrates and cofactors supplied in excess, will tend toward its maximal rate, rather than the *in vivo* rate which ultimately contributes to serum testosterone levels. The *in vitro* rate would be expected to correlate with the hCG-stimulated value, also reportedly decreased in diabetes by a biochemical determination (327), or to the concentration of active enzyme. Thus, the lack of correlation between HSD staining and serum testosterone was

accounted for by methodological concerns and enzyme kinetics.

HSD activity converts pregnenolone to progesterone, and is only one factor in testosterone production. Although HSD is rate-limiting in this process (323), subsequent steps in steroid synthesis and secretion may also have been affected by the different treatments, including streptozotocin, in the present study. This would further reduce a correlation between HSD activity and serum testosterone. Correlation of testosterone levels with the lipid content of Leydig cells suggests that this may be an easier, although indirect, marker of steroidogenic activity.

The difference in HSD activity between Leydig cells from semistarved and diabetic animals was the most obvious indication of a variable response to functional insult in that cell population. In both states, serum testosterone was reduced, but only in diabetes was the HSD staining affected. Whether the preceeding or subsequent steps in steroid production and secretion are subject to the influence of semistarvation more than to diabetes is not known.

It is known, however, that ABP production is reduced in undernutrition (351) and in diabetes (247, 259), but that in diabetes the reduced synthesis is combined with a defect in transport to the epididymis (247). This difference may confound interpretation of the testosterone acting on the germinal epithelium and accessory organs with that measured

in the peripheral circulation. It has also been reported that HSD activity is sensitive to increased heat (322), and this may link the reduced activity in diabetes to the increased vascular density found in those animals.

Possible differences in testosterone catabolism in semistarved (351) and diabetic animals would also influence measured values. That gonadal function in starvation reverts to control similar to prepubertal hypothalamic regulation is well-known (56, 61, 64, 71, 74, 296). Reduced LH support for steroidogenesis in semistarvation would explain the low testosterone levels produced *in vivo*. If the HSD enzyme were reduced in activity, but present in normal concentration, the *in vitro* HSD incubation, with substrate and cofactor in excess, could stimulate control levels of HSD staining in Leydig cells from semistarved animals, as observed in the present study. Alternatively, if the HSD enzyme were reduced in concentration during starvation, differences in earlier production or later catabolism of testosterone would be suggested. These considerations may account for the present finding of different HSD staining in diabetic and semistarved animals, in conjunction with similar serum androgen levels.

In conclusion, the increased HSD activity in insulin-treated diabetic animals when compared to control levels supports the idea of a direct input by insulin in the facilitation of Leydig cell steroidogenesis in Long Evans rats. These results corroborate recent findings of decreased

HSD activity in diabetic Sprague Dawley rats (330), but differ from conclusions of synergy between hCG and insulin necessary for restoration of HSD activity to the control range (206, 330, 353) in that strain.

Thus, while the HSD staining method in the present study was not a precise measure of peripheral testosterone levels, it allowed general quantification of the process. Knowledge of that activity contributed to the understanding of mechanisms compromising Leydig cell function in diabetic and semistarved Long Evans rats.

To improve the HSD method, it could be combined with spectrophotometric measure of the reaction product deposit. This would eliminate the subjectivity of scoring staining results, and the need for addition of high magnification point-counting of reaction product in electron micrographs. Inclusion of the formazan-nitroblue tetrazolium method (340) would also allow average tissue HSD activity to be assessed (325). However, this would prevent the detection of sub- and intra- cellular details of HSD activity, as seen under the electron microscope. Parallel incubations with and without the addition of hCG or LH to the medium would also aid in interpretation of results. In that way, better correlation of HSD activity with testosterone levels may be found.

8. Serum Hormones

While LH, FSH, and testosterone levels are the principal markers of function within the hypothalamo-

pituitary-testicular axis, it is concluded from the present study that their measurement alone cannot give a complete portrait of the details of Leydig cell compromise in diabetic rats. Hormone concentrations, however, highlight the mechanics of compromise.

The evaluation of testosterone gave results consistent with previous work on diabetes induced by streptozotocin injection (176, 181, 189, 208, 223), and on semistarvation (63, 64, 208), while corticosterone levels were not increased in either group of animals. This confirms that the steroidogenic defect is not simply a nonspecific effect of stress on factors that influence Leydig cell function, as suggested by some authors (127).

A measure of estradiol was made in order to determine if an increase in Sertoli cell aromatization (with possibly increased FSH (365) or LH (364) concentrations), was coincident with Leydig cell hypofunction. Estradiol is thought to have a negative local effect on Leydig cell function (367-369) and spermatogenesis (366). The possibility that this mechanism decreases testosterone synthesis was entertained by such a measurement. Estradiol levels in serum were not found above the assay level of sensitivity in any group, and this may have masked any upward trends, considering the normally low levels in male rats. Testicular interstitial fluid may have given more measurable levels of estradiol, as well as estimates of the steroid levels acting directly on Leydig and Sertoli cells

(41).

The decreased testosterone output by Leydig cells from diabetic animals was reflected in their increased lipid content, and their increased mitochondrial areal density. The increased SER and RER compartments and the reduced Golgi fraction from the effects of semistarvation also reflect decreased testosterone output, but with a significant shift of organellar fractions not seen with the effects of diabetes. This suggests that the trend of diabetes to affect Leydig cell ultrastructure in the same direction as semistarvation is either due to milder metabolic compromise despite weight loss parallels, or summons an explanation at least partially distinct from that of starvation. The latter hypothesis is supported by the reduced HSD activity seen in histochemical staining of Leydig cells from diabetic animals, which was not observed in semistarved animals.

In light of increased FSH levels in diabetic and semistarved animals, Sertoli cell aromatization (of testosterone to estradiol) should have been increased. The failure to detect it indicates either that testosterone levels were too low to facilitate this function, that testosterone diffusion across the blood-testis barrier was impaired, or that the Sertoli cell in Long Evans animals has direct requirements for insulin. While insulin facilitation of LH-stimulated Leydig cell function (330, 328) may be mirrored by a corresponding deficit in Sertoli cell function, there is no direct evidence for this. The

qualitative alterations of the tubule basal lamina complex seen in diabetic animals in this study bring the second possibility into play. However, the known dependence of Sertoli cell function on testosterone (243) most likely accounts for the structural alterations seen in the germinal epithelium of diabetic animals. The decrease in Sertoli cell function, including decreased production of inhibin, would secondarily stimulate increased pituitary FSH secretion, by known feedback mechanisms (55-58, 74, 75).

Increased FSH levels in diabetic and semistarved groups indicated that the ability of the hypothalamus to respond to gonadal hypofunction was not absent. This contradicts findings which suggest the hypothalamus in diabetes is unresponsive to altered testosterone levels (208), while the pituitary retains sensitivity to gonadotropin-releasing hormone (124, 125, 226). Although such tertiary hypogonadism was supported by evidence of arcuate nucleus lesions in long-term streptozotocin-induced diabetes in rats (143, 209), the animals used in that study were so old as to overlap with hypothalamic failure which accompanies the normal aging process (344-346, 348, 349).

Increased FSH in the semistarved group also contradicts previous observations of reduced hypothalamic function in starvation (63, 64, 208). Testicular function in starvation has been shown to be accompanied by decreased response to changes in testosterone levels in the serum (64, 208, 351), but usually in conjunction with decreased gonadotropin

concentrations. Whether mature Long Evans animals past the period of peak growth, are not as subject to these endocrine modifications in undernutrition is not known. Strain differences have been documented in many studies (section 9). It is further possible that the food restriction schedule in the present study was insufficient to suppress both LH and FSH secretion.

The unfortunate lack of LH data in the present study leaves undocumented an important factor of Leydig cell regulation in Long Evans animals. In absence of that factor, the possibility of LH being either increased, decreased, or unchanged must be explored. Increased LH levels in the presence of decreased testosterone would implicate the Leydig cell as the site of failure in diabetes. This would suggest that the LH receptor is directly dependent on the presence of insulin, or that insulin facilitates a post-receptor function (for example, cAMP production (353) or mobilization of lipid reserves) in the Leydig cell. In either case, the situation would parallel that of castration or testicular feminization, in that the hypogonadism would be primary in the diabetic Long Evans rat, rather than tertiary as substantiated in Wistar (143, 176-178, 181, 207, 209) and Sprague-Dawley (189) strains. Whether or not the germinal epithelial alterations and steroidogenic failure of the Leydig cell result in reduced fertility remains to be confirmed in the Long Evans strain.

If LH levels were unchanged from control values, the low testosterone concentration in the serum of diabetic animals would indicate a defect in the mechanism of feedback to the pituitary or hypothalamus, and would support secondary or tertiary hypogonadal function, respectively. A report of failure to suppress LH by testosterone implants in insulin-treated diabetic Wistar animals (208) would seem to corroborate the possibility of defective feedback producing hypoandrogenism.

If LH levels were decreased, a supposition which is supported by decreased serum testosterone in the present study, LH production would be dissociated from that of FSH by the pituitary. Separate regulation of LH from FSH secretion from one pituitary cell type has been suggested by the recent finding of intrinsic LHRH-like material in pituitary gonadotrope secretory granules (361) The possibility of this paracrine or "private" regulation of gonadotropin release reiterates the need for detailed receptor and immunocytochemical work on pituitary gonadotropes. That secretory granules within one gonadotrope cell may stain positively for both LH and FSH (269, 360) further complicates the control mechanisms that must govern their separate secretion. This has been reported in acute exertion (103), malnutrition (64, 351), uremia (371), or anesthetic stress (129, 130, 133).

In conclusion, the serum hormone levels measured in the present study were consistent with histological and

ultrastructural findings in Leydig and Sertoli cells from testes of control and diabetic Long Evans rats. While the precise level of origin for the decreased testosterone secretion in diabetes is uncertain, purely secondary or tertiary hypogonadal function appears to have been ruled out.

9. Strain Differences

Markedly different physiological and pathological features have been noted between strains of animals by previous authors. These include: 1)susceptibility to induction of diabetes by streptozotocin (166, 201, 267) and pancreatectomy (338), 2)maximal testosterone production (334), 3)sensitivity of LH receptors on Leydig cells (337), 4)cold adaptation (336), 5)seasonal and circadian hormonal fluctuations (335), 6)the effect of adrenalectomy on ovulation (333), susceptibility to stress (339), and 8)the effect of food deprivation on spermatogenesis in mice (332).

In this project there were distinct differences in responses of Long Evans and Wistar animals in a number of parameters. First, Wistar rats were less sensitive than Long Evans rats to the diabetogenic action of streptozotocin, requiring a higher dose of the drug to produce diabetes. Second, the Wistar animals became more ill once diabetes was induced. This was seen as a significant strain effect on diabetic urine output, and as strain:treatment interaction on diabetic BUN levels. Third,

the Wistar animals were more inclined to respiratory infection. Fourth, Wistar rats became hyperglycemic more often than Long Evans rats after undergoing pancreatectomy.

The finding that streptozotocin-diabetic Wistar rats were uremic, while Long Evans rats were not, makes the latter strain more useful than the former in the study of diabetic infertility. In patients on dialysis, uremia of many etiologies is associated with changes in endocrine, metabolic and structural parameters. These closely resemble the pattern noted in the present study and past reports on the diabetic pituitary-testicular axis.

Uremic patients were reported to have decreased potency and fertility (352, 354, 370-372). Decreased testicular size (373, 374) with seminiferous tubule shrinkage and germinal epithelial thinning (371), maturation arrest, basement membrane thickening and decreased numbers of Leydig cells (373, 374) have also been reported. These biopsy findings were associated wih gynecomastia (352, 374) and raised serum prolactin levels (352), as well as with weight loss, muscle wasting, and anorexia (374). Abnormal glucose tolerance (377), increased growth hormone levels (377) and fasting hyperglucagonemia (378) have also been reported in uremic patients.

Many studies of endocrine parameters showed reduced serum testosterone (352, 370) and reduced testosterone production (370), decreased (352, 359) or increased (354, 370, 371) serum LH, and normal or increased serum FSH levels

(371). Decreased metabolic clearance rates of LH (376) and LHRH (373) were also reported, although normal responses to clomiphene citrate (371), exogenous LHRH and hCG (370) were also noted.

The improvement of fertility indices and endocrine findings toward normal values after dialysis (370) or renal transplantation (371, 372) was thought to indicate primary defects of Leydig cells and of the tubule compartment in the hypogonadism of uremia. The additional possibility of decreased hypothalamic responsiveness to feedback was indicated by an FSH surge before improvement of fertility (371). Therefore, in the present study, without the similar influences of uremia on structure and functions of the pituitary-testicular axis, the effect of diabetes may be much more clearly defined in the Long Evans strain of rat.

Tubule diameter showed treatment:strain interaction, and spermatogenic stage frequency distributions showed differences between control animals from the two strains. While there were significant alterations in lipid droplet size and lipid areal density between control and diabetic Wistar animals, this was likely the effect of small sample size combined with poor fixation. Similarly, the significant strain differences noted in mitochondrial areal and numerical density, lipid size and areal density, and SER and Golgi fractions are also not well-founded. The significantly higher control levels of FSH in Wistar rats than Long Evans animals would appear to be consistent with

previous reports (335).

A further strain difference would appear to involve the presence or absence of lipid droplets in control Sprague-Dawley (189) and Wistar (176-178) rats respectively. This descrepancy is not likely a fixation artifact (section 6). The failure of adequate testicular perfusion in Wistar animals was also notably different from the success in Long Evans rats, and may be a technical reason for the use of the latter strain, if testicular perfusion is deemed necessary for optimal fixation in an ultrastructural study (331). On the other hand, rapid immersion fixation has been found to control and standardize preservation artifact in electron microscopic study of many other tissues (341), including preservation of HSD staining activity (1).

Significantly increased serum corticosterone levels seen in Wistar diabetic animals compared to their controls, produced significant interaction of strain and treatment effects. This indicates the presence of significant stress as an influence in interpreting diabetic pathology in this strain, as found earlier (127) in Sprague-Dawley rats.

The most important strain difference identified by the present investigation is that diabetic hypoandrogenism in Long Evans animals would appear to be primary, while that in Wistar and Sprague-Dawley rats is thought to be tertiary (124, 125, 143, 189, 209, 226), with possible secondary pituitary malfunction (208). This dramatic distinction strongly cautions against direct inference from one strain

to another, as well as extrapolation from rat to human pathology, except in exploration of the realm of the possible.

The apparent primary nature of the deficiency in Leydig cell function during diabetes in the Long Evans strain offers a unique opportunity to study regulation of its LH receptor, and to investigate the presence, attributes, activity, and role of the insulin receptor on the same cell.

10. Model and Interpretation

The use of five groups of Long Evans animals, and orthogonal transformations was found to allow interpretation of data to a much greater depth than would have resulted from a simpler multiple range analyses. The linear expressions for effects of diabetes, semistarvation, streptozotocin treatment and insulin injection are not mathematically unique, as matrix multiplication reveals, and different formulation of these expressions may have resulted in some variation in conclusions. However, the *a priori* definition of the contrast expressions maintains statistical principles, and permits conclusions made with a minimum of experimentwise error.

In the present study, streptozotocin was found to increase final blood glucose and body weight, decrease BUN, and increase the Golgi fraction in Leydig cell cytoplasm. These effects are, to some extent, a result of the exact formulation of the mathematical expression, as it includes

the average of nondiabetic and insulin-treated diabetic streptozotocin injected animals. This allows for a cross-influence of insulin treatment in the final effect, which may have been avoided with alternate orthogonal matrix formulation.

In contrast with a previous report (189), there was no statistically significant streptozotocin effect on serum testosterone levels in the present study. This may be due to a larger group size, a larger number of treatment groups, more rigorous statistical tests, or a possible strain difference in this parameter.

The significance of the drug effects highlights the possibility that streptozotocin may have effects at subdiabetogenic doses, or in animals which are not susceptible to the drug's diabetogenicity. That streptozotocin was found to increase blood glucose may mean that enough of the drug reached the pancreatic beta cells, possibly dependent on islet blood flow (347), to cause only partial damage, without the manifestation of overt signs of diabetes, such as polyuria, or weight loss. The mechanism of streptozotocin effects to decrease BUN and increase the Golgi fraction of the Leydig cell remains unexplained in this study, but the possibility of statistical artifact must not be overlooked.

Although the inclusion of nondiabetic streptozotocin-injected animals prolonged and increased the cost of this work, it was valuable in documenting the lack of that

influence on the many parameters examined. Any further use of the streptozotocin model of diabetes should also include this group, to enable examination of nondiabetogenic streptozotocin cytotoxicity.

SUMMARY

According to the outlined objectives, the following conclusions may be drawn from the results of this study.

(1).A model was designed to enable controls for streptozotocin treatment, diabetes, and semistarvation. No functionally significant streptozotocin effect was found on major parameters studied, using orthogonal contrasts.

(2).A method of labelling $\Delta 5,3\beta$-hydroxysteroid dehydrogenase activity was formulated and applied, showing reduced staining activity in Leydig cells from diabetic animals.

(3).The effects of diabetes were to decrease body weight, seminiferous tubule diameter, serum testosterone, and HSD staining, and to increase interstitial blood vessel density, SER and lipid fractions of the Leydig cells, and serum follicle-stimulating hormone.

(4).The effects of semistarvation were similar to those of diabetes on body weight, serum testosterone and follicle-stimulating hormone, and lipid and SER fractions of Leydig cell cytoplasm, but distinct from those of diabetes on seminiferous tubule diameter, blood vessel density, and HSD staining.

(5).The hypoandrogen state in Long Evans diabetic rats may be due to primary Leydig cell compromise with insulin deficiency, rather than to previous reports of tertiary hypogonadism in diabetic Wistar and Sprague-Dawley rats.

(6).Strain differences suggest that the non-uremic, non-stressed Long Evans strain may be more useful than albino rats in the study of endocrine aspects of impaired reproductive function in diabetic rats.

Appendix 1

To achieve near-normoglycemia in streptozotocin-diabetic rats, four trials of insulin treatment were made. The first three trials used four Long Evans rats made hyperglycemic (blood glucose>16.7mmol/L) by injection of 40mg/kg of streptozotocin intravenously. They were compared to four streptozotocin-injected animals which remained normoglycemic (blood glucose=6.7 ± 0.7mmol/L), and served as controls.

The first trial used daily (0800 hr) subcutaneous injection with 4 IU Lente insulin, and food and water *ad libitum*. The blood sugars were reduced below normal range for 3 hours, but after 1100 hours, were greater than 11mmol/L, and significantly greater than control levels.

The second trial used split doses of Lente insulin (2 IU at 0800hrs, and 2 IU at 1700hrs) and food and water *ad libitum*, with results almost identical to the first trial.

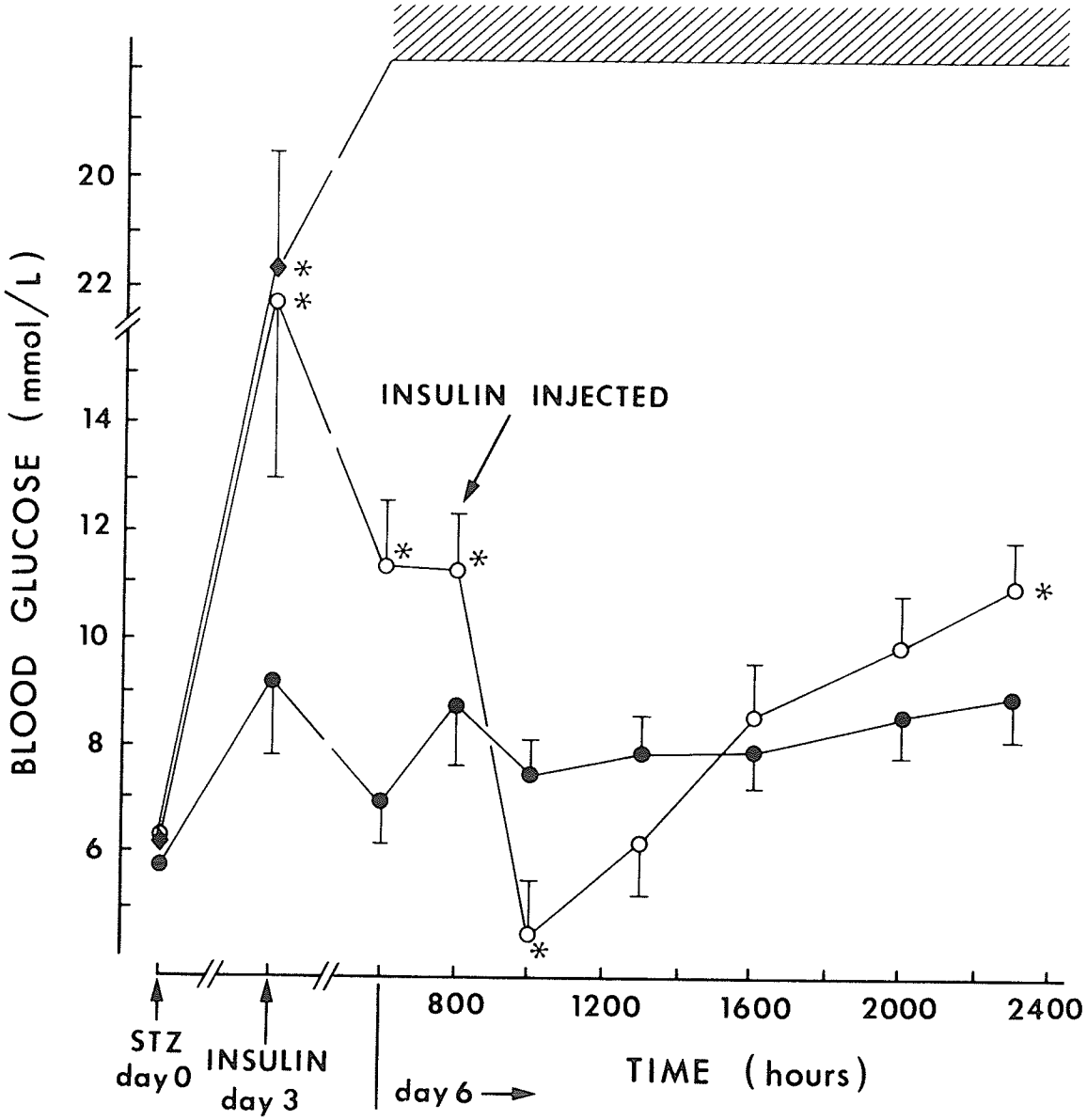
A third trial used 3 IU of Ultralente insulin without food restriction on the same two groups of animals, and a gradual increase of blood glucose occurred from 1200 to 0800 hours the next day.

In the fourth trial, diabetes was induced in six animals, and three stretozotocin-injected but non-diabetic rats served as controls. Diabetes was confirmed at day 3, and insulin treatment begun at that time. In three of the

diabetic animals, 3IU of Ultralente insulin were injected at 0800 hours, and food and water were *ad libitum* only until 1700 hours. The 24-hour sampling period was begun on day 6. Overnight food restriction combined with this insulin schedule gave more acceptable results (Appendix Figure 1). Blood glucose in the insulin-treated diabetic group was significantly below control range at the 1000 hour reading, and significantly above control range for readings from 2200 to 0800 hours, just prior to the next insulin injection. Between 1200hrs and 2200hrs, blood glucose levels were not significantly different from those in non-diabetic streptozotocin-injected rats.

This pattern of glucose control was determined to be acceptable for the purposes of the present study in view of the lack of statistical difference of the cumulative 24-hour average glycemia between the insulin-treated diabetic and the non-diabetic group.

Appendix Figure 1: Graph of 24-hour blood glucose levels in streptozotocin injected diabetic (♦), non-diabetic (●), and insulin-treated diabetic (O) Long Evans rats. Streptozotocin (STZ) injection occurred at day 0, and insulin treatment began on day 3. The 24 hour period indicates day 6 of treatment. Each point represents mean ± standard error of values in three animals. Asterisk (*) indicates significant difference ($p<.05$) from non-diabetic group.


BLOOD GLUCOSE (mmol/L)
20
22
14
12
10
8
6
INSULIN INJECTED
800
1200
1600
2000
2400
STZ
day 0
INSULIN
day 3
day 6
TIME (hours)

Appendix 2

Knowledge of the fertility of streptozotocin-induced diabetes in Long Evans rats would have contributed to the interpretation of the present findings of altered testicular anatomy and steroidogenesis. Fertility of Wistar diabetic rats was reported decreased (181) as assessed by studies of sperm motility and sperm counts. In addition, cycling female rats could be housed with control and diabetic males for a number of cycles, and later examined for implantation and resorption sites in the uterine horns, a techniques also previously utilized (181). Comparison of the fertility determinations between control and diabetic animals could truly quantitate the overall effect of diabetes on reproductive function. The assessment of sperm motility and count, however, is only possible on non-fixed samples, and is subject to the function of seminal vesicles. As the function sex accessory glands would be deleteriously influenced by a reduction of testosterone in diabetic males (61, 181), statistical scoring of these indices could be subject to questions of reliability, and dependent on seminal fluid amounts. Interpretation of the presence or absence of embryo implantation sites as an index of fertility, would be further complicated by the reduction of potency and libido that occur with lowered serum testosterone levels (61, 181), and by autonomic neuropathy which frequently accompanies diabetes mellitus (108-111, 113, 116). For these reasons, study of fertility was not made during this investigation.

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