STUDIES ON THE ROLE OF PROLACTIN IN THE CONTROL OF EPIDIDYMAL STRUCTURE AND FUNCTION IN MALE ALBINO RATS

A THESIS

submitted in fulfilment of the
requirements for the award of the degree
of
DOCTOR OF PHILOSOPHY

By

RAJEEV GAUTAM

DEPARTMENT OF BIOSCIENCES AND BIOTECHNOLOGY
UNIVERSITY OF ROORKEE
ROORKEE-247 667 (INDIA)

JULY, 1991

DEDICATED TO

SMALL AND WHITE
CLEAN AND BRIGHT
THE WONDERFUL WISTAR
WITH COURAGE AND MIGHT
MADE THE UTMOST SACRIFICE
FOR ME ! !

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in this thesis entitled **STUDIES ON THE ROLE OF PROLACTIN IN THE CONTROL OF EPIDIDYMAL STRUCTURE AND FUNCTION IN MALE ALBINO RATS** in fulfilment of the requirements for the award of the Degree of DOCTOR OF PHILOSOPHY, submitted in the Department of Biosciences and Biotechnology of the University is an authentic record of my work carried out during a period from **September, 1986** to **July, 1991** under the supervision of **Dr. Ben M.J. Pereira,** Department of Biosciences and Biotechnology, University of Roorkee, Roorkee.

The matter embodied in this thesis has not been submitted by me for the award of any other degree.

12.07.91
Date :

Rajeev Gautam
(RAJEEV GAUTAM)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge

Ben M. J. Pereira
(B.M.J. PEREIRA)
Lecturer, Deptt. of
Biosciences & Biotechnology

Date : 12.07.91

R. Bahuguna

Head
Department of Bioscience
and Biotechnology
University of Roorkee
ROORKEE-247 667

The Ph.D Viva Voce examination of Sri _____

Research Scholar has been held on _____.

_____ _____

Signature of Supervisor **Signature of External Examiner**

SUMMARY

The intention of this study was to investigate if prolactin had an influence on the epididymis, a region of the male reproductive tract concerned with the functional development of the sperm. The epididymis helps sperm acquire the characteristics of motility and fertilizing ability. This is made possible by the special milieu in which the sperm are bathed while being nurtured within the duct. The composition of this milieu is further dependent on the secretory and absorptive nature of the epididymal epithelium. The nature of the epididymal epithelium is in turn dependent on several factors, both hormonal and non-hormonal. Thus, it seems that one big cascade of events is involved, that finally culminates in the production of functionally developed sperm.

The crux of the problem was to first select an appropriate parameter to assess the functional status of the epididymis and then identify the various factors that influence it. The next step was to investigate if prolactin had a role to play. Sialic acid was one of the parameters that was chosen. Although there is yet no clear appreciation of the significance of sialic acid for sperm function, there is evidence to suggest that the epididymis synthesizes this compound. Experiments involving sham-operated, duct ligated and orchidectomised animals clearly showed that the sialic acid derived from testis could significantly contribute to the levels in the epididymis. Nevertheless, the presence of sialic acid in epididymis even after efferent duct ligation and the higher levels of this compound in cauda than in the caput made it amply clear that the epididymis is capable of synthesizing sialic acid. Moreover, these studies also showed that factors derived from testicular fluid including androgens controlled sialic acid level in the epididymis.

A simple way to see if prolactin is involved is to inject prolactin in animals from an exogenous source (as practised by other investigators) and then look for the impact on the epididymis. But then, it was inferred from the preceeding

experiment that several factors of testicular origin affect sialic acid level in the epididymis. For this reason, prolactin treatment in intact rats was not preferred. The choice now was to use efferent duct ligated or orchidectomised animals for prolactin treatment because all components of testicular origin could be kept away. Accordingly, when prolactin was administered at various doses, the epididymis of orchidectomised and not the duct ligated animals responded to prolactin treatment. The following explanations could be offered. Efferent duct ligation prevents the testicular fluid and spermatozoa from reaching the epididymis but maintains the availability of androgens through general circulation. Orchidectomy, on the otherhand, involves the removal of the primary source of androgens together with all testicular factors known to influence epididymal function. Since the androgens continue to reach the epididymis in duct ligated animals and the epididymis is predominantly an androgen dependent organ, it has been proposed that the action of prolactin is perhaps masked by the much greater effects of androgens in the tissue. In view of these results it was clear that orchidectomised animals would be the most appropriate model to study the effect of prolactin in the epididymis.

Prolactin treatment to orchidectomised rats increased the sialic acid levels in a dose dependent manner. It had been suggested that prolactin directly influences the epididymal sialic acid, perhaps at the level of synthesis. The experiment when repeated with bromocryptine treatment (an ergot alkaloid that reduces circulating levels of prolactin) to orchidectomised rats confirmed that prolactin was involved. It was also clarified that prolactin does not interfere with the assay of sialic acid.

The activity of two representative glycosidases was used as yet another parameter to monitor the effect of prolactin on the epididymis. In this case, the response although positive, was not exactly dose related, implicating that different parameters need not necessarily respond alike to the same treatment. It has

been suggested that the activity of glycosidases measured may represent the net result of an interaction between steroid (androgen) and protein (prolactin) hormone action at each dose of prolactin treatment.

In order to see how long the injected prolactin remains in circulation, the circadian pattern of serum prolactin bioactivity in orchidectomised animals given various doses of prolactin injection was determined. It appeared that the half life of injected prolactin was small, perhaps less than 15 min. In addition, a rebounding phenomenon was observed.

In the first two Chapters prolactin was shown to influence biochemical parameters like the sialic acid and the activity of glycosidases in the epididymis. However, the impact of prolactin on the structure of the epididymal epithelium particularly the principal cells, was not known. For this purpose, both light and electron microscopic studies were performed. From the light microscopic studies it was clear that prolactin had a positive effect on the epididymal epithelium but had no influence on the muscular layer. Electron microscopic studies revealed that prolactin profoundly influenced the ultrastructure of the principal cell. Orchidectomy induced atrophic changes and prolactin treatment to these animals brought about rejuvenation. Thus, prolactin was shown to have a trophic action in the epididymis.

ACKNOWLEDGEMENTS

After the tumult of the thesis preparation one sits down for a while, and thinks what had happened to him, why he is feeling so buoyant as if on moon, and from where in this world this soothing sense of elation has come and settled all over him. This euphoria, at its ease, would be marked with an underlying feeling of indebtness of many a persons, but for whose kind help and guidance right from the word go, I would not have had this herculean task accomplished. If I start demisting my memory and enlisting the names, it would end on an unending note books, but certain individuals deserve citation for their invaluable help, who have remained as the sustaining impetus for the successful completion of this odyssey.

First and foremost, it gives prodigious gratification to enunciate my sincere gratitude to my supervisor Dr. Ben M.J. Pereira, Lecturer, Department of Biosciences and Biotechnology, University of Roorkee. He introduced me to the facinating field of Reproductive Biology. I am greatly indebted to him for his stimulating input of ideas, punctilious and erudite guidance, and perpetual encouragement through out my tenure of research work. His moral support and personal help at various stages have enabled me to complete the work with consummate ease.

I take opportunity to express my sincere regards for Dr. (Mrs) Ritu Barthwal, Head, and other faculty members of the Department of Biosciences and Biotechnology, who have been ever ready to

help me whenever the need so has arisen.

I sincerely express my boundless reverence to my teacher and benefactor **Prof. C.B. Sharma**, who infact awakened and fostered my interest in research. I respect his paternity, coddling for me throughout my research tenure. Whenever I found my thoughts in a demijohn, he only had the opener for it. I am not able to express myself for his infinetude help with etymology, I can only bow my head to him.

During the long stay here, times have been good and bad, moods have been happy, sad and sometimes very sad. But I was fortunate for having the best company of friends in this world, for keeping me always fresh and happy. Their freaky, fun loving, punky living style always kept me staying alive happily. Although it is very difficult to put everyone in Black and White, in my paean particularly **Asoken, Manoj, R.K., Sandeep, Aditya, Anurag, L.P., Tiwary, Pankaj, Ramesh and Sanjeev** had added a stanza each. **Basav and Jaba** are specially thanked for their cooperation and affection. Besides, I am greatly indebted to **Meenakshi** and **Neeti** for their leitmotiv understanding and encouragement. Their generosity, caring had been an anodyne for me in most furry and foggy moments. I enjoyed their ineffable help and affectionable attitude in zonked and inert phases of work. **Thanx Meena thanx Neeti !!**

In addition, I extend my warm acknowledgement to my loving crony **Kush** for walking with me every step for this day. I admire his phlegmatic support. **Thanx Kush !!**

I also acknowledge the help of Mr. Rajiv Juyal and Mr. Vipin Pal for excellent photography. My sincere thanks are due to Mr. Balasubramanian for his untiring efforts in typing this manuscript.

The tireless and selfless efforts put in by Madan in the maintenance of the animals through out the duration of work and countless services rendered by him deserve a special mention.

I am greatful to National Pitutary Agency, M/S Sandoz Chemicals Co and M/S Sigma Chemical Co, for gifting necessary chemicals. My thanks are due to Department of Anatomy, AIIMS, New Delhi, for providing facility for electron Microscopy.

In the end, vocabulary fails me in finding words to accuntiate the cherish, forbearance, immutable encouragement received from my parents and brother-in-law B.K. Kaushik for constantly inspiring me to fulfil my ambition for higher studies.

The assistance of all individuals who have in any way been associated with the completion of this work but not have been mentioned so far, is sincerly acknowledged.

Financial assistance by Council of Scientific and Industrial Research (CSIR), India, is duly acknowledged.

Rajeev Gautam

(RAJEEV GAUTAM)

CONTENTS

LIST OF FIGURES AND TABLE

FIGURES

INTRODUCTION

For many years now endocrinologists are busy evaluating the role of prolactin in the male reproductive system. Although prolactin was at first recognised to be a hormone confined to the females, its importance has now come to be recognized in males as well. The ability of prolactin to affect the growth of male accessory sex glands and spermatogenesis together with the reported coexistence of several male reproductive disorders with disturbed levels of circulating prolactin have been chiefly responsible for research in this direction. One of the disorders that is known to occur is related to sperm maturation, the genesis of which is believed to be in the epididymis. In addition, the demonstration of specific prolactin binding sites in the epididymis of laboratory animals has added further interest and has been the main driving force for initiating the present studies.

The epididymis was once thought to be a mere conduit for sperm passage but now known to create conditions ideal for the maturation of sperm at its proximal region and storage of the same at its distal end. This is made possible by the secretory and absorptive nature of epididymis which carefully controls the composition of the fluid in which these sperm are bathed. Several factors, both hormonal and non-hormonal have been known to modulate the events that occur in the epididymis. While some of major factors have been identified, the impact of several others need to be elucidated. In this regard, very little is known about the role of prolactin and hence the study. A two dimensional approach has been used : one involving the structure

and the other function, to ascertain the influence of prolactin on the epididymis. It is believed that studies of this kind would throw light on the events that take place in the epididymis and how prolactin modulates them.

The objectives of the present studies are as follows:

(*a*) To identify factors that influence the functioning of the epididymis.

(*b*) To generate an appropriate model that would limit the interference from these factors and help in pinpointing the action of prolactin.

(*c*) To identify the effect of exogenous prolactin on the epididymal originates as a means of assessing the functional status of the epididymal duct.

(*d*) To find out the circadian levels of serum prolactin bioactivity in animals treated with prolactin from an exogenous source.

(*e*) To find out the impact of prolactin on the structure of the epididymal epithelium at the light and electron microscopic levels.

(*f*) To suggest the possible mechanism (s) by which prolactin could exert its effects on the epididymal structure and function.

LITERATURE REVIEW

2.1 PROLACTIN

2.1.1 History

Prolactin was discovered by Stricker and Grueter (1928) as a lactogenic substance present in mammalian pituitary extracts some 63 years ago. However, due to its presence in small amounts in the pituitary and to the strong intrinsic lactogenic effect of growth hormone, it was not until 1970 that the existence of prolactin as a separate substance was finally demonstrated (Li *et al.*, 1970). Since then, the entire amino acid sequence of prolactin from several species has been elaborately worked out. Prolactins of different species contain about 190 amino acid residues, lack carbohydrate and have molecular weights some where in the region of 22,000 (Karg and Schams, 1970). The primary amino acid sequence data have been interesting and informative in elucidating evolutionary relationships, but not in terms of insights into the chemical basis of biological function. However, with improved ways of studying tertiary structure, the day seems not far off when the protein chemist would be in a position to explain how prolactin works at the molecular level.

2.1.2 Synthesis, Secretion and Metabolism of Prolactin

In mammals, prolactin is predominantly synthesized by the acidophilic cells of the anterior pituitary gland (Friedgood and Dawson, 1940; Holmes and Ball, 1974). In addition, the decidual cells of the placenta and non-endocrine tumours like the carcinoma cells of the lung and kidney are some of the several extrapituitary sites of prolactin synthesis that have been

reported (Turkington, 1971; Rees *et al.*, 1974; Riddick *et al.*, 1978). Under normal conditions, prolactin is released from lactotrophs into extracellular space by exocytosis (Zimmerman *et al.*, 1974). It is secreted continuously in small amounts. However, like other pituitary hormones, it is also released episodically and has a relatively short half life of 5-10 min in the circulation (Grosvenor and Whitworth, 1979). Prolactin clearance is not a single constant process and may not be relevant for endogenous prolactin levels (van der Gugten and Kwa, 1970).

2.1.3 Factors Effecting Serum Prolactin Levels

Circadian variations, in circulating levels of prolactin have been reported in animals (Clark and Baker, 1964; Koch *et al.*, 1971). Several investigations have also indicated that a sporadic surge in prolactin levels may be influenced by photoperiods (Lincoln and Peet, 1977), stress (Frantz *et al.*, 1972), strenous exercises (Noel *et al.*, 1972; Frantz, 1973), breast manipulation (Kolodny *et al.*, 1972; Noel *et al.*, 1974), sexual intercourse (Frantz *et al.*, 1972) or may even be sleep related (Sassin *et al.*, 1972).

The peripheral level of prolactin in male mammals is comparable to that of non-pregnant non-lactating females (Meites, 1973). The release of prolactin is influenced by a number of factors of hypothalamic origin collectively termed as prolactin inhibiting factors (Talwalker *et al.*, 1963). Besides, prolactin is known to

control its own secretion by an autoregulatory or short loop feed back mechanism (Herbert *et al.*, 1979). It is also shown that transplantation of pituitary to an extra pituitary site in the same animal causes increased prolactin secretion (Everett, 1954; Chen *et al.*, 1970). This has been attributed to the removal of inhibition from central nervous system caused by disconnecting the pituitary from the hypothalamus (Chen *et al.*, 1970). The neuronal control of prolactin secretion involves not only classic neurotransmitters like acetyl choline, noradrenaline, seratonin and dopamine (Clemens, 1976) but also other polypeptides, gamma amine butyric acid, histamine, enkephalins (Mc Cann *et al.*, 1978) and possibly prostaglandins (Ojeda *et al.*, 1978). Serotonergic control is of importance as it is known to alter the circulatin prolactin levels (Koeing *et al.*, 1979).

Another overtopping means of control is through the tubero-infendibular dopaminergic system (Fuxe *et al.*, 1970). This pathway is highly relevant, since ergot alkaloids or dopamine agonists like bromocryptine which act by interfering with dopamine function are extensively used to control circulating levels of prolactin (Flückiger, 1978). Bromocryptine is the drug of choice because at therapeutic doses, rapid and prolonged inhibition of prolactin is brought about (Flückiger and Wagner, 1968). This ergot alkaloid acts at the level of hypothalamus and also on dopamine receptors of pituitary lactotrophs (Flückiger, 1978; Flückiger and Vigournet, 1979). It thus reduces circulating levels by inhibiting transcription and translation of this

hormone (Kinch, 1980; Weinstein *et al.*, 1981) without changing the LH and testosterone concentrations (Harper *et al.*, 1976).

The levels of most hormones are interdependent and happen to be finely tuned and delicately balanced. Prolactin is no exception. Thyroid releasing hormone (TRH) produced in the hypothalamus enhances prolactin release thereby implicating a stimulatory effect of the central nervous system (Jacobs *et al.*, 1971). Estrogen by acting at both the hypothalamic and pituitary levels is considered an important modulating factor in the control of prolactin secretion (Dufy *et al.*, 1979). Testosterone is able to increase peripheral prolactin levels while castration produces the opposite effect (Grosser and Robaire, 1987).

2.1.4 Distribution in Body Fluids

Although prolactin is to a large extent synthesized by the pituitary, its presence has been detected in several body fluids. Like all other hormones, prolactin is perhaps secreted into blood but finds its way into cerebrospinal fluid (Clemens and Sawyer, 1974), antral fluid of ovary (Riddick *et al.* 1978), milk (Gala *et al.*, 1975; Grosvenor and Whitworth, 1976) and in the case of males, even seminal fluid (Sheth *et al.*, 1975; Dericks-Tan *et al.*, 1978). At the present time, the precise functional significance for the presence of prolactin in body fluids is not entirely clear, but it is likely that it has growth promoting effects. Prolactin found in amniotic fluid is derived from decidual tissue and is implicated to be involved in fetoplacental

osmoregulation (Clements *et al.*, 1977).

2.1.5 Synonyms Used in Literature

Prolactin is phylogenetically the oldest polypeptide hormone secreted by the pituitary gland and it subserves a great number of different functions among the vertebrates, many important only to certain species or groups (Nicoll, 1974). At least 85 actions of prolactin have been described in vertebrates and this diversity suggests that unlike other pituitary hormones, it did not become highly specialized early in vertebrate evolution. Its principal functions in teleosts and amphibians are related to osmoregulation and growth, whereas its major role in mammals is concerned with lactation and reproduction with lesser actions on other systems. In view of the number and variety of its actions, several other names like galactin, lactotropin, mammotropin, luteotropin, luteomammotropin, paralactin and versatalin have been proposed but the term prolactin has prevailed (Kurcz, 1971; Nicoll, 1974).

2.1.6 Binding Sites For Prolactin

Like all other water soluble signalling molecules, prolactin also binds to specific receptor proteins on the surface of the target cell they influence. These cell surface receptors bind the signalling molecule (prolactin) with high affinity and convert this extracellular event into an intracellular signal that alters the behavior of the target cell.

Prolactin is known to bind to many tissues like liver (Costlow *et al.*, 1975; Posner *et al.*, 1975), adrenal cortex (Marshall *et al.*, 1979), kidney (Costlow and Mcguire, 1977; Marshall *et al.*, 1979), brain (Walsh *et al.*, 1978), lung (Josimovich *et al.*, 1977), red blood cells and lymphocytes (Lesniak *et al.*, 1977), although discrepancies exist which may reflect species differences. In females, especially mammals, it binds to the mammary gland (Djiane *et al.*, 1979 a,b) and ovary (Saito and Saxena, 1975) as well. Consistent with these findings, the role of prolactin in females has remained undisputed for a long time. The part played by prolactin in puberty and sexual development (Riddle, 1963; Nicoll and Bern, 1971), menstrual cycle (Archer, 1977; Chang, 1978), pregnancy (Smith and Neill, 1976; Yogev and Terkel, 1978) and lactation (Houdebine and Djiane, 1980) have all led to the popular belief that prolactin is essentially a hormone whose importance is confined to the females.

In males, prolactin has not received the attention it deserves. However, in recent years the hormone has become the subject of intensive study for two main reasons. Firstly, binding sites for prolactin have been identified in the testis (both interstitial and germ cells), prostate, seminal vesicles and epididymis (Aragona and Friesen, 1975; Barkey *et al.*, 1977 a,b, 1979 ; Charreau *et al.*, 1977; Orgebin-Crist and Djiane, 1979; Amit *et al.*, 1983). Secondly, the occurrence of reproductive disorders are found to be associated with abnormal levels of circulating prolactin (Hosteller and Plascete, 1977; Bartke, 1980; Perryman

and Thorner, 1981; Buvat *et al.*, 1985; Cohen *et al.*, 1988 ; Gonzales *et al.*, 1989). This has infact made analysis of serum prolactin profiles part of routine practice among clinicians investigating gonadal dysfunction.

2.1.7 Prolactin and Male Reproduction

Gonadal dysfunction in hyperprolactinemia is now widely recognized in males. High levels of prolactin might reduce libido and potency (Thorner and Besser, 1978); impotence is not solely related to low levels of testosterone in hyperprolactinaemic patients (Bartke *et al.*, 1976; Besser *et al.*, 1977; Carter *et al.*, 1978; Franks *et al.*, 1978) and bromocryptine might restore sexual function in impotent hyperprolactinaemic men (Nillius *et al.*, 1978). In patients with galactorrhoea and/ or gynaecomastia, it is not uncommon to find reduced androgen secretion with testis and prostate involution (Carter *et al.*, 1978; Franks *et al.*, 1978), oligospermia (Segal *et al.*, 1976) and even defective sperm maturation (Franks *et al.*, 1978). The pathogenic role of hyperprolactinaemia in hypogonadism is confirmed in men by its reversal when prolactin is lowered either by surgically oblating a prolactinoma or by the use of drugs (Frantz and Kleinberg, 1978). Low circulating levels of prolactin have also been reported to delay puberty, hamper seminal quality and alter serum testosterone (Cohen *et al.*, 1988; Gonzales *et al.*, 1989).

In laboratory animals too a role for prolactin in male fertility

has been clearly demonstrated on a number of occasions. One of the most convincing evidence comes from studies with prolactin deficient hereditary male dwarf mice which are sterile. Injection of highly purified prolactin preparation to these animals had a stimulating action on the fertility (Bartke and Lloyd, 1970). On the other hand, in male rats hyperprolactinemia derived from a transplanted prolactin producing tumor has also been shown to be primarily responsible for hypogonadism (Fang *et al.*, 1974). Other studies involving pituitary transplantation in intact, castrated, castrated-adrenalectomized and hypophysectomized rats have revealed that prolactin exerts a small independent influence on the male reproductive system (Negro-Vilar *et al.*, 1977). Prolactin has been shown to have its action on several accessory sex glands like seminal vesicle and prostate (Bartke, 1967; Grayhack *et al.*, 1967; Bartke and Llyod, 1970; Shah *et al.*, 1976; Negro-Vilar *et al.*, 1977; Rui and Purvis, 1987). Fewer studies have been carried out on other regions of the male reproductive tract like the epididymis. There is probably more to learn on its role in the epididymis particularly in view of the fact that this organ is actively engaged in post-testicular sperm maturational events.

2.2 THE EPIDIDYMIS

The mammalian epididymis was once considered to be a passive conduit through which spermatozoa pass from testis to the ejaculating duct (Hamilton, 1975). It was later found that testicular sperm spent varying periods of time in the epididymis

depending upon the species (Amann *et al.*, 1976; Courot, 1981; Orgebin-Crist and Olson, 1984). During their stay in the epididymis, sperm undergo several morphological, biochemical (Courot, 1981; Orgebin-Crist and Olson, 1984) and physiological changes collectively termed "sperm maturation" (Hamilton, 1975; Voglmayr, 1975). This has led to the logistic belief that processes initiated in the testis are perhaps completed in the epididymis. The analysis of the epididymal fluid collected from various points along the duct radically changed this view. Further investigations revealed that the epididymis is capable of resorption, absorption, synthesis and secretion of several substances thereby carefully controlling the composition of the epididymal fluid in which sperm are bathed. The epididymis is today undoubtedly regarded as a dynamic organ which provides a unique micro-environment conducive for sperm maturation at its proximal region and effective storage at its distal end.

In view of the important role of the epididymis in the development and maintenance of the functional integrity of the spermatozoa, considerable research has been directed in recent years towards understanding the basic physiology of this organ. A number of reviews have been published compiling voluminous data on the morphology, biochemistry and physiology of the epididymis, yet the complex inter-relationship between the epididymal milieu and the spermatozoa is not completely understood. In the survey that follows an attempt has been made to consolidate our current state of knowledge and outline the direction of research

activities in the epididymal area.

2.2.1 Histoarchitecture of the epididymis

The mammalian epididymis is an elongated coiled duct derived from the cellular differentiation and development of the embryonic wolfian duct (Ortiz, 1945; Flickinger, 1969; Alexander, 1972). In adult testicond rodents, the epididymis is located in the scrotum, adjacent and loosely adherent to the testis by tunica albugenia and embedded in a highly vascular fat pad. Further, its lumen opens cranially into the testis via the efferent duct and caudally into the lumen of the vas deferens which in turn opens into the urethica.

Because of the long tubular nature of the epididymis and the variation in the function that is performed at its proximal and distal ends, it would be convenient to divide the epididymis into appropriate segments, before detailed studies are undertaken. Based on characteristics like shape, position on the testis, tubular diameter, height of epithelial cell, histological appearance, ultrastructure of main cell types and functions, many terminologies have been used to refer to specific regions of the epididymis (Nicander, 1957, 1958; Reid and Cleland, 1957; Bishop, 1961; Glover and Nicander, 1971; Hamilton, 1972; Zuncke and Grossrau, 1981). Even within the same animal, say for instance, the rat, the regions of the epididymis have been demarcated very differently (Reid and Cleland, 1957; Hamilton, 1975) making it obligatory to specify the nomenclature adopted. However, for

convenience sake, it is generally agreed that the epididymis of the rat could have three gross points of reference the caput (head), the corpus (body) and cauda (tail) (Brooks *et al.*, 1974).

The histology and ultrastructure of the epididymis has been reported by numerous investigators for a number of species and provides valuable information regarding the regional differentiation (Nicander, 1957; Reid and Cleland, 1957). Interesting correlations between the regional differences in the pattern of innervation and morphologically distinguished types of smooth muscle cells have also been made (Baumgarten *et al.*, 1971). In a way these studies have helped in explaining the local, slow, rhythmic, migratory contractions in the proximal epididymidis and the quiscent state of the distal region (Baumgarten *et al.*, 1971). The pattern of vascularization also relates well with the regional changes in the metabolic activity of cells that line the epididymal duct (Waites and Setchell, 1969; Turner and Johnson, 1973 a,b).

The epididymal duct is lined by pseudostratified epithelial cells with non-motile stereocilia on their free surfaces. The height of the epithelial cells and the length of their stereocilia vary greatly in different regions of the epididymis. The epithelium varies from the tall columnar cell with long stereocilia to the low cuboidal cells with short stereocilia along the duct (Glover and Nicander, 1971; Nicander and Glover, 1973). The cell height is however dependent on the degree of stretch imposed on the tissue by the accumulating spermatozoa and fluids

(Martan and Risley, 1963; Vitale-Calpe and Aoki, 1970).

In most of the species investigated so far, the presence of five cell types viz the principal, basal, halo, apical and clear cells have been shown (Nicander and Glover, 1973; Hamilton, 1975; Ramos and Dym, 1977; Moore and Bedford, 1979 a,b; Abe *et al.*, 1983; Goyal, 1985). These cells differ in their structure, function and distribution along the epididymis (Reid and Cleland, 1957; Hoffer *et al.*, 1973). It appears from the estimates provided by Yeung and Cooper(1982) that the principal (59%) and basal cells (27%) are the most abundant in the rat, a laboratory animal commonly used for experimental work.

The main function of the cell types that line the epididymal epithelium are the resorption and modification of testicular fluid entering the epididymis and the elaboration of epididymal secretion (Moore and Bedford, 1979 a,b; Abe *et al.*, 1983; Goyal 1985). Resorption of fluid increases the concentration of sperm cells and that of secreted impermeant compounds (Setchell and Hinton, 1981; Turner, 1984). The interaction of the secretions with spermatozoa are thought to modulate the functions of spermatozoa (Cooper, 1986; Amann, 1987). Considering the large number of sperm it is unlikely that direct contact with the microvilli of epithelial cells is essential to their maturation.

2.2.2 Resorptive Activity of the Epididymis

The most substantial evidence for the absorptive nature of the epididymis comes from packed cell volume or sperm cell

concentration of luminal fluid in various parts of the tract (Turner *et al.*, 1977; Hunter and Schellpfeffer, 1981; Turner and Cesarini, 1983; Turner, 1984). Sperm cell is considered to be an ideal volume marker since they are not being metabolized and are too large to move between epididymal epithelial cells (Cooper and Hamilton, 1977; Murakami *et al.*, 1984 a,b; Murakami and Nishida, 1985). Both fluid phase and receptor mediated mechanisms of endocytosis have been identified in the epididymis (Morales and Hermo, 1983; Djakiew *et al.*, 1984; Hermo and Morales, 1984). In addition, water soluble compounds placed in the lumen of the epididymis have been recovered from the urine (Shaver, 1954).

2.2.3 Synthetic/Secretory Activity in the Epididymis

Several constituents of the epididymal luminal fluid are found to vary in their concentration along the epididymal duct. While some components like glycerophosphocholine (GPC) have their origin in epididymal tissue (Dawson and Rowlands, 1959; Scott *et al.*, 1963; Hinton and Setchell, 1980; Killian and Chapman, 1980; Hoffman and Killian, 1981), some others like *myo*-inositol and carnitine are selectively accumulated from the blood (Brooks *et al.*, 1973; Lewin and Sulimovici, 1975; Lewin *et al.*, 1978). Whether the high concentration of GPC found in the epididymis has a direct action on spermatozoa is controversial. It might just be that being a secondary metabolite of lecithin metabolism, its accumulation in the epididymal fluid may be merely a reflection of its water soluble property (Levine and Marsh, 1971). The variation in the *myo*-inositol concentration is believed to result

from differential absorption of water along the epididymal duct. In other tissues, inositol has been suggested to act as a second messenger (Berridge and Irvine, 1984; Mitchel, 1986), control protein release (Slaby and Bryan, 1976), behave like growth factors in cell culture (Eagle *et al.*, 1957), maintain epithelial cell cytoskeleton (Chargaff *et al.*, 1948; Murphy *et al.*, 1951; Kirazov and Lagando, 1977) and play a role in osmoregulation (Setchell and Hinton, 1981). However, its precise function in the epididymis has not been established. Carnitine is implicated in transport of fatty acids (Wittles and Hochstein, 1967; Brooks, 1979). Thus, these compounds are ultimately linked to major events that occur in the epididymis: sperm maturation and storage. The role of specific ions has also come to be recognized. By providing and depriving the sperm cells of particular ions, the epididymal epithelium can exert control over the activity of spermatozoa or their interaction with secreted compounds (Hamilton *et al.*, 1976; Au *et al.*, 1978; Jenkins *et al.*, 1983; Turner, 1984).

A large number of proteins are known to be synthesized in the epididymis and the factors that regulate their synthesis are also becoming known (Lea *et al.*, 1978; Brooks and Higgins, 1980; Jones *et al.*, 1980, 1981, 1982; Killian and Chapman, 1980; Hoffman and Killian, 1981; Brooks, 1984). Attempts have also been made to isolate, purify and identify at least some of them. Forward motility protein (FMP) (Brandt *et al.*, 1978; Acott and Hoskins, 1981), sperm agglutination factor (SAF) (Dauchex *et al.*,

1983) and immobilin (Usselman and Cone, 1983) are select examples of such an endeavour. These proteins are directly relevant to the functional characteristics attained by spermatozoa during epididymal transit. Recently, the trend seems to be shifting towards cloning the genes responsible for the proteins, obtaining the gene products and trying to understand their functions in the epididymis (D'Agnostino *et al.*, 1980; Brooks *et al.*, 1986).

The sperm, on the other hand, has little or no capacity for protein synthesis and is perhaps the only cell lacking an elaborate endoplasmic reticulum (Abraham and Bhargava, 1963 a,b; Witkin and Bendrich, 1977). It is now known that proteins are packaged into their membranes in the testis where they are produced (Wilson and Grishold, 1979; Wright *et al.*, 1981). However, it has been repeatedly reported that a majority of these proteins have carbohydrate moieties and undergo extensive remodelling during the passage of the sperm through the epididymis (Vierula and Rajaniemi, 1982; Brooks and Tiver, 1984). Several enzymes involved in such post-translational modification of sperm membrane proteins are believed to be synthesized in the epididymal epithelial cells and secreted into the lumen, enabling them to act on the sperm membranes (Jones and Glover, 1975; Skudlarek and Orgebin-Crist, 1984). Thus, the epididymis not only provides a unique microenvironment but also actively participates in transforming the immature sperm to a mature sperm capable of recognizing and fertilizing ova.

The sperm by virtue of their limited synthetic capacity has to

depend upon the epididymis for its needs. The epididymis also synthesizes lipids which provide their fatty acid side chains as important sources of oxidizable substrate to meet the energy demands of spermatozoa (Voglmayr, 1975; Evans and Setchell, 1979). In addition, phospholipids and cholesterol form constituents of the plasma membrane of the epididymal epithelial cells and thus control such delicate characteristics like fluidity and permeability (Go and Wolf, 1983). This in turn is responsible for the selective passage of substances from and/or to the lumen that ultimately decides the composition of the luminal fluid in regions along the epididymis.

2.2.4 Endocrines and Epididymal Functions

The presence of steroids in the epididymis was first demonstrated by an androgen bioassay (Gallagher, 1928). Since then, it has been established in rats that the proximal epididymis contains more androgens than the distal region (Vreeburg, 1975; Vreeburg *et al.*, 1976; Purvis *et al.*, 1978). In addition, they have shown that among androgens, 5α-dihydrotestosterone is most potent and the concentration of this hormone exceeds that of even testosterone. The testis has been identified as an important source of androgen and its precursors (Aafjes and Vreeburg, 1972). Years of careful experimentation have indicated that these steroids find their way into the epididymis through the testicular fluid (Aafjes and Vreeburg, 1972; Pujol *et al.*, 1976) and blood stream (Back, 1975; Cooper and Waites, 1979, 1980; Cooper, 1980; Turner *et al.*, 1981).

The conversion of acetate into cholesterol has been observed in the epididymis (Hamilton *et al.*, 1969; Hamilton and Fawcett, 1970). The synthesis of other steroids from cholesterol has also been demonstrated. In addition, interconversion of dehydroepiandrosterone and testosterone to androstanediol and androstanedione is known (Frankel and Eik-Nes, 1970). Steroid hydroxylases and 5α-reductase, the enzymes related to metabolism of androgens are found in abundance in the epididymis (McGadey *et al.*, 1966; DJoseland *et al.*, 1973, 1974; Saksena *et al.*, 1976; Pujol and Bayard, 1978; Prakash and Moore, 1982; Scheer and Robaire, 1982). There is also evidence that aromatization of androgens can occur (Kumari *et al.*, 1980). These studies have firmly established that the epididymis is not only capable of synthesizing steroids but also has the potential for generating a variety of active steroid metabolites.

It is by now clear that the epididymis influences spermatozoal maturation and this event is just not an aging process. Whether the steroids secreted into the epididymal lumen interact with sperm is still a subject of controversy. But, it seems likely that the steroids, particularly the androgens influence the spermatozoa in a more indirect way, perhaps by maintaining the structure of epithelial cells that ensure the resorptive and secretory functions of the epididymal epithelium (Hamilton, 1975; Orgebin-Crist *et al.*, 1975; Jones *et al.*, 1979; Moore and Bedford, 1979 b). The role of estrogens, corticosteroids, aldosterone and progestins, however, require further detailed examination

(Orgebin-Crist *et al.*, 1975).

An increasing number of peptides, bioactive in other tissues, have been demonstrated in epididymal tissue by immunocytochemical and biochemical methods. They include β-endorphin (Sharp *et al.*, 1980), enkephalin (Rama Sastry *et al.*, 1982), somatostatin 14 and 28 (Pekary *et al.*, 1984), thyroid releasing hormone (Pekary *et al.*, 1980, 1983), epidermal growth factor (Elson *et al.*, 1984), retinol binding proteins (Ong *et al.*, 1982), angiotensin converting enzyme (ACE) (Cushman and Cheung, 1971), neuropeptide Y (Adrian *et al.*, 1984) and vasoactive intestinal peptide (VIP) (Larsson et al., 1977). The precise role of these peptides is unclear but they seem to be in appropriate positions to modulate contractions of the epididymis or to modify the capillary bed and thus influence sections of the epithelium and their interactions with spermatozoa.

2.3 SCOPE OF THE PRESENT INVESTIGATION

One of the polypeptide hormones whose role in males remains as yet undefined is prolactin. Immunocytochemical staining methods have revealed little or no staining of prolactin in epididymal epithelial cells. On the other hand, tissue sections bind exogenous prolactin (Witorsch, 1978). Binding sites for prolactin have also been reported in laboratory animals like rats (Aragona and Friesen, 1975) and rabbits (Orgebin-Crist and Djiane, 1979). In one study, Jones *et al.* (1983) have shown that prolactin does not augment testosterone stimulated protein synthesis in the rat

caput. But Cooper (1986) has suggested that prolactin may have a function in the control of lactalbumin synthesis in the epididymis. In addition, Reyes *et al.* (1979) have shown that prolactin promotes the uptake of calcium by human epididymal spermatozoa *in vitro*. From these and few other stray reports, it can be inferred that studies concerning prolactin and the epididymis have largely remained neglected.

The action of prolactin on several other male accessory sex organs like seminal vesicle and prostate have received comparatively greater attention (Fransworth, 1972; Kledzik *et al.*, 1975; Negro-Vilar *et al.*, 1977; Jones *et al.*, 1983). It has been suggested that prolactin may bring about its effects either independently or by potentiating the actions of androgens in these organs (Bengmark and Hesselsjo, 1963; Bartke and Lloyd, 1970; Moger and Geschwind, 1972; Thomas and Keenan, 1976). Whatever may be the mechanism of its action, it is now felt that the importance of prolactin in male reproduction can no longer be ignored. Only a systematic approach based both on structure and function of reproductive tissues like the epididymis would help in improving our understanding of how prolactin modulates these characteristics.

CHAPTER 1

THE EFFECT OF PROLACTIN ON EPIDIDYMAL SIALIC ACID CONCENTRATION

3.1 INTRODUCTION

In 1936, Blix isolated in crystalline form the first member of a class of compounds now collectively called "**Sialic acid**". Because of its preparation from salivary gland mucin, it has been given the name sialic acid (Blix *et al.*, 1952). Sialic acid is the group name for acylated derivatives of an amino-deoxynonulosonic acid called neuraminic acid, a nomenclature now in general use.

Ten naturally occurring sialic acids have so far been described. The amino group of neuraminic acid is always substituted by either an acetyl or glycolyl radical. As a rule, sialic acid occurs in nature in glycosidic linkage as structural units of oligosaccharides, homo- and heteropolysaccharides, glycolipids and glycoproteins. Only exceptionally, and in very small amount unbound sialic acid has been found in body fluids and tissues (Svennerholm, 1958).

Sialic acid is widely distributed in animal tissues. Bose *et al.* (1966) have reported that the male reproductive tissues have considerably higher concentration of sialic acids than most other tissues. This has been substantiated and confirmed by the detection of high sialic acid levels in the testis, epididymis, seminal vesicle, prostate, semen and spermatozoa (Bose *et al.*, 1966).

The physiological significance of sialic acid in the epididymis is not clear. At first, it was speculated that sialic acid acts

as a kind of "lubricant" that could facilitate the transport and propulsion of spermatozoa along the epididymal duct (Riar *et al.*, 1973). However, the presence of unusually high levels of N-acetylneuraminidase activity in epididymal fluid has added another dimension to the possible role of sialic acid (Bey, 1965). The removal of sialic acid residue from the membrane of sperm during their transit through the epididymis is reported to be associated with the stabilization of acrosome, change in surface charge and antigenicity of spermatozoa (Bedford, 1963; Yanagimachi *et al.*, 1972; Chulavatnatol *et al.*, 1978; Lea *et al.*, 1978; Moore, 1979; Toowicharanont and Chulavatnatol, 1983; Jones *et al.*, 1985; Moore *et al.*, 1985). A masking-unmasking phenomenon has been suggested (Czuppon, 1984; Hall and Killian, 1987). Several other investigators have shown that sialic acid may not only be involved in sperm maturation but also in events leading upto fertilization (Prasad *et al.*, 1973; Laporte *et al.*, 1975).

The presence of sialic acid in the epididymal epithelium even before the arrival of sperm and fluid from testis (Rajalakshmi and Prasad, 1969) provide indirect evidence that synthesis of this compound takes place in the epididymis. On the basis of cellular content analysis in isolated basal and principal cells, it has convincingly been shown that the epididymis synthesizes sialic acid (Killian and Chapman, 1980). The high sialyl transferase activity of the epididymis may be responsible for the incorporation of these sugars into proteins (Bernal *et al.*, 1980). Further studies have revealed that the synthesis of this

compound in the epididymis is predominantly under the control of androgens (Fournier, 1966; Bose and Kar, 1968; Rajalakshmi and Prasad, 1968; Prasad *et al.*, 1973; Setty *et al.*, 1979).The involvement of several other hormones in modulating the action of androgens has also been suggested (Liao *et al.*, 1965; Fang and Liao, 1971). No studies have as yet been undertaken to establish if prolactin could regulate the sialic acid level in the epididymis. Since the sialic acid level is profoundly influenced by androgens, fluid and sperm derived from the testis (Rajalakshmi and Prasad, 1968, 1971; Gupta *et al.*, 1974; Brooks, 1977), in this chapter, an attempt has been made to analyze the impact of prolactin on epididymal sialic acid in the absence of these over-riding factors.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

All reagents used were of analytical grade. Ovine prolactin (oPRL- 18-NIADDK-NIH, Biopotency 30 I.U./mg) was a generous gift from the National Pituitary Agency, NIADDK-NIH, Bethesda, Maryland, USA. Bromocryptine mesylate was a gift from Ms. Sandoz Chemical Co. Ltd., Switzerland. N-acetylneuraminic acid was provided by Ms.Sigma Chemical Co., USA, under the co-operative allowance program.

3.2.2 Animals

Adult male albino rats (200-250 g body weight) of *Wistar* strain were purchased from commercial sources and were acclimatized

to a 12h L: 12h D lighting schedule. They were fed a standard pelleted diet (Lipton India Ltd.) and water was provided *ad libitum*.

3.2.3 EXPERIMENTS

3.2.3.1 Experiment 1

In all, twenty three animals were utilized in this experiment. One group of 8 animals was bilaterally orchidectomised by surgical means. Bilateral orchidectomy was done under mild ether anaesthesia via a scrotal incision. Each testis and epididymis was located through an incision in the tunica vaginalis and the epididymis was carefully isolated from the testis. A ligature was placed around the testicular artery and vein which was then severed to remove the testis. The epididymis was replaced in the scrotum, the incision closed and Loraxane applied locally. Another group comprising of an equal number of animals had their efferent duct of either side ligated keeping the testis intact. After exposing the testis and epididymis by a procedure just described for orchidectomy, a silk ligature was introduced by a needle through the loose connective tissue between the efferent ductules and the blood vessels near the testis. The ductules were tightly occuluded, the testis carefully returned to the scrotum, the incision closed and Loraxane applied locally. A third group consisting of 7 animals were sham-operated and used as controls. Three weeks later, all animals were killed by cervical dislocation and perfused transcardially.

Perfusion was carried out using a transfusion set (bottle with the polythene catheter containing the fluid suspended at about 150 cm above the animal) at room temperature. After exposing the heart, the ascending arota was cannulated with the polythene catheter of about 1 mm internal diameter through an incision in the left ventricle. Care was taken not to let in air while perfusion was conducted. Animals were perfused with about 50 ml Kreb ringer's solution for 15 min until both the liver and the epididymis turned pale, an indication that perfusion was complete and the epididymis was free from blood.

The organs associated with the male reproductive tract were now excised, cleared of adhering adipose and connective tissue. All organs were weighed on a monopan balance. The weights recorded were rounded up to the nearest milligram. Further, the epididymis was divided into caput, corpus and cauda (Brooks *et al.*, 1974). The tissue was wrapped in aluminium foil and stored at -20°C until it was processed further for sialic acid estimation, the next day. This experiment helps in identifying the factors that influence the epididymal sialic acid levels. From the results, it became clear that several factors of testicular origin influence the level of epididymal sialic acid. Therefore, it was felt necessary that the subsequent experiment should be carried out in the absence of these over-riding factors.

3.2.3.2 Experiment 2

This study was conducted on 38 animals whose efferent duct of both sides were ligated. Fifteen days after ligation was done, the animals were randomly divided into 5 groups. Ovine prolactin was administered subcutaneously, to each group of these animals at doses of 0 μg (8 animals), 50 μg (6 animals), 100 μg (9 animals), 150 μg (8 animals) and 200 μg (7 animals)/100 g body weight, respectively, once daily, for a period of 7 days. Twenty four hours after the last of 7 injections, the animals were killed and perfused as detailed earlier. The sialic acid concentration in the epididymis was estimated.

In another but related experiment, a very similar protocol was used except that the various doses of prolactin were injected in orchidectomised animals instead of duct ligated ones. Forty three animals were randomly divided into 5 groups and each group consisting of 8-10 rats received 0, 50, 100, 150 and 200 μg of ovine prolactin. The dose of the injection specified is per 100 g body weight and was administered, subcutaneously, once daily, for a period of 7 days. Ovine prolactin for injection was prepared initially at a concentration of 2.5 mg/ml in 0.03M $NaHCO_3$ in 0.15 M NaCl (pH 10.8) with very gentle agitation. After solubilization was effected, the pH was lowered to 7.0 by the drop-wise addition of 2 N HCl. This preparation was diluted appropriately to match the dose of ovine prolactin to be administered. The schedule was so arranged that a particular animal received the injection at the same time each day for the 7

day period.

3.2.3.3 Experiment 3

The preceeding experiment showed that the influence of prolactin on the epididymis could be visualized better in orchidectomised animals than in duct ligated ones. Therefore, the present experiment was confined to orchidectomised animals alone. If the exogenous administration of prolactin could initiate a set of responses in the epididymis then it is logical to expect that a depletion of this hormone could bring about opposite effects. To check this hypothesis, bromocryptine at doses of 0 mg, 0.15 mg, 0.30 mg and 0.50 mg/100 g body weight was administered to 15 days post-orchidectomised rats subcutaneously, once daily, for a period of 7 days (bromocryptine has been used by several workers as a drug of choice to reduce the circulating levels of prolactin). In the present study, bromocryptine mesylate was used, 45% ethanol being the vehicle. All together 29 orchidectomised animals were used, 6-9 animals being included in each group given a particular dose of bromocryptine. The sialic acid levels in the epididymal segments of these animals were quantified biochemically.

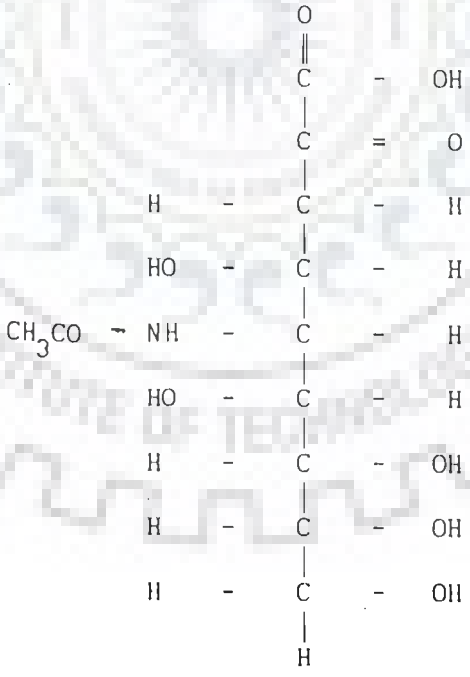
3.2.3.4 Experiment 4

To known concentration of a standard preparation of N-acetylneura -minic acid, was added, 0, 50, 100, 150 or 200 µg of ovine prolactin and the concentration of sialic acid estimated by the Thiobarbutric Acid Assay (Warren, 1959). The impact of prolactin

on the assay procedure of sialic acid was determined by plotting the known value of sialic acid in the presence and absence of ovine prolactin against the actual value obtained by biochemical estimations. Regression analysis was used for the statistical treatment of data.

3.2.4 SIALIC ACID ESTIMATION

The naturally occurring sialic acids are substituted neuraminic acid derivatives (N-acetyl, N-glycol, N,O-diacetylneuraminic acid). These are collectively termed "**Sialic Acids**". The unsubstituted 9 carbon chain is called neuraminic acid (Blix *et al.*, 1957).



N-Acetyl neuraminic acid

In 1957, Waravdekar and Saslaw reported a method for the

measurement of 2-deoxyribose, in which the periodate oxidation product, malon-aldehyde, was coupled with 2-thiobarbituric acid. Weissbach and Hurwitz (1959) have reported that 2-keto-3-deoxy sugar acids could also be assayed by the same method with some modifications. Here the periodate oxidation product was β-formyl pyruvic acid.

Although sialic acids are 2-keto-3-deoxy sugar acids, the amino group is always substituted and should not be reactive in thiobarbituric acid assay. The Waravdekar and Saslaw, and Weissbach and Hurwitz assays on sialic acids do, in fact, lead only to a small amount of color formation.

Warren, 1959 suggested that color formation could be increased considerably by carrying out the periodate oxidation in strong acid solution and by extracting the final colored solution into cyclohexanone. The molecular extinction coefficient thus obtained was 12 times higher than the previously used resorcinol method (Svennerholm, 1957). The thiobarbituric acid is specific enough to permit accurate direct measurement of the sialic acid content of tissues.

3.2.4.1 Reagents

1. Sodium meta periodate : 0.2 M, in 9 M phosphoric acid
2. Sodium arsenite : 10%, in a solution of 0.5 M sodium sulphate-0.1 N H_2SO_4
3. Thiobarbituric acid : 0.6%, in 0.5 M sodium sulphate

All these aqueous solutions were prepared with warming. Solutions were stored at room temperature and were stable for more than a month.

3.2.4.2 Procedure

Tissue was homogenized in 2 ml of distilled water and 1 ml of 1% phosphotungstic acid was added. The resulting precipitate was washed twice with 1 ml of 5 % trichloroacetic acid and dried in a desiccator for 12 hrs. Since the thiobarbituric acid assay measures only free sialic acids, 1 ml of 0.1 N H_2SO_4 was added to the precipitate and digested for 1 h at 80°C to release bound sialic acids without degradation. To 0.2 ml of hydrolysate, 0.1 ml of the periodate solution was added. The tubes were shaken and allowed to stand at room temperature for 20 min. Arsenite solution, 1 ml was added and the tubes were shaken until the yellow-brown color disappeared. Thiobarbituric acid solution, 3 ml, was added, the tubes shaken, capped with a glass bead, and then heated in a vigorously boiling water bath for 15 min. The tubes were then removed and placed in cold water for 5 min. The total 4.3 ml, solution was extracted with an equal volume of cyclohexanone. The tubes were shaken twice and then centrifuged for 3 min in a table top centrifuge at 1000 xg. The clear upper cyclohexanone phase was red and the color was more intense than it was when in water. The organic phase was taken in a 3 ml cuvette (1cm, light path) and optical density was determined at 532 mμ and 549 mμ in a Beckman DU-6 Spectrophotometer. Color production varies linearly with

concentration of N-acetylneuraminic acid (Standard stock 1 μM) over the range usually used, 0.01 to 0.06 μmole. The molecular extinction coefficient is 57,000. The amount of N-acetylneuraminic acid present in a given sample was determined from equation 2 of Warren's formula:

μmoles N-acetylneuraminic acid =

$$0.090 \times OD_{549} - 0.033 \times OD_{532}$$

The constants of equation 2 are calculated as follows:

$$= \left[\frac{\varepsilon_3}{\varepsilon_2\varepsilon_3 - \varepsilon_1\varepsilon_4} OD_{549} - \frac{\varepsilon_4}{\varepsilon_2\varepsilon_3 - \varepsilon_1\varepsilon_4} OD_{532} \right] X4.3$$

Where ε_1 and ε_2 are the molecular extinction coefficients x 10^{-3} of N-acetylneuraminic acid at 532 and 549 mμ, respectively. ε_1 = 26, ε_2 = 57, ε_3 = 133, ε_4 = 48. The final volume of the test solution = 4.3 ml.

3.2.4.3 Interfering Substances

When tissues were subjected to the thiobarbituric acid assay for sialic acid, an absorption maximum was found at 549 mμ, due to sialic acid. However, there was also a second absorption maximum at 532 mμ due to 2-deoxyribose for which a correction has been made since the light absorption of this interfering material at 549 mμ was considerable. Other substances such as unsaturated lipids (Bernheim *et al.*, 1948) may also yield malonaldehyde upon

periodate oxidation and contribute to the optical density at 532 and 549mμ, but the source of the malonaldehyde does not affect the calculations for correction.

Equation 2 corrects for the optical density at 549 mμ which does not derive from N-acetylneuraminic acid. The correction is based upon the observed molecular extinction values of N-acetylneuraminic acid and 2-deoxyribose at 532 and 549 mμ.

3.2.5 STATISTICAL ANALYSIS

The data were subjected to statistical analysis by the Student's t-test. The SEM was calculated by the following formula:

$$SEM = \sqrt{\frac{\Sigma x^2 - \left[\frac{\Sigma x}{n}\right]^2}{n\,(n-1)}}$$

where x = individual observations

n = number of observations

t-values were obtained by using the following equation and compared with the standard table values for Student's t-distribution based on the degrees of freedom (Fischer and Yates, 1948):

$$t = \frac{x_1 - x_2}{S\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where,

$$S = \sqrt{\frac{\sum x_1^2 - \frac{(\Sigma x_1)^2}{n_1} + \sum x_2^2 - \frac{(\Sigma x_2)^2}{n_2}}{n_1 + n_2 - 2}}$$

where n_1 and n_2 denote the number of observations in the two classes being compared (Ostle, 1954).

If the calculated value was more than the table value, it was considered significant at that probability level. The following levels of significance were used $p<0.001$ to $p<0.05$ for significant data and $p>0.05$ for non significant data.

3.3 RESULTS

The effect of bilateral efferent duct ligation and orchidectomy on the weight of organs associated with the male reproductive tract are highlighted in Fig.1. Compared to sham-operated controls, duct ligation significantly decreased the weight of the epididymis ($p < 0.01$) but not that of the seminal vesicle and ventral prostate. On the other hand, the weight of all three organs were substantially lower in the orchidectomised animals than the sham-operated controls.

The sialic acid levels in the caput, corpus and cauda epididymides of sham-operated, duct ligated and orchidectomised rats are presented in Fig.2. When comparisons are made with the sham-operated controls, it is evident that orchidectomy reduces the sialic acid levels in all three segments of the epididymis

but in the duct ligated animals, the reduction is significant only in the caput ($p<0.05$).

The influence of ovine prolactin on the epididymal sialic acid concentration of efferent duct ligated rats is depicted in Fig.3. No significant differences in the sialic acid levels could be observed between prolactin treated animals and the vehicle treated controls.

The impact of exogenous prolactin on the sialic acid profile in the epididymis of orchidectomised rats is shown in Fig.4. It appears that the corpus and caudal epididymal segments are more responsive than the caput to prolactin treatment. The histograms also give the impression that the sialic acid concentration in the epididymis is more or less related to the dose of ovine prolactin administered in orchidectomised animals.

Only the highest dose of bromocryptine (0.5 mg/100g body wt) is able to reduce the sialic acid concentration in the epididymis of orchidectomised animals (Fig. 5). This again is restricted to the distal region of the epididymis ($p<0.05$).

The regression analysis of data to test if prolactin interferes with the thiobarbutric acid assay for sialic acid is presented in fig.6. The slope and intercept of the regression lines obtained for standard preparations of N-acetylneuraminic acid without prolactin are no different from those that in which prolactin has also been included.

Fig. 1. THE EFFECT OF DUCT LIGATION AND ORCHIDECTOMY ON THE WEIGHT OF ORGANS ASSOCIATED WITH THE MALE REPRODUCTIVE TRACT.

The weight of organs (Epi-Epididymis; SV-Seminal Vesicle; VP-Ventral Prostate) of the duct ligated (▪▪▪) and orchidectomised (▪▪▪) groups have been compared with sham-operated controls (▪▪▪). The number of animals included in each group are given in parenthesis. The results are presented as Mean ± SEM (** $p<0.01$ Student's t-test).

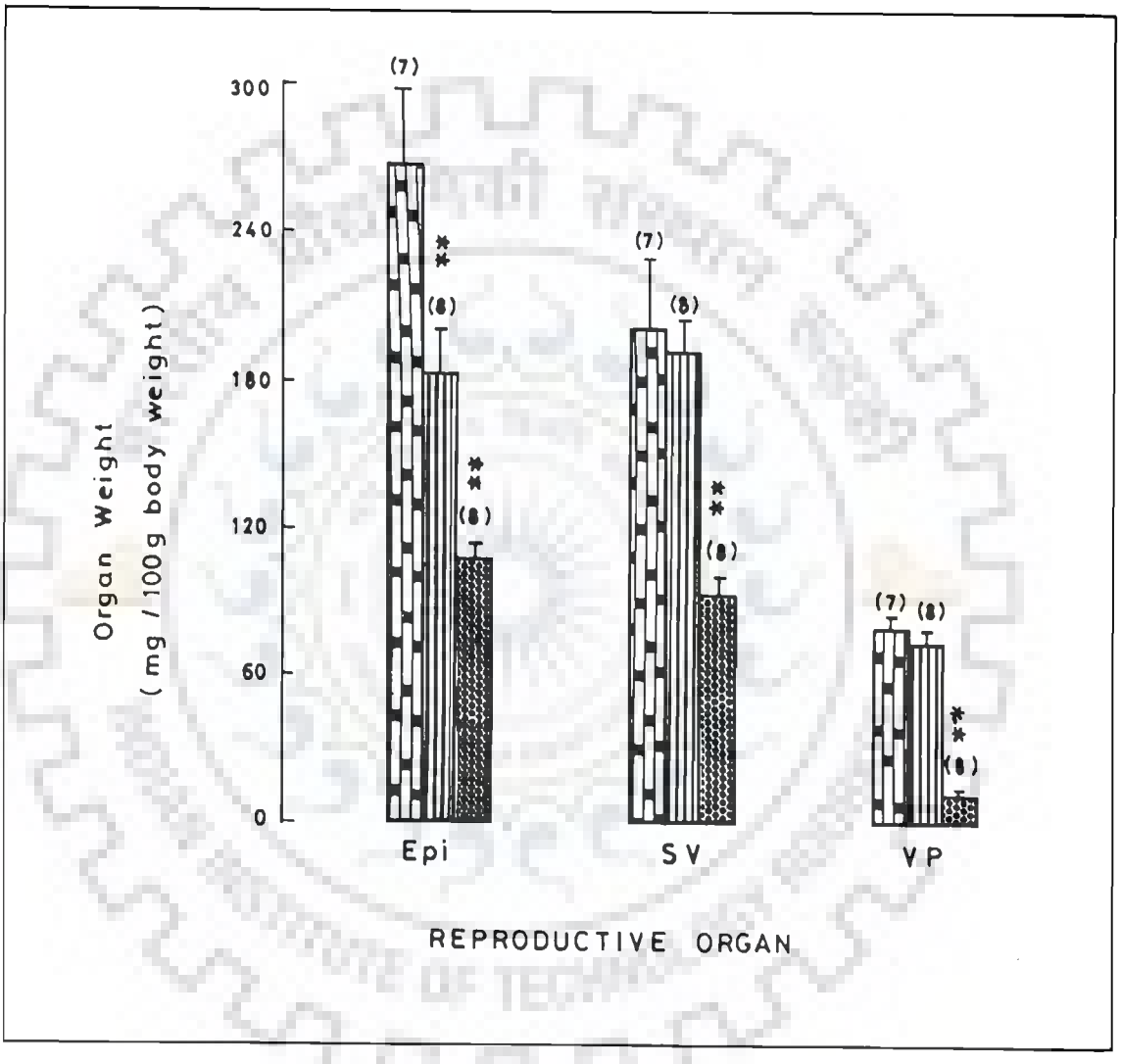

Organ Weight
(mg / 100g body weight)
300
240
180
120
60
0
(7)
(8)
(8)
(7)
(8)
(8)
(7)
(8)
(8)
Epi
SV
VP
REPRODUCTIVE ORGAN

Fig. 1.

Fig. 2. THE EFFECT OF THE DUCT LIGATION AND ORCHIDECTOMY ON THE SIALIC ACID CONCENTRATION IN THE EPIDIDYMAL REGIONS OF MALE RAT.

The values of sialic acid for the sham-operated (◈), duct ligated (◁▯▷) and orchidectomised (◂◇▸) groups of animals are shown in the form of kites, each representing Mean ± SEM. The number of animals included in each group are provided within parenthesis. The impact of duct ligation and orchidectomy on each epididymal region has been analysed by making comparisons with the values obtained for sham-operated controls ($^{*}p<0.05$; $^{*}p<0.01$ Student's t-test).

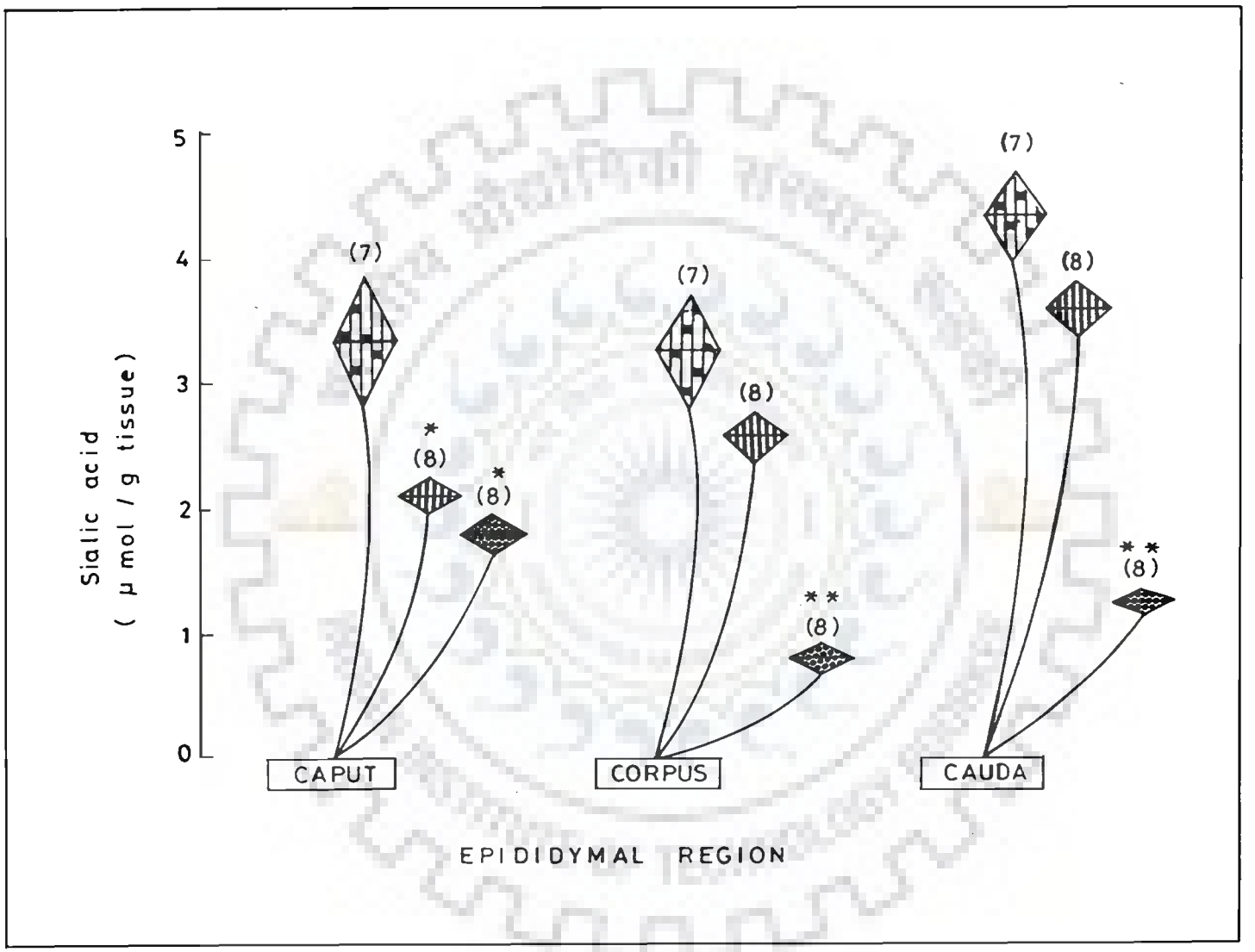

Sialic acid
(μ mol / g tissue)
5
4
3
2
1
0
(7)
*
(8)
*
(8)
(7)
(8)
**
(8)
(7)
(8)
**
(8)
CAPUT
CORPUS
CAUDA
EPIDIDYMAL REGION

Fig. 2.

Fig. 3. EFFECT OF OVINE PROLACTIN (oPRL) ON THE SIALIC ACID CONCENTRATION IN EPIDIDYMAL REGIONS OF EFFERENT DUCT LIGATED RATS.

Fifteen days after the efferent ducts were ligated, the animals were divided into five groups and injected with (▦) Vehicle; (▬) 50 μg oPRL; (▩) 100 μg oPRL; (▨) 150 μg oPRL and (▥) 200 μg oPRL/100 g b wt respectively, once daily, s.c., for a period of 7 days. The concentration of epididymal sialic acid in each of these groups is presented in the histogram as Mean ± SEM. The number of animals included in each group is given within parenthesis. No significant differences in sialic acid levels could be observed between the prolactin treated and vehicle treated controls. (Student's t-test).

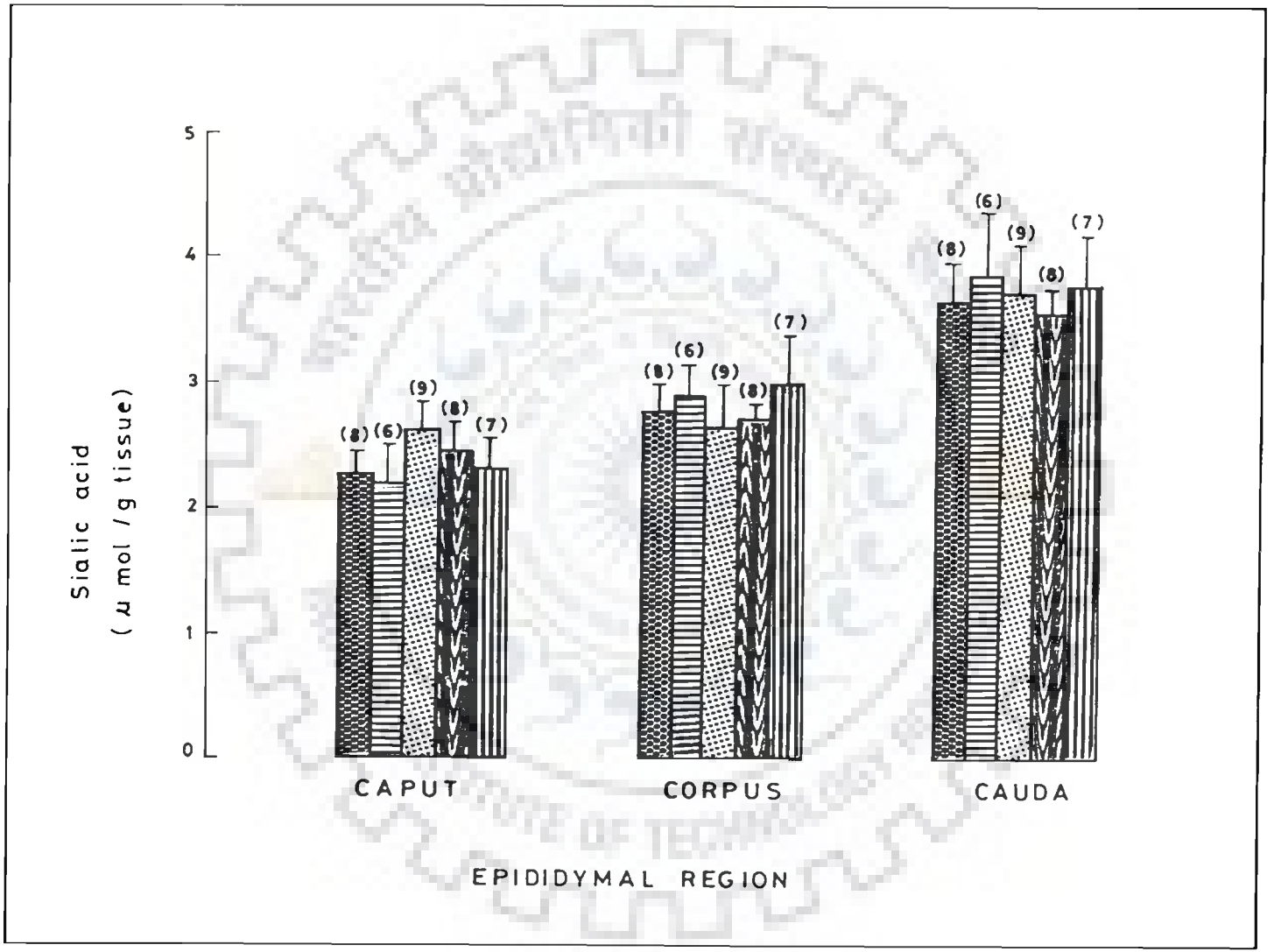

Sialic acid
(μ mol / g tissue)
0
1
2
3
4
5
(8) (6) (9) (8) (7)
(8) (6) (9) (8) (7)
(8) (6) (9) (8) (7)
CAPUT
CORPUS
CAUDA
EPIDIDYMAL REGION

Fig. 3.

Fig. 4. EFFECT OF OVINE PROLACTIN (oPRL) ON THE SIALIC ACID CONCENTRATION IN EPIDIDYMAL REGIONS OF ORCHIDECTOMISED RATS.

Fifteen days after orchidectomy, the animals were divided into five groups and injected with (▬▬▬) vehicle; (▬▬) 50 µg oPRL; (▬▬) 100 µg oPRL; (▬▬) 150 µg oPRL and (▬▬) 200 µg oPRL/100 g b wt respectively, once daily, s.c., for a period of 7 days. The concentration of sialic acid is shown as Mean ± SEM and the number of animals belonging to each group is given within parenthesis. The values obtained for each group of prolactin treated animals have been compared individually with those of vehicle treated controls ($^{*}p<0.05$; $^{**}p<0.01$ Student's t-test).

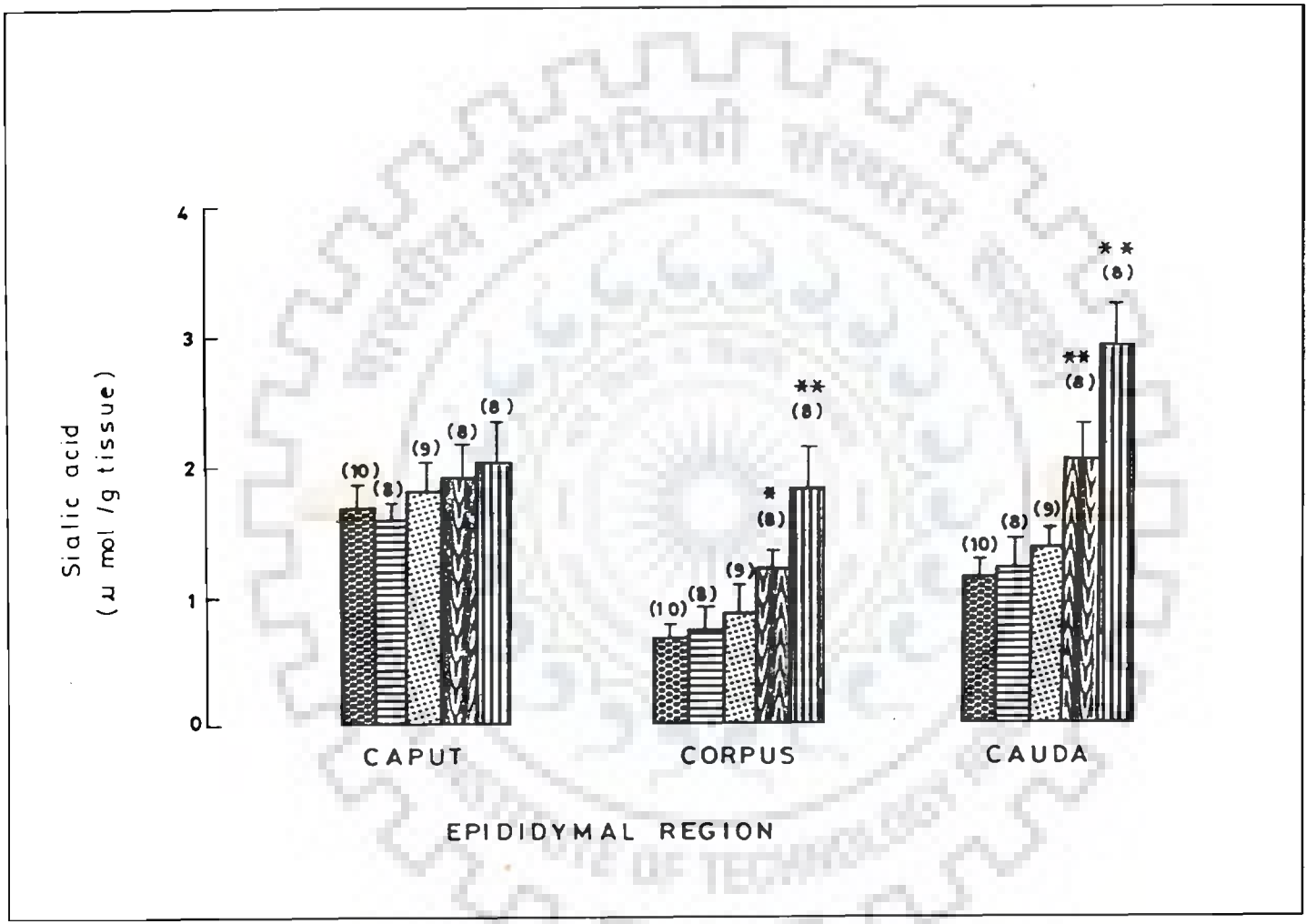

Sialic acid
(μ mol /g tissue)
4
3
2
1
0
(10)
(8)
(9)
(8)
(8)
(10)
(8)
(9)
*
(8)
**
(8)
(10)
(8)
(9)
**
(8)
**
(8)
CAPUT
CORPUS
CAUDA
EPIDIDYMAL REGION

Fig. 4.

Fig. 5. THE EFFECT OF BROMOCRYPTINE ON THE SIALIC ACID CONCENTRATION IN EPIDIDYMAL REGIONS OF ORCHIDECTOMISED RATS.

Fifteen days after orchidectomy, the animals were divided into four groups and injected with (▪▪▪▪▪▪▪▪▪▪) vehicle; (▬▬▬) 0.15 mg; (▪▪▪▪▪▪▪▪) 0.30 mg and (▪▪▪▪▪▪▪▪) 0.50 mg of bromocryptine/100g b wt respectively. The injections were administered, once daily, subcutaneously, for a period of 7 days. The values for epididymal sialic acid obtained are presented as Mean ± SEM in the bar diagrams. The figures within parenthesis represent the number of animals used in each group. The values obtained in each epididymal region of bromocryptine treated animals have been compared individually with the corresponding value of vehicle treated controls ($p<0.05$ Student's t-test).

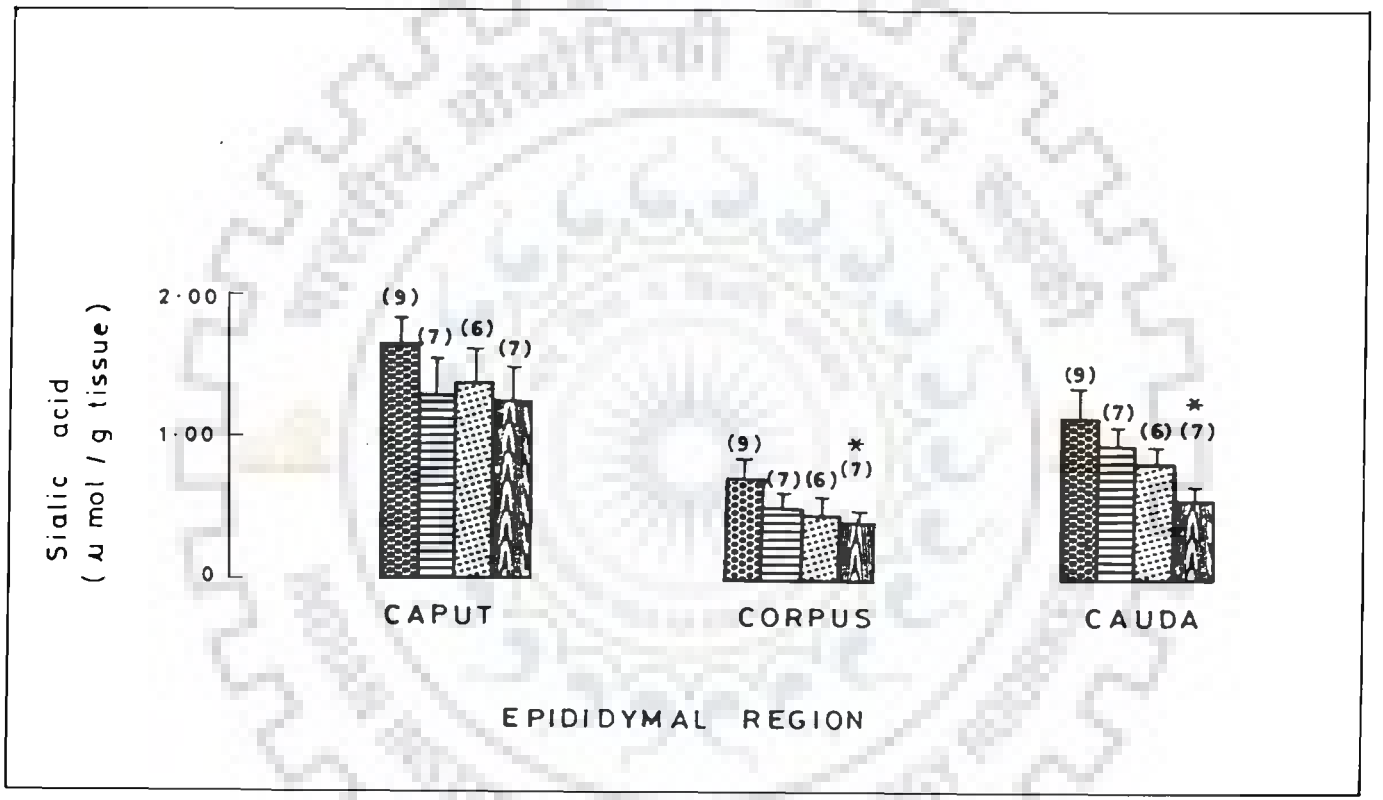
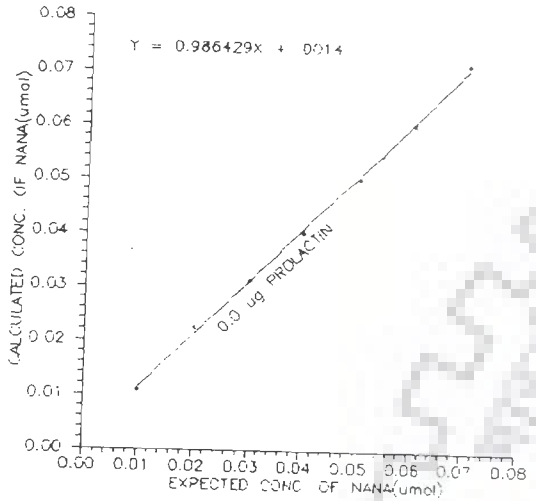
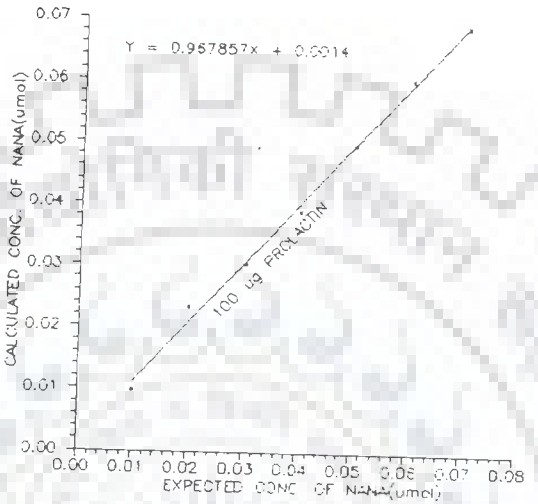

Sialic acid
(μ mol / g tissue)
2·00
1·00
0
(9)
(7)
(6)
(7)
(9)
(7)
(6)
(7)
*
(9)
(7)
(6)
(7)
*
CAPUT
CORPUS
CAUDA
EPIDIDYMAL REGION

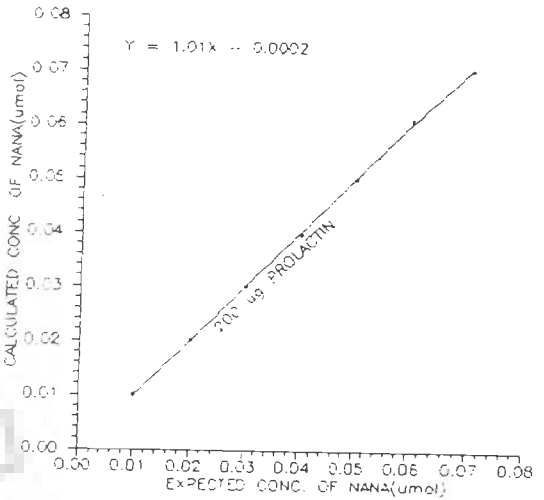
Fig. 5.

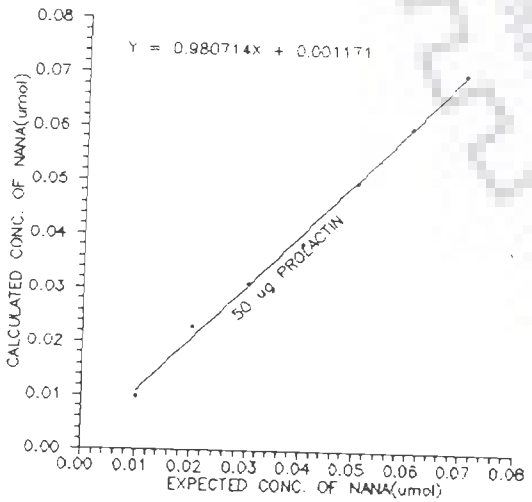
Fig. 6. THE EFFECT OF VARYING CONCENTRATION OF PROLACTIN AND BROMOCRYPTINE ON THE ASSAY OF SIALIC ACID

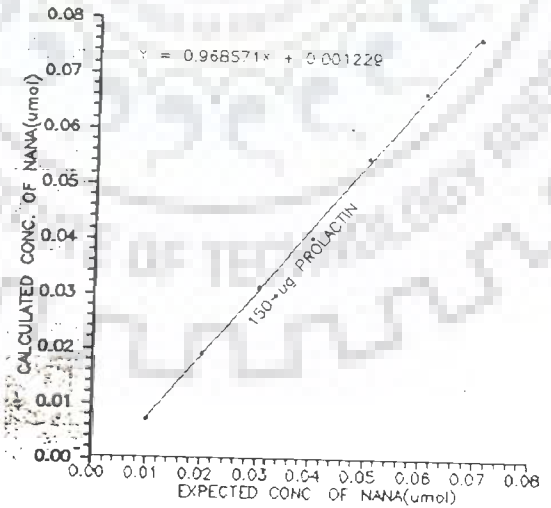
No hormone effect was seen as observed by nonsignificant change in slope and intercept from regression equations for different concentrations. (F-test).


Y = 0.986429x + 0014
CALCULATED CONC. (IF NANA(umol)
EXPECTED CONC OF NANA(umol)
0.0 ug PROLACTIN


Y = 0.957857x + 0.0014
CALCULATED CONC. OF NANA(umol)
EXPECTED CONC OF NANA(umol)
100 ug PROLACTIN


Y = 1.01x - 0.0002
CALCULATED CONC OF NANA(umol)
EXPECTED CONC. OF NANA(umol)
200 ug PROLACTIN


Y = 0.980714x + 0.001171
CALCULATED CONC. OF NANA(umol)
EXPECTED CONC. OF NANA(umol)
50 ug PROLACTIN


Y = 0.968571x + 0.001229
CALCULATED CONC. OF NANA(umol)
EXPECTED CONC OF NANA(umol)
150+ug PROLACTIN

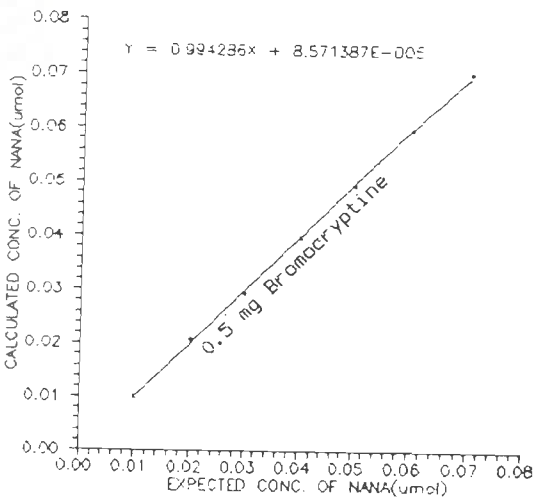

Y = 0.994236x + 8.571387E-005
CALCULATED CONC. OF NANA(umol)
EXPECTED CONC. OF NANA(umol)
0.5 mg Bromocryptine

Fig. 6.

3.4 DISCUSSION

It is a well known fact that the epididymis is a conduit connecting the testis with the vas deferens. It is in this duct that spermatozoa produced in the testis spend varying period of time, depending upon the species, in order to be able to achieve forward progressive motility and fertilizing ability (Amann *et al.*, 1976; Courot, 1981; Orgebin-Crist and Olson, 1984). Thus, the weight of this organ is substantially influenced by the presence of spermatozoa. In addition, the structural integrity of epididymis is to a large extent under androgen control (Brooks, 1977; Hamilton, 1975; Moore and Bedford, 1979b; Orgebin-Crist and Djiane, 1979). Androgens find their way to the epididymis either directly from the testis via the testicular fluid or from circulation through the blood supply (Aafjes and Vreeburg, 1972; Turner *et al.*, 1981; Cooper and Waites, 1979, 1980). Efferent duct ligation prevent spermatozoa and testicular fluid from reaching the epididymis. Therefore, it is not surprising that the weight of the epididymis in the duct ligated animals are substantially lower than the sham-operated controls. Perhaps, the weight of other sex accessories is not much affected by efferent duct ligation because these organs lean more heavily on the blood vascular system for their source of androgens, which is apparently not disturbed. On the otherhand, surgical orchidectomy results in the removal of the primary source of androgens which is of testicular origin. Since the maintenance of most parts of the male reproductive tract is androgen dependent, it is but natural to expect degenerative

changes when androgen support is withdrawn. This accounts for the significant loss in the weight of organs associated with the male reproductive tract in the orchidectomised animals.

The main interest of the present study was to examine the effect of prolactin on epididymis. The sialic acid level in the epididymis was used to monitor the functional status of the organ. In order to use the parameter effectively, it was felt necessary to first identify the factors that normally influence the sialic acid levels in the epididymis. At this stage, it must be mentioned that epididymis comprises of three compartments- the tissue, fluid and spermatozoa. Most early studies concerning sialic acid in the epididymis have dealt with the organ as a whole, making it difficult to interpret data relating to any one compartment (Fournier, 1966; Bose and Kar, 1968; Rlar *et al.*, 1973; Setty *et al.*, 1979). The continuous delivery of spermatozoa and fluid from the testis makes the situation even more complex. In the present studies, comparisons between the sham-operated controls and efferent duct ligated or orchidectomised animals have helped in clarifying if there exist sialic acid of epididymal origin and if so, whether they are under the control of androgens.

The sialic acid level of caput epididymidis are found to be substantially lower in the duct ligated animals than the sham-operated controls. It is also observed that the level of sialic acid in other regions of the epididymis is not severely affected by duct ligation and the concentration of this compound

is higher in the cauda than in the caput. These findings do implicate that epididymis is capable of synthesizing sialic acids. It appears that the testicular fluid and spermatozoa whose entry into the epididymis is curtailed by efferent duct ligation contribute significantly to the level of sialic acid, particularly in the caput. Support to this contention could also be drawn from previous findings that sialic acid is also produced by the testis (Bose *et al.*, 1966) and incorporated in the spermatozoa (Bernal *et al.*, 1980). As mentioned earlier, duct ligation cuts off one of the routes of androgen supply to the epididymis, while orchidectomy ensures the removal of the major source itself thereby depriving the epididymis of androgens. This is reflected in the present studies as all three segments in the epididymis in orchidectomised animals show lower levels of sialic acid than the sham-operated controls. Thus, it may be inferred that the levels of sialic acid in epididymal segments may be influenced by the presence of spermatozoa, testicular fluid and the availability of androgens.

Since several factors of testicular origin are shown to influence the sialic acid level in the epididymis, it was felt necessary to design experiments that would help rule out the interference from these factors. Thus, further experiments involving prolactin treatment were carried out in the duct ligated and orchidectomised animals. In the efferent duct ligated animals, prolactin treatment did not show any effect on the epididymal sialic acid level. On the otherhand, in orchidectomised animals

prolactin administration resulted in an increase in epididymal sialic acid concentration, a response which seems to be dose related. It is interesting to note that the epididymis of duct ligated and orchidectomised animals respond differently to prolactin treatment. Since the epididymis is an androgen dependent tissue, it may be suggested that the effect of prolactin observed in orchidectomised animals is masked by the much greater influence of androgens on the epididymal sialic acid levels in duct ligated animals. However, from these results it is suspected that the effect of prolactin on the epididymal sialic acid may be independent of androgens. This possibility may be true since receptors for prolactin have been reported in the epididymis of rat (Aragona and Friesen, 1975) and other laboratory animals (Orgebin-Crist and Djiane, 1979).

In the preceeding experiment the sialic acid levels in the epididymis of orchidectomised animals were shown to respond to prolactin treatment. To verify if the withdrawal of endogenous prolactin in orchidectomised animals had any effect, an ergot alkaloid bromocryptine was used. The mode of action and pharmacokinetics of bromocryptine have been extensively worked out and it has been shown that bromocryptine reduces circulating levels of endogenous prolactin (Harper *et al.*; 1976 ; Giron-Forest and Schönleber, 1979; Kinch, 1980; Weinstein *et al.*, 1981). Therefore, varying doses of bromocryptine were injected in orchidectomised animals and the epididymal sialic acid concentration determined. Only the highest dose of bromocryptine

used in the study was able to reduce the level of epididymal sialic acid. The results of bromocryptine treatment when viewed together with those obtained for prolactin treatment in orchidectomised animals give the impression that prolactin may have a direct role to play in the epididymis.

It was now felt necessary to investigate the effects of prolactin and bromocryptine on the assay of sialic acid if prolactin interferes positively with the assay, the dose dependent increase in the sialic acid in the epididymis may reflect the higher amount of prolactin in the tissue. If bromocryptine treatment reduces the amount of sialic acid detected, the opposite would be observed. This was highly relevant since binding sites for prolactin have been detected in the epididymis. The analysis of standard preparations of sialic acid in which various amounts of prolactin were added clearly showed that prolactin does not interfere with the assay of sialic acid.

3.5 CONCLUDING REMARKS

1. Efferent duct ligation reduced the weight of the epididymis but not of the seminal vesicle and ventral prostate. The restriction in the entry of spermatozoa and testicular fluid is most likely the cause of this loss in weight.

2. The weights of the epididymis, seminal vesicle and ventral prostate were drastically lower in the orchidectomised animals than in sham-operated controls. The reduction might be due to the withdrawal of androgen support to these organs.

3. From the experiments with sham-operated, duct ligated and orchidectomised animals, it is clear that the sialic acid level in the epididymis is influenced by factors originating in the testis.

4. Prolactin administration to duct ligated animals did not influence the epididymal sialic acid levels. The same treatment to orchidectomised animals resulted in an increase in the epididymal sialic acid which seemed to be dose related. Based on this data, an action of prolactin independent of androgens has been suggested. The lack of any significant response in duct ligated animals may be attributed to the greater effects of androgens which may have masked the effects of prolactin.

5. The action of prolactin is perhaps at the level of sialic acid biosynthesis since the increase in epididymal sialic acid was more or less dose related. Studies with bromocryptine treated orchidectomised animals have provided support to the view that the action of prolactin may be direct.

6. It has also been shown convincingly that prolactin does not interfere with the assay of sialic acid and hence the results obtained could be considered a treatment effect.

CHAPTER 2

THE EFFECT OF PROLACTIN ON EPIDIDYMAL GLYCOSIDASE ACTIVITY

4.1 INTRODUCTION

Over the past few decades the importance of glycosidases in the male reproductive tract has been well recognized. A survey of the distribution of mammalian glycosidases in body tissues has revealed that the epididymis is one of the richest source (Conchie and Hay, 1959; Conchie *et al.*, 1959 a,b). From histochemical studies it is clear that these enzymes are localized in the epididymal epithelium (Rutenberg *et al.*, 1958; Pugh and Walker, 1961; Hayashi, 1967; Moniem and Glover, 1972).

Although the precise function of these enzymes has not been clearly established, there is convincing evidence to show that these enzymes are synthesized in the epididymis, secreted into its lumen and act in the process of sperm maturation (Jones and Glover, 1975; Skudlarek and Orgebin-Crist, 1984, 1986). Pre-existing sperm membrane glycoproteins are reported to be modified during epididymal transit (Killian and Amann, 1973; Bostwick *et al.*, 1980; Jones *et al.*, 1981). In view of the fact that epididymal spermatozoa have limited biosynthetic activity (Abraham and Bhargava, 1963 a,b; Witkin and Bendirch, 1977) and carbohydrate chains associated with sperm surface are not primary gene products (Beyer *et al.*, 1979), it seems most likely that the modification of carbohydrate residues on sperm surface are regulated by glycosidases of epididymal origin (Hamilton and Gould, 1980).

Alterations in the carbohydrate moieties may possibly serve as potential signals for the transfer of biological informations

(Olden *et al.*, 1985). Alternatively, it has been suggested that monosaccharides liberated as a result of hydrolysis may either serve as energy source or be recycled by enzymes involved in the synthesis of complex carbohydrate chains associated with proteins at the sperm surface and in epididymal fluid (Hall and Killian, 1987). At least some of these changes may be linked to sperm capacitation, acrosome retention (Schwartz and Koĕhler, 1979) and even in events such as cumulus dispersion or zona attachment or penetration leading to fertilization (Allison and Hartree, 1970; Gwatkin and Anderson, 1973; Farooqui and Srivastava, 1980; Rodger and Young, 1981; Shur and Hall, 1982; Shalgi *et al.*, 1986).

Like most other enzymes present in the epididymis, the glycosidases are also known to be androgen dependent (Conchie and Mann, 1957; Conchie and Findlay, 1959; Mann, 1964; Mann *et al.*, 1971; Sinowatz *et al.*, 1975). The interplay or involvement of other hormones has, however not received any attention. An attempt has therefore, been made to see if prolactin has a role to play in the control of the epididymal glycosidases.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

All reagents used were of analytical grade. ρ-Nitrophenyl β-D-galactopyranoside and ρ-Nitrophenyl-α-D-mannopyranoside that were used as substrate in enzyme assays were purchased from the CSIR Centre for Biochemicals, V P Chest Institute, Delhi.

4.2.2 Experiment

Male albino rats (200-250 g) of *Wistar* strain were surgically orchidectomised. In this study, only orchidectomised animals were used since it was shown in the earlier chapter dealing with sialic acid that the effects of prolactin are probably masked by the greater effects of androgens in duct ligated animals. Fifteen days after orchidectomy, the animals were injected with either prolactin (0, 50, 100, 150 and 200 μg oPRL/100g body weight) or bromocryptine (0, 0.15, 0.30 and 0.50 mg/100 g body weight as described under section 3.2.3.2 and 3.2.3.3. Ten rats were included in every group receiving a particular dose of prolactin/bromocryptine. Briefly, the injections were administered subcutaneously, once daily, for a period of 7 days. Twenty four hours after the scheduled treatments, the animals were killed and transcardially perfused. The epididymis from these animals was excised and weighed accurately on a single pan balance. β-galactosidase and α-mannosidase activity was assayed in the three epididymal segments (caput, corpus and cauda). The amount of tissue for each epididymal segment from individual animals was not sufficient for determining the enzyme activity. Therefore, the tissue belonging to all animals of a particular group were pooled, the extract of which was used in 5 replicates to determine the activity of β-galactosidase / α- mannosidase.

4.2.2.1 Analytical Methods

Epididymal tissue extracts were initially prepared in water (50 mg/ml). Homogenization was done manually in a mortar

and pestle using a little acid washed sand. The procedure used for enzyme assays was essentially the same as that of Conchie *et al.* (1959a) but with very slight modifications. The assay conditions for two representative glycosidases, β-galactosidase and α-mannosidase used in the present studies were standarized. Accordingly, a pH of 4.8 and a substrate concentration of 2.5 mM ρ-Nitrophenyl β-D-galactopyranoside was used for the assay of β-galactosidase activity (Fig.7.). As is clear from Fig.8., a pH of 5.2 and a substrate concentration of 5 mM ρ-Nitrophenyl α-D-mannopyranoside was optimum for the enzyme α-mannosidase. For both the enzymes, the conversion of the substrate into ρ-Nitrophenol was found to be maximal at 37°C and 20 min incubation time was ideal for this purpose.

4.2.3 ENZYME ASSAY

4.2.3.1 Principle Of The Enzyme Assays

Glycosidases (α-mannosidase and β-galactosidase) are allowed to react with the appropriate ρ-Nitrophenyl glycoside at their optimal pH which is in the acidic range. ρ-Nitrophenol is released from the substrate by enzymatic hydrolysis. This reaction is terminated by the addition of Na_2CO_3 which makes the pH alkaline. The change in pH also results in the development of a yellow color whose intensity could be measured in a Spectrophotometer at 400 nm.

Fig. 7. OPTIMIZATION OF ASSAY CONDITIONS FOR α-MANNOSIDASE FROM RAT EPIDIDYMIS.

a: Hydrolysis of 5mM p-Nitrophenyl α-D-mannopyranoside at varying pH values in 0.2 M sodium acetate-acetic acid buffer, by enzyme from rat epididymis at 37°C for a period of 20 min. Enzyme activities are expressed as μmol of p-Nitrophenol liberated/g tissue/min.

b: Effect of varying the substrate concentration on the rate of hydrolysis of p-Nitrophenyl α-D-mannopyranoside in 0.2 M sodium acetate-acetic acid buffer, pH 5.2, by enzyme from rat epididymis at 37°C for a period of 20 min. Reaction velocities are expressed as μmol of p-Nitrophenol liberated/ min.

c: Effect of incubation time on the hydrolysis of 5 mM p-Nitrophenyl α-D-mannopyranoside in 0.2 M sodium acetate-acetic acid buffer, pH 5.2, at 37°C by enzyme from rat epididymis. Enzyme activities are expressed as μmol of p-Nitrophenol liberated/g tissue/min.

d: Effect of incubation temperature on the hydrolysis of 5 mM p-Nitrophenyl α-D-mannopyranoside in 0.2 M sodium acetate-acetic acid buffer, pH 5.2, by enzyme from rat epididymis for 20 min. Enzyme activities are expessed as μmol of p-Nitrophenol liberated/g tissue/min.

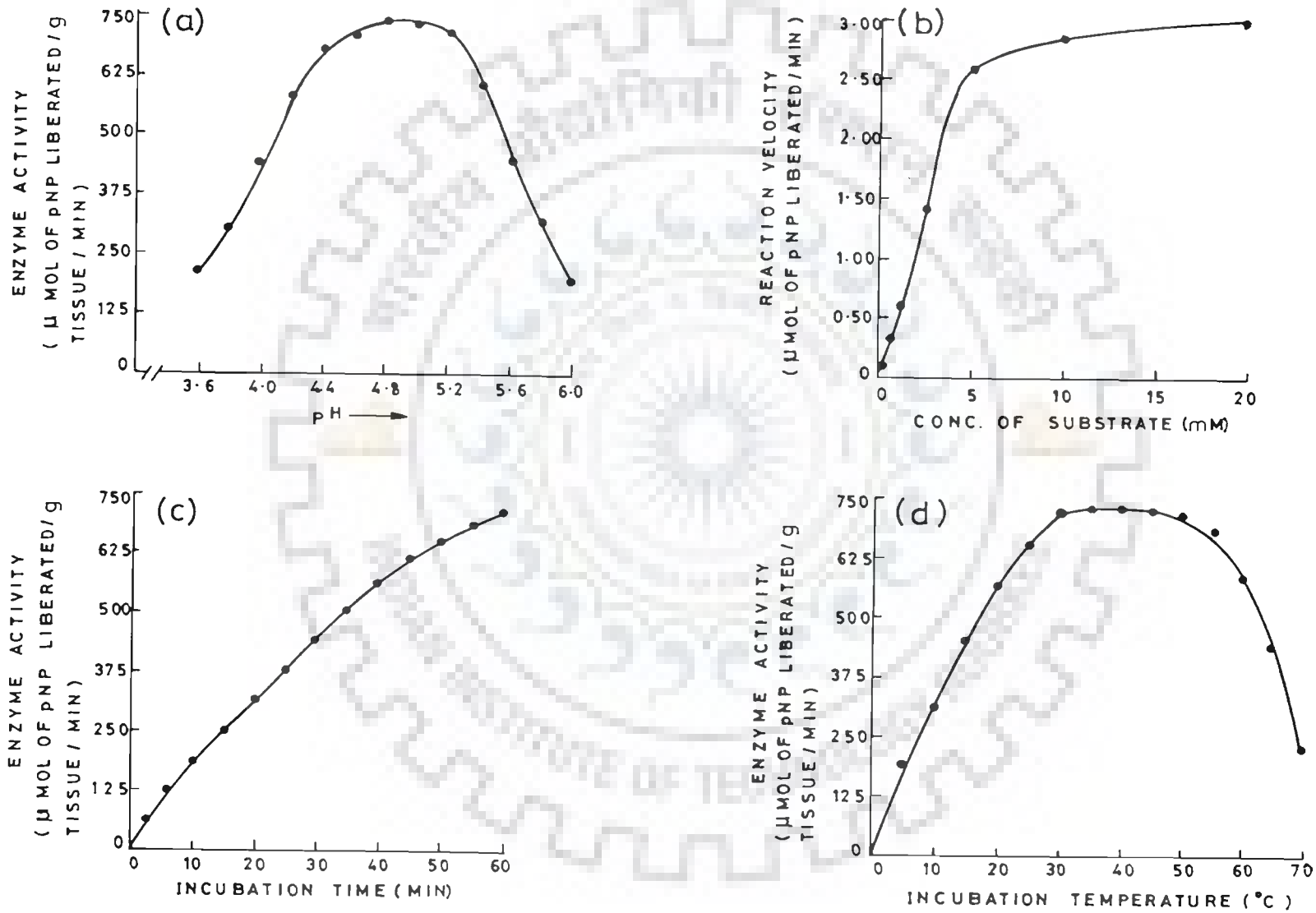

(a)
ENZYME ACTIVITY
(µ MOL OF pNP LIBERATED/g
TISSUE / MIN)
750
625
500
375
250
125
0
3·6
4·0
4·4
4·8
5·2
5·6
6·0
pH
(b)
REACTION VELOCITY
(µMOL OF pNP LIBERATED/MIN)
3·00
2·50
2·00
1·50
1·00
0·50
0
5
10
15
20
CONC. OF SUBSTRATE (mM)
(c)
ENZYME ACTIVITY
(µ MOL OF pNP LIBERATED/g
TISSUE / MIN)
750
625
500
375
250
125
0
10
20
30
40
50
60
INCUBATION TIME (MIN)
(d)
ENZYME ACTIVITY
(µMOL OF pNP LIBERATED/g
TISSUE/MIN)
750
625
500
375
250
125
0
10
20
30
40
50
60
70
INCUBATION TEMPERATURE (°C)

Fig. 7.

Fig. 8. OPTIMIZATION OF ASSAY CONDITIONS FOR β-GALACTOSIDASE FROM RAT EPIDIDYMIS.

a: Hydrolysis of 2.5 mM p-Nitrophenyl β-D-galactopyranoside at varying pH values in 0.2 M sodium acetate-acetic acid buffer, by enzyme from rat epididymis at 37°C for a period of 20 min. Enzyme activities are expressed as μmol of p-Nitrophenol liberated/g tissue/min.

b: Effect of varying the substrate concentration on the rate of hydrolysis of p-Nitrophenyl β-D-galactopyranoside in 0.2 M sodium acetate-acetic acid buffer, pH 4.8, by enzyme from rat epididymis at 37°C for a period of 20 min. Reaction velocities are expressed as μmol of p-Nitrophenol liberated/ min.

c: Effect of incubation time on the hydrolysis of 2.5 mM p-Nitrophenyl β-D-galactopyranoside in 0.2 M sodium acetate-acetic acid buffer, pH 4.8, at 37°C by enzyme from rat epididymis. Enzyme activities are expressed as μmol of p-Nitrophenol liberated/g tissue/min.

d: Effect of incubation temperature on the hydrolysis of 2.5 mM p-Nitrophenyl β-D-galactopyranoside in 0.2 M sodium acetate- acetic acid buffer, pH 4.8, by enzyme from rat epididymis for 20 min. Enzyme activities are expressed as μmol of p-Nitrophenol liberated/g tissue /min.

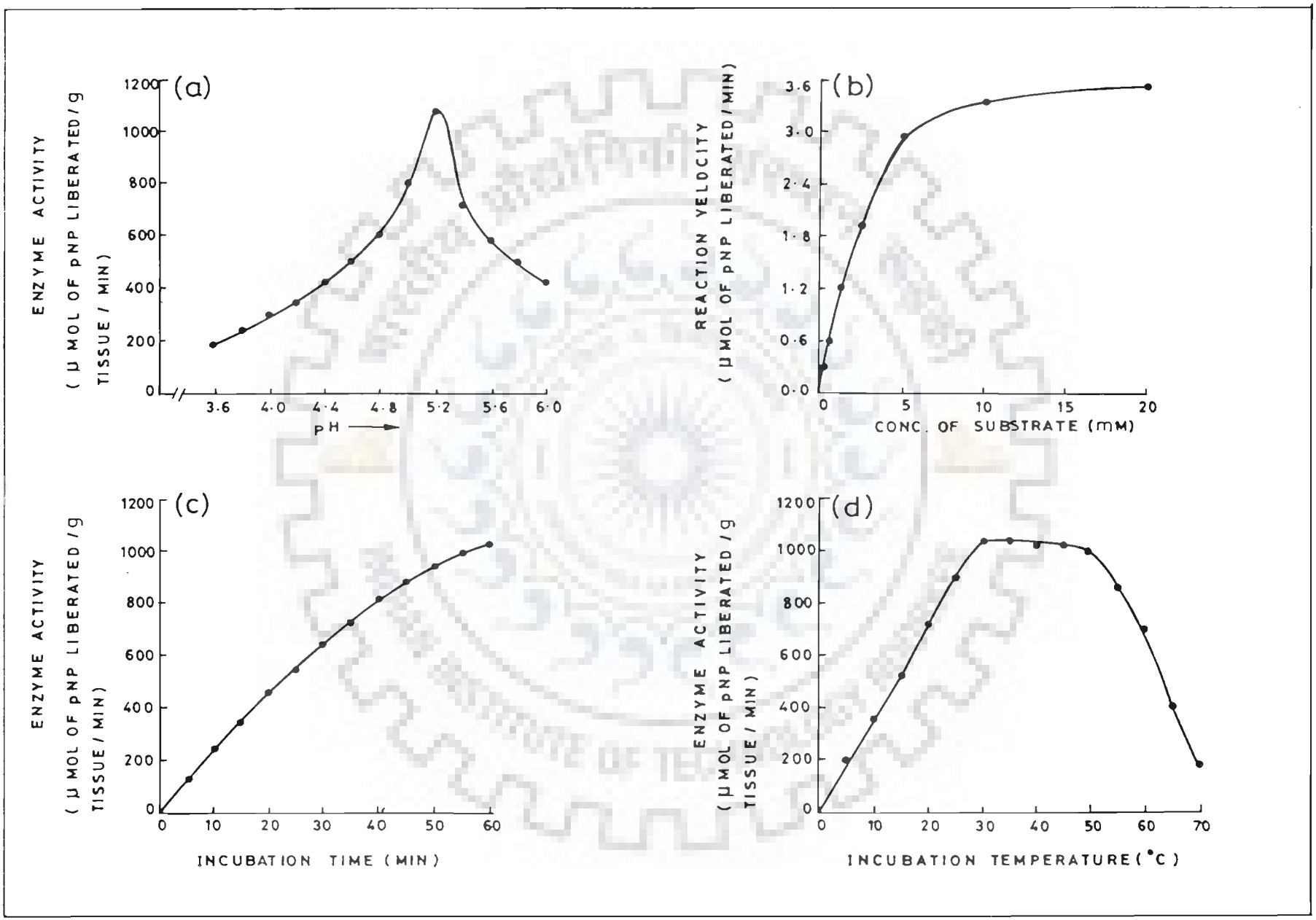

(a)
ENZYME ACTIVITY
(µ MOL OF pNP LIBERATED / g
TISSUE / MIN)
1200
1000
800
600
400
200
0
3·6
4·0
4·4
4·8
5·2
5·6
6·0
pH
(b)
REACTION VELOCITY
(µ MOL OF pNP LIBERATED / MIN)
3·6
3·0
2·4
1·8
1·2
0·6
0·0
0
5
10
15
20
CONC. OF SUBSTRATE (mM)
(c)
ENZYME ACTIVITY
(µ MOL OF pNP LIBERATED / g
TISSUE / MIN)
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
INCUBATION TIME (MIN)
(d)
ENZYME ACTIVITY
(µ MOL OF pNP LIBERATED / g
TISSUE / MIN)
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
70
INCUBATION TEMPERATURE (°C)

Fig. 8.

4.2.3.2 Enzyme Preparations

4.2.3.2.1 Reagents

(A) Sodium acetate-acetic acid buffer : 0.2 M 70 ml of 0.2 M sodium acetate trihydrate, $CH_3COONa.3H_2O$ (mol wt. 136.09) solution (27.22 g/l) was added to 30 ml of 0.2 M acetic acid to make 100 ml 0.2 M sodium acetate-acetic acid buffer (pH 5.0).

4.2.3.2.1 Procedure

A common procedure was adopted for the extraction of β-galactosidase and α-mannosidase from the epididymis. The epididymal segment of interest was homogenized in water (100 mg / ml) in a mortar pestle. The homogenate thus obtained was incubated with an equal volume of 0.2 M sodium acetate buffer, pH 5.0 at 37°C for 1 h. The suspension was centrifuged at 1500 xg for 15 min and the supernatant saved for the enzyme assays.

4.2.3.3 Assay For β-galactosidase (EC 3.2.1.2.3)

4.2.3.3.1 Reagents

(A) Sodium acetate-acetic acid buffer : 0.2 M 59 ml of 0.2 M sodium acetate trihydrate ($CH_3COONa.3H_2O$) solution (27.22 g/l) was added to 41 ml of 0.2 M acetic acid buffer (pH 4.8).

(B) ρ-Nitrophenyl β-D-galactopyranoside: 2.5 mM, in sodium acetate-acetic acid buffer (pH 4.8), freshly prepared.

(C) Standard ρ-Nitrophenol : Stock solution (250 μM)

(D) Sodium carbonate solution : 0.2 M, Na_2CO_3 in distilled water, freshly prepared.

4.2.3.3.2 Procedure

To an aliquot of enzyme preparation (0.3 ml), was added 0.6 ml of 0.1 M sodium acetate buffer (pH 4.8) containing 2.5 mM ρ-Nitrophenyl β-D-galactopyranoside. The mixture was incubated at 37°C. After 20 min, the reaction was stopped by adding 2 ml, of 0.2 M Na_2CO_3. The yellow color developed was read at 400 nm in Beckman DU-6 Spectrophotometer against a blank. The blank was processed just like the other tubes except that the enzyme preparation was added after terminating the reaction with Na_2CO_3. A caliberation curve was made using ρ-Nitrophenol prepared in 0.2 M Na_2CO_3. The enzyme activity is expressed in units where a unit is defined as μmoles of ρ-Nitrophenol liberated at 37°C for 1 h.

4.2.3.4 Assay For α-mannosidase (EC 3.2.1.2.4)

4.2.3.4.1 Reagents

(A) Sodium acetate-acetic acid buffer : 0.2 M

79 ml of 0.2 M sodium acetate trihydrate ($CH_3COONa.3H_2O$) solution (27.22 g/l) was added to 21 ml, of 0.2 M acetic acid buffer (pH 5.2).

(B) ρ-Nitrophenyl β-D-mannopyranoside : 5mM, in sodium acetate buffer (pH-5.2) freshly prepared.

(C) Standard ρ-Nitrophenol : stock solution (250 μm)

(D) Sodium carbonate solution : 0.2 M, Na_2CO_3 in distilled water, freshly prepared.

4.2.3.4.2 Procedure

To 0.3 ml of enzyme extract, 0.6 ml of 0.1 M sodium acetate buffer (pH 5.2) containing 5mM of ρ-Nitrophenyl α-mannopyranoside was added. The enzyme was then allowed to react with the substrate for 20 min at 37°C. The reaction was terminated by the addition of 2 ml Na_2CO_3 (0.2 M). The absorbance was read at 400 nm in a Beckman DU-6 Spectrophotometer against a blank in which the enzyme extract was added to the substrate after terminating the reaction. A standard curve was made using p-Nitrophenol prepared in 0.2 M Na_2CO_3. The activity of the enzyme is expressed in units where one unit represents μmoles of ρ-Nitrophenol liberated at 37°C in 1 h.

4.3 RESULTS

The effect of prolactin on the total activity of β-galactosidase in the three epididymal segments are presented in Fig.9. A dose of 100 μg prolactin produced a significant increase in the β-galactosidase activity in the caput ($p<0.01$), corpus ($p<0.01$) and cauda ($p<0.05$) epididymidis when compared to the control animals given vehicle. The caput responded in the similar fashion to the 150 μg and 200 μg prolactin treatment as well ($p<0.05$). However, these doses had little effect on the corpus

and cauda segments.

The influence of bromocryptine on the epididymal β-galactosidase activity is illustrated in Fig.10. A comparison of the vehicle treated controls with groups given bromocryptine treatments reveal that 0.50 mg treatment has an inhibitory effect in the caput ($p<0.01$), corpus and caudal ($p<0.05$) segments of the epididymis. At the lower doses no significant changes in the activity of this enzyme was evident in any of the epididymal segments.

The action of prolactin on the total activity of α-mannosidase in the epididymis of orchidectomized rats is depicted in Fig.11. Comparison of prolactin treated groups with the vehicle treated controls show that a dose of 100 μg was most effective in stimulating the activity of α-mannosidase in the caput($p<0.01$), corpus and cauda ($p<0.05$) epididymidis. Although the direction of change was same, the positive effect of prolactin was less pronounced at the other doses tried. Regional differences in the degree of response was also seen. For instance, the 150 μg of prolactin treatment significantly increased the enzyme activity in the caput and cauda ($p<0.05$), but not in the corpus.

The impact of bromocryptine on the epididymal α-mannosidase activity is shown in Fig.12. The decrease in the activity of enzyme was statistically significant only in the epididymis of animals that received 0.5 mg of bromocryptine ($p<0.05$). The

Fig. 9. THE EFFECT OF PROLACTIN ON THE TOTAL ACTIVITY OF β-GALACTOSIDASE IN THE EPIDIDYMAL REGIONS OF ORCHIDECTOMISED RATS.

Fifteen days after orchidectomy, animals were injected with ( ) 0 μg; ( ) 50 μg; ( ) 100 μg; ( ) 150 μg and ( ) 200 μg of ovine prolactin/100 g b wt, once daily, subcutaneously, for a period of 7 days. The values represented denote Mean ± SEM of five estimations. Each of the group of animals treated with prolactin is compared with the control group that did not receive hormone treatment ($^{*}p<0.05$, $^{**}p<0.01$ Student's t-test).

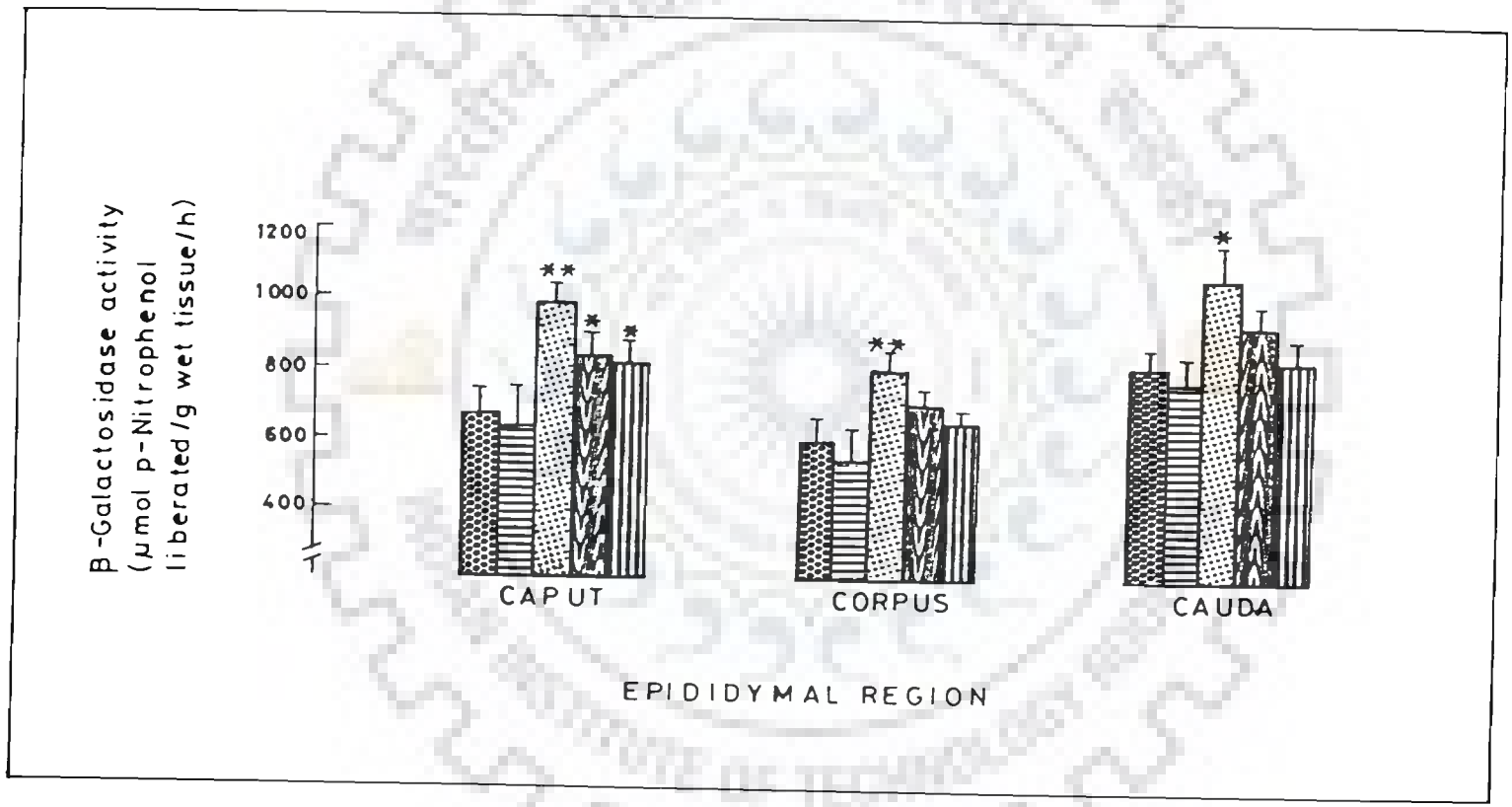

β-Galactosidase activity
(μmol p-Nitrophenol
liberated/g wet tissue/h)
1200
1000
800
600
400
CAPUT
CORPUS
CAUDA
EPIDIDYMAL REGION

Fig. 9.

Fig. 10. THE EFFECT OF BROMOCRYPTINE MESYLATE ON THE TOTAL ACTIVITY OF β-GALACTOSIDASE IN THE EPIDIDYMAL REGIONS OF ORCHIDECTOMISED RATS.

Fifteen days after orchidectomy, animals were injected with (▒▒▒) 0 mg; (▬▬) 0.15 mg; (▒▒▒) 0.30 mg and (▒▒▒) 0.50 mg bromocryptine/100 g b wt, once daily, subcutaneously, for a period of 7 days. The values are represented as Mean ± SEM of 5 estimations. Each group given bromocryptine treatment is compared with control animals that did not receive the drug ($^{*}p<0.05$, $^{**}p<0.01$ Student's t-test).

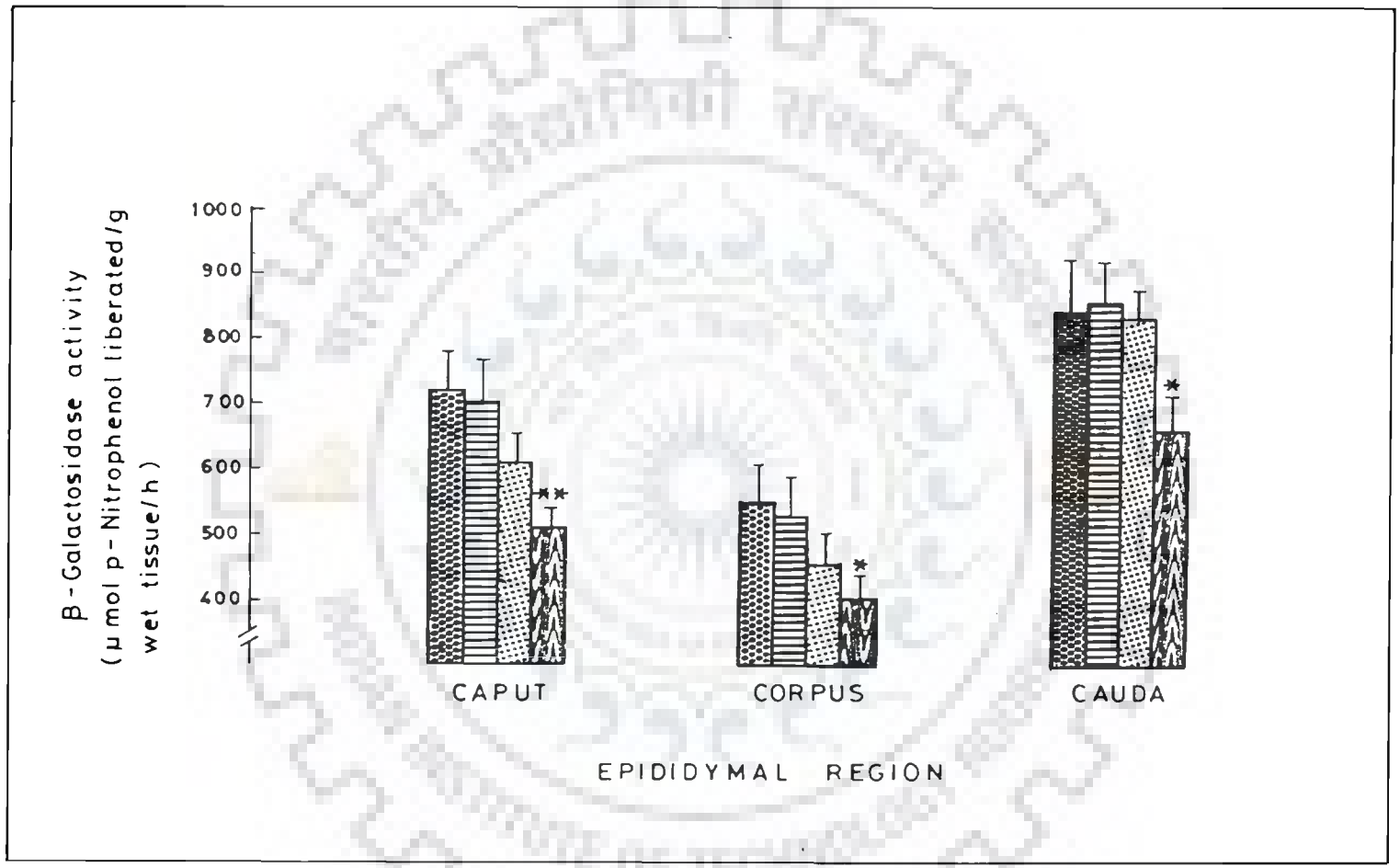

β-Galactosidase activity
(μ mol p-Nitrophenol liberated/g
wet tissue/h)
1000
900
800
700
600
500
400
**
*
*
CAPUT
CORPUS
CAUDA
EPIDIDYMAL REGION

Fig. 10.

Fig. 11. THE EFFECT OF PROLACTIN ON THE TOTAL ACTIVITY OF α-MANNOSIDASE IN THE EPIDIDYMAL REGIONS OF ORCHIDECTOMISED RATS.

Fifteen days after orchidectomy, animals were injected with (▒▒▒) 0 μg; (▬▬) 50 μg; (▒▒▒) 100 μg; (▒▒▒) 150 μg and (▒▒▒) 200 μg of ovine prolactin/100 g b wt, once daily, subcutaneously, for a period of 7 days. The values represented denote Mean ± SEM of five estimations. Each of the group of animals treated with prolactin is compared with the control group that did not recieve hormone treatment ($p<0.05$, * $p<0.01$ ** Student's t-test).

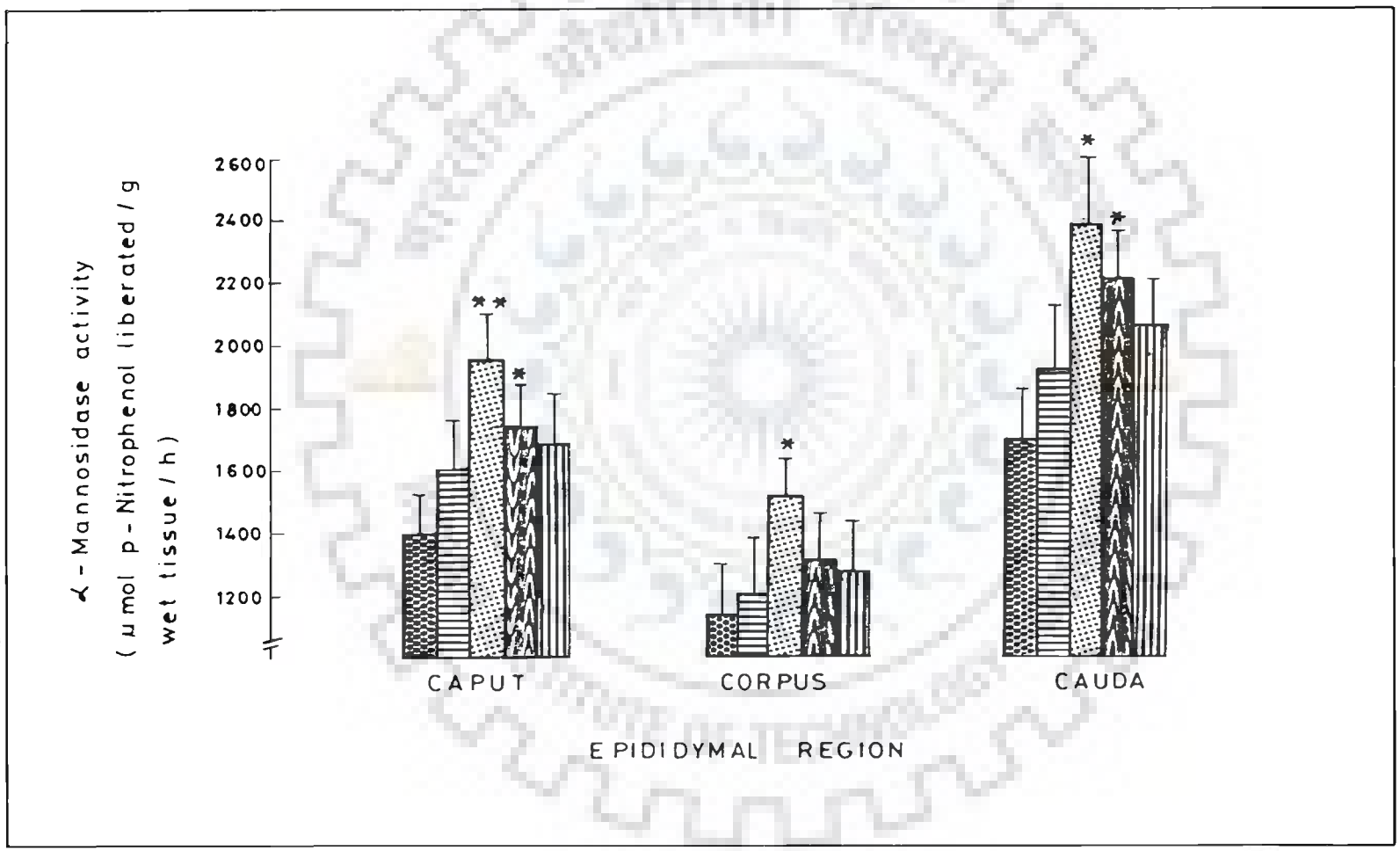

α - Mannosidase activity
(μ mol p - Nitrophenol liberated / g wet tissue / h)
2600
2400
2200
2000
1800
1600
1400
1200
CAPUT
CORPUS
CAUDA
EPIDIDYMAL REGION

Fig. 11.

Fig. 12. THE EFFECT OF BROMOCRYPTINE MESYLATE ON THE TOTAL ACTIVITY OF α-MANNOSIDASE IN THE EPIDIDYMAL REGIONS OF ORCHIDECTOMISED RATS.

Fifteen days after orchidectomy, animals were injected with (▩) 0 mg; (▬) 0.15 mg; (▩) 0.30 mg and (▩) 0.50 mg bromocryptine/100 g b wt, once daily, subcutaneously, for a period of 7 days. The values are represented as Mean ± SEM of 5 estimations. Each group given bromocryptine treatment is compared with control animals that did not receive the drug ($^{*}p<0.05$, $^{**}p<0.01$ Student's t-test).

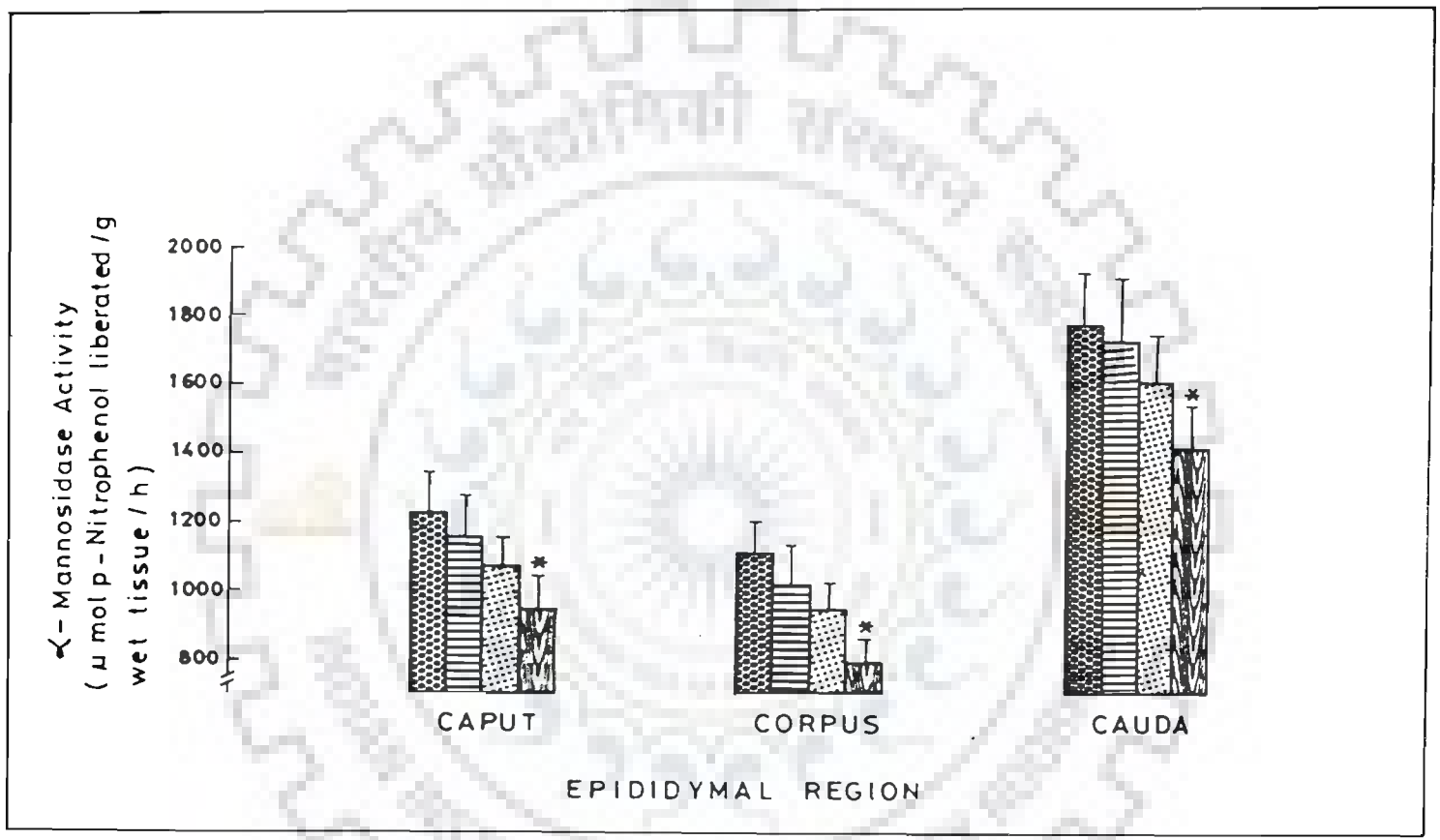

α- Mannosidase Activity
(μ mol p-Nitrophenol liberated/g
wet tissue / h)
2000
1800
1600
1400
1200
1000
800
*
*
*
CAPUT
CORPUS
CAUDA
EPIDIDYMAL REGION

Fig. 12.

lower doses were relatively less effective in reducing α-mannosidase activity.

4.4 DISCUSSION

The glycosidases detected in the epididymis may not only be synthesized in the organ itself but could also be produced in the testis and transported into the epididymis. The latter possibility seems less likely in the present circumstances since the experiments were conducted on orchidectomised animals. Therefore, it could be said with a reasonable amount of certainity that the increase in glycosidase activity observed is the result of prolactin action at the level of the epididymis.

Receptors for prolactin have been detected in the prostate, seminal vesicle and epididymis of laboratory animals (Aragona and Friesen, 1975; Barkey *et al.*, 1977; Orgebin-Crist and Djiane, 1979). Further, studies conducted at different laboratories have shown that prolactin could have a direct action on the sex accessories (Bartke and Lloyd, 1970). In a bid to clarify the condition under which prolactin exerts its action on the sex accessory gland, Negro-Vilar *et al.*(1977) have performed detailed investigations. By a series of pituitary transplantation studies in intact, orchidectomised, adrenalectomized-orchidectomised or hypophysectomized rats, they have shown convincingly the effect of prolactin on the male sex accessory organ in the absence of testicular and adrenal steroids. Thus, the direct action of prolactin in the epididymis may be one among several reasons for

the stimulatory action of prolactin on the activity of epididymal β-galactosidase and α-mannosidase of orchidectomised rats as observed in the present study.

The variation in the threshold for the hormone along the epididymal duct may account for the slight difference in the quantum of increase in enzyme activity observed between the epididymal segments. Nevertheless, the direction of change was the same in all segments and therefore evidence of the treatment effect. The involvement of prolactin is further confirmed by the inhibitory action of bromocryptine on these epididymal glycosidases. This ergot alkaloid is known to bring about its action by reducing the circulating levels of endogenous prolactin (Harper *et al.*, 1976; Kinch, 1980; Weinstein *et al.*, 1981). The results of the present study reveal that a substantial reduction in the glycosidase activity occurs only in animals given 0.50 mg of bromocryptine treatment. Lower doses of this drug were unable to affect the activity of the epididymal enzymes studied. It appears that 0.50 mg is the minimal dose required to reduce circulating prolactin to an extent that can affect epididymal function.

In the previous chapter, prolactin was shown to influence the epididymal sialic acid in a dose dependent manner. However, in the present study, the epididymal response is not directly proportional to the dose of oPRL administered. Thus, it becomes clear that different parameters within the same tissue do not respond alike to prolactin treatment. Obviously, the mechanism

by which prolactin mediates intracellular events to bring about a response in these two cases are not the same.

Earlier investigators have shown convincingly that epididymal glycosidases are androgen dependent (Conchie and Mann, 1957; Conchie and Findlay, 1959; Mann, 1964; Mann *et al.*, 1971; Sinowatz *et al.*, 1975). In the present study, it has come to light that prolactin treatment in orchidectomised rats has increased the epididymal glycosidase activity, but not exactly in a dose related fashion. The presence of binding sites for prolactin in the epididymis and the ability of this organ to synthesize and metabolize androgens (Frankel and Eik-Nes, 1970; Hamilton and Fawcett, 1970; Hamilton, 1971; Hammerstedt and Amann, 1976) gives room for suspicion that the epididymal glycosidase activity may be controlled by both these hormones. Studies on steroidogenic tissues have revealed that prolactin could influence the activity of several enzymes involved in the synthesis and metabolism of androgen (Munabi *et al.*, 1985). Thus, prolactin could indirectly exercise its effect on the epididymal glycosidases by altering the type and potency of the androgen in the tissue.

In addition, it must be emphasised that prolactin is a protein hormone while androgens are steroids. There are basic differences in the mechanism of action of these two categories of hormones since the receptors for steroids are located in the cytoplasm while those for protein hormone reside on the cell surface membranes. It is thus tempting to speculate that

glycosidases measured may represent the net result of an interaction between these two mechanisms at each dose of prolactin treatment.

4.5 CONCLUDING REMARKS

1. Prolactin has a stimulatory effect on the activity of both α-mannosidase and β-galactosidase in the epididymis of orchidectomised rats.

2. Although this effect is not directly proportional to the dose of prolactin administered, the direction of change is the same in all segments and therefore, evidence of a treatment effect.

3. It appears from the quantum of increase in activity that there is a difference in the response of the epididymis to prolactin treatment along the duct.

4. The inhibitory action of bromocryptine on these epididymal glycosidases indicate that prolactin could be at least one part of the control mechanism that regulates enzyme activity.

5. The stimulation of glycosidase activity in a manner not directly related to the dose of injected prolactin suggests that hormones other than prolactin may also be involved.

6. This view is also supported by the known action of prolactin on enzymes that control androgen synthesis and metabolism in steroidogenic tissues. Perhaps protein (prolactin) and steroid (androgens)hormones control the activity of glycosidases by

different mechanisms and the activity measured represents the net result of an interplay between these two mechanisms at each dose of treatment.

CHAPTER 3

THE EFFECT OF PROLACTIN ON SERUM PROLACTIN BIOACTIVITY

5.1 INTRODUCTION

Investigations on the role of prolactin in males have received priority in recent years, for a variety of reasons (Thorner and Besser, 1977; Doherty *et al.*, 1980; Flückiger *et al.*, 1982; Cohen *et al.*, 1988; Gonzales *et al.*, 1989). The demonstration for binding sites of prolactin in the epididymis of several laboratory animals (Aragona and Friesen, 1975; Barkey *et al.*, 1977a,b; Orgebin-Crist and Djiane, 1979) has added interest to the subject. Before proceeding with studies that could help determine the impact of prolactin on the epididymis it is necessary to choose an appropriate model system. Injections of prolactin from an exogenous source have been used in the past to study the effects of prolactin excess on male reproductive tissues (Ueda *et al.*, 1987). Therefore, a similar approach was used in the present study.

Since the epididymis is influenced by several factors originating in the testis, injection of prolactin to intact animals was not found to be suitable. Besides, if prolactin had its action on the testis, then the changes in the testis would be reflected in the epididymis. This would make the identification of the precise action of prolactin on the epididymis difficult. It was therefore, thought that studies involving administration of prolactin to either duct ligated animals or orchidectomised animals could solve the problem. It was found with reference to epididymal sialic acid that the effects of prolactin are clearly discernable in orchidectomised animals rather than duct ligated

ones. It is suspected that since the epididymis is predominantly an androgen dependent tissue, the effect of prolactin is perhaps masked by the much greater effect of androgens in duct ligated animals. Therefore, orchidectomised animals were used as a model to judge the action of prolactin on the epididymis.

Earlier investigations have shown that prolactin from an exogenous source when injected in intact rats has a short half life (Grosvenor, 1967; Chi and Shin, 1978; Diamond *et al.*, 1980). However, the time course of changes in serum prolactin after injecting exogenous prolactin in orchidectomised animals has not been previously examined. Since ovine prolactin was injected in rats, in this chapter, the total serum bioactivity observed at specific time intervals over a 24h period is presented.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

All reagents used were of analytical grade; Ovine prolactin (oPRL18 NIADDK-NIH; Biopotency 30 I.U./mg) was a generous gift from the National Pituitary Agency, NIADDK NIH, Bethesda, Maryland, USA.

5.2.2 Animals

Adult male albino rats of *Wistar* strain (150-200g) were used for the present studies. They were fed a pelleted diet (Lipton India Ltd.) and water was provided *ad libitum*. They were caged in a well ventilated animal house with 12h L : 12h D schedule at a temperature of 28±2°C.

5.2.3 Experimental Design

Orchidectomised animals have been used in the present studies. Fifteen days after orchidectomy, the animals were divided into five groups each consisting of 10 animals. The first group received vehicle and was used as control. The remaining four groups of animals received 50, 100, 150 and 200 μg oPRL/100 g body weight, subcutaneously, once daily, for a period of 7 days. Animals belonging to each group were killed by decapitation at 15 min, 30 min, 2h, 6h or 24h after the last of 7 injections. The blood was collected in a polycarbonate centrifuge tube in an ice bath, immediately clotted in a refrigerator and then centrifuged in a refrigerated centrifuge (20,000 xg for 20 min). The prolactin bioactivity in the serum samples were determined in a bioassay.

5.2.4 Estimation Of Prolactin Bioactivity

The "local micro method", using pigeon hemi-crop sac was followed (Grosvenor and Turner, 1958). Adult common male pigeons weighing 250-300g were obtained from local sources. They were acclimatized to laboratory conditions in an artificially illuminated room (12h L : 12h D schedule). They were provided with a balanced bird feed and water *ad libitum*.

5.2.4.1 Procedure

Two samples to be tested were injected intradermally in 0.1 ml volumes once daily for 4 days over the crop sac at symmetrically opposite sides. The geometrical centre of the crop sac on each

side was permanently marked with a non-toxic dye to ensure the injection of the sample in the same area each day. Twenty four hours after the last injection, each pigeon was killed and the entire crop sac removed. The left and right crop sac were excised and the responding area identified. The caliberation of the assay was done using oPRL (Biopotency 30 I.U./mg, NIH preparation).

Prolactin levels were determined by monitoring the response of the crop sac in terms of relative responding mucosal weight gravimetrically (Nicoll, 1967) and also as a measure of casein/ protein ratio in the responding mucosa biochemically (Turkington *et al.*, 1965).

5.2.4.2 Gravimetric Analysis

The entire hemicrop sac of the pigeon given a particular dose of oPRL was at first excised and weighed. Each hemicrop was stretched slightly, the mucosal layer of the stimulated areas scraped out and weighed. The relative weight of responding area expressed as percentage was plotted against the dose of prolactin injected (Fig.13.)

5.2.4.3 Biochemical Analysis

The responding mucosa from the hemicrop sac was homogenized in alkaline water of pH 7.0-7.5. The homogenate was centrifuged at 2000 xg. The pellet was discarded and the supernatant saved. An aliquot was used for determination of protein (Lowry *et al.*,

1951) and the remaining supernatant was subjected to a drop-wise addition of 20% glacial acetic acid till a thick curd white precipitate resulted. The precipitate was then pelleted, washed with distilled water and resuspended in 0.1N NaOH by boiling in a water bath for 15 min. Protein was estimated in this suspension also. Casein which precipitates in acidic media is a major protein in the cells of responding mucosa which responds to prolactin. Thus, the ratio of casein/total protein was plotted against known concentrations of oPRL injected (Fig.14.). The serum prolactin bioactivity of unknown samples were read from this plot.

5.2.4.4 Determination Of Total Protein

Total protein was estimated by the method of Lowry *et al.*(1951) using Folin Phenol reagent after the alkaline copper treatment. The chemistry of estimation has two distinct steps which lead to the final color with protein : (a) reaction with copper in alkali and (b) reduction of the phosphomolybdic phosphotungstic reagent by the copper treated protein.

5.2.4.4.1 Reagents

Protein standard: 1 mg of bovine serum albumin was dissolved in 1 ml of distilled water and diluted to 5 ml.

Reagent A : 2% Na_2CO_3 in 0.1 N NaOH

Reagent B : 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartrate

Fig. 13. CALIBERATION CURVE OF PROLACTIN BIOACTIVITY OBTAINED FROM THE GRAVIMETRIC RESPONSE OF THE PIGEON CROP SAC TO OVINE PROLACTIN.

(Biopotency of 1 mg oPRL = 30 I.U.). Each value represents Mean ± SEM of 7 determinations. Index of precision (λ) = 0.2269.

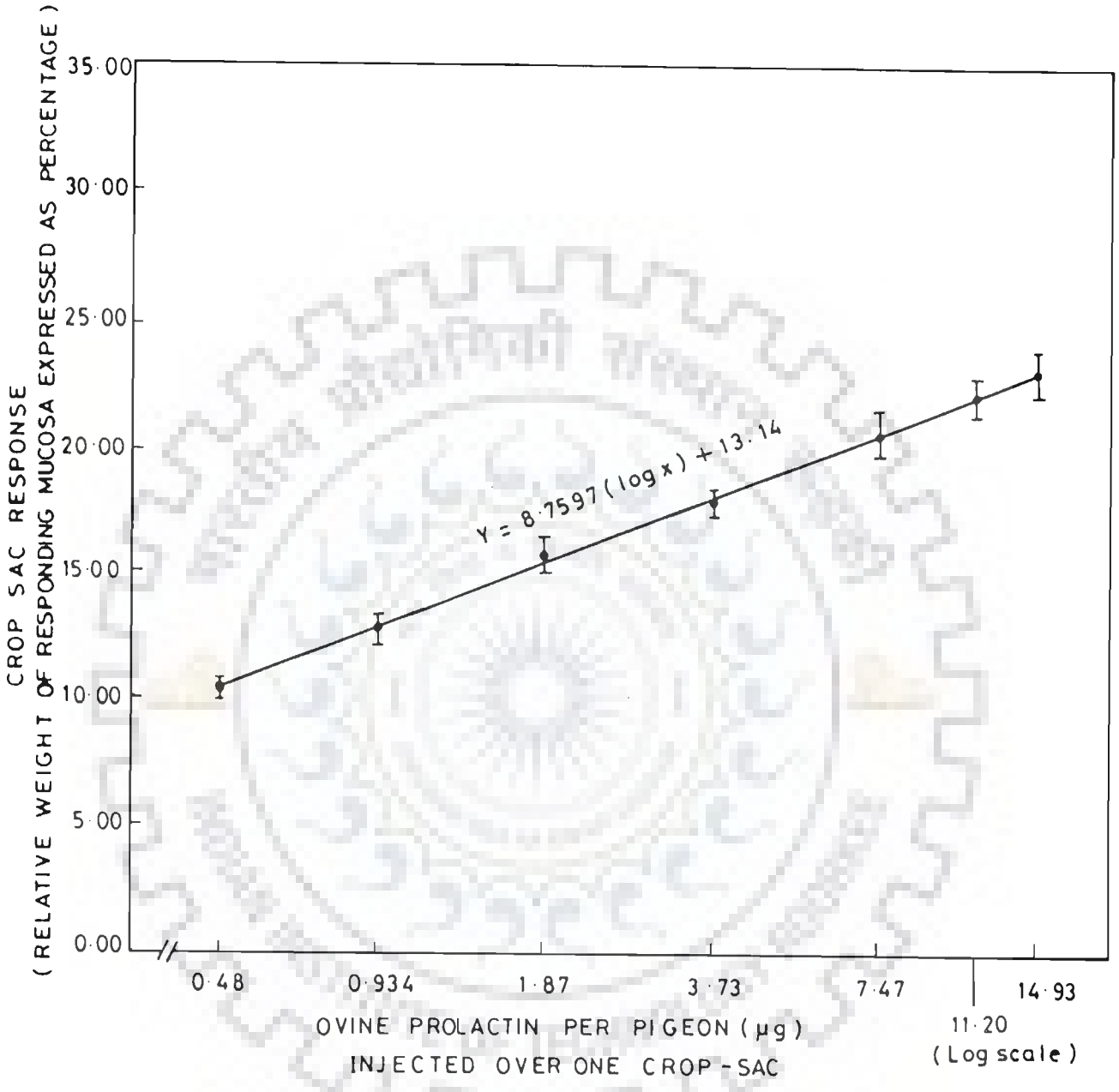

CROP SAC RESPONSE
(RELATIVE WEIGHT OF RESPONDING MUCOSA EXPRESSED AS PERCENTAGE)
35·00
30·00
25·00
20·00
15·00
10·00
5·00
0·00
Y = 8·7597 (log x) + 13·14
0·48
0·934
1·87
3·73
7·47
11·20
14·93
OVINE PROLACTIN PER PIGEON (µg)
INJECTED OVER ONE CROP-SAC
(Log scale)

Fig. 13.

Fig. 14. CALIBERATION CURVE OF PROLACTIN BIOACTIVITY OBTAINED FROM THE BIOCHEMICAL RESPONSE OF THE PIGEON CROP SAC TO OVINE PROLACTIN.

(Biopotency of 1 mg oPRL = 30 I.U.). Each value represents Mean ± SEM of 7 determinations. Index of precision (λ) = 0.1394.

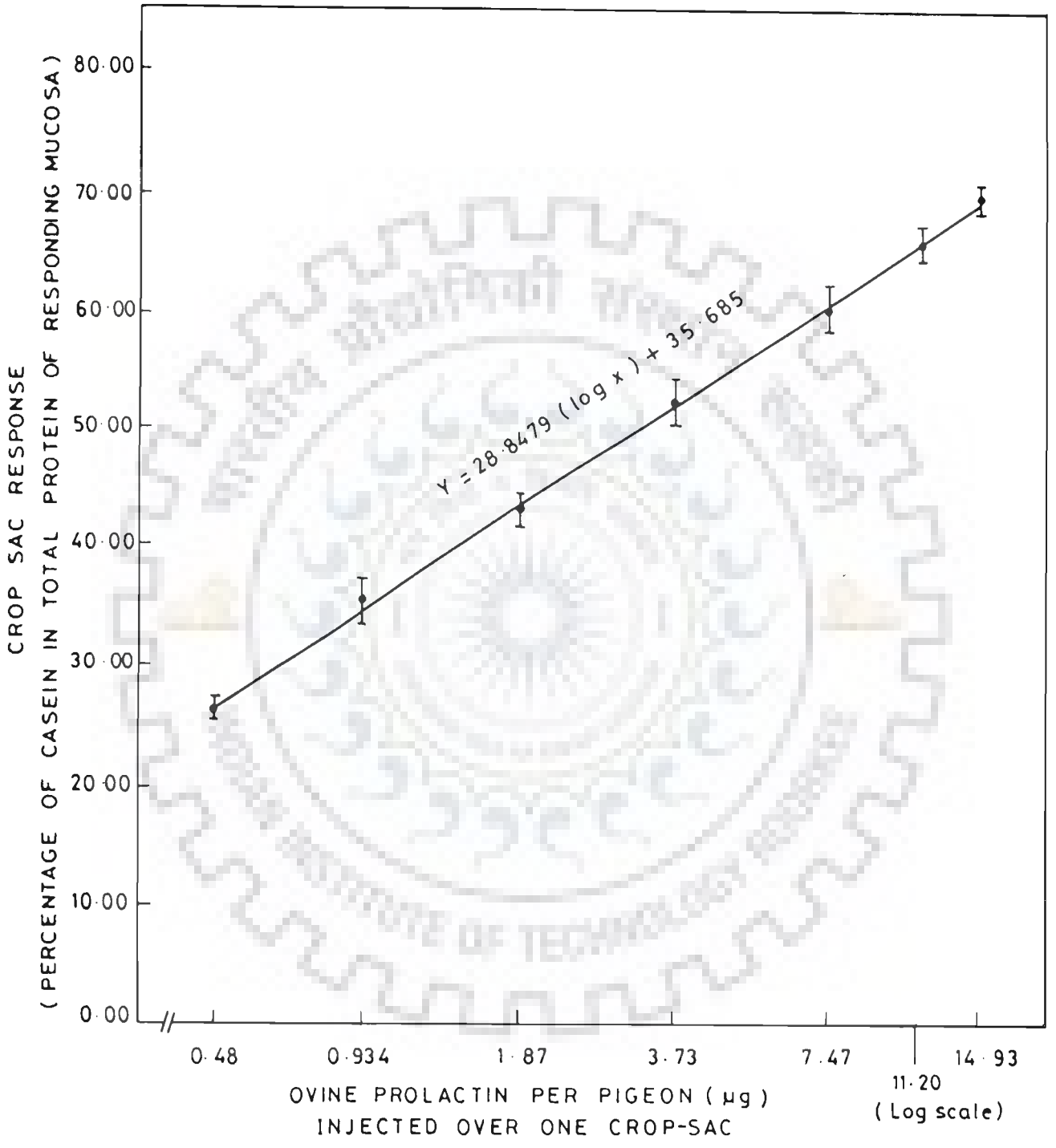

CROP SAC RESPONSE
(PERCENTAGE OF CASEIN IN TOTAL PROTEIN OF RESPONDING MUCOSA)
80·00
70·00
60·00
50·00
40·00
30·00
20·00
10·00
0·00
Y = 28·8479 (log x) + 35·685
0·48
0·934
1·87
3·73
7·47
11·20
14·93
OVINE PROLACTIN PER PIGEON (µg)
INJECTED OVER ONE CROP-SAC
(Log scale)

Fig. 14.

Reagent C : Alkaline copper sulphate solution 50 ml(A)+ 1 ml(B)

Reagent E : Dilute Folin Ciocalteu reagent made to 1 N

5.2.4.4.2 Procedure

A suitable aliquot of sample was taken and the volume made upto 1 ml with 0.1 N NaOH. To this, 1 ml of reagent C was added and mixed. It was allowed to stand for 10 min at room temperature. 0.1 ml of reagent E was added to the tube with immediate mixing. After 30 min at room temperature, the intensity of the color developed was spectrophotometrically read at 690nm on Beckman DU-6 Spectrophotometer.

5.2.5 Validity Of The Bioassay

The caliberation curve, regression formula and index of precision of two types of responses are presented in figures 1 and 2.

A precision profile or response error relationship was made similar to the one suggested by Ekins (1978). Precision was estimated at various points on the standard curve by examining 7 replicates at each point. The precision of measurement (ΔH) of a hormone concentration H may be defined as $\frac{R_H}{(dR/dH)H}$. The lower limit of detection, or sensitivity, of an assay is defined by the precision of measurement of zero hormone (ΔH_o) *i.e.* "sensitivity" constitutes merely a limiting case of precision. ΔH is dependent both on the error incurred in the measurement of the response metameter (ΔR_H), and on the slope of the response curve (dR

/dH)H at the corresponding point. The parameter (R_H /slope) corresponds closely in concept and form, to the "index of precision" (λ) originally proposed by Gaddum (1933) and employed as a measure of performance of this bioassay. However, Gaddum's formulation, relies on assumptions both on constancy of error in the response metameter (homoscedasticity i.e. non-uniformity of error in the response metameter) and on linearity of the response curve (plotted in terms of the logarithm of the dose). Thus λ, which is a measure of the relative error in the measurment of the dose and conventionally regarded as constant for all values of H and therefore as representative of precision of the assay as a whole, was calculated.

Such a test permits the selection of a range critical for the assay. From this, 0.48 µg and 14.93 µg oPRL/bird were taken as the lower and upper limits of sensitivity for the assay respectively, although the range of detection was wider.

In males, the titres of prolactin are lower than in females. Moreover, reduction in serum testosterone is reported to be associated with low circulating levels of prolactin as well (Grosser and Robaire, 1987). In order to "target" the assay in a way that range of samples fall within the limits of sensitivity, 0.3 ml of standard oPRL containing 1 µg was added to 0.7 ml of every test sample (Biopotency of 1 mg standard oPRL =30 I.U.) before the assay was performed. This was later taken into account while computing the results.

It is relevant to mention that 10 rats were used for each group,

two for each time point. The serum from each of these two animals was injected in 2 pigeons. Thus, each data point has four replicates. The same crop sac was used for gravimetric and biochemical assessment of response and the patterns obtained were similar. Statistical evaluation of the data was done by employing Student's t-test.

5.3 RESULTS

The serum level of prolactin bioactivity as ascertained by the pigeon crop sac bioassay using gravimetric and biochemical indices are presented in Fig. 15. and Fig. 16. It is apparent from the data that the prolactin profiles obtained at various time points by both indices are similar. When compared to orchidectomised controls given vehicle, treatment of orchidectomised animals with 50, 100 and 150 µg oPRL did not significantly alter the prolactin bioactivity at 15 and 30 min. However, a higher dose of 200 µg oPRL produced an increase in prolactin bioactivity at 15 min which was lowered at 30 min. At 2h, all groups of animals that received the hormone exogenously showed a significant elevation in their serum prolactin bioactivity when compared to the controls at this time point. However, the rise did not commensurate with the dose of oPRL administered. This spurt in prolactin bioactivity was short lasting with the values returning to control levels at 6h in the 50, 150 and 200 µg oPRL treated groups. The same was accomplished at the 24h interval in the group given 100 µg oPRL. Thus, variations in the time taken to bring about homeostasis in

Fig. 15. EFFECT OF oPRL ADMINISTRATION ON SERUM PROLACTIN BIOACTIVITY IN ORCHIDECTOMISED RATS AS DETERMINED BY GRAVIMETRIC RESPONSE OF PIGEON CROP SAC MUCOSA.

Adult rats (15 days post-orchidectomised were given the following treatment, once daily, s.c., for a period of 7 days : (▭) vehicle; (▭) 50 µg oPRL/100 g b wt; (▭) 100 µg oPRL/100 g b wt; (▭) 150 µg oPRL/100 g b wt and (▭) 200 µg oPRL/100 g b wt. The serum prolactin bioactivity was determined at specific time intervals after the last injection. Each value represents Mean + SEM of 4 determinations. Comparisons have been made with orchidectomised animals given vehicle at the respective time point. The level of significance indicated are $^{*}p<0.05$, $^{**}p<0.01$ (Student's t-test).

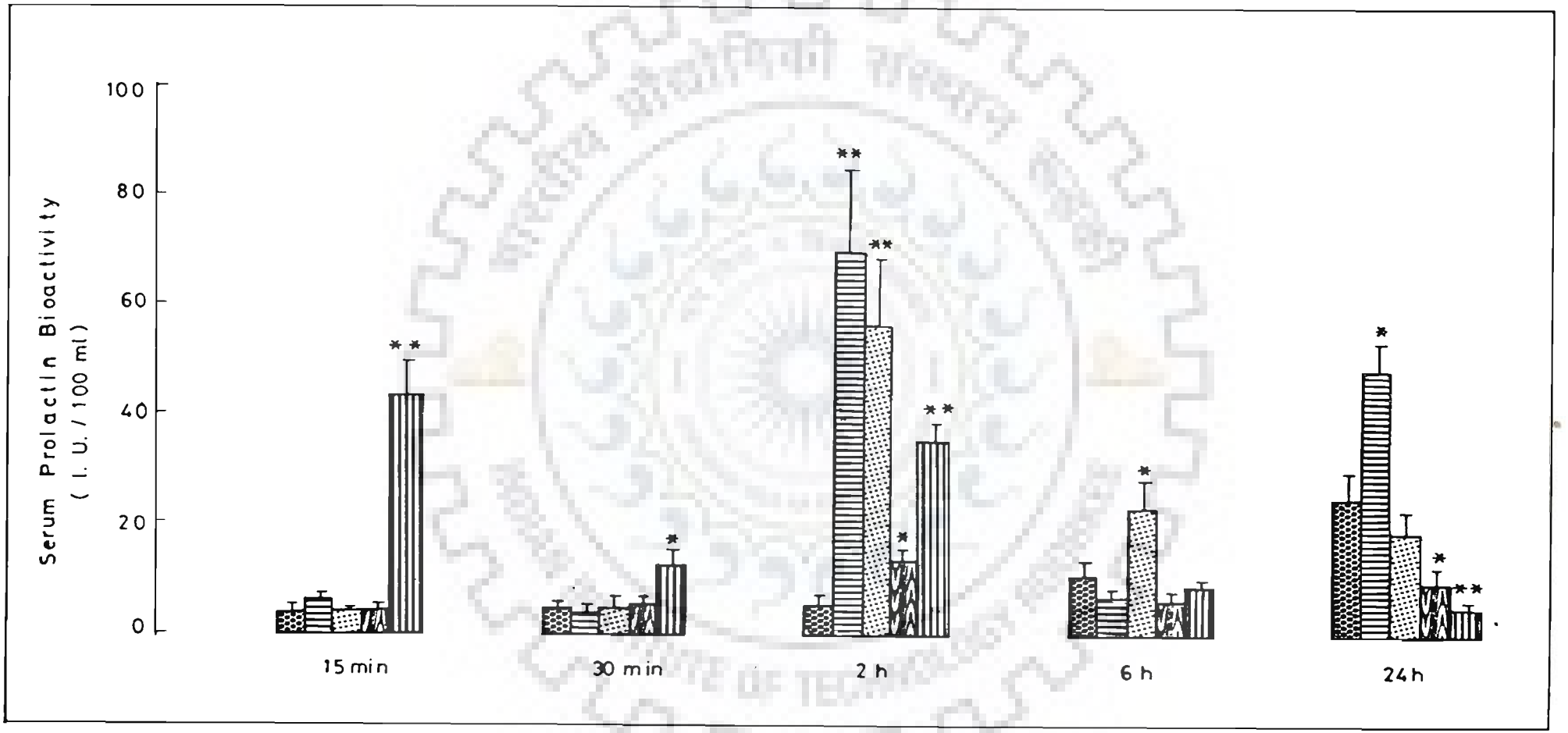

Serum Prolactin Bioactivity
(I. U. / 100 ml)
100
80
60
40
20
0
15 min
30 min
2 h
6 h
24 h

Fig. 15.

Fig. 16. EFFECT OF oPRL ADMINISTRATION ON SERUM PROLACTIN BIOACTIVITY IN ORCHIDECTOMISED RATS AS DETERMINED BY BIOCHEMICAL RESPONSE OF PIGEON CROP SAC MUCOSA.

Adult rats (15 days post-orchidectomised) were given the following treatment, once daily, s.c., for a period of 7 days : ( ) vehicle; ( ) 50 µg oPRL/100 g b wt; ( ) 100 µg oPRL/100 g b wt; ( ) 150 µg oPRL/100 g b wt and ( ) and 200 µg oPRL/100 g b wt. The serum prolactin bioactivity was determined at specific time intervals after the last injection. Each value represents Mean ± SEM of 4 determinations. Comparisons have been made with orchidectomised animals given vehicle at the respective time point. The level of significance indicated are $^{*}p<0.05$, $^{**}p<0.01$ (Student's t-test).

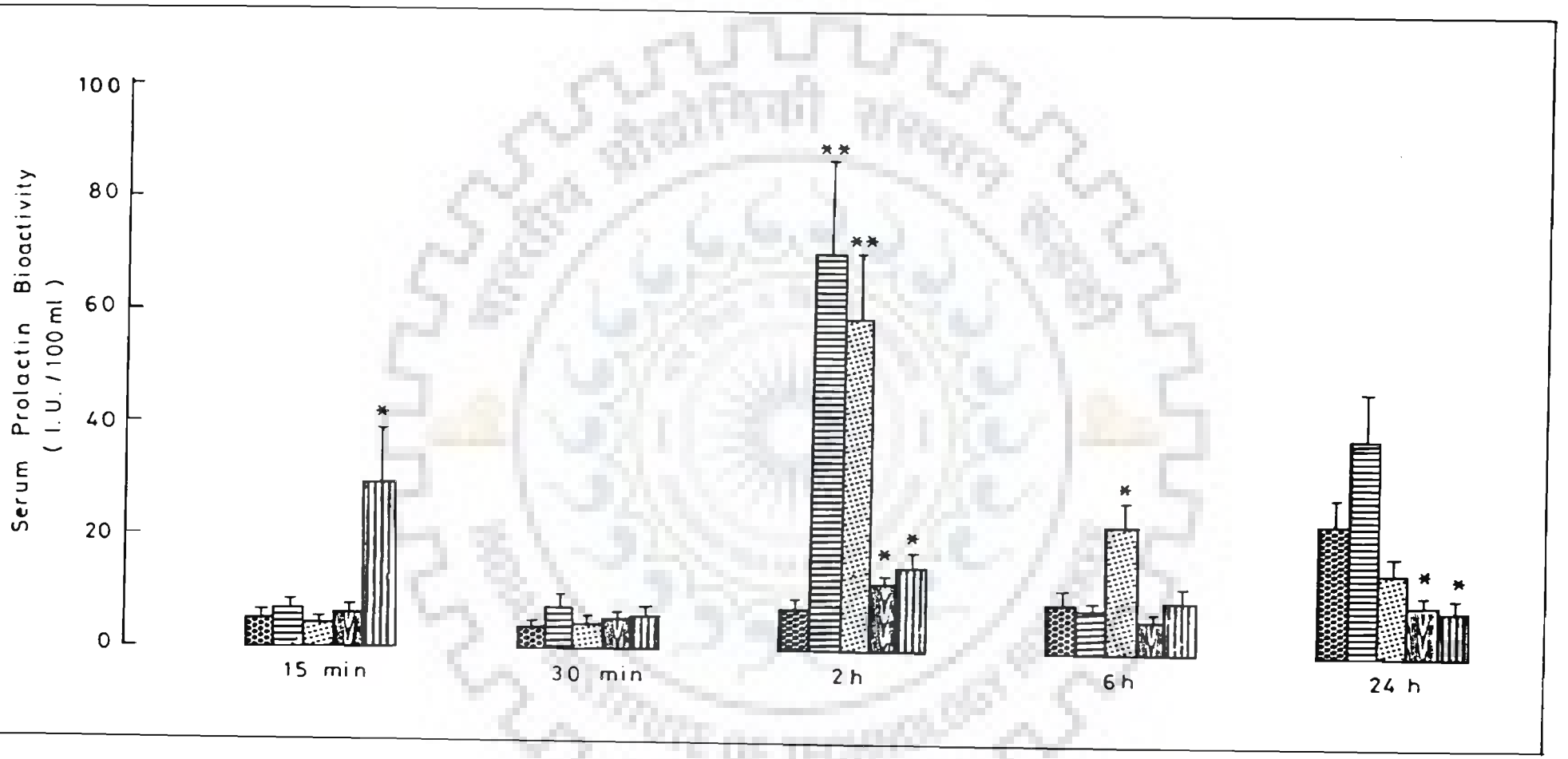

Serum Prolactin Bioactivity
(I.U. / 100 ml)
100
80
60
40
20
0
15 min
30 min
2 h
6 h
24 h

Fig. 16.

circulating levels of prolactin are seen.

5.4 DISCUSSION

In intact male rats, serum prolactin levels have been reported to show a rhythmic pattern with levels reaching a maximum in the late afternoon (Clark and Baker, 1964; Koch *et al.*, 1971). The present experiments were performed on orchidectomised rats and no such pattern was observed. Thus, orchidectomy seems to have disturbed this circadian periodicity. These results however, are comparable to those of Hart (1973), who reported a more or less steady basal level of prolactin with a slow rise and fall over a 24h period in castrated male goats. Studies on male rats have revealed that serum prolactin levels drop starting on day 1 post-castration (Goldman *et al.*, 1971). Orchidectomy was found to reduce not only mean values but also pulse amplitude, and pulse frequency of prolactin (Grosser and Robaire, 1987). Thus, the serum prolactin pattern observed for orchidectomised animals in our studies are in good agreement with those reported by other investigators.

The experiments show that treatment of orchidectomised animals with 50, 100 and 150 µg oPRL does not significantly alter the serum prolactin bioactivity at 15 and 30 min when compared to orchidectomised control given vehicle. In view of the very short half life for oPRL in rats (Grosvenor, 1967; Diamond *et al.*, 1980), it is possible that the exogenous administration of the hormone brings about only a transient rise in prolactin which

is quickly removed from circulation at these doses. This process perhaps takes a longer time when higher doses of oPRL are employed, a situation that is seen in the 200 μg oPRL treated group. At this stage, it is interesting to recall the work of Demarest *et al.*(1986), who have reported that such a transient rise in heterologous prolactin suppresses the release of endogenous prolactin rather rapidly. Therefore, it is suggested that the short half life of oPRL in rats together with a rapid suppression of endogenous prolactin, may account for the serum prolactin profiles observed at 15 and 30 min.

The data also shows a peak in serum prolactin bioactivity at 2h irrespective of the dose of exogenous oPRL administered, which is not present in the orchidectomised controls given vehicle. It is interesting that this serum bioactivity does not commensurate with the dose of oPRL injected. It is suspected that the observed surge perhaps represents a rebound of endogenous prolactin after a period of suppression of the rat pituitary by the exogenously administered oPRL. From the data presented in Fig. 15. and 16, it could be argued that the peak in prolactin bioactivity at 2h was inversely related (rather than unrelated) to the dose of injected prolactin. Although speculative, the following explanation could be offered. With the higher doses injected, the suppression of exogenous prolactin was more profound and prolonged and therefore the rebound occurred either sooner or later than in rats given the lower doses. It appears that the rats injected with the lower doses were sampled closer

to the peak of the postulated rebound of endogenous prolactin. Further, if a rough estimate of the dose of injected oPRL in terms of international units is made (given that the biopotency of 1 mg oPRL = 30 I.U. and assuming that the average weight of the animals used was 200 g, the half life of oPRL in rats was 6-12 min), it can be seen that the values obtained at 2h should be to a large extent derived endogenously. Thus, there is every reason to believe that this rebound is largely due to native prolactin. However, further studies would be required employing radioimmunological assays to discern whether the pattern of bioactivity represents one or the other or both species of the hormone.

Finally, before concluding the following could be highlighted. It is well established that the rat pituitary releases prolactin into circulation in a circadian pattern (Tindall, 1974). The data seem to point that orchidectomy disturbs this pattern. However, injection of prolactin from an exogenous source establishes a cyclic periodicity in the serum prolactin profile. This cyclicity as judged from the time points examined, may be on account of a rebound at 2h and subsequent effort by the animals to bring about homeostasis in the circulating level of the hormone.

It would not be wise to elaborate further on the results obtained at 24h because between the 6h and 24h time points, the animals have been subjected twice to changes in photoperiodicity, once from light to dark and then from dark to light. This is of vital

importance while discussing results since pineal principles are believed to be involved in prolactin synthesis and release (Donofrio and Reiter, 1972). Further support to this view comes from the work of Relkin (1972) and Relkin *et al.* (1972), who have shown that rats kept in constant darkness have lower pituitary prolactin contents and higher circulating prolactin levels than rats kept under a light dark regimen or in constant light and this effect does not occur after pinealectomy. Thus, The situation is complex and not easy to comprehend.

5.5 CONCLUDING REMARKS

1. In orchidectomised controls, the circadian level of serum prolactin bioactivity is more or less constant.

2. At low doses the injected prolactin is quickly removed from circulation, while this process takes more time at higher doses.

3. A surge in prolactin bioactivity is seen in orchidectomised animals around 2h after treatment with exogenous prolactin.

4. This surge appears to be a "rebound" phenomenon following a period of suppression on the pituitary.

5. Photoperiodicity influence serum prolactin bioactivity.

CHAPTER 4

THE EFFECT OF PROLACTIN ON THE STRUCTURE OF EPIDIDYMIS

6.1 INTRODUCTION

In the preceeding chapters an attempt was made to see if prolactin has an influence on epididymal function by monitoring certain biochemical parameters like sialic acid concentration and activity of some glycosidases. Based on these studies and other reports which demonstrated the presence of receptors for prolactin in the epididymis (Aragona and Friesen, 1975; Orgebin-Crist and Djiane, 1979), it was concluded that prolactin could have a direct effect on the epididymis.

It is generally believed that functional changes in organs are often reflected in their structure, more so at the cellular level. The epididymal epithelium profoundly influences sperm maturation by carefully controlling the composition of the fluid in which the sperm are bathed while they are within the duct. This is made possible by the synthetic, secretory and absorptive nature of the epididymis. Details of such a process can be easily visualized in studies concerning epididymal structure. Studies of this kind are therefore important and hence initiated to see if prolactin has a role to play in the epididymis.

Earlier reports have highlighted structural changes in the epididymal epithelium in response to orchidectomy and androgen replacement both at the light and electron microscopic level (Orgebin-Crist and Davies, 1974; Moore and Bedford, 1979 a,b; Goyal, 1983). Studies on the effect of antiandrogen on the epididymis are also available (Wolf, 1986). These and other studies have clearly established that the epididymis is dependent

on androgens for the maintenance of its structure (Fawcett and Hoffer, 1979; Tokano *et al.*, 1981; Goyal, 1983; Nicander *et al.*, 1983). The duct ligation studies have emphasized that the epididymal structure is dependent on several factors originating in the testis (Abe *et al.*, 1984). Fewer investigators have been concerned about the impact of other hormones on the epididymal structure (Pellegrini and Ricciardi, 1983). This research has been confined only to the light microscopic level. Ultrastructural work on the epididymis pertaining to the action of hormones other than androgens are grossly lacking and remains a neglected area. Although the biochemical investigations have revealed that prolactin influences several epididymal parameters, no attempt has so far been made to see what happens in the epididymis at both the light and electron microscopic level and hence this study.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals

All chemicals used were of analytical grade. Embedding kit containing Araldite CY 212, Dodecenyl succinic anhydride (DDSA) HY964, Tri dimethylamino methyl phenol (DMP 30; DY064) was purchased from Fluka Chemical Co. UK.

6.2.1 Animals

Male albino rats of *Wistar* Strain (200-250 g body weight) were used in the present investigation. They were purchased from commercial sources and acclimatized to laboratory conditions for

one week before the start of the experiment. The animals were fed a standard pelleted diet and provided water *ad libitum*. Through out the period of experimentation the animals were subjected to a 12h L: 12h D artificial lighting schedule and maintained in a well ventilated animal house at a temperature of 28 ± 2°C.

6.2.3 Experimental Design

Thirty animals were orchidectomised using the procedure described earlier. Fifteen days later, the orchidectomised rats were divided at random into six groups, each consisting of five animals. One group of animals received vehicle and was used as control. The next four groups were given ovine prolactin (Biopotency, 1mg = 30 I.U.) at doses of 50, 100, 150 and 200 µg / 100 g body weight. The last group was injected with 0.50 mg bromocryptine/100g body weight. For comparison purposes five rats were sham-operated and 15 days later given vehicle treatment. All injections at the specified doses were administered subcutaneously, once daily, for a period of 7 days. The volume of injection was kept to the minimum and care was taken to ensure that a particular animal received the injection at the same time each day. On previous occasions, several doses of bromocryptine were tried and 0.50 mg was found to have maximum effect on the epididymis (**see sections 3.3 and 4.3**) and hence, choosen for this study. The preparation of the ovine prolactin and bromocryptine for injection was the same as mentioned under **sections 3.2.3.2 and 3.2.3.3** . Twenty four hours after the last of 7 injections,

the animals were killed by cervical dislocation.

6.2.4 Fixation of tissue

6.2.4.1 Principle

Fixation is the first step in the preparation of biological specimens for light / electron microscopy. It is a process by which the cell is put to death almost instantaneously using certain chemical compounds (fixatives) which prevent autolysis and preserve various chemical constituents of the cell. This enables the preservation of various organelles of the cell. The main aims of fixation are three : firstly, the preservation of the structure of cells and tissues with minimum or no alteration from the living state. Secondly, protection of cells against alterations during embedding and sectioning and lastly, adequate treatment of cells to withstand subsequent treatments such as staining and exposure to electron beam.

The aldehydes which are used as primary fixatives have the ability to stabilize proteins by creating inter- and intrachain cross-links (Palade, 1952). Acrolein (C_3H_5O) and formaldehyde are also in use particularly when a rapid penetration of the fixative is required. Though 4% formaldehyde which is a monoaldehyde, has been shown to be an effective fixative, when kept for a long time, it gets converted to formic acid which has a deleterious effect on the tissues. Commercial formaldehyde (formalin) has nearly 12 to 15% methyl alcohol which denatures protein. Hence, it has been replaced by paraformaldehyde which

is a polymer of formaldehyde. Glutaraldehyde (dialdehyde) was first used by Sabatini *et al.* (1963) as a primary fixative and was found to be an excellent preservative of fine structures. As a result of aldol condensation, glutaraldehyde molecules can be linked together between neighbouring amino acid chains. Since aldehydes do not react with lipids, a subsequent fixation with osmium tetraoxide (OsO_4) is necessary to prevent loses occurring during dehydration. When reduced, OsO_4 acts as an electron-dense stain which reacts principaly with lipids. Stable glycol osmates are formed from conjugated double bonds, enabling cross-linking between neighbouring hydrocarbon chain to take place (Riemcrsma, 1968).

6.2.4.1.1 Reagents

1. Phosphate buffer : 0.1 M (pH 7.4)

   Stock A : 0.2 M, 3.12 g of sodium dihydrogen ortho-phosphate in 100 ml distilled water.

   Stock B : 0.2 M, 2.83 g of disodium hydrogen phosphate in 100 ml distilled water

9.5 ml of stock A was added to 40.5 ml of stock B and solution was made upto 100 ml. The pH was checked to be 7.4.

2. Kreb ringer's solution :

960 ml, 0.154 M NaCl solution is added to 20 ml, 0.154 M KCl solution and 20 ml, 0.11 M $CaCl_2$ solution to make 1000 ml Kreb ringer's solution.

3. Fixative medium : (David *et al.*, 1973)

20g of paraformaldehyde is added to 250 ml of 0.2M phosphate buffer, pH 7.4 and heated to 60°C to dissolve paraformaldehyde. Volume was made to 480 ml with distilled water and allowed to cool to room temperature. 20 ml of 25% (v/v) glutaraldehyde solution and 12.5 mg of anhydrous $CaCl_2$ was added. Fixative medium was stored at 4°C.

4. Osmium tetraoxide solution : 2% (w/v)

Osmium tetraoxide (Loba Chemical Co.) is available as crystals in sealed ampoules. The label on the ampoule is removed with acetone and then the ampoule is scored with a glass knife. It is then wrapped in aluminium foil and broken open. The crystals, together with the glass, are carefully transferred to a glass-stoppered bottle. Water is added to give a final concentration of 2% (w/v) OsO_4 and stored at 4°C.

6.2.4.2 Vascular Perfusion

Vascular perfusion was carried out transcardially. Perfusion was carried out using a transfusion set (bottle with the polythene catheter containing the fluid suspended at about 150 cm above the animal) at room temperature. The heart of the animal was exposed. The ascending arota was cannulated with a polythene catheter of about 1 mm internal diameter through an incision in the left ventricle. Care was taken not to let in air while perfusion was conducted. As suggested by David *et al.* (1973) the animals were initially perfused with about 50 ml Kreb ringer's solution and

then for 15 min with 0.1M phosphate buffer, pH 7.4, containing the fixation solution (1% glutaraldehyde + 4% paraformaldehyde) and 1 mM $CaCl_2$. At start, the fixative was allowed to flow rapidly for few minutes. Then, the right atrium (auricle) was incised for the outflow of the fixative. The rate of flow was then reduced to about 6 to 8 ml per min. The total body perfusion was performed with 300 to 400 ml of the fixative.

After this procedure the animal was dissected and the epididymis removed. The organ was then divided into caput, corpus and cauda (Brooks *et al.*, 1974). Since the caput and cauda regions of the epididymis perform entirely different functions, the structural changes in these two segments were examined by light and electron microscopy. Other investigators studying the ultrastructure of the epididymis have also restricted themselves to these two regions for the same reasons (Bartsch *et al.*, 1978; Moore and Bedford, 1979 a).

8.2.4.3 Specimen fixation

This step consists of two stages. In the first stage called primary fixation the desired tissue (epididymis) is cut into small pieces (1-2 mm) and further fixed in 2.5% glutaraldehyde overnight at 4°C. The tissues are then washed in 0.1 M phosphate buffer, pH 7.4, thrice with a change after every half an hour. After washing, the tissues were post-fixed in 1% osmium tetraoxide in 0.1 M phosphate buffer, pH 7.4, at 4°C.

6.2.4.4 Dehydration

Epididymal tissues contain about 95% water which needs to be removed to facilitate infiltration with liquid resin (monomer) which is a prerequisite for embedding. This was accomplished by at first washing the tissue in 0.1 M phosphate buffer, pH 7.4, and then passing it through an ascending series of acetone (30%, 50%, 70%, 80%, 90%, 95%) with two changes of 15 min each at 4°C. The dehydration was completed with dry acetone for 15 min at 4°C and two subsequent changes of 30 min each at 25 ± 2°C. Tissues were now cleared of acetone by immersing in toluene for 2h at room temperature.

6.2.5 Infiltration and Embedding

6.2.5.1 Reagents

(1) Infiltration medium

Three different concentrations of infiltration medium were prepared:

(a) 2.5 ml of Araldite CY 212(epoxy monomer) was added to 2.5 ml of DDSA (Dodecenyl succinic anhydride; HY 964) as Hardner and made upto 20 ml with toluene.

(b) 5.0 ml of Araldite CY 212 was added to 5 ml of DDSA and made upto 20 ml with toluene.

(c) 7.5 ml of Araldite CY 212 was added to 7.5 ml of DDSA and made upto 20 ml with toluene.

(2) Araldite CY 212 Embedding Medium

10 ml of Araldite CY 212 was added to 10 ml of DDSA and 0.4 ml of 2,4,6, tridimethyl aminomethyl phenol (DMP-30; DY064) as accelerator.

6.2.5.2 Procedure

The tissues were put in mixture (a) and left overnight at room temperature. Next morning, the tissues were transferred to mixture (b) and kept for 2h. The tissues were once again transferred, this time to mixture (c) for 2h under vacuum and finally left in pure embedding medium excluding DMP-30 at 50°C for 2h. Embedding of tissues was carried out in the Araldite embedding medium including accelerator DMP-30 using beam capsules. The liquid Araldite embedding medium (monomer) containing the tissues was gradually polymerized. This process involved keeping the embedded blocks at 50°C in a special oven for 20h. The temperature was then raised to 60°C which ensured that polymerization was complete by 24h.

6.2.6 Sectioning of tissues for light and electron microscopy

6.2.6.1 Preparation Of Semithin Sections

The blocks were trimmed to pyramid shape by removing all free embedding medium using a pyramitome. Glass knives made out of good glass-strips (Belgian) with the help of a glass-knife marker (LKB) were used for sectioning the blocks. Initially 0.5-2 µm thick sections were made for viewing under an optical microscope.

The sections were cut on a microtome (OMU3, Reichert Jung) and let floating in a pool of 10% acetone. Rust-free stainless steel troughs were used to hold the acetone which were fitted closely round the knife upto the knife edge. The troughs were made water tight using sticking plastic tapes.

6.2.6.2 Staining of Semithin Sections

6.2.6.2.1 Reagents

Toluidene blue (1%):

1 g toluidene blue was dissolved in 100 ml, 0.1 M potassium phosphate buffer. The solution was heated to 90°C for 15 min and then filtered.

6.2.6.2.2 Procedure

Semi thin sections that floated in 10% acetone were lifted with a glass rod and placed on a clean glass slide. The slide was placed on a hot plate at about 80°C and dried. The sections on the microscope slide were covered with the stain and then warmed to 50°C for 5 min (Lewis and Knight, 1977). After washing thoroughly in distilled water to remove unreacted stain, the sections were dried and mounted with DPX using coverslips made of English glass.

The sections thus obtained not only helped in making studies at the light microscopic level but were also useful in ascertaining the state of the embedded tissue. In addition, they also aided in selecting the size, position and area of the cutting face for ultrathin sectioning.

6.2.6.3 Preparation Of Ultrathin Sections

After scanning the sections under the optical microscope, the desired area to be examined under transmission electron microscope was selected and the blocks were further trimmed accordingly. Ultrathin sections were cut on **Reichert Ultracut E** using fresh glass knives. The thickness of ultrathin sections was determined by silver color, an interference color on the liquid in the trough. While viewing, appearance of silver color helps in estimating the thickness of sections at 600-700 A° which was ideal for high resolution work.

The ultrathin sections were floated in water and stretched by exposing them to chloroform. The sections were lifted from below on specially made 200 meshed copper grids. 3% formvar (polyvenyl formaldehyde) in ethylene dichloride was used for coating the grids.

6.2.6.4 Staining Of Ultrathin Sections

To obtain a good contrast a double staining method using uranyl acetate and lead citrate was used.

6.2.6.4.1 Reagents

Uranyl acetate:

Solid uranyl acetate (UO_2AC) (Loba Chemical Co., India) is added to 100 ml double distilled water in a glass-stoppered, brown bottle and the bottle is shaken until the uranyl acetate dissolves. This is done several times over 2-3 h until

saturation conditions are reached. The solution is allowed to stand overnight. The supernatant is then centrifuged for 15 min at 500 xg. The final solution has a pH of 4.0 and stored in the dark.

Lead Citrate:

Lead citrate solution was prepared as suggested by Reynolds (1963). 1.33 g lead nitrate [$(Pb(NO_3)_2$] and 1.76 g sodium citrate [Na_3 $(C_6H_5O_7)$. $2H_2O$] are dissolved in 30 ml double distilled water in 50 ml ground glass stoppered bottle. The solution is shaken vigorously for 30 min. 8 ml, 1 M NaOH is then added together with water to make a volume of 50 ml. The solution is clear and has a pH of 12.

6.2.6.4.2 Procedure

Small amount of uranyl acetate solution was taken in a clean watch glass. Grids carrying the section were placed down onto the stain. A card-board cover was placed over it as the staining is best when carried out in dark. After 15 min each grid was taken and washed in 2 lots of 50% ethanol and 2 lots of distilled water with continuous agitation. Grids were dried on filter paper.

The dry grids were placed onto the lead citrate stain in a watch glass and stained for 10 min. Grids were washed in 0.1N sodium hydroxide and then in 2 lots of double distilled water. Grids were dried and used for further observations.

6.2.7 Electron Microscopy

Ultrathin sections of the caput and cauda epididymidis thus prepared were examined in a Phillips 400 Transmission Electron Microscope at 80 kV. The epididymal duct is lined by an epithelium generally comprising of principal, basal, clear, apical and halo cells (Hamilton, 1975; Yeung and Cooper, 1982). The present study was mainly centered around the principal cells which are identified in the epithelium on the basis of the published descriptions (Sinowatz, 1981; Goyal, 1985; Robaire and Hermo, 1988). Principal cells were choosen because they are the main functional cell type of epididymal epithelium and most responsive to hormone manipulations (Amann, 1987; Robaire and Hermo, 1988). To help in the identification of principal cells and as a source of reference to monitor the effects of various treatments the caput and cauda epididymidis from sham-operated rats given vehicle treatment were also studied.

6.3 RESULTS

6.3.1 Preliminary Observations Showing the Effect of Prolactin on the Structure of Epididymis at the Light Microscopic Level

Orchidectomy brought about dramatic effects on the structure of the epididymis when compared to the sham-operated controls. The prominent features being the shrinkage of epididymal tubules and the disappearance of spermatozoa from the lumen of the duct. The luminal surface of the epithelium also showed involutions. A study of the semithin sections of the epididymis under the

optical microscope gave the impression that the epididymis of castrates was undergoing atrophy. Histoquantitative measurements revealed that the epithelial cell height and the thickness of smooth muscle layer surrounding the epithelium were greatly influenced by factors originating in the testis (Table-1). Orchidectomy decreased the epithelial cell height by about 34% in the caput and 18% in the cauda. The thickness of the smooth muscle on the otherhand was increased in the orchidectomised animals by 9% in the caput and 13% in the cauda epididymidis.

The influence of prolactin and bromocryptine on the epididymal cell height in orchidectomised animals are presented in Fig. 17. In both caput and cauda epididymal segments, prolactin treatment increased the epithelial cell height to values greater than those found in orchidectomised controls injected with vehicle alone. Bromocryptine treatment did not significantly alter the epithelial cell height in orchidectomised animals. Neither prolactin nor bromocryptine had any substantial effect on the smooth muscle thickness surrounding the caput and cauda epididymal tubules of orchidectomised animals Fig. 18.

6.3.2 The Ultrastructure of Principal Cells from the Epididymis of Sham-Operated Animals

The features described are based on electron micrographs of the epididymis obtained from sham-operated animals given vehicle treatment. The principal cell is distinguishable from other cell types of the epididymal epithelium by the presence of stereocilia

TABLE:1 *HISTOQUANTITATIVE MEASUREMENTS OF THE CHANGES IN EPIDIDYMAL STRUCTURE BROUGHT ABOUT BY ORCHIDECTOMY*

Parameters	Epithelial Cell Height			Smooth Wall Thickness		
Epididymal Segments	Sham-operated control	Orchide-ctomised rats	% decrease	Sham-operated control	Orchide-ctomised rats	% decrease
CAPUT	37.14 ± 5.14	24.65 ± 3.80	33.62	10.25 ± 2.10	11.20 ± 2.60	09.26
CAUDA	24.75 ± 4.12	20.35 ± 3.55	17.78	11.10 ± 1.78	12.56 ± 1.78	13.15

Fig. 17. HISTOGRAM SHOWING THE CHANGE IN HEIGHTS OF EPITHELIAL EPITHELIUM FOLLOWING PROLACTIN AND BROMOCRYPTINE TREATMENT IN ORCHIDECTOMISED RATS.

Adult rats (15 days post-orchidectomised) were given the following treatment, once daily, s.c., for a period of 7 days : (▒▒▒) vehicle; (═══) 50 µg oPRL/100 g b wt; (▒▒▒) 100 µg oPRL/100 g b wt; (▒▒▒) 150 µg oPRL/100 g b wt; (▒▒▒) 200 µg oPRL/100 g b wt and (▒▒▒) 0.30 mg BRM/100 g b wt. For ready reference the epithelial cell height of sham-operated rats given vehicle treatment (▒▒▒) has also been provided. Measurements are from 4 animals at 20 different measuring sites throughout transverse cross-section. All comparisons have been made with orchidectomised/animals given vehicle (* $p<0.05$ Student's t-test).

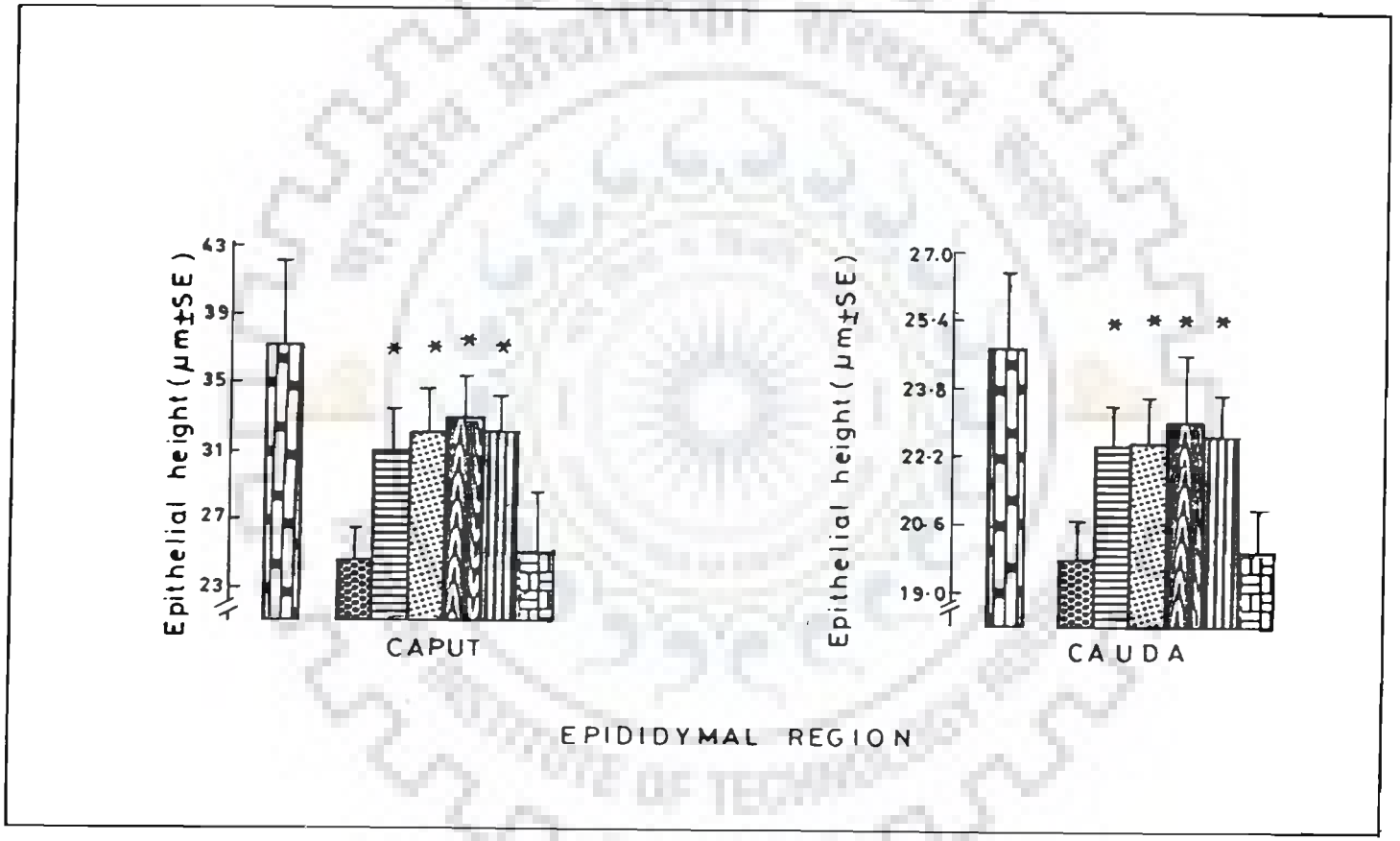

Epithelial height (µm±SE)
43
39
35
31
27
23
CAPUT
Epithelial height (µm±SE)
27.0
25.4
23.8
22.2
20.6
19.0
CAUDA
EPIDIDYMAL REGION

Fig. 17.

Fig. 18. HISTOGRAM SHOWING THE CHANGE IN SMOOTH MUSCLE WALL THICKNESS FOLLOWING PROLACTIN AND BROMOCRYPTINE TREATMENTS IN ORCHIDECTOMISED RATS.

Adult rats (15 days post-orchidectomised) were given the following treatment, once daily, s.c., for a period of 7 days : (▨) vehicle; (▬) 50 µg oPRL/100 g b wt; (▨) 100 µg oPRL/100 g b wt; (▨) 150 µg oPRL/100 g b wt; (▨) 200 µg oPRL/100 g b wt and (▨) 0.30 mg BRM/100 g b wt. For ready reference the epithelial cell height of sham-operated rats given vehicle treatment (▨) has also been provided. Measurements are from 4 animals at 20 different measuring sites throughout transverse cross-section. All comparisons have been made with orchidectomised animals given vehicle (* $p<0.05$ Student's t-test).

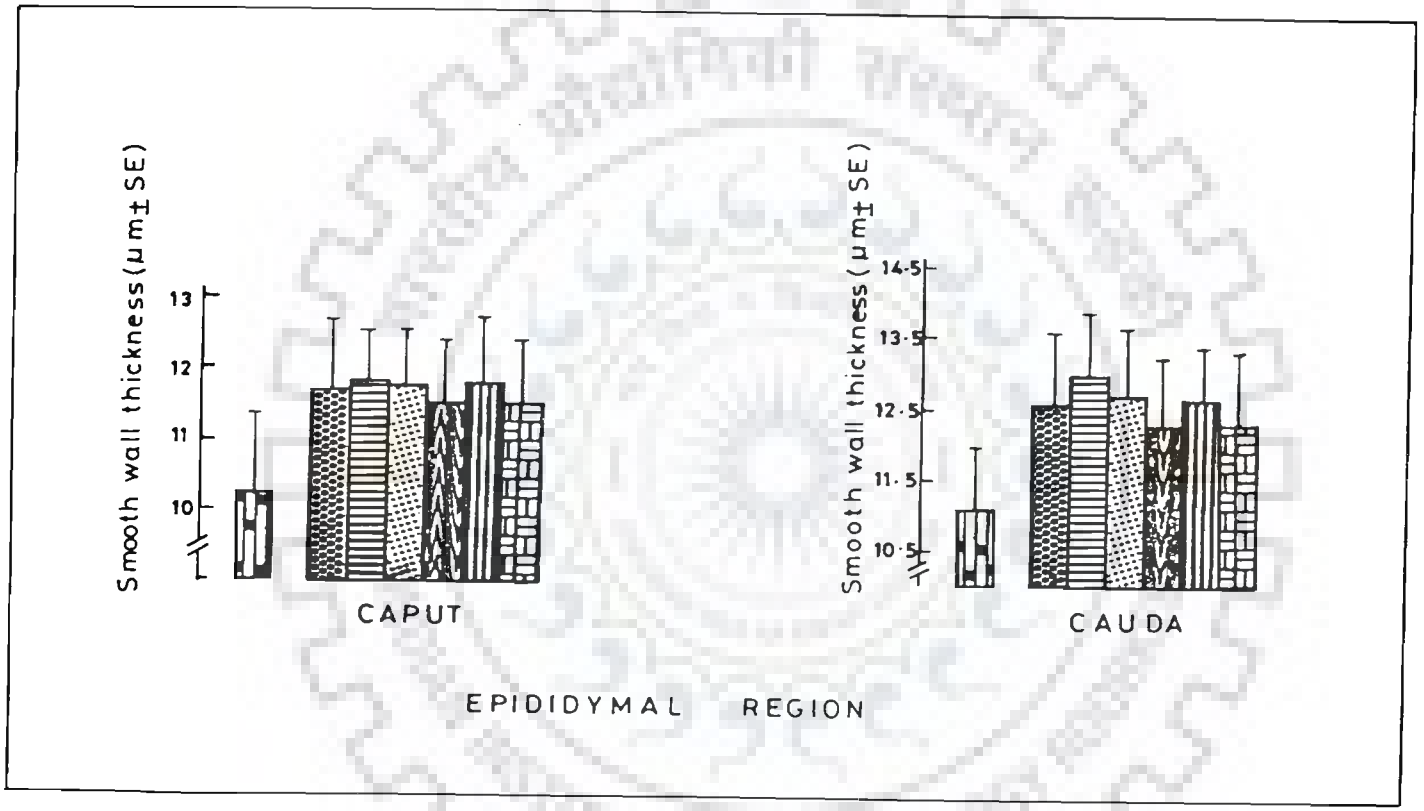

Smooth wall thickness (μm ± SE)
13
12
11
10
CAPUT
Smooth wall thickness (μm ± SE)
14·5
13·5
12·5
11·5
10·5
CAUDA
EPIDIDYMAL REGION

Fig. 18.

that project into the lumen of the duct. They also possess a tall body extending upto the basal lamina and a basally situated nucleus. The synthetic capabilities of the cell are evident from the presence of a prominently large Golgi apparatus, the most striking feature of the principal cell, irrespective of its location along the epididymal duct. As a mark of its absorption function, luminal surface invaginations, coated vesicles, multivesicular bodies and dense bodies are also found. The frequency and distribution of these organelles in the principal cell are distinctly higher than other cell types known to comprise the epididymal epithelium.

In sham-operated animals, the principal cell belonging to the caput can clearly be distinguished from those of the cauda epididymidis by its shape and size. In the caput, the principal cells are columnar and tall while in cauda they become more cuboidal and stumpy. For detailed comparative studies between the caput and cauda epididymidis, the principal cell was divided into a free surfacial region bearing stereocilia projecting into epididymal lumen, a region just under this surface, the supranuclear, the nuclear and the infranuclear regions (Fig. 19-23).

The principal cell in caput and cauda epididymidis appear different at the luminal side. The principal cell in the caput has a long stereocilia while in the cauda it is much shorter. In the region just below the luminal surface, the principal cells have abundant endoplasmic reticulum. The endoplasmic reticulum

Fig. 19. (A) and (B) ELECTRON MICROGRAPHS SHOWING THE SURFACE OF PRINCIPAL CELLS FACING THE LUMEN IN CAPUT AND CAUDA EPIDIDYMIDIS OF SHAM-OPERATED ANIMALS.

(A) Caput x 2100 (B) Cauda x 4500. Lu Lumen, St Stereocilia. Differences in the size of the stereocilia and the contour of the luminal epithelial surface are prominent.

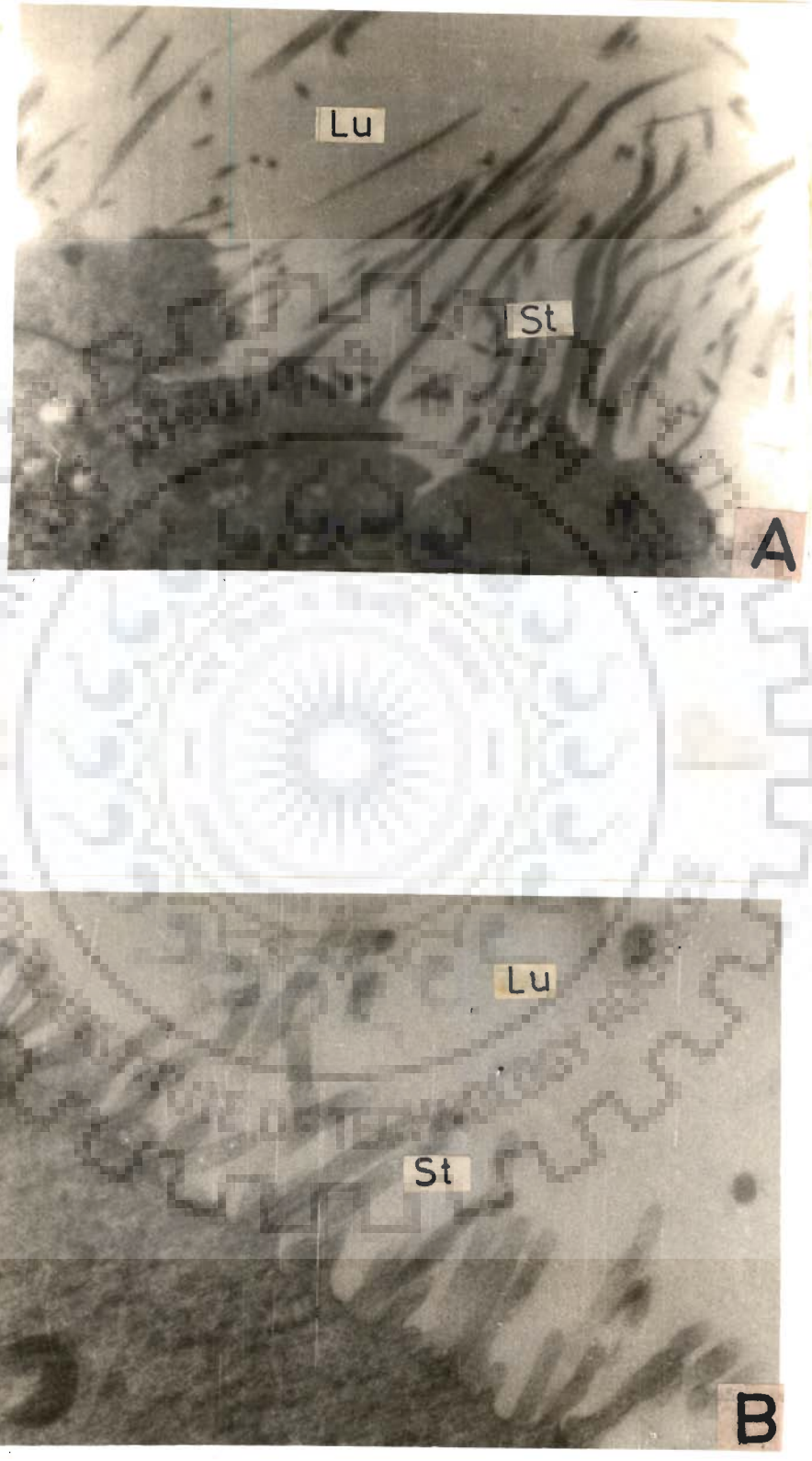


Fig. 19.

Fig. 20. (A) and (B) ELECTRON MICROGRAPHS OF THE PRINCIPAL CELL SHOWING THE SUPRANUCLEAR CYTOPLASM FROM CAPUT AND CAUDA EPIDIDYMIDIS FROM SHAM-OPERATED ANIMALS.

(A) Caput (B) Cauda x 16,000. MVB Multivesicular Bodies, CV Coated Vesicles. Differences in the number of multivesicular bodies and coated vesicles are clearly seen.

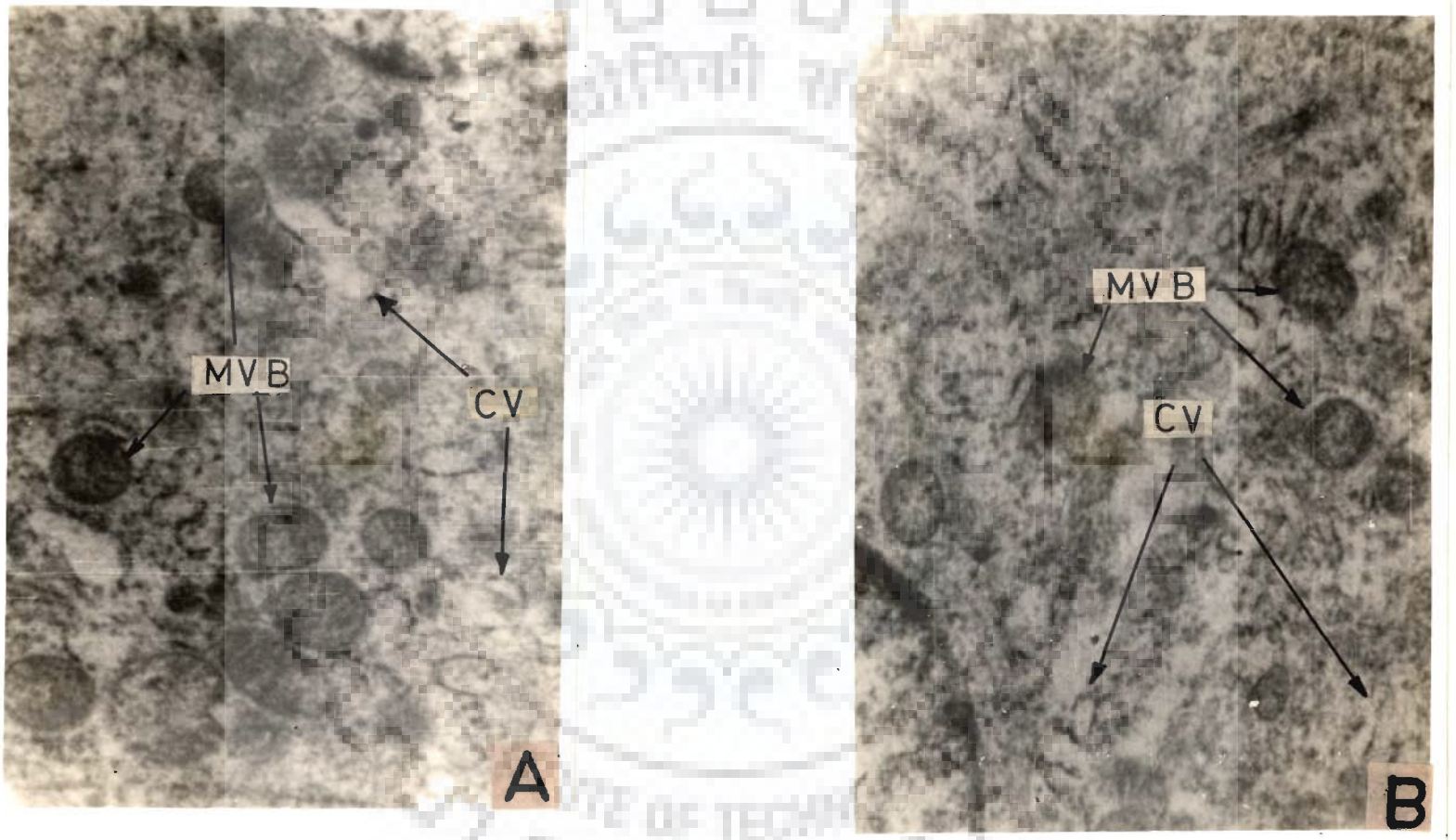


Fig.20.

Fig. 21. (A) and (B) ELECTRON MICROGRAPHS OF THE PRINCIPAL CELL SHOWING THE REGION JUST BELOW THE LUMINAL SURFACE FROM CAPUT AND CAUDA EPIDIDYMIDIS OF SHAM-OPERATED ANIMALS.

(A) Caput x 21,000. ER Endoplasmic Reticulum. The cytoplasm is densely packed with vesicular endoplasmic reticulum showing round profiles (B) Cauda x 21,000. The endoplasmic reticulum is elongated.

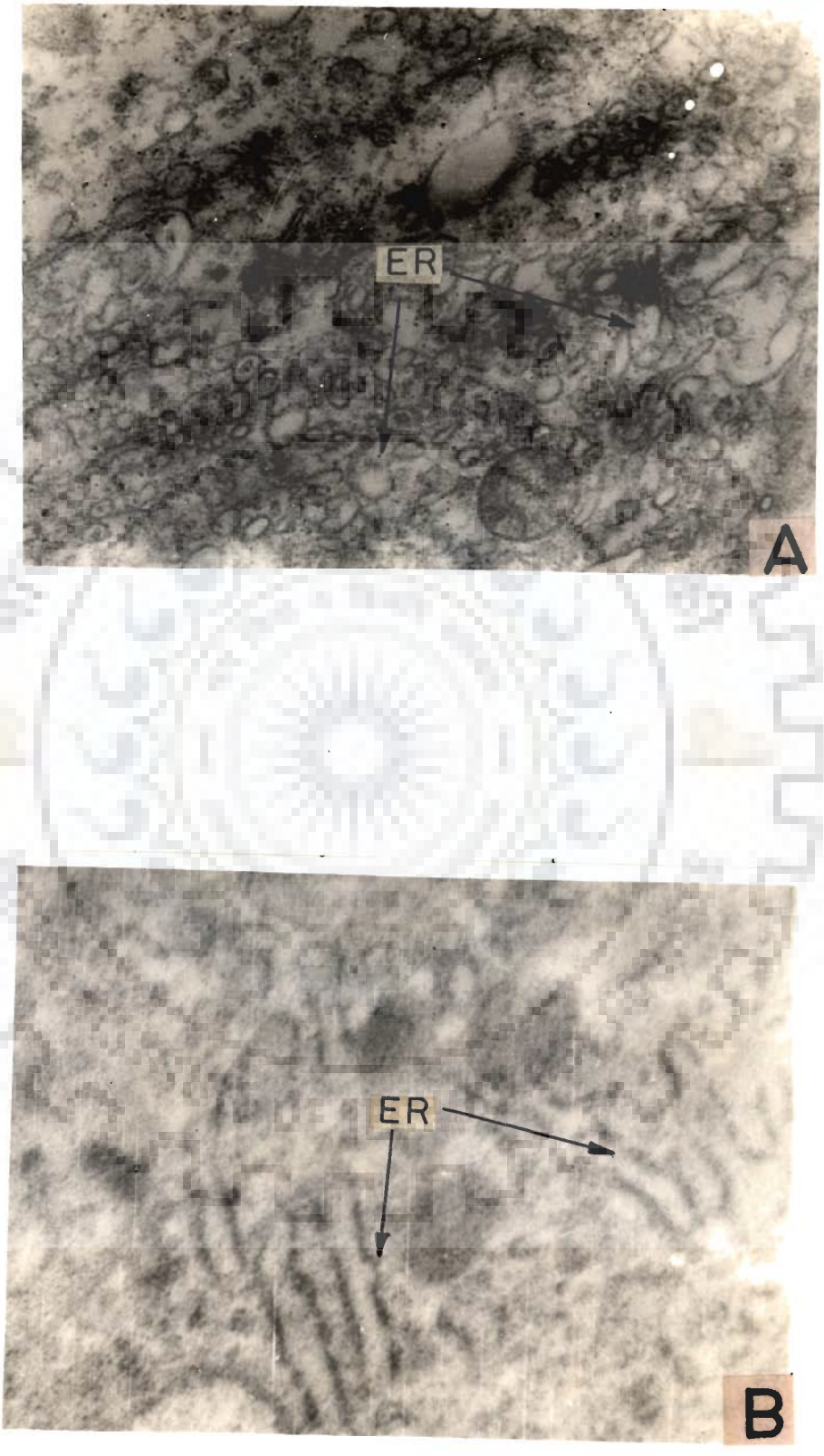


Fig. 21.

Fig. 22. (A) and (B) ELECTRON MICROGRAPHS SHOWING THE NUCLEUS OF THE PRINCIPAL CELL FROM CAPUT AND CAUDA EPIDIDYMIDIS OF SHAM-OPERATED ANIMALS.

(A) Caput x 4500, N Nucleus, Nli Nucleoli. The nucleus is long and elliptical, with the long diameter parallel to the cell axis (B) Cauda x 7400, the nucleus is flattened with the long diameter perpendicular to the cell axis. In both cases the nucleus contains two or more nucleoli.

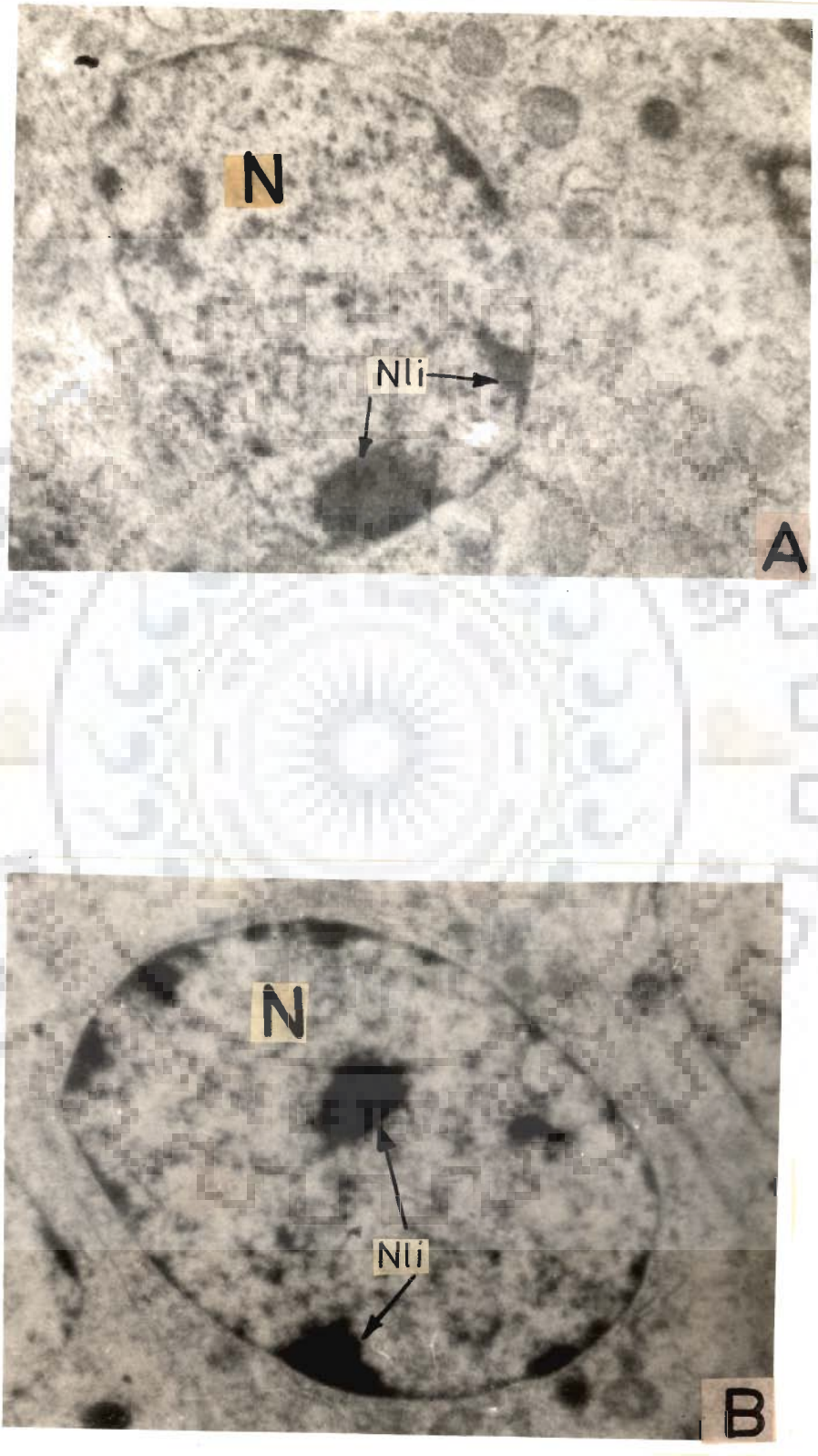


Fig. 22.

Fig. 23. (A) and (B) ELECTRON MICROGRAPHS SHOWING THE INFRANUCLEAR BASAL CYCTOPLASM OF PRINCIPAL CELLS FROM CAPUT AND CAUDA EPIDIDYMIDIS OF SHAM-OPERATED ANIMALS.

(A) Caput x 5800 (B) Cauda x 12,700. N Nucleus, ER Endoplasmic Reticulum. The shape and distribution of the endoplasmic reticulum are not entirely the same in the two regions. The endoplasmic reticulum is V-shaped in caput and U-shaped in cauda.

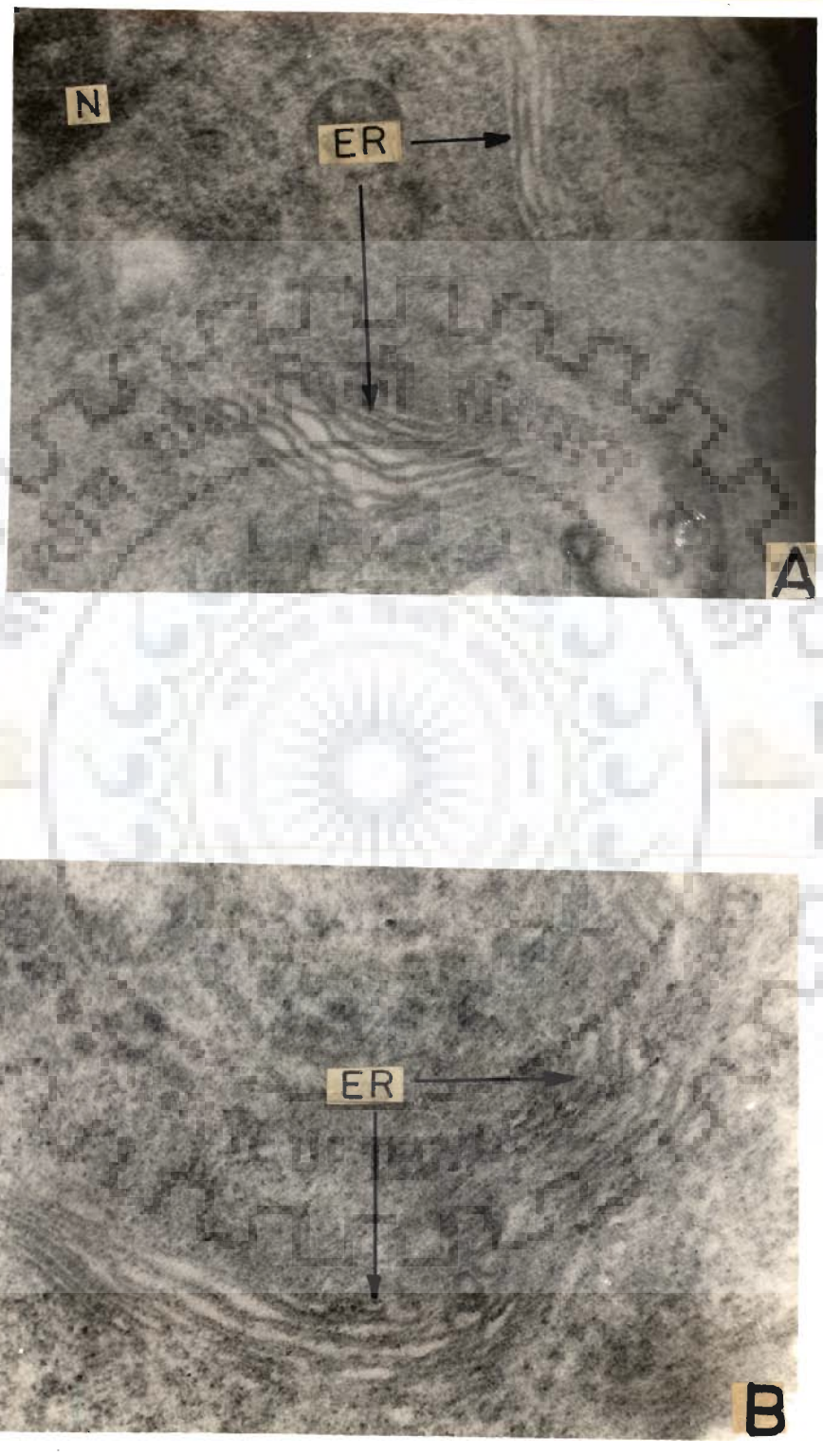


Fig. 23.

from the principal cell of the caput is vesicular with limited number of ribosomes. However, in the cauda the endoplasmic reticulum contains flattened cisternae with ribosomes adhering to their membranes. The supranuclear region of the cell from caput is characterized by a large number of multivesicular bodies and numerous large and small coated vesicles. The cauda has fewer multivesicular bodies but the proportion of large coated vesicles is higher than the small ones. In both the epididymal segments, well defined groups of Golgi bodies are found in this region. The nucleus of principal cell in the caput is long and elliptical, with the long diameter parallel to the cell axis. In the cauda, the same is flattened with the long diameter perpendicular to the cell axis. The infranuclear cytoplasm of the principal cell from caput and cauda contain flattened cisternae of rough endoplasmic reticulum which are frequently arranged in a lamellae form. This arrangement is more or less V-shaped in the caput and U-shaped in the cauda which may be partly due to the columnar, and cuboidal nature of the epithelium in the two segments.

6.3.3 Ultrastructural Changes in the Principal Cells from the Epididymis of Orchidectomised Rats

The fine structure of the principal cells from the caput and cauda epididymis in orchidectomised rats are presented in Fig.24. Following orchidectomy, changes in the orientation of the epithelial cells lining the epididymal duct are seen, sometimes making even the identification of the principal cell from other

Fig. 24. (A) and (B) ELECTRON MICROGRAPHS OF THE PRINCIPAL CELL FROM CAPUT AND CAUDA EPIDIDYMAL EPITHELIUM OF ORCHIDECTOMISED RATS GIVEN VEHICLE TREATMENT.

(A) Caput x 1270 (B) Cauda x 2100. Lu Lumen, PC Principal Cell, N Nucleus, BM Basement Membrane. The surface of the caput epithelial cells facing the lumen of the duct shows infoldings with no stereocilia. The distribution of the cellular organelles appears to be disorganized in both regions. Dense lysosomal aggregations are richly populated in cauda.

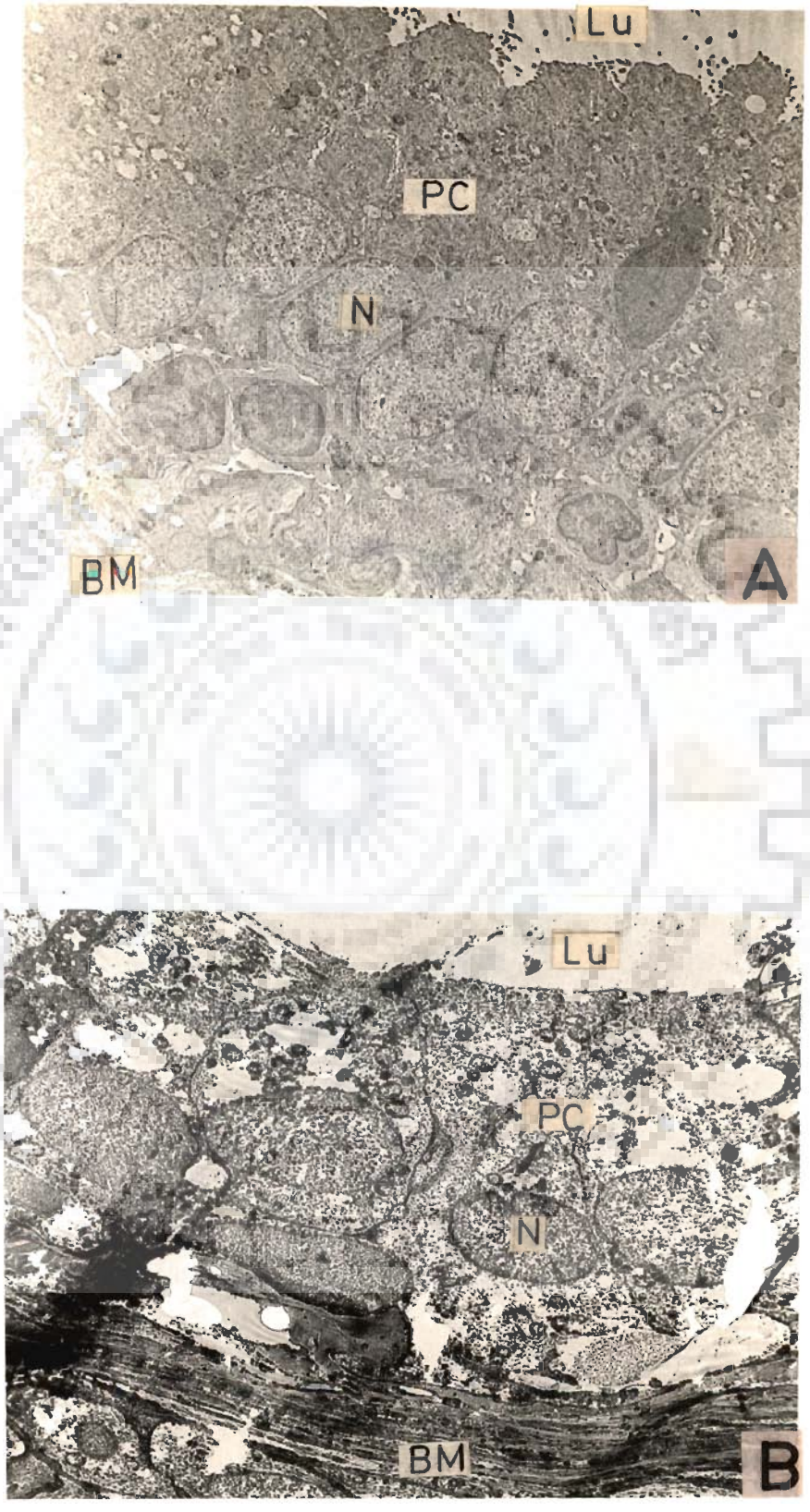


Fig. 24.

cell types difficult. This may be on account of the extensive shrinkage of the epididymal tubule, resulting in the closer packing of cells. A striking feature is the great reduction in the size of the stereocilia in both segments. In most cases, the stereocilia were not seen. Within the confines of the principal cell, many structural changes were observed. These include a reduction in the rough endoplasmic reticulum, a decrease in the volume of Golgi cisternae and the disappearance of secretory vesicles from the cells apex. In addition, the total quantity of cytoplasmic membrane (rER, sER, Golgi) seem to be diminished considerably. The cytoplasmic organelles also become spatially disorganized in relation to each other. These results indicate that androgen withdrawal hampers the production of macromolecules by the principal cells. Other observations like the clustering of coated vesicles in and around the region normally occupied by the Golgi together with the increase in the number of dense bodies and empty vacuoles, suggest that orchidectomy results in increased lysosomal activity. It may be noticed that the principal cells in the cauda epididymidis show changes similar to those in the caput.

6.3.4 Ultrastructural Changes in the Principal Cells from the Epididymis of Orchidectomised Rats given Prolactin Treatment

The fine details of the principal cells from the caput and cauda epididymidis of orchidectomised rats given varying prolactin and bromocryptine doses are presented in Fig.25-28. It must be

Fig. 25. (A) and (B) ELECTRON MICROGRAPHS OF THE PRINCIPAL CELL FROM CAPUT AND CAUDA EPIDIDYMAL EPITHELIUM OF ORCHIDECTOMISED RATS GIVEN 50 μg oPRL TREATMENT.

(A) Caput x 1270 (B) Cauda x 2100. Lu Lumen, PC Principal Cell, N Nucleus, BM Basement Membrane. In supranuclear region of cell from both caput and cauda several cytoplasmic inclusions seem to start appearing. The number of coated vesicles increased and the empty vacuoles reduced in number.

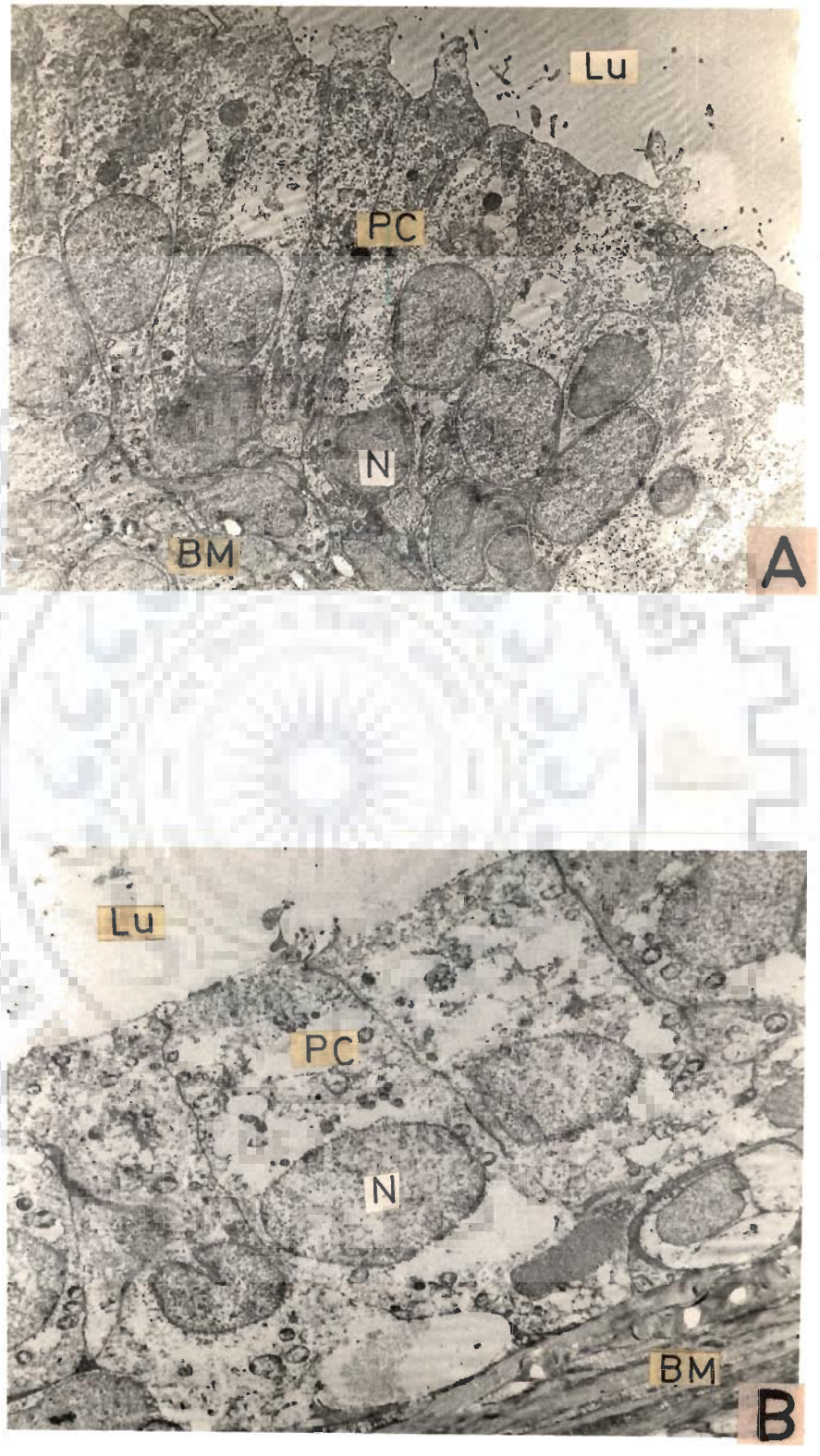


Fig. 25.

Fig. 26. (A) and (B) ELECTRON MICROGRAPHS OF THE PRINCIPAL CELL FROM CAPUT AND CAUDA EPIDIDYMAL EPITHELIUM OF ORCHIDECTOMISED RATS GIVEN 100 μg oPRL TREATMENT.

(A) Caput x 1600 (B) Cauda x 2700. Lu Lumen, PC Principal Cell, N Nucleus, BM Basement Membrane. Numerous cytoplasmic organelles are seen which include relatively large number of secretory vesicles at the apical region in the cell of both caput and cauda. The contour of the epithelial surface exposed to the lumen of the duct appear to be more uniform and infloldings brought about by orchidectomy seems to be straighten out.

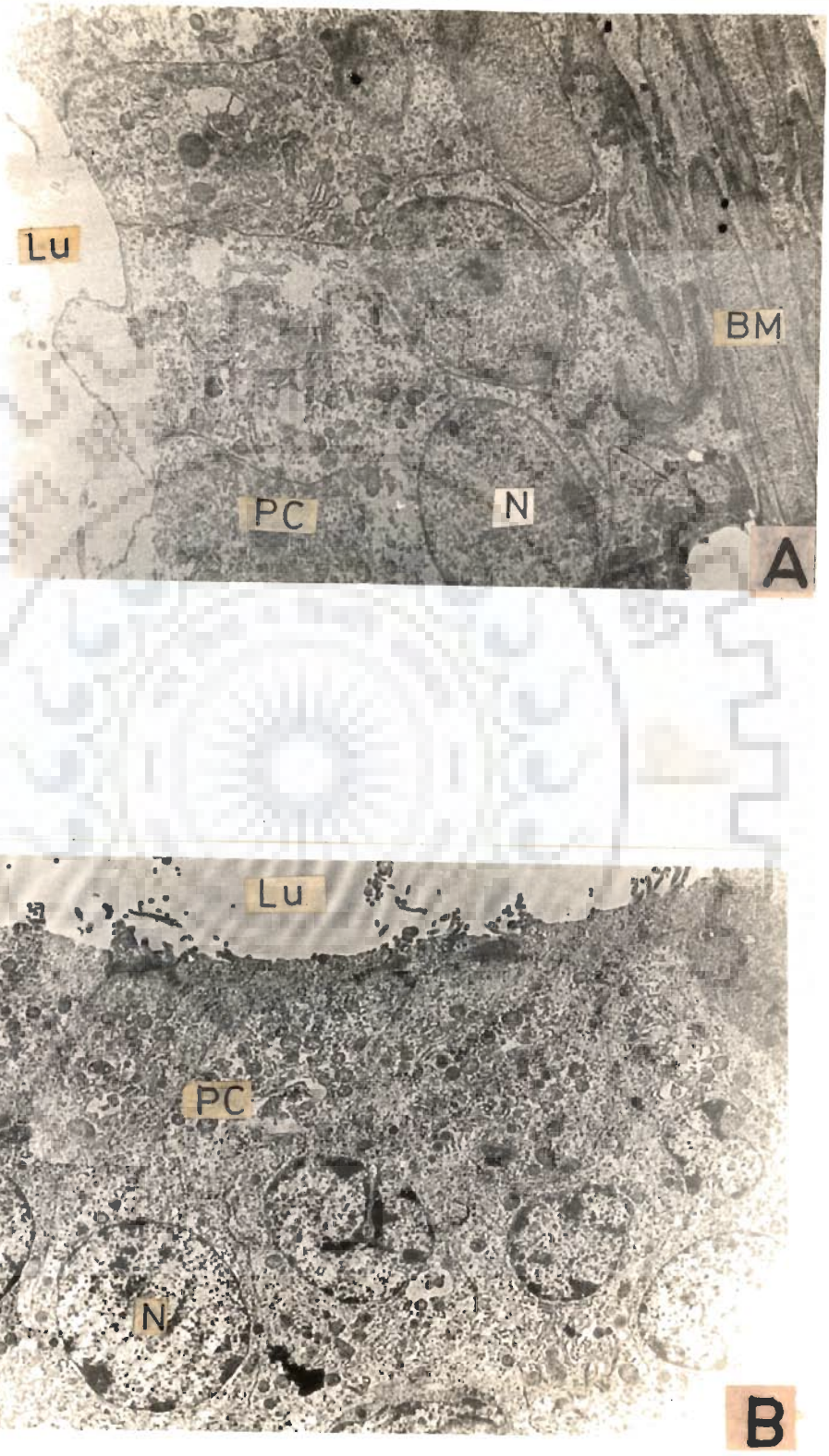


Fig. 26.

Fig. 27. (A) and (B) ELECTRON MICROGRAPHS OF THE PRINCIPAL CELL FROM CAPUT AND CAUDA EPIDIDYMAL EPITHELIUM OF ORCHIDECTOMISED RATS GIVEN 150 μg oPRL TREATMENT.

(A) Caput x 1270 (B) Cauda x 2700. Lu Lumen, PC Principal Cell, N Nucleus, BM Basement Membrane. Prolactin seems to improve the degenerative changes in both the regions as evident by well developed cell organ-elles, large number of secretory vesicles and much reduced number of empty vacuoles throughout the cytoplasm.

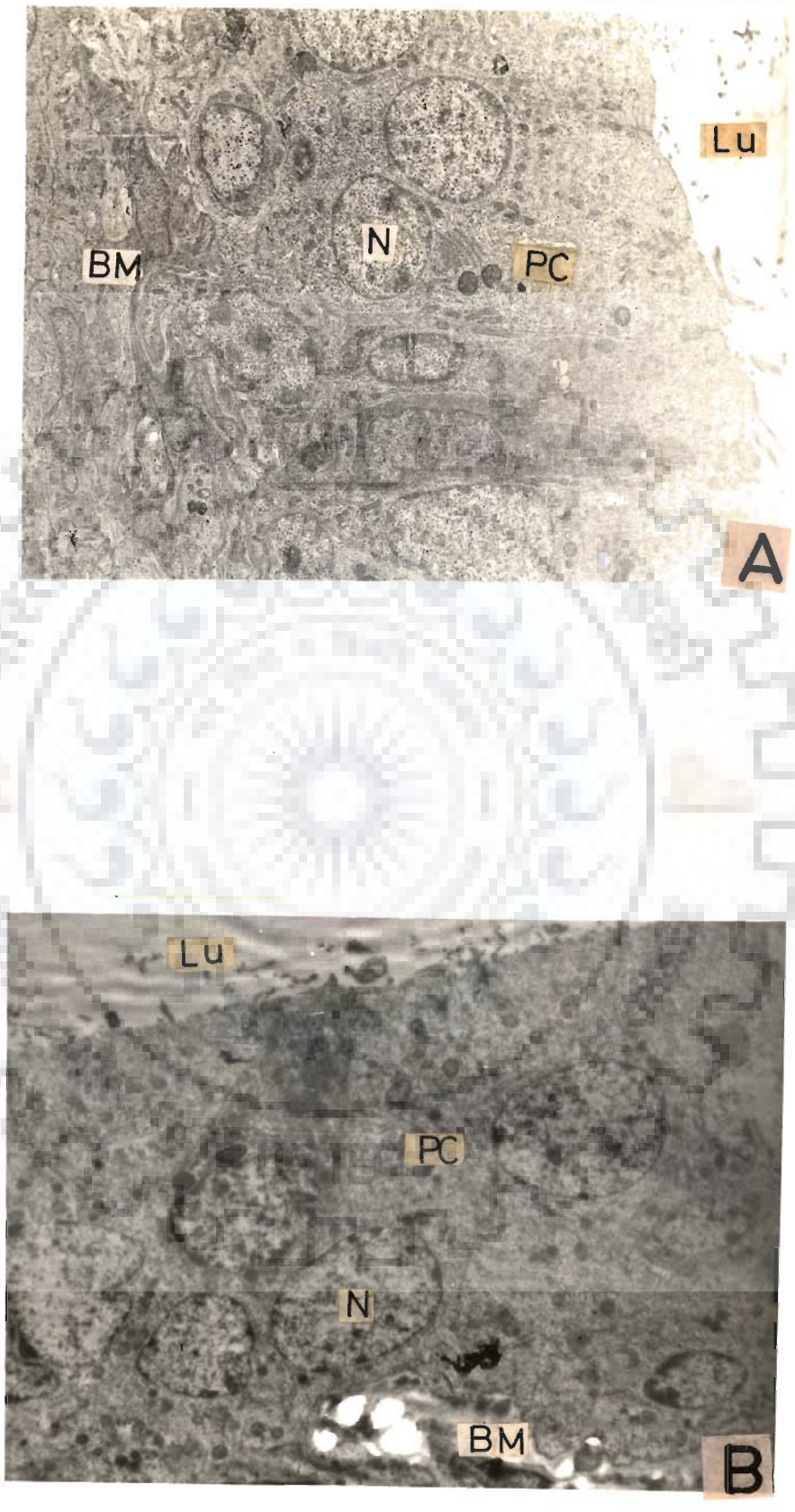


Fig. 27.

Fig. 28. (A) and (B) ELECTRON MICROGRAPHS OF THE PRINCIPAL CELL FROM CAPUT AND CAUDA EPIDIDYMAL EPITHELIUM OF ORCHIDECTOMISED RATS GIVEN 200 μg oPRL TREATMENT.

(A) Caput and (B) Cauda x 1600. The cells appear to contain a large number of coated vesicles, and other organelles. Empty vacuoles are less in number.

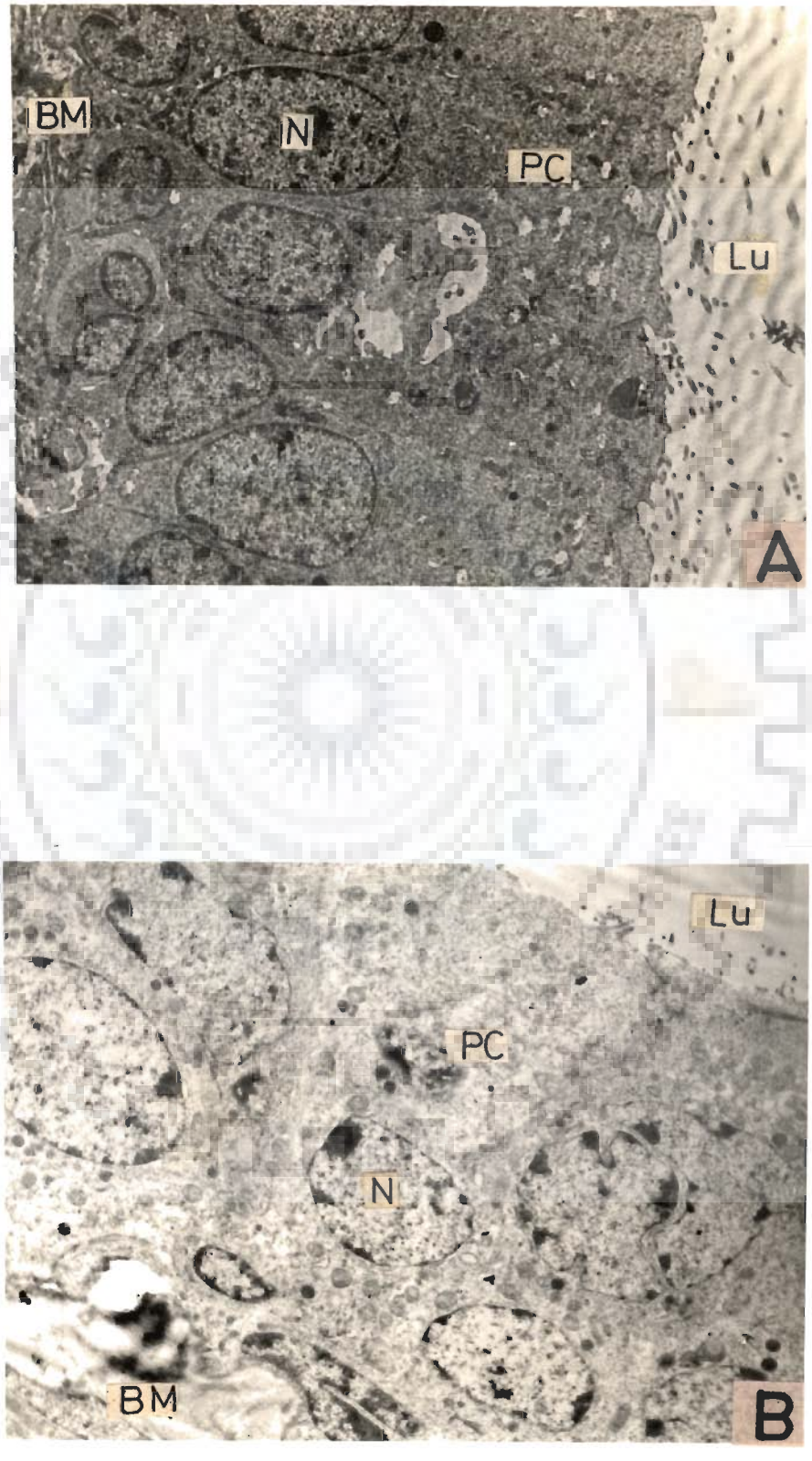


Fig. 28.

mentioned that there was a lot of variation in the response of the individual principal cells in a particular segment of the epididymis of even the same animal, administered a specified dose of prolactin. Nevertheless, it could be observed that the number of principal cells from orchidectomised rats responding to treatment increased with the dose of prolactin employed. The changes in the ultrastructure of the principal cells described below could be attributed to prolactin treatment simply because they were found to occur in all responding cells of animals given prolactin, no matter what the dose was. With the treatment of prolactin to orchidectomised rats several structural elements in the caput and cauda epididymidis seem to be rejuvenated. The shape and orientation of the nucleus and other cell organelles of principal cells were more or less similar to those of sham-operated animals given vehicle treatment, an indication of the positive effect of the hormone. The most remarkable among the changes, however, pertains to the endoplasmic reticulum. The parallel cisternae appeared to have been reorganized and concentrated around the nucleus. Also, the flattened cisternae of rough endoplasmic reticulum had concentric lamellae. The bulk of the apical cytoplasm had a fair distribution of tubular elements. It was pointed out earlier that the volume of Golgi cisternae were considerably reduced in the principal cell of orchidectomised rats when compared to those of the sham-operated animals. Prolactin treatment favoured the normalization of this disturbance in the volume of Golgi cisternae. Such an action of prolactin was found to be more pronounced in the caput than in

the cauda. In short, the organization and orientation of the various cellular organelles within the principal cell is disturbed by orchidectomy and partially restored by prolactin treatment.

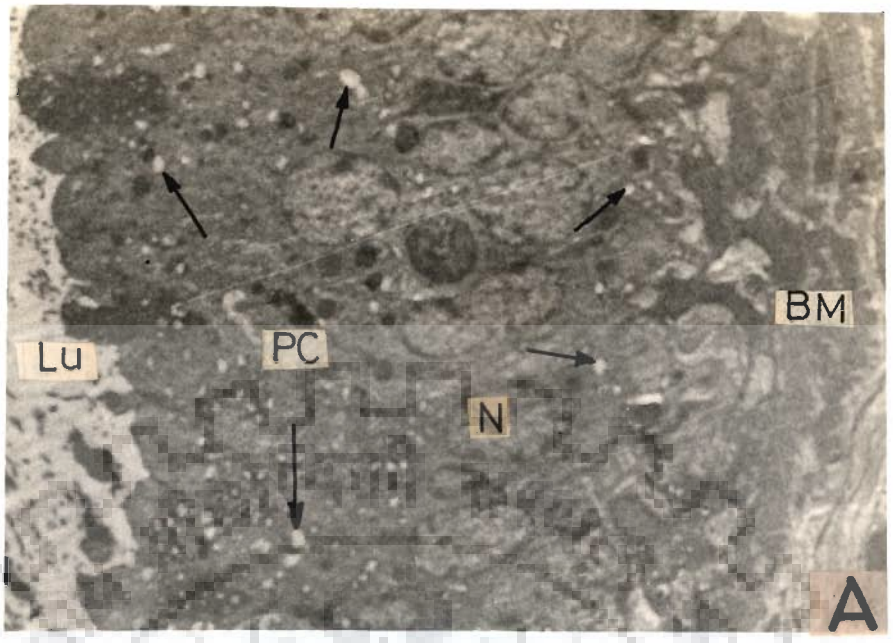
A few other observations that have been made are highlighted. Empty vacuoles, a feature associated with degenerative changes in the principal cell of orchidectomised rats are reduced after prolactin treatment. Dense bodies were found in greater numbers in the principal cell of orchidectomised controls than in their counterparts from orchidectomised rats given prolactin treatment. The number and size of coated vesicles were found to be greater in the principal cell of orchidectomised animals given hormone treatment than in orchidectomised animals treated with vehicle alone. It must be clarified that the structural changes in terms of size, shape and number of cellular organelles described above are based on observations made at considerably higher magnification than those indicated in the electron micrographs.

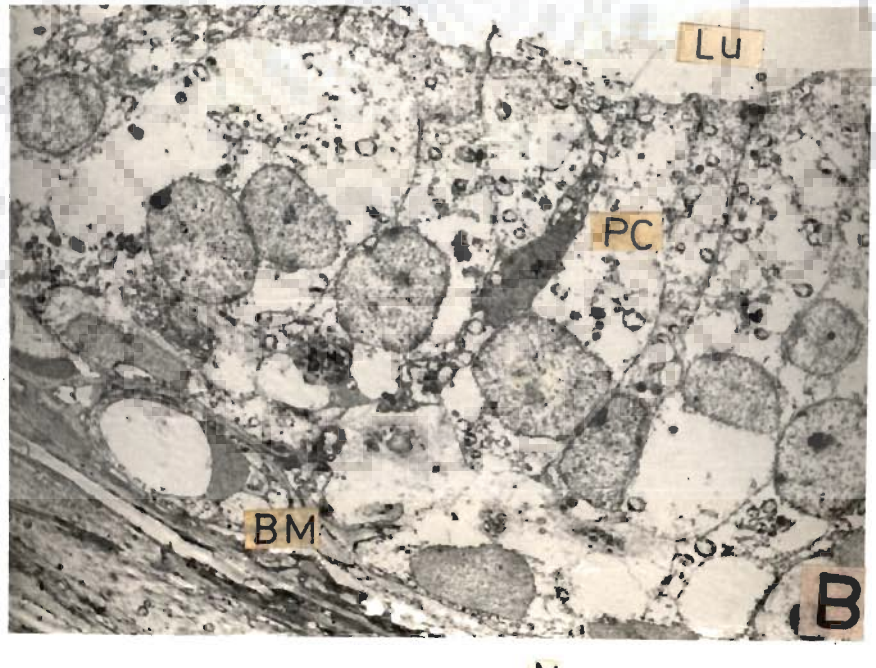
6.3.5 The Ultrastructure of Principal Cells from the Epididymis of Orchidectomised rats after Bromocryptine Treatment

The fine details of the principal cells from the caput and cauda epididymidis of orchidectomised rats given bromocryptine treatment are shown in Fig.29. Apparently, bromocryptine treatment to orchidectomised rats did not produce any substantial effect on the principal cell from either the caput or the cauda epididymidis. The principal cells are to a great extent devoid

Fig. 29. (A) and (B) ELECTRON MICROGRAPHS OF THE PRINCIPAL CELL FROM CAPUT AND CAUDA EPIDIDYMAL EPITHELIUM OF ORCHIDECTOMISED RATS GIVEN 0.50 mg BROMOCRYPTINE TREATMENT.

(A) Caput and (B) Cauda x 1600. PC Principal Cell, N Nucleus, BM Basement Membrane. The principal cell in caput and cauda differ in height and ultrastructural details. The principal cell from both regions appear to resemble the orchidectomised control condition. The cell organelles are lacking but large empty vacuoles are in abundance (Arrows).





N

Fig. 29.

of cytoplasmic organelles and empty vacuoles are abundantly seen. Thus, the features associated with degenerative changes caused by orchidectomy seem to persist even after the bromocryptine was administered to these animals.

6.4 DISCUSSION

Orchidectomy has previously been reported to bring about dramatic changes in the histoarchitecture of the epididymis in several laboratory animals. Decrease in tubular diameter, involution of epididymal epithelium, shrinkage of the tubules, irregular tubular borders and in some cases even the sloughing of epididymal epithelium have been reported (Hoffer and Greenberg, 1978; Moore and Bedford, 1979 a; Goyal, 1983). In the present study, a decrease in the epithelial cell height in orchidectomised animals was seen when compared to sham-operated ones by histoquantitative measurements. This may be attributed to the loss of spermatozoa, fluid and androgens of testicular origin that normally support this organ as has also been suggested by several other investigators (Moore and Bedford, 1979 a; Goyal, 1983; Nicander *et al.*, 1983).

It is interesting to find that this effect of orchidectomy is partially restored when prolactin is injected in orchidectomised animals. Several earlier investigations have also suggested that prolactin has growth promoting effects, in addition to playing a role in maintaining the structure of the epididymis (Bartke *et al.*, 1970; Negro-Vilar *et al.*, 1977; Reddy and

Govindappa, 1986). Based on the data on epithelial cell height of this study, it may be concluded that prolactin administration leads to hypertrophy of epithelial cell. Whether cell proliferation also occurs in these orchidectomised animals could not be established. This action of prolactin could not be convincingly confirmed from the results of the bromocryptine treated animals. The bromocryptine preparation used in this study is known to suppress the synthesis and secretion of prolactin (Kinch, 1980; Weinstein *et al.*, 1981) and thus decrease the circulating levels of this hormone in rats (Lloyd *et al.*, 1975; Weinstein *et al.*, 1981). The results of bromocryptine treatment indicate that the withdrawal of prolactin from circulation in orchidectomised rats does not add to the detrimental effects of orchidectomy on the epididymal epithelium. Although orchidectomy slightly increased the thickness of the smooth muscle layer located between the epididymal tubule, neither prolactin nor bromocryptine treatment to these animals was able to influence this parameter. Thus, it becomes clear that the action of prolactin is confined to the cell of epididymal epithelium.

Once it was established that prolactin influenced the epididymal epithelium, more details were sought from electron microscopic studies. The emphasis of this study was on the principal cells since they have been shown by other workers to be the most active among the epididymal epithelial cells and highly responsive to endocrine manipulations (Klinefelter and Amann, 1980; Amann,

1987; Amann *et al.*, 1987; Robaire and Hermo, 1988). Important secretions of the epididymis have been traced to the principal cells (Hoffer *et al.*, 1973). In addition, *in vitro* studies have also proved that the principal cell is involved in the synthesis and secretion of GPC, sialic acids and a number of proteins (Killian and Chapman, 1980). The fine structure of the epididymal duct from the sham-operated animals bear testimony to the fact that principal cells are actively involved in this process. The presence of an elaborate system of endoplasmic reticulum and Golgi apparatus could be taken as an evidence for such a premise. Besides, synthesis and secretion of substances the principal cells are also concerned with absorptive function of the epididymis (Moore and Bedford, 1979a,b; Yeung and Cooper, 1982). Dense bodies, multivesicular bodies, coated and uncoated vesicles are extensively found in the principal cells and could be taken as a direct evidence for the operation of a system that absorbs and transports biomolecules in these cells.

Examination of electron micrographs of the caput and cauda epididymidis from orchidectomised and sham-operated animals clearly reveals that androgens and perhaps the factor(s) present in testicular fluid support the maintenance and functioning of the principal cells. This is reflected in the poor organization and spatial orientation of the cellular organelles in the principal cells from both the caput and cauda epididymidis of orchidectomised rats. Several organelles involved in the synthesis, secretion and absorptive functions of the principal

cells become scarce and the cytoplasm instead shows a large number of empty vacuoles. The empty vacuoles may represent contracted or segmented Golgi lamellae as suggested by Honjin *et al.*, 1960 and Yamaoka *et al.*, 1983. This sort of a situation is seen in both the caput and cauda and probably represents degeneration of the duct on account of increased lysosomal activity. These results are in good agreement with those of Moore and Bedford (1979 a) who have shown by orchidectomy experiments that the absorptive and secretory function of principal cells are androgen dependent. Just as in present study, the principal cells of the caput and cauda epididymidis have been shown to undergo a variety of changes suggestive of ceasation of macromolecule production and an increase in their lysosomal component. The development of rough endoplasmic reticulum in principal cells appears to be dependent on the testicular fluid contained in the lumen of epididymis, because the endoplasmic reticulum regresses after efferent duct ligation (Fawcett and Hoffer, 1979; Nicander *et al.*, 1980). thus, there seems little doubt that vital components of the testicular fluid including androgens are essential for the maintenance of epididymal structure.

Orchidectomy produced degenerative changes in almost all the principal cells belonging to both the caput and cauda epididymal segments. This is understandably so since the principal cells are known to be highly dependent on androgens (Moore and Bedford, 1979 a). Although receptors for prolactin have been

detected in the epididymis (Aragona and Friesen, 1975; Orgebin-Crist and Djiane, 1979), it appears that prolactin is not as potent as androgen in this organ. Thus, when prolactin treatment was given to orchidectomised rats, not all the principal cells from the two epididymal segments responded. However, the population of cells that responded positively increased with the dose of prolactin administered. Based on the structural details of this population of principal cells it appears that prolactin treatment revived the structure of the principal cells that was damaged by orchidectomy.

Despite the variation in the number of responding cells, the nature of at least some changes seems to be similar in the principal cells of all prolactin treated orchidectomised rats, irrespective of the dose administered. The reestablishment of an elaborate endoplasmic reticulum and Golgi apparatus seem to indicate that the principal cells have regained their synthetic and secretory activity. The occurrence of multivesicular bodies, dense bodies, coated and uncoated vesicles in the principal cells of prolactin treated orchidectomised animals is an indication that processes such as transport and absorption of macromolecules are taking place. All evidences thus seem to suggest that prolactin treatment rejuvenates the principal cells in terms of both structure and function in the epididymis of orchidectomised animals. However, when bromocryptine, a drug that is known to reduce endogenous prolactin was administered to orchidectomised rats, the fine structure of the principal cells in both

epididymal segments was not significantly changed from those of the orchidectomised control that did not receive the drug. It is difficult to say if bromocryptine further deterioated the effect of orchidectomy on the principal cells. But, it is quite clear that the drug did not act in a manner that could be considered beneficial.

It is well recognized that in the rat, sperm maturation occurs in the caput and sperm storage in the cauda (Hamilton, 1975). Inspite of being involved in entirely different functions, it is interesting to note that the principal cells from these two segments respond to orchidectomy and subsequent treatment with prolactin or bromocryptine in a more or less identical fashion. Thus, it is clear that the action of prolactin is not restricted to any one segment of the epididymis.

The present study demonstrates a tropic action of prolactin on the epididymis of orchidectomised rats. From the results it is clear that at least in the absence of a major source of androgen, prolactin may play an important role in maintaining the structural integrity of the epididymis. However, the physiological significance of this action of prolactin is not clear at the present time.

6.5 CONCLUDING REMARKS

1. The effect of prolactin on the structure of the epididymis has been studied with particular emphasis on the principal cells.

2. Transmission electron microscopic studies of the epididymal epithelium lend support to the view that the principal cells are involved in secretory and absorptive functions.

3. The degenerative changes occurring in the epididymis of orchidectomised rat indicate that the maintenance of structural integrity of the principal cells depends to a great extent on factors originating in the testis.

4. Prolactin treatment to orchidectomised animals provides a positive stimulus for the revival of the structure and function of the principal cells that were thrown into disarray by orchidectomy. The population of principal cells that responded to prolactin treatment increase with the dose of hormone administered.

5. Bromocryptine treatment to orchidectomised animals was without effect.

6. Prolactin had a similar effect on both the caput and cauda epididymidis.

By using orchidectomised rats in the present study as an appropriate model, it was possible to demonstrate that the observed structural changes in the principal cells were due to prolactin and not factors originating in the testis. The studies have also shown that prolactin plays an important role in the maintenance of epididymal structure at least when a major source of androgen is absent.

REFERENCES

1. Aafjes JH and Vreeburg JTM (1972) Distribution of 5α-dihydrotestosterone in the epididymis of bull and boar, and its concentration in rat epididymis after ligation of efferent testicular ducts, castration and unilateral gonadectomy. *J Endocr* **53**: 85-93.

2. Abe K, Takano H and Ito T (1983) Ultrastructure of the mouse epididymal duct with special reference of the principal cells. *Arch histol jap* **46**: 51-68.

3. Abe K, Takano H and Ito T (1984) Interruption of the luminal flow in the epididymal duct of the corpus epididymidis in the mouse, with special reference to differentiation of the epididymal epithelium. *Arch histol jap* **47(2)**: 137-147.

4. Abraham KA and Bhargava PM (1963a) Nucleic acid metabolism of mammalian spermatozoa. *Biochem J* **86**: 298-307.

5. Abraham KA and Bhargava PM (1963b) The uptake of radioactive amino acids by spermatozoa. *Biochem J* **86**: 308-313.

6. Acott TS and Hoskins DD (1981) Bovine sperm forward motility protein: binding to epididymal spermatozoa. *Biol Reprod* **24**: 234- 240.

7. Adrian TE, Gu J, Allen JM, Tatemoto K, Polak JM and Bloom SR (1984) Neuropeptide Y in the human male genital tract. *Life Sci* **35**: 2643-2648.

8. Alexander NJ (1972) Prenatal development of the ductus epididymidis in the rhesus monkey. The effects of fetal castration. *Am J Anat* **135**: 119-134.

9. Allison VF and Hartree EF (1970) Lysosomal enzymes in the acrosome and their possible role in fertilization. *J Reprod Fert* **21**: 501-515.

10. Amann RP (1987) Function of the epididymis in bulls and rams. *J Reprod Fertil (Suppl)* **34**: 115-131.

11. Amann RP, Johnson L, Thompson DL and Pickett BW (1976) Daily spermatozoal production, epididymal spermatozoal reserves and transit times of spermatozoa through the epididymis of the rhesus monkey. *Biol Reprod* 15: 586-592.

12. Amann RP, Marengo SR and Brown DV (1987) Steroidogenesis and testosterone metabolism in cultured principal cells from the ram epididymis. *J Androl* 8: 238-246.

13. Amit T, Barkey RJ and Youdim MBH (1983) Effect of prolactin, testosterone and estrogen on prolactin binding in the rat testis, prostate, seminal vesicle and liver. *Mol Cell Endocr* 30: 179-187.

14. Aragona C and Friesen HG (1975) Specific prolactin binding sites in prostate and testis of rats. *Endocrinology* 97: 677-684.

15. Archer DF (1977) Current concepts of prolactin physiology in normal and abnormal conditions. *Fert Steril* 28: 125-134.

16. Au CL, Nagl HK, Yeung CH and Wong PYD (1978) Effect of adrenalectomy and hormone replacement on sodium and water transport in the perfused rat cauda epididymis. *J Endocr* 77: 265-266.

17. Back DJ (1975) The presence of metabolites of ^{3}H-testosterone in the lumen of the cauda epididymidis of the rat. *Steroids* 25: 413-420.

18. Barkey HWG, Worgut TJ, Santen RJ, Jefferson LS and Bardin CW (1977) Effect of prolactin on nuclear androgens in perfused male accessory sex organs. In: *Testis in Normal and Infertile men*. Eds P Troen and HR Nankin, Raven Press, New York. pp 379-385.

19. Barkey RJ, Shani J, Amit T and Barzilai D (1977a) Specific binding of prolactin to seminal vesicle, prostate and testicular homogenates of immature, mature and aged rats. *J Endocr* **74**: 163-173.

20. Barkey RJ, Shani J, Amit T and Barzilai D (1977b) Characterization of the specific binding of prolactin to binding sites in the seminal vesicle of the rat. *J Endocr* **80**: 181-189.

21. Barkey RJ, Shani J, Barzilai D (1979) Regulation of prolactin binding sites in the seminal vesicle, prostate gland, testis and liver of intact and castrated adult rats: effect of administration of testosterone, 2-bromo-α-ergocryptine and fluphenazine. *J Endocr* **81**: 11-18.

22. Bartke A (1967) Influence of pituitary homografts on the weight of seminal vesicle in castrated mice. *J Endocr* **38**: 195-196.

23. Bartke A (1976) Pituitary-testis relationship: Role of prolactin in the regulation of testicular function. *Progr Reprod Biol* **1**: 136-150.

24. Bartke A (1980) Role of prolactin in reproduction in male mammals. *Fed Proc* **39**: 2577-2581.

25. Bartke A and Lloyd CW (1970) The influence of pituitary homografts on the weight of sex accessories in castrated male mice and rats and on mating behaviour in male mice. *J Endocr* **46**: 313-320.

26. Bartke A, Smith MS, Michael SD, Peron FG and Dalterio S (1976) Effects of experimentally-induced chronic hyperprolactinemia on testosterone and gonadotropin levels in male rats and mice. *Endocrinology* **100**: 182-186.

27. Bartsch G, Oberholzer M, Holliger O, Weber J, Weber A and Rohr HP (1978) Sterology: a new quantitative method to study epididymal function. *Andrologia* 10: 31-42.

28. Baumgarten HG, Holstein AF and Rosengren E (1971) Arrangement, ultrastructure and adrenergic innervation of smooth musculature of the ductuli efferentes, ductus epididymidis and ductus deferens of man. *Z Zellforsch* 120: 37-79.

29. Bedford, JM (1963) Changes in the electrophoretic properties of rabbit spermatozoa during passage through the epididymis. *Nature* 200: 1178-1180.

30. Bengmark S and Hesselsjo R (1963) The combined effect of prolactin and androsterone on the growth of rat seminal vesicle *in vitro*. *Urol Int* 16: 387-390.

31. Bernals A, Torres J, Reyes A and Rosado A (1980) Presence and regional distribution of sialyltransferase in the epididymis of the rat. *Biol Reprod* 23: 290-293.

32. Bernheim F, Bernheim MLC and Wilbur KM (1948) The reaction between thiobarbituric acid and the oxidation products of certain lipids. *J Biol Chem* 174: 257-264.

33. Berridge MJ and Irvine RF (1984) Inositol triphosphate, a novel second messenger in cellular signal transduction. *Nature (London)* 312: 315-317.

34. Besser GM, Thorner MO and Wass JAH (1977) A prolactinemia-hypogonadism syndrome-medical treatment. In: *Endocrinology, Vol II Experta Medica*. Ed VHT James, Oxford, Amsterdam. pp 353-357.

35. Bey E (1965) The electrophoretic mobility of sperm cells. In: *Cell Electrophoresis*, Ed EJ Ambrose, Churchill, London. pp 142-151.

36. Beyer TA, Rearick JI, Paulson JC, Prieels JP, Sadler JE and Hill RL (1979) Biosynthesis of mammalian glycoproteins: glycosylation pathways in the synthesis of the non reducing terminal sequences. *J Biol Chem* **254**: 12531-12541.

37. Bishop DW (1961) Biology of spermatozoa. In: *Sex and Internal Secretions, Vol II, 3rd Edn*, Ed WC Young, Williams and Wilkins Co, Baltimore. pp 707-796.

38. Blix G (1936) *Z Physiol Chem* 240: 43.

39. Blix G, Svennerholm L and Warner I (1952) The isolation of chondrosamine from gangliosides and submaxillary mucin. *Acta Chem Scand* 6: 358-362.

40. Blix FG, Gottschalk A and Klenk E (1957) Proposed nomenclature in the field of neuraminic acid and sialic acid. *Nature* 179: 1088.

41. Bose AR and Kar AB (1968) Distribution of sialic acid in the genital organs of male rhesus monkeys: effect of castration and replacement therapy. *Curr Sci* 37: 168-169.

42. Bose AR, Kar AB and Dasgupta PR (1966) Sialic acid in the genital organs of the male rat. *Curr Sci* 35: 336-337.

43. Bostwick EF, Bently MD, Hunter AG and Hammer R (1980) Identification of a surface glycoprotein on porcine spermatozoa and its alteration during epididymal maturation. *Biol Reprod* 23: 161-169.

44. Brandt H, Acott TS, Johnson DJ and Hoskins DD (1978) Evidence for an epididymal origin of bovine sperm forward motility protein. *Biol Reprod* **19**: 830-835.

45. Brooks DE (1977) The androgenic control of the composition of the rat epididymis determined by efferent duct ligation or castration. *J Reprod Fert* **49**: 383-385.

46. Brooks D E (1979) Biochemical environment of maturing spermatozoa. In: *The Spermatozoon.* Eds DW Fawcett and JM Bedford, Urban and Schwarzenberg, Baltimore. pp 23-34.

47. Brooks DE (1984) Epididymal functions and their hormonal regulation. *Aust J Bio Sci* 36: 205-221.

48. Brooks DE and Higgins SJ (1980) Characterization and androgen dependence of proteins associated with luminal fluid and spermatozoa in rat epididymis. *J Reprod Fert* 59: 363-375.

49. Brooks DE and Tiver K (1984) Analysis of surface proteins of rat spermatozoa during epididymal transit and identification of antigens common to spermatozoa, rete testis fluid and cauda epididymal plasma. *J Reprod Fert* 71: 249-257.

50. Brooks DE, Hamilton DW and Mallek AH (1973) The uptake of L-(methyl -^{3}H) carnitine by the rat epididymis. *Biochim Biophys Res Commun* 52: 1354-1360.

51. Brooks DE, Hamilton DW and MallcK AH (1974) Carnitine and glycerylphosphorylcholine in the reproductive tract of the male rat. *J Reprod Fert* 36: 141-160.

52. Brooks DE, Means AR, Wright EJ, Singh SP and Tiver KK (1986) Molecular cloning of the cDNA for two major androgen-dependent secretory proteins of 18.5 kilo daltons synthesized by the rat epididymis . *J Biol Chem* 26: 4961-4965.

53. Buvat J, Lemaire A, Buvat-Herbaut M, Fourlinnie JC, Racadot A and Fossati P (1985) Hyperprolactinemia and sexual function in men. *Hormone Res* 22: 196-203.

54. Carter JN, Tyson JE, Tolis G, van Vliet S, Faimen C, Friesen HG (1978) Prolactin-secreting tumors and hypogonadism in 22 men. *N Engl J Med* 299: 847-852.

55. Chang RJ (1978) Normal and abnormal prolatin metabolism. *Clin Obstet Gynecol* 21: 125-138.

56. Chargraff E, Stewart RN and Magasanik B (1948) Inhibition of mitotic poisoning by *meso*-inositol. *Science* 108: 556-558.

57. Charreau EH, Attramadal A, Torjesen PA, Purvis K, Calendra R and Hansson V (1977) Prolactin binding in rat testis: specific receptors in interstitial cells. *Mol Cell Endocr* 6: 303-307.

58. Chen CL, Amenomori Y, Lu KH, Voogt JL and Meites J (1970) Serum prolactin levels in rats with pituitary transplants or hypothalamic lelsions. *Neuroendocrinology* 6: 220-227.

59. Chi HJ and Shin SH (1978) The effect of exposure to ether on prolactin secretion and the half life of endogenous prolactin secretion. *Endocrinology* 102: 1614-1620.

60. Chulavatnatol M, Eksittikul T and Toowicharanont P (1978) Control of epididymal sperm motility and approach to male fertility regulation. *Int J Androl Suppl* 2: 629-638.

61. Clark RH and Baker BL (1964) Circadian periodicity in the concentration of prolactin in the rat hypophysis. *Science NY* 143: 375-376.

62. Clemens JA (1976) Neuropharmacological aspects of the neural control of prolactin secretion. In: *Hypothalamus and endocrine functions*, Eds F Labrie, J Meites and G Palletner, Plenum Press, New York. pp 283-302.

63. Clemens JA and Sawyer ED (1974) Identification of prolactin in cerebrospinal fluid. *Exp Brain Res* 21: 399-402.

64. Clements JA, Reyes FL, Winter JSD and Faimen C (1977) Studies on human sexual development IV. Fetal pituitary and serum and amniotic fluid concentrations of prolactin. *J Clin Endocr Metab* 44: 408-413.

65. Cohen H, Sabbagh I, Guillaumot P, Bertrand J, Robertson M and Friesen HG (1988) Relationship between bioassay and radioimmunoassay measurement of prolactin in the IPL rat, a hypoprolactinemic rat strain. *Biol Reprod* **34**: 84-90.

66. Conchie J and Findlay J (1959) Influence of gonadectomy, sex hormones and other factors, on the activity of certain glycosidases in the rat and mouse. *J Endocr* **18**: 132-146.

67. Conchie J and Hay AJ (1959) Mammalian glycosidases: 2 properties of α-mannosidase and β-galactosidase from rat epididymis. *Biochem J* **73**: 327-334.

68. Conchie J and Mann T (1957) Glycosidases in mammalian sperm and seminal plasma. *Nature* **179**: 1190-1191.

69. Conchie J, Findlay J and Levvy GA (1959a) Mammalian glycosidases: Distribution in the body. *Biochem J* **71**: 318-325.

70. Conchie J, Findlay J and Levvy GA (1959b) Glycosidases in some farm animals. *Nature* **183**: 615-616.

71. Cooper TG (1980) The general importance of proteins and other factors in the transfer of steroids into the rat epididymis. *Int J Androl* **3**: 333-348.

72. Cooper TG (1986) Function of the epididymis and its secretory products. In: *The Epididymis, Sperm Maturation and Fertilization*. Springer-Verlag, Berlin, Heidelberg, New York, London, Paris, Tokyo. pp 117-217.

73. Cooper TG and Hamilton DW (1977) Phagocytosis of spermatozoa in the terminal region and gland of the vas deferens of the rat. *Am J Anat* **150**: 247-268.

74. Cooper TG and Waites GMH (1979) Factors affecting the entry of testosterone into the lumen of the cauda epididymidis of the anesthetized rat. *J Reprod Fert* **56**: 165-174.

75. Cooper TG and Waites GMH (1980) Role of proteins in the transport of steroids into the epididymis. **In:** *Testicular Development, Structure, and Function*, Eds A Steinberger and E Steinberger, Raven Press, New York. pp 299-304.

76. Costlow ME and McGuire WL (1977) Autoradiographic localization of the binding of ^{125}I-labelled prolactin to rat tissues *in vitro*. *J Endocr* 75: 221-226.

77. Costlow ME, Buschow RA, McGuire WL (1975) Prolactin stimulation of prolactin receptors in rat liver. *Life Sci* 17: 1457-1466.

78. Courot M (1981) Transport and maturation of spermatozoa in the epididymis of mammals. **In:** *Epididymis and Fertility: Biology and Pathology*, Eds C Bollack and A Clavert, Karger, Munich. pp 67-79.

79. Cushman DW and Cheung HS (1971) Concentrations of angiotensin converting enzyme in tissues of the rat. *Biochim Biophys Acta* 250: 261-265.

80. Czuppon AB (1984) Biochemical characterization of a human spermatozoal sialoglycoprotein with respect to antigenicity masking by its sialic acid moieties. *Biochem Int* 8: 9-18.

81. Daucheux JL, Paquignon M and Combarnous Y (1983) Head - to - head agglutination of ram and boar epididymal spermatozoa and evidence for an epididymal antagglutinin. *J Reprod Fert* 67: 181-189.

82. D'Agnostino A, Jones R, White R and Parker MG (1980) Androgenic regulation of messenger RNA in rat epididymis. *Biochem J* 190: 505-512.

83. David GFX, Herbert J and Wright GDS (1973) The ultrastructure of the pineal ganglion in the ferret. *J Anat* 115(1): 79-97.

84. Dawson RMC and Rowlands IW (1959) Glyceryl phosphoryl choline in the male reproductive organs of rats and guinea pigs. *Quart J Exp Biol* **44**: 26-34.

85. Demerast KT, Riegle GD and Moore KE (1986) The rapid tonic and the delay induction component of prolactin induced activation of tuberoinfundibular dopaminergic neurons following the systemic administration of prolactin. *Neuroendocrinology* **43**: 291-299.

86. Dericks-Tan JSE, Bradler H and Taubert HD (1978) Evaluation of proteohormone concentrations in serum and seminal fluid and its relationship with sperm count. *Andrologia* 10: 175-182.

87. Diamond EJ, Khan S, Paul MA, Koprak S and Hollander VP (1980) Chronic administration of pharmacological doses of ovine prolactin decreases hormone disapperance rate. *Endocrinology* 106: 952-958.

88. Djakiew D, Byers SW and Dym M (1984) Receptor-mediated endocytosis of alpha 2-macroglobulin and transferrin in rat caput epithelial cells *in vitro*. *Biol Reprod* 31: 1073-1085.

89. Djiane J, Clauser H and Kelley PA (1979a) Rapid down-regulation of prolactin receptors in mammary gland and liver. *Biochem Biophys Res Common* 90: 1371-1378.

90. Djiane J, Delouis C and Kelley PA (1979b) Prolactin receptors in organ culture of rabbit mammary gland: effect of cycloheximide and prolactin. *Proc Soc Exp Biol Med* 162: 342-345.

91. Djoseland O, Hansson V and Haugten HN (1973) Androgen metabolism by rat epididymis. 1. Metabolic conversion of ^{3}H-testosterone *in vivo*. *Steroids* 21: 733-783.

92. Djoseland O, Hansson V and Haugten HN (1974) Androgen metabolism by rat epididymis 2. Metabolic conversion of ^{3}H-testosterone *in vitro*. *Steroids* 23: 397-410.

93. Doherty PC, Smith MS and Bartke A (1980) Hypoprolactinemia and reproductive functions in male rats. Effects of adrenalectomy and bromocryptine. *Proceedings of 62 annual meeting of Endocrinological Society*, pp 704-708.

94. Donofrio RJ and Reiter RJ (1972) Depressed pituitary prolactin levels in blinded anosmic female rats: role of the pineal gland. *J Reprod Fert* **31**: 159-162.

95. Dufy B, Vincent JD, Fleury H, Du Pasquier P, Gourdji D and Tixier-Vidal A (1979) Dopamine inhibition of action potentials in prolactin secreting cell line is modulated by estrogen. *Nature* **282**: 855-857.

96. Eagle H, Oyama VI, Levy M and Freeman AE (1957) *Myo*-inositol as an essential growth factor for normal and malignant human cells in tissue culture. *J Biol Chem* **226**: 292-293.

97. Ekins RP (1978) General principles of hormone assay. **In:** *Hormone assay and their clinical applications*, Eds JA Loraine and ET Bell, Churchill and Livingstone, Edinburgh. pp 1-72.

98. Elson ED, Browne CA and Thorburn GD (1984) Identification of epidermal growth factor like activity in human male reproductive tissues and fluids. *J Clin Endocr* **58**: 589-594.

99. Evans RW and Setchell BP (1979) Lipid changes during epididymal maturation in ram spermatozoa collected at different times of the year. *J Reprod Fert* **57**: 197-203.

100. Everett JW (1954) Luteotrophic function of autografts of the rat hypophyses. *Endocrinology* **54**: 685-690.

101. Fang S and Liao S (1971) Androgen receptors. Steroid- and tissue- specific retention of a 17β-hydroxy-5α-androstan-3-one-protein complex by the cell nuclei of ventral prostate. *J Biol Chem* **246**: 16-24.

102. Fang VS, Refetoff S and Rosenfield RL (1974) Hypogonadism induced by a transplantable, prolactin producing tumor in male rats: *Hormonal and Morphological Studies* **95**: 991-998.

103. Farooqui AA and Srivastava PN (1980) Isolation of β-N-acetyl hexosaminidase from rabbit semen and its role in fertilization. *Biochem J* **191**: 827-834.

104. Fawcett DW and Hoffer AP (1979) Failure of exogenous androgen to prevent regression of the initial segments of the rat epididymis after efferent duct ligation or orchidectomy. *Biol Reprod* **20**: 162-181.

105. Fischer RA and Yates F (1978) In: *Statistical Tables for Biological Agricultural and Medical Research*, Oliver and Boyd, London.

106. Flickinger CJ (1969) Fine structure of the Wolffian duct and cyto differentiation of the epididymis in fetal rats. Z *Zerforsch* **96**: 344-360.

107. Flückiger E (1978) Ergot alkaloids and the modulation of hypo-thalamine function. In: *Pharmacology of the hypothalamus*, Eds B Cox, ID Morris and AH Weston, Macmillan, London. pp 137-160.

108. Flückiger E and Vigouret JM (1979) Drugs, dopamine and pituitary secretion. In: *Medicinal Chemistry, VI, Proceedings of 6th International Symposium on Medicinal Chemistry*, Ed MA Sinkins, Costworld, Oxford. pp 469-477.

109. Flückiger E and Wagner HR (1968) 2 Br-α-ergokryptin: Beeinflussung von fertilitat and laktation bei der Ratte. *Expertienti* **24**: 1130-1131.

110. Flückiger E, del Pozo E and von WerderK (1982) Non-tumoral hyperprolactinemia. In: *Monographs on Endocrinology*, Vol 23, *Prolactin physiology, pharmacology and clinical findings*, Eds F Gross, M M Grumbach, A Labhart, M B Lipsett, T Mann, LT Samuels and J Zander, Springer-Verlag, Berlin, Heidelberg, New York. pp 102-136.

111. Fournier S (1966) Repartition de l'acide sialique dans l'appareil genital rat *Wistar* d'adulte normal et castre. *Comptes Rendus Soc de Biol* 160: 1087-1090.

112. Frankel AI and Eik-Nes KB (1970) Testosterone and dehydroepiandrosterone in the epididymis of the rabbit. *J Reprod Fert* 23: 441-445.

113. Franks S, Jacobs HS, Martin N and Nabarro JDN (1978) Hyperprolactinemia and impotence. *Clin Endocr* 8: 277-287.

114. Fransworth WE (1972) Prolactin and the prostate. In: *Prolactin and Carciogenenis*, Eds AR Boyns and K Griffiths. Alpha Omega Alpha, Cardiff, Wales. pp 217-228.

115. Frantz AG (1973) The regulation of prolactin secretion in humans In: *Frontiers in Neuroendocrinology*, Eds WF Ganong and L Martini, Oxford University Press, New York. pp 337-374.

116. Frantz AG and Kleiberg DL (1978) The pathophysiology of hyperprolactinemic states and the role of newer ergot compounds in their treatment. *Fed Proc* 37: 2192-2196.

117. Frantz AG, Kleinberg DL and Noel GL (1972) Studies on prolactin in man. *Recent Prog Horm Res* 28: 527-573.

118. Friedgood HB and Dawson AB (1940) Physiological significance and morphology of the carmine cell in the cat's anterior pituitary. *Endocrinology* 26: 1022-1031.

119. Fuxe K, Hokfelt T and Jonsson G (1970) Participation of central monoaminergic neurons in regulation of anterior pituitary secretion. In: *Neurochemical aspects of Hypothalamic Functions*, Eds M Martin and J Meites, Academic Press, New York. pp 61-83.

120. Gaddum JH (1933) *Spec Rep Ser Med Res Coun* Lond, No. 183.

121. Gala RR, Singhakowinta A and Brennan MJ (1975) Studies on prolactin in human serum, urine and milk. *Horm Res* 6: 310-320.

122. Gallagher TF (1928) Distribution of testicular comb growth stimulating principles in tissues. *Am J Physiol* 87: 447-449.

123. Giron-Forest DA and Dieter Schönleber W (1979) Bromocryptine methanesulphonate. In: *Analytical profiles of Drug Substances*. 8: 47-81.

124. Glover TD and Nicander L (1971) Some aspects of structure and function of mammalian epididymis. *J Reprod Fert Suppl* 13: 39-50.

125. Go KJ and Wolf DP (1983) The role of sterols in sperm capacitation. *Adv Lipid Res* 20: 317-330.

126. Goldman BD, Grazia YR, Kamberi IA and Porter JC (1971) Serum gonadotropin concentrations in intact and castrated neonatal rats. *Endocrinology* 88: 771-775.

127. Gonzales GF, Velasquez G and Garcia-Hjarles M (1989) Hyperprolactinemia as related to semen quality and serum testosterone. *Arch Androl* 23(3): 259-266.

128. Goyal HO (1983) Histoquantitative effects of orchidectomy with and without testosterone enanthate treatment on the bovine epididymis. *Am J Vet Res* 44(6): 1085-1090.

129. Goyal HO (1985) Morphology of the bovine epididymis. *Am J Anat* 172: 155-172.

130. Grayhack JT and Lebowitz JN (1967) Effect of prolactin on citric acid of lateral lobe of prostate of Sprague-Dawley rat. *Invest Urol* 5: 87-94.

131. Grosser PM and Robaire B (1987) Modulation of plasma LH, FSH, and prolactin patterns by castration or castration and partial testosterone-replacement in adult male rats. In: *Cell Biology of Testis and Epididymis,* Eds MC Orgebin-Crist and BJ Danzo, Ann New York Acad Sci. 513: 332-336.

132. Grosvenor CE (1967) Disappearance rate of exogenous prolactin from serum of female rats. *Endocrinology* 80: 195-200.

133. Grosvenor CE and Turner CW (1958) Assay of lactogenic hormone. *Endocrinology* 63: 530-534.

134. Grosvenor CE and Whitworth NS (1979) Secretion rate and metabolic clearance rate of prolactin in the rat during mid- and late-lactation. *J Endocr* 82: 409-415.

135. Gupta G, Rajalakshmi M, Prasad MRN and Moudgal NR (1974) Alteration of epididymal function and its relation to maturation of spermatozoa. *Andrologia* 6: 35-44.

136. Gwatkin RBL and Anderson OF (1973) Effect of glycosidase inhibitors on the capacitation of hamster spermatozoa by cumulus cells *in vitro*. *J Reprod Fert* 35: 565-567.

137. Hall JC and Killian GJ (1987) Changes in rat sperm membrane glycosidase activities and carbohydrate and protein contents associated with epididymal transit. *Biol Reprod* 36: 709-718.

138. Hamilton DW (1971) Steroid function in the mammalian epididymis. *J Reprod Fert Suppl* 13: 89-97.

139. Hamilton DW (1972) The mammalian epididymis. In: *Reproductive Biology*, Eds H Balin and S Glasser, Excerpta Medica Monograph, Amsterdam, pp 268-337.

140. Hamilton DW (1975) Structure and function of the epithelium lining and ductuli efferentes, ductus epididymidis and ductus deferens in the rat. **In:** *Handbook of Physiology, Section VII, Endocrinology,* **Vol 5,** *Male reproductive system,* Eds DW Hamilton and RO Greep, Am Physiol Soc, Washington DC, pp 259-301.

141. Hamilton DW and Fawcett DW (1970) *In vitro* synthesis of cholesterol and testosterone from acetate by rat epididymis and vas deferens. *Proc Soc Exp Biol (NY)* **133**: 693-695.

142. Hamilton DW and Gould RP (1980) Galactosyltransferase activity associated with rat epididymal spermatozoan maturation. *Anat Rec* 196, 71a.

143. Hamilton DW, Jones AL and Fawcett DW (1969) Cholesterol biosynthesis in the mouse epididymis and ductus deferens: abiochemical and morphological study. *Biol Reprod* 1: 167-184.

144. Hamilton DW, Olson GE and Beeuwkes R (1976) Epididymal physiology and sperm maturation. **In:** *Progress in Reproductive Biology* **Vol I,** Ed PO Hubinot and S Karger, Basel, pp 62-73.

145. Hammerstedt RH and Amann RP (1976) Interconversion of steroids by intact bovine sperm and epididymal tissue. *Biol Reprod* **15**: 686-694.

146. Harper ME, Danutra V, Chandler JA and Griffiths K (1976) The effect of 2-bromo-α-ergocryptine (CB 154) administration on the hormone levels, organ weights, prostatic morphology and zinc concentration in the male rat. *Acta Endrocrinol (Copenh)* 83: 211-224.

147. Hart IC (1973) Basal level of prolactin in goat blood measured throughout a 24 h period by a rapid double antibody solid phase radioimmunoassay. *J Dairy Res* 40: 235.

148. Hayashi M (1967) Comparative histochemical localization of lysosomal enzymes in rat tissues. *J Histochem Cytochem* **15**: 83-92.

149. Herbert DC, Ishikawa H and Ronnels EG (1979) Evidence for the autoregulation of hormone secretion by prolactin. *Endocrinology* **104**: 97-100.

150. Hermo L and Morales C (1984) Endocytosis in non - ciliated epithelial cells of the ductuli efferentes in the rat. *Am J Anat* **171**: 59-74.

151. Hinton BT and Setchell BP (1980) Concentrations of glycerophosphocholine and free inorganic phosphate in the luminal fluid of the rat testis and epididymis. *J Reprod Fert* **58**: 401-406.

152. Hoffer AP and Greenberg J (1978) The structure of the epididymis, efferent ductules and ductus deferens of the guinea pig: A light microscope study. *Anat Rec* **190**: 659-678.

153. Hoffer AP, Hamilton DW and Fawcett DW (1973) The ultrastructure of the principal cells and intraepithelial leucocytes in the initial segment of the rat epididymis. *Anat Rec* **175**: 169-202.

154. Hoffman DS and Killian GJ (1981) Isolation of epithelial cells from the corpus epididymis and analysis for glyceryl phosphoryl choline, sialic acid and protein . *J Exp Zool* **217**: 93-102.

155. Holmes RL and Ball JN (1974) *The Pituitary Gland: A comparative account*. Cambridge University Press, London. pp 26-53.

156. Honjin R, Uozu T and Nakanishi A (1960) Electron microscopic studies on the epithelial cells of the epididymis, with special reference to the morphological changes of the intracellular ultrastructures induced by experimental section of the pelvic nerve and castration. *Report 3 On the effect of castration on the intracellular ultrastructure,* Juzenkaishi, Univ Kanazawa, **64**: 281-288.

157. Hostetter MW and Piacsete B (1977) The effect of prolactin deficiency during sexual maturation in the male rat. *Biol Reprod* **17**: 574-577.

158. Houdebine L-M and Djiane J (1980) Effect of lysomotropic agents and of microfilament and microtubule disurpting drugs on the activation of casein-gene expression by prolactin in the mammary gland. *Mol Cell Endocr* **17**: 1-15.

159. Hunter AG and Schellpfeffer DA (1981) Concentrations of sperm, protein and a sperm membrane glycoprotein within boar epididymal luminal fluids. *J Anim Sci* **52**: 575-579.

160. Jacobs LS, Synder PJ, Wilber JP, Utiger RD and Daughaday WH (1971) Increased serum prolactin after administration of synthetic TRH in man. *J Clin Endocr Metab* **33**: 996-998.

161. Jenkins AD, Lechene CP and Howards SS (1983) The effect of estrogen administration *in vivo* on the elemental composition of intraluminal fluids of the seminiferous tubules, rete testis and epididymis of the rat. *J Androl* **4**: 272-275.

162. Jones R and Glover TD (1975) Interrelationship between spermatozoa, the epididymis and epididymal plasma. In: *The Biology of the Male Gamete.* Eds JG Duckett and PM Racey, Suppl No 1 to the Biol *J Linnaen Soc* **7**: 367-384.

163. Jones R, Hamilton DW and Fawcett DW (1979) Some effects of testosterone, 5α-dihydrotestosterone, and 5α-androsterone-3α, 17β-diol on the morphology of the rabbit epididymis and prostate. *J Ultrastruct Res* 67: 194-208.

164. Jones R, von Glos KI and Brown CR (1980) The synthesis of a sperm - coating protein in the initial segment of the rat epididymis is stimulated by factors in testicular fluid. *IRCS Med Sci* 8: 56.

165. Jones R, Pholpramool C, Setchell BP and Brown CR (1981) Labeling of membrane glycoproteins on rat spermatozoa collected from different regions of the epididymis. *Biochem J* 200: 457-460.

166. Jones R, Fournier-Delpech S and Willadson SA (1982) Identification of androgen-dependent proteins synthesized *in vitro* by the ram epididymis. *Reprod Nutr Develop* 22: 495-504.

167. Jones R, Riding PR and Parker MG (1983) Effects of prolactin on testosterone - induced growth and protein synthesis in rat accessory glands. *J Endocr* 96: 407-416.

168. Jones R, Brown CR, von Glos KI and Gaunt SJ (1985) Development of a maturation antigen of the plasmamembrane of rat spermatozoa in the epididymis and its fate during fertilization. *Exp Cell Res* 156: 31-44.

169. Josimovich JB, Merisko K, Boccella L and Tobon H (1977) Binding of prolactin by fetal rhesus cell membrane fractions. *Endocrinology* 100: 557-563.

170. Karg H and Shams D (1970) Action of prolactin. In: *Proceedings of the International Symposium on Lactation Nottingham,* Ed IR Falconer, Butterworth, London, Sydney, Washington, pp 233-237.

171. Killian GJ and Amann RP (1973) Immunoelectrophoretic characterization of fluid and sperm entering and leaving the bovine epididymis. *Biol Reprod* 9: 489-499.

172. Killian GJ and Chapman DA (1980) Glycerylphosphocholine, sialic acid and protein in epithelial cells isolated from the rat caput epididymis by elutriation. *Biol Reprod* 22: 846-850.

173. Kinch RA (1980) The use of bromocryptine in obstretics and gynecology. *Fert Steril* 33(5): 463-470.

174. Kirazov EP and Lagnado JR (1977) Interaction of *myo*-inositol with brain microtubules. *FEBS Lett* 81: 173-178.

175. Kledzik GS, Marshall S, Campbell GA, Gelato M and Meites J (1975). Effects of castration, testosterone, estradiol, and prolactin on specific prolactin binding activity in ventral prostate of male rats. *Endocrinology* 98: 373-379.

176. Klinefelter GR and Amann RP (1980) Metabolism of testosterone by principal cells and basal cells isolated from the rat epididymal epithelium. *Biol Reprod* 22: 1149-1154.

177. Koch Y, Chow YF and Meites J (1971) Metabolic clearance and secretion rates for prolactin in the rat. *Endocrinology* 89: 1303-1308.

178. Koeing JI, Mayfield MP, McCann SM and Krulich L (1979) Stimulation of prolactin secretion by morphine: role of the central serotonergic system. *Life Sci* 25: 853-864.

179. Kolodny RC, Jacobs LS and Daughaday WH (1972) Mammary stimulation causes prolactin secretion in non - lactating women. *Nature* 238: 284-286.

180. Kumari GL, Allag IS, Das RP and Datta JK (1980) Regional differences in steroidogenesis and hormone levels in the epididymis and vas deferens of adult rats. *Int J Androl* 3: 267-281.

181. Kurcz M (1971) Prolactin. In: *Polypeptide Hormones. Proceedings of the Fourth Congress of the Hungarian Society of Endocrinology and Metabolism,* Part V, *Prolactin, Publishing House of the Hungarian Academy of Sciences,* Budapest. pp 231-246.

182. Laporte P, Guillet J and Peyre A (1975) Les sialoproteines epididymaires actions de quelques facteurs exogenes et endogenes. *Ann Biol Anim Biochem Biophys* 15: 669-683.

183. Larsson LI, Fahrenkrug J and Schaffalitzky de Muchadell OB (1977) Occurrence of nerves containing vasoactive intestinal polypeptide immunoreactivity in the male genital tract. *Life Sci* 21: 503-508.

184. Lea OA, Petrusz P and French FS (1978) Purification and localization of acidic epididymal glycoprotein (AEG): a sperm coating protein secreted by the rat epididymis. *Int J Androl Suppl 2*: 592-607.

185. Lesniak MA, Gorden Pand Roth J (1977) Reactivity of non-primate growthhormones receptors on cultured human lymphocytes. *J Clin Endocr Metab* **44**: 838-849.

186. Levine N and Marsh DJ (1971) Micropuncture studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tubules, the epididymis and vas deferens. *J Physiol* **213**: 557-570.

187. Lewin LM and Sulimovici S (1975) The distribution of radioactive *myo*-inositol in the reproductive tract of the male rat. *J Reprod Fert* **43**: 355-358.

188. Lewin LM, Yannai Y and Kraicer PF (1978) The distribution of radioactive *myo*-inositol in the reproductive tract of the male rat. The blood-testis barrier to *myo*-inositol. In: *Cyclitols and Phosphoinositides*. Eds WW Wells and F Eisenberg, Acad Press, New York. pp 317-322.

189. Lewis PR and Knight DP (1977) Staining methods for sectioned material. In: *Practical Methods in Electron Microscopy*, vol V *part I*. Ed AM Glauert, Elsevier/North Holland, Amsterdam, pp 1-311.

190. Li CH, Dixon JS, Lo TB, Schmidt KD and Pankov YA (1970) Studies on pituitary lactogenic hormone. XXX. The primary structure of the sheep hormone. *Arch Biochem Biophys* **141**: 705-731.

191. Liao S, Leininger KR, Sagher D, Barton RW (1965) Rapid effect of testosterone on ribonucleic acid polymerase activity of rat ventral prostate. *Endocrinology* 77: 763-765.

192. Lincoln GA and Peet MJ (1977) Photoperiodic control of gonadotrophin secretion in the ram: a detailed study of the temporal changes in plasma levels of FSH, luteinizing hormone and testosterone following an abrupt switch from long to short days. *J Endocr* **74**: 355-367.

193. Lloyd HM, Meares JD and Jacob J (1975) Effect of oestrogen and bromocryptine on *in vivo* secretion and mitosis in prolactin cells. *Nature* 255: 497-498.

194. Lowry OH, Rosenbrough AL, Farr AK and Randall AJ (1951) Protein measurement in Folin-Phenol reagent. *J Biol Chem* **193**: 265-275.

195. Mann T (1964) *The biochemistry of semen and of the male reproductive tract. Ed 2.* Methuen, London.

196. Mann T, Rowson LEA, Beronos S and Karagiannidis A (1971) The androgenic potency of dihydrotestosterone and testosterone in the bull, and the effect of growth hormone on the bovine seminal plasma vesicle. *J Endocr* **51**: 707.

197. Marshall S, Bruni JF and Meites J (1979) Effects of hypophysectomy, thyroidectomy, and thyroxine on specific prolactin receptor sites in kidneys and adrenals of male rats. *Endocrinology* **104**: 390-395.

198. Martan J and Risley PL (1963) The epididymis of mated and unmated rats. *J Morph* **113**: 1-15.

199. McCann SM, Krulich L, Ojeda SR, Negro-Vilar A and Vijayan E (1978) Neurotransmitters in the control of anterior pituitary function. In: *Central Regulation of the Endocrine System.* Eds K Fuxe, T Hokfelt and R Luft, Plenum Press, New York, London. pp 329-347.

200. McGadey J, Baillie AH and Ferguson MM (1966) Histochemical utilization of hydroxysteroids by the hamster epididymis. *Histochemie* 7: 211-217.

201. Meites J (1973) Control of prolactin secretion in animals. In: *Human Prolactin*, Eds JL Pasteels and C Robyn, Excerpta Medica, Amsterdam, pp 105-118.

202. Michell B (1986) Profusion and confussion. *Nature (Lond)* 319: 176-177.

203. Moger WH and Geschwind II (1972) The action of prolactin on sex accessory glands of the male rat. *Proc Soc Exptl Biol Med* **141**: 1017-1021.

204. Moniem KA and Glover TD (1972) Comparative histochemical localization of lysosomal enzymes in mammalian epididymides. *J Anat* **111**: 437-452.

205. Moore HDM (1979) The net surface charge of mammalian spermatozoa as determined by isoelectric focussing changes following sperm maturation, ejaculation, incubation in the female tract, and after enzyme treatment. *Int J Androl* 2: 449-462.

206. Moore HDM and Bedford JM (1979a) The differential absorptive activity of epithelial cells of the rat epididymis before and after castration. *Anat Rec* 193: 313-328.

207. Moore HDM and Bedford JM (1979b) Short-term effects of androgen withdrawal on the structure of different epithelial cells in the rat epididymis. *Anat Rec* 193: 293-312.

208. Moore HDM, Hartman TD Brown AC, Smith CA and Ellis DH (1985) Expression of sperm antigens during spermatogenesis and maturation detected with monoclonal antibodies. *Expl Clin Immunogenet* 2: 84-96.

209. Morales C and Hermo L (1983) Demonstration of fluid-phase endocytosis in epithelial cells of the male reproductive system by means of horseradish peroxidase colloidal gold complex. *Cell Tiss Res* 230: 503-510.

210. Munabi AK, Mericq V, Gelato MC, Koppelman MCS, Albertson BD, Loriaux DL and Cassorla F (1985) The effect of prolactin on rat testicular steroidogenic enzyme activities. *Horm Metabol Res* 17: 47-48.

211. Murakami M and Nishida T (1985) Phagocytosis of spermatozoa and latex beads by epithelial cells of the ampulla vasis deferntis of the rabbit a combined SEM and TEM study. *Arch histol jap* 48: 269-278.

212. Murakami M, Nishida T, Iwanaga S and Shiromoto M (1984a) Scanning and transmission electron microscopic evidence of epithelial phagocytosis of spermatozoa in the terminal regions of the vas deferens of the cat. *Experientia* 40: 958-960.

213. Murakami M, Iwanaga S, Nishida T and Alba T (1984b) Phagocytosis of latex beads by the epithelial cells in the terminal region of the vas deferens of the cat: SEM and TEM study. *Andrologia* 16: 548-553.

214. Murphy MR, De Lam HH and Charagraff E (1951) Specific inhibition by *meso*-inositol of the colchicine effect on rat fibroblasts. *Exp Cell Res* 2: 165-177.

215. Negro-Vilar A, Saad WA and McCann SM (1977) Evidence for a role of prolactin in prostate and seminal vesicle growth in immature male rats. *Endocrinology* 100: 729-737.

216. Nicander L (1957) On the regional histology and cytochemistry of the ductus epididymidis in rabbits. *Acta Morphol Neerl - Scand* 1: 99-118.

217. Nicander L (1958) Studies on the regional histology and cytochemistry of the ductus epididymidis in stallions, rams and bulls. *Acta Morphol Neerl - Scand* 1: 337-362.

218. Nicander L and Glover TD (1973) Regional histology and fine structure of the epididymal duct in the golden hamster (Mesocricetus auratus). *J Anat* 114: 347-364.

219. Nicander L, Plöen L and Osman DI (1980) Epithelial degeneration in the epididymal head after efferent duct ligation in rats, rabbits and bulls. *Annu Conf Soc Study Fertil (Oxf)* 59.

220. Nicander L, Osman DI, Ploen L, Bugge HP and Kvisgaard KN (1983) Early effects of efferent ductule ligation on the proximal segment of the rat epididymis. *Int J Androl* 6: 91-92.

221. Nicoll CS (1967) Bioassay of prolactin. Analysis of the pigeon crop sac response to local prolactin injections by an objective and quantitative method. *Endocrinology* 80: 641-655.

222. Nicoll CS (1974) Physiological actions of prolactin. In: *Handbook of Physiology, Sect. 7,* **Vol IV,** *Part 2,* Eds E Knobil and WH Sawyer, American Physiological Society, Bethesda MD, pp 253-292.

223. Nicoll CS and Bern HA (1971) On the actions of prolactin among the vertebrates: is there a common denominator ? *Ciba Foundtion Symposium on Lactogenic Hormones,* Eds GEW Wolstenholme and J Knight, Churchill-Livingstone, London, pp 299-324.

224. Nillius SJ, Bergh T, Lundberg PO, Stahle J and Wide L (1978) Regression of a prolactin - secreting pituitary tumor during long - term treatment with bromocryptine. *Fert Steril* 30: 710-712.

225. Noel GL, Suh HK, Stone JG and Frantz AG (1972) Human prolactin and growth hormone release during surgery and other conditions of stress. *J Clin Endocr* 35: 840-851.

226. Noel GL, Suh HK and Frantz AG (1974) Prolactin release during nursing and breast stimulation in postpartum and non postpartum subjects. *J Clin Endocr Metab* 38: 413-423.

227. Ojeda SR, Jameson HF and McCann SM (1978) Effects of indomethacin and prostaglandin injections on plasma prolactin and growth hormone level in rats. *Endocrinology* 102: 531-539.

228. Olden K, Bernard BA, Humphries MJ, Yeo T, Yeo K, White S, Newton SA, Bauer HC and Parent JB (1985) Funtion of glycoprotein glycans. *TIBS* **114**: 72-82..

229. Ong DE, Crow JA and Chytill F (1982) Radioimmunochemical determination of cellular retinol and cellular retinoic acid-binding proteins in cytosols of rat tissues. *J Biol Chem* 257: 13385-13389.

230. Orgebin - Crist MC and Davies J (1974) Functional and morphological effects of hypophysectomy and androgen replacement in the rabbit epididymis. *Cell Tiss Res* 148: 188-201.

231. Orgebin - Crist MC and Djiane JD (1979) Properties of prolactin receptors from the rabbit epididymis. *Biol Reprod* 21: 135-139.

232. Orgebin - Crist MC and Olson GE (1984) Epididymal sperm maturation. In: *The Male in Farm Animal Production*, Ed M Courot, Martinus - Nijhoff, Boston, US, pp 80-102.

233. Orgebin - Crist MC, Danzo BJ and Davies J (1975) Endocrine control of the development and maintenance of sperm fertilizing ability in the epididymis. In: *Handbook of Physiology, Section II Endocrinology*, vol. 5, *Male Reproductive system*, Eds RO Greep and DW Hamilton, Am Physiol Soc, Washington DC, pp 319-330.

234. Ostle V (1954) In: *Statistics in Research*, Oxford and IBH Publications, New Delhi.

235. Ortiz E (1945) The embryological development of Wolffian and mullerian ducts and accessory reproductive organs of the golden hamster (Cricetus auratus). *Anat Rec* 92: 371-389.

236. Palade GE (1952) A study of fixation for electron microscopy. *J Exp Med* 95 285.

237. Pekary AE, Meyer NV, Vaillant C and Hershman JM (1980) Thyrotropin - releasing hormone and a homologous peptide in the male rat reproductive system. *Biochem Biophys Res Commun* 95: 993-1000.

238. Pekary AE, Ross R, Kernion JOE and Hershman JM (1983) Human reproductive tissues contain thyrotropin releasing hormone (TRH) and TRH homologous peptides. *J Androl* **4**: 408-414.

239. Pekary AE, Yamada T, Sharp B, Bhasin S, Swerdloff RS and Hershman JM (1984) Somatostatin - 14 and - 28 in the male reproductive system. *Life Sci* **34**: 939-946.

240. Pellegrini A and Ricciardi MP (1983) Histological and histochemical observations on the testis and epididymis of the albino rat treated with ACTH 1-39 and dexamethasone. *Int J Tiss Reac V* **(1)**: 41-45.

241. Perryman RL and Thorner MO (1981) The effects of hyperprolactinemia on sexual and reproductive function in men. *J Androl* **5**: 233-242.

242. Posner BI, Kelly PA and Friesen HG (1975) Prolactin receptors in rat liver: possible induction by prolactin . *Science* **188**: 57-59.

243. Prakash A and Moore HDM (1982) Localization of Δ^5- 3β- and 17β- hydroxysteroid dehydrogenase activity in the efferent ducts, epididymis and vas deferens of the rabbit, hamster and marmoset monkey. *J Reprod Fert* **66**: 95-100.

244. Prasad MRN, Rajalakshmi M, Gupta G and Karkum T (1973) Control of epididymal function. *J Reprod Fert Suppl* **18**: 215-222.

245. Pugh D and Walker PG (1961) The localization of N - Acetyl glucosaminidase in tissues. *J Histochem Cytochem* **9**: 242-250.

246. Pujol A and Bayard F (1978) 5α-reductase and 3β-hydroxysteroid oxidoreductase enzyme activities in epididymis and their control by androgen and rete testis fluid. *Steroids* **31**: 485-493.

247. Pujol A, Bayard F, Louvet JP and Boulard C (1976) Testosterone and dihydrotestosterone concentrations in plasma, epididymal tissues, and seminal fluid of adult rats. *Endocrinology* **98**: 111-113.

248. Purvis, Calandra R, Sander S and Hansson V (1978) Androgen binding proteins and androgen levels in the human testis and epididymis. *Int J Androl* **1**: 531-548.

249. Rajalakshmi M and Prasad MRN (1968) Changes in the sialic acid content of the accessory glands of the rat. *J Endocr* **41**: 471-476.

250. Rajalakshmi M and Prasad MRN (1969) Changes in the sialic acid in the testis and epididymis of the rat during the onset of puberty. *J Endocr* **44**: 379-385.

251. Rajalakshmi M and Prasad MRN (1971) Alterations in sialic acid in the epididymis of the pubertal rat, in response to changes in functional activity of the testis. *J Reprod Fert* **24**: 409-413.

252. Rama Sastry BV, Janson VE, Owens LK and Tayeb OS (1982) Enkephalin- and substanceP-like immunoreactivities of mammalian sperm and accessory sex glands. *Biochem Pharmacol* **31**: 3519-3522.

253. Ramos AS and Dym M (1977) Fine structure of the monkey epididymis. *Am J Anat* **149**: 501-532.

254. Reddy KVR and Govindappa S (1986) Prolactin induced advancement in structural organisation of accessory sex organs during pubertal transition in albino rats. *Ind J Exp Biol* **24**: 356-362.

255. Rees LH, Bloomfield GA, Rees GM, Corrin B, Franks LM and Ratchiffe JG (1974) Multiple Hormones in a bronchial tumor. *J Clin Endocr Metab* **38**: 1090-1097.

256. Reid BL and Cleland KW (1957) The structure and function of the epididymis. I. The histology of the rat epididymis. *Aus J Zool* 5: 223-246.

257. Relkin R (1972) Effect of the variations in environmental lighting on pituitary and plasma prolactin levels in the rat. *Neuroendocrinology* 9: 278-284.

258. Relkin R, Adachi M and Kahan SA (1972) Effects of pinealectomy and constant light and darkness on prolactin levels in the pituitary and plasma and on pitutary ultrastructure of the rat. *J Endocr* 54: 263-268.

259. Reyes A, Parra A, Chavarria ME, Goicoechea B and Rosado A (1979) Effect of prolactin on the calcium binding and/or transport of ejaculated and epididymal human spermatozoa. *Fert Steril* 31:669-672.

260. Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J Cell Biol* 17: 208-212.

261. Riar SS, Setty BS and Kar AB (1973) Studies on the physiology and biochemistry of mammalian epididymis. Biochemical composition of epididymis. A comparative study. *Fert Steril* 24: 355-363.

262. Riddick DH, Luciano AA, Kusmik WF and Maslar IA (1978) *de novo* synthesis of prolactin by human decidua. *Life Sci* 23: 1913-1922.

263. Riddle O (1963) Prolactin in vertebrate function and organization. *J Natl Cancer Inst* 31: 1039-1110.

264. Riemersma JC (1968) Osmium tetraoxide fixation of lipids for electron microscopy: a possible reaction mechanism. *Biochim Biophys Acta* 152: 718-727.

265. Robaire B and Hermo L (1988) Efferent ducts, epididymis and vas deferens: structure, functions and their regulation. In: *The Physiology of Reproduction*, **Vol 1**, Eds E Knobil and JD Neill, Raven Press, New York, pp 999-1089.

266. Rodger JC and Young RJ (1981) Glycosidase and cumulus dispersal activities of acrosomal extracts from opossum (marsupial) and rabbit (eutherian) spermatozoa. *Gamete Res* **4**: 507-514.

267. Rui H and Purvis K (1987) Independent control of citrate production and ornithine decarboxylase by prolactin in the lateral lobe of the rat prostate. *Mol Cell Endocr* **52**: 91-95.

268. Rutenberg AM, Rutenberg SH, Monis B, Teague R and Seligman AM (1958) Histochemical demonstration of β-galactosidase in rat. *J Histochem Cytochem* 6: 122-129.

269. Sabatini DD, Bensch K and Barnett RJ (1963) Cytochemistry and electron microscopy. The preservation of cellular structure and enzymatic activity by aldehyde fixation *J Cell Biol* **17**: 19-58.

270. Saito T and Saxena BB (1975) Specific receptors for prolactin in the ovary. *Acta Endocr (copenh)* **80**: 126-137.

271. Saksena SK, Lau IF and Chang M (1976) The inhibition of the conversion of testosterone into 5α-dihydrotestosterone in the reproductive organs of the male rat. *Steroids* **27**: 751-757.

272. Sassin JF, Frantz AG, Weitzman ED and Kapen S (1972) Human prolactin: 24 hour pattern with increased release during sleep. *Science* **177**: 1205-1207.

273. Scheer H and Robaire B (1982) Characterization of epididymal Δ^4-5α-reductase. *Ann NY Acad Sci* **383**: 496-498.

274. Schwartz MA and Koehlar JK (1979) Alterations in lectin binding to guinea pig spermatozoa accompanying *in vitro* capacitation and the acrosomal reaction. *Biol Reprod* **21**: 1295-1307.

275. Segal S, Polishuk WZ and Ben-David M (1976) Hyperprolactinemic male infertility. *Fert Steril* **27**: 1425-1427.

276. Scott TW, Wales RG, Wallace KC and White IG (1963) Composition of ram epididymal and testicular fluid and the biosynthesis of glyceryl phosphoryl choline by the rabbit epididymis. *J Reprod Fert* 6: 49-59.

277. Setchell BP and Hinton BT (1981) The effects on the spermatozoa of changes in the composition of luminal fluid as it passes along the epididymis. In: *Epididymis and Fertility: Biology and Pathology*. Eds C Bollack and A Clavert, S Krager, London, *Progr Reprod Biol* 8: 58-66.

278. Setty BS, Riar SS and Kar AB (1979) Androgen control of epididymal function in rhesus monkey and rabbit. *Fert Steril* **28**: 674-681.

279. Shah GV, Desai RB and Sheth AR (1976) Effect of prolactin on metabolism of human spermatozoa. *Fert Steril* **27**: 1292-1294.

280. Shalgi R, Matityahu A and Nebel L (1986) The role of carbohydrates in sperm - egg interaction in rats. *Biol Reprod* **34**: 446-452.

281. Sharp B, Pekary AE, Meyer NV and Hersham JM (1980) β-endorphin in male rat reproductive organs. *Biochem Biophys Res Commun* **95**: 618-623.

282. Shaver SL (1954) The role of stereocilia in removing India ink particles from the lumen of the rat epididymis. *Anat Rec* **119**: 177-185.

283. Sheth AR, Mugatvala PP, Shah GV and Rao SS (1975) Occurrence of prolactin in human semen. *Fert Steril* 26: 905-907.

284. Shur BD and Hall GN (1982) A role for mouse sperm surface galactosyltransferase in sperm binding to the egg zona pellucida. *J Cell Biol* 95: 574-579.

285. Sinowatz F (1981) Ultrastrukturelle und enzymhistochemische Utersuchungen am ductus epididymidis des Rindes. *Zbl Vet Med C Anat Histol Embryol (suppl)* 32: 1-99.

286. Sinowatz F, Bamberg E and Stockl W (1975) Histochemical localization of N-acetylglycosaminidase in the bull accessory sex glands. IRCS Medical Science *(Biochem Reprod Obstet Gynecol Vet Sci)* 3: 179.

287. Skudlarek MD and Orgebin-Crist MC (1984) Glycosidase synthesis and secretion in epididymal cells. *J Cell Biol* 99: 256.

288. Skudlarek MD and Orgebin-Crist MC (1986) Glycosidases in cultured rat epididymal cells: enzyme activity, synthesis and secretion. *Biol Reprod* 35: 167-178.

289. Slaby F and Bryan J (1976) High uptake of *myo*-inositol by rat pancreatic tissue *in vitro* stimulates secretion. *J Biol Chem* 251: 5078-5086.

290. Smith MS and Neill JD (1976) Termination at midpregnancy of the two daily surges of plasma prolactin initiated by mating in the rat. *Endocrinology* 98: 696-701.

291. Stricker P and Grueter R (1928) Action du lobe anterieur de l' hypophyse sur la mont'ee laiteuse. *CR Soc Biol, Paris* 99: 1978-1980.

292. Svennerholm L (1957) Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. *Biochim Biophys Acta* 24: 604-611.

293. Svennerholm L (1958) Quantitative estimation of sialic acids. III. An anion exchange resin method. *Acta Chem Scand* 12: 547-554.

294. Takano H, Abe K and Ito T (1981) Changes in the mouse epididyamis after ligation of the ductuli efferentes or proximal epididymal duct: qualitative and quantitative histological studies. *Acta anat nippon* 56: 79-90.

295. Talwalker PK, Ratner A and Meites J (1963) *In vitro* inhibition of pituitary prolactin synthesis and release by hypothalamic extracts. *Am J Physiol* 205: 213-218.

296. Thomas JA and Keenan EJ (1976) Prolactin influences upon androgen action in male accessory sex organs. In: *Cellular Mechanisms Modulating Gonadal Hormone Action*, Eds RL Singhal and JA Thomos, University Park Press, Baltimore, pp 425-470.

297. Thorner MO and Besser GM (1977) Hyperprolactinemia and gonadal function. Results of bromocryptine treatment. In: *Proceedings of the Serono Symposium* vol 11, *Prolactin and human reproduction*, Eds PG Crosignani and C Robyn, Academic Press London, New York, San Francisco. pp 285-302.

298. Thorner MO and Besser GM (1978) Bromocryptine treatment of hyperprolactinaemic hypogonadism. *Acta Endocrinol* 88 *suppl* 216: 131-146.

299. Tindall JS (1974) Hypothalamic control of secretion and release of prolactin. *J Reprod Fert* 39: 437-461.

300. Toowicharenont P and Chulavatnatol M (1983) Measurement of amionic sites of rat epididymal spermatozoa using tritiated polycationized ferritin. *J Reprod Fert* 69: 303-306.

301. Turkington RW (1971) Ectopic production of prolactin. *N Engl J Med* 285: 1455-1458.

302. Turkington RW, Juergens WG and Topper YJ (1965) Hormone dependent synthesis of casein *in vitro*. *Biochem Biophys Acta* **111**: 573-576.

303. Turner TT (1984) Resorption versus secretion in the rat epididymis. *J Reprod Fert* **72**: 509-514.

304. Turner TT and Cesarini DM (1983) The ability of the rat epididymis to concentrate spermatozoa. Responsiveness to aldosterone. *J Androl* **4**: 197-202.

305. Turner TT and Johnson AD (1973a) The metabolic activity of the bovine epididymis. I. Utilization of glucose and fructose. *J Reprod Fert* **34**: 201-213.

306. Turner TT and Johnson AD (1973b) The metabolic activity of the bovine epididymis. II. Utilization of acetate, succinate, pyruvate, lactate and glucose. *J Reprod Fert* **35**: 445-451.

307. Turner TT, Hartmann PK and Howards SS (1977) *In vivo* sodium and potassium, and sperm concentrations in the rat epididymis. *Fert Steril* **28**: 191-194.

308. Turner TT, Cochran RC and Howards SS (1981) Transfer of steroids across the hamster blood testis and blood epididymal barriers. *Biol Reprod* **25**: 342-348.

309. Ueda T, Takeyama M, Namiki M, Okuyama A, Aono T and Matsumoto K (1987) Effects of hyperprolactinemia on FSH on LH - induced activities of 17 β-oxidoreductase 5 α-reductase and 17 hydroxylase in hypophysectomized rat testis. *Endocr Japon* **34(4)**: 4577-585.

310. Usselman MC and Cone RA (1983) Rat Sperm are mechanically immobilized in the caudal epididymis by "immobilin" a high molecular weight glycoprotein. *Biol Reprod* **29**: 1241-1253.

311. van der Gugten AA and Kwa HG (1970) Disappearance rate of bovine prolactin from plasma of female rats studied at intervals of up to 160 minutes by radioimmunoassay. *Acta Endocrinol (Copenh)* **64**: 718-725.

312. Vierula M and Rajaniemi H (1982) Epididymal maturation of the surface protein structure of mammalian spermatozoa. *Medical Biology* 60: 323-327.

313. Vitale - Calpe R and Aoki A (1970) The hamster cauda epididymidis after coitus. *Acta Anat* 77: 98-108.

314. Voglmayr JK (1975) Metabolic changes in spermatozoa during epididymal transit. In: *Handbook of Physiology, Section VII, Endocrinology,* **Vol. 5,** *Male Reproductive system,* Eds DW Hamilton and RO Greep, Am Physiol Soc, Washington DC, pp 437-451.

315. Vreeburg JTM (1975) Distribution of testosterone and 5α-dihydrotestosterone in rat epididymis and their concentrations in efferent duct fluid. *J Endocr* 67: 203-210.

316. Vreeburg JTM, Bielske and Ooms M (1976) Maturation and survival of spermatozoa in the epididymis of pregnenolone treated hypophysectomized rats. *Endocrinology* 99: 824-830.

317. Waites GMH and Setchell BP (1969) Physiology of the testis, epididymis and scrotum. In: *Advances in Reproductive Physiology,* **Vol 4,** Ed A McLaren, Logos Press, London, pp 1-63.

318. Walsh RJ, Posner BI, Kopriva BM and Brawer JR (1978) Polactin binding sites in the rat brain. *Science* 201: 1041-1042.

319. Waravdekar VS and Saslav LD (1957) *Biochim Biphys Acta* **24**: 439.

320. Warren L (1959) The thiobarbituric acid assay of sialic acids. *J Biol Chem* 234: 1971-1975.

321. Weinstein D, Schenker JG, Gloger I, Slonim JH, DeGroot N, Hochberg AA and Folman R (1981) The mode of action of bromocryptine. *FEBS Lett* **126(1)**: 29-32.

322. Weissbach A and Hurwitz J (1959) J Biol Chem **234**: 705.

323. Wilson RM and Griswold MD (1979) Secreted proteins from rat sertoli cells. *Exp Cell Res* 123: 127-131.

324. Witkin SS and Bendirch A (1977) DNA synthesizing activity in normal human sperm: location and characterization of the endogenous reaction. *Exp Cell Res* **106**: 47-54.

325. Witorsch RJ (1978) Immunohistochemical studies of prolactin binding in sex accessory organs of the male rat. *J Histo Cyto* **26**: 565-580.

326. Wittels B and Hochstein P (1967) The identification of carnitine palmityltransferase in erythrocyte membranes. *J Biol Chem* **242**: 126-130.

327. Wolf M (1986) Light and electron microscopy of human epididymis after estrogen- antiandrogen treatment. *Andrologia* **18(3)**: 280-291.

328. Wright WW, Musto NA, Mather JT and Bardin CW (1981) Sertoli cells secrete both testis-specific and serum proteins. *Proc Natn Acad Sci US* 78: 7565-7569.

329. Yamaoka J, Yamamoto K, Urabe N and Nagatani Y (1983) Osmium - impregnation patterns of the golgi complex in the epididymal epithelial cells of castrated and testosterone injectedmice. *J Cell Sci* **59**: 71-79.

330. Yanagimachi R, Noda YD, Fujimoto M and Nicolson GL (1972) The distribution of negative surface charges on mammalian spermatozoa. *Am J Anat* **135**: 497-520.

331. Yeung CH and Cooper TG (1982) Ultrastructure of the perfused rat epididymis: effect of luminal sodium ion concentration. *Cell Tiss Res* 226: 407-425.

332. Yogev L and Terkel J (1978) The temporal relationship between implantation and termination of nocturnal prolactin surges in pregnant lactating rats. *Endocrinology* **102**: 160-165.

333. Zimmerman EA, Defendine R and Frantz AG (1974) Prolactin and growth hormone in patients with pituitary adenomas: A correlative study of hormone in tumor and plasma by immunoperoxidase technique and radioimmunoassay. *J Clin Endocr Metab* 38: 577-585.

334. Zuncke VV and Grossrau R (1981) Morphology and zonaldivision of the ductus epididymidis of rats. I. Investigations of semithin sections. *Anat Anz (Jena)* **149**: 409-424.

* Due to the nonavailability of the references, various review articles were consulted and used as cross references.