INVESTIGATION OF GALACTOSYLTRANSFERASE AND α-LACTALBUMIN-LIKE PROTEINS IN MAMMALIAN REPRODUCTIVE TRACTS

by

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This thesis is dedicated to the memory of my loving grandparents

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ABSTRACT

The studies undertaken in this thesis concern the functions of galactosyltransferase (GalTase) and α-lactalbumin (α-lac) in mammalian reproduction. The following results have been obtained.

(a) GalTase activity is commonly present in the epididymal plasma of various mammalian species examined including mouse, rat, rabbit, ram and boar. Under the assay conditions which inhibited pyrophosphatase and alkaline phosphatase activities, the levels of the activities in different species were similar. However, it was found that the pyrophosphatase and alkaline phosphatase activities, which inhibit GalTase activity, of epididymal plasma differed very much between these species. This suggests that under physiological conditions, the GalTase activity of epididymal plasma is inhibited to very different degrees between these species. Therefore, GalTase may not have an important function at least in the epididymal plasma of the species with high pyrophosphatase and alkaline phosphatase activities in the plasma.

(b) Soluble GalTase activity is present in the ewe uterine lumen. Comparisons of the kinetic parameters of the uterine luminal enzyme and those of the serum enzyme of ewe suggest that the luminal enzyme is not transported from blood. This study is an initial investigation of soluble GalTase in the lumen of female reproductive tract. When further investigations are carried out, the comparisons between the enzyme status in oviduct lumen and that in uterine lumen in different species should yield important data concerning the function of GalTase in sperm-zona pellucida binding.

(c) Both rat sperm and ram sperm can bind to mouse zona pellucida. GalTase activity has been detected only in the sperm preparation of ram but not rat using the current method. UDP-galactose does not show specific inhibition on the binding of either rat or ram sperm to mouse zona. These results do not suggest that GalTase mediates the sperm-zona binding in rat and ram.

(d) There is no α-lac-like activity detectable in epididymal extracts of various mammals including rat. Two essential problems have been found to cause experimental artefacts in the previous reports about α-lac-like proteins. These results invalidate the suggestions from some of the previous reports about the special functions of GalTase in epididymal plasma under the regulation of α-lac-like proteins, and suggest that the epididymal plasma GalTase may just catalyze the galactosylation of glycoproteins or glycolipids as the enzyme does elsewhere (not including those in milk) in the body.

DECLARATION

I hearby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan if accepted for the award of a PhD degree.

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Tang, Y., the late Brooks, D. E., the late Snoswell, A. M. and Setchell, B. P. (1990). α-lactalbumin-like activity in mammalian epididymal fluid. Proc. Aust. Soc. Reprod. Biol., 22, abstract 63.

Tang, Y., the late Brooks, D. E., the late Snoswell, A. M. and Setchell, B. P. (1991). The effect of UDP-galactose on the sperm-zona pellucida binding between mouse, rat, and ram sperm and mouse eggs. Proc. Aust. Soc. Reprod. Biol., 23, abstract 35.

Tang, Y., the late Brooks, D. E., the late Snoswell, A. M. and Setchell, B. P. (1991). Examination of products of rat epididymal extract α-lactalbumin-like activity assays. Proc. Aust. Soc. Reprod. Biol., 23, abstract 123.

ABBREVIATIONS

cAMP	cyclic adenosine monophosphate
AR	acrosome reaction
ATP	adenosine triphosphate
BSA	bovine serum albumin
CDP	cytidine diphosphate
CPM	counts per minute
CTP	cytidine triphosphate
EDTA	ethylenediaminetetraacetic acid
FITC	fluorescein isothiocyanate
GalTase	galactosyltransferase
GlcNAc	*N*-acetylglucosamine
GTP	guanosine triphosphate
hCG	human chorionic gonadotrophin
HVPE	high-voltage paper electrophoresis
ITP	inosine triphosphate
IU	international unit
IVF	*in vitro* fertilization
K_M	Michaelis-Menten constant
α-Lac	α-lactalbumin
LacNAc	*N*-acetyllactosamine
M_r	relative molecular mass
NAD^+	nicotinamide adenine dinucleotide
PBS	phosphate buffered saline
Pi	inorganic phosphate
PMSG	pregnant mare serum gonadotrophin
PPi	inorganic pyrophosphate

SD	standard deviation
SE	standard error
TLC	thin layer chromatography
TPP	thiamine pyrophosphate
UDP	uridine diphosphate
UMP	uridine monophosphate
dUMP	deoxyadenosine monophosphate
UTP	uridine triphosphate
ZP	zona pellucida or zonae pellucidae

GENERAL INTRODUCTION

The studies of this thesis concern the functions of galactosyltransferase (GalTase) and α-lactalbumin (α-lac) in mammalian reproduction.

GalTase and α-lac are two functionally closely related proteins in mammals. GalTase catalyzes the transfer of galactose to various acceptors, and α-lac stimulates GalTase activity towards glucose, but inhibits the enzyme activity towards GlcNAc. GalTase is widely distributed in mammalian tissues, while α-lac was believed to be present only in milk and mammary glands, where it combines with GalTase to form lactose synthetase.

The interest to study the functions of these proteins in mammalian reproduction was aroused by the following factors. (a) GalTase activity was detected in rat rete testis plasma and epididymal plasma. (b) α-lac-like proteins were reported to be present in epididymal plasma and on the sperm surface of some mammals, mainly the rat. These proteins were reported not only to be able to stimulate the transfer of galactose by GalTase to glucose, but also to stimulate the transfer to myo-inositol. It is known that glucose is hardly detectable in mammalian epididymal plasma, while inositol is abundant in rat epididymal plasma. It was speculated that GalTase in epididymal plasma might have some unusual functions under the regulation of α-lac-like proteins. (c) Independently, mouse sperm surface GalTase was reported to be the receptor of sperm for zona pellucida in mouse sperm-zona binding. It was therefore speculated that α-lac-like proteins might also be involved in fertilization, e.g., as a decapacitation factor.

The purposes of the studies of this thesis were as follows: (a) to investigate whether GalTase and α-lac-like proteins were widely present in epididymal plasma of various mammalian species, and the functions of epididymal plasma GalTase and α-lac-like proteins in reproduction; (b) to investigate whether GalTase was also present in the luminal plasma of female reproductive tract, where sperm capacitation and

fertilization occur; and (c) to investigate whether GalTase was also the sperm's receptor for zona in other species than mouse.

In this thesis, the general literature is reviewed in Chapter 1, while specific background knowledge is introduced in individual experimental Chapters. Materials and methods used in the work of more than one experimental chapters are introduced in chapter 2, otherwise, they are introduced in individual experimental chapters. Chapter 3-6 are experimental chapters. The results of each experimental chapters are discussed in the same chapter, and an overall discussion of the relations between the results from the individual chapters is presented in Chapter 7.

CHAPTER 1
LITERATURE REVIEW
(To December 31, 1992)

1.1 GENERAL FEATURES OF RELEVANT SECTIONS OF THE MAMMALIAN REPRODUCTIVE TRACT

1.1.1 Epididymis, Epididymal fluid and Epididymal sperm

1.1.1.1 Epididymis

The epididymis is a single highly convoluted duct connecting the ductus efferens from testis and the ductus deferens (vas deferens) at its two ends, and closely applied to testis surface (Ashdown, 1987; Setchell and Brooks, 1988). The epididymis is usually divided into four segments: the initial segment, where the efferent ducts empty, and, from proximal to distal, caput, corpus, and cauda epididymidis (Setchell and Brooks, 1988). The epididymal epithelium contains a variety of cell types including principal, narrow, clear, basal and halo cells. Principal cells are the predominant cell type. Each cell type varies as a proportion of the total population at different positions along the duct (Robaire and Hermo, 1988). Principal cells are known to be capable of secretion of a variety of low and high molecular weight compounds into the epididymal lumen (Jones, 1989).

1.1.1.2 Epididymal Fluid

The rete testis fluid enters the epididymis from the testis through the efferent ducts. The composition of the rete testis fluid (see Setchell, 1970) is altered extensively by absorptive and secretory activity of epididymal epithelium (Setchell and Brooks, 1988).

Epididymal fluid contains water, ions, small organic molecules, proteins and sperm. There is general lack of reducing sugars in epididymal fluid. This, however, may because of the immediate metabolism of the sugars by sperm (Brooks, 1983a; Setchell and Brooks, 1988). Certain small organic molecules in particular high concentrations are variously present in the epididymal fluid of mammals (Robaire and Hermo, 1988; Jones, 1989). For example, in rat and hamster, inositol concentration gradually rises along the epididymal duct and is in high concentration in cauda epididymal fluid (Setchell and Brooks, 1988; Jones, 1989). The composition of epididymal plasma proteins is both proportionally and qualitatively different from those of serum and rete testis fluid, and the composition of the proteins at different epididymal segments are also different from each other (Robaire and Hermo, 1988). However, considerable amount of proteins from rete testis fluid, including certain of serum origin, remain in the epididymal plasma throughout the duct (for examples, see Brooks and Higgins, 1980; Hamilton, 1981). Galactosyltransferase (GalTase) and α-lactalbumin (α-lac) -like proteins have been reported to be present in the epididymal fluids of some mammals (see Section 3.1 and Section 6.1 respectively).

1.1.1.3 Major Epididymal Secretory Proteins in the Rat

Epididymal secretory proteins have been studied mostly in the rat. Several major rat epididymal secretory proteins have been reported (Cameo and Blaquier, 1976; Lea et al., 1978; Garberi et al., 1979; Brooks and Higgins, 1980; Faye et al., 1980; Jones et al., 1980; Brooks, 1981; 1982; 1983b; 1985; Lea and French, 1981; Jones and Brown, 1982; Wong and Tsang, 1982; Brooks and Tiver, 1984; Mongkolsirilieat and Chulavatnatol, 1984). Among them, four proteins are most commonly observed, although their M_r may be slightly different in the reports of different authors. In this thesis, these proteins will be referred to as they are in the report of Jones et al. (1980), which is more directly related to the work of chapter 6 of this thesis. In their report, these proteins are namely M_r 18,500, 19,000, 23,000 and 32,000 proteins respectively, according to their migration on SDS-polyacrylamide gel

electrophoresis. In the reports of Brooks and his colleagues (e.g., Brooks and Higgins, 1980; Brooks, 1982), there is one more protein of M_r 30,000. Molecular cloning has revealed that the M_r 18,500 and 19,000 proteins form a pair of proteins with an apparently identical primary amino acid sequence (Brooks et al., 1986a), and the similar situation exists for the M_r 30,000 and the 32,000 proteins (Brooks et al., 1986b). These proteins are secreted at various regions of rat epididymis (in addition to the above references, also see Kohane et al., 1980; 1983; Moore et al., 1990). It is well known that their secretions are androgen-dependent (in addition to the above references, also see D'Agostino et al., 1980; 1983; Kohane et al., 1983; Brooks et al., 1986a; b; Brooks, 1987a; b; Moore et al., 1990). The M_r 23,000 protein exists in both testis and epididymis (Jones and Brown, 1982; Brooks, 1985), and is also present in tissue extracts, but not fluids, of testis and epididymis of some other mammals (Brooks, 1985). The *M*r 32,000 proteins are known to be glycoproteins (Garberi et al., 1979; Brooks and Higgins, 1980; Faye et al., 1980; Lea and French, 1981; Brooks et al., 1986b). However, the M_r 18,500 and 19,000 proteins seem not to be glycosylated (Brooks et al., 1986a). All these proteins are acidic proteins with the isoelectric points about 5 (Garberi et al., 1979; Jones, et al., 1980; Faye et al., 1980). The M_r 18,500, 19,000 and 23,000 proteins have been reported to possess α-lac-like activity (see Section 6.1). However, the clones of the 18,500 protein (Brooks, 1987b) show that it belongs to the a_{2u}-globulin superfamily, suggesting that its possible function is to transport retinoids within the male reproductive tract (Brooks, 1987b). The clones of the M_r 32,000 proteins display significant sequence homology with those of yeast carboxypeptidase Y (Brooks et al., 1986b) and also with the cysteine-rich metal-binding region of ferredoxin (Charest et al., 1988), suggesting a metal binding role. It has been reported that these proteins associate with sperm surface when the sperm pass through the epididymis (Faye et al., 1980; Lea and French, 1981; Jones and Brown, 1982; Wong and Tsang, 1982; Brooks and Tiver, 1983, 1984).

1.1.1.4 Sperm Maturation in the Epididymis

Mammalian testicular sperm are both infertile and immotile, they acquire these abilities during their passage through the epididymis and attain their full potential for these abilities after they reach the cauda epididymis or nearby sections in a variety of animals (Yanagimachi, 1988; Jones, 1989). The plasma membrane of testicular spermatozoa undergoes extensive remodelling in the epididymis by a variety of mechanisms. The mechanisms can be classified into alteration of existing components; addition or coupling of new macromolecules; and removal of some macromolecules (Yanagimachi, 1988; Jones, 1989). The changes of glycoproteins are dominant in the remodelling. Other changes includes those in lipid and net surface charge etc. (Yanagimachi, 1988). Of the different mechanisms, the addition or deletion of carbohydrate moieties to sperm membrane protein or lipid acceptors has been the most studied (Jones, 1989).

1.1.2 Uterus and Uterine Fluid

1.1.2.1 Uterus

The uterus usually consists of two uterine horns, a body, and a cervix. Each uterine horn is connected with an oviduct at the top, and the cervix connects to the vagina (Nalbandov, 1976; Hafez, 1987). There are three layers of tissue in the uterine wall, the internal serous membrane, i.e., the endometrium; the middle muscular layer, i.e., the myometrium; and the external serous membrane (Nalbandov, 1976). The function of the myometrium is to contract at some specific physiological stages, e.g., oestrus and parturition, while the endometrium is responsible for formation of the uterine fluid and the placenta (Nalbandov, 1976; McRae, 1988). The endometrium contains the luminal epithelium, uterine glands, and connective tissue, i.e., stroma (Finn and Porter, 1975). The uterine epithelium is a simple epithelium composed of columnar cells which are joined apically by tight junctions. The complexity of these tight

There is a wide range of morphology of mammalian uterus due to different degrees of fusion of the uterine horns, the four common types are as follow. (1) In mouse, rat, rabbit and guinea pig, the uterus is of the duplex type. This type of uterus has two cervixes, but no uterine body, the uterine horns are completely separated. (2) In pig and insectivores, the uterus is of the bicornuate type. This type of uterus has one cervix and a body which is very small comparing with the horns. (3) The uteri of cow, ewe, mare and some other animals are of the bipartite type. This type of uterus has one cervix and is featured by a prominent body. In cow and ewe, but not mare, the two horns are separated by a septum. (4) The uterus of primates is of the simplex type, which has one cervix and a very prominent body, but no horns (Nalbandov, 1976)

junctions varies with reproductive hormone status (McRae, 1988). The uterine glands are branched, coiled tubular structures lined with epithelium and open onto the endometrial surface (Hafez, 1987).

1.1.2.2 Uterine Fluid

The term uterine fluid (UF) will be used in this thesis to refer to the uterine luminal fluid, which is formed by both transduction of blood serum and secretion of uterine endometrium. Both of the activities vary in accordance with the reproductive hormone status (McRae, 1988). During the luteal phase of the menstrual cycle in primates and during implantation of majority of mammals, the UF also contains decidual cells from endometrium decidual transformation (Finn and Porter, 1975).

It is known that ions, monosaccharides, amino acids and proteins can be transported from blood to uterine fluid (McRae, 1988). The transduction can be extracellular depending on the permeability of the tight junctions between the epithelial cells, or transcellular involving pinocytotic vesicles. Proteins in general are transported into the uterine fluid through the latter pathway. The transduction of the blood serum is selective, only certain molecules can be transported to UF and some substances can be concentrated in UF. There are both specific and non-specific transduction. The uterine endometrium also synthesizes and secretes a variety of proteins (McRae, 1988).

The volume and biochemical composition of UF, as well as the amount and the ratio of the UF proteins, depend on the reproductive hormone status (Hamner, 1971; Murray et al., 1972; Wolf and Mastroianni, 1975; Harrison et al., 1976; Moffatt et al., 1987). The UF proteins during the oestrous cycle and early pregnancy are partially serum proteins, but also some specific uterine secretory proteins (Daniel, 1971; Hamner, 1971; Shirai et al., 1972; Roberts and Parker, 1974; Wolf and Mastroianni, 1975; Roberts G.P. et al., 1976a; b; Martin et al., 1988).

Among the UF proteins are some enzymes. Some of the enzyme activities are elevated in UF compared with those in serum (Roberts and Parker, 1974; Roberts G.P. et al., 1976a; b; Roberts R.M. et al., 1976; Hansen et al., 1985), and the

However, Fawcett (1958) discribed the acrosome as consisting of three parts: the equatorial segment, the principal segment overlying the nucleus, and the apical segment protruding rostrally beyond the edge of the nucleus. This is particularly relevant to some rodents with a pronounced apical segment, such as guinea pig.

elevation reported is often, though not always, during early pregnancy before implantation. Such enzymes reported are mostly lysosomal-like enzymes (Hansen et al., 1985) including mostly glycosidases (see the above references for elevated enzyme activities in UF). The most elevated enzyme activities are different in different species, for example, they are ß-*N*-acetylglucosaminidase and ß-*N*-acetylgalactosamindase in cattle and sheep respectively (Roberts and Parker, 1974). At least some of the enzymes are secreted by the endometrium (Hansen et al., 1985).

1.2 EVENTS AND CHARACTERISTICS OF FERTILIZATION IN MAMMALS

1.2.1 Structures of Mammalian Spermatozoon and Egg

1.2.1.1 Structure of Mammalian Spermatozoon

The mammalian spermatozoon, as in other animals, has two main components, the head and the tail, though the size and the shape of the head and the length of the tail vary greatly among species. The sperm tail is composed of a highly ordered complex of microtubes, fibres and surrounding mitochondria, and is the motile part of the cell, whereas, the sperm head is the place where gamete recognition and interaction happen (Setchell, 1982; Eddy, 1988).

As shown in Fig. 1.1., the sperm head is mostly occupied by nucleus. The outmost membrane is the plasma membrane which covers the whole sperm. Under the plasma membrane and covering the anterior portion of the nucleus is the acrosome which is a membrane bound, caplike structure. The acrosome can be divided into two segments, the front acrosome cap and the equatorial segment (Yanagimachi, 1981; Setchell, 1982; Eddy, 1988). The acrosome contains many enzymes, including a variety of hydrolyzing enzymes, which are discharged during the acrosome reaction

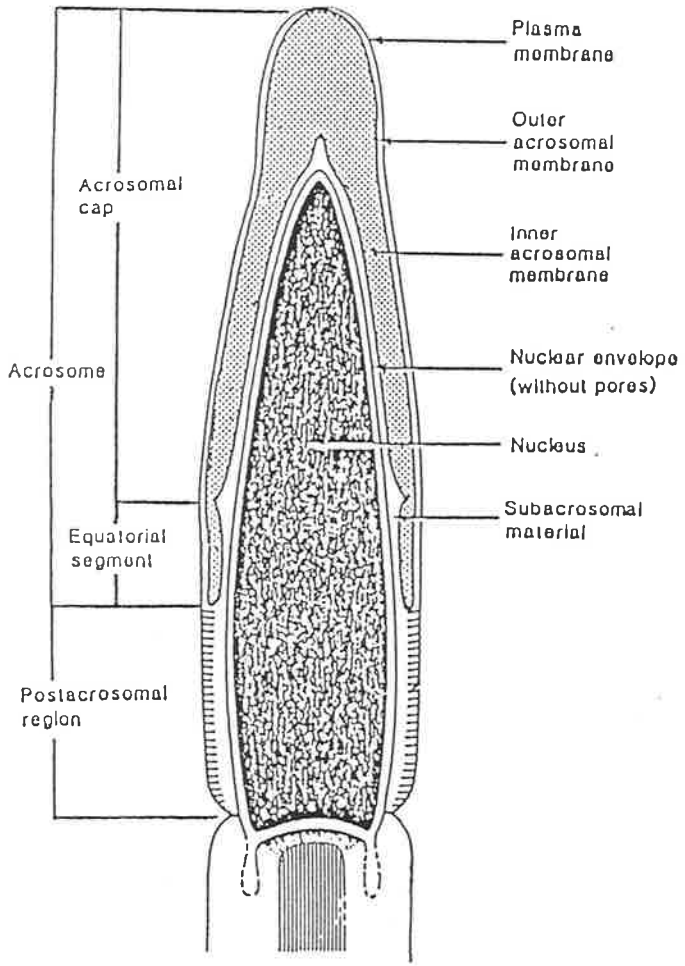


Fig. 1.1 Diagram of a sagittal section of a mammalian sperm head. (Modified from Yanagimachi, 1981).

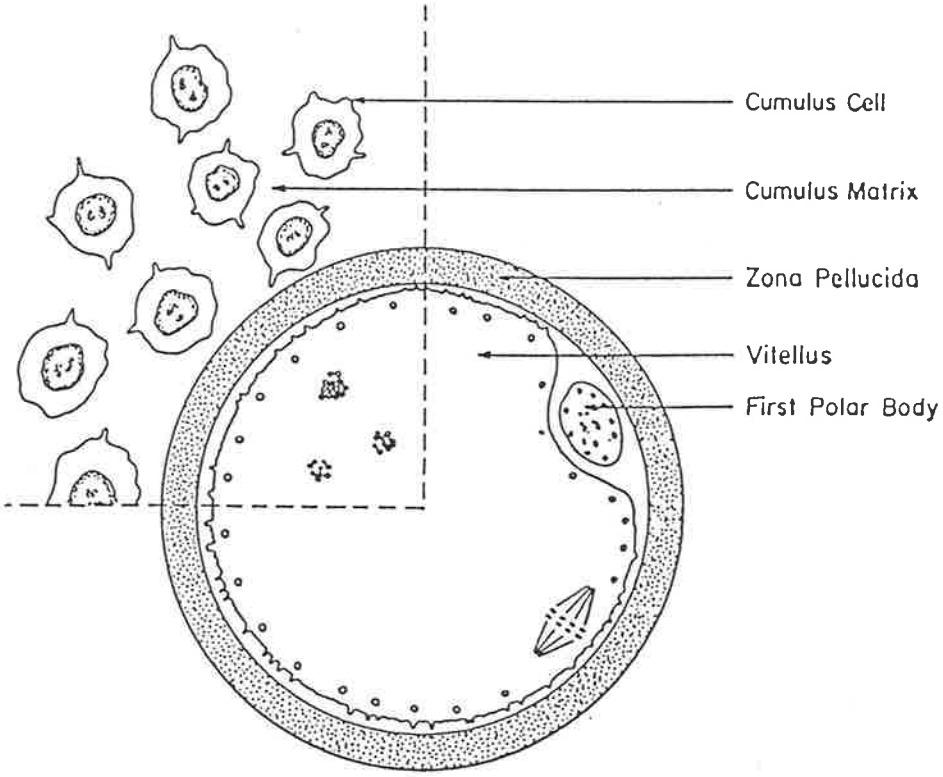


Fig. 1.2 Diagram of a cross-section of a mammalian egg with its associated cumulus oophorus. (Modified from Gwatkin, 1977).

(AR). The enzymes which are unique to the mammalian sperm acrosome are acrosin and hyaluronidase. Both of them are major constituents of the acrosome (Yanagimachi, 1988; Eddy, 1988). A special feature of the sperm plasma membrane is that it is divided into sharply delineated regional domains that differ in composition and function. The division of the domains usually corresponds to the difference of the sperm structure covered by the plasma membrane (Eddy, 1988).

1.2.1.2 Structure of Mammalian Egg

The structure of a mammalian egg is shown in Fig. 1.2. In mammals, the egg vitellus is bounded by a plasma membrane, which is surrounded by the zona pellucida (ZP). The ZP is a thick layer of relatively homogeneous matrix of randomly arranged fibrillogranular strands composed primarily of glycoproteins. In most mammals, in the recently ovulated egg, ZP is surrounded by several thousand cumulus cells which have relatively large spaces between each other and are embedded in a gelatinous matrix (Gwatkin, 1977; Wassarman, 1988).

It is necessary to bear in mind that in strict terminology, an unfertilized egg should be called an oocyte, and only a fertilized oocyte should be called an egg (Wassarman et al., 1985), though "egg" is commonly used to refer to both of them.

1.2.2 General Events of The Process of Mammalian Fertilization

The introduction of this section is summarized from the book of Gwatkin (1977), the review of Saling (1989), and especially the review of Yanagimachi (1988) about mammalian fertilization, except where other authors are quoted.

Fertilization is the process by which sperm and oocyte unite to form a zygote, the true beginning of a new individual (Wassarman et al., 1985). Mammalian fertilization happens normally in the isthmus of oviduct. However, fertilization can occur outside the body in culture medium and *in vitro* fertilization (IVF) is a very

important method for fertilization studies.

1.2.2.1 Sperm Capacitation

Capacitation is an essential event involving preparation of epididymal matured sperm for fertilization. It is usually defined as the process during which mammalian spermatozoa acquire the ability to fertilize homologous eggs. It is generally accepted that only capacitated sperm can penetrate through the cumulus oophorus and undergo the AR. Capacitation was first described by Austin (1951; 1952) and Chang (1951) who observed that the epididymal matured sperm have to stay in female reproductive tract for some time before they are able to fertilize an egg, they . After the IVF technique was developed, it was shown that sperm of many mammalian species could be capacitated *in vitro* in culture medium. There is no visible structural or ultrastructural change during capacitation. However, it is believed that a very important part of capacitation is to remove or alter substances absorbed onto or integrated into the sperm plasma membrane during epididymal maturation of sperm and/or ejaculation. Some other reported changes include the changes in adenylate cyclase/protein kinase systems, metabolism, intracellular ions and nucleus. The times required for capacitation are different in different species both in *vivo* and *in vitro* . For examples of *in vitro* capacitation time, see Section 4-2.2.2. For examples of the time *in vivo*, less than 1 hr, about 2-3hrs and about 1.5 hr for mouse, rat and ram sperm capacitation respectively were estimated. Capacitated sperm can be reversibly decapacitated by recoating the sperm with various seminal plasma glycoproteins, which are called decapacitation factors.

1.2.2.2 Sperm Acrosome Reaction

In mammals, only capacitated sperm can undergo the physical (AR), which involves multiple fusions between the outer acrosomal membrane and the overlying plasma membrane enabling the acrosome contents to discharge through the fenestrated

Λ There are also reports about the link of sperm hyperactivation to sperm intracellular calcium level (e.g., Suarez et al., 1993) and that to sperm moving through the viscous oviduct fluid (e.g., Suarez et al., 1991).

membranes. The mechanism of the AR includes the following events in sperm: increased Ca^{2+} level; elevated intracellular pH; H^+ and K^+ efflux, Na^+ influx; and elevated cAMP concentrations. The AR generally occurs during the binding of the sperm on zona surface, while it can also happen, especially in some species, before sperm-zona binding. More details about the relationship between AR and sperm-zona binding are introduced in Section 1.2.2.4. The AR of mammalian sperm can be induced not only by natural inducer(s), but also by some other substances or conditions which alter the ion permeability of the plasma membrane over the acrosomal cap of capacitated sperm. The AR has at least two functions: first, it enables the sperm to penetrate through the zona; and second, to fuse with egg plasma membrane.

Hyperactivation of sperm is a physiological phenomenon associated with the AR, which has been reported in many species including mouse and sheep. The phenomenon is that sperm, either observed *in vitro* or *in vivo*, start to move very actively before the AR happens. *In vitro*, the components of the medium including Ca^{2+}, cAMP etc. influence the initiation and maintenance of hyperactivated motility of sperm. The hyperactivated motility seems to enable the sperm to swim strongly and not only to migrate through the isthmus of the oviduct, but also to pass the egg investments, especially the ZP. Λ

1.2.2.3 Sperm Penetration through the Cumulus Oophorus

Only capacitated and acrosome-intact sperm can pass through the cumulus. Motility is essential for sperm penetration of the cumulus. Contrary to previous opinion, it is now believed that hyaluronidase is not essential for cumulus penetration, but may assist the penetration; and that it is the sperm surface hyaluronidase, but not that in the acrosome, which is responsible. Actually, the necessity for specific surface components for cumulus penetration has been challenged. Rather, the evidence is more consistent with the opposite view, that particular components present on the uncapacitated and acrosome-reacted sperm restrict their entrance into the cumulus, though this could involve different components on these two

types of cells.

1.2.2.4 Sperm Binding to Zona Pellucida

In vivo, sperm bind to zona after penetrating the cumulus oophorus. It is known that in mouse, capacitation is not necessary for sperm binding to zona *in vivo* , however, an AR cannot be induced in the uncapacitated sperm bound to zona (see the review of Saling, 1989).

The time resolution of sperm-zona attachment and binding has been studied mostly in mouse and hamster *in vitro*. Hartmann et al. (1972) reported that in golden hamsters, the first step of association between sperm and ZP was loose, and could be disrupted by pipetting the gametes. This association was not temperature dependent, nor species specific, since it occurred at 2°C, and the golden hamster sperm also attached to the eggs of mouse and rat. The second step of association between sperm and egg was a relatively tenacious union, which happened 30-40min after the loose association and was temperature dependent and species specific. The authors termed these two kinds of associations as attachment and binding respectively. Schmell and Gulyas (1980) also reported similar observations in mouse. However, in their report, the binding increased linearly during a 0-90min incubation period after insemination, while the attachment plateaued from 15min during the same period. In studies of *in vitro* sperm-zona binding, two methods are usually used to get rid of the attached sperm and leave the bound sperm on the zona. One is to transfer the gametes through sperm free medium (usually three times) by pipetting, and the other is to centrifuge the gametes in density gradients (Saling et al., 1978).

There have been some different time courses of sperm-zona binding observed in mouse and hamster (Hartmann et al., 1972; Hartmann and Hutchison, 1974; 1975; Saling et al., 1978; Schmell and Gulyas, 1980; Florman et al., 1982; Soldani and Rosati, 1987). The time curves can be classified into two types. In one type, the binding level continuously increases with time until it reaches a plateau. The mouse sperm-zona binding result of Schmell and Gulyas (1980) introduced above

belongs to this type. Also, both Saling et al. (1978) and Florman et al. (1982) showed that mouse sperm-zona binding plateaued about 15min post-insemination during a 30min incubation time.

The other type is a two phase sperm-zona binding curve. Soldani and Rosati (1987) carried out experiments with both capacitated and uncapacitated mouse sperm and obtained similar time curves of zona binding from them. In their report, an initial peak of binding appeared at about 10min after addition of sperm, followed by a substantial decrease in binding level at 20-25min, then a rise in the binding level at about 40min to a plateau about 1.5 times higher than the first peak. If eggs were fixed with glutaraldehyde and then incubated with sperm, the first binding phase was observed with no intermediate decrease in binding level. Only the first phase of the binding was reversed by EGTA when tested with capacitated sperm. When the gametes were examined ultrastructurally, capacitated sperm on the surface of non-fixed eggs began to display reacted acrosomes at about 20min. after gamete mixing, while uncapacitated sperm did not have an AR during the observation period of over 2hrs. With glutaraldehyde-fixed eggs, acrosome-reacted sperm were never observed regardless of their capacitation status. Actually, similar observation of two phase binding was also reported earlier in hamster with capacitated sperm (Hartmann and Hutchison, 1974), though it was only observed at very high sperm concentrations (Hartmann and Hutchison, 1975).

Saling (1989) has interpreted the above results of Soldani and Rosati as follows. The initial high-affinity binding is between the plasma membrane of acrosome-intact sperm and ZP (primary binding) which is followed by the occurrence of the AR when there is a brief, dramatic decrease in affinity. The second high affinity binding is between acrosome reacted sperm and the ZP (secondary binding). This author has also proposed that it is due only to experimental perturbation that the altered affinities are observed, and that under normal circumstance binding and then re-binding would not occur, instead, the acrosome-reacting sperm would remain associated with ZP, though briefly at low affinity, until the high-affinity second phase of binding is operational.

- Proacrosin is the zymogen of acrosin. In vitro, proacrosin is converted to acrosin and some other intermediates when being incubated with ZP glycoproteins, fucoidin, or DNA (Eberspaecher et al., 1991).

This interpretation seems plausible in terms of the time relationship between the binding of acrosome-intact sperm to ZP and the AR. However, it does not explain why uncapacitated sperm also have two phases of binding. Regardless whether or not this interpretation for the results of Soldani and Rosati (1987) is valid, the concept of primary binding and secondary binding is accepted and used in this thesis.

The acrosome status of sperm when they make their contact with zona in various mammals has been reviewed by Saling (1989). The binding of acrosome-intact sperm to ZP has been generally observed in all the species reviewed, including mouse, hamster, guinea pig, rabbit, human, boar, bull and ram; ZP induced AR has been reported in some of these species including mouse, hamster, rabbit, human and bull. The ability of acrosome-reacted sperm to bind to ZP appears to vary amongst species probably with mouse and guinea pig as the two extremes. In mouse, only acrosome intact sperm initiate binding to ZP, whereas in guinea pig, both acrosome-intact and acrosome-reacted sperm can initiate binding to ZP with the latter form most commonly observed.

1.2.2.5 Sperm Penetration through Zona Pellucida

Sperm need to complete the AR before penetrating the ZP. When an acrosome-reacted spermatozoon passes through the zona, the acrosome cap is lost leaving only the equatorial segment and the inner acrosome membrane. Understanding about how the sperm of eutherian mammals pass through the zona is still limited. Rigorous sperm tail movement seems essential, but assistance from enzymes is still required. Hyaluronidase released from the acrosome digests the hyaluronic acid in the outer regions of the zona. Acrosin hydrolyzes specific zona glycoproteins. Some of the acrosin and possibly some other enzymes remain bound to the inner acrosomal membrane and cleave zona molecules as the inner acrosomal membrane is pushed against the zona by sperm movement.

The fusion between the gametes triggers the exocytosis of cortical granuels and the block of polyspermy. The discharged cortical granule contents induce the zona reaction, i.e., they alter the physical and chemical characteristics of the ZP in such a way that sperm can no longer penetrate it. However, in a few mammal species, e.g., the rabbit, the zona reaction is absent. The fusion also induces changes in vitelline membrane, so that it prevents sperm entry of the membrane.

1.2.2.6 Sperm-Egg Fusion

Upon passing through the zona, the sperm head crosses the perivitelline space and attaches to the plasma membrane of the vitellus. Then the sperm gradually enters the vitellus and the sperm plasma membrane becomes part of the egg plasma membrane. The fusion starts from the plasma membrane covering the equatorial segment of the sperm. In IVF only acrosome-reacted sperm can fuse with zona-free eggs, and the acrosome-intact ones cannot, no matter whether or not they are capacitated.

Once sperm and egg are fused, a series of biological reactions happen in the egg, and finally the duplicated chromosomes of sperm and egg mingle, marking the end of fertilization and the beginning of embryonic development.

1.2.3 Molecular Mechanisms in Sperm-Zona Binding

There is no doubt nowadays that sperm-zona binding is mediated by the recognition of special molecules, usually called receptors, on the sperm surface and the ZP surface respectively.

1.2.3.1 Receptors of Zona Pellucida for Sperm

Mouse ZP is the most thoroughly studied ZP in mammals. Significant progress has been made especially with respect to its macromolecular composition, structure, biosynthesis and purification. Most of the research work has been carried out by Wassarman and his colleagues. The following introduction about mouse ZP proteins is summarised from the reviews of Wassarman et al. (1985) and Wassarman (1988) except where other authors are quoted.

Mouse ZP protein contains three glycoproteins, ZP1, ZP2 and ZP3 with average M_r of 200,000, 120,000 and 83,000 respectively. All the three glycoproteins

are sulphated, probably on their oligosaccharides. Each glycoprotein represents a unique polypeptide species. ZP2 and ZP3 are monomers, while ZP1 is a dimer. ZP2 and ZP3 are present in about same amounts, whereas the ZP1 amount is much lower. ZP1, ZP2 and ZP3 all contain N-linked complex-type oligosaccharides, and at least ZP2 and ZP3 contain O-linked oligosaccharides.

It has been known for a decade that ZP3 is the glycoprotein that accounts for all sperm receptor activity present in ZP for the primary sperm-zona binding. It is also known that the receptor activity is associated only with the ZP3 O-linked oligosaccharides having a M_r of 3900, but not with the N-linked oligosaccharides or the polypeptide chain. It has been further suggested that galactose, located in an α-linkage at the nonreducing terminus of the O-linked oligosaccharides, is at least one of the sugar determinates essential for the sperm receptor activity of ZP3 (Bleil and Wassarman, 1988). ZP3 also serves as AR inducer and this function depends on both its O-linked oligosaccharides and glycopeptides. Recently, Leyton and Saling (1989a) suggested that ZP3 triggers the AR by aggregating sperm surface receptors, ie. by cross-linkage of ZP3 polypeptide to the sperm plasma membrane components recognized by ZP3. The role for ZP2 is as the secondary receptor for sperm following the AR.

During zona reaction after fertilization, ZP2 and ZP3 are modified, ZP3 loses its sperm receptor and AR-inducer activities and ZP2 decreases its solubility.

Porcine ZP is the second mostly intensively studied ZP. Porcine ZP also contains three glycoprotein species, one with M_r of 82,000, and the other two with the same M_r of 55,000 (Hedrick and Wardrip, 1987; Yurewicz et al., 1987). Actually, there is every indication that this same format is also followed by ZP glycoproteins from mammals other than mouse and pig (Wassarman, 1988). Recently, one of the M_r 55,000 glycoproteins has been shown to bind to porcine sperm plasma membrane (Peterson et al., 1991). Porcine ZP glycoproteins contain both N- and O-linked saccharide chains (Hedrick and Wardrip, 1987; Yurewicz et al., 1987). N-linked oligosaccharide chains of porcine ZP glycoproteins have recently been analysed (Mori et al., 1991). It has been found that these sugar chains are of bi-, tri- and tetraantennary

complex type with a trimannosyl core which is mostly fucosylated, that 26% of the chains contain *N*-acetyllactosamine repeating structures in their outer chains, and that, as a characteristic feature, 39% of the chains contain *N*-acetylglucosamine residues at their nonreducing termini.

1.2.3.2 Receptors of Sperm for Zona Pellucida

Although many candidates for sperm's receptor for ZP have been suggested, and some of them have been intensively studied, in contrast to present knowledge about receptors of ZP for sperm, the precise nature of these molecules has not been well defined. This is probably because the composition, reactions and functions of the surface of sperm head are much more complex than those of ZP. Efforts have been made in this section to classify the candidates according to the suggested mechanism of their interactions with ZP's receptors.

In addition to the following review of this section (1.2.3.2), readers attention is also directed to the review about sperm-egg recognition molecules by Jones (1990), which includes a detailed review about the identification and function of some of the following introduced molecules.

1.2.3.2.1 Saccharides Possibly Involved in Sperm-Zona Pellucida Binding

In some of the individual studies, the inhibition of more than one type of saccharide residue on *in vitro* sperm-zona binding of a same species is reported. This, in turn, may support the suggestions of different candidates for sperm's receptor.

In hamster, fertilization is not inhibited by the majority of monosaccharides and homopolysaccharides of fucose, galactose, glucose and mannose etc., whereas, heteropolysacchatides containing fucose (e.g., fucodin) and galactose inhibit sperm-zona binding without inhibiting sperm-egg fusion (Ahuja, 1982; 1985). Fucoidin is especially effective as an inhibitor. Acetyled amino acid sugars of glucose,

galactose, and mannose also inhibit the binding. Several glycoproteins with oligosaccharide chains terminating in galactose (asialo-fetuin and asialo-orosomucoid), *N*-acetylgalactosamine (asialo-submaxillary mucin), and *N*-acetylgluctosamine are also potent inhibitors of sperm-zona binding, but not sperm-egg fusion. However, the natural (not desialated) forms of these glycoproteins (fetuin, orosomucoid and submaxillary mucin) are non-inhibitory. Lambert (1984) reported that in two mouse species, α-methylmannose and sialic acid inhibited sperm-zona binding. In the report of Shalgi et al. (1986), among various monosaccharides tested for inhibition of rat sperm-zona binding, α-melthymannose was most effective, D-mannose was the next and L-fucose was the third; while fucoidin, but not mannan, was a more potent inhibitor.

L-fucose and fucose containing saccharides, especially fucoidin have also been reported to inhibit sperm-zona binding in many other mammals including guinea pig (Huang et al., 1982; Huang and Yanagimachi, 1984); human (Huang et al., 1982); mouse (Yamagata, 1985) and pig (Peterson et al., 1984). Fucoidin also strongly inhibits the sperm-egg binding in the sea urchin (Vacquier and Moy, 1977; Glabe et al., 1982).

1.2.3.2.2 Lectin-Like Proteins

Sea urchin is one of the best studied examples of gamete interaction. In this species, the sperm binding to the egg vitelline layer is mediated by bindin, an acrosome surface protein, with a lectin-like activity (Vacquier and Moy, 1977; Glabe et al., 1982). In mammals, there is some evidence indicating the possible involvement of lectin-like activities in sperm-zona binding.

(1) Zona-Pellucida-Binding and Fucose-Binding Proteins of Boar Sperm

In the pig, fucoidin strongly inhibits sperm-zona binding (Peterson et al., 1984); and fucose conjugated to HR-peroxidase, but not the other HR-peroxidase conjugated carbohydrates, binds well to sperm (Töpfer-Petersen et al., 1985; 1987). Correspondingly, a M_r 53,000 protein and a group of proteins with M_r around 20,000

(low M_r proteins) of boar sperm have been found to be both ZP binding (e.g., Brown and Jones, 1987; Hanqing et al., 1991) and fucose binding (e.g., Töpfer-Petersen et al., 1985; Jones, 1987). The M_r 53,000 protein has been identified as proacrosin (Jones and Brown, 1987; Jones et al. 1988). Strong similarities exist between proacrosin and bindin (see Discussion in Jones et al. 1988). The fucose-binding proteins are localized to two sites on sperm: the apical crescent in acrosome-intact sperm, and the acrosomal matrix in acrosome-reacted sperm (Friess et al., 1987a; b). Evidence suggests that the 53,000 protein is localized intra-acrosome (Jones et al, 1988). It was proposed that the initial recognition of the sperm and zona is established by the fucose binding protein located on the apical crescent, probably the low M_r proteins, then AR is induced and the M_r 53,000 protein is exposed, which mediates the secondary binding (Töpfer-Petersen et al., 1987; Hanqing et al., 1991). However, evidence of corresponding ZP's receptor is still needed to certify finally that binding of these proteins to ZP is mediated by its terminal fucose residues.

(2) Rabbit Sperm Autoantigen

Rabbit sperm autoantigens (RSA) are a family of M_r 14,000-19,000 proteins, which are located at the border of the post acrosomal segment and equatorial segment, including the medial bulge in the equatorial segment in acrosome-intact sperm, and are concentrated in the medial bulge in acrosome-reacted sperm (Esaguy et al., 1988). RSA recognize several carbohydrate residues including fucose (O'Rand et al., 1988). Monoclonal antibodies directly against RSA block rabbit sperm-zona binding. However, it has been observed that the distribution of RSA on sperm (Esaguy et al., 1988) is more restricted than the distribution of all zona binding proteins (O'Rand and Fisher, 1987) suggesting that other zona-binding receptors in addition to RSA are present in rabbit sperm (Esaguy et al., 1988).

Actually, except the low M_r proteins of porcine sperm and the RSA introduced above, ZP binding proteins with M_r less than 20,000 have also been found in

the sperm of mouse (O'Rand et al., 1985) and human (O'Rand et al., 1985; Shabanowitz and O'Rand, 1988). It has been suggested that the proteins of this group are likely to be analogues to bindin (O'Rand, 1988).

(3) Galactosyl Receptor Protein

In the rat, a galactosyl receptor protein has been identified on epididymal sperm plasma membrane overlying a region of the acrosome (Abdullah and Kierszenbaum, 1989). The presence of this galactosyl receptor seems to correspond to the suggestion of Bleil and Wassarman (1988) that in the mouse the galactose terminus of ZP3 is at least one of the sugar terminates essential for sperm-zona binding; and to the report that some galactose containing saccharides inhibit sperm-zona binding in the hamster (see Section 1.2.3.2.1). This galactose receptor protein was identified using antibodies to liver asialoglycoprotein receptor which had binding affinity to desialized serum proteins. Accordingly as mentioned in Section 1.2.3.2.1 above, some asialoglycoproteins inhibit the sperm-zona binding in the hamster. The above data may suggest the possibility that galactosyl receptor protein may be involved in mammalian sperm-zona recognition.

1.2.3.2.3 Enzymes

It is suggested that enzymes and enzyme-like proteins from several families are involved in fertilization. Enzyme-like proteins refers to those proteins which have the binding activities, but not the catalytic activities of the corresponding enzymes. Nevertheless, even when sperm-zona binding is mediated by a enzyme, the activity of the enzyme involved is most likely a lectin-like activity.

(1) Proteinase Inhibitor-Sensitive Sites

It has been reported that various proteinase inhibitors block sperm-zona interaction in the hamster and mouse (Hartmann and Hutchison, 1974; Saling, 1981; Aarons et al., 1984; Poirier et al., 1986). In the mouse, two sites sensitive to proteinase

inhibitor have been reported. Saling (1981) found that sperm-zona binding, but not penetration and sperm-egg fusion, was inhibited by trypsin inhibitors. This trypsin inhibitor-sensitive site is also sensitive to a synthetic serine proteinase inhibitor. However, it does not react with substrates of trypsin / acrosin (Benau and Storey, 1987). The other proteinase inhibitor-sensitive site of mouse sperm is known to be a M_r 15,000 membrane protein (Aarons et al., 1984; Poririer et al., 1986), which inhibits sperm-zona binding (Poirier et al., 1986). This protein serves as a binding site for a seminal vesicle proteinase inhibitor, and both inhibitors and substrates of trypsin / acrosin, but does not hydrolyse the substrates (Aarons et al., 1984).

(2) Glycosyltransferase

The role of mouse sperm surface galactosyltransferase (GalTase) in fertilization has been intensively studied, and is introduced in "General Introduction" of chapter 4.

The possible involvements of sialyltransferase (Durr et al., 1977) and fucosyltrasferase (Apter et al., 1988) in mouse fertilization have also be suggested.

(3) Glycosidase

α-D-mannosidase has been reported to be present on the sperm plasma membrane of rat (Tulsiani et al., 1989) and human (Tulsiani et al., 1990). The possible role of mannosidase in sperm-zona binding has been discussed by the authors based on the following observations. Firstly, D-mannose and α-methylmannoside inhibit rat sperm surface mannosidase (Tulsiani et al., 1989). Correspondingly, as indicated in Section 1.2.3.2.1, these substances inhibit the sperm-zona binding in rat and mouse. $Man_5GlcNAc$, which inhibits sperm surface mannosidase of mouse and human, also inhibits mouse sperm-zona binding, and the two inhibitions in the mouse have a similar concentration dependence (Tulsiani et al., 1990). Secondly, treatment of the zona intact egg with concanavalin A (a mannose specific lectin) prevents sperm-egg binding in hamsters (Oikawa et al., 1974). Thirdly, treatment of zona-intact rat eggs with Jack

bean mannosidase causes nearly complete inhibition of sperm-egg binding (Shalgi et al., 1986).

1.2.3.2.4 Signal Transduction Proteins

The signal transduction mechanism is the latest mechanism reported to be involved in mammalian fertilization in terms of sperm-zona binding and induction of AR, though its involvement in sperm-egg interaction in sea urchins has been demonstrated earlier (see the review of Garbers, 1989). Maybe it is not surprising to know that AR is induced under this mechanism considering the great similarity between AR and some of the early biochemical responses of other cells to hormones, growth factors, transforming factors etc.

Proteins similar to guanine nucleotide binding inhibitory proteins have been reported to be present on the sperm of all mammalian species examined including mouse, bovine and human (Kopf et al., 1986), and have been suggested to play a role in mediating AR (Endo et al., 1987; 1988). With respect to sperm-zona binding, it has been suggested that protein tyrosine kinase (PTK) or substrates for PTK are involved in mouse sperm-zona binding as follows.

One class of cell surface signal transduction receptor is PTK (Hunter and Cooper, 1985; Yanden and Ullrich, 1988). Binding of ligand to the extracellular domain of this type of receptor stimulates the intrinsic PTK activity of its intracellular domain leading to phosphorylation of tyrosine on the PTK itself (autophosphorylation), which is the most usual situation (Hunter and Cooper, 1985; Yarden and Ullrich, 1988), and/or phosphorylation of tyrosines on other cellular substrates. The phosphorylation of tyrosines induces biochemical cascades which may result in a variety of effects including increased pH, increased Ca^{2+}, activation of the phosphoinositide cycle and exocytosis. Some evidence suggests that ligand-stimulated receptor aggregation is the initial step required for PTK activation (Yarden and Ullrich, 1988).

Leyton and Saling (1989a) reported a M_r 95,000 phosphotyrosine containing protein (P95) of mouse sperm, the phosphotyrosine level of which increased

with capacitation and doubled with incubation of capacitated sperm with ZP. In the same study, two proteins with M_r 95,000 and 42,000 were identified to be ZP3 binding proteins from solublized uncapacitated sperm briefly exposed to Ca^{2+} to increase their zona binding activity. The phosphotyrosine containing proteins have been localized to the acrosomal region of the sperm head (Leyton and Saling, 1989a). This is in correspondence with the location of the M_r 56,000 ZP3 binding proteins reported by Bleil and Wassarman (1986). Evidence from immunological and gradient fraction studies, together with the possibly identical localization, suggest the possibility that the P95 and the M_r 95,000 zona-binding protein are the same protein (see the review of Saling, 1991). Based on the above results, Saling (1991) hypothesized that ZP3 is the activating ligand which binds to the domain of P95 on sperm surface where it aggregates a number of P95 molecules and triggers the phosphorylation of the tyrosine residues of P95. It is not yet known if P95 is a PTK itself. In another report of Leyton and Saling (1989b), it was suggested that ZP3 triggers the AR by aggregation of sperm surface receptors. Therefore, it seems possible that P95 is also the sperm surface receptor responsible for AR.

1.2.3.2.5 Other Proteins

The use of the term "other proteins" means that these substances have not been further classified; it does not mean they do not belong to any of the above classes.

(1) PH-20

PH-20 is a guinea pig sperm membrane protein with M_r about 60,000 that appears to exist in two distinct populations, PH-20_{pm}, located on the surface of post-acrosomal segment, and PH-20_{am}, on the luminal surface of the inner acrosome membrane (Cowan et al., 1986; Phelps and Myles, 1987; Primakoff et al., 1988). Monoclonal antibodies directly against PH-20 block guinea pig sperm-zona binding in a concentration dependent manner (Primakoff et al., 1985). Whereas acrosome-intact guinea pig sperm do bind to ZP, the antibody seems only to inhibit the interaction

between acrosome-reacted sperm and ZP (Myles et al., 1987). The result of special enzyme treatment of sperm suggests that PH-20 is a protein anchored to the membrane via phosphatidylinositol (Phelps et al., 1988). It is known that some of the proteins which are membrane-anchored via phosphatidylinositol are involved in cellular adhension.

(2) M_r 56,000 ZP3-binding protein

Bleil and Wassarman (1990) has recently reported the identification of a ZP3 binding protein of M_r 56,000, localized to the heads of acrosome-intacted sperm in mouse.

1.2.4 Species Specificity

In invertebrates with external fertilization, species specificity mainly depends on sperm-egg recognition, though incompatibility of embryo development may also play a role (Minor et al., 1989). However, in mammals, species specificity exists at many levels including the mating behaviour; the structure of the female reproductive tract through which sperm have to swim to meet the eggs; the special circumstances where sperm and egg meet etc.. Therefore the question arises whether or not the sperm-egg recognition in mammals is highly species specific. A large number of studies, mostly with *in vitro* fertilization techniques, have shown that all the steps of sperm-egg interaction have only ~~more or less~~ limited species specificity.

Bedford (1977) tested the interspecies sperm-zona binding in several mammalian species including human, monkey, rabbit, guinea pig, mouse and hamster with uncapacitated sperm. Sperm from most species generally bound to the zona of foreign eggs with the exception of human and guinea pig sperm. Human sperm did not bind to foreign zona from any of the above species, nor that from baboon, but bound to the zona of apes. Guinea pig sperm showed obvious binding only to the zona of monkey; but bound poorly to rabbit and mouse zona and did not bind to hamster zona.

These results were supported by some later reports. Yanagimachi (1981) offered data on sperm-zona binding between both acrosome-intact and acrosome-reacted hamster sperm and the zona of guinea pig, mouse and rat. Swenson and Dunbar (1982) showed that both capacitated and uncapacitated mouse sperm bound to the isolated zona of rabbit and pig; also both capacitated and uncapacitated rabbit sperm bound the isolated zona of mouse and pig. The high species specificity of human and guinea pig sperm were also indicated respectively by Yanagimachi et al. (1979) and Yanagimachi (1981). Moreover, Fournier-Delpech et al. (1982) reported the binding of uncapacitated ram sperm to rat zona. Both binding of mouse sperm to rat zona (Hanada and Chang, 1978; Du, 1988) and rat sperm to mouse zona (Du, 1988) were demonstrated. Although the species specificity of sperm-zona binding is limited, the affinity of heterologous binding is usually lower than that of the homologous binding even in the same genus (Lambert, 1984; Roldan et al., 1985). However, exceptionally, Du (1988) showed that both the affinity of the binding of mouse sperm to rat zona and that of the rat sperm to mouse zona were higher than the affinity of the binding of rat sperm to rat zona *in vitro*.

Penetration of sperm through the ZP seems to be the most prominent step of species specificity in mammalian sperm-egg interaction (Yanagimachi, 1977). However, the species specificity in penetration is also not absolute. Rat zona can be penetrated by mouse sperm at a rate of 0.9 - 6.3% (Hanada and Chang, 1978), and by hamster sperm at 0.3% (Barros, 1968). As many as 20-21% of the zona of mouse egg can be penetrated by deer mouse sperm (Fukuda et al., 1979). The penetration of bovine zona by ram sperm has been obtained at a rate varying between 10 and 84% (Slavik et al., 1990).

Sperm-egg fusion appears to be less species specific than sperm-zona interaction (Yanagimachi, 1977). For example, mouse sperm which penetrate the rat zona only at a very low rate, can readily enter zona-free rat eggs (Hanada and Chang, 1972; Pavlok, 1979). Similarly, human sperm which cannot penetrate hamster zona can enter zona-free hamster eggs (Yanagimachi et al., 1976). However, although mouse zona allow binding of foreign sperm of many different species, the plasma membrane of

The above literature review has introduced the mammalian reproduction background relevant to the studies in this thesis. The following review is about the two proteins studied in this thesis, galactosyltransferase and α-lactalbumin.

mouse egg only permits the fusion of mouse sperm (Yanagimachi, 1981). Again the affinity in homologous sperm-egg fusion is higher than that in heterologous sperm-egg fusion (Yanagimachi, 1981).

1.3 GALACTOSYLTRANSFERASE AND α-LACTALBUMIN

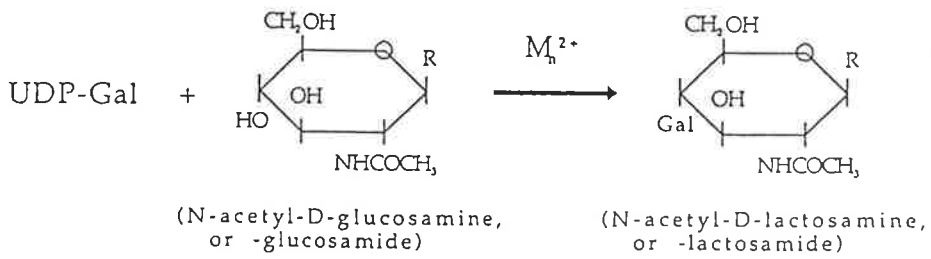
1.3.1 General Introduction

1.3.1.1 Enzyme Reaction and Terminology of Galactosyltransferase

Galactosyltransferase (GalTase) catalyzes the following general reaction:

UDP-galactose + HO-acceptor ----> Galactose-O-acceptor + UDP

Nearly 20 different linkages between galactose and acceptors have been described; they may be between galactosyl and other sugars, or amino acids, or lipids (Beyer et al., 1981, Beyer and Hill, 1982). The most common linkage is between galactose and β–*N*–acetylglucosamine (GlcNAc). GlcNAc can be a free sugar or a non-reducing terminus of a saccharide chain, which can be in all classes of glycoconjugates. Galactose β1->4 GlcNAc is the most common sequence of the linkage between galactose and GlcNAc. However, galactose β1->3 GlcNAc has also been described in glycoproteins, glycolipids and oligosaccharides, and galactose β1->6 GlcNAc in glycoproteins. GlcNAc β1, 4 GalTase has been reported in a number of studies. The reaction catalyzed by this enzyme is



where R can be an -OH or an oligosaccharide (Magee et al., 1973). The oligosaccharide can be alone or in a glycoprotein or a glycolipid (Ram and Munjal, 1985). Neither GlcNAc (β1->3) GalTase, nor GlcNAc (β1->6) GalTase have been detected. Many GalTases transferring galactose to GlcNAc were reported without characterized linkages. The GalTase discussed in the following review generally refers to UDP-galactose : GlcNAc GalTase.

1.3.1.2 Relationship between Galactosyltransferase and α-Lactalbumin

α-Lactalbumin (α-lac) was identified as a milk constituent long before its role as a regulatory protein of the GalTase was revealed by Brew et al. (1967). Prior to the report of Brew et al. (1967), a considerable amount of relevant knowledge had been accumulated as follows. Bovine milk lactose synthetase was known to catalyse the following reaction

UDP-D-galactose + D-glucose ---> lactose + UDP. (1)

GlcNAc was known to be also able to serve as an acceptor for galactose, but at a much lower efficiency (Babad and Hassid, 1966). It was known that lactose synthetase was simply an UDP-galactose : glucose GalTase, but composed of two proteins, namely protein A and B, neither of which could catalyze the above reaction separately (Brodbeck and Ebner, 1966). The B protein was known to be an α-lac (Brodbeck et al., 1967).

Brew et al. (1968) further reported that the A protein was an UDP-galactose : GlcNAc GalTase, which catalyze the following reaction:

UDP-galactose + GlcNAc ---> *N* -acetyllactosamine (LacNAc)+ UDP (2)

Glucose was only about 1.5% as effective as GlcNAc for the enzyme. However, in the presence of α-lac, the reaction (2) was inhibited, while the reaction (1) was preferentially catalyzed.

It is known now that α-lac only regulates the above reactions (1) and (2). it does not regulate the activity of UDP-galactose : GlcNAc GalTase towards other acceptors (see Section 1.3.3.3), nor the reactions catalyzed by other GalTases (Ram and

Munjal, 1985). The modification of the substrate specificity of GalTase by α-lac is an unique example of enzyme modification (Brew et al., 1968; Strous, 1986).

Following the report of Brew et al. (1968), many investigations were carried out on GalTase and α-lac from lactose synthetase , especially the enzyme from bovine milk. These early studies offered considerable general knowledge about these two proteins.

The papers about GalTase in this review are mainly those about the GalTases of milk and serum. GalTases from other specific sources relevant to the work of this thesis are introduced in the individual experimental chapters.

1.3.2 Distribution of Galactosyltransferase and α-Lactalbumin

In mammals, GalTase activity is widely distributed in various parts of the body in soluble or membrane bound forms. The soluble enzymes have been found in milk, colostrum, amniotic fluid, serum, cerebrospinal fluid, urine (Schachter & Roseman, 1980) and male reproductive tract fluids (see Section 3.1). The membrane-bound enzymes can be inside or on the surface of cells. They are most commonly found to be intracellular in an association with the Golgi apparatus, and the Golgi apparatus-bound GalTase exists in most of the cells (Schachter and Roseman, 1980). The enzymes have also been reported to be associated with smooth endoplasmic reticulum and mitochondria (Ram and Munjal, 1985). Examples of the enzyme association with cell surface, i.e., cell plasma membrane, are GalTases on sperm (Shur & Bennett, 1979; Lopez et al., 1985), uterine epithelial cells (Dutt, 1987) and fibroblasts (Patt and Grimes, 1974).

α-lac is synthesized only by mammary glands and secreted into milk (Schanbacher and Ebner, 1970, Bell et al., 1976).

1.3.3 Enzymatic Properties of Galactosyltransferase

1.3.3.1 Regulation of α-Lactalbumin on Galactosyltransferase Reaction

The basic regulation of α-lac on the substrate specificity of GalTase has been introduced in Section 1.3.1.2. In a further investigation about the relationship between GalTase and α-lac, Klee and Klee (1970) demonstrated that in fact, α-lac regulated the utilization of glucose and GlcNAc in the same way, i.e., when the concentrations of these sugars were low, α-lac stimulated the reactions; whereas when the concentrations were high, it inhibited the reactions. However, the concentration of glucose for the stimulation of its utilization was much higher than that of GlcNAc, the utilization of glucose and GlcNAc were stimulated when their concentrations were lower than 10mM and 1mM respectively. These effects have been confirmed by later studies, although the subsequently reported concentrations at which the stimulation or inhibition occured were sometimes different (Morrison and Ebner, 1971; Bell et al., 1976).

The kinetic mechanism of the regulation of α-lac on the activity of GalTase to transfer galactosyl to glucose and GlcNAc has been studied. At low concentrations of these sugars (e.g., concentrations lower than 10mM and 1mM respectively as mentioned above), α-lac stimulates the reactions by lowering the K_M values of the enzyme for these sugars; (Klee and Klee, 1970). Under normal assay conditions (i.e., when glucose concentration is not very high and GlcNAc concentration is not very low), α-lac greatly lowers the K_M of GalTase for glucose (Fitzgerald et al., 1970; Klee and Klee, 1970) and slightly increases the Vmax of the reaction (Morrison and Ebner, 1971). Under these conditions, α-lac also lowers the K_M of GalTase for GlcNAc, however its dominant effect on this reaction is lowering the Vmax (Morrison and Ebner, 1971).

Mitranic et al. (1988) reported the interaction of bovine milk GalTase with lipid and α-lac as follows. At low α-lac concentrations, the stimulation of GalTase activity towards glucose was sigmoidal. This sigmoidal relationship was eliminated by natural lipids such as phosphatidylcholine and phosphatidylethanolamine, detergents

such as Triton X-100, or an aggregated form of α-lac generated by crosslinking α-lac with dithiobissuccinimidylpropionate. It was suggested that these substances presented a hydrophobic surface necessary for lactose synthesis activity by the enzyme. In the presence of excess α-lac, the stimulatory effect of lipid was lost. This suggested that a large amount of α-lac was able to displace lipid from the enzyme. It was indicated that full activity of GalTase towards glucose required both α-lac and lipid, and optimal enzyme activity was obtained when the ratio of lipid / α-lac / enzyme was 60 : 6 : 1.

A special characteristic of α-lac is that its function of modifying GalTase is extremely heat-resistant, even withstanding boiling for 20min at pH 7.4 without losing its activity (Brodbeck and Ebner, 1966).

1.3.3.2 Metal Ion Requirement of Galactosyltransferase

GalTase requires Mn^{2+} for its catalytic activity. Babad and Hassid (1966) reported that Mg^{2+} and Ca^{2+} could activate bovine milk lactose synthetase when glucose was used as acceptor, although they were much less efficient than Mn^{2+}. However Co^{2+}, Na^{+}, K^{+}, or NH_4^{+} showed only little or no activation of the enzyme. Powell and Brew (1976a) reported that for GalTase in EDTA-treated bovine colostrum , some other cation ions could replace Mn^{2+}, although with lower efficiency, when GlcNAc was used as the acceptor. The efficiency was in the order of Zn^{2+}, Fe^{2+} > Cd^{2+} > Co^{2+} > Fe^{3+}. The ions Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Sn^{2+}, Ni^{2+}, Al^{3+}, Cr^{3+}, and Ni^{3+} did not activate the enzyme. In the same report, it was suggested that there were two metal binding sites on the GalTase. One was a tight binding site for Mn^{2+}, the other is a site at which Ca^{2+} and Sr^{2+} could replace Mn^{2+}. It was suggested that the first binding site was required for enzyme activity, while the second involved in UDP-galactose binding. Gmeiner (1988) investigated the substitution of Co^{2+} for Mn^{2+} on bovine milk and serum GalTase activities towards a group of 6 glycoproteins with GlcNAc as a terminus (e.g., ovalbumin) as well as towards GlcNAc. When pure milk enzyme was used, Co^{2+} activated the enzyme equally well as Mn^{2+} for both protein acceptors and GlcNAc. The result obtained from both crude and purified GalTase of serum were

similar. In these cases, Co^{2+} was a good substitute for Mn^{2+}, although generally less efficient, when the protein acceptors were used; however, when GlcNAc was used, the Co^{2+} activity was less then 8% of the Mn^{2+} activity. Hudgin and Schachter (1971) reported the activating function of various metal ions on porcine serum GalTase. When desialized and degalactosylated α_1-acid glycoprotein was used as an acceptor, the radioactivity (CPM) in the enzyme product in the assays with various ions were: Mn^{2+}, 4140; Co^{2+}, 582; Cu^{2+}, 429; Ba^{2+}, 293; Mg^{2+}, 212; Cd^{2+}, 124; and Ca^{2+}, 93. When GlcNAc was used, the CPMs were: Mn^{2+}, 4280, Ba^{2+}, 1885, Co^{2+}, 539, Ca^{2+}, 230, Cd^{2+}, 206, Mg^{2+}, 173, and Cu^{2+},106. In contrast, Wagner and Cynkin (1971) reported that the crude enzyme of rat serum had a absolute requirment for Mn^{2+}, not replaceable by Mg^{2+} or Ca^{2+} when ovalbumin was used as acceptor. Fraser and Mookerjea (1976) also found that the absolute requirement of Mn^{2+} by purified GalTase of rat serum, which could not be replaced by Ca^{2+}, Mg^{2+}, Zn^{2+}, or Co^{2+} when desialized and degalactosylated fetuin was used as acceptors. Bella et al. (1977) reported that Mn^{2+} was also the only effective metal ion among those tested for purified human plasma GalTase when either GlcNAc or glucose was used as an acceptor, though the tested metal ions were not listed.

1.3.3.3 Acceptor Specificity of Galactosyltransferase

With reference to the acceptor specificity of GalTase, Schanbacher and Ebner (1970) investigated many substances for bovine milk GalTase. Among their results were that β1,.4 glycosides of GlcNAc, such as di-, tri-, tetra-GlcNAc, GlcNAc-*N*-acetylmuramic acid and ovalbumin were also good galactosyl acceptors, and that α-lac did not appreciably inhibit the transfer of galactose to them. Schanbacher and Ebner (1970) reported the following observations. For bovine milk GalTase, in addition to the derivatives of GlcNAc, some other β-linked glucosides could also serve as acceptor substrates, but the enzyme activity was much lower with them than with GlcNAc. Generally α-lac had no effect on them. Certain α1->4 linked GlcNAc glycosides were poor acceptors in the presence of α-lac, but were not acceptors in its absence. In

contrast, Bell et al. (1976) reported that α-lac had inhibitory effects when dimers or tetramers of GlcNAc was used as the acceptor substrate for bovine milk GalTase. However, they suggested that the inhibition of α-lac on the GalTase activity towards GlcNAc polymers was different from that of α-lac on the GalTase activity towards free GlcNAc, it was a competitive inhibition rather than a change in the K_M and Vmax. Geren et al. (1976) reported that O-glycosidic analogies of GlcNAc were good substrates for bovine milk GalTase, and as the substituted groups became more hydrophobic, the affinity of the binding of these analogy was increased. The extreme example was that the K_M value of *N*-acetyl-5-bromoindolyl-ß-glucosaminde was 2000 times lower than the K_M value of GlcNAc. In the review of Ram and Munjal (1985), it was indicated that human plasma GalTase also use various substances with terminal GlcNAc as acceptor, and α-lac had no significant effect on the use of these substance by the enzyme.

1.3.3.4 Inhibition of Galactosyltransferase Activity

The substrate inhibition by either glucose or GlcNAc occurs at lower concentration in the presence of α-lac than in its absence (Klee and Klee, 1970). Glucose has no inhibitory effect on GlcNAc reaction in the absence of α-lac, and in fact, it may stimulate the overall reaction slightly; however, GlcNAc inhibits glucose utilization in the presence of α-lac (Klee and Klee, 1970). UDP-galactose concentration affects the K_M of GalTase for glucose in the presence of α-lac (Fitzgerald et al., 1970). Dial-UDP is a competitive inhibitor binding to GalTase (Powell and Brew, 1976b). Babad and Hassid (1966) reported that when glucose was used as an acceptor, lactose synthetase was inhibited by the following compounds in the order of effectiveness: PPi, ITP, UTP, UDP, Pi, UMP, TPP, and CTP. Little or no inhibition obtained with uridine, dUMP, ATP, GTP, galactose 1-phosphate, galactose or lactose.

1.3.3.5 Interactions of Galactosyltransferase with α-Lactalbumin, Mn^{2+}, UDP-Galactose and Acceptors

A number of studies have been carried out on the interactions of GalTase, α-lac , Mn^{2+}, UDP-galactose, and substitutes mainly using bovine milk GalTase. Initially, an ordered sequential mechanism was suggested by the kinetics of the GalTase reactions, in which the substances bound to GalTase in the order of Mn^{2+}, UDP-galactose, (a-lac), and acceptor (references in Bell et al., 1976). However, Bell et al. (1976) proposed a mechanism of a random equilibrium, in which Mn^{2+} must first bind to the enzyme, while UDP-galactose, acceptor, and α-lac were then added to the enzyme·Mn complex randomly. Ram and Munjal (1985) have carefully reviewed the studies of the interactions. The studies were mostly carried out with bovine milk GalTase and using a difference spectroscope. The following are the main points considered by the authors. Mn^{2+} alone can bind to GalTase, and free UDP-galactose can bind to the enzyme·Mn complex. Many observations suggest that Mn^{2+} is required for significant binding of UDP-galactose or UDP to GalTase, and the tyrosine or tryptophan residues are involved in the binding of UDP-galactose or UDP to the enzyme·Mn complex. It is apparent that the binding of Mn^{2+} and UDP-galactose precedes GlcNAc reaction with the enzyme. The *N*-acetyl group appears essential for the binding, while the $-CH_3$ group does not show any function in the binding. GalTase may have a hydrophobic pocket near or containing the binding site for GlcNAc. The presence of UDP can facilitate the interaction of GalTase and α-lac, however, UDP alone may not be as effective as GlcNAc or glucose in forming the GalTase·α-lac complex. Some tryptophan residues are involved in the interaction of GalTase and α-lac. These residues do not interact with UDP bound to GalTase and are not located in the region which undergoes a conformational change induced by the interactions with UDP and GlcNAc. On the other hand, the tyrosine residues involved in the interactions with UDP and GlcNAc are not affected by the interaction with α-lac. The reaction of GalTase and α-lac does not involve the uracil ring of UDP.

1.3.3.6 K_M Values of Galactosyltransferase

The K_M values of GalTases of various sources of milk and blood for different reactants in the enzyme reaction have been given in a number of reports, which are summarized in Table 1.1.

Table 1.1 Apparent K_M values of GalTase from various blood and milk sources associated with Mn^{2+}, UDP-galactose, GlcNAc and glucose.

Purified GalTase was used in all the reports. The K_M values from the assays with GlcNAc as an acceptor appear in regular type, while those from the assay with glucose as an acceptor are in bold type. The K_M values for GlcNAc were those determined in the absence of α-lac while the K_M values for glucose were in the presence of α-lac.

Source of GalTase	K_M (mM)				Reference
	Mn^{2+}	UDP-galactose	GlcNAc	Glucose	
Bovine milk			3.6		Klee and Klee, 1970
			8.3		Schanbacher and Ebner, 1970
	0.20	**0.050**		**2.5**	Morrison and Ebner 1971
Human milk	0.083	0.072	7.4		Khatra et al., 1974
Human plasma	0.40	0.024	3:9	**2.9**	Bella et al., 1977
		0.052 **0.0090**	1.5	**1.8**	Paquet and Moscarello, 1984

1.3.4 Chemical Aspects of Galactosyltrasferase and α-Lactalbumin

Mr of GalTases from some milk and blood sources have been reported as follows: bovine milk, two (Magee et al., 1973) or three (Barker et al., 1972) bands of

42,000-43,000, (49,000), and 54,000-58,000 respectively (SDS-PAGE); bovine serum, 47,800 (sedimentation equilibrium, Turco and Heath, 1976); human milk 55,000 (SDS-PAGE, Berger et al., 1983); and human serum, 49,000 (Fujita-Yamaguchi and Yoshida, 1981) or 50,000 (Berger et al., 1983) (SDS-PAGE). The membrane bound GalTases appear usually to have higher M_r. For example, the GalTase from Golgi membranes of lactating mammary glands have a major band of M_r 69,000 and a minor band of 53,000 (Smith and Brew, 1977); and the M_r of pig thyroid microsomal GalTase is 74,000 (Bouchilloux, 1979). More data about the M_r of various GalTases are in the review of Strous (1986).

GalTases from various sources (eg milk, serum, membrane) have some differences in amino acid composition (Ram and Munjal, 1985). Bovine milk GalTase is known to be a glycoprotein and its carbohydrate content is 12-13% of its net weight (Trayer and Hill, 1971).

The reported values of M_r of bovine α-lac are between of 14,000-15,000 (Hopper and Mckenzie, 1973; Brodback et al., 1967; Schmidt and Ebner, 1971). The M_r and amino acid sequences of α-lac from other mammals, including sheep, goat, pig and human are similar to those of bovine α-lac (Schmidt and Ebner, 1971). The amino acid sequence of α-lac is homologous to that of hen egg white lysosome (Brew et al., 1967).

1.3.5 Biological Functions of Galactosyltrasferase

As already introduced in Section 1.3.1.2, GalTase is one of the two protein components of lactose synthetase, which synthesize lactose in milk.

GalTase catalyzes the galactosylation of glycoproteins or glycolipids at non-reducing GlcNAc residues. Almost all eukaryotic proteins destined for secretion or as components of either an intracellular membrane system or plasma membrane are glycoproteins with a galactose at the penultimate or ultimate position of their N-linked

oligosaccharide chains. (Strous, 1986). The Golgi apparature has been identified as the subcellular site for the sequential addition of at least some of the galactose moieties to glycoproteins (Ram and Munjal, 1985).

During cell interactions, cell surface GalTase, as some other cell surface glycosyltransferase, may participate in adhesion by binding to specific substrates on adjacent cells or in extracellular matrix (Shur and Roth, 1975; Strous, 1986; Shur, 1989). It has been suggested that mouse sperm surface GalTase binds to the terminal *N*-acetylglucosaminyl residue in zona glycoprotein during sperm-zona binding; this is further introduced in Chapter 4.

1.3.6 Some Methods Used in Studies of Galactosyltransferase and α-Lactalbumin

1.3.6.1 Purification and Stabilization of Galactosyltransferase

(1) Purification

It was only after the advent of affinity chromatography columns that GalTase was highly purified. Andrew (1970) firstly utilized an α-lac-agarose column for GalTase purification. Barker et al. (1972) then developed UDP-hexanoalmine-agarose (UDP-agarose) and GlcNAc-agarose columns for this purpose. Barker et al. (1972) compared the above three absorbents and found that UDP-agarose had a much larger capacity, but was less specific, for GalTase than the other two absorbents. They indicated that when purifying GalTase from milk, UDP-galactose seemed best suited for adsorbing the enzyme from whey, while α-lac-agarose and GlcNAc-agarose were more suitable for further purification.

(2) Stabilization

Unlike many other enzymes (Bergmeyer et al., 1974), purified GalTase is very unstable (Fraser and Mookerjea, 1976; 1977), and shows a pronounced

tendency to aggregate (Khatra et al., 1974). Many substances have been used to stabilize the purified GalTase. Among them, Triton X-100 (e.g., Fraser and Mookerjea, 1976; 1977), albumin (e.g., Khatra et al. 1974), and GlcNAc (e.g., Trayer and Hill, 1971; Barker et al., 1972; Shur and Neely, 1988) have been commonly used. The membrane-bound GalTase is normally extracted with Triton X-100 to release most of the enzyme (Ram and Munjal, 1985). Khatra et al. (1974) showed that GalTase activity towards GlcNAc increased with the concentration of bovine serum albumin between the range of 0-1mM.

1.3.6.2 Assay Methods for Galactosyltransferase Activity and α-Lactalbumin Activity

1.3.6.2.1 Assay Methods

Although GalTase or α-lac activity can be assayed by spectrophotometrically measuring the amount of UDP produced in the reaction (Schanbacher and Ebner, 1970; Morrison and Ebner, 1971), it has been commonly assayed by the more sensitive and simpler radioenzyme assay method, which was initially used by Babad and Hassid in 1966.

The radioenzyme method for GalTase activity assay and that for α-lac activity assay are similar. Mn^{2+} is required for enzyme activity. Trace amounts of UDP-galactose with ^{3}H or ^{14}C labelled galactose is added to non-radioactive UDP-galactose at an optimal concentration in the assay media. The pH of the medium is usually buffered to about 7.4. In GalTase assay, the acceptor used is usually GlcNAc or ovalbumin (e.g., Bell et al., 1976). The GalTase activity is determined by measuring the radioactivity of LacNAc, or of galactosylated ovalbumin formed in the assay. In α-lac assay, GalTase needs to be present in the medium. The acceptor can be glucose or GlcNAc (e.g., Brew et al., 1968). When glucose is used as an acceptor, the radioactive lactose is measured, which increases with the α-lac activity; while when GlcNAc is used, radioactive LacNAc is measured, which decreases with the α-lac activity.

When GlcNAc or glucose is used as a receptor in the assays, as in the

work of this thesis, two methods are commonly used for separating the product, LacNAc or lactose, from the remaining UDP-galactose. One uses cation ion exchange column, e.g., AG 2-X8 column, to retard the residual UDP-galactose and allow LacNAc or lactose to be eluted (e.g., Brew et al., 1968; Hamilton, 1980). The other uses high-voltage paper electrophoresis in 1% (w/v) tetraborate borate buffer (e.g., Babad and Hassid, 1966; Shur and Bennet, 1979). In this system, the assay product LacNAc or glucose remains near the origin, while UDP-galactose migrates much further towards the cation electrode.

1.3.6.2.2 Some Factors Affecting the Assays

Two factors most commonly affect the assay of GalTase or α-lac, both by degrading UDP-galactose.

One affecting factor includes nucleotide pyrophosphatases and alkaline phosphatases, which are present in many tissues or body fluids of mammals (Mookerjea and Yung, 1975; Vessey and Zakim, 1975; Spik et al., 1979). The pyrophosphatases decompose UDP-galactose into galactose 1-phosphate, which are further hydrolyzed into galactose by the phosphatases. A method used to control such degradation is to add a variety of nucleotide mono-, di- and triphosphates to serve as competitive inhibitors of pyrophosphatases (Mookerjea and Yung, 1975; Vessey and Zakim, 1975). Lau and Carlson (1981) also reported that nucleotide pyrophosphatase in rat intestinal mucosa homogenate was Zn^{2+}-dependent. It was inhibited by preincubation of enzyme preparation with EDTA to remove Zn^{2+}, and addition of Mn^{2+} for the GalTase activity assay in the same preparation did not reactivate the pyrophosphatase.

The other affecting factor is Mn^{2+}, which causes the decomposition of UDP-galactose into galactose 1,2-cyclic phosphate and UMP. The decomposition is concentration (Nunez and Barker, 1976; Spik et al., 1979) and pH dependent (Nunez and Barker, 1976). Measured between 0 and 40mM Mn^{2+} (at pH 7.3), the decomposition increased linearly (Spik et al., 1979). Measured between pH values of 6.5 and 7.9 (at 10 and 20mM Mn^{2+}), the decomposition increased dramatically.

Comparing with other divalent cations of the effect on the decomposition of UDP-gal, the order of $Mn^{2+} > Co^{2+} > Zn^{2+} > Ni^{2+} > Ca^{2+} > Cu^{2+}$ was reported by Nunez and Barker (1976). However, Mn^{2+} and Mg^{2+} were equally effective as reported by Spik et al.(1979).

CHAPTER 2
GENERAL MATERIALS AND METHODS

2.1 MATERIALS

Bovine milk galactosyltransferase (GalTase), bovine milk α-lactalbumin (α-lac), bovine serum albumin (BSA), galactose, lactose, *N*-acetylglucosamine (GlcNAc), and *N*-acetyllactosamine (LacNAc) were purchased from Sigma (St. Louis, MO, USA). UDP-galactose and galactose 1-phosphate from Boehringer (Sydney, Australia). UDP-[^{3}H]galactose and UDP-[^{14}C]galactose were supplied by New England Nuclear (Bedford, MA, USA). AG 1-X8 (200-400 mesh, acetate form) and AG 2-X8 (200-400 mesh, Cl^- form) anion exchange resins were obtained from Bio Rad (Sydney, Australia). Sodium tetraborate was from Ajax Chemicals (Adelaide, Australia). The paper used for high-voltage paper electrophoresis was 3MM paper from Whatman (Maidstone, England). Other chemicals and organic solvents used were all of analytic reagent grade.

The purities of both radio active and non-radioactive UDP-galactose were checked from time to time by paper chromatography following the method of Spik, et al. (1979) in the system of ethyl acetate / pyridine / glacial acetic acid / H_2O, 5 : 5 : 1 : 3.

2.2 METHODS

2.2.1 Galactosyltransferase Assay and α-Lactalbumin Assay

Both assays were carried out in 50μl medium. The medium components in both assays included Hepes, pH 7.4 or Mes, pH 6.5 (50mM), $MnCl_2$ (4-20mM as indicated in different chapters), UDP-galactose (0.4-2mM as indicated in different

chapters), UDP-[^{3}H]galactose or UDP-[^{14}C]galactose (0.008-1.6μCi as indicated in different chapters), GlcNAc or glucose (25mM). For α-lac assay, bovine milk GalTase was added to the medium. BSA was used to bring the protein concentrations of all the samples in each α-lac activity assay to the same level. The reaction was incubated at 36°C for certain times as indicated in different chapters and the reaction was stopped by putting the samples on ice.

The incubated reaction mixture were either directly applied to cation ion exchange columns or treated for high-voltage paper electrophoresis (HVPE). The ion exchange column used was 0.5 x 2cm AG 1-X8 or AG 2-X8 columns. The remaining UDP-galactose was bound to the column, and the neutral sugar product, LacNAc or lactose, was washed with 2 x 0.5ml H_20. The washings were mixed with scintillation fluid and counted for radioactivity. In the current experiments, after incubation the enzyme reaction was stopped by putting samples on ice, not by EDTA which has been used elsewhere. This was because that EDTA was found to affect the re-use of the AG 2X-8 columns, i.e., some retarded radioactive sugars were irregularly eluted down when the column was re-used. However, the AG 2X-8 resin used in the work of this thesis was about 10 years old, and no studies were undertaken on new AG 1X-8 or AG 2X-8 resin.

The samples for HVPE were mixed with 200μl methanol and 85μl chloroform (methanol : chloroform : water = 60 : 25 : 15) (Jones et al, 1986). After vortexing and centrifugation, the proteins were precipitated at the bottom of the Eppendorf tubes, and the supernatants were transferred to new Eppendorf tubes and dried in a vacuum centrifuge. The dried samples were then redissolved in 15μl H_2O and applied to 55 x 16cm Whatman 3MM paper at positions 20cm from the edge of the anion electrode side and 2cm apart from each other. The paper was subjected to high-voltage electrophoresis in 1% sodium tetraborate at 2000V for 75min. Lactose, LacNAc galactose, UDP-galactose, galactose 1-phosphate were used as standards. The UDP-galactose was located by 245nm UV light. The other sugars were stained by silver nitrate (Trevelyan et al., 1950). Towards the anion electrode, LacNAc and lactose

migrated a short distance with a R_f value of 0.13 and 0.19 respectively, galactose migrated much further with a R_f value of 0.60, and UDP-galactose and galactose 1-phosphate migrated farthest with R_f values of 0.85 and 0.89 respectively. The paper of each sample at the position corresponding to the relevant sugar was cut for counting radioactivity in scintillation fluid, the total activity of each lane was also counted. The following equation was used to calculate the amount of the products formed by the enzyme:

$$\text{nmole sugar product} = \text{nmole UDP-galactose in assay medium} \times \frac{\text{radioactivity at the position of the sugar product}}{\text{total radioactivity}}$$

In the previous reports using HVPE for assay of GalTase activity in epididymal fluid or in sperm, the incubated samples were not extracted to obtain sugar solutions without proteins or sperm (e.g., Shur and Hall, 1982a; b). However, it was found in the present experiments that the use of the unextracted samples caused difficulty in sample application, especially when rat epididymal fluid was used in the assay, which was extremely viscous after concentration. The proteins in the samples also affected the result of the electrophoresis.

2.2.2 Assay of Protein Concentration

The Coomassie blue method of Sedmak and Grossberg (1977) was followed for estimation of protein concentration, and BSA was used as the standard.

CHAPTER 3
GALACTOSYLTRASFERASE ACTIVITY IN EPIDIDYMAL PLASMA AND RETE TESTIS PLASMA OF SOME MAMMALS

3.1 INTRODUCTION

Galactosyltransferase (GalTase) activity was reported to be present in the epididymal plasma of rat (Hamilton, 1980) and mouse (Shur and Hall, 1982a), and in rat rete testis plasma (Hamilton, 1980). The enzyme activity was also detected on the sperm surface of some mammalian species (see "General Introduction" to Chapter 4), and it was reported that the enzyme was the sperm's receptor for zona pellucida in sperm-zona binding in mouse (see "General Introduction" to Chapter 4). Meanwhile, proteins similar to α-lactalbumin (α-lac), that modifies the substrate specificity of GalTase and had been thought to be unique in mammary gland and milk (see Section 1.3), were reported to be present in epididymis mainly in the rat, and also in some other mammals. It was reported that epididymal α-lac-like activities could stimulate the activity of GalTase not only towards glucose but also towards inositol (see Section 6.1). Therefore, it was considered that GalTase in the male reproductive tract probably had some special important functions in reproductive physiology in mammals. However, most of the relevant reports were of experiments with rodents. The studies in this chapter were planned to establish whether GalTase activity was also present in the male reproductive tract plasma of some other mammals, and if so, to compare the levels of the activity in various species to see whether there were any differences which were possibly related to reproductive functions.

3.2 MATERIALS AND METHODS

3.2.1 Literature Review of the Method

At the time that the experiments of this chapter were carried out, the only available report concerning the assay of GalTase activity in mammalian testicular and epididymal plasma was that of Hamilton (1980) using the rat. In this report, rat rete testis fluid was collected from the extratesticular portion of the rete by piercing with a needle and collecting with vinyl tubing 12-18 hr after the ligation of the ductus efferens. Rat epididymides were perfused with Krebs Ringer phosphate buffer and cut under paraffin oil after removal from the animals. The epididymal fluid was oozed out under gentle pressure with forceps and collected with microhematocrit pipettes. The sperm in the fluids were removed by centrifugation. In this report, GalTase activity in rete testis plasma or epididymal plasma was assayed in a medium containing 80mM Tris-HCl of pH 7.5, 2mM UDP-[^{14}C]galactose, 40mM $MnCl_2$, 40mM *N*-acetylglucosamine (GlcNAc) in a total volume of 75µl. Incubation was carried out at 35^{0}C. These assay conditions were optimised for the GalTase of rete testis plasma , but not for the enzyme of epididymal plasma. An AG 2-X8 column was used for sugar separation.

3.2.2 Chemicals

Avertin was made up as follows; 5g methylbutanol (Ajax chemicals, Sydney, Australia) and 3ml tribromoethanol (Aldrich, Milwaukee, Wisconsin, USA) were dissolved in 20ml absolute ethanol, then 0.9% NaCl (w/v) was used to make up the volume to 250ml. The sources of the chemicals used for GalTase assay were as introduced in Section 2.1.

3.2.3 Animals

All the animals used were sexually mature. Mice, rats and rabbits were obtained from the Central Animal House of the University of Adelaide. Testes and epididymides of rams, bulls and boars were collected from the abattoirs of the South Australian Meat Corporation and stored on ice.

3.2.4 Methods

3.2.4.1 Sample Collection

Mice and rats were anaesthetized with ether and decapitated to release blood, rabbits were killed by injection of an overdose of Avertin intravenously. Cauda epididymal fluids were collected immediately after the animals were killed: the vas deferens was connected to the exit tube of a peristaltic pump and saline was pumped into the cauda epididymis, so that epididymal fluid exuding from a needle puncture on the epididymal duct could be collected with a Pasteur pipette. In mouse, the pumping was very difficult since the vas deferens is very narrow, so an extremely slow speed had to be used in this species. The testis and epididymis of rams, bulls, and boars were placed on ice soon after being obtained and cauda epididymal fluids were collected after 1-1.5hr, which was the time required for transport to the laboratory. A small area of the tunica albuginea of the cauda epididymides was removed, the exposed cauda epididymal tubules were punctured at points where there were no visible blood vessels, and the fluids squeezed out by forceps and collected by Pasteur pipette. The epididymal fluids were centrifuged in a microcentrifuge for 2min at 4°C and the supernatant was separated. After collection, the protein concentration of the plasma of individual animal was measured. Then the enzyme assay of the plasma was carried out.

Rat rete testis fluid was collected following the method in Hamilton's report (1980). Rete testis fluids of rams, bulls and boars were collected by piercing the extratesticular portion of the rete of the tissue.

3.2.4.2 Assay of Galactosyltransferase Activity and Pyrophosphatase and Phosphatase Activities

The methods for cauda epididymal plasma GalTase assay were as described in Section 2.2.1 with some modifications. Pyrophosphatase and phosphatase activities were determined in the same assay as GalTase activity. The assays were carried out in two ways. (a) The assay medium contained 50mM Hepes, pH7.0, 20mM $MnCl_2$, 25mM GlcNAc, 0.4mM UDP-galactose containing 8nCi UDP-[^{14}C]galactose, and cauda epididymal plasma containing 150μg protein in a total volume of 50μl. 8nCi UDP-[^{14}C]galactose gave 12 x 10^3 CPM when counted in scintillation fluid. The assay medium was incubated at 37^{0}C for 1hr. AG1-X8 chromatography was used for separation of the products. Maximally, 90% of the total radioactivity could be recovered from the column in neutral sugars produced from UDP-galactose by enzymes. The difference between the amounts of radioactive products of each sample in the presence and absence of GlcNAc was taken as the GalTase activity. Radioactivity in the absence of GlcNAc minus radioactive background, (which was the radioactivity in the eluate from sample without epididymal plasma), was taken as the pyrophosphatase and phosphatase activities. (b) The assay mixture contained 50mM Mes, pH6.5, 20mM $MnCl_2$, 25mM GlcNAc, 2mM UDP-galactose containing 1.6μCi UDP-[^{3}H]galactose, and cauda epididymal plasma containing 100μg protein in a total volume of 50μl. The assay mixture was incubated at 37^{0}C for 20min. High-voltage paper electrophoresis (HVPE) was used to separate the different sugars in the assay medium at the end of the incubation. Samples incubated at 0^{o}C were used as controls for both GalTase activity and pyrophosphatase and phosphatase activities. Method (a) was mainly used in the early assays to survey the GalTase activity as well as the pyrophosphatase and phosphatase activities, and the inhibition of the latter activities on the former activity in various species. Method (b) was used in the later assays to compare quantitively the GalTase activity and the pyrophosphatase and phosphatase activities in different species.

GalTase activity in rete testis plasma was assayed with method (a) as above.

3.2.4.3 Assay of Protein Concentration

The Coomassie blue method was used (see Section 2.2.2).

3.3 RESULTS

3.3.1 Assay Method

3.3.1.1 Pyrophosphatase and Phosphatase Activities in Cauda Epididymal Plasma

It was found that pyrophosphatase and phosphatase activities in cauda epididymal plasma caused interference, which was very severe in some species, with the GalTase activity assay of the plasma (see Section 1.3.6.2.2). Therefore, attempts were made to find a proper way which could control the effects of these activities on the GalTase activity, but did not bias the comparison of the GalTase activity in different species.

(a) Pyrophosphatase and phosphatase activities in cauda epididymal plasma of various species

Pyrophosphatase and phosphatase activities were present in the cauda epididymal plasma of all the species used, and the activities in various species were very different. This can be seen in Fig. 3.1 and Section 3.3.2. The activities in the order high to low were boar > ram > rabbit > mouse > rat.

(b) Effect of the pyrophosphatase and phosphatase activities on the GalTase activity in cauda epididymal plasma

ATP was used in the assays to demonstrate the relationship between the pyrophosphatase and phosphatase activities and the GalTase activity in various species

under the assay conditions of method (a). In the rat (Fig. 3.1a), the pyrophosphatase and phosphatase activities did not have significant inhibitory effects on the GalTase activity. 1mM ATP was enough to inhibit most of the pyrophosphatase activity, while it only slightly stimulated the GalTase activity. ATP had an inhibitory effect on the GalTase activity at concentrations higher than 2mM, and dramatically inhibited the GalTase activity at a concentration of 6mM. The result from rabbits (Fig. 3.1b) was between those of rats and rams. In the ram (Fig. 3.1c), in the absence of ATP, all the UDP-galactose was degraded and GalTase activity was not detectable. Although ATP at 1mM started to inhibit the pyrophosphatase activity and stimulate the GalTase activity, only at 3mM and above (measured to 6mM), did it inhibit most of the pyrophosphatase activity and stimulate the GalTase activity to a maximum. In the boar, (Fig. 3.1d) even in the presence of 3mM ATP, all the UDP-galactose was still completely degraded and GalTase activity was not detectable. Only from 4mM, was ATP able to have some inhibitory effect on the pyrophosphatase activity and did GalTase activity start to be detectable.

(c) Effects of pH on the pyrophosphatase and phosphatase activities and the GalTase activity in cauda epididymal plasma

The effect of pH, over the range of 6.0-7.5, on the pyrophosphatase and phosphatase activities of the epididymal plasma of the rat, rabbit, ram and boar was examined. In each species, these activities increased with rising pH, and the most pronounced increase was between 6.5 and 7.0. GalTase activities in the epididymal plasma of the ram and the boar over the pH range of 6.0-7.5 were also measured to determine the optimum pH, since it was noticed that at pH 7.4 and 7.0 respectively, the GalTase activity in these species was severely inhibited by the pyrophosphatase and phosphatase activities. The changes of both pyrophosphatase and phosphatase activities and GalTase activity with pH in the epididymal plasma of ram and boar are shown in Fig. 3.2 a and b. In both species, the GalTase activity was greatly inhibited when pH was higher than 7, because of the dramatic increase of the pyrophosphatase and

Table 3.supplement Effects of some factors on the pyrophosphatase and phosphatase (P) activities and the GalTase activity (G) in ram and boar epididymal plasma

Vales are mean ± SE of CPM. Triplicate samples were assayed. Method (a) was used for the assay.

Animals		Ram		Boar	
Enzymes		P	G	P	G
	None	14204 ± 435	n.d.	14431 ± 568	n.d.
	CDP-choline	542 ± 56	914 ± 67	4057 ± 120	389 ± 70
Factors (5mM)	CTP	531 ± 71	1121 ± 99	1798 ± 122	815 ± 59
	GTP	683 ± 64	834 ± 78	3988 ± 191	378 ± 57
	NAD^+	487 ± 53	1103 ± 79	2015 ± 146	792 ± 81

n.d., not detectable.

The pyrophosphatase and phosphatase activities of samples with and without EDTA-treatment were 14175 ± 541CPM and 14323 ± 246CPM respectively in ram, and 14369 ± 430 CPM and 14256 ± 611CPM respectively in boar (mean ± SE, n=3).

phosphatase activities. Actually in the boar, the GalTase activity was not detectable when pH was higher than 7. Method (a) was used for the assay with some modifications as described in the legend of the figures.

(d) Effects of other factors on the pyrophosphatase and phosphatase activities and the GalTase activity in epididymal plasma

In preliminary experiments, other nucleotides besides ATP including CTP, GTP and CDP-Choline, and NAD^+ were also tested as possible inhibitors of the pyrophosphatase activity in cauda epididymal plasma in ram and boar. All of them showed some inhibitory effect on the pyrophosphatase activity and some stimulation of the GalTase activity. The results are shown in Table 3.supplement.

Lau and Carlson (1981) reported that nucleotide pyrophosphatase in rat intestinal mucosa homogenate was Zn^{2+}-dependent. It was inhibited by pre-incubation of the enzyme preparation with EDTA to remove Zn^{2+}, and addition of Mn^{2+} to the GalTase assay in the same preparation did not reactivate pyrophosphatase. Ion chelation was tried following their method with ram and boar cauda epididymal plasma in the current experiments. However, inhibition of pyrophosphatase activity was not observed, presumably because of the differences between the pyrophosphatases from different tissues and / or different animals. Λ

3.3.1.2 Assay Conditions for Comparison of Galactosyltransferase Activities in Various Species

Method (b) was used to compare the GalTase activities in various species. In the assay of this method, low pH buffer (pH 6.5) and high UDP-galactose concentration (2mM) were used. Epididymal plasma containing 100μg protein was used and the incubation time was 20min. Samples from rats and boars were chosen to examine the inhibitory effects of pyrophosphatase and phosphatase activities on GalTase activity in epididymal plasma under the assay conditions, since they are the species with the lowest and highest pyrophosphatase and phosphatase activities respectively in

epididymal plasma in the species used. In the rat (Fig. 3.3 a), the pyrophosphatase and phosphatase activities at pH 6.5 were very low, and addition of ATP further inhibited these activities. ATP did not stimulate the GalTase activity and actually seemed to slightly inhibit it. In the boar (Fig 3.3 b), at pH 6.5, the pyrophosphatase and phosphatase activities were still high. Increased concentration of ATP between 2-8mM increasingly inhibited these activities, and at 8mM it inhibited by about 60%. However, over the whole range of concentrations, ATP did not stimulate the GalTase activity. These results suggested that at pH 6.5, 2mM UDP-galactose allowed non-competitive consumption by both the pyrophosphatase and phosphatase and the GalTase in all the species used. Therefore, under the above assay conditions, the pyrophosphatase and phosphatase activities did not have inhibitory effects on the GalTase activities in these species.

The effect of concentration of epididymal plasma protein and that of incubation time on the GalTase activity in epididymal plasma was examined using boar epididymal plasma. Under the assay conditions of method (b), both the GalTase activity of boar epididymal plasma containing 100μg protein (Fig. 3.4) and the activity at a 20min of incubation time (Fig. 3.5) were within the ranges of linear increase of the enzyme activity.

3.3.2 Comparison of Galactosyltransferase Activities (together with Comparison of Pyrophosphatase and Phosphatase Activities) in Cauda Epididymal Plasma of Various Species

The cauda epididymal plasma GalTase activities in various mammals are shown in Table 3.1. Method (b) was used for the assays. There were no statistical differences between the GalTase levels in the various species.

The pyrophosphatase and phosphatase activities of the samples were determined in the same assay for GalTase activity. The activities of the boar were significantly higher ($P < 0.01$) than those of the other species; the activities of the ram

were significantly higher ($P < 0.05$) than those of the mouse, rat and rabbit; and the activities of the rabbit were significantly higher ($P < 0.05$) than those of the rat.

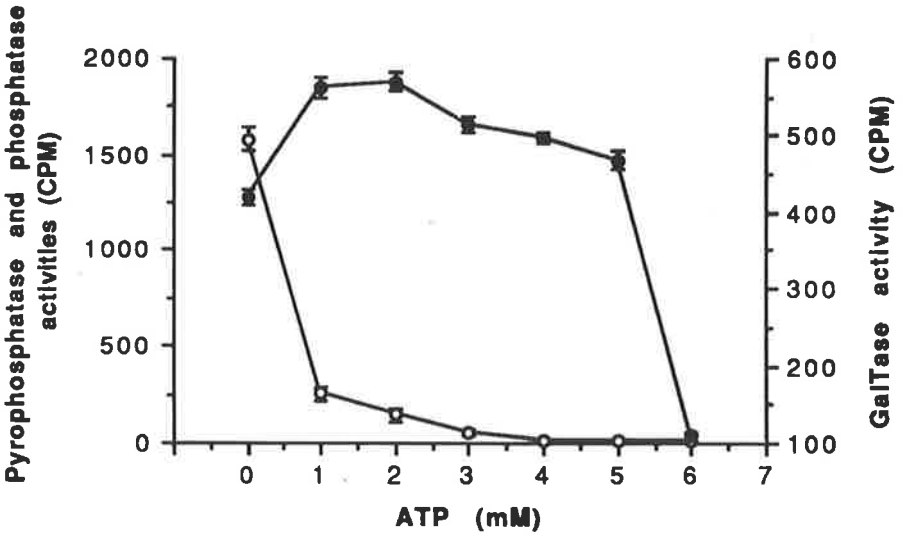
3.3.3 Comparison of Cauda Epididymal Plasma Galactosyltransferase and Serum Galactosyltransferase

Hamilton (1980) reported that in the rat rete testis plasma GalTase was more resistant than the serum enzyme to pre-heating (50°C, 1hr). A similar comparison was made between the epididymal plasma and serum GalTases in the ram.

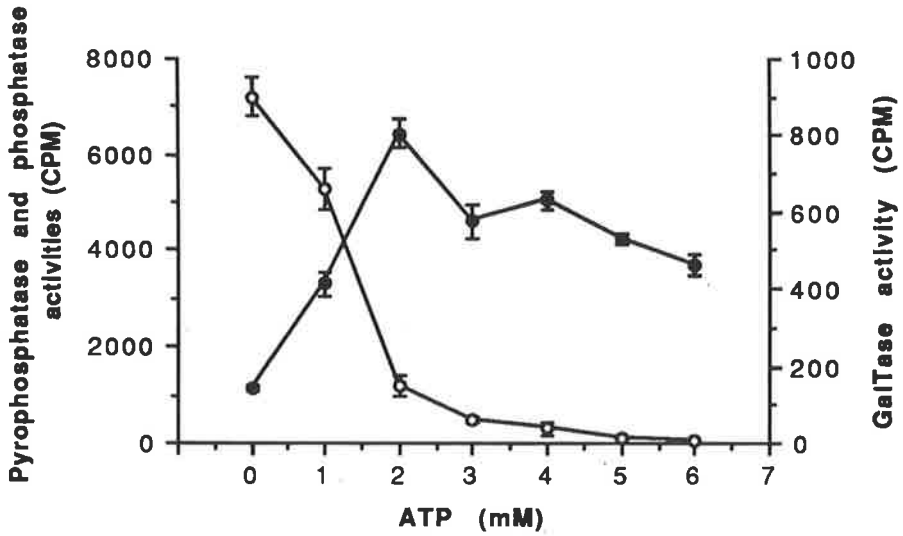
Ram cauda epididymal plasma and serum were incubated at 50°C for 0.5 or 1 hr prior to the assay of GalTase activity, another subset was kept on ice for the same period as control (Fig. 3.6). After 0.5hr and 1hr of pre-heating, the activity of serum GalTase reduced by 78.1 and 97.6% of the controls respectively, while the activity of epididymal plasma enzyme reduced only by 56.9 and 78.0% respectively. The differences between the enzyme activity of the epididymal plasma and that of the serum after both 0.5hr and 1hr pre-incubation were significant at 5% level.

3.3.4 Galactosyltransferase Activity in Rete Testis Plasma

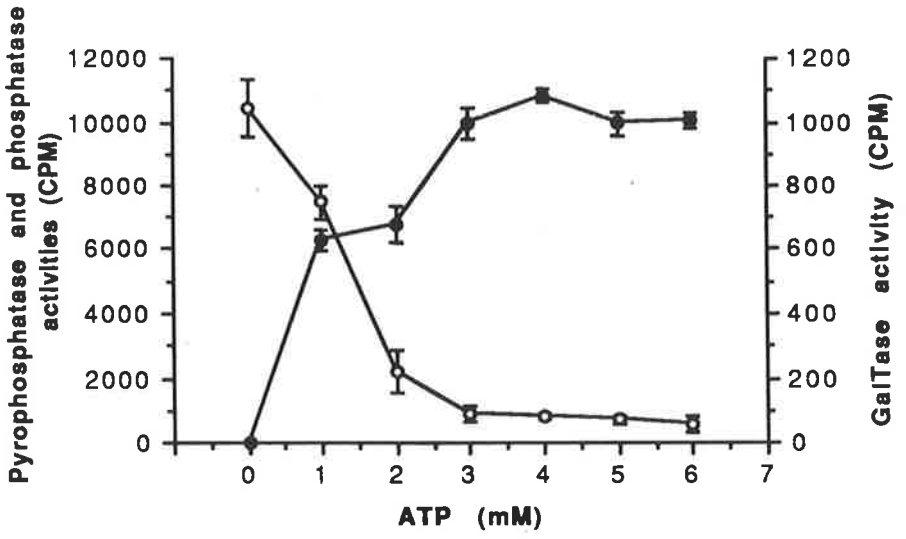
GalTase activity was present in the rete testis plasma of all the species examined (the rat, ram, bull and boar). Because of the small amounts of rete testis fluid available, no further study on the enzyme in this plasma was carried out. Pyrophosphatase and phosphatase activities in rete testis plasma in all the species were either not detectable, or very low, compared with the same activities in the cauda epididymal plasma of the same species. .


Pyrophosphatase and phosphatase activities (CPM)
2000
1500
1000
500
0
600
500
400
300
200
100
GalTase activity (CPM)
0 1 2 3 4 5 6 7
ATP (mM)

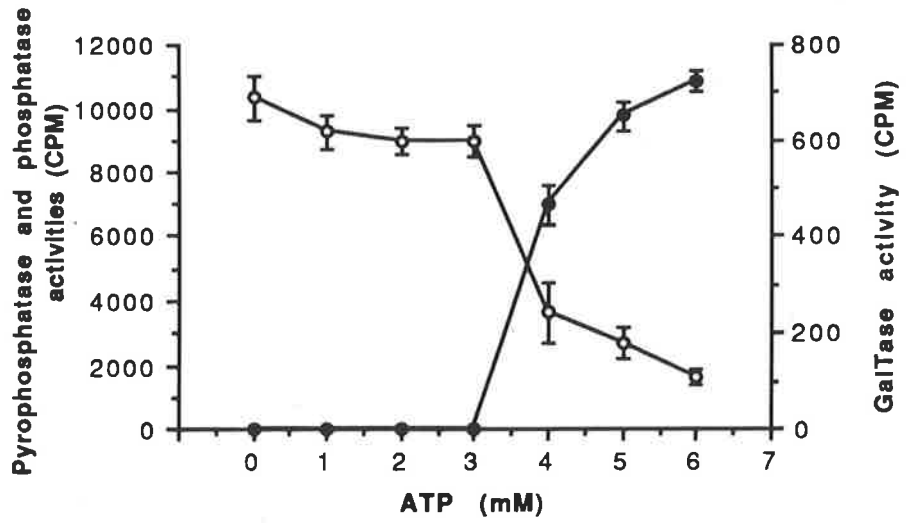
a


Pyrophosphatase and phosphatase activities (CPM)
8000
6000
4000
2000
0
1000
800
600
400
200
0
GalTase activity (CPM)
0 1 2 3 4 5 6 7
ATP (mM)

b

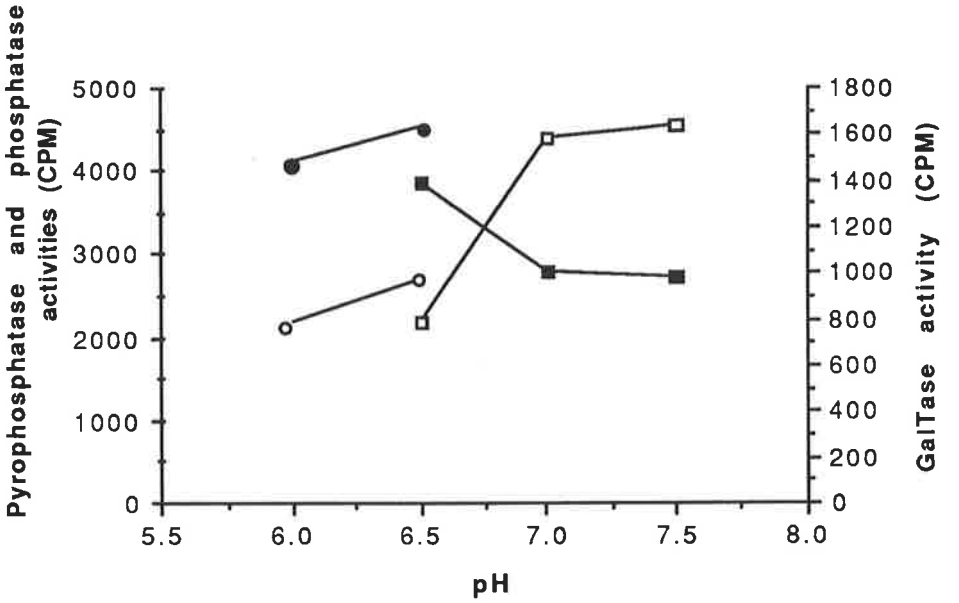


c

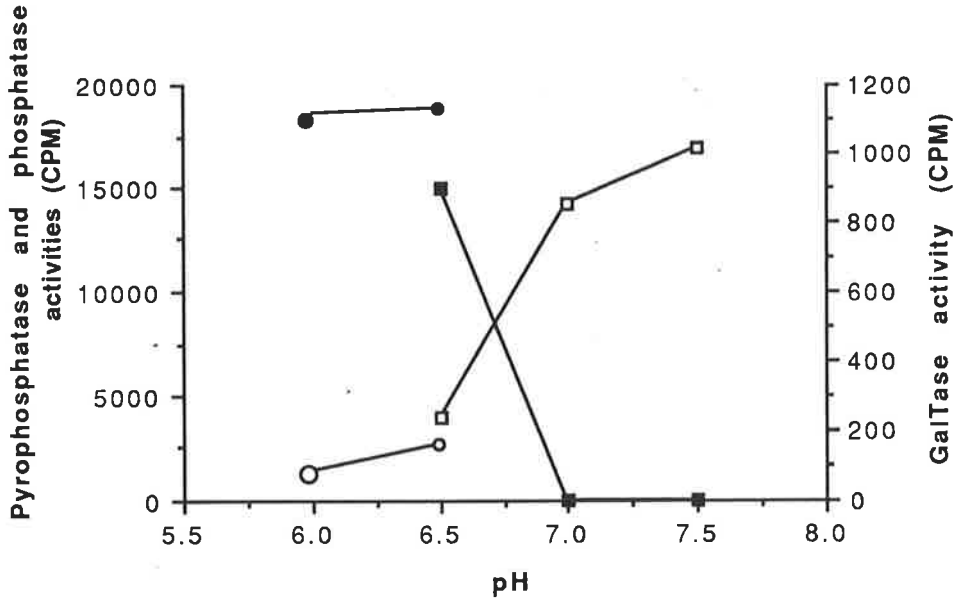


d

Fig. 3.1 Effect of ATP on the pyrophosphatase and phosphatase activities (o) and the GalTase activity (•) in cauda epididymal plasma of rat (a), rabbit (b), ram (c) and boar (d). Method (a) was used for the assays. Each point is the mean of three assays of samples from different individuals, with the SE shown as a vertical line.

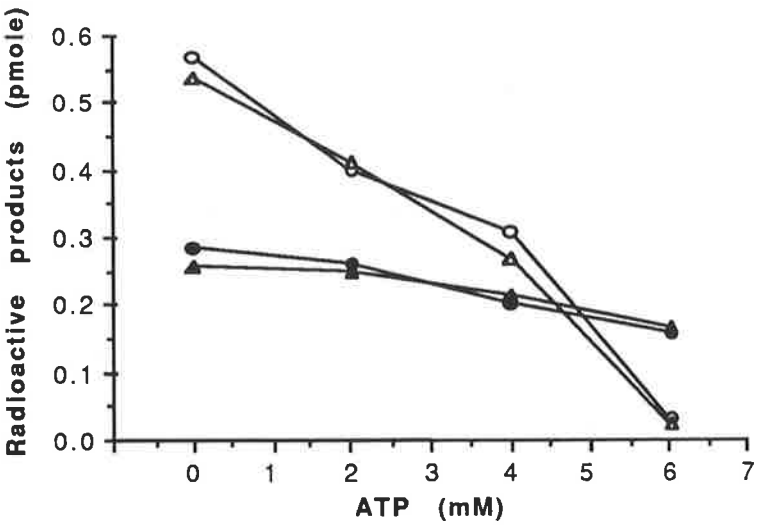


a

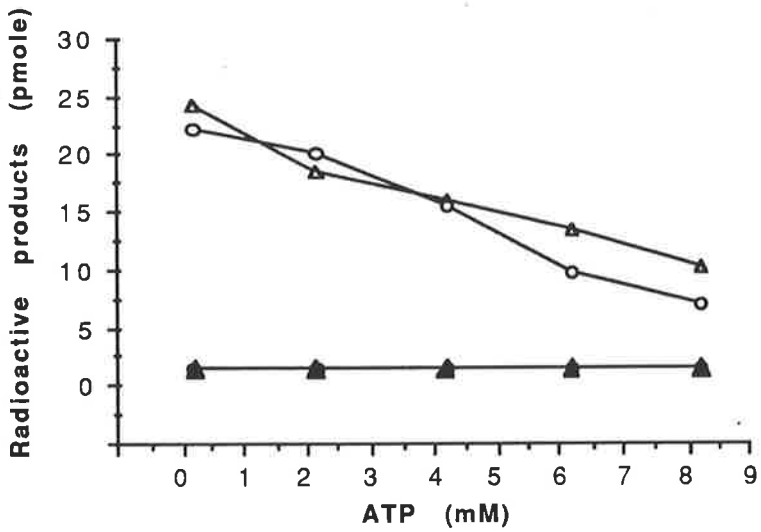


b

Fig. 3.2 Effect of pH on the pyrophosphatase and phosphatase activities (o and □) and the GalTase activity (● and ■) in cauda epididymal plasma of ram (a) and boar (b). The assay medium contained 50mM buffer of varying pH (● and o for Mes, ■ and □ for Hepes), 1mM UDP-galactose, 0.02μCi UDP-[^{14}C]galactose, and 2mM ATP. The other assay conditions and the method for isolating the assay products were the same as those of method (a). Each figure is the representative of two separate assays of samples from different indiviuals.



a



b

Fig. 3.3 Effect of ATP on the pyrophosphatase and phosphatase activities (o and Δ) and the GalTase activity (● and ▲) in duplicate samples of cauda epididymal plasma of rat (a) and boar (b) under the assay conditions of method (b).

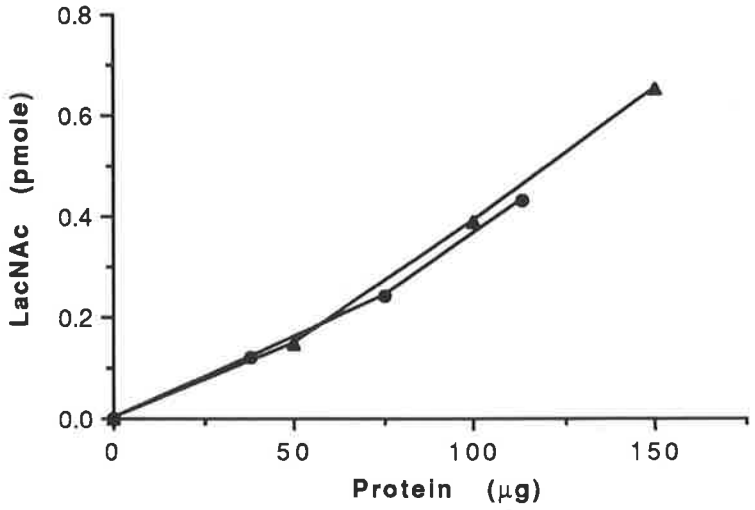


Fig. 3.4 Effect of protein concentration of cauda epididymal plasma on the GalTase activity in boar cauda epididymal plasma under the assay conditions of method (b). Two assays of samples from different individuals (● and ▲) were carried out.

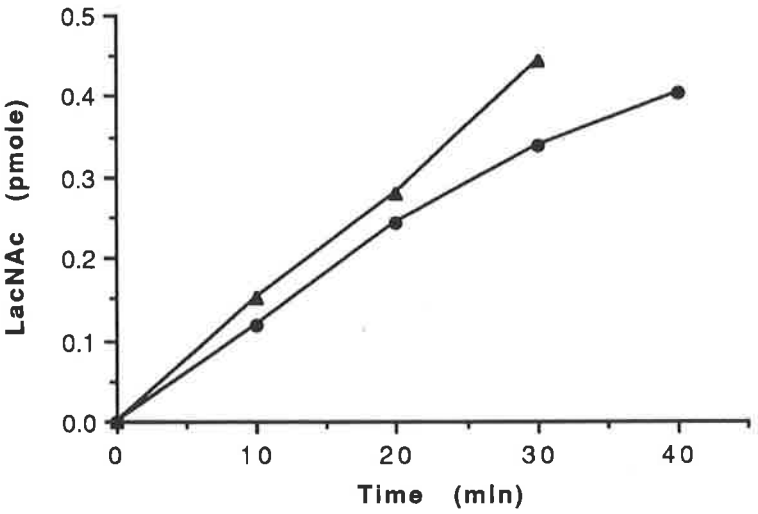


Fig. 3.5 Effect of incubation time on the GalTase activity in boar cauda epididymal plasma under the assay conditions of method (b). Two assays of samples from different individuals (● and ▲)were carried out.

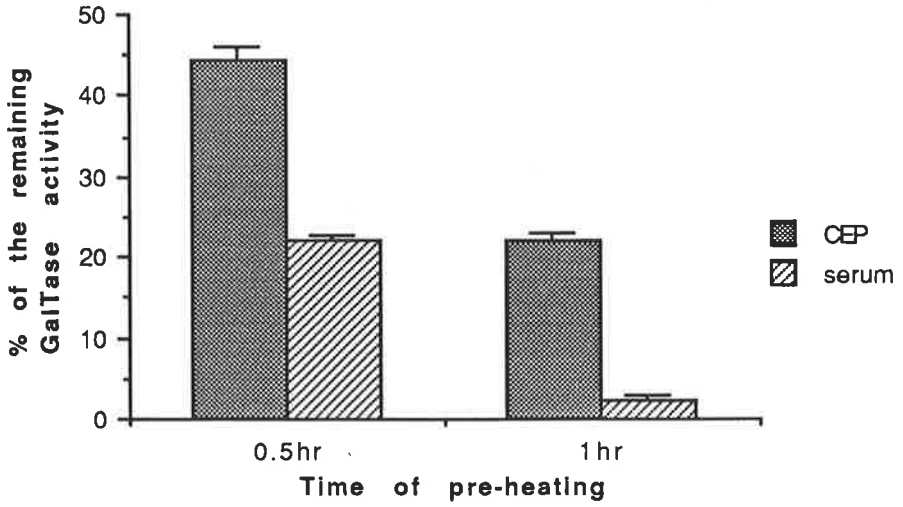


Fig. 3.6 Comparison of the effects of pre-heating at 50°C on the GalTase activity of ram cauda epididymal plasma (CEP) and ram serum. Samples were pre-heated for 0.5hr and 1hr respectively. GalTase activities after preheating were expressed as a percentage of those samples kept on ice for the same period. Method (b) was used for the assays. Each column is the mean of 3 assays with samples from different individuals and the error bars represent SE. The difference between the enzyme activity of the epididymal plasma and that of the serum after both 0.5hr and 1hr pre-incubation were significant at 0.05% level.

Table 3.1 Comparisons of GalTase activity and pyrophosphatase and phosphatase (PPase and Pase) activities in epididymal plasmas of various mammals (Mean ± SE).

Method (b) was used for the assay.

Analysis of variance was used for statistical analysis. There was difference between the species with different letters, but not the species with the same letter at 5% significance level (*) or 1% level (**).

Animals		Mouse	Rat	Rabbit	Ram	Boar
Numbers		5 (groups)	6	8	8	10
Enzyme activities (pmole product / μg protein·20min) and statitical comparisons	GalTase *	6.1 ± 2.1 a	3.2 ± 0.5 a	7.0 ± 1.5 a	4.5 ± 0.4 a	4.4 ± 0.8 a
	PPase & Pase * **	6.0 ± 1.9 a a	3.0 ± 0.1 ab a	54.4 ± 6.6 ab b	106.8 ± 17.0 c b	220.3 ± 12.1 d c

3.4 DISCUSSION

In the present study, it was shown that GalTase activity was present in the rete testis plasma and epididymal plasma of all the species examined. This suggests that GalTase is commonly present in both rete testis plasma and epididymal plasma of mammals.

There are different ways of comparing enzyme activities in various species. The procedure adopted will depend on the purpose of the comparison as well as the limitation of time and cost of the experiments. For example, to compare the activities of purified enzymes from various species under optimal assay conditions may be an accurate way assessing activity. However, such comparisons may lack physiological relevance and would usually be time consuming and expensive. In the present study, the GalTase activities, together with pyrophosphatase and phosphatase activities, of epididymal plasma from various species were compared under the same conditions. It appears that the results were physiologically relevant.

The comparison of the GalTase activities in the epididymal plasma of various mammals under the assay conditions of method (b) showed that there was no statistical difference between all the species used at 5% significance level (Table 3.1). This was in contrast to the comparison of the pyrophosphatase and phosphatase activities in the epididymal plasma between these species (Table 3.1). Pyrophosphatase and phosphatase activities were especially high in the boar, moderately high in the ram and then the rabbit, and very low in the rat and mouse. However, it needsto be born in mind that the GalTase activity was measured under conditions under which the inhibitory effect of pyrophosphatase and phosphatase was controlled. It also needsto be born in mind that the pyrophosphatase and phosphatase activities were measured at pH 6.5 which was actually inhibitory to these activities, and under optimal conditions for these activities, their differences between these species may be much greater. It is known that the pH of the epididymal plasma of the species used are between 6.8-7.0 (Jones, 1978). It was shown in the current results (Fig. 3.1 a-d) that at pH 7 and when

the concentration of UDP-galactose was not very high, the inhibitions of GalTase activity by pyrophosphatase and phosphatase activities in epididymal plasma were correlated to the levels of the later activities in the species used and were very different between the species.

According to the results of the effect of pH (Section 3.3.1.1 (c)), the phosphatase in the epididymal plasma which was involved in the hydrolysis of galactose 1-phosphate was an alkaline phosphatase (see Fernley, 1971). It is known that the activities of both alkaline phosphatase and acid phosphatase of epididymal plasma differ greatly between different mammalian species (Jones, 1978). The order of the pyrophosphatase and phosphatase activities of the species in the present study is similar to that of the alkaline phosphatase activities of the same species reported by Jones (1978), except that in Jones' report, the activity in rabbit is higher than that in ram. In Jones' report, the difference of the alkaline phosphatase activities between boar and rabbit or ram was much greater than the difference of the pyrophosphatase and phosphatase activities between these species in the present study. However, as mentioned above, this may be because the current conditions for assay of pyrophosphatase and phosphatase activities were actually inhibitory for them. Therefore, it is most likely that the degree of degradation of UDP-galactose by epididymal plasma in a species is mainly decided by the level of the alkaline phosphatase in the same plasma of the species.

The functions of the GalTases in rete testis plasma and epididymal plasma are not known. According to the general function of GalTase, they may be involved in galactosylation of molecules in the plasma or on sperm surface. Hamilton and Gould (1982) reported the enzymatic galactosylation of rat caput epididymal sperm membrane glycoproteins, especially two proteins of M_r 37,000 and 23,000 respectively. The galactosylation occurred mostly on the sperm tail, primarily at the mid-piece. The M_r 37,000 protein was suggested to be the same as that reported by Olson and Hamilton (1978) which was detectable using galactose oxidase-NaB[^{3}H]$_4$ technique only on sperm of cauda epididymidis but not of caput epididymidis. Therefore, the

authors proposed that the M_r 37,000 protein was a nascent glycoprotein in the plasma membrane of rat caput epididymal sperm, and the maturation of the sperm involved the galactosylation of this protein. However, the authors also indicated that it was not clear whether sperm surface GalTase or epididymal plasma GalTase was responsible for the galactosylation.

The present results suggest that the GalTase levels of epididymal plasma in various species are similar when the interference of pyrophosphatase and phosphatase are controlled, but under physiological conditions, the levels of the enzyme activity available for function are extremely different. It is expected that the GalTase activity is readily available in epididymal plasma in mouse and rat, but hardly so in ram and especially in boar. Therefore, it seems that at least in some species the epididymal plasma GalTase activity is not important. It is difficult to suggest at this stage whether the difference of the availability of the GalTase activity between species is correlated with functional difference. As mentioned above, the activities of both acid and alkaline phosphatases in epididymal plasma vary greatly between species (Jones , 1978). There is the possibility that the differences of the pyrophosphatase activity and/or the activities of the phosphatases in epididymal plasma of different species are related to the difference of the requirement of GalTase activity and probably also the activities of some other enzymes in the plasma. If this is the case, GalTase is expected to be important in the epididymal plasma of the species with low pyrophosphatase and alkaline phosphatase activities in the same plasma, but not in the other species. For example, if the function of epididymal plasma GalTase in rat is to galactosylate sperm surface glycoproteins related to sperm maturation, there may be no such galactosylation involved in the maturation of the sperm of boar. However it is also possible that the evolution of the levels of the pyrophosphatase and/or the phosphatases in epididymal plasma of each species has been according to the requirement of these enzymes themselves in the species, but not related to GalTase or other enzymes. If so, it does not seem that GalTase has important functions in mammalian epididymal plasma.

The discussion concerning the α-lac-like proteins as introduced in Section 3.1 is given in Chapter 7.

The effect of heating samples at 50°C prior to assay was determined for epididymal plasma GalTase and the serum enzyme of ram. Hamilton (1980) has reported that rete testis plasma GalTase was more resistant than the serum enzyme to pre-heating (50°C, 1hr) in the rat. The present results (Fig. 3.6) show that in the ram the epididymal plasma GalTase is also more resistant than the serum enzyme to pre-heating at 50°C. Therefore, the GalTase in epididymal plasma is possibly derived from rete testis fluid but not serum. As indicated by Hamilton (1980), the difference between the soluble GalTase in male reproductive tract lumen and that in serum in response to pre-heating may not be of physiological importance, since the temperature of mammalian testis and epididymis is usually lower than body temperature.

The functions of pyrophosphatase and phosphatase in epididymal plasma are not clear. One possibility is that the epididymis is the place to secrete alkaline phosphatase for the requirement in the later stage of reproductive physiology. It is known that the high concentration of alkaline phosphatase in boar seminal plasma is mainly produced at cauda epididymal plasma (Einarsson et al., 1976).

The present results indicates that considerably greater pyrophosphatase and phosphatase activities in epididymal plasma compared with rete testis plasma may be common in mammals. This may be because the epididymis is the place to produce these enzymes for later requirement as mentioned above. This may also be because of some other physiological reasons.

CHAPTER 4
SOME STUDIES ABOUT GALACTOSYLTRANSFERASE AND MAMMALIAN SPERM-ZONA PELLUCIDA BINDING

GENERAL INTRODUCTION

It has been reported that galactosyltransferase (GalTase) is present on the surface of mouse sperm, and that the sperm surface GalTase is the sperm's receptor for zona pellucida (ZP) in mouse. The reports have been mainly from Shur and co-workers.

Initially, Shur and Bennett (1979) reported that GalTase activity, but not the activities of eight other enzymes, on the sperm surface of T/t-locus mutant mice (*t* sperm) was more than 2-5 times of that of wild-type mice. They suggested that the increased GalTase activity on *t* sperm was due to a deficiency of GalTase inhibition relative to normal sperm according to the following observations. When wild-type and *t* sperm were mixed and assayed for GalTase activity, the wild-type sperm inhibited *t* sperm GalTase activity by 80%. Pretreatment of wild-type or *t* sperm with antiserum directed against T/t-locus antigen stimulated the GalTase activity of either sperm 2- to 15- fold. It is known that T/t-locus mutant mice have diverse defects in sperm function and embryonic morphogenesis, however, one of their characteristics is a preferential fertilizing ability of their sperm compared with wild-type sperm (for review see Bennett, 1975). Therefore, it was claimed in their report that increased availability of GalTase on *t* sperm was at least partly responsible for their preferential fertilizing ability. These authors also suggested that the GalTase activity was not present on the plasma membrane overlapping the acrosome, since the acrosome status did not affect the assay of the sperm GalTase activity. They suggested that the GalTase was primarily present on the postacrosomal plasma membrane.

Subsequently, the roles of sperm surface GalTase in sperm capacitation and sperm-zona binding in mouse were investigated. In the following introduction, all the sperm-zona binding experiments mentioned were carried out *in vitro*. Shur and Hall (1982a) demonstrated that surface GalTase of uncapacitated sperm was loaded with poly *N*-acetyllactosaminyl substrates. As a consequence of capacitation in Ca^{2+} containing medium, these polylactosaminyl substrates were released and the sperm surface GalTase was exposed for binding to the ZP. Capacitation can be mimicked in the absence of Ca^{2+}, either by washing sperm in Ca^{2+}-free medium, or by pretreating sperm with an antiserum which reacted with both a class of poly-*N*-acetyllactosamine glycoconjugates and sperm. They also showed that high M_r polylactosaminyl glycosides, but not low M_r ones, inhibited the binding of capacitated sperm to ZP by competing for the sperm surface GalTase. They called these high M_r glycosides "decapacitation factors".

Shur and Hall (1982b) reported the following observations to suggest that mouse sperm surface GalTase was the sperm's receptor for ZP. α-lactalbumin (α-lac) (see Section 1.3.1.2) dose-dependently inhibited sperm-zona binding. Covalent linkage of UDP-dialdehyde, which is an inhibitor of GalTase, to sperm greatly inhibited sperm-zona binding, while pretreatment of eggs with UDP-dialdehyde did not affect the binding. UDP-dialdehyde caused inhibition of sperm surface GalTase, but had no inhibition of 5 other enzymes and only slight inhibition of one other enzyme on the sperm surface. Heat-solubilized or pronase-digested ZP inhibited sperm-zona binding, and these treated ZP, as well as the intact ZP, could be galactosylated by sperm with UDP-galactose. Lopez et al. (1985) offered some further evidence about the receptor function of the GalTase as follows. Purified bovine milk GalTase when untreated, inhibited sperm-zona binding, but had no effect when heat-inactivated or in the absence of Mn^{2+}. UDP-galactose, but not UDP-glucose, inhibited sperm-zona binding in a dose-dependent manner, and also dissociated preformed sperm-zona binding. Both the inhibition and the dissociation were time-dependent, they were more effective over short times (e.g., 5min) than over prolonged times (e.g., 27min) of incubation. The dissociation was also temperature-dependent, the bound sperm-zona was resistant to

dissociation at cool temperatures (e.g., 22°C). The dissociation did not occur when the sperm were immobile. Finally, monospecific anti-GalTase IgG and its Fab fragments, but neither preimmune IgG nor anti-mouse brain IgG, produced a dose-dependent inhibition of sperm-zona binding and concomitantly blocked sperm GalTase catalytic activity. The same authors also indicated that GalTase inhibition of sperm-zona binding was not due to steric blocking, since sperm-zona binding was inhibited by approximately 50% when they used ZP which were galactosylated with GalTase first and then washed.

The same authors also suggested that the sugar residue on ZP bound by the sperm GalTase was a *N*-acetylglucosamyl residue, since pretreatment of eggs with ß-*N*-acetylglucosamindase inhibited sperm-zona binding by 86%. They also suggested that some of the ZP *N*-acetylglucosamyl residues were masked by galactose, suggestive of conventional *N*-acetylglucosamyl linkages (galactose -> *N*-acetylglucosamine (GlcNAc)), since pretreatment with ß-galactosidase increased the binding by 55%.

When the mouse sperm surface GalTase was labelled by anti-GalTase IgG and then biotinylated goat anti-rabbit IgG / fluoresceinated avidin, the label was found at a discrete plasma membrane domain on the dorsal surface of the anterior head overlying the intact acrosome, where mouse sperm initiates binding to the zona (Lopez et al., 1985). However, this result did not agree with the location of the GalTase suggested earlier by Shur and Bennett (1979) (see above) according to the results of enzyme activity assay. Scully et al. (1987) investigated at which stage of spermatogenesis GalTase is initially detectable on the spermatogenic cell surface by indirect immunofluorescence and by direct enzymatic assay. They showed that GalTase was present on the surface of all spermatogenic cells assayed, and that during differentiation, GalTase progressively redistributed from an initially diffuse and uniform localization on the surface of primary spermatocytes to a restricted plasma membrane domain overlying the dorsal aspect of the mature acrosome. With epididymal sperm, they showed, with the immunofluorescence method, that the GalTase changed subtly from a granular appearance over the acrosome of caput sperm to become more intense

and uniform on caudal sperm. Lopez and Shur (1987) also showed by the same technique that during the acrosome reaction (AR), mouse sperm surface GalTase was redistributed to the lateral surface of the sperm head. They also demonstrated that 90% of the sperm GalTase activity was retained during the AR, while removal of plasma membranes by nitrogen cavitation released GalTase activity from the sperm surface. Shur and Neely (1988) indicated that mouse sperm surface GalTase was an externally oriented, integral plasma membrane component, according to their results with immunofluorescence observations of sperm with different biochemical treatments.

Shur and Neely (1988) purified mouse sperm GalTase from solubilized whole sperm, and partially characterized this enzyme, which has a M_r of 60,000. The purified GalTase had a inhibitory effect on mouse sperm-zona binding.

Furthermore, Macek et al. (1991) reported that mouse sperm AR was induced by the aggregation of the sperm surface GalTase. Their evidence was that neither UDP-galactose, GlcNAc, α-lac nor anti-GalTase Fab, each of which showed inhibition of mouse sperm-zona binding, could induce AR in mouse sperm, while anti-GalTase IgG or anti-GalTase Fab cross linked with goat anti rabbit IgG induced the AR. The above results agreed well with the mechanism for ZP-induced AR proposed by Leyton and Saling (1989a) that aggregation of sperm surface receptors by ZP3 induces AR. Study from this laboratory suggested that AR could be induced by the aggregation of another sperm surface protein, P95 (see Sailing, 1991).

It is well known that mouse ZP contains 3 glycoproteins, ZP1, ZP2 and ZP3; that ZP3 is the receptor for sperm in mouse sperm-zona binding; that the receptor activity is associated with the O-linked oligosaccharides of ZP3; and that ZP2 serves as the secondary receptor after the sperm AR (see Section 1.2.3.1). In the letter of Miller et al. (1992) to Nature about the complementarity between sperm surface GalTase and ZP3 mediated mouse sperm-zona binding, some new results were presented. It was shown that only ZP3, but not ZP1 and ZP2, was the substrate of the sperm surface

GalTase, while all the three glycoproteins were substrates for bovine milk GalTase. This demonstrated a more stringent substrate specificity of the sperm GalTase. Each molecule of ZP3 can be galactosylated by 2.6 galactose residues, indicating the multiple binding sites of ZP3 for GalTase and supporting the early report from the same laboratory that the aggregation of sperm surface GalTase induced AR (Macek et al., 1991). Selective enzymatic removal of the oligosaccharides from ZP3 showed that the GalTase-binding oligosaccharides on ZP3 were O-linked but not N-linked. The selective binding of sperm GalTase to ZP3 oligosaccharides was shown to be required for mouse sperm-zona binding, but galactosylated ZP3 had significantly less sperm-receptor activity. GalTase binding sites of ZP3 were removed by digestion with *N*-acetylhexosamindase, but not α-galactosidase, nor ß-galactosidase. The reactions between the GalTase, which was redistributed on the lateral surface of the head of acrosome reacted sperm and the zona glycoproteins included the following. The GalTase of the acrosome reacted sperm no longer recognized ZP3 or had lower affinity for ZP3. Ionophore treatment, which induced 50% of the acrosome-intact sperm to undergo AR, gave a 50% reduction in GalTase galactosylation of ZP3; and less than 20% acrosome-intact sperm resulted in barely detectable ZP3 labelling. GalTase on acrosome-reacted sperm still retained normal activity towards other substrates, indicating that the reduced binding to ZP3 was specific. GalTase did not bind to ZP2 or ZP1, suggesting that the enzyme was not involved in secondary binding, as had been suggested by Saling (1989). Finally, it was demonstrated that ZP from fertilized eggs could no longer serve as substrate for sperm GalTase, though they still had abundant substrate for non-sperm GalTase, showing that the loss of specific recognition by sperm-receptor activity was correlated with a loss of specific sperm GalTase substrate. It was suggested *N*-acetylglucosamindase released from cortical granules at fertilization inactivated ZP3 by specifically moving the oligosaccharides recognized by sperm GalTase, but not non-sperm GalTase, since it is known that cortical granules in eggs of some species, including mouse, contain *N*-acetylglucosamindase as the principal glucosidase.

Shur and co-workers have also reported the presence of sperm surface GalTase activity in some mammalian species other than the mouse, including the human (Miller et al., 1991), guinea pig (Primakoff, Myles and Shur, unpublished observation, in Lopez et al., 1985), rabbit (Dunbar and Shur, unpublished observation, in Miller et al., 1991), and boar (Peterson and Shur, unpublished observation, in Miller et al., 1991). The human sperm were prepared in a different way for surface GalTase activity assay. The method used included washing sperm for 3 times in medium with balanced salts and bovine serum albumin (BSA) and capacitating sperm for 2hr in medium containing 3mg/ml BSA or for 6hr in medium containing 35mg/ml BSA. The sperm preparation under these conditions were believed to be > 95% acrosome intact (Miller et al., 1991). Detection of GalTase activity in the plasma membrane fractions of both equine and bovine sperm was also reported (Fayrer-Hosken et al., 1991).

There have been some reports from other groups directly or indirectly related to the above results of Shur and his colleagues. GalTase activity has been reported to associate with some other mammalian sperm. It has been immunologically labelled on the surface of rat sperm head (Okuno, 1986); biochemically detected in the total membrane fraction of ejaculated human sperm (Humphreys-Beher and Backwell, 1989); and immunologically detected in the proteins isolated from a sub-population of high buoyant density of ejaculated human sperm (Sullivan et al., 1989). However, Tulsiani et al. (1990) reported the following results obtained from human sperm. Little GalTase activity could be detected either in the sperm fraction of semen separated by Percoll gradient centrifugation, or in the purified plasma membrane of the sperm. Presence of protease inhibitors did not increase the GalTase in a membrane fraction of the sperm, which eliminated the possibility that the catalytic domain of an integral GalTase of the sperm plasma membranes was cleaved by protease(s) during the isolation of the membranes.

Benau and Storey (1988) investigated the relationship between the GalTase site and the trypsin inhibitor-sensitive site (see section 1.2.3.2.3 (a)) on mouse sperm surface. They concluded that the two types of site are independent in binding

their specific zona ligands, but are close enough for steric perturbation of the enzyme activity of one site by macromolecules bound to the other. Among their evidence was the dose-dependent inhibition of 12.5-100μM UDP-galactose on the sperm-zona binding with close correlation to GalTase activity.

DeGeyter et al. (1989) showed that bovine α-lac inhibited both hyperactivated motility and ZP binding ability of mouse sperm, while sperm-zona binding could be partially restored by lowering the α-lac concentration. They suggested that α-lac mimicked mouse sperm decapacitation.

Benau et al. (1990) also further investigated whether the mouse sperm surface GalTase site behaved as a noncatalytic or a catalytic site during sperm-zona binding and found the following results. With GlcNAc as the acceptor substrate in the reaction, increasing concentrations of Mn^{2+} in the range of 0.1-10mM increased the sperm surface GalTase activity. Correspondingly, in the presence of 0-2mM Mn^{2+}, sperm-zona binding was inhibited in a concentration-dependent manner. At 1.25mM Mn^{2+}, at which the GalTase activity was at 65% Vmax, 50% inhibition of sperm-zona binding occurred. They predicted that in the absence of Mn^{2+}, UDP-galactose and glycoprotein with GlcNAc terminal residues competitively inhibited sperm-zona binding; the presence of Mn^{2+} causes a change in the site from substrate binding conformation to catalytic conformation which is not favourite for sperm-zona interaction.

Kawai et al. (1991) showed that SDS solubilized plasma membrane of mouse sperm inhibited the mouse sperm-zona binding. In contrast to the opinion of Benau et al.(1990), they considered that the inhibition was not due to GalTase, since the sperm-zona binding was inhibited even in the absence of Mn^{2+}.

Bleil and Wassarman (1988) reported that galactose, located in an α-linkage at the nonreducing terminus of the O-linked oligosaccharides, was at least one of the sugar determinates essential for the sperm receptor activity of ZP3. They showed that purified ZP3 and ZP3-derived O-linked oligosaccharides treated with either α-galactosidase or galactose oxidase lost their inhibitory activity on sperm-zona binding, and that in the later case, the activity could be restored by treatment with sodium

borohydride. However, this α-galactose is not expected to be the substrate of mouse sperm surface GalTase, since Miller et al. (1991) reported that treatment of ZP3 by α-galactosidase did not remove the GalTase binding site on ZP3. Bleil and Wassarman (1988) also reported that treatment of the ZP3 and its O-linked oligosaccharides with ß-*N*-acetylglucosamindase did not result in the loss of their inhibitory activity. This result did not support the findings of Shur and Hall (1982b) on the effect of ß-*N*-acetylglucosamindase treatment of ZP on mouse sperm-zona binding.

Fayrer-Hosken et al. (1987) reported the effect of UDP-hexoses and GalTase on rabbit sperm-zona binding using *in vivo* capacitated sperm. When the sugar concentration was increased from 50 to 3,000 μg/ml (i.e., 0.08 to 5mM), UDP-galactose stimulated sperm-zona binding and sperm penetration through the zona, while UDP-glucose inhibited them. When $(NH_4)_2SO_4$-stored ZP was used, the sperm-zona interaction was also stimulated by UDP-galactose, and inhibited by UDP-glucose. Alpha-amylase reversed the inhibition of sperm treated with 500μg/ml UDP-glucose/ml but not that with 2,500μg/ml UDP-glucose. UDP alone had no effect on the sperm binding to or the sperm penetration through the $(NH_4)_2SO_4$-stored ZP. Pre-treatment of the $(NH_4)_2SO_4$-stored ZP with GalTase and UDP-galactose decreased the sperm penetration through the zona, using the same ZP without treatment or with GalTase or UDP-galactose treatment alone as controls.

Berger et al. (1989) reported that preincubation of porcine eggs with ß-*N*-acetylglucosaminidase almost abolished sperm-zona binding, while preincubation of the eggs with ß-galactosidase did not affect binding.

Mori et al. (1991) showed that 39% of N-linked, neutral oligosaccharides in porcine ZP glycoproteins terminated with GlcNAc residues. They also reported a preliminary observation that α-lac inhibited the sperm-zona binding in pigs.

Humphreys-Beher and Blackwell (1989) reported that human sperm which demonstrated an inability to penetrate ZP free hamster eggs *in vitro* had lower GalTase activity than those from a penetration-positive group. A unique allele variant

was identified on the DNA of the penetration-negative group by hybridization with a putative human DNA clone suggesting the possible association between the mutations and the gene for GalTase in this group (Humphreys-Beher and Blackwell, 1989). It was also reported from the same laboratory (Humphreys-Beher et al., 1990) that anti-GalTase antibodies were detected in the serum of patients with anti-sperm antibodies when compared with control people. However, Ronquist (1987) reported that there was no significant difference in the activities of fucosyltransferase, GalTase and sialyltransferase in seminal plasma samples from normospermic and oligospermic men, though a significant correlation was noted between seminal plasma GalTase activity and semen volume only in normospermic men.

The studies of this chapter were to investigate whether GalTase was also involved in sperm-zona binding of mammalian species other than the mouse. Only primary binding was considered, since GalTase was reported to be the sperm receptor for zona in mouse only in the primary, but not the secondary binding. In the following description of the present results, "sperm-zona binding" means especially the primary binding. The rat and ram were chosen for the current study. The rat is a species closely related to the mouse in evolution. Therefore, it was considered that if GalTase is involved in sperm-zona binding of any other species, the rat would be a likely one. The ram was chosen as a convenient, much less related species. Studies in Part 1 (4-1) examined whether GalTase activity was present in the preparations of rat sperm and ram sperm. Studies of Part 2 (4-2) dealt with the methods of sperm-zona binding of rat sperm and ram sperm to mouse zona. Studies in Part 3 (4-3) investigated whether the bindings of rat sperm and ram sperm to mouse zona were inhibited by UDP-galactose.

PART 1 (4-1)
EXAMINATION OF GALACTOSYLTRANSFERASE ACTIVITY IN SPERM PREPARATIONS OF THE RAT AND RAM

4-1.1 INTRODUCTION

Sperm surface GalTase has been an important characteristic in the study of the role of GalTase in mouse sperm-zona binding. Initial attention to GalTase for its role in mouse fertilization was actually raised by the unusual high level of GalTase activity of T/t-locus mutant mice, the sperm of which have a preferential fertilizing ability (Shur and Bennett, 1979). Also, the measurement of sperm GalTase activity has usually been the first step in investigating the role of this enzyme in fertilization in other species (see "General Introduction" of this chapter). In this part (4-1) of this chapter, GalTase activities in the preparations of rat and ram sperm were measured. Mouse sperm were used as a control. Acrosome status of rat and ram sperm at different stages during sperm preparation and sperm surface GalTase assay was also examined.

4-1 2 MATERIALS AND METHODS

4-1.2.1 Animals

F1 hybrid mice and Porton rats were obtained from the Central Animal House of the University of Adelaide. Epididymides of Merino rams were collected from the abattoirs of the South Australian Meat Corporation. All animals used were sexually mature.

4-1.2.2 Methods

4-1.2.2.1 Examination of Sperm Surface Galactosyltransferase Activity

4-1.2.2.1.1 Sperm Preparation

The method for mouse sperm preparation of Shur and co-workers (e.g., Shur and Bennett, 1979; Lopez et al., 1985) was basically followed. However, different methods to collect sperm were used in some of the experiments with rat sperm and in the experiments with ram sperm.

The buffer used for sperm washing contained 128mM NaCl, 5.4mM KCl, and 10mM $MnCl_2$ in 20mM Hepes, pH 7.4.

(1) Mouse Sperm

For each assay, 10 mice were killed under ether anaesthesia by decapitation to release blood. Cauda epididymides were collected and the blood in visible blood vessels was removed. The tissue was minced in 5ml sperm preparation buffer at room temperature to allow the sperm to be released and spread. The supernatant was then transferred to the cold (4 °C) and passed through a layer of 37μm mesh nylon cloth, which was rinsed with cold sperm washing buffer. The sperm were then washed three times by centrifugation at 1,200g for 10min at 4°C to remove epididymal luminal plasma, 30ml buffer being used in each washing. Then the sperm were resuspended in a small volume of buffer, and sperm concentration and percentage of other types of cells were counted using a haemocytometer (Assistant, West Germany).

(2) Rat Sperm

Rat sperm were collected by two methods as follows.

(a) Sperm collection by mincing epididymis. The sperm were collected in the same way as the method for mouse sperm preparation. One rat was used for each assay.

(b) Sperm collection by pumping sperm from epididymal duct. The vas deferens was cut at the position about 3cm from epididymis and the fluid inside was squeezed out. The duct was cannulated with plastic tubing, which was thinned at the end over a bunsen flame. Sperm washing buffer was pumped into the cauda epididymides with a peristaltic pump at a rate about 0.2ml/min, and cauda epididymal fluid was allowed to escape from a needle puncture in the epididymal duct and collected with a Pasteur pipette into 5ml sperm washing buffer at room temperature. The collection was carried out on one side after the other. After the sperm were well suspended in the buffer, the tubes were put on ice. Two rats were needed for each assay.

After collection, the sperm were treated in the same way as for mouse sperm. However, the centrifugation of the washing was at 500g for 10min. This was because of the observation that rat sperm were more likely to be damaged by centrifugation.

(3) Ram Sperm

Epididymides were collected from freshly killed animals and transported to the laboratory within 1hr at a temperature of about 20-25°C. A small area of the tunica albuginea of a cauda epididymis was cut off, the exposed cauda epididymal tubes were punctured at points without visible blood vessels, and the fluid (up to 0.5ml) which oozed out was collected with a Pasteur pipette into sperm washing buffer at room temperature. The sample was then processed in the cold and treated as described for rat sperm.

4-1.2.2.1.2 Assay of Sperm Surface Galactosyltransferase Activity

The assay conditions for all the species used were basically the same as those described by Shur and co-workers (e.g., Shur and Bennett, 1979; Lopez et al., 1985). The assays were carried out in 50µl medium containing 20mM Hepes, pH 7.4, 150mM NaCl, 5mM KCl, 10mM $MnCl_2$, 0.3mM UDP-galactose and 3µCi UDP-

[^{3}H]galactose, and 30mM *N*-acetylglucosamine (GlcNAc). In the assays, 0.25-1.5 x 10^6 mouse or rat sperm, or 0.25-1.5 x 10^7 ram sperm were used. Mouse sperm and rat sperm were much longer than ram sperm, therefore, the maximum concentrations of the sperm suspension of these two species which could be used in the assay was much lower than that of the ram. The incubation was carried out at 37°C for 1hr. Parallel samples incubated at 0°C were used as background control. Three measurements for each species were carried out.

4-1.2.2.2 Assessment of Acrosome Status

(1) Rat Sperm

Rat acrosomes were labelled by a modification of the technique of Ellis et al. (1985) using monoclonal antibody WS 18.6. WS 18.6 hybridoma culture supernatant was obtained by courtesy of Dr T. D. Hartman, Institute of Zoology, London, England. Sperm at different stages of the preparation and assay for sperm surface GalTase activity were fixed in 95% ethanol. Drops of each fixed sample were repeatedly applied and dried on a slide until enough sperm were obtained. Following three washings of the slides in phosphate buffered saline (20mM phosphate buffer, 150mM NaCl) (PBS) pH 7.4, the samples on the slides was covered with hybridoma WS 18.6 at 10 times dilution in PBS and incubated for 1hr at room temperature. The slides were then washed three times in PBS. The following procedure was carried out under conditions which avoided light as much as possible. The samples on slides were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti mouse IgG at concentration of 10μg/ml in PBS for 30min at room temperature. The slides were then rinsed twice with PBS and twice with distilled water, then air dried. The slides were mounted with a 9:1 mix of glycerol and PBS, pH 8.0. The samples were assessed at 500 x magnification with epifluorescence using a Leitz microscope and an I 2/3 filter block.

When a comparison was carried out between the sperm preparation with the pumping method and that with the mincing method, in each experiment, one

epididymis of a rat was used for the pumping and the other for mincing. Experiments were repeated 3 times.

(2) Ram Sperm

The method of Shams-Borhan and Harrison (1981) was followed for the assessment of acrosome status of ram sperm. Samples were fixed by addition of equal volume of 2% (v/v) glutaraldehyde in 0.165M cacodylate, pH 7.3. The fixed samples were mounted on slides and covered by cover slides which were then sealed with mounting medium. The samples were scored at 500 x magnification with phase-contrast illumination using a Leitz microscope. Sperm from three rams were scored separately.

The slides were previously coded and scored blind. One hundred sperm on each slide were scored.

4-1.2.2.3 Purification of Mouse Sperm by Percoll Gradient Centrifugation

Preliminary experiments with purification of mouse sperm by Percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation was carried out as follows. The starting density was 40%, which was made by mixing undiluted Percoll, 10 times concentrated sperm washing buffer, and H_2O in a ratio of 4:1:5. The diluted percoll preparation (9.5ml) was centrifuged in a 50Ti rotor of Beckman ultracentrifuge at 30,000g for 15min to establish a gradient. Mouse sperm were collected as usual and washed once with sperm washing buffer by centrifugation at 1,200g for 10min at 4°C. The precipitated sperm were resuspended in 0.5ml buffer and the suspension was layered onto the top of the preformed gradient. The tube were centrifuged in a Beckman TJ-6 centrifuge at 350g for 20min at 4°C. By this method, the sperm band was at the position of 38-39% of Percoll. Red blood cell were more concentrated in the higher concentrations, and epithelial cells were more in the lower concentrations. However, the white blood cells and/or lymph cells spread through the whole gradient. The sperm fraction was further washed twice with washing buffer by centrifugation at 1,200g for 10min at 4°C and

then used for GalTase activity assay. In the final sperm preparation, the number of the red blood cells and the total number of the other types of cells were lower than 1% respectively.

4-1.3 RESULTS

4-1.3.1 Galactosyltransferase Activity in Sperm Preparation

The lowest detectable amount of GlcNAc, the product of GalTase, in the current experiments was 20pmole.

(1) Mouse Sperm

GalTase activity of mouse sperm preparation was measured as the control for the enzyme activity in rat and ram sperm preparations. The activity is shown in Table 4-1.1. Of total cell number, 4% red blood cells, 2% white blood cells and/or lymph cells, and 1% epithelia cells were observed as contamination.

(2) Rat Sperm

(a) Sperm preparation with the mincing method. When rat sperm were collected by mincing the epididymis, the GalTase activity of the sperm preparation was similar to that of mouse sperm preparation (Table 4-1.1). Of total cell number, 4% red blood cells, 2% white blood cells and/or lymph cells, and 2% luminal epithelia cells were observed.

(b) Sperm preparation with the pumping method. When rat sperm were collected by pumping, no other type of cells were observed in the preparation. However, there was no GalTase activity detectable in this preparations under the present assay conditions. Experiments (assays) were repeated 3 times.

(3) Ram Sperm

The preparation of ram sperm avoided contamination with blood cells. However, epithelia cells and lymph cell-like cells, both accounting about 0.01% of total cell number, were observed. GalTase activity of the sperm preparation is presented in Table 4-1.1.

Table 4-1.1 GalTase activity in sperm preparations of different species.
Values are pmole GlcNAc produced / hr of 3 experiments (mean ± SE)

Sperm number (mouse and rat, $x10^{-6}$, ram, $x10^{-7}$)	0.25	0.5	1.0	1.5
Mouse	96.1 ± 14.4	187.3 ± 23.9	308.7 ± 41.4	501.4 ± 71.2
Rat, mincing	72.5 ± 9.9	120.3 ± 22.3	278.7 ± 28.1	465.9 ± 18.4
Rat, pumping	Not detectable			
Ram	63.5 ± 8.9	117.2 ± 18.1	159.7 ± 31.4	342.9 ± 47.2

4-1.3.2 Acrosome Status of Rat Sperm and Ram Sperm during the Procedure for Sperm Surface Galactosyltransferase Assay

(1) Rat Sperm

By labelling with monoclonal antibody WS 18.6, 3 types of acrosome status of rat sperm could be observed as shown in Fig. 4-1.2.

(a) Intact acrosomes: the acrosomes were labelled as a bright and sharp crescent image.

(b) Damaged acrosomes: the labelled acrosomes had a damaged appearance, e.g., the fluorecence appeared patchy and was usually thinner and dimmer.

(c) No acrosomes: there was virtually no labelling.

There was no difference between the sperm collected by pumping and those collected by mincing in acrosome status at different stages of the sperm preparation and GalTase activity assay. After collection, about 85% of the sperm were

acrosome intact, while only about 30% of the sperm remained acrosome intact after washing and before incubation for GalTase assay. Most of the sperm (>95%) either had damaged acrosome or had lost their acrosome by the end of the incubation. The results are shown in Table 4-1.2.

(2) Ram Sperm

The standards for scoring intact and reacted acrosome were as described by Shams-Borhan and Harrison (1981). As shown in Table 4-1.2, about 95% of the sperm were acrosome-intact after collection; about 70% remained acrosome intact after washing and before incubation; and about 35% of the sperm still had intact acrosomes at the end of the incubation.

Table 4-1.2 Acrosome status of rat and ram sperm at different stages of the sperm preparation and assay for GalTase activity

Values are percentage of intact acrosomes from 3 animals (mean ± SE)

Stage of the experiment	After collection	After washing	After incubation
Rat, mincing	85.3 ± 5.2	33.5 ± 4.6	3.2 ± 1.7
Rat, pumping	82.6 ± 8.7	31.2 ± 5.2	2.3 ± 1.2
Ram	94.8 ± 2.7	71.4 ± 4.6	34.6 ± 5.8

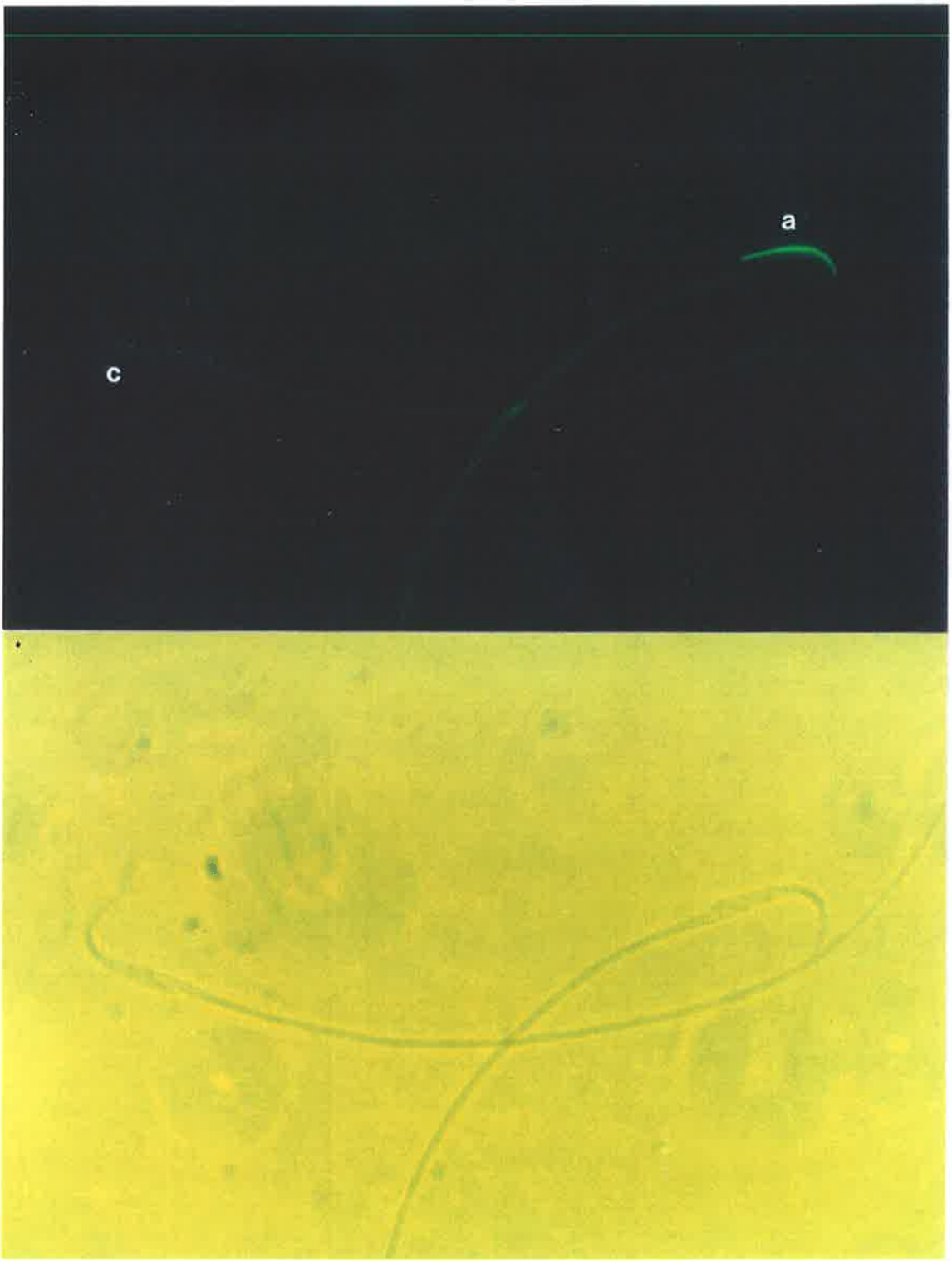
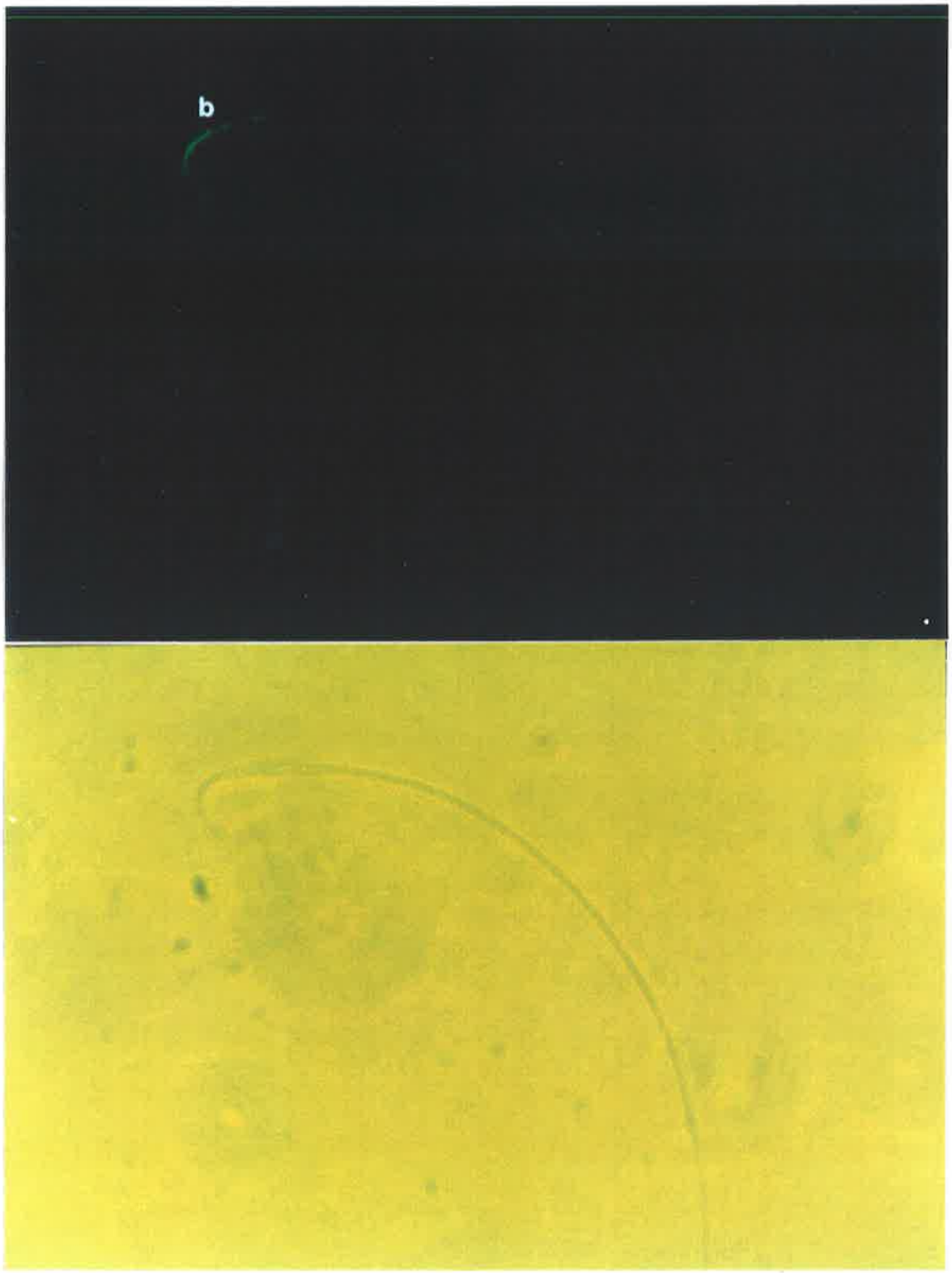


Fig. 4-1.2 $\frac{1}{2}\frac{3}{4}$

Various acrosome status labelled by monoclonal antibody 18.6. Paired epifluorescent and phase contrast micrographs (1and 2, and 3 and 4 respectively). (a) The acrosome was a bright and sharp crescent image. (b) The acrosome had a damaged appearance. (c) There was virtually no labelling on the acrosome. Only acrosomes of type (a) were considered as intact acrosomes.


b

4-1.4 DISCUSSION

The great difference in GalTase activity between the rat sperm preparation obtained by mincing and that obtained by pumping was unexpected. The enzyme activity with the former method was about the same level as that of mouse sperm preparation, while the activity with the latter method was not detectable. There was no difference between the acrosome status of the two sperm preparations at various stages of sperm preparation and assay for GalTase activity. However, the preparation from the mincing method, but not that from the pumping method contained a certain amount of contaminating cells including red blood cells, white blood cells and epithelial cells. The mouse sperm preparation also contained the same type of contaminating cells. Therefore, the possibility existed that all or part of the measured GalTase activity in the preparations of mouse sperm and rat sperm collected by mincing was from the contamination.

In order to gain some evidence about the this possible importance of the contaminating cells, preliminary experiments using purification of mouse sperm by Percoll gradient centrifugation were carried out. GalTase activity in the sperm preparation after Percoll purification is shown in Table 4-1.3. The result was compared with that of sperm preparation without percoll gradient purification in the same table.

Table 4-1.3 GalTase activity in mouse sperm preparation with and without Percoll gradient purification.

Values were pmole GlcNAc produced / hr. Values of the activity of the sperm preparation with Percoll gradient purification were from 2 experiments, and those of the preparation without Percoll purification were from 3 experiments (mean ± SE).

Sperm number	(x 10^{-6})	0.5	1	1.5
GalTase activity in sperm preparation	Percoll purified	50.3, 28.4	98.6, 61.4	145.1, 99.9
	Unpurified	187.3 ± 23.9	308.7 ± 41.1	501.4 ± 71.2

It can be seen that GalTase activity was lower, but still appreciable, in the mouse sperm preparation purified by Percoll gradient centrifugation compared with the sperm preparation without the purification. This result supported the possibility that some at least of the GalTase activity measured in mouse and rat sperm preparation was derived from the contaminating cells. However, the experiments with Percoll gradient purification were only preliminary ones, many improvements, e.g., better separation, are necessary for more results.

Another possibility was that sperm absorbed GalTase from blood plasma when the sperm preparation was contaminated by blood. However, this possibility seemed less likely. Firstly, it is known that the epididymal plasma contains considerable amount of GalTase (see result of Chapter 3). Unless there is some special difference related to sperm absorption between the soluble GalTase of blood and the enzyme in epididymal plasma, there was no reason that the sperm absorbed the enzyme only from blood but not from epididymal plasma. Secondly, the sperm were thoroughly washed before being used for enzyme activity assay, unless the enzyme was absorbed tightly, it should have been removed.

If the GalTase activity measured in preparations of mouse sperm and mincing-collected rat sperm was from contamination, the next question will be what is the exact source of the activity. Available references relevant to this are fragmentary. It is known that GalTase is contained in the membranes of human red blood cells (Kim et al., 1972) and human platelets (Bosmann, 1971; references in review of Ram and Munjal, 1985; Rodriguez et al., 1987; 1988), though it is not clear whether the enzyme is present on the outside or inside surface. Platelets are known to be able to actively adhere to cells, e.g., monocytes, in the cell preparations (Perussia et al., 1982). It is also known that human and mouse monocytes and platelets can release GalTase in culture (Hopper et al, 1985; 1986). GalTase is also known to be present in at least some lymphocytes and epithelial cells (e.g., Sakamaki, et al., 1989), and the enzyme is present in the plasma membrane of some epithelial cells (e.g., Morita et al., 1986; Dutt et al., 1987). It was not examined whether platelets were present either free or on the

surface of sperm or other cells in the sperm preparation. Such examination may be necessary in further investigations. It may also be necessary to examine whether blood cells and epididymal epithelial cells demonstrate any GalTase activity respectively at concentrations similar to those found as contamination in the sperm preparation used for GalTase assay.

No GalTase activity was detectable in rat sperm obtained with the pumping method, although the acrosome status of this sperm preparation was the same as that of the sperm obtained with the mincing method. However, there was a possibility that the true sperm-associated GalTase activity, but not the enzyme activity from contamination, in the sperm preparation was below the detectable limit of the method currently used. If the activity was on plasma membrane over the acrosome or in the acrosome contents, only 30% of the sperm would be expected to contain the activity, since 70% had lost their acrosomes before the assay. Alternative methods may need to be employed for further investigation. For example, purified plasma membrane of rat sperm may be used for GalTase assay.

However, GalTase activity was detected in the ram sperm preparation, although the activity was much lower than those measured in mouse sperm and rat collected by mincing. The ram sperm preparation contained only very low percentages of epithelial cells and lymph cell-like cells, at about 200 times lower concentration than the levels of the same types of cells in mincing-collected rat sperm preparation. Therefore, although the concentration range of ram sperm used for GalTase assay was 10 times as high as those of mouse and rat sperm, it seemed unlikely that the major GalTase activity measured in the ram sperm preparation was from the other cell types. However, it needs to be further clarified exactly where the GalTase activity is located in ram sperm. This was because that in 30% of the sperm before the assay, the acrosome was already damaged or absent; and 35% more were damaged or lost during the incubation for the assay. Therefore, the GalTase activity could be from either the acrosome contents or the inner acrosome membrane as well as the sperm plasma membrane.

Shur and Bennett (1979) reported that in the assay of mouse sperm surface GalTase, 97% of the wild type of sperm lost their acrosome within 10 min of incubation, though the acrosome status just before the incubation was not indicated. They also reported that mouse sperm in buffer without cation had over 70% intact acrosomes. However, such sperm preparations had the same level of GalTase activity as those preincubated in 10mM $MnCl_2$ for 0.5hr on ice and then washed to remove the acrosome membrane. The authors' interpretation of the identical GalTase activities in the two sperm preparations was that the GalTase was probably on the postacrosomal plasma membrane. However, the possibility that the GalTase was on the inner acrosome membrane appears not to have been considered. The sperm preparation used in the above report contained 5% red blood cells as contamination (other types of cells were not mentioned). According to the present results with rat sperm, it seemed that there was also a possibility that the GalTase activity measured in the mouse sperm preparation in the above report was at least partially from contamination. Such a possibility seems to be able to explain the identical GalTase activity in preparations of acrosome-intact sperm and acrosome-absent sperm in that report.

In the current experiments, monoclonal antibody WS 18.6 was used to label rat sperm for acrosome status. It specifically labelled the acrosome region and clearly showed different acrosome status. FITC-conjugated *Pisum sativum* agglutinin (FITC-PSA) was also tried as a probe for rat acrosome status. This lectin probe works well with sperm of humans, monkeys and pigs (Cross and Meizel, 1989). However, it did not specifically label the acrosome region but labelled the whole head of rat sperm (results not shown).

PART 2 (4-2)
STUDIES OF BINDINGS OF RAT AND RAM SPERM TO MOUSE ZONA PELLUCIDA

4-2.1 INTRODUCTION

It is known that rat sperm can bind to mouse zona (see Section 4-2.2.2 (2). Therefore, it was interesting to know whether GalTase was involved in the binding between rat sperm and mouse zona. This was because it is extremely difficult to consistently achieve rat sperm-rat zona binding *in vitro* (see Section 4-2.2.2 (2)). Therefore the fact that rat sperm can relatively easily bind to mouse zona may be used for an initial investigation on the role played by GalTase in rat sperm-zona binding. If GalTase is also the receptor of the sperm for zona in rat, it is very likely to mediate the binding of rat sperm to mouse zona as well. GalTase activity was not detected under the current conditions in uncontaminated rat sperm preparation (Part 1 of this chapter). However, there was a possibility that the amount of rat sperm surface GalTase required for sperm-zona binding was less than the limit for the detection of the method currently used.

It has been shown in Part 1 (4-1) that GalTase activity was detectable in a ram sperm preparation. The question naturally arose whether GalTase was involved in the sperm-zona binding of sheep as well as in mouse. So as to chose a convenient system for the initial study, an attempt was made to see whether ram sperm could bind to mouse eggs, and if so, to study further the possibility of the involvement of GalTase in the binding. Again, if GalTase was involved in ram sperm-zona binding, the binding between the ram sperm and mouse zona was most likely to be mediated by GalTase as well.

The study described in this Part (4-2) was to establish and/or characterize the experimental methods for the above purposes, which included the following. (a) the effects of sperm concentration and incubation time on the binding of rat sperm to mouse zona. (b) Trials and characterization of the binding between ram sperm and mouse zona. (c) Characterization of mouse sperm-zona binding as a control for the study of the role of GalTase in the sperm-zona bindings of the other two species.

4-2.2 LITERATURE REVIEW OF MAMMALIAN *IN VITRO* FERTILIZATION (IVF)

The literature reviewed in this section was mainly literature up to 1989, when the experiments of this part were carried out. IVF in sheep has become routine in Adelaide in recent years, therefore, the recent sheep IVF study in Adelaide is also reviewed.

IVF is a very important method in the studies of gamete interaction. Methods for IVF have been developed for many mammalian species including most laboratory animals, most farm animals and the human since the first successful IVF in rabbits in 1959 by Chang. This review includes the critical parameters for the success of IVF and some characteristics of IVF in the mouse, rat and sheep. It will emphasize *in vitro* sperm-zona binding according to the studies in this chapter.

4-2.2.1 Parameters of IVF Methodology

Rogers (1978) gave a comprehensive summary of the critical parameters for IVF, which is reorganised and modified with some supplementary explanations as follows.

(1) Culture Medium

Culture medium is of utmost importance. It can be divided into undefined media and chemically defined media. The undefined media are those containing biological fluid(s) and/or compound(s), such as fallopian tube fluid, cumulus cells or matrix, epididymal fluid, serum etc.. Undefined media have been much less used since the success of in vitro capacitation and fertilization in chemically defined medium in the mouse (Toyoda et al., 1971a; b). However, commercially available tissue culture media, such as Ham F-10 and TCM 199, supplemented with blood serum or follicular fluid are still commonly used in IVF of some species such as human and sheep (Bavister, 1986; Yanagimachi, 1988; also see Section 4-2.1.2.3).

Chemically defined media are more commonly used now. Many of them are modified Krebs-Ringer's solution and some are modified Tyrode's solution supplemented with energy sources and albumin. Certain components are necessary and important in the defined medium and need attention. Ca^{2+} is essential for sperm motility, capacitation and fertilization (Kaplan and Kraicer, 1978; Saling et al., 1978; Huneau and Crozet, 1989). Glucose, lactate and pyruvate are usually considered to be proper energy sources (also see Bavister, 1981; 1986), although, Neill and Olds-Clarke (1988) have argued that lactate has actually a negative effect on both capacitation and fertilization in the mouse. Albumin plays an essential role in the achievement of IVF in some species (eg. the hamster and mouse), though the mechanism is not yet clear (also see Bavister, 1981; 1986). Osmolarity and pH of the medium also need attention.

(2) Sperm Preparation

(a) Sperm source. Both cauda epididymal sperm and ejaculated sperm can be used for IVF. In general, epididymal sperm are able to fertilize eggs *in vitro* at a higher rate and/or much more easily than ejaculated sperm in various species (also see Yanagimachi, 1988).

(b) Washing. When unwashed sperm are used, the introduction of epididymal or seminal fluid may affect the result. Washing may allow the sperm to

capacitate, or, on the other hand, weaken the sperm and make them less capable of fertilization.

(c) Contamination. The volume of epididymal or seminal fluid introduced into the culture medium, when unwashed sperm are used, may indicate the contamination level of the medium by the male reproductive tract fluid.

(3) Egg Preparation

The source of eggs can influence the results, and so can the conditions under which they are collected. Eggs can be superovulated, naturally ovulated, ovarian and immature, matured in culture etc., and can be ZP free, ZP intact or cumulus surrounded.

(4) Time and Sperm Concentration of Preincubation

Preincubation time refers to the time period during which sperm are incubated prior to the combination with eggs. During this time, sperm can capacitate or, alternatively, be weakened. Sperm concentration during preincubation can affect the rate of capacitation.

(5) Insemination Conditions

If sperm are added to eggs, they are further diluted and AR may therefore be stimulated.

(6) Time and Conditions of Incubation

Incubation time refers to the time period when sperm and eggs are incubated together. This time is important for estimating the status of the sperm and the sperm-egg interaction (for example, see Soldani and Rosati, 1987 and Saling, 1989). Final sperm concentrations at incubation directly affect IVF result. Temperature and gas phase also need to be considered.

4-2.2.2 *In Vitro* Fertilization in the Mouse, Rat and Sheep

(1) Mouse

The mouse is the most commonly used species in IVF experiments. The culture media used for IVF in the mouse are usually modified Krebs-Ringer Solutions. The optimal osmolarity is that provided by 94-126 mM NaCl (Miyamoto and Chang, 1973). However, preincubation of sperm in medium with elevated NaCl concentrations can at least improve the speed of capacitation (Oliphant and Brackett, 1973). Calcium concentration is usually about 1.7 mM (Miyamoto and Ishibashi, 1975). The optimal final sperm concentrations, judged by penetration rate, vary in different reports probably because of differences in mouse strain (Iwamatsu and Chang, 1971; Parkening and Chang, 1976; Fraser, 1977) and culture media (Wolf and Inoue, 1976) used. A sperm concentration of 1-6 x 10^5/ml (Toyoda et al., 1971a; b; Tsunoda and Chang, 1975; Wolf and Inoue, 1976) is usually accepted as optimal, though the concentration range which gave high fertilization rates reported by Fraser and Drury (1975) is much higher. Over the range of 10^3 to 10^6 sperm/ml, the sperm-zona binding has been found to be related to sperm concentration (Wolf and Inoue, 1976). Preincubation of mouse sperm is usually carried out at a concentration of 10^7-10^6 / ml (Toyoda et al., 1971b; Wolf and Inoue, 1976; Florman et al., 1982; Neill and Olds-Clarke, 1988). One hour is enough for sperm capacitation (Wolf and Inoue , 1976; Florman et al., 1982; Soldani and Rosati, 1987).

There have been several different types of time curves reported for mouse sperm-zona binding. Schmell and Gulyas (1980) claimed that sperm-zona binding increased linearly during the incubation period of 90min. Saling et al. (1978) and Florman et al. (1982) reported that sperm-zona binding reached a maximum level about 15min after insemination and remained at the same level during the rest of the 30min of incubation time. As already introduced in 1.2.2.4, Soldani and Rosati (1987) observed a type of two phase sperm-zona binding with an initial peak at about 10min after sperm addition and a second higher plateau at 40min with a low binding level

between at 20-25 min. Saling (1989) suggested that the low level of binding between two high levels in the report of Soldani and Rosati (1987) was due to experimental perturbation at the stage that sperm were having acrosomal reaction and had low affinity to ZP.

(2) Rat

The culture media used for IVF in the rat are similar to those used in the mouse, except that the $CaCl_2$ concentration is elevated to 3.4 mM, which greatly increases the fertilization rate in rats (Kaplan and Kraicer, 1978). Niwa and Chang (1974a) demonstrated that final sperm concentration of 0.5-1.5 x 10^6/ml was optimal for sperm penetration, the same authors (1974b) also showed that capacitation of rat sperm during preincubation was better achieved at a similar low sperm concentration (0.7-1.2 x 10^6/ml) than at a high concentration of 9.6-14.9 x 10^6/ml. In the report of Shalgi et al. (1983), at least 3.5hr preincubation was required for capacitation of ejaculated sperm recovered from rat uterus; and the number of sperm bound to an egg increased with incubation time and reached a peak at 30min. It was extremely difficult to achieve success in rat IVF. It was 11 years after the success of *in vitro* capacitation and fertilization in mouse that the first successful *in vitro* capacitation and fertilization in rat was reported (Toyada and Chang, 1974). Following the several reports from Chang's laboratory, there have been limited number of reports about rat IVF, mostly from Shalgi and co-workers. However, they regularly used sperm isolated from the uteri of mated adult rats (e.g., Shalgi et al., 1981, 1983, 1986). Du (1988) carried out some experiments on rat sperm-zona binding with epididymal sperm. However, successful binding was achieved only with one batch of male rats (personal communication). The difficulty of IVF in the rat may be because rat sperm are particularly fragile as discussed in Cardullo and Cone (1986).

As already mentioned in Section 1.2.4, Du (1988) demonstrated that rat sperm can cross-bind to mouse zona, and the affinity of the binding was even higher than that of the binding between rat sperm and rat zona. He showed that in the same

assay using rat sperm culture medium and at sperm concentration of 0.4 x 10^6, the number of rat sperm bound to one rat zona was only 2.0 ($\pm$ 0.4, n=18) (mean $\pm$ SE, number of eggs used), while the number of mouse sperm bound to one rat zona was 22.2 ($\pm$ 2.9, n=18). However, the effects of sperm concentration and incubation time on the cross binding have not been examined.

(3) Ram

Successful IVF in farm animals has lagged far behind most of the laboratory animals and human. There have only been small number of reports about IVF in sheep and even fewer reports of stable and satisfactory results. The methods used in these reports vary widely. In the report of Bondioli and Wright (1983), modified Krebs-Ringer medium as used for rat IVF (Toyoda and Chang, 1974) was employed. Ejaculated sperm were washed twice by centrifugation in the medium and then preincubated for 3hr, the final sperm concentration for fertilization was 1 x 10^6 sperm/ml and the sperm-egg mixture was incubated under a 5% CO_2 in air at 37°C. A 5% penetration rate without further cleavage was obtained. In the same report it was found that high ionic strength (126-129mM NaCl) did not improve the penetration rate in sheep, in contrast to findings in rabbits (Brackett and Oliphant, 1975) and cattle (Brackett et al., 1980). High percentage (over 60%) of fertilized egg and births of living young were obtained in some later work (Cheng, 1985; Cheng et al., 1986; Crozet, et al., 1987; Fukui et al., 1988 a; b; Huneau and Crozet, 1989). Two kinds of methods have been employed in these studies. One involves Cheng's (1985) method and its slight modifications (Fukui et al., 1988a; b), and the other is the method of Crozet et al. (1987). In both methods, fresh ejaculated sperm were used. In Cheng's method, TCM 199 is used with heat inactivated sheep serum and some other supplements. Semen is maintained undiluted at 20°C for 4hr before washing, and sperm are preincubated at 20°C for 30 to 40min at a concentration of 2 x 10^8/ml after washing. In Crozet et al.'s method, modified defined medium of Brackett and Oliphant (1975) is used also with heat-inactivated sheep serum. Sperm are allowed to swim up for 2hr after being washed

by centrifugation and then further incubated for 5-6hr at 38.5°C at a concentration of 1 x 10^7/ml. The final sperm concentration in both methods is 1 x 10^6/ml and incubation temperature is about 39°C under 5% CO_2 in air, or under air if Hepes is added to the medium as in Crozet et al.'s method. Furthermore, Huneau and Crozet (1989) have demonstrated that elevated Ca^{2+} concentration (to 8.74 mM from 2.74 mM) increases the fertilization rate.

In recent years, IVF in sheep has been routinely used by Dr R.F. Seamark, Department of Obstetrics and Gynaecology, University of Adelaide, Dr S.K. Walker, Department of Agriculture, South Australian Gorvenment and their co-workers in their research to improve the relationship between sheep IVF and the practice of sheep production. They basically follow the method of Crozet et al. (1987) and Fukui et al. (1988, a; b) (personal communication from Dr S.K. Walker). The status of IVF technology in the sheep was recently reviewed by Walker (1992).

There have also been a few reports about cross fertilization *in vitro* with ram sperm. Binding of ram sperm with rat oocytes was reported by Fournier-Delpech et al. (1982). Their method involved simply diluting epididymal sperm in culture medium to a final concentration of 5 x 10^6/ml and incubating the sperm-egg mixture at 35°C in a humid atmosphere for 30-45min. The medium used was TCM 199 with Hepes buffer. High rate of penetration of cattle ova by ram sperm has also been reported (Slavik et al., 1990). The culture medium used was TCM 199 supplemented with extra Ca^{2+} and energy source and buffered by Hepes. The method was very similar to that of Crozet et al. (1987), except that sperm were only allowed to swim up for 30-40 min and there was no further preincubation of the sperm.

4-2.3 MATERIALS AND METHODS

4-2.3.1 Materials

Human chorionic gonadotrophin (hCG) and hyaluronidase (28IU/mg) were purchased from Sigma (St. Louis, MO, USA). Pregnant mare serum gonadotrophin (PMSG) was Folligon from Intervet International (Boxmeer, Holland). Paraffin oil was from Laboratory Chemicals (Sydney, Australia). All the other chemicals used were analytic reagent grade.

The incubator used for culture medium equilibrating, sperm capacitation, sperm-zona binding etc. was a humidified incubator at 37°C with an atmosphere containing 5% CO_2. It will be simply mentioned as a CO_2 incubator in the following text. The micropipette used for egg collection was made from a 5.8 X 0.6 disposable Pasteur pipette (Chase Instruments Corp, Glen Falls, NY, USA). The thin part of the pasture pipette was pulled out over a bunsen flame to a fine bore which allowed the passage of only one egg (for the pipette for collecting free eggs) or one egg with bound sperm (for the pipette for collecting eggs with bound sperm) at each time. The other end of the Pasture pipette was connected to a soft rubber tube which was connected to a plastic tip filled with cotton wool as a mouth piece.

4-2.3.2 Preparation of Culture Medium

The same culture medium was used for the binding of mouse and rat sperm to mouse eggs, as that discussed by Toyoda and Chang (1974) except that the concentration of $CaCl_2$ was 3.4mM instead of 1.7mM. The culture medium used for ram sperm was the same as the above except that the NaCl concentration was 20mM higher. This was because it was observed that the higher NaCl concentration improved the binding of ram sperm to mouse eggs by 40%. The medium used for washing ram sperm before culture was the same as the culture medium for ram sperm, except that $CaCl_2$ was omitted.

Four stock solutions, A, B, C and D were prepared as shown in Table 4-2.1.

Table 4-2.1 Stock solutions for the media used for sperm-zona binding.

Stock solutions	Component	Concentration in stock (g/100ml)	Concentration in final preparation (mM)
Stock A	NaCl	10.50	94.6 for mouse or rat sperm 114.6 for ram sperm
	KCl	0.71	4.78
	KH_2PO_4	0.32	1.19
	$MgSO_4 \cdot 7H_2O$	0.59	1.19
	Glucose	2.00	5.56
	Na lactate	7.4ml of 60% syrup	21.6
	Streptomycin sulphate	0.10	50μg/ml
	Penicillin G	0.12	100IU/ml
Stock B	$NaHCO_3$	2.10	25.1
	Phenol red	0.005	
Stock C	$CaCl_2 \cdot 2H_2O$	5.24	3.42
Stock D	Na pyruvate	0.51	0.50

Solution A and C were prepared every 3 month, while solution B and D were prepared every 2 weeks.

For the final preparations, the stock solutions were diluted as shown in Table 4-2.2.

Table 4-2.2 Final preparations of sperm-zona binding culture medium.

Stock solutions	Culture medium for mouse and rat sperm	Culture medium for ram sperm	Washing medium for ram sperm
Stock A	1.0 ml	1.0 ml	10.0 ml
Stock B	1.0 ml	1.0 ml	10.0 ml
Stock C	0.096 ml	0.096 ml	---
Stock D	0.108 ml	0.108 ml	1.08 ml
1M NaCl	---	0.20 ml	2.0 ml
H_2O	7.796 ml	7.596 ml	76.92 ml
BSA	40 mg	40 mg	400 mg
Total volume	10 ml	10 ml	100 ml

Double-distilled water was used for the preparation of all solutions and media. The final solution was sterilized by passing it though a 0.45μm filter membrane (Millipore Corp., MA, USA). The sterilized culture medium can be stored at 4°C for a week.

Twelve to twenty-four hours before use, the culture medium and washing medium (for ram sperm) were gassed with 5% CO_2 : 5% O_2 : 90% N_2 until the phenol red became very light pink-orange coloured, and then sterilized by the filter again.

Sterilized Petri dishes of 35 x 10mm (Kayline Plastics, Adelaide, Australia) were used for insemination. Culture medium of 0.1-0.5ml was placed in the centre of the dishes and immediately covered with paraffin oil. The paraffin oil was sterilized by autoclave for 15min at 1-2 atmospheres and gassed with 5% CO_2 : 5% O_2 : 90% N_2 for 5-10min before use. The dishes were then placed in a CO_2 incubator for equilibration overnight. For binding assay with ram sperm, 50-100ml of the washing medium covered with paraffin oil was also equilibrated overnight.

The whole process was carried out under sterile conditions.

4-2.3.3 Preparation of Mouse Eggs

Swiss mice 23-26 days old obtained from the Central Animal House, University of Adelaide were used. Superovulation was induced by injecting intraperitoneally 6IU PMSG 64hr and 6IU hCG 16-18hr before collecting the eggs.

The mice were killed by cervical dislocation. The oviducts were dissected out and briefly washed in a small volume of culture medium, and then placed in 2ml 0.1% hyaluronidase in PBS containing 4mg/ml BSA in a 35 x 10mm Petri dish. The eggs surrounded by cumulus cells were released into the hyaluronidase solution by tearing the swollen ampulla with a 27-gauge syringe needle, and incubated for 5min to dissociate the cumulus cells from the eggs. The tubal remnants were discarded. Then, the morphologically normal eggs, free of cumulus cells, were selected, passed though 4 petri dishes of clean culture medium, and finally transferred to the culture medium (usually 0.4ml) under paraffin oil, which had been equilibrated in the CO_2 incubator overnight. In the above manipulations, the eggs were collected by a micropipette. The eggs were then stored in the incubator for use in the sperm-zona binding assay. The final yields were usually 20-40 eggs from each mouse. All the procedures were carried out at 35-37°C. The preparation of the eggs was carried out during the time of capacitation of mouse and rat sperm, but before the preparation of ram sperm.

4-2.3.4 Preparation of Sperm

The culture medium used in the preparation of sperm were equilibrated in a CO_2 incubator in petri dishes under paraffin oil overnight as described in 4-2.3.2.

(1) Preparation of Mouse Sperm

F1 hybrid (C57 ♂ x ABLB/c ♀) mice and LACA mice of 3-4 weeks old obtained from Central Animal House, University of Adelaide were used. Usually one mouse was used for each assay. The animal was killed by cervical dislocation. A piece

of cauda epididymidis was dissected out and placed in 0.4ml of culture medium immediately. It was briefly torn with forceps and a 27-gauge syringe needle, and then placed in the incubator for 5min to allow the sperm to disperse in the medium. An aliquot of the concentrated sperm suspension was diluted with 90µl of 10% formalin in 0.5M phosphate buffer (pH 7.4) and counted using a haemocytometer (Assistant, West Germany). For capacitation, a sperm suspension of 1-4 x 10^7 sperm/ml in 0.5ml culture medium was incubated in a CO_2 incubator for 1-2hr before insemination. The sperm suspension was then diluted in 100µl culture medium to a concentration suitable for sperm-zona binding assay, as indicated in the legends of figures.

(2) Preparation of Rat Sperm

Porton rats, 5-8 month old, from Central Animal House, University of Adelaide were used. Rat sperm were prepared in the same way as mouse sperm. However, the sperm were incubated in a suspension of 0.3-0.8 x 10^6 in 100µl culture medium for 4-5hr for capacitation. The same suspension was used for sperm-zona binding assay.

Rat sperm were much more vulnerable than mouse sperm to various factors of the environment, e.g., temperature, pH, composition of culture medium. Therefore, more care need to be taken to ensure the success of their capacitation and binding to zona, and to avoid the experimental error caused by the environmental factors.

(3) Preparation of Ram Sperm

Epididymides of Merino rams were obtained from abattoirs soon after being separated from the body and transferred to the laboratory at 20-25°C within 1hr. A small piece of the cauda epididymidis was briefly minced in 5ml of medium and left in the medium for 10min to let the sperm to spread in the medium. Large tissue pieces were then taken out by forceps and smaller pieces were filtered out by fine nylon cloth. The sperm suspension was centrifuged in a 10ml sterile plastic tube in a TJ-6 centrifuge

of Beckman at 600g for 5min. Half millilitres of the pellet was transferred to 5ml washing medium, and after the sperm had spread though the medium, the suspension was centrifuged at 600g for 5min. The pellet was re-suspended in 5ml of the washing medium and the washing was repeated twice. Finally, the pellet was resuspended in 1ml of the culture medium, the sperm were counted, and an aliquot of the suspension was transferred to 0.5ml of the culture medium covered by paraffin oil in petri dish. This suspension was further diluted in 100µl culture medium to concentrations appropriate for sperm-zona binding assay, depending on the binding ability of the sperm. The concentrations used are indicated in the legends of figures.

4-2.3.5 Sperm-Zona Pellucida Binding Assay

Ten to twenty eggs were added to the 100µl sperm suspension for the binding assay, prepared as described above. Incubation was carried out in a CO_2 incubator for various times, then the eggs bound with sperm were fixed.

Two methods were used to fix the bound sperm and eggs. The general method used was the "stop-fix" technique essentially following that described by Saling et al. (1978) which involved the centrifugation of the eggs with bound sperm through a discontinuous dextran gradient. A dextran gradient was set up in an Eppendorf tube by adding with microsyringe 50µl culture medium, then 25µl 1.8% dextran and finally 25µl 2.25% dextran containing 2.5% glutaraldehyde. The eggs were gently transferred to the culture medium layer of the gradient by a micropippette, and the tubes were centrifuged in a Beckman TJ-6 centrifuge at 100g for 90sec. The eggs were then recovered from the bottom of the tubes using a micropipette.

The other method used was the washing method used elsewhere (e.g., Schmell and Gulyas, 1980; Soldani and Rosati, 1987). Eggs with bound sperm were passed through three dishes of clean culture medium by a micropipette and then fixed in 1.5% glutaraldehyde. This method was used to confirm the results obtained by the "stop-fix" method.

The recovered eggs with bound sperm were then mounted on microscope slides and covered with cover slides. Each cover slide had a little vaseline on 3 sides, so that the eggs would not be pressed. The bound sperm on individual eggs were counted under a microscope using phase contrast optics at a 200-400 x magnification.

4-2.4 RESULTS

4-2.4.1 Binding of Mouse, Rat and Ram Sperm to Mouse Zona

The eggs associated with sperm were fixed after 10 min of incubation by both the "stop-fix" method and the washing methods. Using either method, it was shown that both rat sperm and ram sperm could bind to mouse zona. The numbers of sperm bound to a zona from the same dish fixed by these two methods were similar (e.g., 46 $\pm$ 2.9 vs 41 $\pm$ 3.5 (mean $\pm$ SE), n=5, in the mouse). The bindings of rat sperm to mouse zona and of ram sperm to mouse zona are shown in Fig. 4-2.1 and 4-2.2 respectively.

4-2.4.2 Effect of Sperm Concentration on the Binding of Mouse, Rat, and Ram Sperm to Mouse Zona

The relationship between mouse, rat and ram sperm concentrations and binding are shown in Fig. 4-2.3, 4-2.4 and 4-2.5, respectively. It can be seen that with the sperm of each species, the binding level increased with the increase of sperm concentration within the measured range (0-0.75 x 10^6 of F1 mouse sperm, 0-1 x 10^6 of rat sperm and 0-1 x 10^6 of ram sperm)

4-2.4.3 Effect of Incubation Time on the Binding of Mouse, Rat, and Ram Sperm to Mouse Zona

The effect of incubation time on the binding of FI and LACA mouse sperm to mouse zona are shown in Fig. 4-2.6 and 4-2.7 respectively. The time courses of binding by the sperm of these two mouse strains were different. The binding of the F1 sperm increased with the time without any obvious decrease within the 45min studied. However, the binding of LACA sperm firstly peaked at about 10-15min and then decreased to form a trough between 20-40min, followed by a recovery to the maximum measurement time, 50 or 55 min.

As shown in Fig. 4-2.9a, b and c, when rat sperm bound to mouse zona, the general patten of the binding was that the initial plateau or peak appeared between 10 to 17min, followed by a trough between 20 and 30min, and then a second rise. However, the changes afterwards did not appear to be consistent in the assays carried out. The second rise either lasted for 10min (4-2.8a) or appeared only briefly (4-2.8b and c). The second plateau or peak was either about the same (4-2.8a and c) or lower (4-2.8b) than the first one. It seemed that after the second drop the binding rose again around 50min.

The effect of the time on the binding of ram sperm to mouse zona is shown in Fig. 4-2.9a and b. There was again a first peak between 10 to 15min, followed by a drop with the lowest point at about 20min. There then followed a second rise peaking around 30min. After the second peak, no consistent changes could be observed in the two assays, and it seemed that the binding level became very variable.

4-2.4.4 Binding Affinities of Mouse, Rat and Ram Sperm to Mouse Zona

The respective binding affinities of mouse, rat and ram sperm to mouse zona were compared. At the same sperm concentrations, the binding level of F1 mouse sperm to mouse zona was the highest, followed by those of LACA mouse sperm and rat sperm. The binding level of the ram sperm was the lowest (Table 4-2.3).

Table 4-2.3 Binding affinities of mouse, rat and ram sperm to mouse zona.

The same sperm concentration of 4 x 10^5 was used in the assay of each species. 10-15 eggs were used in each assay. Values were number of sperm bound/egg (mean ± SE).

Sperm	F1 mouse	LACA mouse	Rat	Ram
Mean + SE	61.5 ± 5.4	42.5 ± 4.4	39.2 ± 5.7	28.0 ± 4.8
Number of assays	4	3	4	4

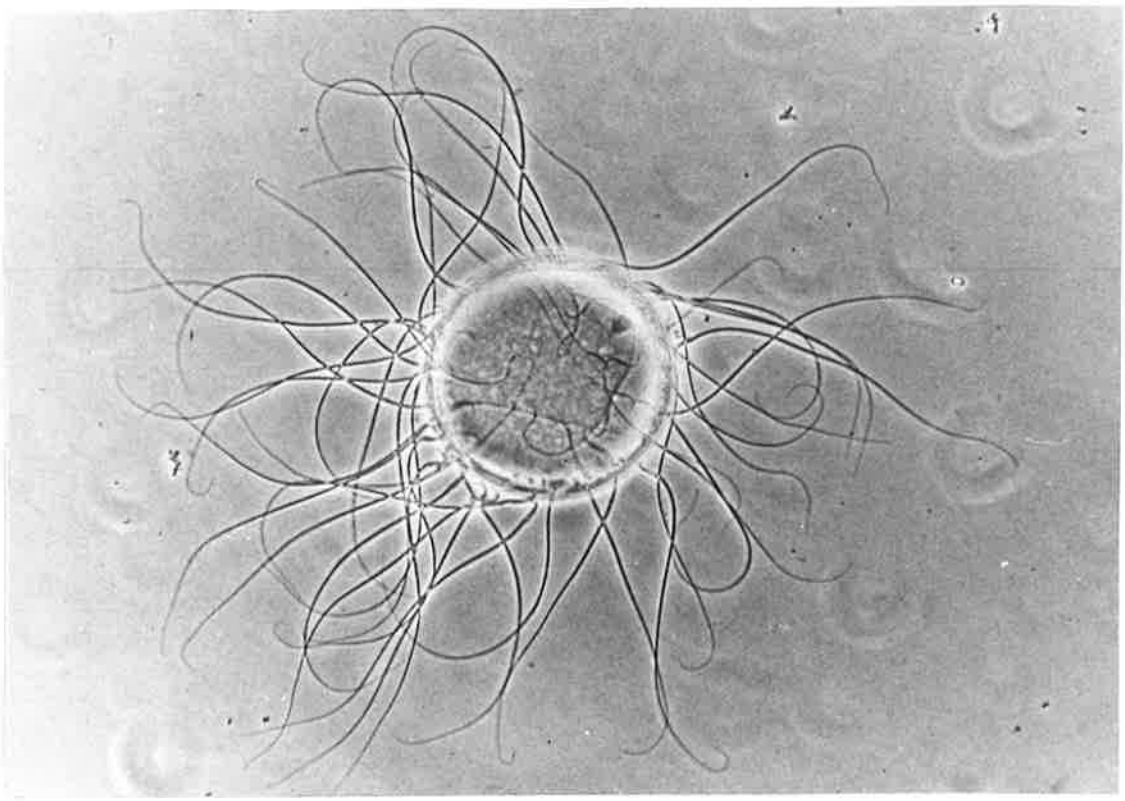


Fig. 4-2.1 Binding of rat sperm to mouse zona.

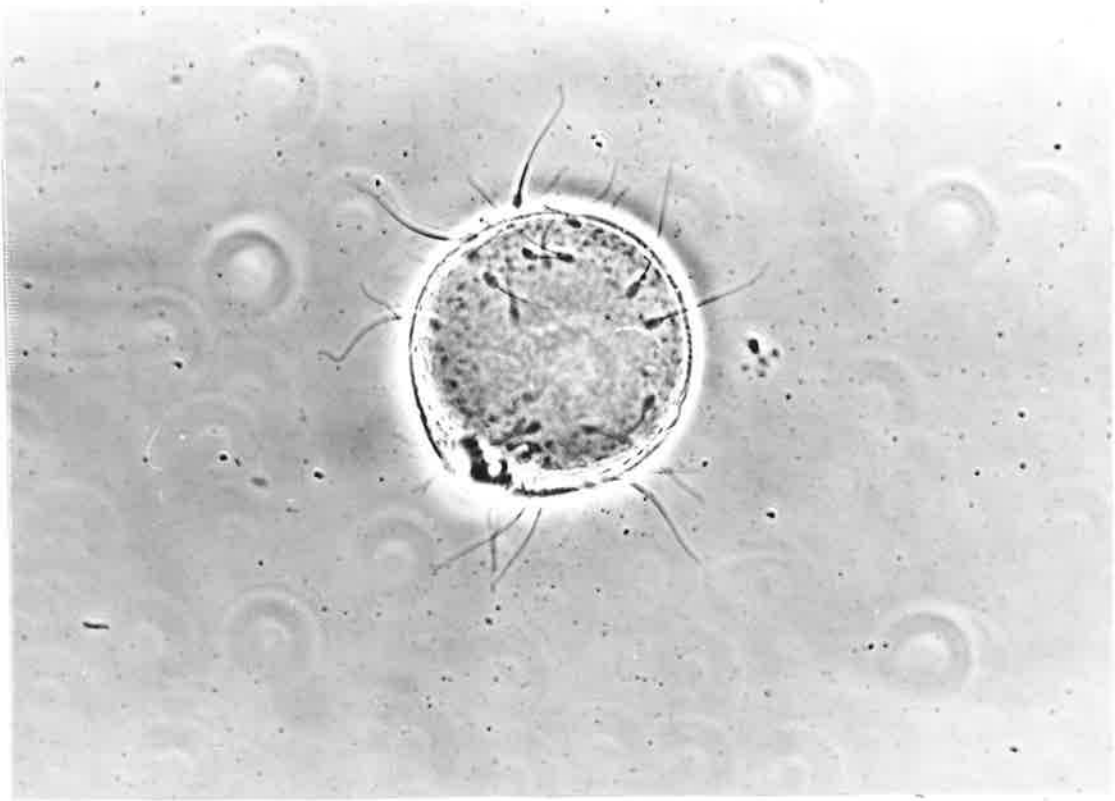


Fig. 4-2.2 Binding of ram sperm to mouse zona.

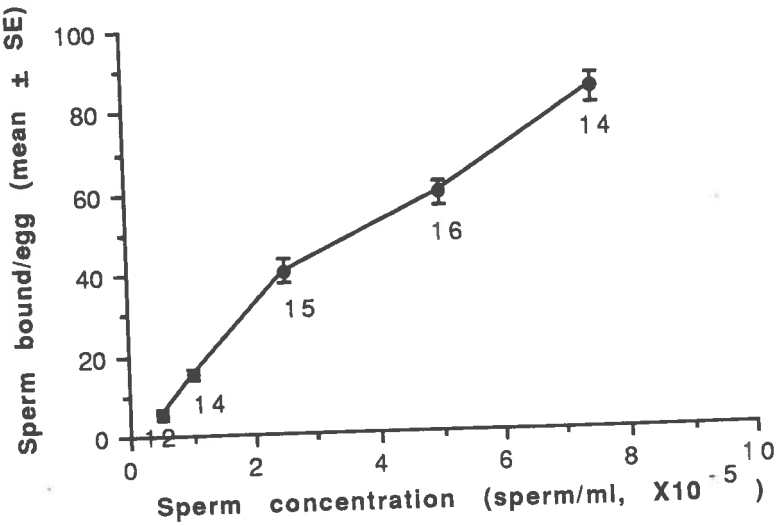


Fig. 4-2.3 Effect of sperm concentration on mouse sperm-zona binding. Sperm of F1 mouse were used. Incubation time was 15min. Number given at each point is the number of eggs used for that point.

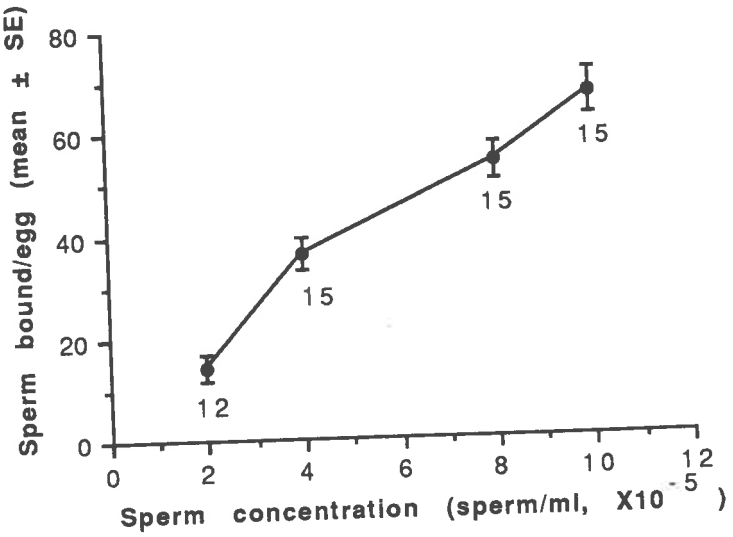


Fig. 4-2.4 Effect of sperm concentration on the binding of rat sperm to mouse zona Incubation time was 15min. Number labelled at each point is the number of eggs used for that point.

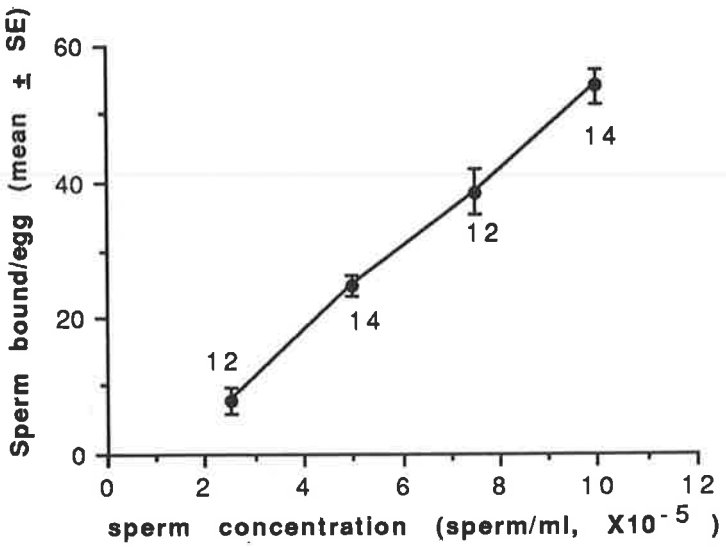


Fig. 4-2.5 Effect of sperm concentration on the binding of ram sperm to mouse zona. Incubation time was 15min. Number given at each point is the number of the eggs used for that point.

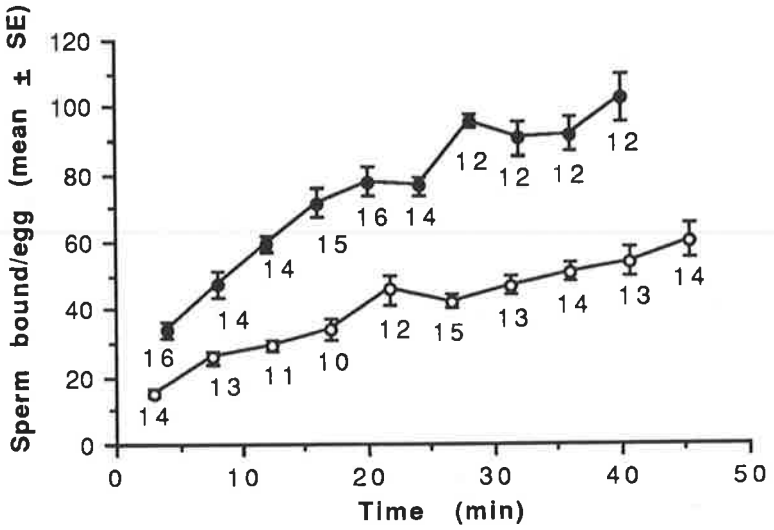


Fig. 4-2.6 Effect of incubation time on the binding of F1 mouse sperm to mouse zona. Two assays with gametes from different individuals were carried out. The sperm concentrations in the assays were 3 x 10^5/ml (•) and 1.5 x 10^5/ml (o) respectively. Number given at each point is the number of eggs used for that point.

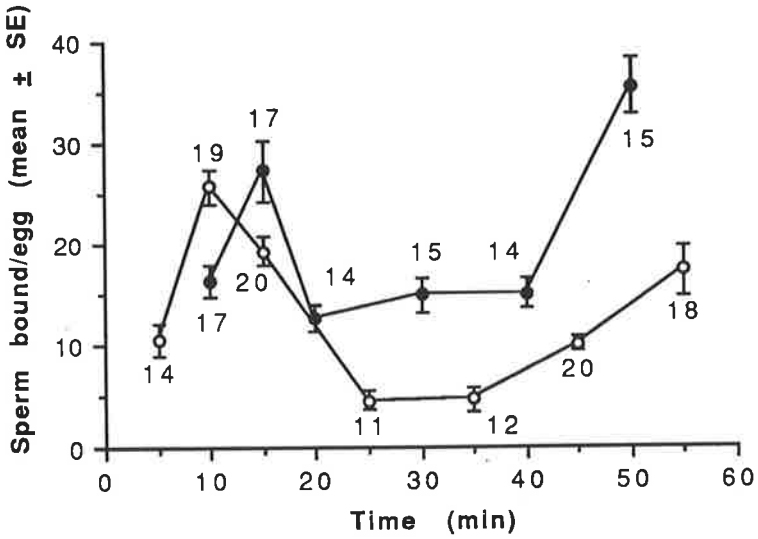


Fig. 4-2.7 Effect of incubation time on the binding of LACA mouse sperm to mouse zona. Two assays with gametes from different individuals were carried out. The sperm concentrations in the assays were 3 x 10^5/ml (•) and 1.5 x 10^5/ml (o) respectively. Number given at each point is the number of eggs used for that point.

Fig. 4-2.8 Effect of incubation time on the binding of rat sperm to mouse zona. Three assays with gametes from different individuals were carried out. The sperm concentrations in assay (a), (b) and (c) were 3.5, 4 and 6 x 10^5/ml respectively. Number given at each point is the number of eggs used for that point. (Next page)

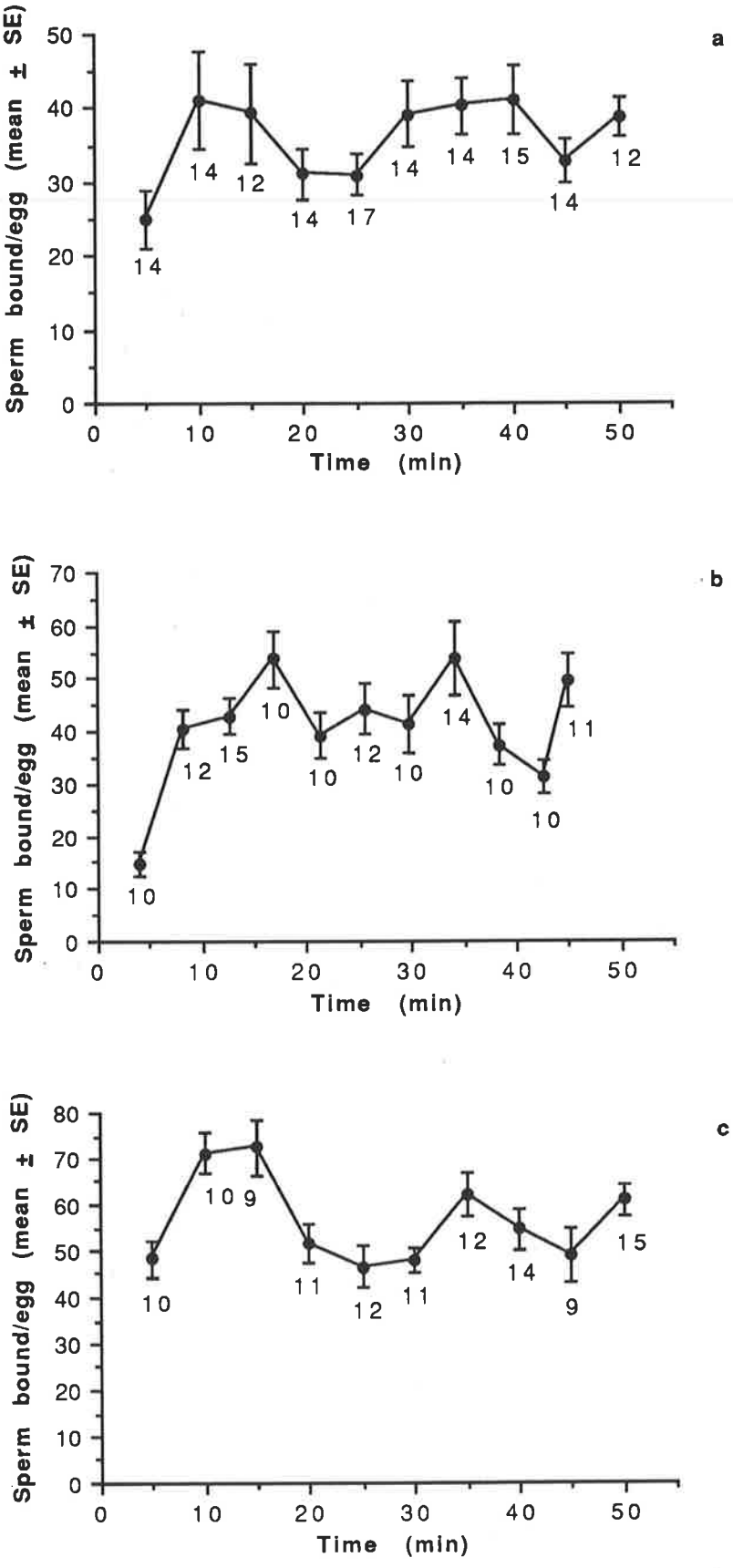


Fig. 4-2.8 (The legend of Fig. 4-2.8 is on last page)

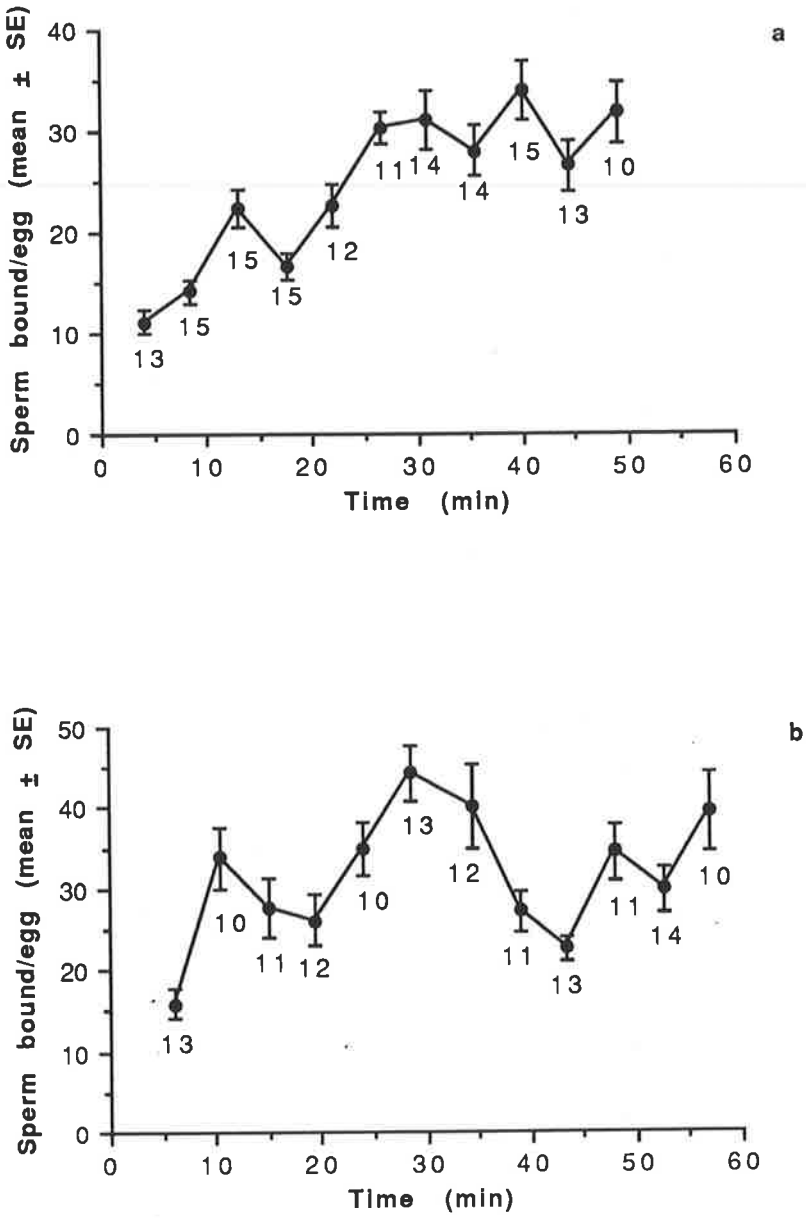


Fig. 4-2.9 Effect of incubation time on the binding of ram sperm to mouse zona. Two assays with gametes from different individuals were carried out. The sperm concentrations in assay (a) and (b) were 2.5 and 5 x 10^5/ml respectively. Number given at each point is the number of eggs used for that point.

4-2.5 DISCUSSION

The procedures of mouse and rat sperm preparation in the current study including the step for capacitation of these sperm. Because of the limits of finance and time, no attempt was made to capacitate ram sperm following the latest procedures described in Section 4-2.2.2 (3). Instead, ram sperm washed in Ca^{2+}-free medium were used. The reasons for using such sperm were as follows. (a) According to Shur and Hall (1982a), capacitation of mouse sperm could be mimicked by washing sperm in Ca^{2+}-free medium. It was suggested that the washing eliminated the poly *N*-acetyllactosamine substrates covering the GalTase of mouse sperm surface. Therefore, if GalTase is also involved in ram sperm-zona binding as in mouse, it should be possible to capacitate ram sperm also by washing them with Ca^{2+} free medium as well. (b) If the ram sperm were actually not capacitated, this may not be important. This is because that although it is not clear whether the zona-binding molecules of uncapacitated sperm are different from those of capacitated sperm, some studies having shown identical binding characteristics with these two kinds of sperm. For example, Peterson et al. (1985) reported that antibodies (Fab) against partially purified zona-binding proteins from plasma membrane of uncapacitated boar sperm strongly inhibited the binding of both capacitated and uncapacitated boar sperm to porcine zona. In fact, many studies about the receptor of boar sperm for porcine zona have been carried out with uncapacitated sperm (e.g., Töpfer-Petersen et al., 1985; Peterson and Hunt, 1989; Hanqing et al., 1991). A zona binding protein with M_r 53,000 was shown to be in the extractions of both capacitated and uncapacitated sperm (Brown and Jones, 1987). This protein is also a fucose-binding protein (see Section 1.2.3.2.2 (1)). Correspondingly, it was shown that fucoidin, a fucose-containing saccharide, inhibited the binding of uncapacitated boar sperm to porcine zona (Peterson et al., 1984). Leyton and Saling (1989b) reported the following results. Both capacitated and uncapacitated mouse sperm contained a M_r 95,000 protein which was a tyrosine kinase (substrate) and was most probably also a ZP3-binding protein. After either the incubation for capacitation or

incubation which maximized zona-binding ability but did not capacitate sperm (in Tris/NaCl buffer containing Ca^{2+}, at 37°C), the phosphotyrosine level of the *M*r 95,000 protein increased substantially.

Two methods, gradient centrifugation and washing, were used to fix the associated sperm and egg to ensure the association examined was sperm-zona binding, but not the attachment of sperm to zona (see Schmell and Gulyas, 1980; Saling et al., 1978).

The work presented here showed for the first time that ram sperm could bind to mouse zona, though the binding between ram sperm and rat zona was previously reported (Fournier-Delpech et al., 1982).

The binding levels of all the species used increased with sperm concentration in culture medium within the range measured. This showed the quantitative reliability of the assay of each species with respect to sperm concentration.

The increase in the binding of the F1 mouse sperm to mouse zona increased continuously with time during the 45min observation agrees with the observation of Schmell and Gulyas (1980). There were some common characteristics about the effects of incubation time on the binding of the sperm of LACA mouse, rat and ram to mouse zona. There was an early peak or plateau before 20min of incubation, followed by a trough, and then a second peak or plateau. However, the time of the second rise differed among the species; and there was no regular pattern of change after the second rise of the sperm binding in rat or ram (the second rise in binding of LACA mouse sperm occurred at the end of the period of observation). This high-low-high type of change of binding with extension of the incubation time was similar to that reported by Soldani and Rosati (1987) (or see Section 1.2.2.4). As detailed in Section 1.2.2.4, Saling (1989) suggested that the low phase of the binding was the stage of sperm acrosome reaction. However, in the present study, ram sperm were possibly not capacitated. Nevertheless, the irregular pattens of bindings of rat and ram sperm to mouse zona after the second rise in binding level might reflect the difficulty in the further interaction between rat and ram sperm to mouse zona after the primary binding. As

already mentioned in Section 4-2.2.2 (1), to explain that low phase binding was only observed in some reports but not others, Saling (1989) suggested that it is due only to experimental perturbation that the altered affinities are observed; and that under normal circumstances the loss of binding and re-binding would not occur, rather the reacting sperm would remain associated with the zona. In the current study, the same care was taken in processing the eggs associated with sperm of all the species used. Therefore, there was the possibility that the binding of the sperm of LACA mouse, rat and ram were more readily experimentally perturbed than was the binding of F1 mouse sperm. In support of this suggestion, the present result showed that the binding affinity of F1 mouse sperm to zona was higher than those of sperm from LACA mouse, rat or ram.

The sperm binding affinities to mouse zona seemed to descend in the order of F1 mouse > LACA mouse and rat > ram. It seemed that the zona binding abilities of sperm from different strains in a same species could be quite different. The binding affinity of the sperm of LACA mouse was much lower than that of F1 mouse sperm. Information is rare about the general degree of variation of binding affinities of the sperm from different strains of a same species; and it is not clear how well the strain of each species used in the current study represented the general affinity of the species. If the current results from F1 mouse, rat and ram sperm are good representatives for the general situation of their respective species, the differences between the affinities of the species may be easily explained by the evolutional relations between these species.

PART 3 (4-3)
EFFECTS OF UDP-GALACTOSE ON THE BINDING OF RAT OR RAM SPERM TO MOUSE ZONA PELLUCIDA

4-3.1 INTRODUCTION

As stated in 4-2.1, it is interesting to known whether GalTase also mediates the bindings of rat sperm and ram sperm to mouse zona. On the basis of reliable assay methods for the bindings of mouse, rat and ram sperm to mouse zona (Part 2 of this chapter), the effects of UDP-galactose on the bindings of rat and ram sperm to mouse zona were examined. The effect of UDP-glucose on mouse sperm zona binding was also carried out as a control for non-specific effect of UDP-hexose. UDP-galactose is the donor substrate of GalTase (see Section 1.3.1). It has been reported to inhibit mouse sperm-zona binding, a finding which was one of the important results to suggest that GalTase was the receptor of sperm for zona in mouse (see "General Introduction" of this chapter).

4-3.2 MATERIALS AND METHODS

4-3.2.1 Animals

The sources of mouse, rat and ram sperm and that of mouse eggs were the same as those introduced in Part 2 (4-2). F1 mice but not LACA mice were used in the binding assays of this part (4-3) of the chapter.

4-3.2.2 Assay of the Effect of UDP-galactose on Sperm-Zona Binding *In Vitro*

The culture medium used was as introduced in Section 4-2.3.2 with the addition of 0.1mM $MnCl_2$. The procedures of sperm preparation, egg preparation and

in vitro sperm-zona binding were the same as described in Section 4-2.3. The sperm concentrations used in the binding assays of mouse, rat and ram were 1.5-4 x 10^5, 2-6 x 10^5 and 0.4-1 x 10^6, respectively. These concentrations were chosen in order to obtain bindings of about 20-50 sperm per egg, a rate for both reliable result and reasonably easy counting. 12-15 eggs were used in each assay. The incubation time of the assays was 10min.

4-3.3 RESULTS

4-3.3.1 Inhibition of UDP-Galactose on Mouse Sperm-Zona Binding

The effect of UDP-galactose at a concentrations between 0 and 5mM on mouse sperm-zona binding was examined. In this range, UDP-galactose had a concentration-dependent inhibitory effect on the binding (Fig 4-3.1).

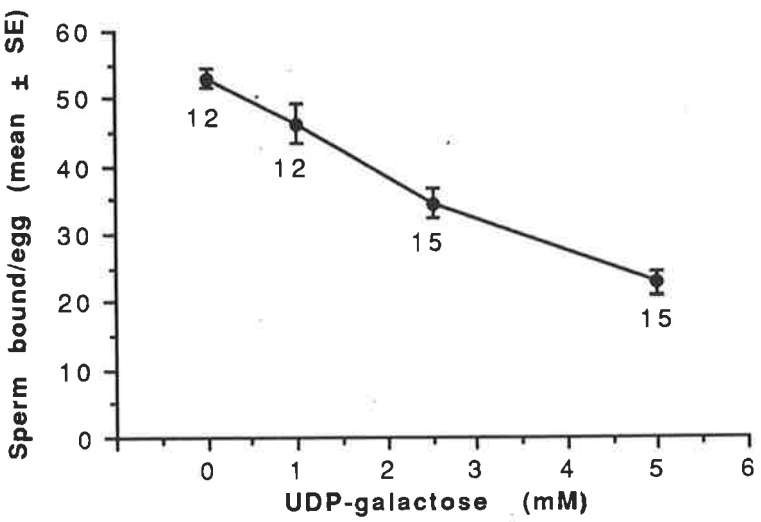


Fig 4-3.1 Effect of UDP-galactose on mouse sperm-zona binding, which was assayed as described in Section 4-3.2.2. Number given at each point is the number of the eggs used for that point.

4-3.3.2 Effects of UDP-galactose on the Binding of Mouse, Rat and Ram Sperm to Mouse Eggs

There were three dishes in each assay as follows. (a) The dish contained 2.5mM UDP-galactose; (b) the dish contained 2.5mM UDP-glucose, which was used as the control for non-specific effect of UDP-hexose; and (c) the dish contained no UDP-sugar, which was used as the control for normal binding level. The results are shown in Table 4-3.1.

Table 4-3.1 Effects of UDP-galactose on the binding of mouse, rat and ram sperm to mouse zona.

The concentration of UDP-hexoses in the assays was 2.5mM. Twelve to fifteen eggs were used for each treatment in each assay.

Within each species, between the same letters following the values of mean ± SE, $P > 0.05$; Between a and b, $P < 0.05$; and between a and c, $P < 0.01$. Data were analysed by the method of analysis of variance for random block design with each assay blocked.

Animal	Treatment					Number of assays
	UDP- galactose		UDP- glucose		No UDP-hexose	
	Binding* Mean ± SE	Inhibition %	Binding* Mean ± SE	Inhibition %	Binding* Mean ± SE	
Mouse	29.2 ± 6.0 c	30.1	40.5 ± 7.1 a	0.7	42.8 ± 7.8 a	10
Rat	30.1 ± 3.6 b	24.8	32.3 ± 3.2 b	19.3	40.1 ± 3.4 a	15
Ram	26.8 ± 3.4 a	-6.8	23.3 ± 3.7 a	7.2	25.1 ± 5.2 a	10

* Number of sperm bound/egg.

At the concentration of 2.5mM, UDP-glucose did not have significant inhibitory effect on mouse sperm-zona binding ($P > 0.05$), while UDP-galactose significantly inhibited the binding ($P < 0.01$). Both UDP-galactose and UDP-glucose at 2.5mM significantly inhibited the binding of rat sperm to mouse zona ($P < 0.05$). Neither UDP-galactose nor UDP-glucose at 2.5mM had significant effect on the binding of ram sperm to mouse zona ($P < 0.05$).

4-3.4 DISCUSSION

The experiments of this part (4-3) of the chapter dealt only with the primary sperm-zona binding. As indicated in the "General Introduction" of this chapter, the secondary binding was not considered in the current study.

In the present results, UDP-galactose specifically inhibited mouse sperm-zona binding. This agrees with the result of Lopez et al. (1985).

UDP-galactose also inhibited the binding of rat sperm to mouse zona, but UDP-glucose inhibited the binding as well, and there was no significant difference between the inhibitory effects of these two UDP-hexoses. The results suggested that, at the concentration at which UDP-galactose specifically inhibits mouse sperm-zona binding (2.5mM), both UDP-galactose and UDP-glucose had non-specific inhibitory effects on the binding of rat sperm to mouse zona. This possibility seems more likely, when one considers the fact that rat sperm are very fragile and susceptible to various types of stimulation. However, on the other hand, there still exists the possibility that the specific inhibitory effect of UDP-galactose was overshadowed by the non-specific inhibitory effect of the UDP-hexoses. In further studies, the effects of other substances, which are involved in GalTase reaction, on the binding of rat sperm to mouse zona or on rat sperm-zona binding may need to be investigated to prove the current result from using UDP-hexoses.

Neither UDP-galactose nor UDP-glucose had any effect on the binding of ram sperm to mouse zona. These results suggest the following points. (a) GalTase is not involved in the binding of ram sperm to mouse zona, and therefore, is very unlikely to be involved in the sheep sperm-zona binding. (b) Ram sperm are more resistant to the stimulation by UDP-hexoses than rat sperm.

The incubation time in the current experiments was 10min. This was because that Lopez et al. (1985) reported that the inhibition of UDP-galactose on sperm-zona binding was time-dependent, it was more effective at short times (e.g., 5min) than longer times (e.g., 27min). This was also because that according to the results in Part 2

of this chapter, 10min was within the period assumed to be so called "primary binding" in the assays with the sperm from all the three species used.

In current experiments, $MnCl_2$ was added to the culture medium. This was because that it has been suggested that Mn^{2+} is required for the binding of UDP-galactose to bovine milk GalTase (see Section 1.3.3.5). The concentration of $MnCl_2$ used in the current experiments was 0.1mM. This was because of the observation that $MnCl_2$ at concentrations of 1mM and 0.5mM had inhibitory effects on mouse sperm-zona binding, but not at 0.2mM.

GENERAL DISCUSSION OF CHAPTER 4

In the present study, there was no evidence to support the possibility that in the rat, as in the mouse, GalTase is the receptor on sperm for the zona in sperm-zona binding. GalTase activity was not detected in uncontaminated rat sperm preparations. UDP-galactose did not show specific inhibition on the binding of rat sperm to mouse zona. However, as already discussed in Part 1 and Part 3 of this chapter, further investigations through alternative avenues may be necessary.

Although GalTase activity was detected in ram sperm preparation, UDP-galactose had no effect on the binding of ram sperm to mouse zona. This result suggests that GalTase is not involved in the binding of ram sperm to mouse zona, and it may not be involved in ram sperm-zona binding as well. The localization of GalTase on ram sperm still needs to be established.

Although there has been plenty of evidence as introduced in the "General Introduction" of this chapter suggesting that GalTase is the receptor of sperm for zona in the mouse, a basic question seems to have been ignored. As introduced in Section 1.3.3.5, it has been strongly suggested that Mn^{2+} is required for the binding of bovine milk GalTase and its substrates. However, it is also known that neither mouse *in vitro* fertilization, nor the inhibition of mouse sperm-zona binding in some previous reports (e.g., Lopez et al., 1985; Benau and Storey, 1988) required the presence of Mn^{2+} in the medium. A possibility is that mouse sperm surface GalTase is different from the bovine milk enzyme with respect to the interaction of the enzyme with its substrates. Indeed, Benau et al. (1990) proposed the following model of the reaction of mouse sperm surface GalTase. In the absence of Mn^{2+}, the GalTase was in a binding conformation, since UDP-galactose and glycoproteins with GlcNAc terminal residuals inhibited mouse sperm-zona binding in the absence of Mn^{2+}. However, the presence of Mn^{2+} caused a change of the enzyme from substrate binding conformation to catalytic conformation

which was unfavourable for sperm-zona binding, since Mn^{2+} inhibited the sperm-zona binding in a concentration-dependent manner. However, evidence directly from the examination of the conformation change is necessary to support the above proposal.

CHAPTER 5
GALACTOSYLTRANSFERASE IN EWE UTERINE FLUSHINGS
---DETECTION AND KINETIC COMPARISONS WITH SERUM GALACTOSYLTRANSFERASE

5.1 INTRODUCTION

Galactosyltransferase (GalTase) has been reported to be present in male reproductive tract fluids and on the sperm surface in mammals, and the sperm surface GalTase has been suggested to be involved in sperm capacitation and sperm-zona pellucida binding in the mouse (see Section 3.1 and Introduction of Chapter 4). Therefore, it is interesting to see whether GalTase is also present in female reproductive tract, i.e., uterus and oviduct, where capacitation and fertilization occurs. Uterine flushings from ewes were chosen for the initial study on this topic, since it is relatively easy to collect reasonable amount of proteins from the flushings.

It is known that proteins can be transported from blood to uterine fluid in mammals (see Section 1.1.2.2). Therefore, after detecting soluble GalTase activity in the uterine flushings, its kinetic parameters were compared with those of ewe serum GalTase as an initial investigation about the source of the enzyme in the uterine lumen.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of Uterine Flushings

Each ewe was put into a cradle, anaesthetized by Nembutal (Boehringer Ingelheim, NSW, Australia) via the jugular vein and an endotracheal tube inserted,

followed by clipping and washing of the belly area. Then the sheep was tilted headdown so that the gastrointestinal tract moved away from the pelvic area. A mid-line incision approximately 10cm long through the skin and peritoneum was made between the mammary glands and the umbilicus. The uterus and ovaries were exteriorized through the incision. A hole was made in the base of one uterine horn using the point of artery clamps. A Foley catheter (Sherwood Medical, St Loise, Missouri, USA) was pushed into the uterine lumen just before the bifurcation of the uterus body and its balloon was inflated to hold the catheter in place and to block that uterine horn. Then a Tom Cat catheter (Sherwood Medical) was put into the fimbria and fed along the Fallopian tube for 3-4cm. The uterine horn was flushed with 10ml Dulbecco's Modified Eagle Medium (Gibco Laboratories, Life technology Inc, Grand Island, NY, USA) through the Tom Cat catheter, the uterus was gently massaged, and the fluid was collected though the Foley catheter into a sterile yellow-capped tube. This flushing procedure was repeated for the other uterine horn.

The collected flushings were stored on ice, centrifuged at 140,000g for 1hr at 4°C immediately after the completion of the operation to remove the cell debris etc. Pooled uterine flushings after centrifugation were concentrated with Diaflo YM5 membrane and then with Centricon 10 microconcentrator (Amicon, Danvers, MA, USA, molecular wight cut-off 5,000 and 10,000 respectively) at 4°C to a protein concentration of about 150mg/ml, which was measured by the Coomassie blue method (see Section 2.2.2). The final preparation was stored at -20°C until use.

5.2.2 Preparation of Ewe Serum

Blood was collected in a glass tube from the jugular vein of each ewe. The blood was allowed to stand at room temperature for 3hr to clot and then at 4°C for 1hr, and centrifuged at 1,300g for 20min at 4°C. The supernatant serum was collected and stored at -20°C before use.

5.2.3 Galactosyltransferase Activity Assay

The sources of the chemicals for GalTase activity assay were as described in Section 2.1.

The general assay conditions for GalTase activity in ewe uterine flushings or ewe serum were as follows. The reaction mixture contained 50mM Mes, pH 6.5, 1mM UDP-galactose together with 0.3μCi UDP-[^{3}H]galactose, 20mM *N*-acetylglucosamine (GlcNAc), 4mM $MnCl_2$, and ewe uterine flushings containing 30μg proteins, or 10μl ewe serum, the protein concentration of which was 45mg/ml measured by Coomassie blue method introduced in Section 2.2.2. The mixture was incubated at 37°C, for 20min. AG 2-X8 column separation as described in Section 2.2.1 was used in most of the assays. However, high-voltage paper electrophoresis (see Section 2.2.1) was used to determine the effects of pH on the enzyme activity to avoid interference of high pyrophosphatase and alkaline phosphatase activities at high pH with the assays.

5.3 RESULTS

5.3.1 Reactions of Galactosyltransferase in Ewe Uterine Flushings to α-Lactalbumin

The reactions of the GalTase of ewe uterine flushings to α-lactalbumin (α-lac) were the typical ones of UDP-galactose : GlcNAc GalTase. In the presence of α-lac, the enzyme activity to transfer galactose from UDP-galactose to GlcNAc was inhibited; while that to transfer the galactose to glucose was stimulated (Fig. 5.1).

5.3.2 Effect of UDP-galactose Concentration

The effects of UDP-galactose on the GalTase activity in the uterine flushings and that in the serum are shown in Fig. 5.2 a and b. The K_M values for the uterine and serum enzymes were 0.16mM and 0.11mM respectively.

5.3.3 Effect of *N*-acetylglucosamine Concentration

The effects of GlcNAc concentration on the GalTase activity in the uterine flushings and that in the serum are shown in Fig. 5.3 a and b. The K_M value for the GalTase in the uterine flushings was 5.8mM, while that for the serum enzyme was 4.0mM. The enzyme activities from both sources started to be inhibited at 20mM GlcNAc.

5.3.4 Effect of Mn^{2+} Concentration

Fig 5.4 (1) a and b show the effects of the Mn^{2+} concentration on the GalTases in the uterine flushings and the serum. The K_M values for the enzymes from these two sources were similar, 0.14mM and 0.17mM respectively.

High Mn^{2+} concentrations measured to 10mM and 12mM respectively did not inhibit the enzyme activity from the uterine fluid or that from the serum (Fig. 5.4 (2) a and b).

5.3.5 Effects of Various Divalent Cations

Ba^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+} were examined for their effects in replacement of Mn^{2+} on the GalTase activity in the uterine flushings and that in the serum. The enzyme activities from each source in the presence of these metal ions were compared with, and are shown as percentages of that in the presence of Mn^{2+} (Fig. 5.5). For stimulating the GalTase activities from both sources, the order of activity was $Zn^{2+} > Co^{2+} > Mg^{2+} > Ca^{2+}$. However, the percentages of the activities of these metal ions were higher when used for the GalTase in the uterine flushings than for the serum enzyme. Actually, no activity of Ca^{2+} for the serum GalTase was detectable. Ba^{2+} did not have activity for either the uterine GalTase or the serum enzyme.

5.3.6 Effect of pH

The pH for maximum activity of the GalTase in the uterine flushings was 8-8.5 (Fig. 5.6 a), and that for maximum activity of the serum GalTase was 9 (Fig. 5.6 b).

5.3.7 Effect of Incubation Temperature

When the assay mixture was incubated at 32, 37, 42, 46 and 50°C respectively, the changes of the GalTase activity in the uterine flushings and that in the serum were similar, the activities from both source reached maximum at 42-46°C, though afterwards the activity in the serum seemed to drop more quickly than that in the uterine flushings (Fig 5.7 (1) and (2)).

5.3.8 Comparison of the Galactosyltransferase Activities Normalized to Protein Concentrations in the Uterine Flushings and in the Serum of Ewes

The specific GalTase activity of the uterine flushings was much higher than that of the serum. The values of CPM/μg protein of them were 207.8 ± 18.6 and 16.3 ± 1.4 (mean + SD, n=6 of each) respectively.

5.3.9 Effects of Protein Concentration and Incubation Time

Under the present assay conditions, both GalTase activity of 30μg proteins of the uterine flushings and that of 10μl serum (see Section 5.2.3) were within the linear ranges of the enzyme activity curves against the amount of the sample (Fig. 5.8 a and b). The incubation time chosen (20min) was also within the linear ranges of the activity curves of the enzymes from both sources (Fig. 5.9 a and b).

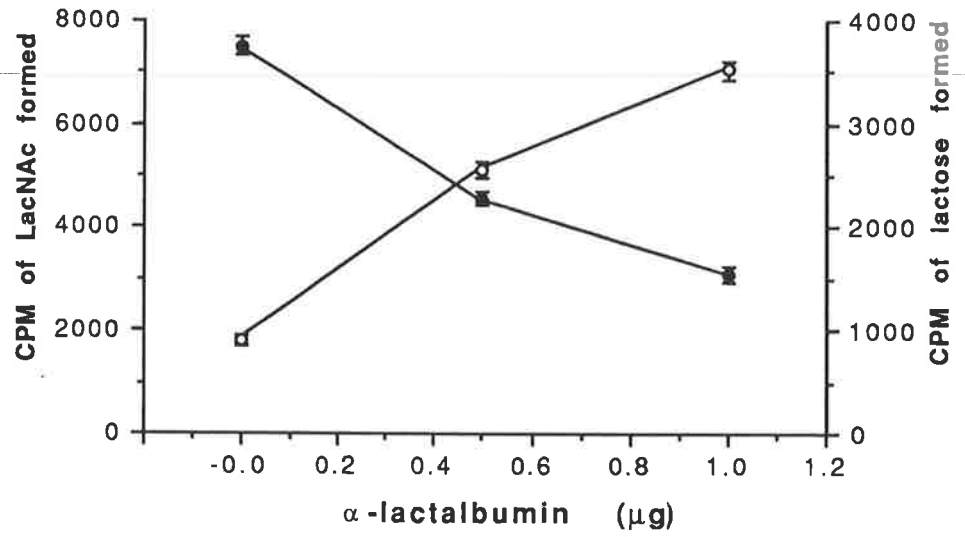


Fig. 5.1 Effects of α-lactalbumin on the galactosyltransferase activities in ewe uterine flushings with *N*-acetylglucosamine (●) and glucose (O) as the acceptors respectively. Triplicate samples were assayed for each point. SE values are shown as the vertical bars.

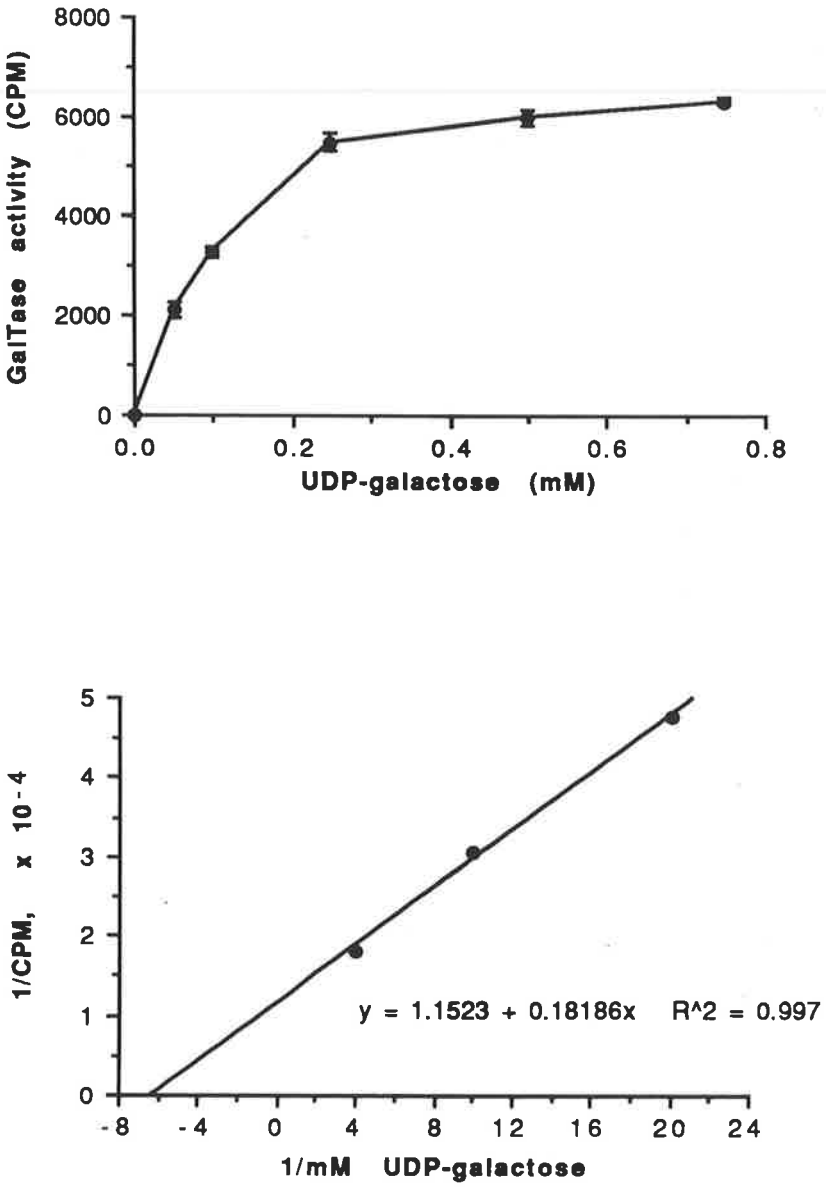


Fig. 5.2 a Effect of UDP-galactose concentration on the galactosyltransferase activity in ewe uterine flushings. Triplicate samples were assayed for each point. SE values are shown as the vertical bars. The bottom figure is the Lineweaver-Burk plot of the data.

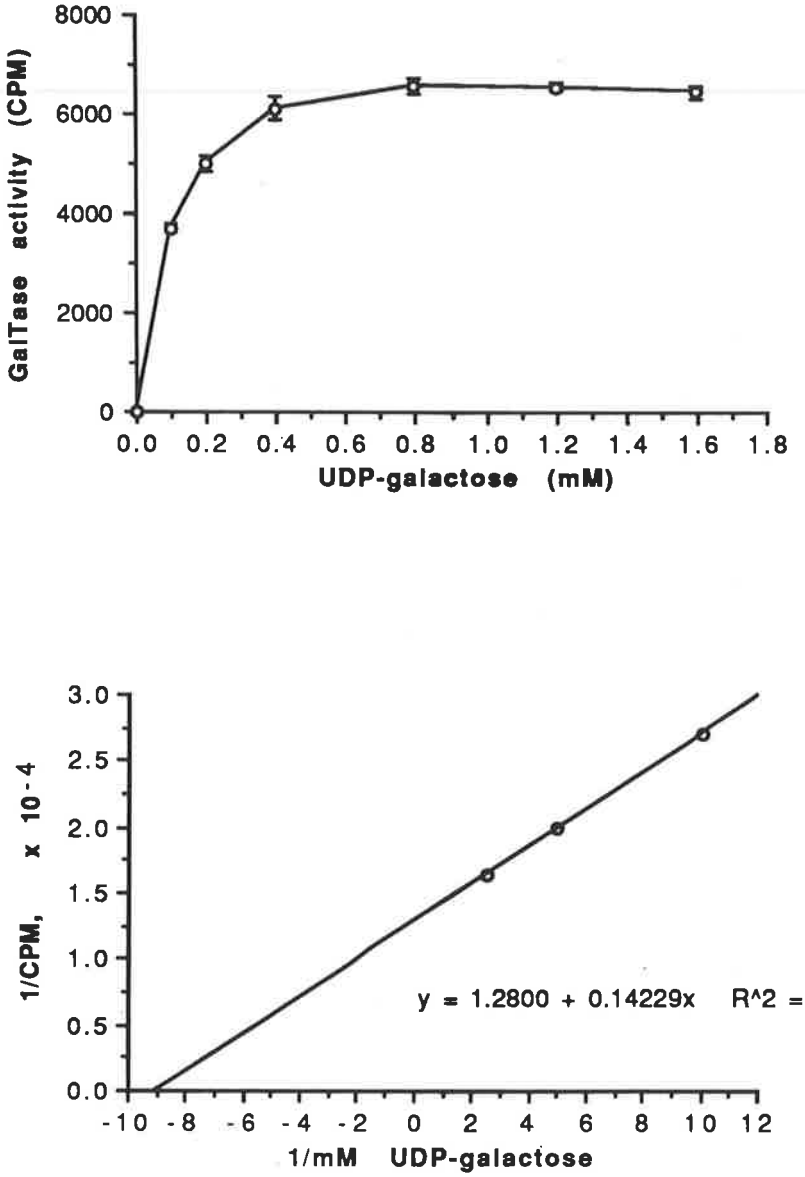


Fig. 5.2 b Effect of UDP-galactose concentration on the galactosyltransferase activity of ewe serum. Triplicate samples were assayed for each point. SE values are shown as the vertical bars. The bottom figure is the Lineweaver-Burk plot of the data.

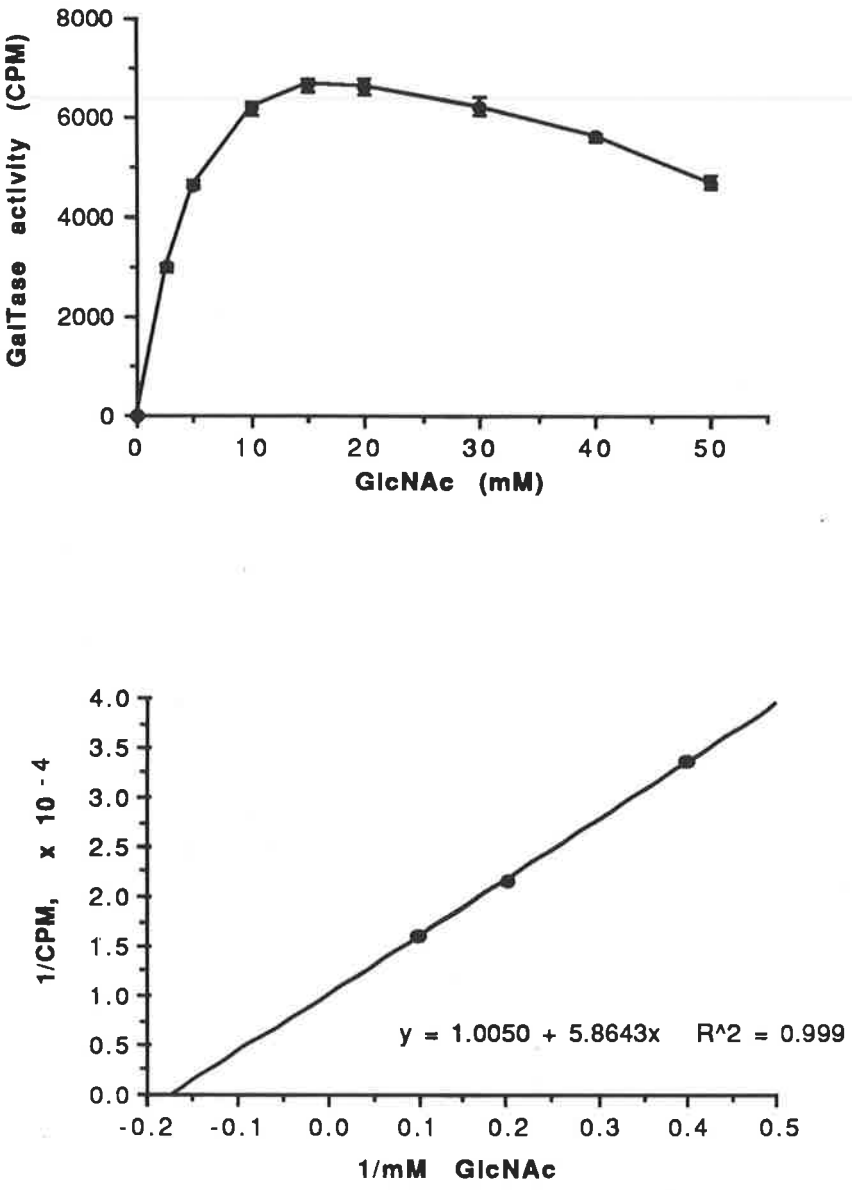


Fig. 5.3 a Effect of *N*-acetylglucosamine concentration on the galactosyltransferase activity in ewe uterine flushings. Triplicate samples were assayed for each point. SE values are shown as the vertical bars. The bottom figure is the Lineweaver-Burk plot of the data.

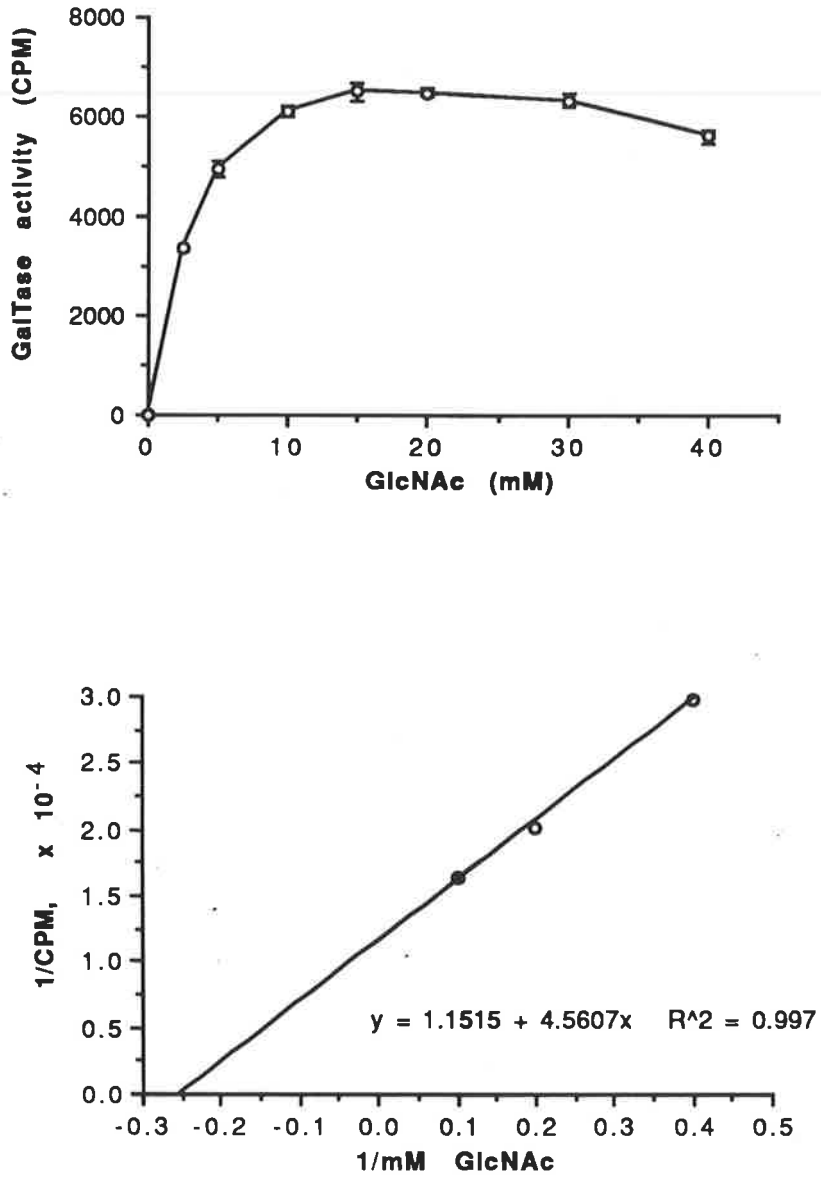


Fig. 5.3 b Effect of *N*-acetylglucosamine concentration on the galactosyltransferase activity of ewe serum. Triplicate samples were assayed for each point. SE values are shown as the vertical bars. The bottom figure is the Lineweaver-Burk plot of the data.

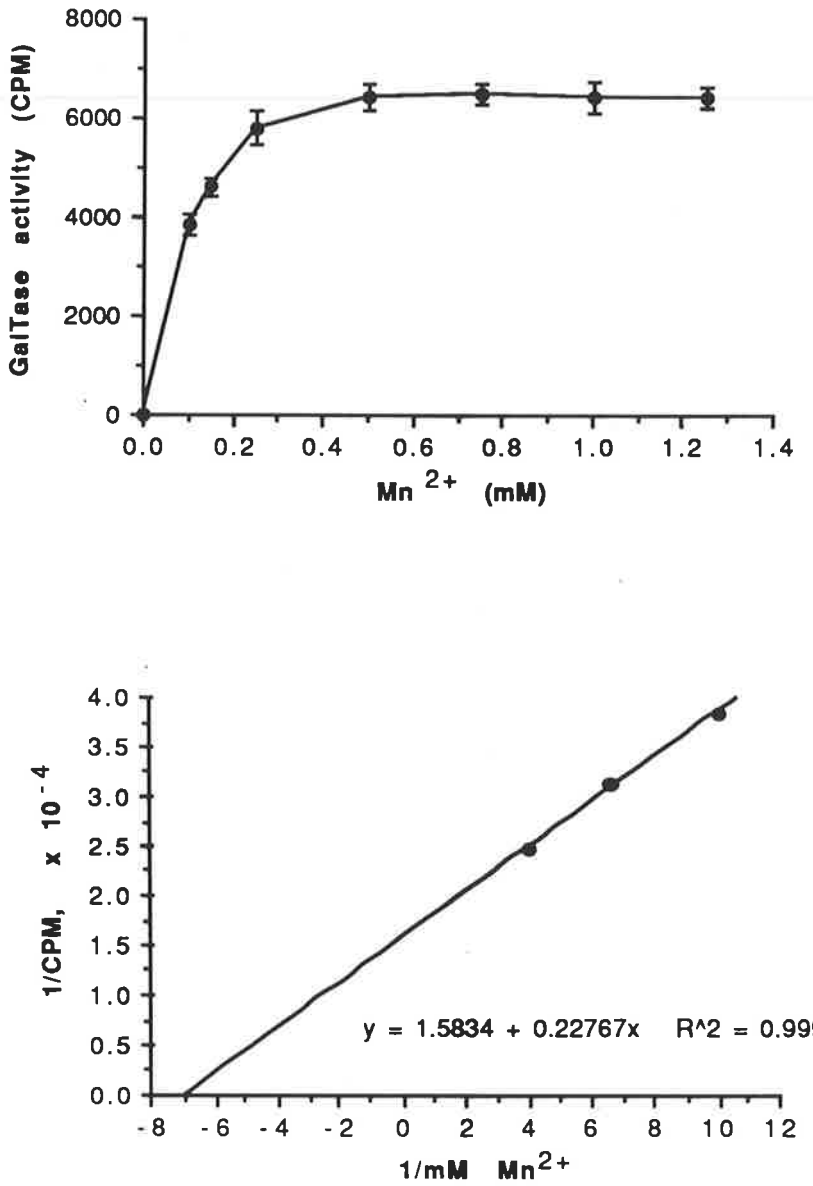
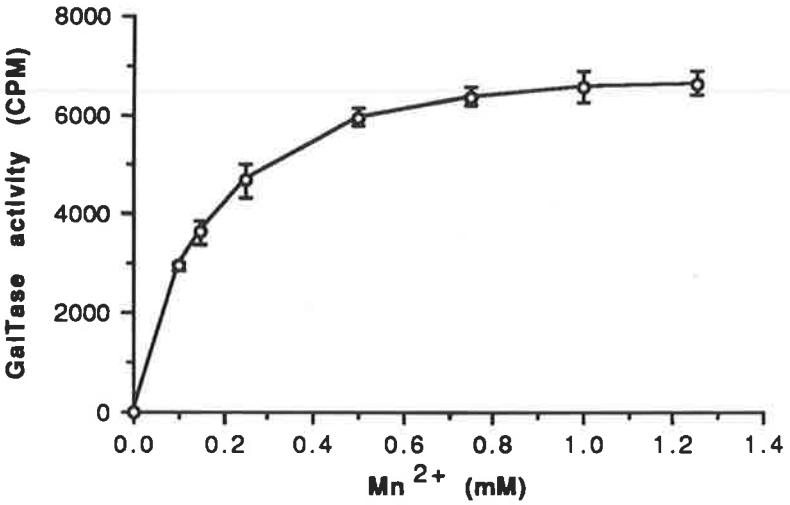


Fig. 5.4 (1) a Effect of Mn^{2+} concentration on the galactosyltransferase activity in ewe uterine flushings. Each point is the average of three assays. SE values are shown as the vertical bars. The bottom figure is the Lineweaver-Burk plot of the data.



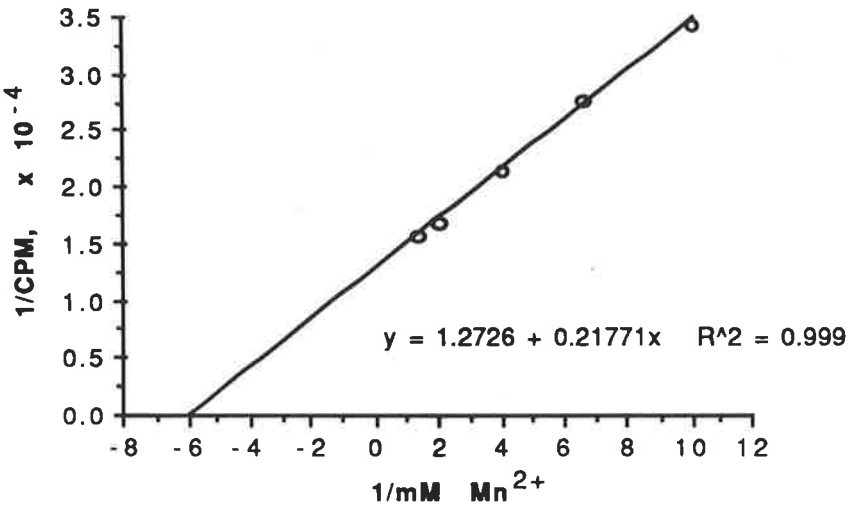
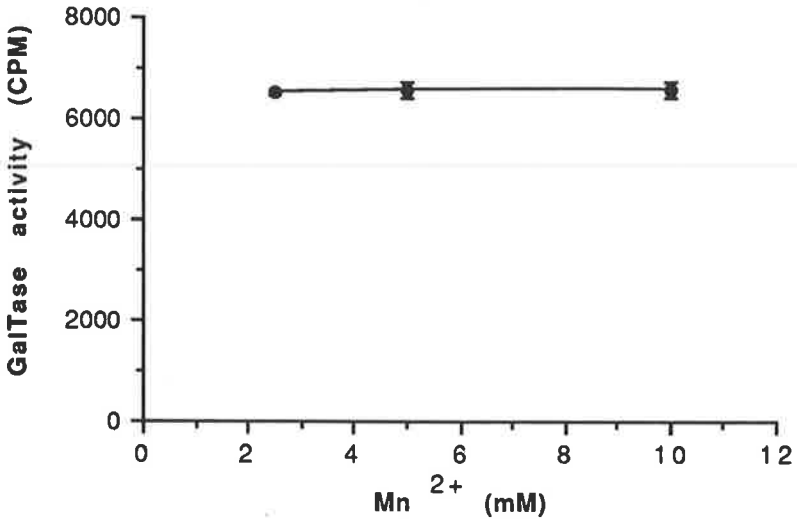
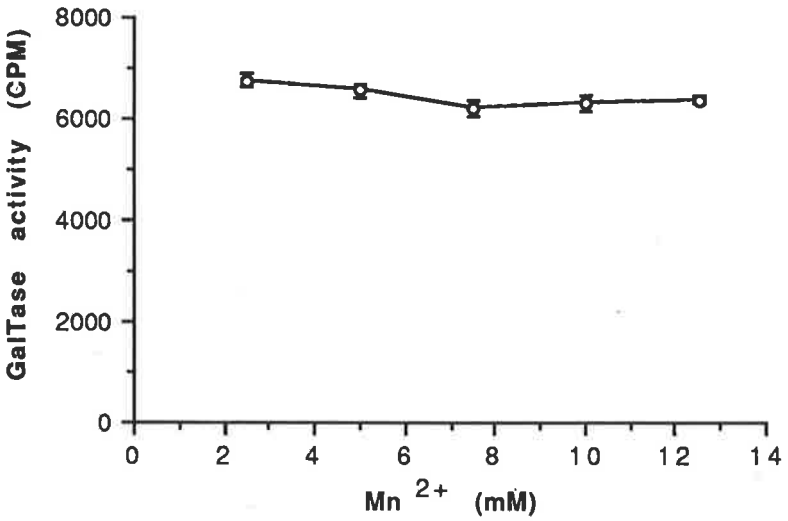


Fig. 5.4 (1) b Effect of Mn^{2+} concentration on the galactosyltransferase activity of ewe serum. Each point is the average of three assays. SE values are shown as the vertical bars. The bottom figure is the Lineweaver-Burk plot of the data.



a



b

Fig. 5.4 (2) Effect of high Mn^{2+} concentration on the galactosyltransferase activity in the uterine flushings (a) and that of the serum (b) of ewes. Triplicate samples were assayed for each point. SE values are shown as the vertical bars.

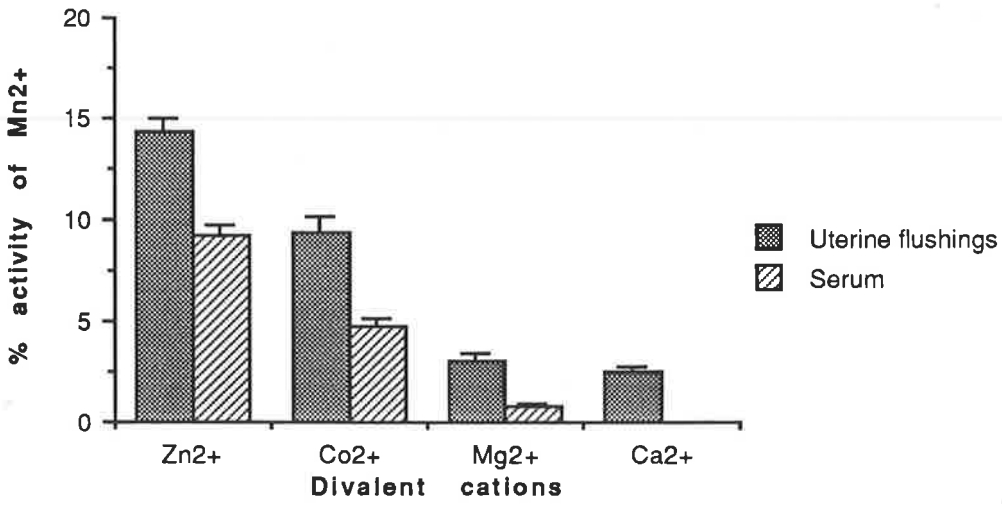


Fig. 5.5 Effect of various divalent cations on the galactosyltransferase activity in the uterine flushings and that of the serum of ewes. Each column represents the average value of triplicate assays, and the vertical bars represent SE values. The effect of Ba^{2+} was also examined, but it did not have activity for either the GalTase in the uterine flushings or the enzyme in the serum of ewes.

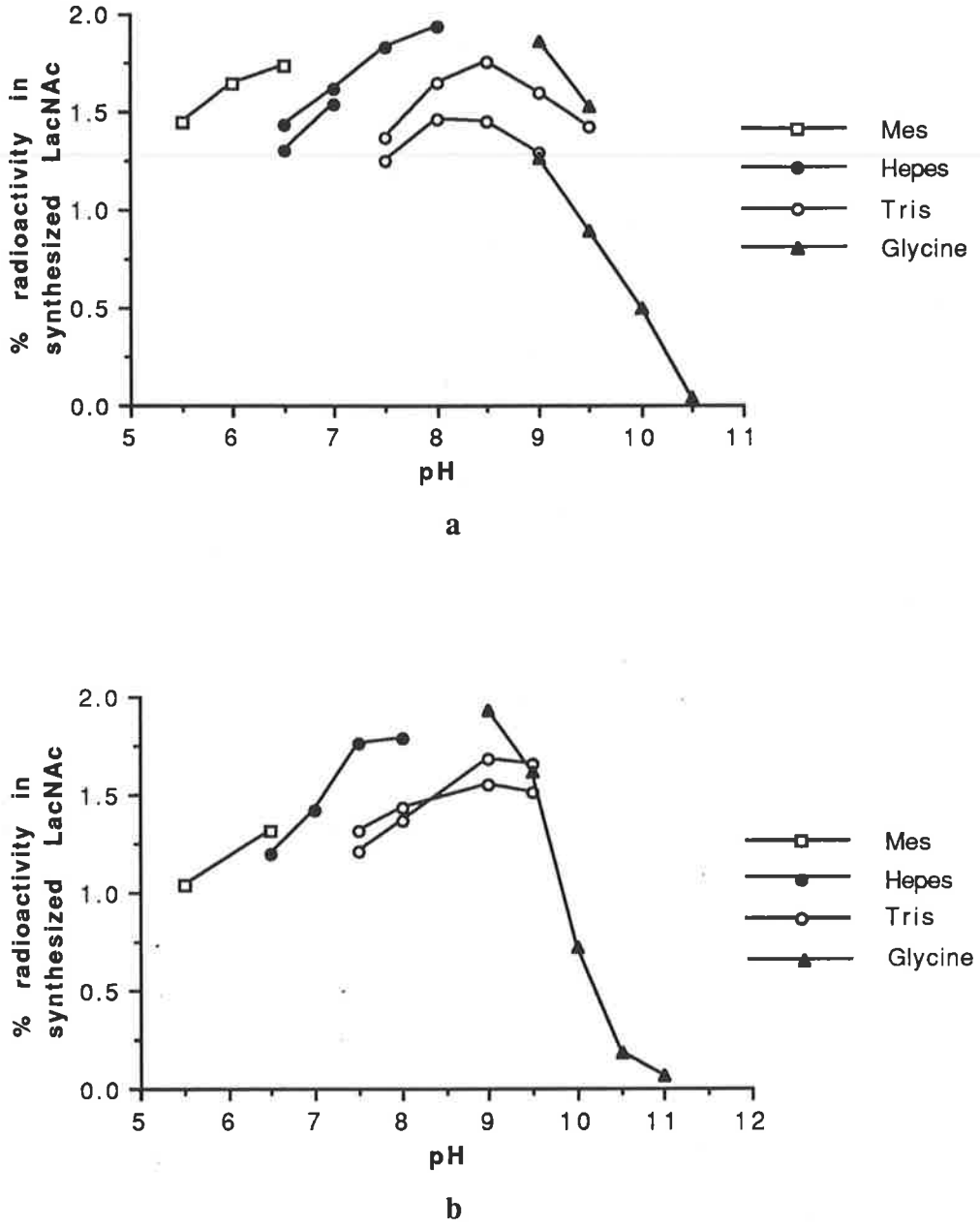
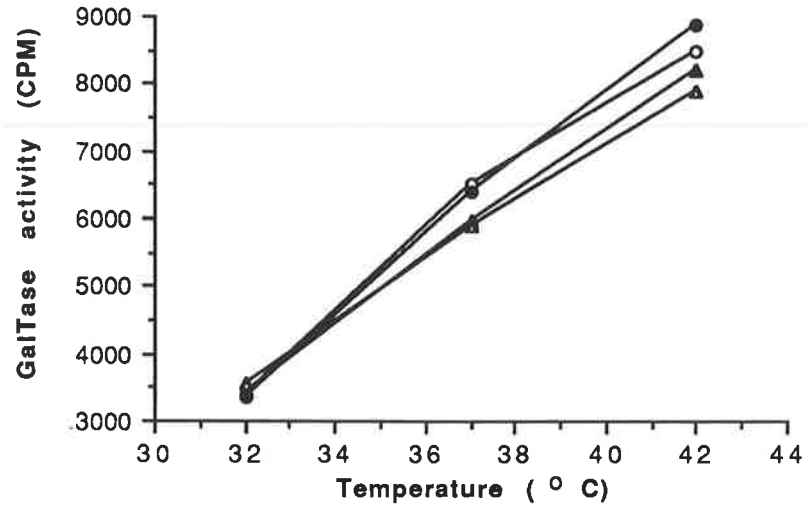
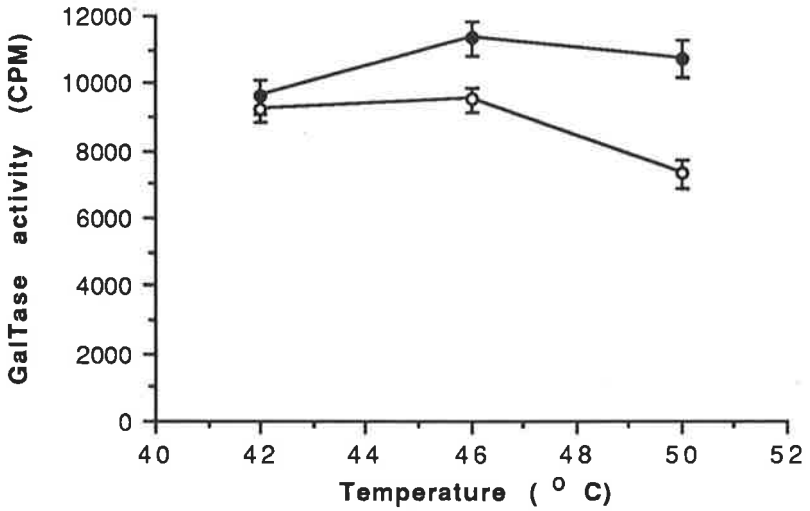


Fig. 5.6 Effect of pH on the galactosyltransferase activity in the uterine flushings (a) and that of the serum (b) of ewes. Buffers of 50mM were used in the assay. The curves were gathered from individual assays.



(1)

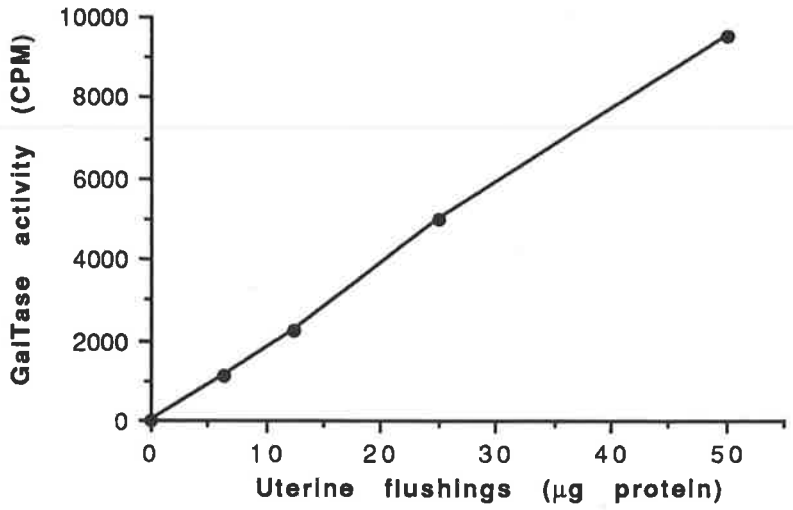


(2)

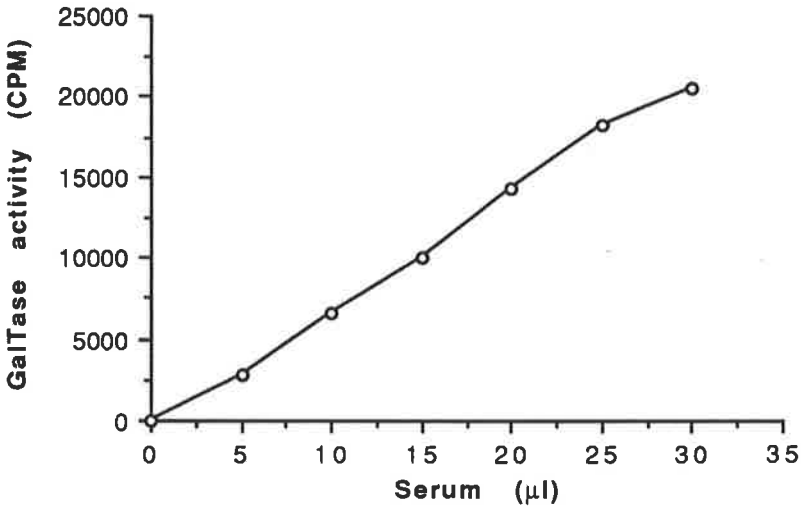
Fig. 5.7 Effect of incubation temperature on the galactosyltransferase activity in the uterine flushings (solid symbols) and that of the serum (open symbols) of ewes.

(1) Temperature range from 32°C to 42°C. Two assays (circle and triangles respectively) with both samples were carried out.

(2) Temperature range from 42°C to 55°C. Each point is the average of three assays, and the vertical bars represent SE values.

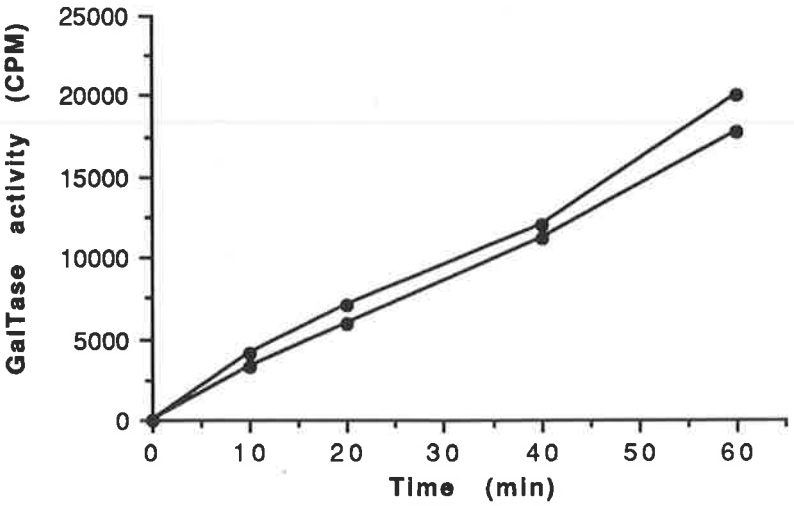


a

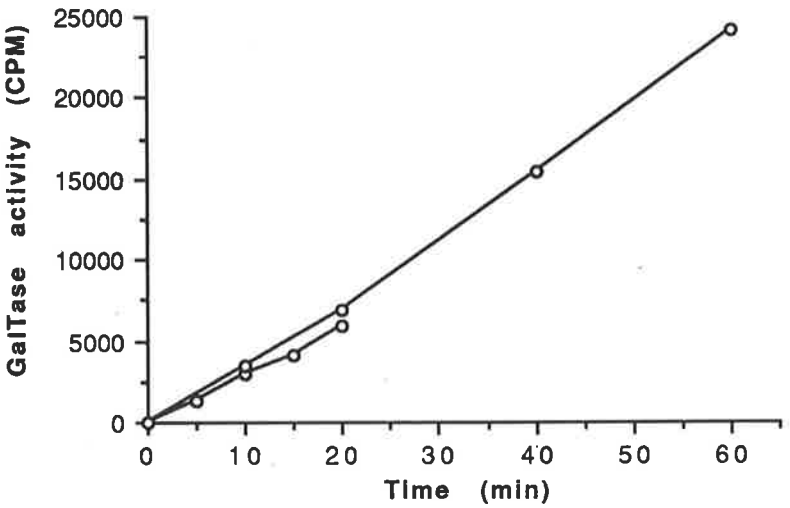


b

Fig. 5.8 Effect of the amount of the sample on the galactosyltransferase activity in the uterine flushings (a) and that of the serum (b) of ewes.



a



b

Fig. 5.9 Effect of incubation time on the galactosyltransferase activity in the uterine flushings (a) and that of the serum (b) of ewes. Two assays of each sample were carried out.

5.4 DISCUSSION

This study has shown that a soluble UDP-galactose : GlcNAc GalTase is present in ewe uterine fluid.

As introduced in Section 5.1, it has been suggested that GalTase is the sperm's receptor for zona in the mouse and probably also in some other mammals. As a study to obtain relevant information about the above possibilities from the investigation of GalTase activity in female reproductive tract, the present study is only an initial exploration. In a further study, it will be interesting to see whether GalTase is present in ewe oviductal fluid, and whether in the oviductal and uterine fluids respectively of other mammals, especially mouse. Such comparisons of GalTase status may give valuable information regarding the role of GalTase in sperm-zona binding. Further discussion regarding this aspect is given in Chapter 7.

Several enzymatic characteristics of the GalTase in ewe uterine flushings were similar to those in ewe serum. These included the K_M value of Mn^{2+}, the order of various divalent cations in replacement of Mn^{2+} for the stimulation of the enzyme activity, and the effect of pH and that of incubation temperature on the enzyme activity. However, the K_M values of UDP-galactose and GlcNAc for the GalTases from the two sources were quite different, though GlcNAc started to inhibit the enzyme activities from the two sources at similar concentrations. These may indicate that the GalTase of the uterine flushings is different from that of the serum. However, it needs to be borne in mind, when considering either the similarities or differences, that the GalTase activities from both samples were measured in crude preparations, and the substances in these two kinds of preparations might have different effects on the assay results. A possible affecting factor was the combination of pyrophosphatase and alkaline phosphatase activities, which were found in the present study to be present in both the uterine flushings and the serum of ewes. These activities were partially inhibited by the pH (6.5) of the assay medium used in the present study, being only about 50% of those at pH 7.5 in both samples. However, under the present assay conditions, these

activities in 30μg proteins of the uterine flushings were about three times as high as those in 10μl of the serum, and the level of these activities was about the same as that of the GalTase activity in the uterine flushings. Therefore, it could be because of the effect of the pyrophosphatase and phosphatase activities that the K_M value of UDP-galactose for the GalTase of the uterine flushings was higher than that for the serum enzyme. This study was only an initial investigation of the source of uterine luminal GalTase. Further studies, such as enzyme purification, M_r determination, immunological location and cDNA cloning may be necessary to identify the source of this enzyme in the uterine flushings.

The K_M values of Mn^{2+}, UDP-galactose and GlcNAc for the serum GalTase of ewe were respectively within the ranges of the reported K_M values of purified GalTases from human plasma and rat serum (see Table 1.1). The present result showed that ovine serum GalTase could have activity, though lower, with several other divalent cations instead of Mn^{2+} when GlcNAc was used as an acceptor. Among the examined ions, Zn^{2+} and Co^{2+} were the most active ones. It is different from human serum GalTase which has an absolute requirement of Mn^{2+} for its activity (see Section 1.3.3.2). This is also different from porcine serum GalTase, where Ba^{2+} is the most active cation after Mn^{2+} among several active divalent cations including Co^{2+}, Mg^{2+} and Ca^{2+} (see Section 1.3.3.2).

The specific activity of GalTase in uterine flushings was much higher than that of serum. This might be related to the abundance of albumin in serum.

Although the present study was carried out with ewes, it is possible that soluble GalTase activity is also present in the uterine lumen of other mammalian species. The function of the GalTase in uterine lumen is unknown, it is possible that it is involved in glycosylation, but no data are available on this yet. A suggestion for the study of the function of the enzyme in future is to compare its total activity in different hormone status.

CHAPTER 6
INVESTIGATIONS OF α-LACTALBUMIN-LIKE ACTIVITY IN MAMMALIAN EPIDIDYMIS

6.1 INTRODUCTION

α-Lactalbumin (α-lac) and its relationship with galactosyltransferase (GalTase) has been introduced in Section 1.3. Since it had been thought that α-lac was present only in milk and mammary tissue, considerable interest was aroused by the report that α-lac-like activity was present in rat epididymal fluid. Hamilton (1981) reported that fraction III, which was a protein fraction of M_r lower than 30,000 obtained from Sephadex G-75 chromatography, had α-lac-like activity: it stimulated the transfer of galactose by GalTase from UDP-galactose to glucose, while inhibiting the transfer to *N*-acetylglucosamine (GlcNAc). When the eluates of unbound substances from the AG 2-X8 columns of the assay with glucose as an acceptor were applied to silica thin layer chromatography (TLC) to identify the product, a distinct lactose peak was obtained from the samples with fraction III, but not the samples without fraction III. Different from the milk or mammary gland α-lac, fraction III appeared to stimulate the transfer of galactose by GalTase from UDP-galactose to myo-inositol. SDS polyacrylamide gel electrophoresis showed that fraction III mainly contained proteins of M_r lower than 35,000, though there were some minor bands with higher M_r up to 67,000. Fraction III contained major bands at M_r about 18,000, 19,000, 24,000 and 32,000 respectively. As introduced in Section 1.1.1.3, the M_r 18,000 (or 18,500), 19,000 and 32,000 proteins are epididymis secretory proteins, and the M_r 24,000 (or 23,000) protein is secreted by both testis and epididymis. Isolated by ion exchange chromatography, there were two protein peaks in fraction III, both containing α-lac-like activity. On electrophoresis of SDS polyacrylamide gel, both peak fractions contained M_r 18,000

and 19,000 bands, and the fraction at the higher ion concentration also contained a band of M_r 24,000. This finding actually followed the initial report of the detection of GalTase activity in mammalian (rat) rete testis fluid and epididymal fluid (Hamilton, 1980). In the discussion (Hamilton, 1981) about the function of α-lac-like proteins in rat epididymal fluid, the ability of these proteins to stimulate the GalTase to transfer galactose to inositol was considered, because of the absence of glucose and the presence of myo-inositol at a high level in the same fluid. The product of this reaction would be galactinol (a disaccharide of galactose and inositol). However, it was also indicated that no disaccharide containing galactose and inositol had been identified. Inositol in sperm membrane phosphatidylinositol was also considered to be possible a galactose acceptor. It was also speculated that a lactose synthetase like complex could regulate glycosylation of epididymal fluid proteins or of proteins on the sperm cell surface.

The above finding was apparently confirmed in a number of other laboratories. Jones and Brown (1982) reported that the purified M_r 18,500, 19,000 and 23,000 proteins, but not the purified M_r 32,000 protein from rat epididymal fluid, stimulated GalTase activity to transfer galactose to glucose, the M_r 23,000 protein had highest activity, though still eight times less than bovine mammary gland α-lac. The M_r 23,000 protein was also reported to stimulate the transfer of galactose to inositol. Examined by the antisera against the proteins, it was found that the M_r 18,500 and 19,000 proteins were associated with the plasma membrane of epididymal sperm, and the M_r 23,000 protein was associated with the plasma membrane of both testicular and epididymal sperm. It was suggested that proteins showing α-lac-like activity associated with the plasma membrane of sperm might function to regulate the sequential removal and addition of sugar residues on membrane-bound glycoprotein. The possibility that inositol functioned as a galactose receptor *in vivo* was also suggested.

Qasba et al. (1983) reported α-lac-like activity in rat epididymis homogenate, from which GalTase activity was eliminated by passing the homogenate though a GlcNAc-Sepharose column. This preparation was reported to stimulate the transfer of galactose by GalTase to glucose, and equally well to myo-inositol. The

products of both reactions, lactose and galactinol were further characterised by paper chromatography, though results were not shown. It was also reported that using a rat mammary gland α-lac cDNA clone as a hybridization probe, RNA sequences homologous to α-lac mRNA from rat epididymis were detected. Agreeing with the previous reports (Hamilton, 1981; Jones and Brown, 1982), it was suggested in this report that α-lac-like proteins may interact with GalTase in transferring galactose to myo-inositol or some other sugars linked to membrane-bound glycoproteins on sperm surface. Byers, Qasba, Paulson and Dym (1984) reported the immunocytochemical localization of α-lac-like proteins in male reproductive tract with a primary antiserum raised against highly purified rat milk α-lac. It was reported that α-lac-like molecule(s) was localized in principal cells from the proximal caput epididymis, germ cells from the seminiferous epithelium and epididymal sperm. In the following review from the same laboratory (Byers, Dym, Hewlett and Qasba, 1984) about α-lac-like proteins in the male reproductive tract, more information about α-lac-like proteins in addition to the above were given. It was claimed that α-lac-like activity was also detected in extracts from mouse and rabbit epididymis and human serum, in terms of the stimulation of transfer of galactose to glucose. However, these preparations failed to stimulate the transfer of galactose to myo-inositol. Amino acid compositions of the two forms of rat mammary α-lac analysed by Qasba and Chakrabartty (1978) and those of the M_r 18,500, 19,000 and 23,000 proteins analysed by Jones et al. (1980) were compared, and high similarity was shown. It was also indicated that, similar to authentic α-lac, the α-lac-like activity in the epididymis was also stable at high temperature (about 80^0C for 10min).

Limpaseni and Chulavatnatol (1986) reported that a major sialoglycoprotein of M_r 21,000 from rat epididymal fluid possessed α-lac-like activity. It stimulated the transfer of galactose to glucose and inhibited the transfer of galactose to GlcNAc. The amino acid composition of this protein was compared with that of the M_r 19,000 protein reported by Jones et al. (1980), and they were similar. It was proposed that this sialoglycoprotein is a sialylated form of an α-lac-like protein.

In this introduction, the completely independent reports of Shur and his colleagues during the similar period (Shur and Hall, 1982a; b; Lopez et al., 1985; also see Section 1.3) about the relationship of GalTase and fertilization need to be mentioned as part of the background of the studies about α-lac-like proteins. This group reported that GalTase is the sperm surface receptor for the receptor molecule on zona pellucida during sperm-zona binding in mouse, and one of the important pieces of evidence was that α-lac inhibited sperm-zona binding. These reports were obviously relevant to the reports about the presence of α-lac-like proteins in the epididymis, and raised a new possibility of the function of these proteins, i.e., decapacitating the sperm. Therefore, the possibility of the involvement of α-lac-like proteins in fertilization was discussed in the latter reports about α-lac-like proteins (Qasba et al., 1983; Limpaseni and Chulavatnatol, 1986). In the discussion by Qasba et al. (1983), it was further indicated that both sperm capacitation and acrosome reaction are Ca^{2+}-dependent phenomena in many animals, and it was known that α-lac has two binding sites for Ca^{2+}. Also, the possibility of α-lac as a decapacitation factor was introduced in some reviews about fertilization (Yanagimachi, 1988, Jones, 1989).

Based on the above knowledge, McLaughlin and Shur (1987) studied the effect of epididymal plasma on sperm capacitation and sperm-zona binding in mouse, and examined the presence of α-lac-like protein(s) in mouse epididymal plasma. They reported that immotile caput epididymal sperm were able to bind to the zona if they are first washed free of caput epididymal plasma, while the binding of mature cauda epididymal sperm to the zona were inhibited by the caput plasma. The caput epididymal plasma was shown to contain α-lac-like activity as well as GalTase activity. Therefore, this report suggested a possible relationship between α-lac-like proteins and sperm-zona binding, though the authors indicated that whether GalTase and / or α-lac-like proteins were the only putative inhibitory components in caput epididymal plasma was not clear.

However, in a report about a M_r 22,000 protein in male reproductive tract, Brooks (1985) indicated that no α-lac-like activity could be detected in this protein by an spectrophotometric method, though the protein was "similar in several aspects to

the α-lac-like proteins of epididymal fluid". This protein actually should be the same protein as the M_r 24,000 protein in Hamilton's report (1981) and the M_r 23,000 protein in the report of Jones and Brown (1982). However, because this was only briefly mentioned in the introduction of the report of Brooks (1985), and not as a major result, not much attention was paid to this finding.

The original plan for this part of work was to examine firstly if α-lac-like proteins were widely present in the epididymal fluid of different mammals, especially farm animals, and if it was also present in other body fluids of mammals; and secondly, what was the function of α-lac-like proteins in male reproduction system. However, α-lac-like activity could not be detected in epididymal plasma of any of the species used, including the rat. Consequently, the apparent α-lac-like activity in rat epididymal plasma was re-examined. My results showed that no α-lac-like activity was detectable in rat in either crude epididymal plasma, or a low M_r protein fraction of epididymal extract as in the original report of Hamilton (1981), or a fraction of 40-60% ammonium sulphate precipitated rat epididymal proteins as in the report of Qasba et al. (1983) and Byers, Dym, Hewlett and Qasba (1984). It was concluded that there could be some experimental artefacts in the previous reports about α-lac-like proteins. Preliminary reports of these studies were presented to the Australian Society for Reproductive Biology (Tang et al., 1990).

While part of this work was being written for publication, Moore et al. (1990) reported that the M_r 18,000 protein from the rat epididymis that apparently modified GalTase activity had no structural similarity to rat milk α-lac. Also, reports from another laboratory (Hölpert and Cooper, 1990a; b) indicated that when the assay conditions were optimised for GalTase activity, no α-lac activity could be demonstrated in epididymal homogenates or in unfractioned epididymal fluid. The present results confirm these latter findings with respect to the fractions of epididymal luminal fluid as originally studied by Hamilton (1981). Part of the results of this chapter have been published (Tang, 1992)

6.2 MATERIALS AND METHODS

6.2.1 Animals

Adult male Albino Wistar rats were obtained from the Central Animal House of the University of Adelaide.

6.2.2 Chemicals

Sephadex G-75 was purchased from Pharmacia (Uppsala, Sweden). Chemicals for SDS-polyacrylamide gel electrophoresis were purchased from Sigma (St. Louis, MO, USA). Protein standards for Sephadex G-75 chromatography and for SDS-polyacrylamide gel electrophoresis were obtained either from Sigma or from Pharmacia. For gas chromatography, trimethylsily imidazole and pyridine were obtained from Sigma, and SE-30 from Alltech (Sydney, Australia). Ammonium sulphate and Triton X-100 were from BDH chemicals (Melbourne, Australia). The sources of the chemicals used for GalTase activity assay were as introduced in Section 2.1. Other chemicals were either from Sigma or were analytic reagent grade from Ajax Chemicals (Sydney, Australia).

6.2.3 Methods

6.2.3.1 Preparation of a Low M_r Protein Fraction of Rat Epididymal Extract

6.2.3.1.1 Collection of Rat Epididymides

Rats were anaesthetized with ether and then decapitated so that as much blood as possible was removed from the tissues. Epididymides were removed and fat was carefully cleaned off.

6.2.3.1.2 Preparation of a Low M_r Protein Fraction by Sephadex G-75 Chromatography

Hamilton's procedure (1981) was followed to prepare this protein fraction. Rat epididymides were minced with scissors in Sephadex G-75 chromatography buffer on ice and then centrifuged twice at 46,000g, 4°C, for 45min to remove tissue and cell debris. The supernatant containing epididymal luminal plasma was passed through glasswool to remove remaining fat.

All the following procedures were carried out at 4°C. The epididymal extract was chromatographed on Sephadex G-75 (1.5 x 73 cm) in 80mM Tris-HCl and 150mM NaCl, pH 7.2 at a rate of 30 ml/hr. About 5 ml extract was applied and 2ml fractions were collected. Fractions containing the proteins less than M_r 30,000 were collected and concentrated with Diaflo YM5 membrane (Amicon, Danvers, MA, USA, molecular weight cut-off 5,000). The column was calibrated before use with molecular weight standards of proteins including BSA (67,000), ovalbumin (43,000), carbonic anhydrate.(29,000), chymotrypsinogen A (25,000), and α-lac (14,400).

6.2.3.1.3 Elimination of the Remaining GalTase Activity in the Low M_r Protein Fraction

Two methods were used to eliminate the remaining GalTase activity in the low M_r protein fraction from Sephadex G-75 chromatography.

(a) Heating. The fraction was dialysed in cellulose tubing (Selbys, Melbourne, Australia, molecular weight cut-off 6,000 - 8,000) against 50mM Hepes and further concentrated by Centricon 10 microconcentrator (Amicon, molecular weight cut off 10,000), then heated in a water bath at 60°C for 10min before assay. This procedure was used because GalTase is heat-labile, while α-lac is extremely heat stable (see Results section, also Section 1.3.3.1). Furthermore α-lac-like proteins from the epididymis have also been reported to be able to resist heat treatment at least of 80°C for 10min (Byers, Dym, Hewlett and Qasba, 1984), or 60°C for 30min (McLaughlin and Shur, 1987).

(b) UDP-agarose affinity chromatography. The whole procedure was carried out at 4°C. The fraction was dialysed in cellulose tubing against 50mM Hepes, 25mM $MnCl_2$, pH7.2, and then passed through a 3ml UDP-agarose column in the same buffer at 4°C to retain the GalTase. The eluate of the unbound substances was concentrated by a Centricon 10 microconcentrator for assays of α-lac-like activity. The affinity of the column was checked with bovine milk whey, the column was washed with the same buffer as the above and the GalTase was eluted with 25mM Hepes, 25mM EDTA, and 5mM GlcNAc, pH 7.2 (Appendix Fig. 6.1).

6.2.3.2 Preparation of Epididymal Plasma from Various Mammalian Species

The methods for preparation of epididymal plasma of the rat, rabbit, ram and boar were as described in Section 3.2.4.1. The plasma was heated at 60 °C for 10min before it was assayed for α-lac-like activity assay

6.2.3.3 Preparation of a Protein Fraction of Rat Epididymal Extract by Ammonium Sulphate Precipitation

The protein fraction was prepared following the method of Qasba et al. (1983). A sample of about 5g rat epididymides was homogenized in 15ml Tris-HCl buffer, pH 7.4 containing 10mM $MgCl_2$ and 1% Triton X-100. Proteins of the homogenate precipitated by 40-60% saturated ammonium sulphate were dialysed against 20mM Tris-HCl, pH 7.4 containing 10mM $MgCl_2$, and then passed through a GlcNAc -Sepharose column (1 x 3 cm) equilibrated with the same buffer to remove GalTase activity. The affinity of the column was checked with bovine milk whey following the method of Barker et al. (1972), and the profile is shown in Appendix Fig. 6.2. The proteins washed from the column with the same buffer for equilibration were collected and concentrated by Centricon 10 microconcentrator for SDS-polyacrylamide gel electrophoresis and α-lac-like activity assay.

6.2.3.4 α-Lactalbumin-Like Activity Assay

Commercial bovine milk GalTase was used as the enzyme source and it was further purified by UDP-agarose affinity chromatography to remove the contaminating α-lac (1-2% of the total protein content). The column was washed and the enzyme was eluted as above (6.2.3.1.3 (b)). The purified enzyme was dialysed and stored in 50mM Hepes and 2mg/ml BSA.

(1) Assay of α-Lactalbumin-Like Activity in the Low M_r Protein Fraction

In 50μl medium, 6munits of the further purified GalTase were used for lactose synthesis and 0.16munits for *N*-acetyllactosamine (LacNAc) synthesis except where otherwise indicated. The other components in the incubation medium were 50mM Hepes, pH 7.2, 4mM $MnCl_2$, 0.4mM UDP-galactose, 0.2μCi UDP-[^{3}H]galactose, 25mM glucose or GlcNAc, 1mg/ml BSA and different amounts of proteins of the sample as given in the figures and table. BSA was also used to bring the protein concentrations of all the samples in each α-lac-like activity assay to the same level. The reaction medium was incubated at 36°C for 1.5 hr, and the reaction was stopped by putting the samples on ice.

The samples were then applied to AG 1-X8 columns for product isolation (see Section 2.2.1). The eluates of unbound substances were mixed with scintillation fluid and counted for radioactivity. The difference between the amounts of radioactivity in the presence and absence of an acceptor at the same protein level of the low M_r fraction was taken to be the GalTase activity. When the products of the assay were analysed by high-voltage paper electrophoresis (HVPE), the eluates from the AG 1-X8 columns were further processed as described in Section 6.2.3.5.

(2) Assay of α-Lactalbumin-Like Activity in the Epididymal Plasma of Various Mammalian Species

The basic assay conditions for GalTase activity in the epididymal plasma of various mammalian species described in Section 3.2.4.2 (method (b)) were adopted.

However, 6munits of the further purified GalTase was added to the assay medium, and glucose or inositol was used as the acceptor. The incubation was carried out at 36°C for 1.5 hr.

The samples were then applied to HVPE for separation of the sugars in the medium (see Section 2.2.1). When glucose was used as the acceptor, lactose was used as a standard for GalTase product; while when inositol was an acceptor, galactinol was used. Galactinol was obtained as described in Section 6.2.3.6.

(3) Assay of α-Lactalbumin-Like Activity in the Protein Fraction of Rat Epididymal Extract Precipitated by 40-60% Saturated Ammonium Sulphate

The assay was carried out under the same conditions as those for the low M_r protein fraction of rat epididymis described above, except that Tris, instead of Hepes was used as a buffer.

6.2.3.5 High-Voltage Paper Electrophoresis Analysis of the Product(s) in the α-Lactalbumin-Like Activity Assays of the low M_r Protein Fraction

Incubated samples of the low M_r protein fraction for α-lac-like activity assay were chromatographed on AG 1-X8 columns (see Section 6.2.3.4 (1)). The eluates of unbound substances were dried by a vacuum centrifuge and then applied to HVPE to identify the radioactive material(s). The HVPE was carried as described in Section 2.2.1, but 0.03μCi L-[^{14}C]-lysine together with 20μg non-radioactive L-lysine was added to each eluate obtained from the column. The reason for adding L-[^{14}C]-lysine in the assay medium was as follows. When assay samples are directly applied to HVPE without previous AG 1-X8 chromatography, the procedural losses can be monitored by the total radioactivity in the electrophoresis lane for each sample. However, the samples used for analysis of the product(s) in the present experiments were eluates of unbound substances from AG 1-X8 columns containing different amounts of radioactivity. Therefore, the same amount of radioactive lysine was added

to each sample to monitor the procedural losses during sample application etc. The non-radioactive lysine, which migrates towards the anion electrode, was added to stabilize the migration of the trace amount of radioactive lysine. The position of lysine was examined both by radioactive counting of the [^{14}C]-lysine and by ninhydrin stain (Ersser and Smith, 1976) of the non-radioactive lysine. The radioactivity of each assay product was calculated as percentage of the radioactivity of lysine in the same sample. The difference of the percentages at the position of lactose or LacNAc between the corresponding samples with and without an acceptor was taken as a measure of GalTase activity. The difference of the percentages at the position of galactose between samples with and without the protein fraction was taken as a measure of pyrophosphatase and phosphatase activity.

6.2.3.6 Galactinol Standard

The galactinol standard used in α-lac-Like activity assays was [^{3}H] galactinol produced by pea UDP-galactose : myo-inositol GalTase. The enzyme was prepared following the method of Frydman and Neufeld (1963). Briefly, fresh pea seeds were ground in cold medium of 50mM phosphate, pH 7.0, 0.5M sucrose and 50mM ß-mercaptoethanol. Supernatant solution was collected after centrifugation. 30-50% saturated ammonium sulphate precipitation of the supernatant was dissolved in 10mM Tris-HCl, pH 7.5 dialysed against the same buffer and clarified by centrifugation. A 5ml portion of this fraction was fractionated further by the sequential addition of 2.5, 1, 1.5, and 5ml saturated and neutralized solution of ammonium sulphate. The precipitates were dissolved in 10mM Tris-HCl, pH 7.5, and dialysed against the same buffer. The third fraction, which showed highest activity of the UDP-galactose : myo-inositol GalTase, was used as the enzyme source for galactinol synthesis. When galactinol was needed as a standard, the third fraction was incubated in the same assay medium for the assay samples but without bovine milk GalTase in the

medium. The assay mixture was then applied to the HVPE in the same way as the assay samples. The position of the radioactive product was considered to be that of galactinol.

In later stage of the experiments, purified galactinol was kindly offered by Dr C. E. Ballou, University of California, Berkely, USA. The migrated position of the purified galactinol in the HVPE was exactly the same as the position of the radioactive product in the reaction of pea GalTase. The R_f value of galactinol was 0.17, between those of GlcNAc and lactose (see Section 2.2.1).

6.2.3.7 Gas Chromatography Examination of Degradation Product of UDP-galactose by Rat Epididymal Preparation

Supernatant of rat epididymal plasma was prepared as for Sephadex G-75 chromatography (see Section 6.2.3.1.2), and used for assay of degradation of UDP-galactose. The supernatant containing 12mg proteins was incubated in medium of 50mM Hepes, 4mM $MnCl_2$ and 0.4mM UDP-galactose in a total volume of 1.5ml. After incubation at 36°C for 1.5 hr, the sample was applied to an AG 1-X8 column, and the column was eluted with 2 x 0.5ml H_2O. The eluate was dried by a vacuum centrifuge and the sugars were extracted by the same method used for preparing the sample for HVPE (see Section 2.2.1). 0.2mg each of galactose and galactitol (dulcitol), the cyclic derivative of galactose as inositol is of glucose, were used as the standards.

Sugars were analysed as their trimethylsilyl derivatives using gas chromatography by the method of Holligan and Drew (1971) and Ford (1979) with xylitol as an internal reference. 0.2ml xylitol solution of 1mg/ml in 20% methanol was added to each sample of epididymal extract or sugar standard. The samples were then completely dried in a 1ml glass vial in a vacuum centrifuge. 0.1ml of the derivatizing reagent, a mixture of trimethylsilyl imidazole and pyridine (1:2, V/V) was added to each sample. The vial was capped with a plastic cap and vortexed rapidly for a few seconds to dissolve the sugars, and left overnight to allow the reaction to complete. 2µl of the sugar standard or 10µl of the epididymal sample was used for the assay. The sample

was injected onto a 6 foot x 2mm glass column packed with 3% SE-30 on 100/120 mesh Gas Chrom Q. The temperature of the gas chromatography started at 140°C, using a temperature gradient of 4°C/min up to 220°C, then remained for 20min at this temperature. The injection temperature was 225°C and the flame ionization detector temperature was 235°C. The carrier gas flow rate was 20cm^3/min.

6.2.3.8 SDS-Polyacrylamide Gel Electrophoresis and Gel Staining

The electrophoresis was carried out on 8-15% gradient slab gel prepared as described by Van Blerkom (1978). The gels were applied to 200V until the blue marker reaches the bottom of the gel.

After electrophoresis, the gels were stained by Coomassie blue, the diffusion staining method in the Pharmacia book "Polyacrylamide gel electrophoresis" (Uppsala, Sweden) was followed.

6.3.2.9 Assay of Protein Concentration

Coomassie blue method was used (see Section 2.2.2)

6.3 RESULTS

6.3.1 Investigation of α-Lactalbumin-Like Activity in Epididymal Plasma of Different Mammals

When epididymal plasma of rat, rabbit, ram or boar was assayed for α-lac-like activity, neither lactose nor galactinol was detected by HVPE in any of the species when glucose and inositol were used as the acceptor respectively.

6.3.2 Investigation of α-Lactalbumin-Like Activity in the Low M_r Protein Fraction of Sephadex G-75 Chromatography

6.3.2.1 The Low M_r Protein Fraction from Sephadex G-75 Chromatography

The Sephadex G-75 chromatography profile of rat epididymal extract and the fraction of the low M_r proteins are shown in Fig. 6 1. SDS-polyacrylamide electrophoresis (Fig. 6.2) shows that though there was still some small amount of high M_r proteins, the low M_r fraction contains mainly proteins of M_r less than 30,000. It had three major groups of bands. I believe the middle band in the first group which had a M_r 24,000 corresponded to the 24,000 (Hamilton, 1981) or 23,000 (Jones and Brown, 1982) or 22,000 (Brooks 1985) band in previous reports; the double bands of the middle group, M_r 18,500 and 17,500 respectively corresponded to the 19,000 and 18,000 (Hamilton, 1981) or 19,000 and 18,500 (Jones and Brown, 1982) bands in previous reports (Hamilton, 1981; Jones and Brown, 1982; White et al., 1987, Moore et al., 1990). However, the two bands in the last group, with M_r 14,500 and 13,500 respectively were more abundant than bands of about these M_r in previous reports (Hamilton, 1981; Jones and Brown, 1982). This might be because of the cellulose tubing for dialysis and Amicon membranes for concentration used in the current experiments were with very low molecular weight cut-off and tended to retain more of the low M_r proteins, which might contain some molecules produced by proteolytic enzymes as well as natural components of the epididymal extract. Nevertheless, the bands with M_r lower than 17,500 are not important to this study, their M_r being lower than those of the α-lac-like proteins reported.

At least 75% of the GalTase activity in the original epididymal extract was eliminated by the Sephadex G-75 chromatography. The low M_r protein fraction from the column still contained GalTase activity about 1,500 CPM / 100μg fraction protein when GlcNAc was an acceptor; but no detectable GalTase activity when glucose was an acceptor. The fraction also contained about 15% of the pyrophosphatase and phosphatase activities of that in rat epididymal extract before Sephadex G-75

chromatography, determined by the difference between the acceptor-free samples with and without the low M_r fraction proteins.

Heating in a water bath at 60°C for 10 min completely inactivated both bovine milk GalTase and GalTase in the fraction. However, this temperature or even higher temperatures (80°C, 10min) had no effect on bovine milk α-lac activity, and boiling for 30 min only reduced the activity by 25%. Treatment of 60°C for 10min inactivated 92% of the pyrophosphatase and phosphatase activities in the fraction.

Chromatography with UDP-agarose eliminated 80-90% of the GalTase activity in the fraction. It also reduced the pyrophosphatase and phosphatase activities in the fraction by 80-90%. SDS-polyacrylamide electrophoresis showed the same patterns of the fraction before and after UDP-agarose chromatography.

6.3.2.2 Assay of α-lac-Like Activity in the Low M_r Protein Fraction

(1) With Glucose as an Acceptor

There was more radioactivity in the AG 1-X8 column eluates as the amount of the proteins of the low M_r fraction was increased with both the heat treated preparation (Fig. 6.3a) and the UDP-agarose chromatographed preparation (Fig. 6.3b). However, with the same protein concentrations but without glucose, there was a similar increase of radioactive products. This means that GalTase activity, the difference between the samples with and without glucose, remained independent of the amount of the fraction proteins. The lowest detectable concentration of α-lac in this assay without proteins of the low M_r fraction was 40ng/ml. The presence of the proteins from the low M_r fraction reduced the sensitivity of the assay, and at a protein concentration of 4mg/ml, the lowest detectable amount was 60ng/ml.

(2) With *N*-Acetylglucosamine as an Acceptor

Only the heat treated fraction was used in these assays, since the fraction treated by passing it through an UDP-agarose column still contained detectable GalTase

activity towards GlcNAc. There was no inhibitory effect of the fraction proteins on the GalTase activity (Fig. 6.4). The lowest detectable concentration of α-lac in the assay was 160ng/ml. The presence of the fraction proteins from the low M_r fraction did not reduce the sensitivity.

6.3.2.3 High-Voltage Paper Electrophoresis Analysis of the Product(s) in the α-Lactalbumin-like Activity Assays of the Low M_r Protein Fraction

HVPE of AG 1-X8 column eluates showed that the increased radioactivity with the increased amount of proteins from the low M_r fraction in the assay with either glucose or GlcNAc as an acceptor co-migrated with galactose (Table 6.1), which can be produced from UDP-galactose by pyrophosphatases and phosphatases (Spik et al. 1979).

Table. 6.1 High-voltage paper electrophoresis of AG 1-X8 column eluates of α-lactalbumin-like activity assay of the low M_r protein fraction with glucose or GlcNAc as an acceptor.

Values given are radioactive products as percent of radioactive lysine (CPM). The results given are from two individual assays.

Acceptor	Product co-migrating with	Low M_r fraction			α-lac
		0	1mg/ml	3mg/ml	20μg/ml
galactose	lactose	11.4, 8.1	11.0, 7.6	10.6, 8.1	506.3, 502.9
	galactose		2.2, 3.2	6.7, 10.6	
GlcNAc	LacNAc	10.6, 11.0	10.4	10.1, 11.0	1.9, 1.1
	galactose		1.5	4.9, 4.4	

6.3.2.4 Gas Chromatography Examination of Degradation Product of UDP-galactose by Rat Epididymal Preparation

The purpose of this part of work was to see whether galactitol was a side product in the α-lac-like activity assay of rat epididymal preparation.

Triplicate samples of either the epididymal sample or each of the sugar standards were chromatographed, and consistent results were obtained. Comparing the peaks of the epididymal sample (Fig. 6.5 c) with those of the sugar standards (Fig. 6.5 a and b), there was no galactitol present in the AG 1-X8 column eluate of the assay of degradation of UDP-galactose by the epididymal preparation; however, the eluate did contain the three peaks (peak 3, 4 and 5 of Fig. 6.5 c) at the same positions as those of galactose. This further confirmed that galactose was the degradation product of UDP-galactose by epididymal extract. The eluate of the epididymal sample also contained three other significant peaks (peak 1, 2 and 6 of Fig 6.5 c). According to the sugar standards chromatographed on the same column with exactly the same method by Mr Y. Gao, Department of Plant Physiology, Waite Agricultural Research Institute, peak 7 corresponds to the position of inositol, which is rich in rat epididymal plasma (see Section 1.1.1.2). However, the other two peaks (1 and 2) were not identified.

6.3.3 Stimulation of Galactosyltransferase Activity by Bovine Serum Albumin

In the early stages of my experiments, it was found that when the medium for α-lac-like activity assay contained no other protein than GalTase, addition of proteins of the low M_r fraction did stimulate GalTase activity as reported by Hamilton (1981). However, the two following factors were also found. (a) The stimulation occurred in the assay with either glucose (in the presence of α-lac) or GlcNAc as an acceptor. This was in contrast to Hamilton's results that these proteins stimulated the GalTase activity towards glucose, but inhibited the enzyme activity towards GlcNAc. (b) The ranges of the protein concentrations for the linear increase of the GalTase activities in both reactions were the same, which were at least 10 times lower than that of

the GalTase activity towards glucose reported by Hamilton (1981). These results suggested that the stimulation of the GalTase activity might be non-specific. Consequently, the effect of BSA as a non-specific protein was examined. The activities of bovine milk GalTase increased with BSA concentration in the medium when either glucose (in the presence of α-lac) or GlcNAc was used as an acceptor, and reached a plateau at the concentration of 1mg/ml in both reactions (Fig. 6.6). The present result with glucose as an acceptor agreed with the earlier observation by Khatra et al. (1974).

6.3.4 Investigation of α-Lactalbumin-Like Activity in the Protein Fraction of Rat Epididymal Extract Precipitated by 40-60% Saturated Ammonium Sulphate

8-15% gradient SDS-polyacrylamide gel electrophoresis of the fraction of 40-60% saturated ammonium sulphate precipitated rat epididymal proteins after passing the GlcNAc-agrose column is shown in Fig. 6.7. It can be seen that comparing with the M_r of the α-lac-like proteins reported by other groups (Hamilton, 1981; Jones and Brown, 1982; Limpaseni and Chulavatnatol, 1986), this fraction did not contain proteins around M_r 18,000-19,000, though it gave a band at M_r 24,000. However, the M_r 24,000 protein was only a minor component of this fraction, since there were many other proteins in the fraction. Most of the other proteins were of M_r higher than 43,000, while there was a pronounced band at M_r 14,500.

In current study, this protein fraction after passing the GlcNAc agrose column still contained some GalTase activity. There was no α-lac-like activity detectable under the current assay conditions when either glucose, or inositol, or GlcNAc was used as an acceptor for GalTase.

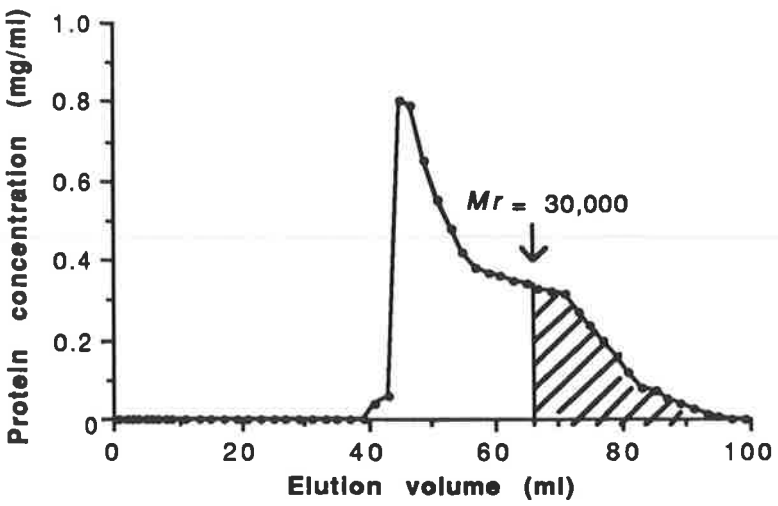


Fig. 6.1 Profile of Sephadex G-75 chromatography of rat epididymal extract. The shaded section represents the low M_r protein fraction.

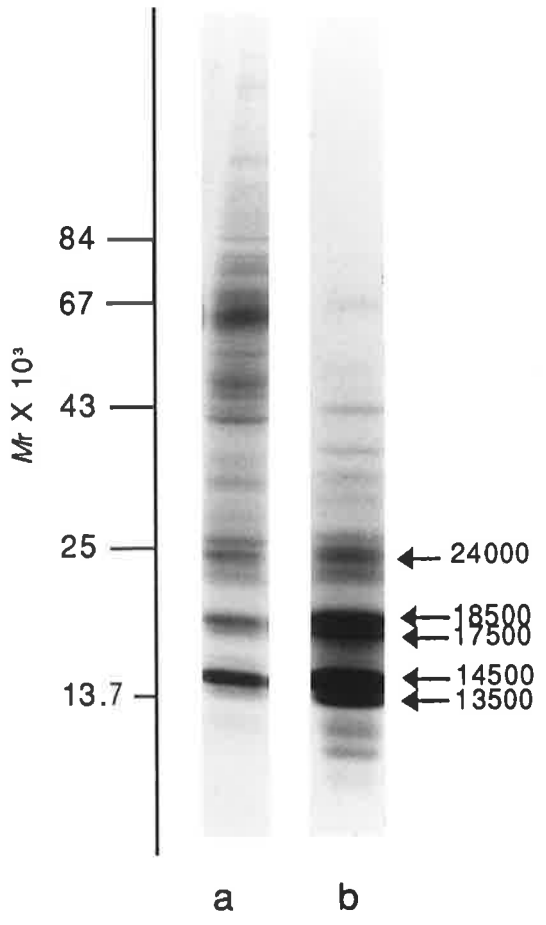
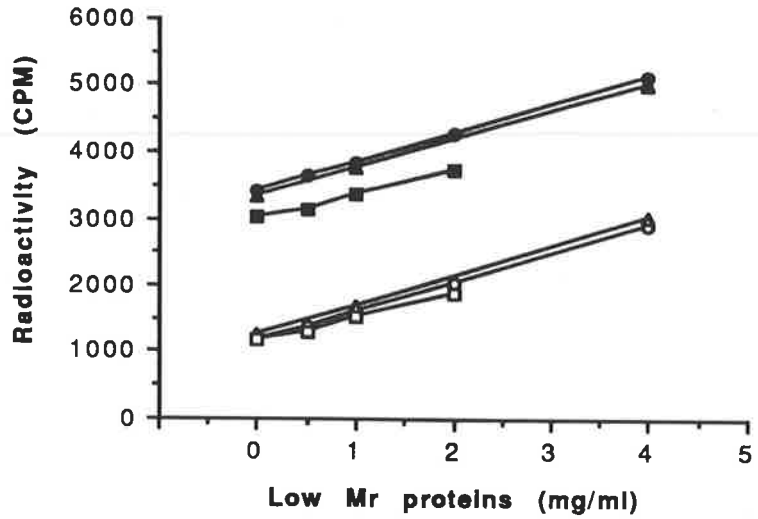
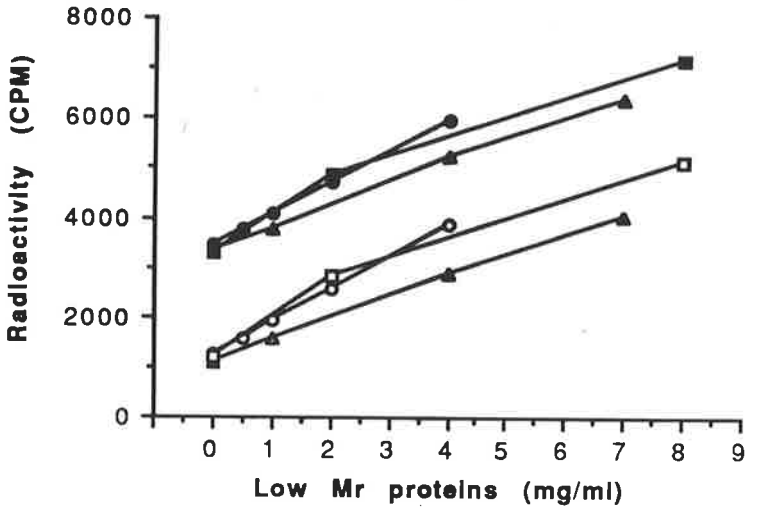


Fig. 6.2 8-15% gradient SDS-polyacrylamide gel electrophoresis of rat epididymal extract before Sephadex G-75 chromatography (a), and the low M_r protein fraction after Sephadex G-75 chromatography (b).



a



b

Fig. 6.3 Unbound radioactivity eluted from AG 1-X8 columns in 3 separate assays of α-lac-like activity of the low M_r protein fraction of rat epididymal extract in the presence (● ■ ▲) and absence (o □ Δ) of glucose. Endogenous GalTase was inactivated by heat (a) or removed by affinity chromatography (b), respectively. The assay conditions were as described in Section 6.2.3.3 (a).

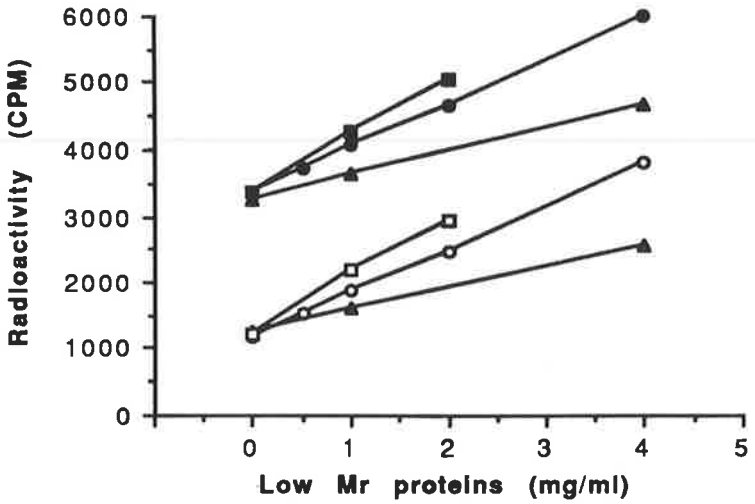
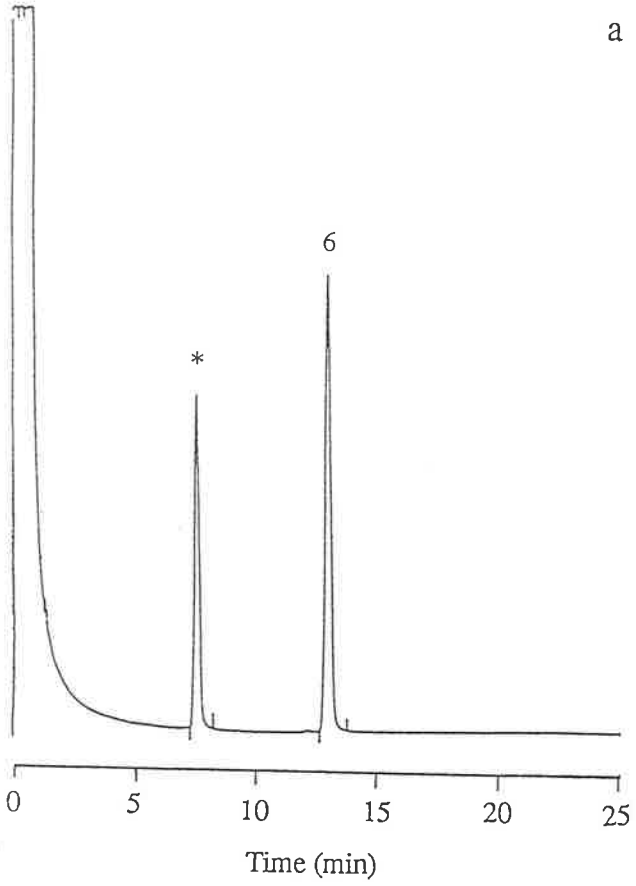


Fig. 6.4 Unbound radioactivity eluted from AG 1-X8 columns in 3 separate assays of α-lac-like activity of the low M_r protein fraction of rat epididymal extract in the presence (● ■ ▲) and absence (o □ Δ) of GlcNAc. Endogenous GalTase was inactivated by heat. The assay conditions were as described in Section 6.2.3.3 (a).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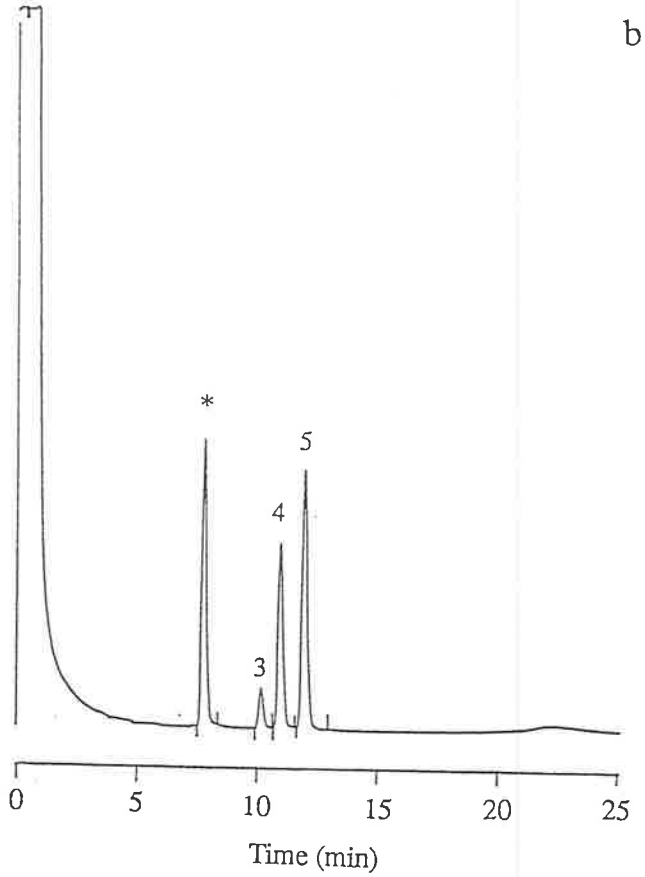
25
Time (min)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Time (min)

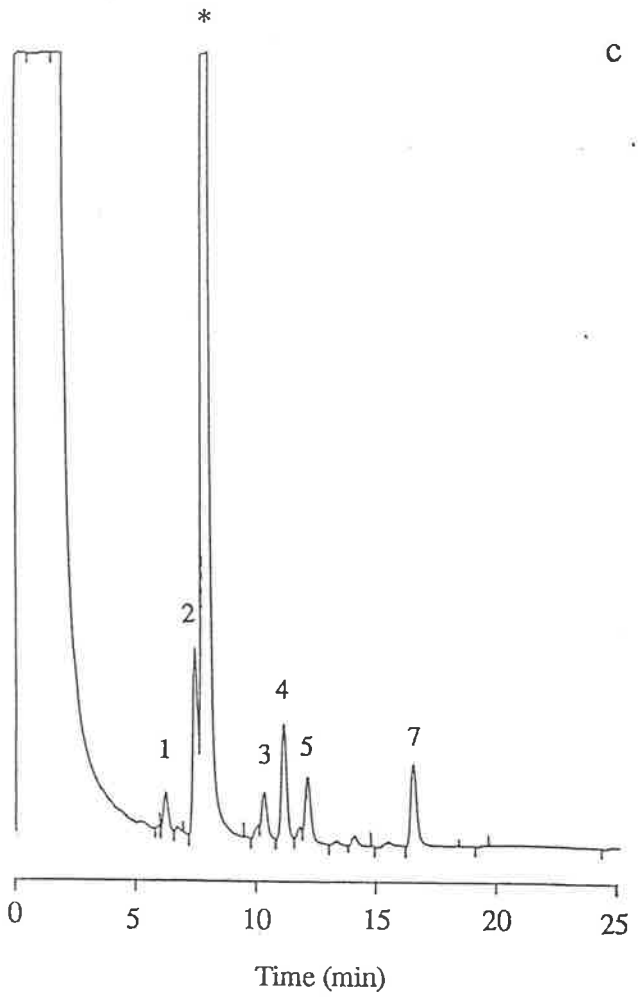


Fig. 6.5 Profiles of gas chromatography of galactitol (a); galactose (b); and the mixture of the assay of the degradation of UDP-galactose by rat epididymal extract (c). The peak with " * " is the internal reference, xylitol. In Fig. 6.5 c, peak 3, 4 and 5 correspond to the positions of the three galactose peaks; peak 7 corresponds to the position of inositol (see Section 6.3.2.4). Peaks 1 and 2 are not identified.

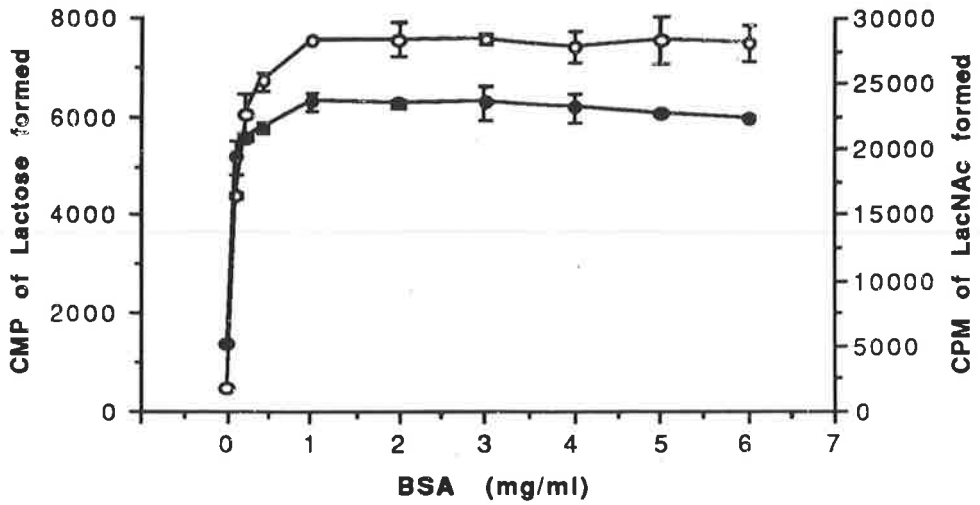


Fig. 6.6 The stimulation of bovine milk GalTase activities towards glucose (○) and GlcNAc (●) by BSA. 0.25munits of bovine milk GalTase was used in each assay, and 250ng bovine α-lac was present in the assay with glucose as an acceptor. Other assay conditions were the same as those for α-lac-like activity assay described in Section 6.2.3.3 (a). Each point is the mean of three assays, with the SD shown as a vertical line.

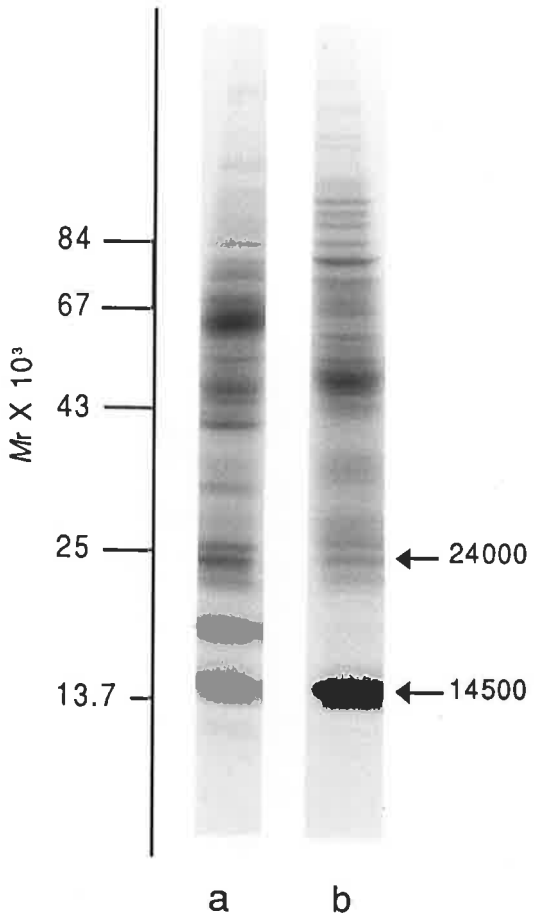
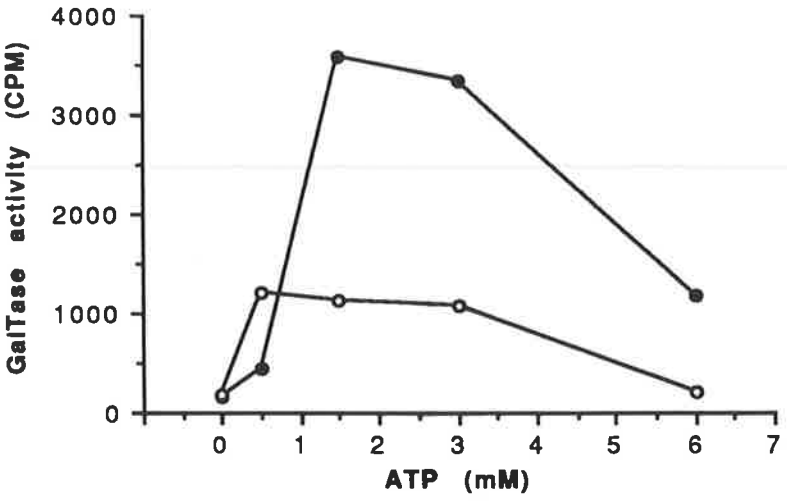
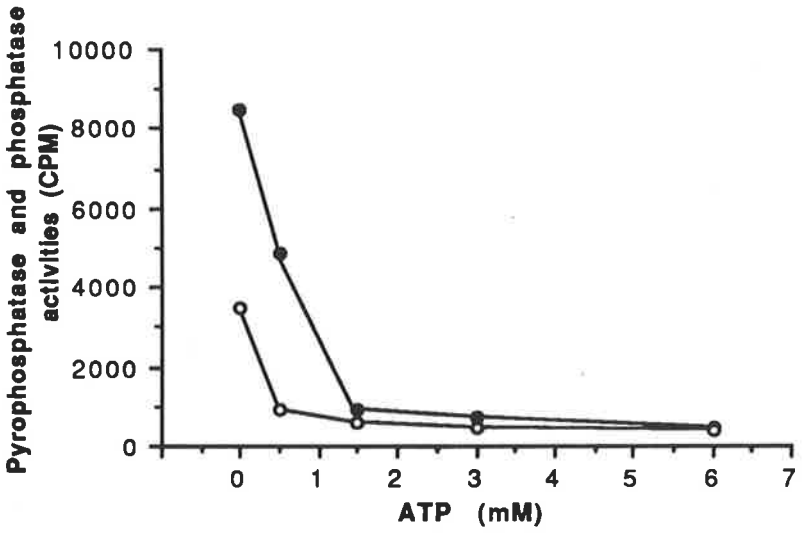


Fig. 6.7 8-15% gradient SDS-polyacrylamide gel electrophoresis. Lane a, crude rat epididymal extract prepared as described in Section 6.2.3.1.2. Lane b, protein fraction of 40-60% ammonium sulphate precipitation of rat epididymal extract after passing GlcNAc-agrose column as described in Section 6.2.3.7.

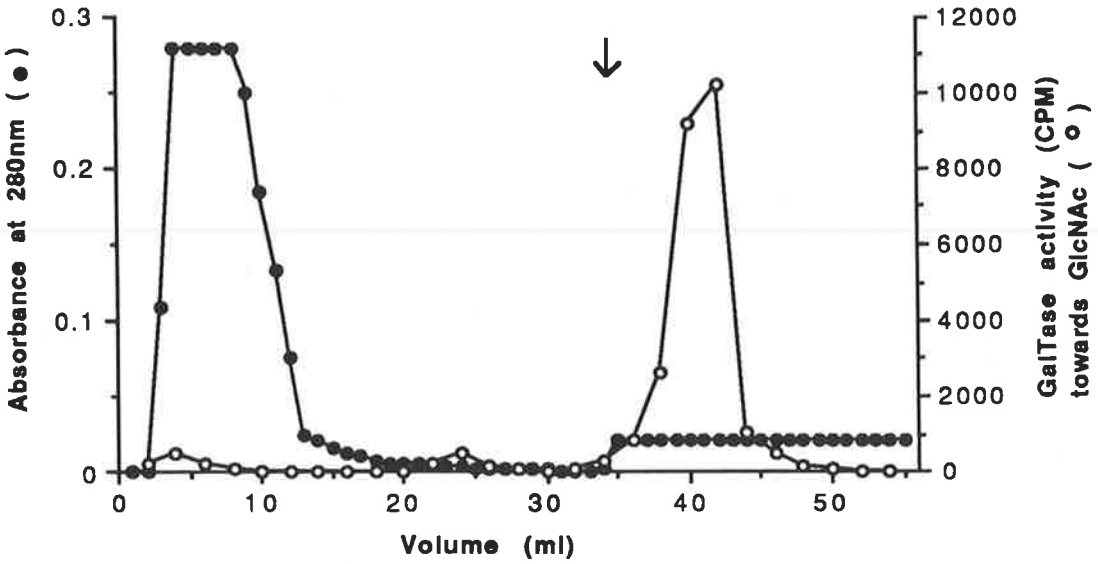


a

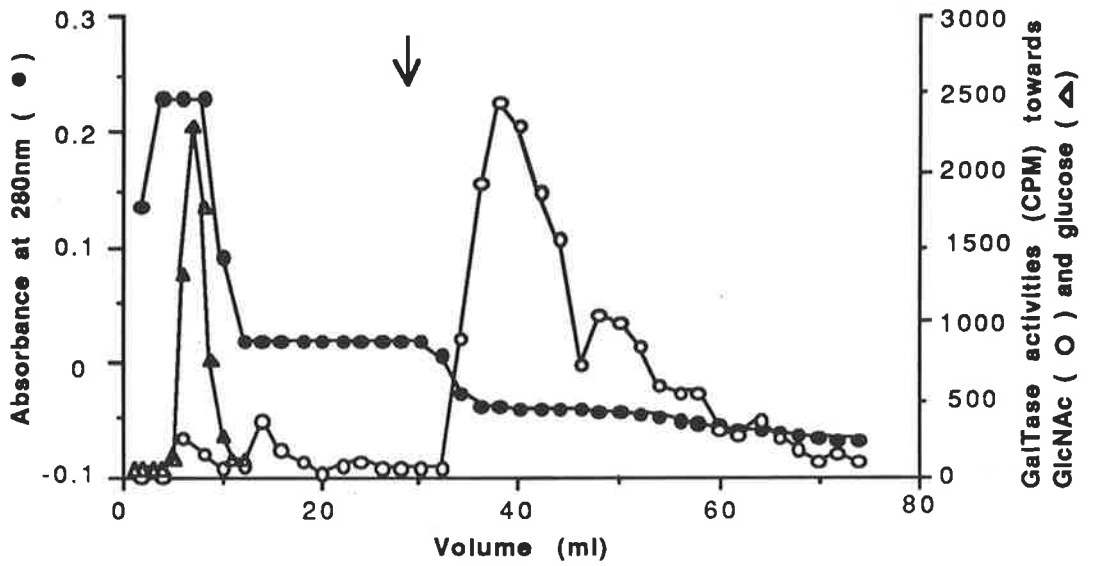


b

Fig 6.8 The inhibition of pyrophosphatase and phosphatase activities (a) and stimulation of GalTase activity (b) in rat epididymal extract. 2mg/ml (o) and 5mg/ml (●) of rat epididymal extract proteins were used. The assay conditions were as described in 6.2.3.3 (a), GlcNAc was used as an acceptor.



Appendix Fig. 6.1 Profile of UDP-agrose chromatography of bovine milk whey. The arrow indicates the position where elusion buffer was applied.



Appendix Fig. 6.2 Profile of GlcNAc-agrose chromatography of bovine milk whey. The column was equilibrated with 25mM Na cacodylate, pH 7.4, containing 25mM $MnCl_2$, 0.5mM UDP and 1mM ß-mecaptoethanol, and washed after sample application with the same buffer. The GalTase activity was eluted with 25mM Na cacodylate, pH 7.4, containing 5mM GlcNAc and 1mM ß-mecaptoethanol. The arrow indicates the position where elusion buffer was applied.

6.4 DISCUSSION

The present results showed that no α-lac-like activity is detectable in the low M_r protein fraction of rat epididymal extracts, and that there were two experimental artefacts in the original report (Hamilton, 1981).

One experimental artefact was that the α-lac-like activity assay lacked proper controls. It is known that pyrophosphatase and phosphatase activities are present in many tissues or body fluids of mammals, that they can degrade UDP-galactose into galactose 1-phosphate and finally galactose (see Section 1.3.6.2.2), and that AG 1-X8 does not separate galactose from lactose or GlcNAc, which are the products of GalTase. Therefore, it is important to have controls for pyrophosphatase and phosphatase activities in the assay when unpurified protein preparations containing these activities are used and AG 1-X8 resin is used for separation. Each sample should have a control with the same amount of the preparation, but no acceptor. Otherwise, when glucose is used as an acceptor, the radioactive galactose will not be distinguished from radioactive lactose, and the pyrophosphatase and phosphatase activities will be considered as GalTase activity. The present results showed that with proper controls, the low M_r protein fraction neither stimulated GalTase activity when glucose was used as an acceptor (Fig. 6.3), nor inhibited GalTase activity when GlcNAc was used as an acceptor (Fig. 6.4), though the total radio activity in the eluates of the AG 1-X8 columns in both assays increased with the amount of the proteins of the low M_r fraction; it was also shown from HVPE that the increased radioactivity in AG 1-X8 eluate with the increase of the proteins of the low M_r fraction co-migrated only with galactose. An anomaly concerns the clear indication in Hamilton's paper (1980) that the only significant radioactive product, when glucose was used as an acceptor, emerging from the ion exchange column, co-migrated with lactose on silica TLC, using a system which should have been adequate to separate lactose and galactose (Gauch et al, 1979). However, in the present study, the radioactivity collected from the ion exchange column partially co-migrated with lactose and partially with galactose using HVPE. Hölpert and

Cooper (1990b) also showed that when rat epididymal homogenate was tested with glucose as an accceptor, radioactive product(s) co-migrated only with galactose on silica TLC under non-optimal conditions, and with both galactose and lactose under optimal conditions. Therefore I believe that the "α-lac-like activity" detected when glucose and inositol was used as an acceptor in Hamilton's report (1981) was actually pyrophosphatase and phosphatase activities. However, it is difficult to explain the inhibition of GalTase activity by fraction III in Hamilton's report (1981), though Hölpert and Cooper (1990b) suggested that the enzyme activity was inhibited by pyrophosphatase and phosphatase activities.

Purified bovine milk GalTase was used as the enzyme source in some of the assays of α-lac-like activity in Hamilton's report (1981). The other experimental artefact was that the activity of the purified GalTase was not optimized by non-specific proteins in the assay. This problem was also present in some of the other previous reports about α-lac-like activity (Limpaseni and Chulavatnatol, 1986; McLaughlin and Shur, 1987; and probably Qasba et al., 1983). The present results showed that purified GalTase required certain amount (about 1mg/ml in concentration) of non-specific proteins in the assay medium to optimize its activity (Fig. 6.6). The result of the assay with glucose as an acceptor agreed with that of Khatra et al. (1974). In most of the above reports, only assays with glucose as an acceptor were carried out when purified GalTase was used, and in none of them did the assay medium contain non-specific proteins before the epididymal proteins were added. In this situation, the "α-lac-like activity" detected was most possibly the non-specific stimulation of the enzyme activity". However, in the report of Limpaseni and Chulavatnatol (1986), the inhibition of purified epididymal protein on the activity of purified GalTase towards GlcNAc was also demonstrated, which can not be explained by the known experimental artefacts.

The above experimental artefacts cannot explain the "α-lac-like activity" in the report of Jones and Brown (1982), who examined the α-lac-like activity of purified epididymal proteins and used rat rete testis fluid as the enzyme source. Rat rete testis fluid had been previously shown to contain GalTase activity but not

pyrophosphatase and phosphatase activities (Hamilton, 1980). Hölpert and Cooper (1990b) suggested that the "α-lac-like activity" in their report might be the non-specific activation of GalTase by hydrophobic epididymal proteins of rat epididymis, since Mitranic et al. (1988) reported that GalTase can be activated via an interaction with a hydrophobic domain of the enzyme.

Qasba et al. (1983) and Byers, Dym, Hewlett and Qasba (1984) reported α-lac-like activity in a rat epididymal protein fraction of 40-60% saturated ammonium sulphate. The protein composition of this fraction was not reported. In the present study, this was examined by SDS-polyacrylamide gel electrophoresis. Comparing with the M_r of the α-lac-like proteins reported by other groups (Hamilton, 1980; Jones and Brown, 1982; Limpaseni and Chulavatnatol, 1986), this fraction contained a M_r 24,000 protein only as a minor component, but not proteins with M_r around 18,000-19,000. This fraction was also devoid of α-lac-like activity in the current study. Because the evidence about "α-lac-like proteins" in the previous work of the 40-60% saturated ammonium sulphate protein fraction was not as significant as that of the low M_r protein fraction, the current study did not concentrate on this fraction.

The present results confirm the findings of Hölpert and Cooper (1990b) who however did not examine the fraction of epididymal luminal fluid with M_r less then 30,000 as originally studied by Hamilton (1981). There are some major differences between the present study and that of Hölpert and Cooper (1990b). Firstly, these authors used crude rat epididymal extract, while protein fraction of M_r less then 30,000 of rat epididymal fluid was used in the present work. I started to examine the α-lac-like activity in this fraction after failing to detect any α-lac-like activity in crude epididymal fluids of several mammals including the rat. This was because the M_r range of this fraction is close to the M_r reported for α-lac-like proteins, which were between 18,500 and 24,000 (Hamilton, 1981; Jones and Brown, 1982; Limpaseni and Chulavatnatol, 1986), and that three major bands of this fraction were reported to be α-lac-like proteins (Hamilton, 1981; Jones and Brown, 1982). Therefore, more conclusive results should be obtained by the use of this fraction. Secondly, the GalTase activity in this fraction

for α-lac-like activity assay was inactivated or substantially eliminated. It seems important to eliminate as many disturbing factors as possible from the assay to reduce the complexity of the assay system. Thirdly, HVPE was used to separate the assay products from AG 1-X8 columns to confirm further my results obtained from using AG 1-X8 column and identify the radioactive product which was increased with the increased amount of the fraction proteins. HVPE has the advantage of completely separating the GalTase products (Lactose or LacNAc) from all the other radioactive molecules in the assay system. HVPE was used in one of the previous reports of α-lac-like activity (McLaughlin and Shur, 1987). The present HVPE results have given evidence which directly disagrees with their results. In addition, α-lac-like activity in the fraction of 40-60% ammonium sulphate precipitated rat epididymal proteins as described by Qasba et al. (1983) was also examined to support the results obtained from the study of the low M_r protein fraction.

In order to optimize the assay condition for GalTase, Hölpert and Cooper (1990a, b) did not only use BSA, but also Triton X-100 and ATP in their assay medium. In the present assays, Triton X-100 was not used, because detergent was not used in the sample preparation and the sample was dialysed against assay buffer before being used. ATP was also not used. This was because the pyrophosphatase and phosphatase activities in the assays were only the activities left from Sephadex G-75 chromatography and either heat treatment or UDP-agrose chromatography, and did not inhibit GalTase activity under the assay conditions. In addition, my results showed that the effects of ATP, probably also other nucleotide inhibitors, on pyrophosphatase and phosphatase activities and GalTase activity in the epididymal preparations were different at different protein concentrations of the same sample (Fig. 6.8). Therefore, I tended not to use them in the assays to avoid the necessity of more complex assay control system.

It was reported that rete testis fluid of the galactose-fed rat contain a large amount of galactitol (Middleton, 1973). Therefore, gas chromatography was used to examine whether galactose degraded from UDP-galactose was further translated to

galactitol by rat epididymal plasma under the conditions for α-lac-like activity assay. However, there was no galactitol identified. This may suggest that the enzyme to change galactose to galactitol is only present in testis, but not epididymal plasma of rat.

CHAPTER 7
GENERAL DISCUSSION

The results presented in different experimental chapters have been discussed in detail in the individual chapters. In this chapter, the relations between the results of the different chapters are discussed.

The existence of α-lactalbumin (α-lac)-like proteins in epididymal plasma of the rat and some other mammals was reported from several laboratories following the report about the detection of GalTase in rat epididymal plasma. These reports aroused great interest about whether GalTase in mammalian epididymal plasma had some unusual functions under the regulation of the α-lac-like proteins, e.g., the function of transferring galactose to inositol or to sperm membrane phosphatidylinositol (See Section 6.1). However, it has been shown in the present study (Chapter 6), as well as in the report of Hölpert and Cooper (1990b), that α-lac-like activity is not detectable in mammalian epididymal plasma, and the previously reported "α-lac-like activity" was actually due to experimental artefacts. I think this has greatly weakened the special attraction of epididymal plasma GalTase in research. This enzyme is likely to just be an enzyme catalyzing the galactosylation of glycoproteins or glycolipids as most of the other UDP-galactose : GlcNAc GalTases elsewhere (not including those in milk) in the body. More detailed discussion about the possible function of epididymal plasma GalTase has been given in Chapter 3.

It was originally planned in the present study to investigate whether there was any correlation between the levels of GalTase, α-lac-like proteins and inositol in various mammals. Obviously, this plan was no longer meaningful after the finding that there are no α-lac-like proteins in mammalian epididymal plasma. However, the investigation of the epididymal plasma GalTase activity in various mammals, which was carried out before the study of α-lac-like proteins, suggested another interesting possibility that epididymal plasma GalTase has a function only in the species which have

low pyrophosphatase and alkaline phosphatase activities in the same plasma, but not in the species which have high activities of the later enzymes (Chapter 3). I think it is worth to have further investigations carried out along this line.

The report of the existence of α-lac-like proteins in the mammalian epididymal tract was also once considered to be an exciting finding related to the mechanism of mammalian fertilization, since it was independently reported that mouse sperm surface GalTase was the sperm's receptor for zona in mouse sperm-zona binding. The possibility that α-lac-like proteins were decapacitation factors was naturally considered (see "General Introduction" of Chapter 4). In fact, α-lac-like activity was then reported to be present in mouse epididymal plasma and it was suggested that it was possibly related to mouse fertilization (see Section 6.1). However, since "α-lac-like proteins" had never been involved as an evidence for the zona receptor function of sperm surface GalTase, the finding that the activities of these proteins were reported based on experimental artefacts had no great effect on the study in this aspect.

In the study of sperm-zona binding, the present result of the effect of UDP-galactose on mouse sperm-zona binding (Part 3, Chapter 4) agreed with the result of Shur and co-workers (see "General Introduction" of Chapter 4) that UDP-galactose specifically inhibited the binding, which is one of the important pieces of evidence that sperm surface GalTase is the sperm's receptor for zona pellucida in mouse sperm-zona binding in the study of Shur and co-workers. However, the present result of the effect of UDP-galactose on the binding of rat or ram sperm to mouse zona suggested that GalTase is not the receptor on the sperm of these species for zona binding (Part 3, Chapter 4). On the other hand, the current investigation about the sperm surface GalTase activity suggested that there might be some experimental artefact in the measurement of sperm surface GalTase activity in the work of Shur and co-workers (Part 1, Chapter 4). In order to gain some information relevant to the study of the involvement of GalTase in mammalian sperm-zona binding from female reproductive tract, where sperm capacitation and fertilization occur, the experiments of Chapter 5 were carried out as an initial investigation. The results have shown that a soluble

GalTase is present in ewe uterine lumen. Further investigation to determine whether the same enzyme is also present in ewe oviduct lumen, and uterine and oviduct lumina in other species, especially the mouse, may offer some important relevant information. If mouse sperm surface GalTase is involved in sperm zona binding, logically, the enviroment of the binding, i.e., the mouse oviduct plasma is expected to contain no or a very low level of GalTase, so that the GalTase receptors on zona surface are not masked by the competitive binding of the enzyme in the fluid. Considering the difficulties in obtaining oviduct and uterine fluid from the mouse, tissue culture may be used. It may also be able to collect mouse uterine fluid by ligation of the uterine horn.

GalTase is only one of the candidates reported in mouse or in mammals for sperm's receptor for zona pellucida in sperm-zona binding (see Section 1.2.3.2). Although, there has been much evidence reported from Shur and co-workers to support the zona receptor function of mouse sperm surface GalTase, the exact molecule on mouse zona to serve as GalTase acceptor has not been identified (see "General Introduction" of Chapter 4). A similar situation applies to some of the other candidates, e.g., the M_r 95,000 phosphotyrosine containing protein of mouse sperm, and the zona-binding and fucose-binding protein of boar sperm (see Section 1.2.3.2). At present, none of the candidates has been fully proven to be the sperm's receptor for zona. It seems that to convincingly identify such a receptor, a great amount of delicate work may be needed and it may have to await the availability of more advanced biological techniques. It is possible that sperm-zona binding process in each species is mediated by more than one system, and it is the combination of these systems that decides the species specificity of the binding. However, except for the fact that many candidates have been reported to mediate sperm-zona binding, there is hardly any other evidence available now to support this hypothesis. To my best knowledge, the only report relevant is from Benau and Story (1988) about the relationship between the GalTase site and the trypsin inhibitor-sensitive site on mouse sperm surface. I once heard that it is more difficult to explore the surface of a sperm than the surface of the moon, and hope it is not true.

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