RECEPTOR-MEDIATED ENDOCYTOSIS OF TESTICULAR TRANSFERRIN BY GERMINAL CELLS OF THE RAT TESTIS

by

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This Work is Dedicated to
my Mother and Father
for the
Love and Discipline They Imparted

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ABSTRACT

The present study examines events of the Sertoli cell iron delivery pathway following the secretion of diferric testicular transferrin (tTf) in to the adluminal compartment of the rat seminiferous epithelium. The unidirectional secretion of tTf by Sertoli cells was verified, in vivo, and it was shown that this protein is internalized by adluminal germ cells. It was further determined by Scatchard analysis that this internalization was mediated by high affinity transferrin binding sites on the surface of round spermatids, numbering 1453/cell and displaying a $K_d=0.6x10^{-9}$ M. Northern blot analysis of RNA isolated from adluminal germ cells, namely spermatocytes, round spermatids and elongating spermatids, indicated that these cells expressed Tf receptor mRNA and ferritin mRNA in levels inversely related to their stage of maturation. Finally it was determined that following binding and internalization in round spermatids, Tf became associated with the endosomal compartment and was recycled back to the cell surface. This study illustrates the immediate fate of tTf once it is secreted by the Sertoli cell. Thus, diferric tTf binds to Tf receptor on the surface of adluminal germ cells,

is internalized by receptor-mediated endocytosis and the apotTf-Tf receptor complex is recycled back to the cell surface where apoTf is released into the adlumenal fluid.

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RÉSUME

Nous avons étudie la secrétion de la transferrine testiculaire (tTf) par les cellules de Sertoli et son incorporation par les cellules germinales localisees dans le compartiment adluminale de l'epithelium séminifère du rat. Nous avons d'abord confirme une secrétion unidirectionnelle de la tTf vers la lumiere des tubes séminifères, in vivo, et ensuite nous avons montré que les cellules germinales incorporent cette protéine par endocytose. Une analyse Scatchard nous permet de conclure que cette endocytose est favorisee par la presence de récepteurs specifiques a la surface des jeunes spermatides spheriques. On evalue a 1453 le nombre approximatif de ces récepteurs de grande affinité (K_d=0.6 X 10^{-9} M) à la surface de chaque cellule. L'analyse du mRNA par la methode Northern Blot révèle que les spermatocytes et les spermatides sphériques ou allongées possedent tous les mRNA responsables de la synthese de la ferritine et des récepteurs de la transferrine mais en quantite decroissante au cours de la differentiation de ces cellules. Nous avons pu également observer qu'apres son endocytose par les jeunes spermatides, la tTf est incorporee dans des endosomes pour être par la suite

recyclee vers la surface de ces cellules. Donc la tTf diferrique secretee par les cellules de Sertoli se lie aux récepteurs présents à la surface des cellules germinales. Elle est endocytée par ces cellules pour être incorporee dans des endosomes vraisemblablement sous forme d'un complexe apotTf-récepteur qui est par la suite retourné vers la surface des cellules ou l'apotTf est libéree dans la lumiere du tube séminifère.

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INTRODUCTION

Iron is clearly a vital nutritional requirement for all mammalian cells. Transferrin (Tf) is known to be the major systemic transporter of iron in mammals (Morgan, 1981). Each Tf molecule delivers two atoms of iron to cells via a process of receptor-mediated endocytocis which involves a single high-affinity binding site (Karin and Mintz, 1981; Bleil and Bretscher, 1982; Harding et al., 1983; Klausner et al., 1983b). Curiously, however, the ligand, rather than finding its fate along the degradative pathway, as is often the case with receptor mediated events, is recycled back to the plasma membrane with and still bound to its receptor (Dautry-Varsat et al., 1983). During internalization, the iron atoms dissociate from the Tf molecule and are readily absorbed by the cell. Upon recycling to the cell surface, the apoTf (Tf devoid of iron) is released into the circulation to acquire more iron and the Tf receptor once again becomes available to bind another Tf molecule and begin a new cycle.

As in other cell systems, Tf is probably the major source of iron for all the cells comprising the male reproductive organs. The structure, however, of the basic functional unit of the male testis, the seminiferous tubule and its epithelium, is such that not all cell types are exposed to this systemic source of iron. Tight junctional complexes between adjacent Sertoli cells, a permeability

barrier known as the blood-testis-barrier (BTB), restricts passage of blood-borne macromolecules into the adluminal compartment of this epithelium (Dym and Fawcett, 1970). Thus only Sertoli cells and germ cells of the basal compartment of the seminiferous epithelium are able to bind systemic Tf (sTf) (Morales et al., 1986b). It is now understood, however, that Sertoli cells may supply adluminal germ cells, namely spermatocytes, round spermatids and elongated spermatids, with iron by secreting diferric testicular Tf (tTf) into the adluminal compartment thus allowing these cells to sequester their complement of iron (Morales et al., 1987b).

Presumably these haploid germ cells, which exist in a very unique extracellular environment (Fawcett, 1975; Waites, 1977), carry Tf binding sites and express the necessary intracellular mechanisms to store iron. If so and given that these special conditions appear nowhere else in the organism, one may ask whether the nature of the endocytic pathway in these cells diverges from the model which has been observed and accepted to exist in somatic cells. Moreover one may also question the purpose of a recycling pathway if released apoTf is essentially wasted in the ejaculate. Free iron is not known to be a constituent of adluminal fluid (Fawcett, 1975).

The present work explores the hypothesis that adluminal germ cells of the rat seminiferous epithelium, instead of recycling tTf, possess an alternative pathway that involves

the targetting of the tTf-Tf receptor complex to the lysosomal compartment. In so doing the objective of this thesis also includes the characterization of the iron acquisition mechanism in adluminal germ cells, a process that is still poorly understood but which may involve receptor-mediated endocytosis of tTf.

LITERATURE REVIEW

Section 1 Transferrin: Biochemistry and Function

In the same way that today's mammalian organisms must supply all their cells with a very dependable and uninterrupted flow of oxygen, so they must ensure that each cell's vital requirement for iron is efficiently met. Evolution has accomplished this by developing a class of transport molecules termed transferrins by Holmberg and Laurell in 1947. Since then Tf has been clearly demonstrated to be the major systemic iron transporter in mammals (Aisen and Listowsky, 1980).

The Tf molecule is a 77 kd glycoprotein made up of a single polypeptide backbone of approximately 680 amino acids in length and one to two carbohydrate side chains. This complement of carbohydrate comprises 6% of the molecule's mass but its function is unknown (Morgan, 1981). In terms of tertiary structure the chain folds into two similar, but not identical, globular domains: The N-terminal domain and the C-terminal domain (Huebers and Finch, 1984). Each domain contains one high affinity binding site for iron with a K_d range of 10^{-19} to 10^{-20} mol/l thus making Tf a highly efficient carrier (Huebers and Finch, 1984). This affinity approaches zero, however, if an anion, largely believed to be bicarbonate, does not cooperate with ferric iron in complexing

to the Tf binding site (Gelb and Harris, 1980).

Another prerequisite for optimal binding of iron to Tf is an appropriate pH. This pH-dependence is evident when a solution of diferric Tf (dTf), which characteristically has a salmon-pink colour, is followed spectrophotometrically, at 460-465 nm, as it is acidified (Surgenor, 1949). Binding, which is maximal above pH 7, begins to drop off at pH 6.5 and approaches zero at pH 4.5 as evidenced by a gradual loss of the solution's colour (Morgan, 1981). This lowering of pH causes the iron atoms and bicarbonate molecules to be displaced by three hydrogen atoms which bind at, as yet, unknown locations of the Tf molecule (Crichton and CharloteauxWauters, 1987).

Besides acting as an iron transporter, Tf may function in some secondary roles as well such as in growth and differentiation of cell lines (see review by Khun et al., 1990), clearing the organism of deleterious metals (Morgan, 1981) and acting as a bacteriostatic agent (Schade and Caroline, 1946). In all cases no additional function beyond its ability to efficiently deliver iron has been conclusively attributed to Tf itself (see review by Khun et al., 1990; Perez-Infante and Mather, 1982).

Transferrin is synthesized in a number of tissues including brain, stomach, placenta, spleen, kidney, muscle,

heart and testis (Aldred et al., 1987). In most of these cases it is secreted as an alternate source of Tf to the main lieu of synthesis, the liver. In the testis, tTf is also destined to function as an iron transporter, but in contrast to the other sites, it is never secreted into the blood stream but ultimately ends up in the ejaculate (Morales et al., 1987b).

Transferrin is not the only molecule which binds iron. Lactoferrin is normally secreted by neutrophils during inflammatory states and is found at high levels in lactating women. Under normal physiological conditions, lactoferrin is present at very low plasma concentrations and is not considered to play any significant role in iron transport (Morgan, 1981). Haptoglobin (Laurell and Granvall, 1962) and hemopexin (Muller-Eberhard and Morgan, 1975) clear haemoglobin and haem from the plasma to the liver, respectively, but are not involved in any delivery of iron to cells. Ovotransferrin, found in bird yolk sac, is also known to bind iron but only in a bacteriostatic capacity. Finally vitellogenin, which is not known to be expressed in mammals (Ali and Ramsay, 1974; Morgan, 1975) has been shown to have an iron-transport role in birds, fish and some reptiles (Wallace and Bergink, 1974; Dessauer, 1974; Craik, 1978). Therefore, for all intents and purposes, Tf can indeed be considered as the main transporter of iron in mammals.

Section 2 Iron: Function and Storage

As suggested above, iron is as important a nutrient to cells as is oxygen. Its central role in life processes is surely linked to its electron exchange capabilities and multiple oxidation states (Bergeron, 1986). This is evidenced by a vital role in biological redox reactions, serving as the prosthetic group in cytochromes, catalases, as cofactor of ribonucleotide reductase, a rate-limiting enzyme in DNA synthesis etc.

Available sources of iron in the body include the liver and the intestinal epithelium but by far the largest source of iron for Tf binding is that resulting from the breakdown of senile erythrocytes (Morgan, 1981). Iron derived from this degeneration is readily picked up by Tf since, under normal physiological conditions, the total iron-binding capacity of plasma plus interstitial Tf is only 1/3 saturated (Morgan, 1981).

Iron's access to cells is achieved by binding to its carrier protein Tf. This efficient delivery system was developed by mammals, in part, because iron, if not bound to Tf, is present in plasma as $Fe(OH)_3$ which is insoluble and metabolically inactive (Bergeron, 1986). By complexing with Tf, iron transiently adopts its carrier's solubility and distribution characteristics thus making itself universally available.

Once internalized, iron becomes associated with its intracellular storage protein, ferritin. This 480 kd protein consists of 24 subunits of two sizes, 19 kd (L) and 21 kd (H), in proportions varying from tissue to tissue and with iron load (Munro et al., 1988; Theil, 1987). Ferritin, which is a ubiquitous and highly conserved protein, is known to play a pivotal role in intracellular iron metabolism (Campbell et al., 1989). Not only is it known to act as a storage site but is considered as critical in preventing potential oxidative damage in the cytosol by rapidly binding internalized iron (Campbell et al., 1989). Levels of intracellular ferritin mRNA are regulated by extracellular iron supply (Zahringer et al., 1976). In fact, according to a rapidly expanding body of data, iron has been suggested to simultaneously regulate levels of Tf receptor mRNA and ferritin mRNA through a well integrated negative feedback loop (Mullner et al., 1989).

It is clear that an adequate iron supply is required by most proliferating cell systems studied, especially during events requiring the synthesis of nucleic acids. During the course of spermatogenesis it has been shown that a number of cell divisions occur, including 2 meiotic divisions (Leblond and Clermont, 1952b). Iron is undoubtedly required for these events to take place.

Section 3 The Transferrin Receptor

The Tf receptor is expressed ubiquitously since the cellular need for iron is universal. Perhaps the only exception to this is in terminally differentiated cells such as the mature erythrocyte. The Tf receptor protein is a 180-200 kd disulfide-linked homodimer. Each 95 kd subunit consists of a 760 amino acid backbone containing two, and rarely three, N-linked glycan units and is post-translationally modified by the addition of two palmitate moieties (Omary and Trowbridge, 1981). The amino-acid sequence of the receptor shows no strong homology to any other known protein (McClelland et al., 1984).

Also, as determined by analysis of partial sequence data of a cDNA clone for murine Tf receptor, this integral membrane protein has a single transmembrane sequence of 28 amino acids (Stearne et al., 1985), a 65 residue amino-terminal cytoplasmic domain and a long (672 amino acid) extracellular domain which houses its high affinity binding site (McClelland et al., 1984), the K_d of which generally falls within the nanomolar range. This reverse orientation in the plasma membrane designates the Tf receptor as type-II which, like the asialoglycoprotein receptor (ASGP receptor) for example, specifies the transmembrane region as the signal sequence for membrane insertion in lieu of a cleavable amino terminal signal sequence (see review by Steer and Ashwell, 1990). The cytoplasmic domain, which has been found to be highly

conserved from specie to specie (Stearne et al., 1985), is now thought to be important for high efficiency endocytosis (Rothenberger et al., 1987).

In the human, the Tf receptor is encoded by a single copy gene of about 32 kb, (Kuhn et al., 1984) present on chromosome 3, (Goodfellow et al., 1982) which gives rise to a major 5.0 kb mRNA species containing a 2.28 kb coding region and an unusually long but highly conserved non-coding region (Kuhn et al., 1984). This 3' untranslated region is widely believed to be of regulatory significance for Tf receptor mRNA levels in the chicken (Chan et al., 1989), mouse (Stearne et al., 1985) and human (Kuhn et al., 1984). Since the homology between human and rat in these two regions has been reported at 82% and 90% respectively, it has been suggested that the same may apply in the rat (Roberts and Griswold, 1990).

A model for regulation which is presently receiving widespread acceptance suggests that iron availability governs the relative levels of both Tf receptor mRNA and ferritin mRNA (Casey et al., 1988). According to this proposal, a so-called cytoplasmic iron responsive element (IRE) simultaneously interacts with this regulatory region on the 3' untranslated region of the Tf receptor mRNA and with an analogous region on the 5' untranslated end of the ferritin mRNA molecule. The IRE is proposed to interact with both corresponding regions to raise Tf receptor mRNA levels and simultaneously lower

ferritin mRNA levels when iron is in low extracellular concentration and vice-versa when iron is in abundant supply (Mullner et al., 1989). In vivo studies conducted by Schulman et al. (1989) concur in these results.

Each Tf receptor subunit is capable of binding one Tf molecule (Turkewitz and Schwartz, 1988). This binding, as with most receptor-ligand interactions is pH-dependent (Goldstein et al., 1979). The diferric state of the Tf molecule is also a prerequisite for binding since the receptor's affinity for apoTf (Tf devoid of iron) is 10 to 30 times lower than for dTf (Morgan et al., 1981). This would therefore ensure that cells only bind those Tf molecules containing a full iron complement.

Section 4 The Endocytic Apparatus and Transferrin Routing

(a) Receptor-Mediated Endocytosis

RME is the process by which ligands are specifically bound by a cell-surface receptor, internalized and packaged into plasma membrane vesicles (see review by Gruenberg and Howell, 1989). This should be distinguished from pinocytosis or fluid-phase endocytosis and phagocytosis. The former, which is known to occur at the apical aspect of the Sertoli cell, is a constitutive process generally defined by the non-selective uptake of extracellular fluid by small smooth-

surfaced invaginations of the plasma membrane (Steinman et al., 1978; 1983). Phagocytosis, which is also practiced by Sertoli cells, can be described as the ingestion of particles, membrane-bound entities or even expansive regions of membrane from the surface of the phagocytosing cell (Steinman et al., 1983). Although neither of these processes are generally thought to involve a receptor-mediated event (see review Steer and Ashwell, 1990), some preliminary studies have suggested that Sertoli cell may be a notable exception (Morales et al., 1985).

Cell surface receptors, depending on the specific ligand, may be randomly distributed on the plasma bilayer or occur in clusters in discrete invaginations of the membrane referred to as coated pits (Besterman and Low, 1983). The first step of RME is for dTf molecules to bind to their receptors and for these complexes to cluster into clathrin-coated pits (Hopkins and Trowbridge, 1983; Ciechanover et al., 1983b) (Fig.1); This is an essential but ill-understood step for internalization by RME (Pastan and Willingham, 1985; Wileman et al. 1985). It is clear however that most ligands, including Tf, interact with their receptor in a highly pH-dependent fashion (Goldstein et al., 1979). Thus ligand binding initiates RME, but depending on the R-L system in question, not necessarily internalization (Goldstein et al., 1985). In the case of the Tf receptor, internalization occurs constitutively and as such its endocytosis is independent of

ligand binding (Wileman et al., 1985; Geuze et al., 1986; Forgac, 1988). The regulatory signal controlling constitutive endocytosis is thought to be localized to the highly conserved cytoplasmic tail of the Tf receptor molecule. A tetrapeptide located in this region has recently been suggested by Collawn et al.(1990) to be necessary for high efficiency constitutive endocytosis to occur.

(b) The Endosomal Compartment

Once internalized, the clathrin-coated vesicles lose their coat and are thought to either fuse with one another to form an early endosome (Yamashiro et al., 1984) or, alternatively, to individually fuse with a preexisting early endosome near the cell periphery. Whatever the case may be, the subsequent step following internalization is the rapid localization of the R-L complex with what's been called the endosomal compartment, a compartment generally defined as a network of tubules and vesicles that form a reticulum within the periphery of the cytosol (Geuze et al., 1983) (Fig.1).

Whether structures that make up this compartment are permanent or transient organelles is the fundamental difference between the vesicular model of receptor trafficking proposed by Palade, 1975, and the maturational model (see review by Helenius et al., 1983). According to the vesicular model, early and late endosomes are expected to contain resident proteins in addition to the molecules which are in

transit. According to the maturational model, early endosomes are constantly being formed by the fusion of incoming vesicles with each other. This early endosome then matures while being translocated within the cell and receives Golgi components to become a late endosome, and eventually a lysosome (see review by Gruenberg and Howell, 1989) (Fig.1). In short, once molecules destined for reutilization have, at some point, sorted into the recycling pathway, the rest of the membrane and its contents become the next stage in the pathway. Therefore resident proteins are not expected to be present in early and late endosomes. Clearly the available biochemical and functional data indicate that early and late endosomes are successive stations of the endocytic system (see review by Gruenberg and Howell, 1989). The question of resident proteins however has not been satisfactorally answered to date. Stations of the pathway, for the sake of clarity, will herein be referred to as "steps".

(c) Sorting and Recycling of the Tf-Tf Receptor Complex

The next step following internalization and localization to the endosomal compartment is for R-L complexes to be "sorted". Sorting is the process by which the various R-L complexes and their components become targetted to other compartments or back to the cell surface. Located within the endosomal compartment is thought to be a region where at least most sorting occurs. This region is generally characterized as being receptor rich (Willingham and Pastan, 1985).

Recently some researchers have been able to identify, amongst a heterogeneous population of structures, a morphologically distinct system of thin anastomosing smooth-surfaced tubules with vesicles exhibiting various degrees of detachment (Geuze et al., 1983). These authors designated this structure by the acronym CURL (compartment of uncoupling of receptor and ligand) a name by which it is still widely referred today (Fig.1).

They observed that tubular aspects of this structure were in fact receptor-rich whereas vesicular aspects were devoid of receptor but instead showed ligand enrichment (Yamashiro et al., 1984). This observation indicated to them that sorting was indeed occuring in this structure. In the case of Tf administration to cells, Tf-Tf receptor complex enrichment is also seen in the tubular structures together with other receptors. Furthermore it was speculated that sorting for the transcytotic, degradative, receptor recycling and diacytotic pathways was coordinated through CURL. Whether the early endosome matures into CURL, fuses with other endosomes to become CURL (Gruenberg and Howell, 1989) or fuses with a preexisting CURL is not clear (Yamashiro et al., 1984).

Some have reported that the early endosome and CURL are one and the same (Stoorvogel et al., 1987) and that sorting may in fact occur at the early endosomal step. In these

experiments labeled ASGP and dTf were observed to be internalized and to rapidly colocalize to tubulo-vesicular structures identified as the early endosomal step but whose morphology was said, in an earlier study, to be very reminiscent of CURL (Geuze et al., 1983). At this point it was further observed that the two R-L complexes were immediately segregated which was taken to indicate that sorting was occuring at this early step in the endocytic pathway. This suggestion was corroborated by independent double-labelling studies in hepatocytes using ASGP and polymeric IgA where both ligands were observed to colocalize to early endosomes (within minutes) and segregate towards their respective destinations (Courtnoy, 1989).

A number of cell surface entities undergo a process of recycling where the receptor, or R-L complex in the case of the Tf-Tf receptor complex, once their intracellular function has been performed, are returned to the cell surface. From which point in the endocytic pathway recycling is initiated, however, is currently a matter of considerable controversy. The recycling kinetics for the Tf-Tf receptor complex can be very rapid (Goldstein et al., 1985; Wileman et al., 1985): For example, half-times of 3-5 minutes have been calculated for the release of apoTf from cells such as rabbit reticulocytes (Iocopetta and Morgan, 1983), Chinese hamster ovary (CHO) cells (Schmid et al., 1988) and hepatocytes (Ciechanover et al., 1983b). Since these kinetics are

consistent with recycling occuring soon after internalization, they have lead many to suggest that recycling of the Tf-Tf receptor complex may be occuring at the early endosomal stage (Schmid et al., 1988; Mueller and Hubbard, 1986; Hopkins and Trowbridge, 1983). According to the CURL model, the tubular segments, therefore, which contain the apoTf-Tf receptor complex and other receptors would then recycle to the cell surface within the tubular structures (Geuze et al., 1983). Indeed structures fitting this description containing receptor devoid of ligand have been observed closely associated with the plasma membrane (Willingham et al., 1984).

Therefore the morphological and the kinetic data both seem to point to the idea that at least recycling and perhaps sorting could be occuring at the early endosomal stage or at the step very shortly after internalization. Evidence that this is the case for the Tf-Tf receptor complex comes from Hopkins. He found that the vast majority of complexes, which had not embarked on an alternative recycling pathway, were indeed recycled to the cell surface directly from peripheral endosomal vesicles (Hopkins, 1983a). Whether these peripheral endosomal vesicles corresponded to early endosomes was not considered by the authors which may reflect the controversy which now surrounds the earliest steps of endocytosis.

The mechanisms regulating differential processing are unknown although some have suggested that sorting information

is contained within the amino-acid sequence of membrane receptors including Tf receptor (Harding et al., 1983; Ciechanover et al., 1983a). An apparently important contributor to the sorting process is the acidification of the endosomal compartment (Dautry-Varsat et al., 1983). This occurs as a result of ATP-dependent proton pumps which become activated during the formation of the endosome (see review by Steer and Ashwell, 1990) (Fig.1). Once the step or compartment of receptor enrichment has been achieved, the pH has been shown to drop to approximately 5.5 (Dautry-Varsat et al. 1983). These authors suggested that at this pH, most R-L complexes dissociate from one another as a result of a pH-dependent conformational change in the receptor that alters affinity allowing sorting to take place (Tietze et al., 1982; Mellman et al., 1986).

The apoTf-Tf receptor complex however is unique in its behaviour at this stage. Compartment acidification has been shown to be necessary and sufficient to cause the dissociation of Tf-bound iron atoms (Ciechanover et al., 1983c). These somehow diffuse through the vesicle membrane and presumably bind to ferritin through an unknown pathway, but it does not, however, cause apoTf to dissociate from its receptor (Klausner et al., 1983a; Dautry-Varsat et al., 1983). The complex remains intact because the dissociation rate of apoTf from the Tf receptor at pH 4.8-5.5 is much reduced compared to that at neutral pH. This unusual behaviour may be due to a

conformational change in the receptor and/or Tf and is suggested to be the reason why the apoTf-Tf receptor is sorted with other recycling receptors and returned to the cell surface (Klausner et al., 1983b; Dautry-Varsat, 1983) where dissociation is very rapid, $T_{1/2}$= 17 seconds (Ciechanover et al., 1983c). Indeed inhibitors of compartment acidification such as monensin prevent iron release from the Tf molecule and may in fact cause Tf-Tf receptor complex accumulation in endosomes (Stein and Sussman, 1986).

(d) Tf-Tf Receptor Complex Recycling and the Trans-Reticular Golgi

In addition to the main short recycling pathway, numerous reports describe a second longer recycling route involving elements of the Golgi apparatus, particularly the trans-reticular Golgi (TRG) (Willingham and Pastan, 1985;see review by Gruenberg and Howell, 1989) (Fig.1). In fact an increasing number of biochemical studies are compatible with a second slow recycling pathway that would not represent the main recycling route for Tf-Tf receptor complexes (see review by Khun et al., 1990).

This is consistent with resialation studies using asialotransferrin. Regoeczi et al. (1982) observed in the rat hepatocyte that asialoTf was recycled in a resialated form and concluded that the Golgi must be part of the Tf-Tf receptor recycling pathway since sialyltransferase is confined to this organelle. The results however also revealed that resialation

was incomplete and followed very slow kinetics (Regoeczi et al., 1982). Although there seems to be evidence for the existence of both pathways (Hopkins, 1983b; Yamashiro et al., 1984; Mueller and Hubbard, 1986), there also seems to be strong indications that the TRG associated pathway is relatively minor (Sibille et al., 1986; Ajioka et al., 1986).

(e) The Degradative Pathway

The final step of the endocytic pathway for a number of ligands such as asialoglycoproteins, insulin, epidermal growth factor (EGF), low density lipoprotein (LDL) (Dautry-Varsat et al., 1983) and some receptors such as that for EGF (see review by Steer and Ashwell, 1990), in the case of down-regulation, will follow the degradative pathway (Fig.1). The exception is the apoTf-Tf receptor complex which escapes this route (Willingham et al., 1983; Ciechanover et al., 1983b) with missorting reported to sometimes result in less than 0.3% degradation (Fuller and Simons, 1986). Although it is clear that the degradative pathway is not a characteristic step of apoTf-Tf receptor recycling, there is controversy concerning the steps of the degradative pathway for the other ligands and receptors.

There is agreement that as a result of sorting the ultimate fate of these ligands is degradation in the lysosomal compartment. According to the CURL model, the vesicular part of CURL, which was shown to be rich in ligand and receptors

destined for degradation, somehow becomes a secondary lysosome (Geuze et al., 1983). It is unclear, however, what the steps are between CURL and the lysosomal steps. Schmid et al. (1988) maintain that the late endosome is functionnally distinct and preceeds the lysosome step. A structure that may be intermediary between these two steps and possibly correspond to this "transformation" of CURL is the multivesicular body (MVB) (Felder, 1990). Since the MVB and late endosome are often described to have similar features, such as a complex internal membranous component, it would seem that these designates either refer to the same structure or to two distinct compartments related in a precursor/product fashion (see review by Gruenberg and Howell, 1989).

In support of this notion Griffiths et al. (1988) have claimed that entities destined for down-regulative degradation such as the EGF receptor sequentially pass from the late endosomal step to then localize to the multiple vesicles within the MVB. This MVB would then be involved in certain processing events leading to the lysosomal compartment step. The exact nature of these "processing" steps remains unclear. However, they have been shown to include progressive acidification, the development of acid-phosphatase positivity concomitant with a progressive loss of cation-dependent mannose-6-phosphate receptor (CI-MPR) activity (see review by Gruenberg and Howell, 1989), both of which seem to be characteristic of lysosomal hydrolase targetting from the Golgi.

(f) Summary of the Tf-Tf Receptor Complex Endocytic Route

It is therefore generally understood that the endocytic and recycling pathways can be defined as an orderly itinerary of discrete membrane bound compartments through which R-L complexes circulate and are differentially processed according to their specific function (Hubbard, 1989). The progressive acidification of steps or compartements of the endocytic pathway, from the earliest endosome to the lysosome, is nevertheless seen to support the idea of functional heterogeneity of the various steps (Merion et al. 1983; Kielian et al., 1986; Murphy et al., 1983).

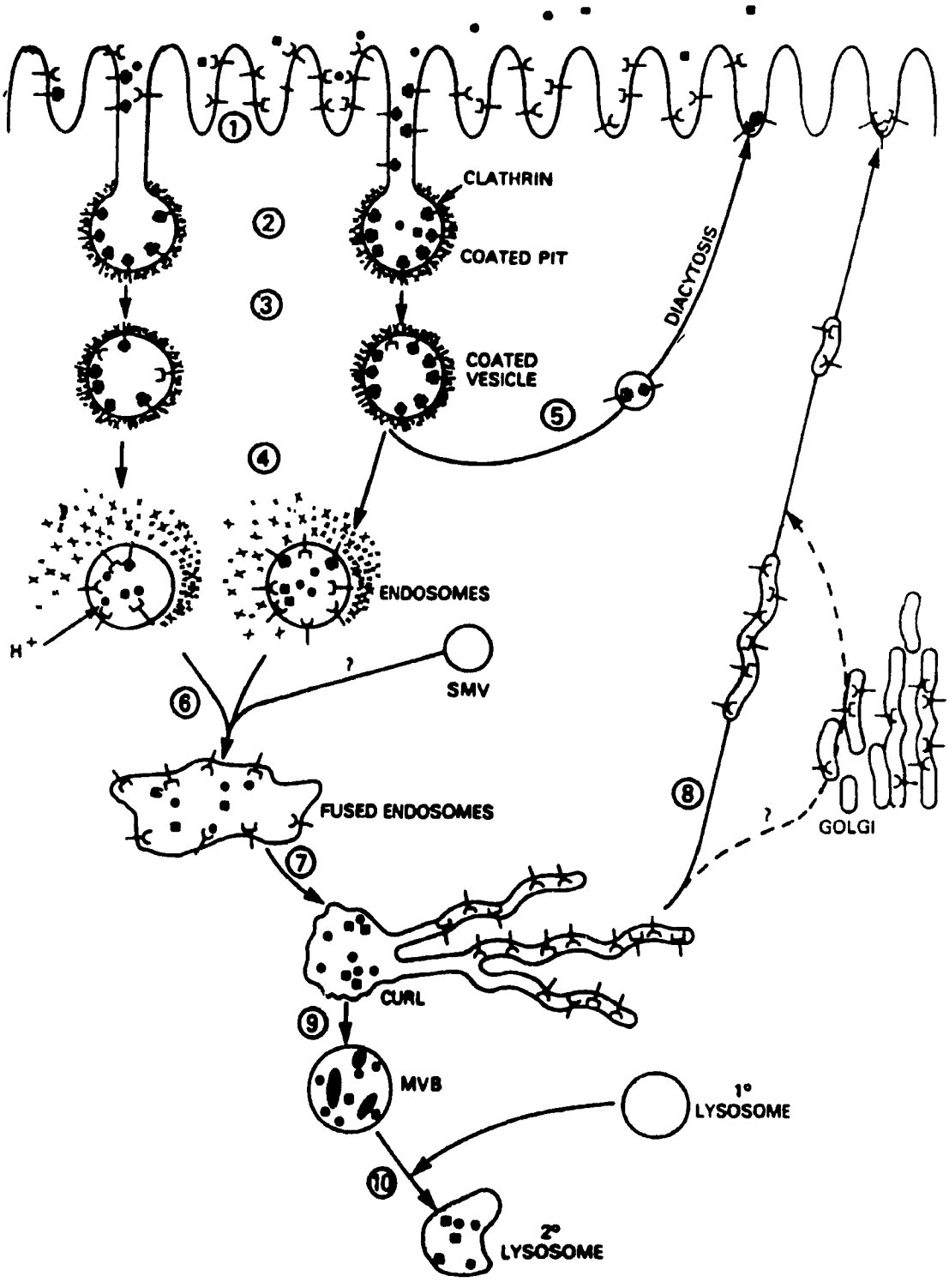
The specific pathway followed by the Tf-Tf receptor complex, according to current understanding, can be summarized in (Fig.1): 1. Like many of the ligands mentioned, Tf binds to its receptor in a pH-dependent manner and is constitutively internalized, along with other R-L complexes, by the inbudding of clathrin-coated pits. 2. The internalized vesicles containing the R-L complexes, including the diferric Tf-Tf receptor complex, lose their clathrin coats and become associated with the endosomal compartment. 3. Proton-pump dependent acidification of the early endosome or CURL causes: a) the two iron atoms bound to the Tf molecule to dissociate and b) all ligands except for apoTf to dissociate from their receptors. 4. Within the endosome or CURL, sorting of the contents results in the apoTf-Tf receptor complex being directed along either or both of two recycling pathways i.e.

the proposed short pathway from the endosomal step or the proposed pathway through the TRG. 5. At no time is the apoTf-Tf receptor complex targetted to the lysosomal compartment as are a number of ligands.

It is notewhorthy that a novel new proposal has recently been put forth by Hopkins et al. (1990) to explain the endocytic pathway. They used fluorescently labeled Tf and EGF to follow their intracellular route in living cells using video recording at low light. Instead of observing the label moving through discrete endocytic compartments, rather, they seemed to show that the endosomal apparatus consisted of a continuous tubular reticulum through which pulses of receptors move at high speed. Their model therefore suggests that the endosomal apparatus is a permanent but very dynamic organelle where processes do not occur at discrete steps but continuously and in a highly integrated manner (Hopkins et al., 1990). No supporting data is yet available to corroborate these claims but it shall be interesting to see the types of developments that research in this direction may bring to the field.

FIGURE 1

Schematic drawing depicting structures and pathways presently being considered as part of receptor-mediated endocytosis. (1) Circulating ligands (solid circles, solid squares) bind to plasma membrane receptors. (2) The ligand-receptor complexes cluster in coated pits, which invaginate (3) to form coated vesicles. Very rapidly, the coated vesicle loses its clathrin coat and becomes an endosome (4). Some ligand-receptor complexes return to the surface membrane (diacytosis) (5). The endosomes fuse with each other and/or other smooth membraned vesicles (SMV) (6). During release of the clathrin coat, membrane fusion, or both, protons are pumped into the intravesicular space and initiate dissociation of ligand from receptor. The majority of ligands remain within the acidified vesicular portion of the compartment of uncoupling of receptor and ligand (CURL) (7). The unoccupied receptor molecules concentrate in the membranes of tubular structures of CURL, which mediate the recycling of receptors back to the surface membrane (8). The recycling may involve a transient pathway through portions of the Golgi complex. The ligand molecules and some receptors within the vesicular portion of CURL enter multivesicular bodies (MVB) (9), which ultimately fuse with primary lysosomes (10). Degradation of the contents occurs in the resultant secondary lysosomes. The transferrin-transferrin receptor complex has been observed to be involved in all steps except (9) and (10). (Adapted from Steer,C.J. and Ashwell, G., 1990).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COATED PIT
COATED VESICLE
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ENDOSOMES
H+
SMV
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GOLGI
CURL
MVB
1° LYSOSOME
2° LYSOSOME

Section 5 The Rat Seminiferous Epithelium

(a) Epithelial Structure

The rat testis consists of 25 to 30 tubules measuring approximately 350 μm in diameter and an average of 32.2 cm in length, all contained within a thick fibrous capsule, the tunica albuginea (Clermont and Huckins, 1961). Each tubule is an anastomotic loop which is continuous with the rete testis which in turn is connected to the ductuli efferentes and the epididymis. The space between the tubules is called the interstitium and contains the testosterone-secreting Leydig cells, blood vessels, nerves and lymphatics. The seminiferous tubule consists of a limiting membrane composed of one to two layers of myoid cells, a lining epithelium and a central lumen (Fig.2).

The epithelium lining the tubules has been termed the seminiferous epithelium and is made up of two basic cellular components: Sertoli cells, a somatic non-proliferative component which is made up of a single, terminally differentiated cell type and a spermatogenic component which is composed of a population of proliferating germinal cells at various phases of a complex differentiation process.

The somatic component of the seminiferous epithelium, the Sertoli cell, extends from the limiting membrane to the lumen and is known to be evenly spaced along the length of the

seminiferous tubule. In a tubular cross section the spermatogenic cells give the epithelium a stratified appearance which displays three general germ cell types from LM to lumen: spermatogonia which rest on the limiting membrane, spermatocytes and spermatids (Fig.2).

Finally the epithelium is divided into two compartments by virtue of junctional specializations that link adjacent Sertoli cells (Dym and Fawcett, 1970) (Fig.2): The basal compartment containing spermatogonia and young spermatocytes and the adluminal compartment containing later spermatocytes, spermatids and spermatozoa. The functional significance of these junctional complexes will be discussed later in this section.

(b) Spermatogenesis

Spermatogenesis is the process by which diploid spermatogonial stem cells differentiate into haploid spermatozoa. Upon close examination of a tubular cross section from a mature rat, one can identify one to two superimposed layers or generations of spermatogonia, spermatocytes and spermatids which gives the epithelium the stratified appearance. These generations however do not occur at random and in a given tubule cross section, in the rat, one will generally always see the same generations associated with one another. Such a cellular association is called a stage. In the rat 14 such stages have been defined, each composed of

several characteristic generations of germ cells, a generation being defined as a group of cells at the same step of development (Leblond and Clermont, 1952b).

If one, for example, observes, in time, a given cross-section of a seminiferous tubule, one will witness the successive appearance of each of the 14 cellular associations or stages. This constitutes one cycle of the seminiferous epithelium (Leblond and Clermont, 1952b) (Fig.3). The duration of one cycle, that is the time required for a spermatogenic generation to mature into another, is species-specific and seemingly invariable for that species. In the case of the rat the cycle time is 12.9 days. Hence the time it takes for a spermatogonia, committed to differentiation, to become a spermatozoon is 51.6 days in the rat (Clermont and Harvey, 1965).

Finally spermatogenesis can be divided into three distinct phases: 1. Where spermatogonia undergo a series of mitotic divisions which give rise to spermatocytes and spermatogonial stem cells which in turn renew the spermatogonial population. 2. Where primary and secondary spermatocytes undergo two successive meiotic divisions giving rise to haploid spermatids. 3. Where spermatids follow a dramatic nineteen step differentiation process termed spermiogenesis to finally give rise to mature spermatozoa.

(c) Spermiogenesis

As can be seen in Fig.3, the stages of the cycle of the seminiferous epithelium are indicated by roman numerals (I to XIV). The "steps" of spermiogenesis however are indicated in Arabic numerals (1 to 19) (Fig.4). Spermiogenesis involves the transformation of young haploid germ cells or early round spermatids into mature spermatozoa. Because the nuclear changes involved in spermiogenesis are relatively distinct, the steps describing this process, as proposed by Leblond and Clermont (1952b) are considered by these authors as the most important guide for the classification of the cycle of the seminiferous epithelium.

For the purposes of description, the 19 steps of spermiogenesis were divided, by the above authors, into 4 phases: The Golgi, cap, acrosome and maturation phases (Leblond and Clermont, 1952a). The Golgi and cap phases (steps 1-7) are characterized, at the light microscope level, by a well demarcated Golgi zone, the appearance of proacrosomic granules associated with the nucleus and the eventual elaboration of the cap-like acrosomal system. At the antipode one can also see the assembled centrioles and the early development of the growing flagellum. Germ cells which have evolved to this stage are generally referred to as round spermatids (Leblond and Clermont, 1952b).

During the acrosome phase (steps 8-14), the nucleus and covering acrosomic system undergo their most dramatic morphological transformation: As the nuclear contents condense and form a more compact mass, the shape of this organelle takes on a species-specific shape. In the rat the nucleus acquires a falciform or sickle shape and remains closely associated with the acrosomic system. At the caudal end of the cell, the bulk of the cytoplasm is seen to decentralize and accumulate around the flagellum which is attached to the nucleus and is continuing to elongate. The Golgi apparatus becomes displaced from the perinuclear region and relocates to the cytoplasmic lobule. Germ cells which have evolved to this stage are generally referred to as elongated spermatids. The maturation phase (steps 15-19) consists of the final morphological transformations which result in the formation of the mature spermatozoa (step 19). At this time the spermatozoon sheds its periflagellar cytoplasm to yield the residual body and is finally released into the tubule lumen in a process called spermiation (Leblond and Clermont, 1952b).

(d) Sertoli Cells: Structure and Function

Extending from the limiting membrane to the tubule lumen, the Sertoli cell is a columnar cell with numerous apical and lateral sheat-like processes which envelope nearby spermatogenic cells. Its nucleus is either ovoid or piriform

with a prominent nucleolus and one or more characteristic deep infoldings. Sertoli cells constitute the framework of the seminiferous epithelium thus lending a tubular cross section its highly ordered stratified appearance. Generations of spermatogenic cells are continuously pushed through the interstices of Sertoli cell cytoplasmic extensions as they differentiate and are eventually extruded into the tubular lumen as mature spermatozoa (Fig.2).

For the most part, adjacent Sertoli cell cytoplasmic processes are separated by a space of 150-200 Å (Dym and Fawcett, 1970). However, at the level of the the junctional complexes constituting the BTB, they approach to within an exceedingly close 20 Å forming an exclusion site that is morphologically unique (Dym and Fawcett, 1970) (Fig.2). The BTB forms a belt-like structure parallel to the tubule limiting membrane which defines the compartmentalized character of this epithelium and constitutes an impermiable barrier to virtually any blood-borne element (Dym & Fawcett, 1970).

The functional significance of the BTB is three fold: 1. It acts as a permiability barrier that allows Sertoli cells to create an adluminal extracellular environment that is conducive to germ cell maturation (Tuck et al., 1970; Skinner and Griswold, 1983). 2. It acts in a preventive role by disallowing the passage of germ cell proteins which would be

identified as foreign by the immunological system (Setchell, 1980). 3. It has a dynamic role which permits the passage of clones of preleptotene spermatocytes from the basal to the adluminal compartment at the level of the junctional complexes (Dym and Fawcett, 1970).

The Sertoli cell has also been shown to function as a phagocyte. Immediately preceding their release into the tubular lumen step 19 spermatids shed a globular mass of excess cytoplasm, or residual body (RB), which is quickly phagocytosed by the apical Sertoli cell (Fig.2). Besides RBs the Sertoli cell was also shown to regulate the area of its own adluminal plasma membrane via the same mechanism. Morales et al. (1985) demonstrated that lysosomes formed as a consequence of fluid-phase endocytosis fused with the endosome containing the RB resulting in the degradation and absorption of its elements (Morales et al., 1986a).

The Sertoli cell's primary role, it is thought, and the one which is by far the most studied is its involvement in the maintenance of spermatogenesis (see review by Griswold et al., 1988b). That is, it appears that Sertoli cells function in a nurturing capacity and thus, like a mother, provide most, if not all, the means of survival for germ cells. The manner in which Sertoli cells fulfill this role and the nature of these means is presently the subject of intense study. This role of Sertoli cells still remains poorly understood.

In the study of the seminiferous epithelium it has become clear that developing germ cells are invariably dependent on iron for their maturation. This dependence is thought to be linked, as is the case in proliferating cell lines in general, to the synthesis of important iron containing enzymes and cofactors necessary for fundamental processes such as nucleic acid synthesis and respiration. Those germ cells located in the adlumenal compartment however do not have access to iron bound to sTf due to the presence of the BTB. It has been shown that Sertoli cells as well as spermatogonia express receptor for Tf and thus are capable of providing for their own iron needs (Morales and Clermont, 1986b). The Sertoli cell therefore, acting in its nurturing capacity, must possess a mechanism for supplying adlumenal germ cell with iron.

The Sertoli cell is known to secrete a number of proteins including ceruloplasmin, sulfated glycoprotein 1 and 2, androgen binding protein and tTf (Griswold, 1988a). These adlumenally secreted proteins are thought, at least in part, to mediate the Sertoli cell's role in the maintenance of spermatogenesis (Griswold, 1988a). Testicular Tf, which comprises up to 15% of Sertoli cell protein secretion (Skinner and Griswold, 1980), is currently being implicated in a developing model of iron delivery to adlumenal germ cells (Fig.5). In this model the Sertoli cell is described as the gateway mediating the flow of iron from the plasma to the

adlumenal germ cells with tTf functioning as the means by which the Sertoli cells accomplish this task.

Morales and Clermont (1986) have shown that Sertoli cells in vivo are capable of receptor-mediated endocytosis (RME) at their basal aspect (Fig.5). Here, it was shown, that Tf receptors bind diferric sTf and the complex is internalized. They went on to show that, in a similar way to other systems, [^{125}I]-Tf then localized to a discrete endosomal compartment. The pulse of labeled apoTf was then observed to be quickly released into the medium with intermediate steps apparently involving small spherical and tubular-shaped vesicles visible at the electron microscope level. At no time was the label seen to enter an acid phosphatase-positive compartment or lysosomes. In order to ascertain whether this putative endocytic pathway was in fact distinct, native ferritin was simultaneously administered but instead injected into the lumen of the seminiferous tubule via the rete-testis. The native ferritin was observed to internalize by fluid-phase endocytosis at the lumenal pole of the Sertoli cell and to localize to a separate endosomal compartment which eventually evolved into MVBs and secondary lysosomes (Morales and Clermont, 1986b).

It was first shown by Skinner et al. (1984) that cultured Sertoli cells synthesized and secreted a tTf that was essentially identical to sTf and possibly the product of the

same gene. Some controversy arose concerning this preliminary finding when Lee et al. (1986) argued that Sertoli cells, in vivo, do not normally produce Tf and that culture conditions artificially activated the Tf gene. Hence they claimed that the results obtained by the above researchers were artifactual to *in vitro* conditions. In response Morales et al. (1987a) employed an in vivo system where paraffin-embedded sections of rat testis were subject to both liquid and in situ hybridization with cRNA probes specific for tTf mRNA. They demonstrated that exclusively Sertoli cells labeled positively for Tf mRNA. Together with supporting data, derived from the same series of experiments it was concluded that Sertoli cells do in fact express tTf.

Once iron detaches from Sertoli-cell-internalized sTf, presumably in the endosomal compartment, it is not known how it crosses the endosomal wall nor what its immediate fate is. It is known that Sertoli cells in culture express ferritin but it is not known whether or how iron interacts with it. Nevertheless the available data strongly suggests that Sertoli cells use iron, sequestered from sTRF, to supply adluminal germ cells, employing tTf as the vehicle (Fig.5). In an elegant series of in vivo experiment by Morales et al. (1986b; 1987b) it was in fact shown that newly synthesized [^{35}S]-labeled tTf was secreted in a vectorial fashion towards the adluminal compartment. It was concomitantly shown that [^{55}Fe], internalized as sTf at the basal pole of the Sertoli

cell, also demonstrated the same unidirectional flow. The conclusion therefore was that iron, acquired from sTf, became attached to newly synthesized tTf and was secreted adluminally (Fig.5). A crucial step in this process however still remains undetermined and that is the mechanism by which tTf is coupled to iron. Is this process mediated through ferritin, through another iron chelator or is the iron directly chelated by newly synthesized tTf? New and creative investigative techniques shall be required to answer this question.

As shown in Fig.5 the steps involved in the suggested pathway for adluminal germ cell iron delivery are: 1. Sertoli cells internalize diferric sTf at their basal pole by receptor-mediated endocytosis, 2. Iron is liberated and the apoTf-Tf complex is recycled back to the interstitial space where apoTf is released, 3. The iron atoms are somehow coupled to tTf which is then released into the adluminal compartment. According to this developing model therefore, adluminal germ cells must be capable of utilizing iron supplied to them in the form of tTf although there exists no evidence to directly support this.

Early work by Holmes et al. (1983) indicated that Tf, in vitro, specifically bound Sertoli cells, early, mid and late pachytene spermatocytes with K_d values in the nM range. Sylvester and Griswold (1984) soon after supported these findings in an indirect immunofluorescence study showing that

Tf receptor was expressed on germ cells ranging from primary spermatocytes to early spermatids. Using northern blot analysis, Roberts and Griswold (1990) further demonstrated that mRNA for Tf receptor was expressed in spermatocytes and elongating spermatids but this time failed to detect this mRNA species in round spermatids. In contrast immunohistochemical studies carried out by Brown (1985) do show Tf receptor to be expressed on the surface of round spermatids in addition to other germ cell classes. Furthermore he observed that the immunolabeling of the Tf receptor decreased as spermatocytes matured into round spermatids. He found no labeling in elongated spermatids or mature spermatozoa. Therefore there seems to be conflicting results concerning the final steps of the iron delivery pathway to adluminal germ cells.

Sylvester and Griswold (1984), using indirect immunofluorescence techniques, suggested that tTf may bind to spermatids and perhaps somewhat to spermatozoa. No immunolabeling for Tf was seen on spermatocytes. They explained this discrepancy with their above Tf receptor results, where spermatocytes were shown to carry Tf receptor mRNA, by postulating that Sertoli cells may selectively target tTF to different germ cell populations depending on the stage of the cycle (Leblond and Clermont, 1952b).

The work presented herein will attempt to clarify the above and propose what the final steps of this iron delivery model may be following the release tTf by the Sertoli cell.

(e) Sertoli Cell Regulation and Testicular Transferrin

It's been mentioned that Sertoli cells play a central role in the maintenance of spermatogenesis. One approach that is presently being taken to clarify the nature of this role is to determine how certain factors may regulate Sertoli cell function. It has been known for some time that the hormones FSH and testosterone and nutritive factors such as retinol and iron are necessary for the control and maintenance of spermatogenesis respectively. Early work by Clermont and Harvey (1965) confirm that while hormones such as gonadotropins and androgens act in a permissive role, they do not influence the rate of spermatogenesis per se. Furthermore the effect of testosterone on spermatogenesis was investigated and similar conclusions were drawn (Clermont and Harvey, 1967).

Recently the regulatory roles of some of these substances, vis-a-vis Sertoli cell protein synthesis, have been assessed. Roberts and Griswold (1990), in experiments where various combinations of FSH, testosterone, insulin and retinol were administered to Sertoli cells in culture, reported that while Tf receptor mRNA levels were unaffected by the treatment, tTf mRNA levels were "sensitive" to hormones. In another recent study it was shown that retinol, which is thought to interact with Sertoli cells, modulated levels of tTf mRNA in Sertoli cells in vivo (Hugly and Griswold, 1987; Morales and Griswold, 1991). The molecular nature of this

interaction remains to be shown (Erikson et al., 1987).

Studies involving iron as a possible regulator of Sertoli cell function have also been carried out. In conditions of iron deprivation Sertoli cells in culture were found to express levels of Tf receptor mRNA that were 7 times higher than control cultures. Levels of tTf mRNA and ferritin mRNA, however, remained unaffected by these conditions (Roberts and Griswold, 1990). These results may indicate that the mechanism, involving the mutually counterbalancing regulation of Tf receptor mRNA and ferritin mRNA by iron and IRE, is not applicable to Sertoli cells.

The above experimental results imply that each of the agents mentioned are important for spermatogenesis because they influence Sertoli cell secretion patterns of proteins known to be important for the maintenance of this process (Griswold, 1988a). The question of the Sertoli cell's role in spermatogenic regulation, however, still remains unanswered. Attention has therefore been turned to the suspected association of germ cell and germ cell-Sertoli cell interactions with Sertoli cell function and regulation of spermatogenesis.

During the course of their experiments, Roberts and Griswold (1990) made the observation that the increased Tf receptor mRNA response to iron deprivation paralleled that in

a control proliferating cell line. It is generally known that cells entering a proliferating state express higher levels of Tf receptor compared to non-proliferating cells (Enns et al., 1988). Since Sertoli cells are non-proliferative in primary culture, it is possible, according to these authors, that this relatively dramatic response to iron deprivation was associated to the Sertoli cell's role in iron delivery to germ cells. Stallard and Griswold (1990) conducted studies to address this point. They showed that Sertoli cells, cocultured with germ cells, expressed 3 times higher levels of Tf mRNA relative to control Sertoli cell in cultures as demonstrated by solution hybridization and Northern blot analysis. This result was taken to suggest that germ cells, perhaps via secretions such as paracrine factors, somehow control iron flow through the regulation of Sertoli cell function (Stallard and Griswold, 1990). Another similar study where Sertoli cells were cocultured with enriched populations of germ cells also demonstrated that this treatment markedly increased secretion of tTf. Furthermore spent media of these germ cell populations exhibited the same effect thus reenforcing the idea of germ cell-regulation of Sertoli cell function (Le Magueresse et al., 1988).

Based on these observations the notion of germ cell-regulation of Sertoli cell function has started to gain popularity and studies recently carried out to explore these possibilities appear to be positive. It has been observed

that levels of Sertoli cell proteins are differentially regulated according to the stages of the cycle of the seminiferous epithelium (Morales et al., 1987a; Roberts and Griswold, 1990). It was demonstrated that tTf is expressed at its highest level, by Sertoli cells, at stages XIII and XIV of the cycle while levels of SGP-2, another Sertoli cell secretion product used as a control, remained unchanged. This surge exactly coincided with the two successive meiotic divisions of spermatocytes (Morales et al. 1987a) implying once again that germ cells appear to regulate their own supply of iron by influencing Sertoli cell expression of tTf and Tf receptor.

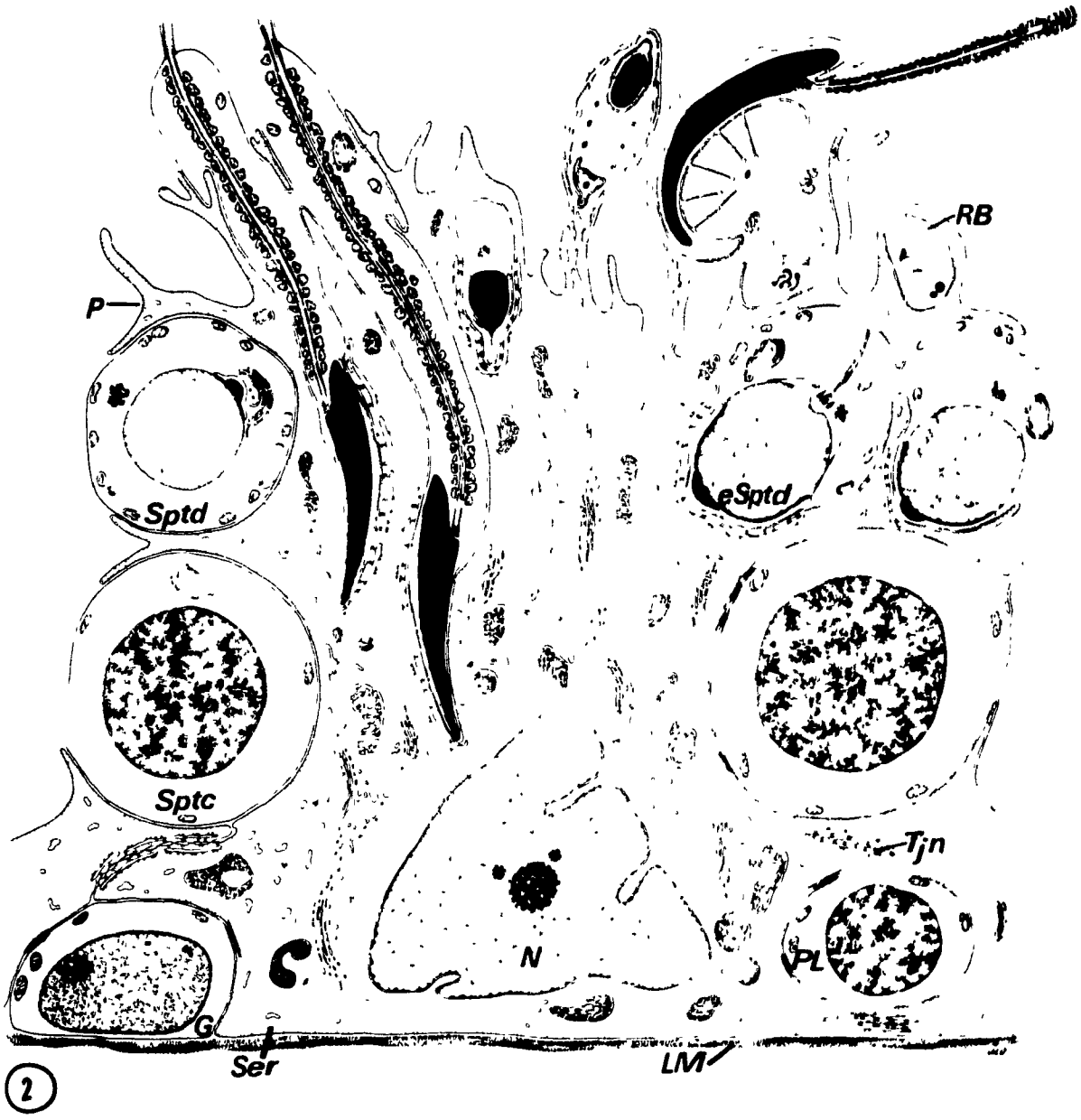
Stage-specific regulation of Tf receptor mRNA has also been documented (Roberts and Griswold, 1990). In addition to confirming the above results using northern blot analysis of Sertoli cells in culture, these authors observed that Sertoli cell Tf receptor mRNA expression peaked at stages VII-VIII while that in total germ cells remained unchanged. Hence the increase appeared to be specific to Sertoli cells. It was therefore suggested that the Sertoli cell undergoes a period of iron loading during stages VII-VIII and an intense period of iron delivery at stages XIII-XIV when pre-meiotic spermatocytes presumably most require iron. These authors have also reported that Tf production by Sertoli cells seems to correlate linearly with germ cell number, implying perhaps that Sertoli cell tTf levels are limited by the number of

post-meiotic germ cells a testis can support (Griswold et al., 1987).

The emerging picture therefore, is that indeed hormones and essential nutritive factors are important for the maintenance of spermatogenesis and that their cellular target is most cases the Sertoli cell. The regulation of the expression of Sertoli cell proteins however, which are considered important for spermatogenesis, appears to be, according to data amassed to date, most clearly attributable to spermatogenic cells.

FIGURE 2

Schematic diagram of the structure of the rat seminiferous epithelium. The base of the seminiferous epithelium consists of three to four layers of myoid cell called the limiting membrane. Resting on the limiting membrane are various types of spermatogonial stem cell and non-proliferating Sertoli cells. Sertoli cells are tall irregularly-shaped somatic cells which extend to the tubular lumen and send numerous fine cytoplasmic processes in the interstitial spaces between surrounding germinal cells. Adjacent Sertoli cells are linked by tight junctional complexes which divide the seminiferous epithelium into two physically and functionally different compartment: The basal compartment which houses the various spermatogonia stem cells and the adluminal compartment which contains spermatocytes and spermatids. **Ser**, Sertoli cell; **N**, Sertoli cell nucleus; **G**, spermatogonia; **PL**, preleptotene spermatocyte; **Sptc**, spermatocyte; **Sptd**, spermatid; **eSptd**, elongating spermatid; **P**, Sertoli cell process; **LM**, limiting membrane; **Tjn**, tight junctional complexes; **RB**, residual body.



(2)

FIGURE 3

Schematic diagram depicting the spermatogenic cell associations of the 14 stages of the cycle of the seminiferous epithelium in the rat. Each column, designated by a Roman numeral, represents spermatogenic cell types which constitute the cellular associations found in cross-sections of seminiferous tubules. Steps in the development of spermatids (or spermiogenesis), which are used to identify the stages of the cycle, are designated by Arabic numerals 1 to 19. Letter initials designate the various maturational states of spermatogonia and spermatocytes: A_1, A_2, A_3 and A_4, four generations of type A spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocyte; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; Di, diplotene spermatocytes; II, secondary spermatocytes. Capital M designates mitotic divisions of spermatogonia. The duration of each stage, in hours, is also given.

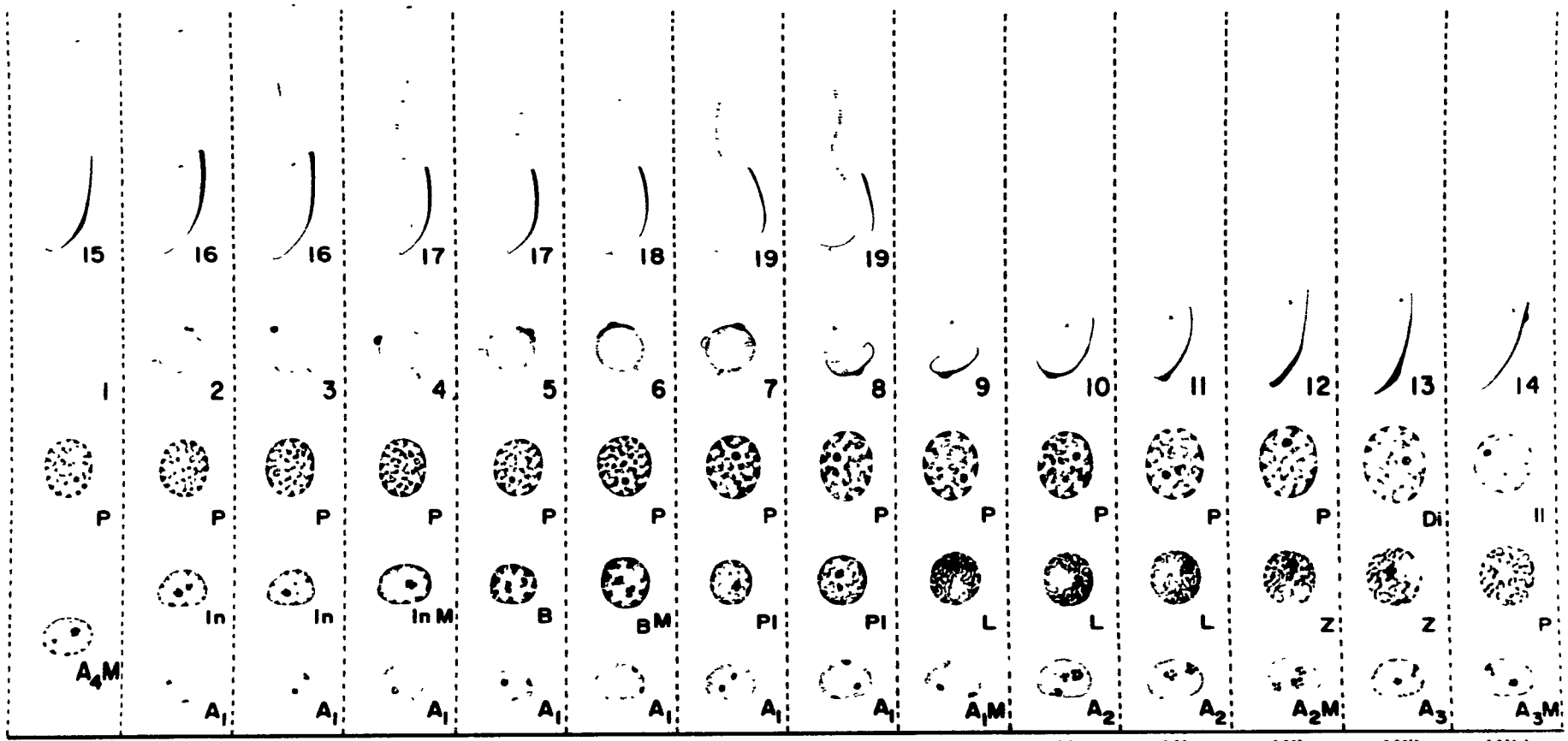

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FIGURE 4

Schematic representation of spermiogenesis in the rat illustrating the gradual differentiation of young spermatids into mature spermatozoa. The 19 steps, which are defined by changes in the structure of the nucleus and acrosome, can be divided into 4 phases: The **Golgi phase** (steps 1-3), the **cap phase** (steps 4-7), the **acrosome phase** (steps 8-14) and the **maturation phase** (steps 15-19). The numbers given to each of the steps correspond to those proposed in the classification of Leblond and Clermont (1952). **RB**, residual body.

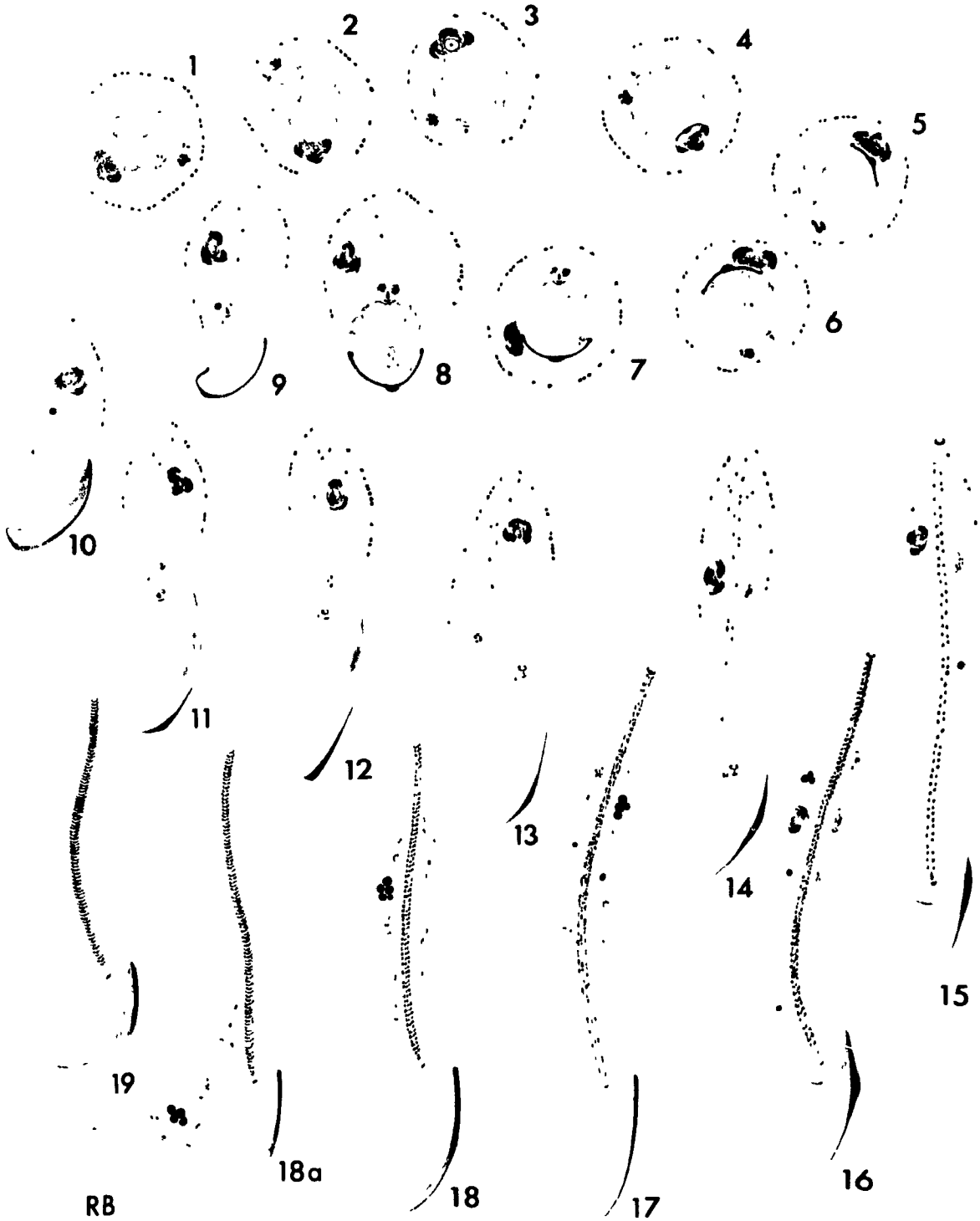
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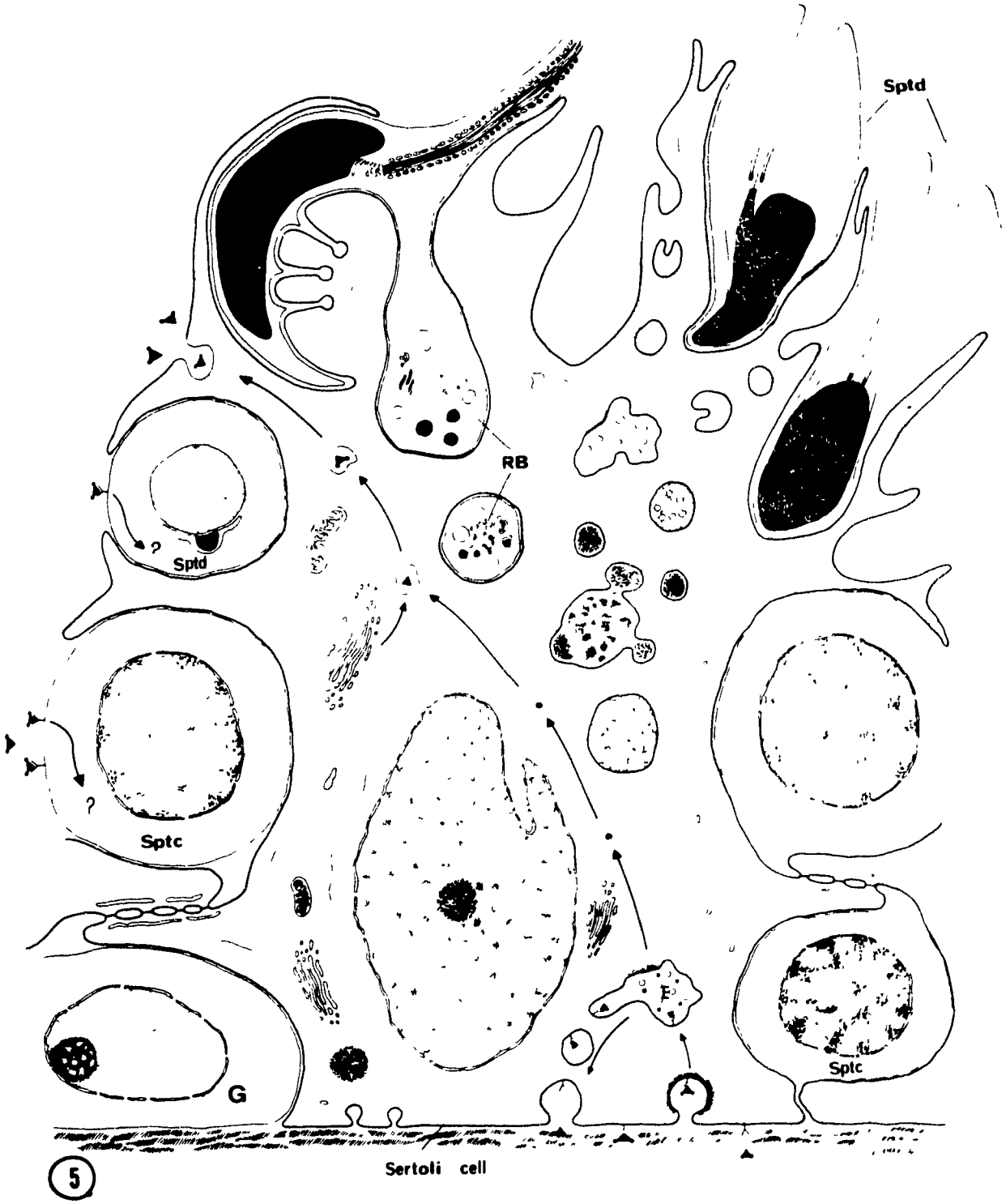
FIGURE 5

Schematic representation of the iron delivery pathway in Sertoli cells of the rat seminiferous epithelium as it was previously understood. At the lower aspect of the drawing is the first step of iron delivery: Receptor-mediated endocytosis of diferric serum transferrin (Tf) at the basal pole of the Sertoli cell and recycling of the apoTf-Tf receptor complex back to the basal plasma membrane. Proton pump acidification of the endosome containing the internalized complex results in the release the two iron atoms from each Tf molecule. It is then known that these atoms become associated with testicular Tf. The number and nature of the steps between the release of iron and its coupling to testicular Tf still remains to be determined. At the upper aspect of the drawing it is shown that the diferric testicular Tf is secreted by the Sertoli cell into the adluminal compartment. Its fate, once secreted, remained unclear. (▲), serum/testicular transferrin; (•), iron; **G**, spermatogonia; **Sptc**, spermatocyte; **Sptd**, spermatid; E, endosome; RB, residual body.



5

MATERIALS and METHODS

1. Isolation of Germ Cells from Mature Rat

a) **Germ cell preparation for mRNA isolation:** One adult male Sprague-Dawley rat was sacrificed by CO_2 inhalation and the testis removed by a scrotal incision. The testis were then washed with Hanks Balanced Salt Solution (HBSS), decapsulated and lightly minced. The tissue was then suspended in 10 ml HBSS containing 4 mg collagenase, 6.64 mg DNase, 6 mM sodium pyruvate and 2 mM lactate. The suspension was then incubated at 37°C for 10 minutes followed by an additional 15 minutes incubation period with 18 mg trypsin, all the time with intermittent agitation. The supernatant containing the germ cells was then spun at 700xg for 5 minutes and the cells resuspended in 10 ml HBSS containing 18 mg trypsin inhibitor and 6.64 mg DNase.

The cell suspension was then spun again at 700xg for 5 minutes, resuspended in 1% BSA-HBSS and sequencially filtered through 80 μm and 35 μm mesh to remove cellular debris. It was finally layered into a staput chamber and left to sediment for 2 hours in a 400 ml linear 1-4% BSA density gradient with HBSS. Twenty 10 ml fractions were collected and each examined by contrast-phase microscopy for identification and assessment of germ cell-type percentage per fraction. Only those fractions containing pachytene spermatocytes, round spermatids

(steps 1-8) and elongated spermatids (steps 9-18) in purities of at least 90% were pooled and used to isolate their respective contents of RNA.

b) **Germ cell preparation for binding assays:** The tissue was removed from the animal and processed in the same way as above. It was however suspended in 10 ml HBSS containing 1300 Kunitz/ml DNase, 350 units/ml collagenase, 1 mg/ml trypsin inhibitor, 6 mM sodium pyruvate and 2 mM lacate. The suspension was then incubated as above for 20-30 minutes with continuous agitation. It was then filtered as above and directly layered into a similar gradient containing 50 mg trypsin inhibitor, 6 mM sodium pyruvate, 2 mM lactate and also left to sediment for 2 hours. Fractions were collected, identified, their percentages assessed as above and finally put on ice until the experiment begun.

2. RNA Isolation

All RNA samples were obtained from freshly isolated tissue and processed as specified above. The RNA was extracted by dissolving the tissue in 1 ml of lysis buffer (5 M guanidine monothiocyanate, 10 mM EDTA, 50 mM Tris-HCl, 8% beta-mercapto-ethanol, pH 7.5) and homogenizing the mixture with a polytron homogenizer. Once dissolved the homogenate was precipitated with 25 volumes of 4 M $LiCl_2$ at -20°C overnight. The mixture was then spun at 14000xg for 30 minutes at 4°C and the pellet resuspended in 1 ml

homogenization buffer (150 mM NaCl, 0.5% SDS, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5). In order to dissolve protein away from the nucleic acid 100 μg/ml proteinase K was added and the solution incubated at 45°C for 30 minutes.

The resulting proteinacious debris was then removed by three consecutive equal volume phenol/chloroform (50 ml chloroform, 50 ml phenol + an overlay of 10 mM Tris pH 8, 1 mM and 0.4% beta-mercapto-ethanol) extractions of the nucleic acid. The resulting extract was then precipitated with 2.5 volumes of ethanol for 24 hours at -20°C, spun at 14000xg rpm for 20 minutes and the pellet finally resuspended in resuspension solution (30% diethyl pyrocarbonate-treated water, 69% ethanol, 1% NaCl)n and stored at -70°C (Cathala et al. 1983).

3. mRNA Blotting

1.2% agarose-formaldehyde gels, used in all cases, were run in an LKB electrophoresis bath in running buffer containing 10% HEPES-EDTA and 16.2% formaldehyde. The samples were mixed with sample buffer, which contained formaldehyde, formamide and HEPES-EDTA, denatured at 60°C for 10 minutes and stained with 5 μl bromophenol blue. Following 15-20 hours at 20 volts, the gels were washed with 1X SSC and transferred to a nylon membrane in an LKB 2016 VACUGENE vacume transfer unit. The membrane was then washed, dried until lightly moist and treated with ultraviolet light to crosslink the RNA to the

nylon matrix.

4. Northern Blot Analysis

In order to establish favourable hybridization conditions, the membrane was soaked in 30 ml of hybridization buffer (0.2g BSA, 100 ml formamide, 80 ml NaH_2PO_4, 800 µl 250 mM EDTA and 10g SDS) in a hybridization chamber (Hoefer Scientific Instruments) and incubated at 60°C for 24 hours. Pre-hybridization buffer was then withdrawn from the chamber and replaced with 30 ml of fresh hybridization buffer containing $[^{32}P]$-labelled nick-translated cDNA for Tf receptor or in vitro transcribed ferritin L-chain mRNA labeled with $[^{32}P]$-UTP. The chamber was then resealed and incubated at 42 °C for 24 hours. The nylon was then removed from the chamber washed to remove non-specifically bound activity from the membrane and placed over x-ray film at -70°C and developed after 1 week.

5. Densitometric Analysis

Quantitative interpretation of x-ray radioautographs was carried out by assessing the relative density of bands by densitometric scanning of each band. In all cases three scans were made of each band and averaged to obtain an accurate result. A Zeineh Soft Lazer Scanning Densitometer (Biomed Instruments, Inc.) was used for the measurements.

6. cDNA Probes Preparati,on

The pcD-TR1 plasmid obtained from the laboratory of Dr. L. Kuhn (Kuhn et al., 1984), containing the 4.9 kb Tf receptor cDNA sequence, was nick-translated and labeled with [32P]-ATP (Dupont-NEM) according to the specifications of a kit from BRL, Maryland, USA. Unincorporated radioactivity was removed by G75 Sephadex column chromatography.

7. cRNA In Vitro-Transcribed Probes

An SP-65 plasmid containing the cDNA sequence for ferritin L-chain in its ECoR1 site, obtained from the laboratory of Dr. H. Munro (Brown et al., 1983), was linearized by treatment with Hind III restriction enzyme and transcribed at 37°C for 2 hours in transcription buffer containing SP6 polymerase (Promega) in the presence of [^{3}H]-UTP according to the procedure of Morales et al, 1987. The resulting radiolabeled cRNA probe was then successively extracted by phenol/chloroform and precipitated with ethanol at -20°C.

8. Plasmid Amplification and Isolation

Competent E.coli bacteria transfected with our plasmid were stored at -70°C in 15% glycerol. Prior to plasmid isolation, the bacteria was submitted to two chloramphenicol amplifications and grown overnight in broth at 37°C. The cells were pelleted for 5-10 minutes at 2500xg and resuspended in 1.28 ml of solution I (5 mM glucose, 10 mM EDTA, 2 mg/ml

lysozyme and 25 mM Tris pH 8) and incubate on ice for 15 minutes. Then 2.56 ml of solution II (0.2 N NaOH, 1% SDS) was added, mixed gently and incubated on ice for a further 5 minutes or until the mixture became clear. 1.92 ml of solution III (3 M sodium acetate pH 4.8) was then added, mixed gently and again incubated on ice for 20 minutes. The solution was then centrifuged at 7500xg for 20 minutes and the supernatant precipitated with 5.6 ml of isopropanol for 2 hours at -20°C. After another spin at 7500xg for 20 minutes the pellet now containing the plasmid was washed with 2 ml of 100% ethanol and spun down once again under the same conditions.

The resulting pellet was dissolved in 1 ml $T_{50}A_{150}$ (150 mM NaCL and 50 mM Tris, pH 7.5), precipitated overnight at-20°C, spun down for 15 minutes at 1200xg, washed with 2 ml 100% ethanol, pelleted again and air dried. The plasmid pellet was then resuspended in 1 ml $T_{50}A_{150}$, incubated at 37°C for 30 minutes with 20 µl/ml RNase and incubated with 40 µl/ml proteinase K for a further 30 minutes. Three successive equal volume phenol/chloroform extractions were performed to obtain pure plasmid DNA. Sodium acetate was then added to a final concentration of 300 mM in addition to 2.5 volumes of 100% ethanol, the solution left for 1 hour at -20°C and the DNA pelleted at 12000xg for 15 minutes.

The pellet was thouroughly dissolved in 4 ml TE buffer (1 mM Tris and 25 μM EDTA). To this was added 4.3 g of $CsCl_2$, 10 mg/ml ethidium bromide and the R_1 adjusted to 1.3895-1.3900. A vertical gradient was then formed using a VTi 65 Beckman rotor for 21 hours at 60000xg. The plasmid DNA band was collected, cleansed of contaminating ethidium bromide and the solution extracted with SSC-saturated isopropanol (20X SSC: 3 M NaCl and 0.3 M sodium citrate-mixed with 2 volumes isopropanol and the upper phase used) until clear. The resulting extract was then precipitated at -20°C for 2 hours with 0.7 volume isopropanol following the addition of 2 volumes water and 0.1 volume 3 M sodium acetate. The DNA was then finally pelleted, washed and precipitated as before two more times and resuspended in the appropriate buffer for utilization.

9. Transferrin Labeling

Diferric Tf was prepared by disolving 10 mg lyophilized rat Tf (Organon Teknika Inc., Scarborough, Ontario) in 1 ml buffer (0.25 M Tris-Cl, pH 8, .06 M $NaHCO_3$) and was then incubated with 5 mM ferrous citrate for 3 hours at room temperature. Removal of free iron was not necessary for our purposes. [^{125}I]-Tf was prepared by reacting 50 μg dTf with 2 mCi Na[^{125}I] for 30 seconds using the chloramine T method. The reaction was halted by the addition of sodium metabisulfite. All tracers made were calculated to have a

specific activity of between 5-7 μCi/μg. Labeled protein was separated from free radioactivity by G25 Sephadex column chromatography.

10. Polarized Secretion of Testicular Transferrin

The following experiment was performed to confirm that tTf is secreted by Sertoli cells, in a polarized fashion towards the seminiferous tubule lumen.

An adult rat was anesthetized and the portal circulation and the testes exposed via an incision along the abdominal midline. The hepatic artery and portal vein were tied off as 1 mCi [^{35}S]-methionine was injected subcapsularly into each testis. The incision was then sutured and the animal given 5 ml of warm glucose i.p.. Body temperature was maintained at 37°C with the aid of a cervo-regulated heat lamp connected to a rectal temperature probe.

One hour later the testes were removed and rinsed with HBSS. Intertubular fluid (TIF) was collected by placing the decapsulated testes into 5 ml of ice-cold HBSS. 5 minutes thereafter the liquid was retained and the tubule mass washed two more times with ice-cold HBSS and the three 5 ml washes pooled as TIF. The intratubular fluid (SNF) was collected by extruding the washed tubules through a 20-gauge needle and the resulting sheared tubules spun at 17000xg. The resulting supernatant was pooled with 1 ml of HBSS as SNF and the

tubule pellet washed once more with HBSS and homogenized with cold IMPB (10 mM Tris pH 7.4, 150 mM NaCl, 2 mM PMSF, 5 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate). The homogenate was then centrifuged at 17000xg and the supernatant fluid taken to be solubilized cells. Each fraction (TIF, SNF solubilized cells) was preincubated with Pansorbin for 30 minutes and then immunoprecipitated with rabbit anti-rat Tf antibody, run on SDS-PAGE and the gels fluorographed (Chasteen and Williams, 1981).

11. Internalization of [^{35}S]-labeled Testicular Transferrin

The following experiment was carried out to determine if newly synthesized tTf, secreted by Sertoli cells in vivo, is internalized by adlumenal germ cells of the rat seminiferous epithelium.

The animal was processed as above except that once the testes were removed they were minced and the various germ cell populations separated as above. Also according to this procedure pachytene spermatocytes and round spermatids were identified and their fractions pooled. This suspension was then treated with 0.1 mg/ml trypsin to remove all specific and non-specific Tf activity from the cellular surface. The cells were then homogenized in cold IMPB, centrifuged at 17000xg and the supernatant immunoprecipitated with rabbit anti-Tf antibody. Finally the pellet was run on SDS-PAGE and the gel fluorographed (Chasteen and Williams, 1981).

12. Electron Microscope Radioautography

The following experiment was undertaken to ascertain whether Tf is internalized by adlumenal germ cells of the rat seminiferous epithelium. Two male adult Sprague-Dawley rats were anesthesized and a scrotal incision made to expose the internal genetalia. [^{125}I]-labeled Tf was then microinjected through the rete testis into each testis. At 5, 15 and 30 minutes thereafter one animal was sacrificed and the testes perfused through the abdominal aorta with 2.5% glutaraldehyde buffered in sodium cacodylate (0.1 M, pH 7.4) containing 0.5% $CaCl_2$.

After perfusion, the testes were removed and immersed in the same fixative for 1 hour at 4°C and then dehydrated in alcohol and embedded in epon. Thin sections were cut, placed on 1% celloidin-coated glass slides, and coated with a monolayer of Ilford L4 nuclear emulsion with an automatic coating instrument. After a 3 month exposure period at 4°C, electron microscope radioautographs were developed with a freshly prepared solution physical developer, preceded by gold latensification for fine silver grains. Sections were counter-stained with uranyl acetate and lead acetate.

13. Kinetic Study of Transferrin Internalization and Recycling in Round Spermatids

The purpose of the following experiment was to determine, in vitro, whether round spermatids isolated from rat seminiferous epithelium recycle or degrade internalized Tf.

Round spermatids were isolated as described in Section 1, part b and the suspension divided according to cell number in 15 aliquots of 3 million cells or 5 times replicates of 3 tubes/cell suspensions. The volume of each aliquot was brought up to 3 ml with incubation media IM (6mM sodium pyruvate, 2mM lactate and trypsin inhibitor in HBSS) and kept on ice. Excess [^{32}P]-labeled Tf (50 μl containing approximately 1 X 10^4 cpm) of specific activity 5-7 μCi/μg was then added to each cell suspension and left to bind, on ice, for 6 hours.

Excess radioactivity was then washed off the cells with ice-cold IM and the aliquots then slowly warmed to 37°C for 10 minutes to allow internalization of the [^{125}I]-Tf to take place. At the end of this time period 0.1 mg/ml pronase was then added to each aliquot to remove all surface-specific and non-specific activity then washed once again. The suspensions were then cooled to 4°C.

Each replicate was then incubated at 37°C for various lengths of time, rapidly cooled to 4°C and spun down at 3000xg for 5 minutes at 4°C; Three samples of the supernatant were then taken, TCA precipitated, spun down, and the resulting supernatants separated from pellets and each counted. In summary there were three replicates (aliquots) per time point (5 time points) and three supernatant samples taken per aliquot.

14. Scatchard Analysis of Transferrin Binding Assay in Round Spermatids

This experiment was conducted to ascertain the characteristics of Tf receptor expressed on the surface of isolated round spermatids.

Testicular tissue from two adult male Sprague-Dawley rats was processed and loaded onto two 400 ml BSA-HBSS density gradients as described in Section 1, part b. Also as described in this section cellular fractions were collected, identified and counted. For the purposes of this experiment, however, only fractions containing round spermatids in relative purities exceeding 90% were retained and pooled.
In order to establish an optimal cell number for the binding study, a dilution assay was performed. It consisted of aliquoting these round spermatids into groups of 4 tubes with final cell concentrations ranging from 1 to 10 million cells/ml. Two replicates/group coincubated with excess cold Tf (2μg/ml) and 22000 cpm of [^{125}I]-labeled Tf to a final volume of 0.5 ml to assess non-specific binding and the other 2 replicates of the same group coincubated only with 22000 cpm of [^{125}I]-labeled Tf to assess specific ligand binding.

The ligand was then allowed to bind to its cell surface receptor for 6 hours at 4°C. The tubes were then diluted to 3 ml with ice-cold IM (see Section 11) and spun down at 1000xg for 5 minutes at 4°C. The media was then removed and the resulting pellets are then counted, non-specific counts subtracted from specific ones, to determine

which cell concentration has incurred the greatest degree of specific binding.

Secondly it was necessary to pre-establish the optimal binding time: This was carried out as above except that the cell number was held constant at 4 million and the incubation time varied from 1 to 16 hours. Finally the binding study was performed with an optimal cell concentration of 8 million cells/ml and an optimal incubation period of 6 hours. In order to obtain a reliable Scatchard plot, 8 datapoints were considered desirable (Scatchard, 1949). This required the isolation of at least 96 million round spermatids at high purity since there were to be 3 replicates per datapoint at 4 million cells each. This requirement was satisfied and the cells were incubated in IM (with the addition of 0.5% BSA) at a final volume of 0.5 ml with 22000 cpm of [^{125}I]-labeled Tf, specific activity 5-7 μCi/μg. Final concentrations of cold transferrin were: 0, 1, 3, 10, 30, 100, 300 and 2000 ng/ml as an excess to assess non-specific binding. Following binding all tubes were diluted to 3 ml with ice-cold IM (plus 0.5% BSA) and spun down at 1000xg for 5 minutes at 4°C. The pellets were then counted for 10 minutes and the raw data analysed and plotted with the use of GraphPad InPlot, a standard graphics computer program.

RESULTS

1. Polarized Secretion of Testicular Transferrin by Sertoli Cells

This experiment was carried out to verify whether Sertoli cells invariably secrete tTf apically into the adlumenal compartment of the seminiferous epithelium.

De novo protein synthesis in the testes of the rat was labeled with [^{35}S]-methionine while at the same time sTf access to the systemic circulation was mechanically excluded by porta-hepatic ligation. Each of the TIF, SNF and cellular fractions were isolated, immunoprecipitated with rabbit anti-rat Tf antibody and run on SDS-PAGE and fluorographed. Immunoprecipitates of spent media from Sertoli cells in culture incubated with [^{35}S]-methionine were also run on the same gel to serve as a source of molecular markers.

The resulting fluorograph revealed major bands running at 77 Kd which corresponded to immunoprecipitated [^{35}S]-labeled tTf. These were associated, however, only with the intratubular fraction (SNF) and the cellular fraction (cells) but not with the extratubular fraction (TIF) (Fig.6). This experiment reconfirmed that tTf activity only became associated with cellular and tubular fluid elements of the rat seminiferous epithelium but not with interstitial elements of the testis by virtue of the strict apically-directed secretion of tTf by Sertoli cells.

2. Germ Cell Internalization of [^{35}S]-labeled Testicular Transferrin

Following the same experimental protocol, [^{35}S]-methionine was injected into the testis and the liver excluded from the circulation. In this case however the testicular tissue was processed for germ cell separation on a staput velocity sedimentation gradient at 4°C. Only high purity fractions of spermatocytes and round spermatids were pooled together, treated with trypsin, homogenized and immunoprecipitated with rabbit anti-rat Tf antibody. The pellet was run on SDS-PAGE and the gel fluorographed. Sertoli cells in culture coincubated with [^{35}S]-methionine were treated as above and the spent media run to serve as molecular weight markers on the gel.

The fluorograph shows that the [^{35}S]-labeled protein, precipitated by the anti-Tf antibody, ran in a 77 Kd band as indicated by the Sertoli cell markers. This corresponds to newly synthesized tTf secreted by the Sertoli cell and internalized by the isolated germ cells (Fig.7).

3. Scatchard Analysis of Transferrin Binding to Round Spermatids

Round spermatids were again used for this experiment only because they can be isolated in sufficient quantities to carry out a proper binding assay. Tf receptor type(s), number and their dissociation constant was assessed by subjecting the equilibrium binding data to Scatchard analysis.

Preliminary dilution experiments were first performed to establish an optimal cell concentration and an optimal binding time for the actual binding study. In the first case an increasing number of cells were incubated with a constant quantity [^{125}I]-labeled Tf (specific activity 5-7 μCi/μg) (Fig.8). This experiment shows that a cell number of 4 X 10^6 cells/reaction mixture constituted the optimal level of binding amongst the cell numbers examined. This range extended only to 6 X 10^6 cells since but a limited number of cells are isolatable at one time.

The second dilution experiment involved incubating a fixed number of cells with a fixed amount of labeled Tf for increasing lengths of time. Maximal binding was achieved at 6 hours and remained relatively constant thereafter until T=15 hours (Fig.9). Binding may or may not have been enhanced after this time but due to the rapid deterioration observed in these cells in vitro, the lowest possible binding time of 6 hours was selected.

The binding assay was therefore carried out for 6 hours with 4 X 10^6 cells/reaction tube and the resulting data interpreted according to the method of Scatchard by plotting the ratio of specifically bound [^{125}I]-Tf and free ligand against the concentration of bound ligand (Fig.10). The linear nature of the plot suggests that a single receptor type is expressed on the surface of round spermatids under these

conditions. The earliest datapoint was somewhat removed from the plot and was considered to have resulted from random error. Nevertheless a correlation coefficient of 0.9 was calculated, taking all points into consideration. Mathematical interpretation of the slope data reveals an apparent K_d of 0.6×10^{-9} thus indicating a high affinity binding site. According to the x-intercept the number of Tf binding sites present on the surface of each round spermatid is approximately 1453.

4. Transferrin Receptor mRNA and Ferritin L-Chain mRNA Expression in Rat Whole Testis of Increasing Ages

The expression of Tf receptor mRNA and ferretin L-chain mRNA in rat whole testis was examined by northern blot analysis. Total testicular RNA was isolated from rats aged 10 to 45 days and each sample suspended in an equal volume of water. Concentrations of RNA and record of the most advanced cell type at each age is listed in Table I. Five μl of each sample was then fractionated on a 1.2% agarose-formaldehyde gel and transferred onto a nylon membrane where the samples were crosslinked to the matrix with U.V. light.

Two separate membranes produced as above were used for the hybridization experiment. The first blot was hybridized with a [^{32}P]-labeled nick-transcribed cDNA probe specific for Tf receptor mRNA and the second was hybridized with a [^{32}P]-UTP-labeled in vitro-transcribed cRNA probe specific for ferritin L-chain mRNA. The blots were then washed free of

TABLE I

ISOLATION OF RNA FROM PREPUBERTAL TESTES

Sample no.	Age	Most Advanced Cell-Type	mg Total RNA/ testis	μg/μl
1	10	Pachytene Spermatocyte	0.7	1.4
2	15	Early Pachytene	0.7	1.4
3	20	Late Pachytene, Diplotene	1.1	2.2
4	25	Step 1-3 Spermatid	0.9	1.8
5	30	Step 6-8 Spermatid	1.7	3.4
6	35	Step 13 Spermatid	2.0	4.0
7	40	Step 16-17 Spermatid	2.9	5.8
8	45	Step 18 Spermatid	2.7	5.4

non-specifically bound radioactivity and autoradiographed on X-ray film for 3-5 days.

Both RNA species were found to be expressed at each age-specific stage, hence continuously throughout testicular developement (Fig.11,12). Furthermore the rates of expression of each specie were shown to increase linearly with testicular age as illustrated by densitometric analysis of the autoradiographs as (Fig.13,14).

5. Relative Expression Rates of Transferrin Receptor mRNA and Ferritin L-Chain mRNA in Adluminal Germ Cells

The relative expression rates of Tf receptor mRNA and ferritin L-chain mRNA in adluminal germ cells of the rat were examined by northern blot analysis using radiolabeled probes specific for each mRNA transcript. Spermatocytes, round spermatids and elongated spermatids were isolated at < 90% purity by staput velocity sedimentation on a 1-4% BSA gradient in HBSS. In the case of the elongated spermatid fractions, contamination was chiefly by residual bodies. The collected fractions were identified by phase-contrast microscopy and the high-purity fractions of each of the above cell types pooled. The three resulting cell suspensions were then counted and the RNA extracted from the spun-down pellet of each cell type. Concentrations of RNA obtained from each sample was determined by spectrophotometric absorption at 260 nm.

The RNA-equivalent of 10 X 10^6 cells from each cell type sample was fractionated on a 1.2% agarose-formaldehyde gel and transferred onto a nylon membrane. The blot was then hybridized with a [^{32}P]-labeled nick-translated cDNA probe specific for Tf receptor mRNA, washed and placed over an X-ray film for autoradiography for 3-5 days. The same blot was then striped of this probe by high-temperature boiling and rehybridized with a [^{32}P]-UTP-labeled in vitro-transcribed cRNA probe specific for ferritin L-chain mRNA which was autoradiographed in a similar way as before.

As seen in Figs.15 and 16, both mRNA species demonstrated a decreasing expression rate as adlumenal germ cells mature. Densitometric analysis of each autoradiograph illustrates this linear decrease (Figs.17,18). The relative decrease in the case of Tf receptor mRNA was observed to be sharper.

6. Recycling of Transferrin in Round Spermatids

The internalization and subcellular fate of tTf by germ cells was examined in isolated round spermatids by incubating these cells with [^{125}I]-Tf. According to the aforementioned procedure, cell suspensions of [^{125}I]-Tf-loaded round spermatids at 4°C were sequentially and slowly warmed to 37°C for various lengths of time. Each replicate, at the end of its incubation period, was fast-cooled in freezing point-depressed water to halt any possible recycling that may have

taking place. The tubes were then immediately spun down at 4°C. Triplicate samples of each resulting supernatant was then taken and TCA-precipitated. Precipitable radioactivity was taken to be intact "recycled" [^{125}I]-Tf and non-precipitable radioactivity was taken to be free [^{125}I] liberated from the degradation of the internalized Tf in a putative lysosomal compartment.

It was found that radioactivity localized to the precipitable fraction thus indicating that Tf, internalized by isolated round spermatids in vitro, do recycle the internalized Tf. The radioactivity contained in each replicate was averaged and plotted against time (Fig.19). The resulting Tf recycling profile shows that a progressively increasing amount of [^{125}I]-Tf was externalized by the round spermatids with time. It also indicates that recycling achieved a constant rate at approximately 10 minutes. In an attempt to account for the earliest recycled Tf molecules, the experiment was repeated and time points before 10 minutes were examined. The resulting data was similar to the first but failed to detect any radioactivity, above background levels, prior to 5 minutes.

7. Internalization and Subcellular Localization of [^{125}I]-labeled Transferrin in Adluminal Germ Cells

In order to deliver [^{125}I]-Tf to adluminal germ cells in vivo, [^{125}I]-Tf was microinjected through the rete testis into

the lumen of the seminiferous tubuls. At times of 5, 15 and 30 minutes following injection one animal was sacrificed by fixation and thin sections of testicular tissue were processed for electron microscope radioautography.

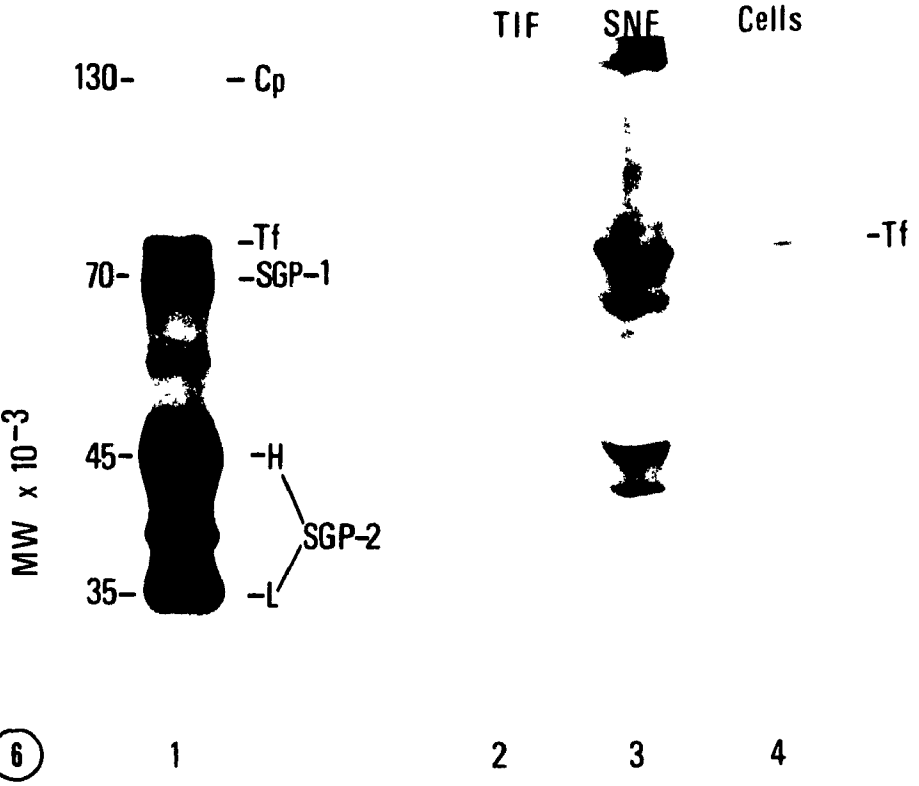
At 5 minutes following injection, the radioautographic grains appear to be associated with the plasma membrane of the spermatids (Fig.20). At 15 minutes following injection, the label was internalized by the spermatids and localized to electron-lucent, membrane-bound vesicles that were very reminiscent of endosomes. Often the label was seen in close proximity to the plasma membrane associated with what may be small tubular vesicles (Fig.21). It could not be determined whether the Tf was entering or exiting the cell, which in the latter case would be indicative of recycling in this germ cell type. At 30 minutes following injection, the labeled Tf seemed to have completely exited the cell and the adlumenal space which again may be indicative of recycling (Fig.22).

FIGURE 6

Fluorograph of polyacrylamide gel electrophoresis of radiolabeled newly synthesized testicular transferrin in the rat testis. An adult rat was anesthesized and the liver exposed by an abdominal incision. The hepatic artery and portal vein were ligated to exclude the liver from the circulation immediately after 1 mCi [^{35}S]-methionine was injected intratesticularly. The animal was sacrificed one hour later. At this time the intertubular (TIF) fluid and the intratubular (SNF) fluid were collected and the remaining cells were washed and extracted with immunoprecipitation buffer. Immunoprecipitation with rat anti-transferrin antibody reveals that testicular transferrin is associated with **SNF** and **cell pellets** but not with **TIF**. Lane 1 represents control radiolabeled proteins secreted by rat Sertoli cells in culture used as molecular markers.

FIGURE 7

Fluorograph of polyacrylamide gel electrophoresis of radiolabeled transferrin immunoprecipitated with rat anti-transferrin antibody from germ cell homogenate. After ligating out the liver from the circulation, [^{35}S]-methionine was injected intratesticularly and the animal sacrificed one hour later. Lane 2 shows immunoprecipitated testicular transferrin from isolated germ cell pellets containing pachytene spermatocytes and round spermatids previously treated with trypsin to remove non-specifically bound transferrin from the plasma membrane. Lane 1 represents control radiolabeled proteins secreted by rat Sertoli cells in culture used as molecular markers.


TIF
SNF
Cells
130-
– Cp
-Tf
70-
-SGP-1
MW x 10-3
45-
-H
SGP-2
35-
-L
-Tf
6
1
2
3
4

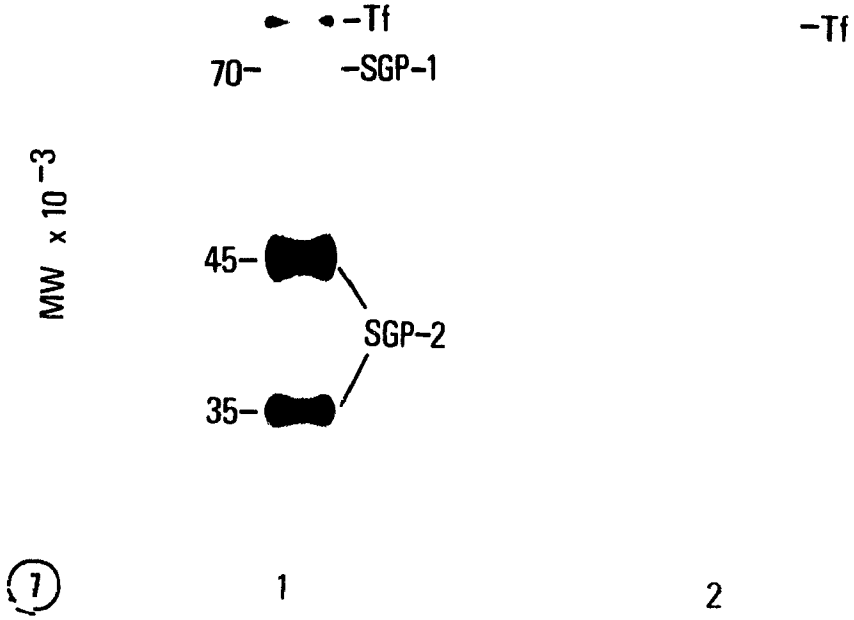

-Tf
-Tf
70-
-SGP-1
MW x 10-3
45-
SGP-2
35-
7
1
2

FIGURE 8

Dilution experiment to determine optimal cell number for the binding assay. Round spermatids were isolated, in the presence of trypsin inhibitor, by staput velocity sedimentation on a 1-4% BSA density gradient in HBSS. The cells were then aliquoted in increasing concentrations in four-tube replicates and incubated with 22000 cpm [^{125}I]-transferrin (specific activity 5-7 μCi/μg) for 6 hours with continuous agitation at 4°C. Two tubes of each replicate were also incubated with excess cold transferrin to measure non-specific binding (NSB). Following the incubation the tubes were diluted with 3 mls ice-cold medium, spun down, the pellet counted and NSB subtracted.

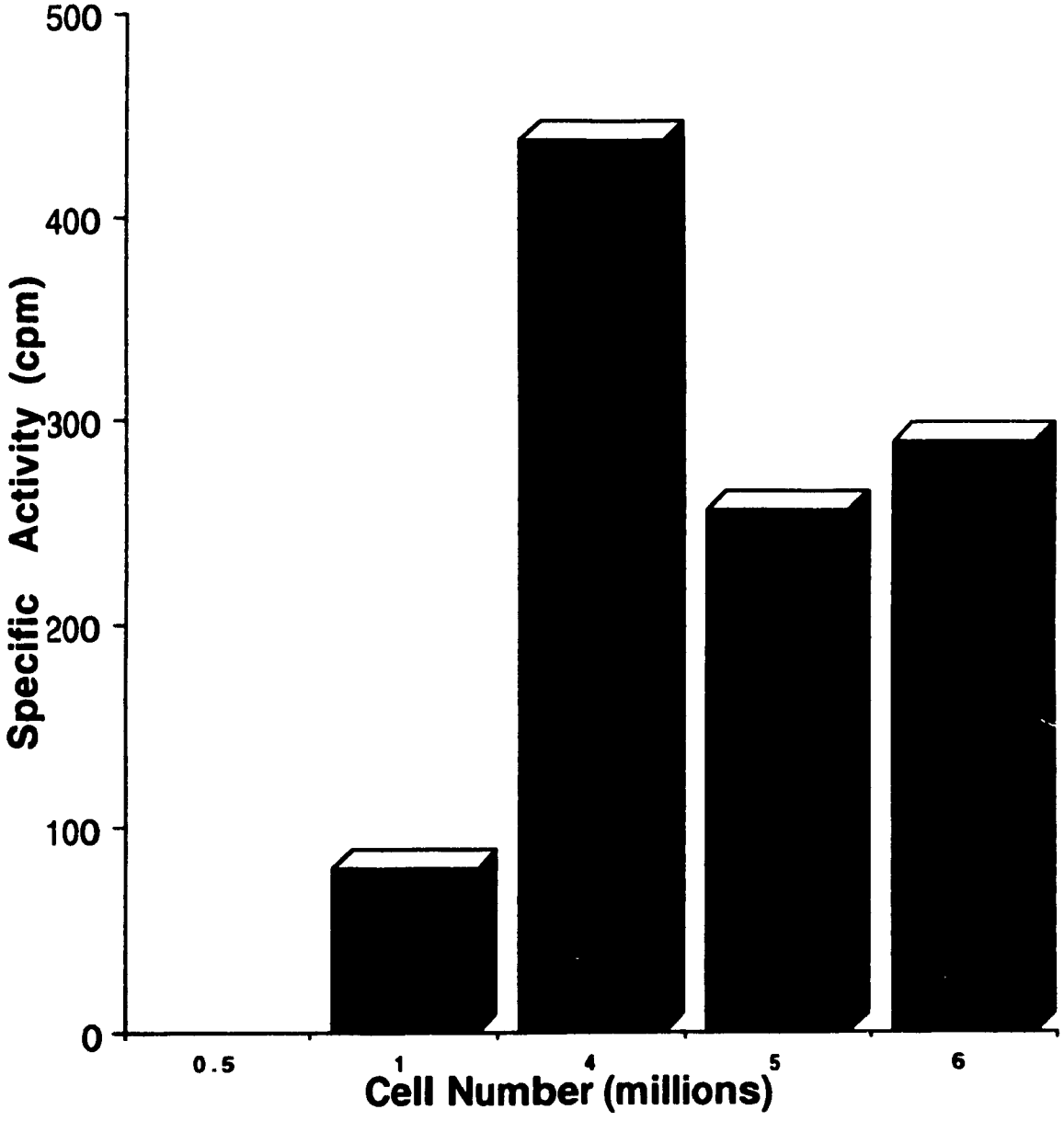

500
400
300
200
100
0
Specific Activity (cpm)
0.5
1
4
5
6
Cell Number (millions)

FIGURE 9

Dilution experiment to determine optimal binding time for the binding assay. Round spermatids were isolated, in the presence of trypsin inhibitor, by staput velocity sedimentation on a 1-4% BSA density gradient in HBSS. The cells were then equally distributed in four-tube replicates at a concentration of 8 X 10^6 cell/ml and incubated with 22000 cpm [^{125}I]-transferrin for increasing numbers of hours with continuous agitation at 4°C. Two tubes of each replicate also contained excess cold transferrin to ascertain non-specific binding (NSB) for each replicate. After each incubation period the tubes were diluted with 3 mls ice-cold medium, spun down, the pellet counted and NSB subtracted.

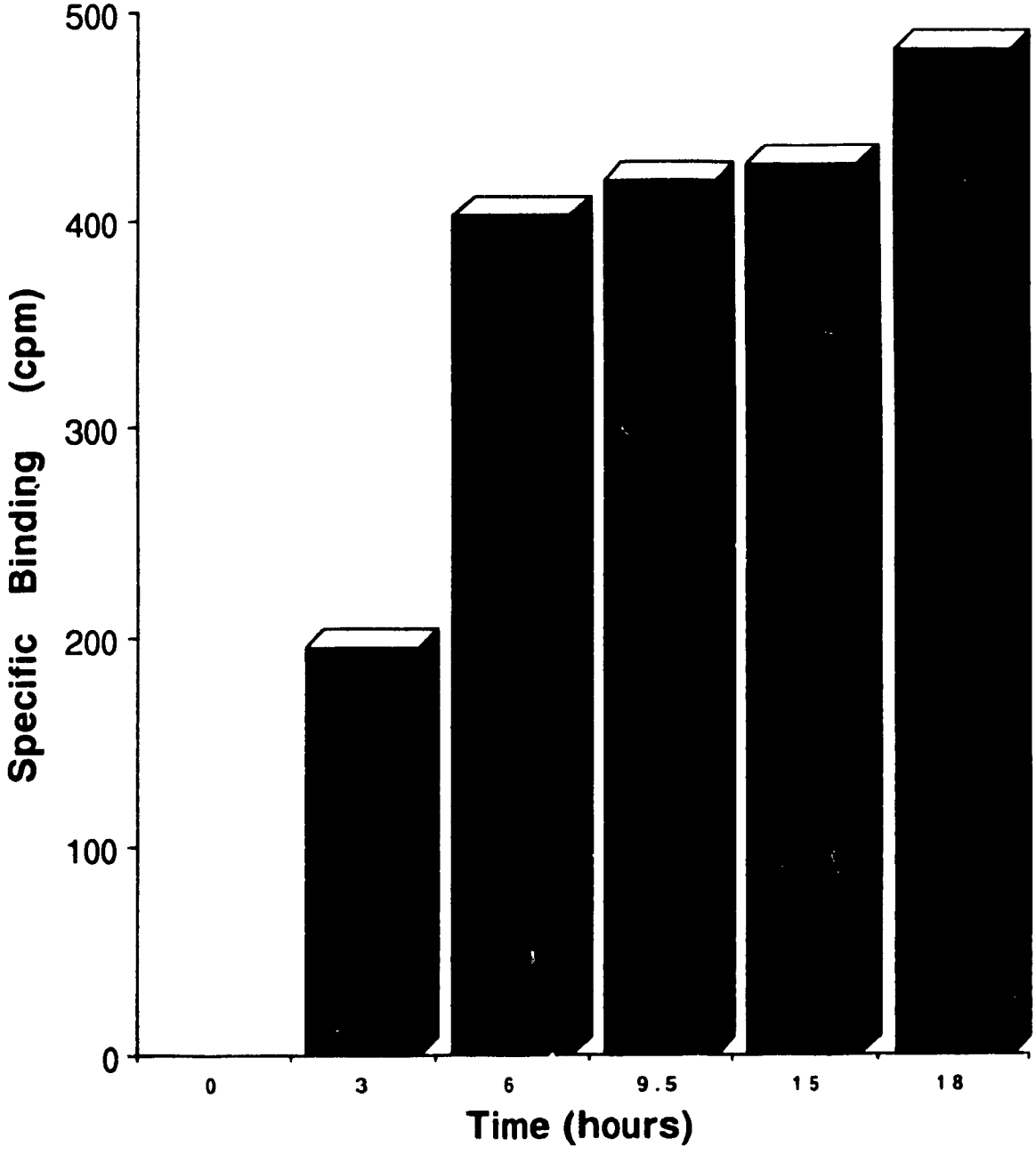

500
400
300
200
100
0
Specific Binding (cpm)
0
3
6
9.5
15
18
Time (hours)

FIGURE 10

Scatchard analysis of transferrin binding to round spermatids. Round spermatids were isolated in the presence of trypsin inhibitor and at high purity by staput velocity sedimentation on a 4% BSA-HBSS density gradient. The cells were then aliquoted in 8 replicates of 3 tubes each at a concentration of 8 X 10^6/ml and incubated with 22000 cpm [^{125}I]-labeled rat transferrin for 6 hours at 4°C. Final cold transferrin concentrations were 0, 1, 3, 10, 30, 100, 300 and 2000 ng/ml to assess non-specific binding (NSB). Following the binding period tubes were diluted to 3 mls with ice-cold medium containing 0.5% BSA. The suspensions were finally spun down, counted and the raw data interpreted using the method of Scatchard.

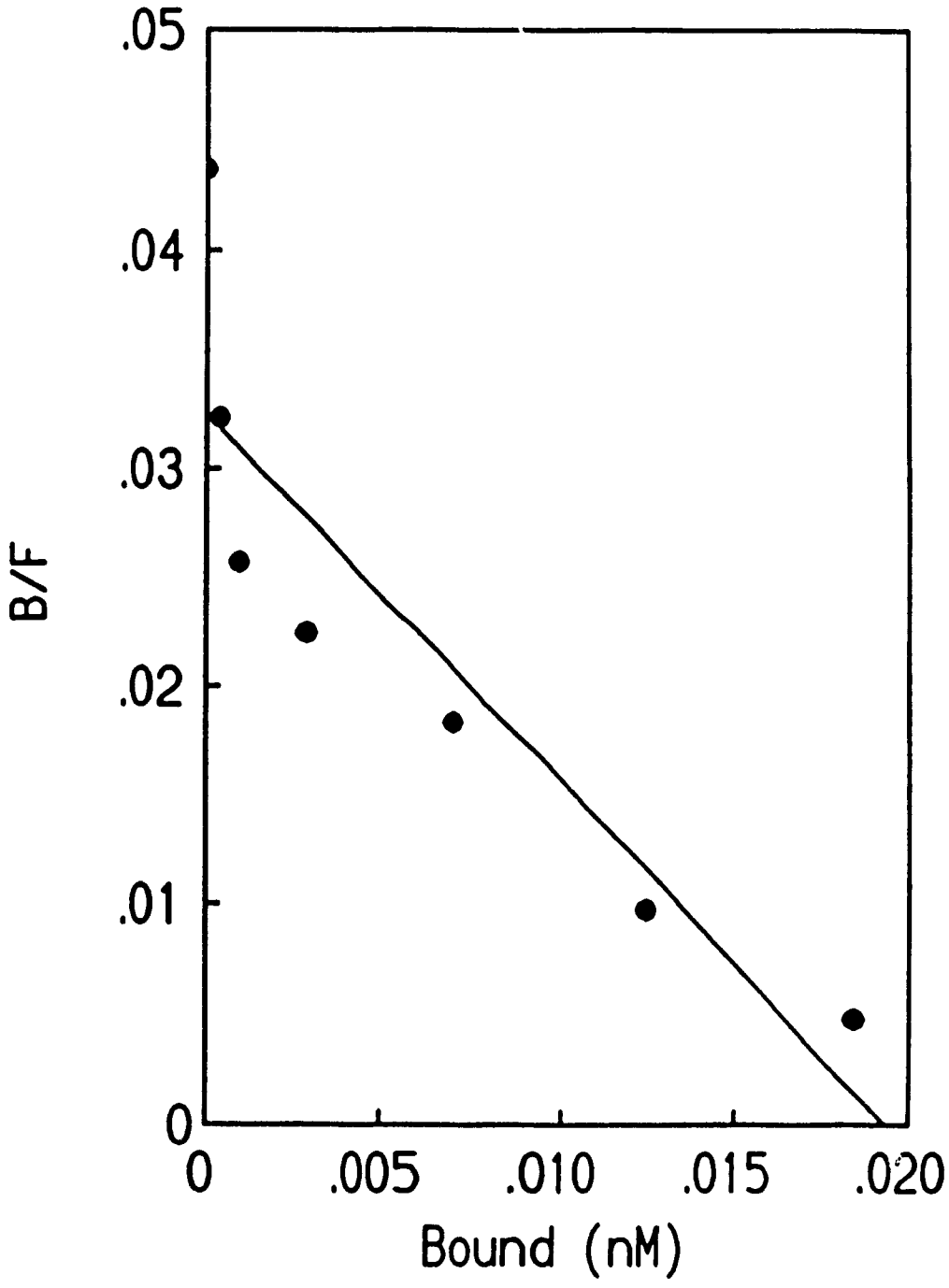
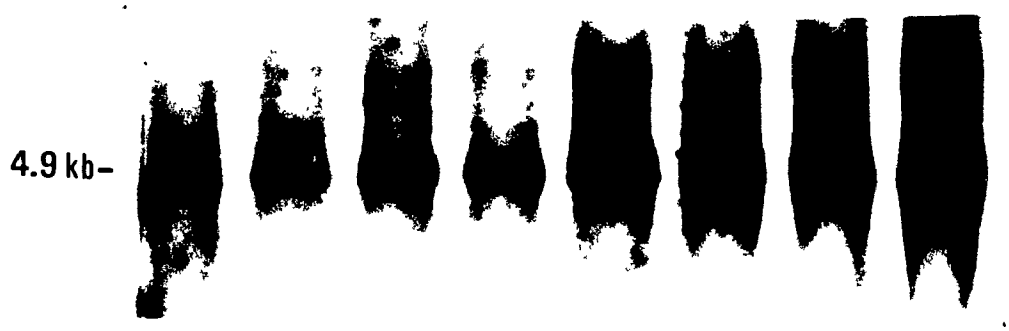

.05
.04
.03
.02
.01
0
B/F
0
.005
.010
.015
.020
Bound (nM)

FIGURE 11

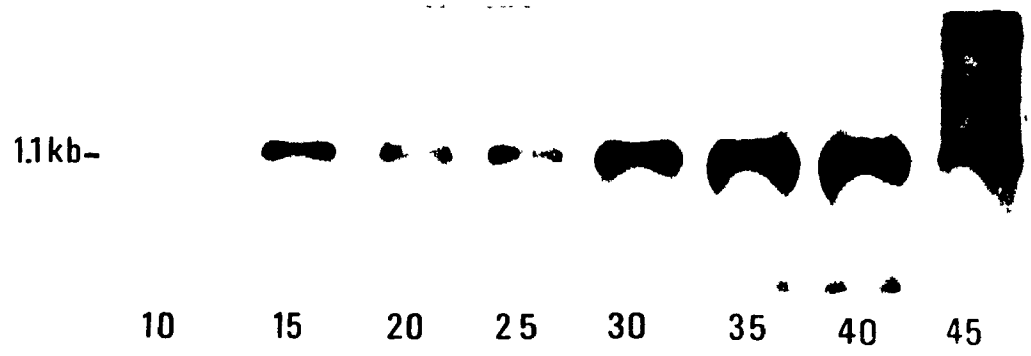
Northern blot analysis of testes mRNA isolated from rats aged 10, 15, 20, 25, 30, 35, 40 and 45 days (lanes 1 to 8). The RNA was isolated from whole testis and equal volumes (5 μl) were fractionated so that the intensity of the resulting bands could be as relative to the total amount of specific mRNA per testis. The lanes represent a radioautograph of a blot probed with a [^{32}P]-labeled nick-transcribed cDNA probe specific for transferrin receptor mRNA.

FIGURE 12

Northern blot analysis of testes mRNA isolated from rats aged 10, 15, 20, 25, 30, 35, 40 and 45 days (lanes 1 to 8). The RNA was isolated from whole testis and equal volumes (5 μl) were fractionated so that the intensity of the resulting bands could be visualized as relative to the total amount of specific mRNA per testis. The lanes represent a radioautograph of a blot hybridized with a [^{32}P]-UTP-labeled cRNA probe transcribed from an SP65 linearized vector containing the L-chain ferritin cDNA.



(11)



(12)

FIGURE 13

Variations in the levels of transferrin receptor mRNA per testis of increasing ages expressed as relative hybridization. The columns represent densitometric values obtained by scanning the northern blot radioautograph (Fig.11) with a densitometer. The readings indicate a significant increase in the levels of this mRNA specie as the seminiferous epithelium matures. The increasing values correlate well with the successive appearance of pachytene spermatocytes, round and elongated spermatids.

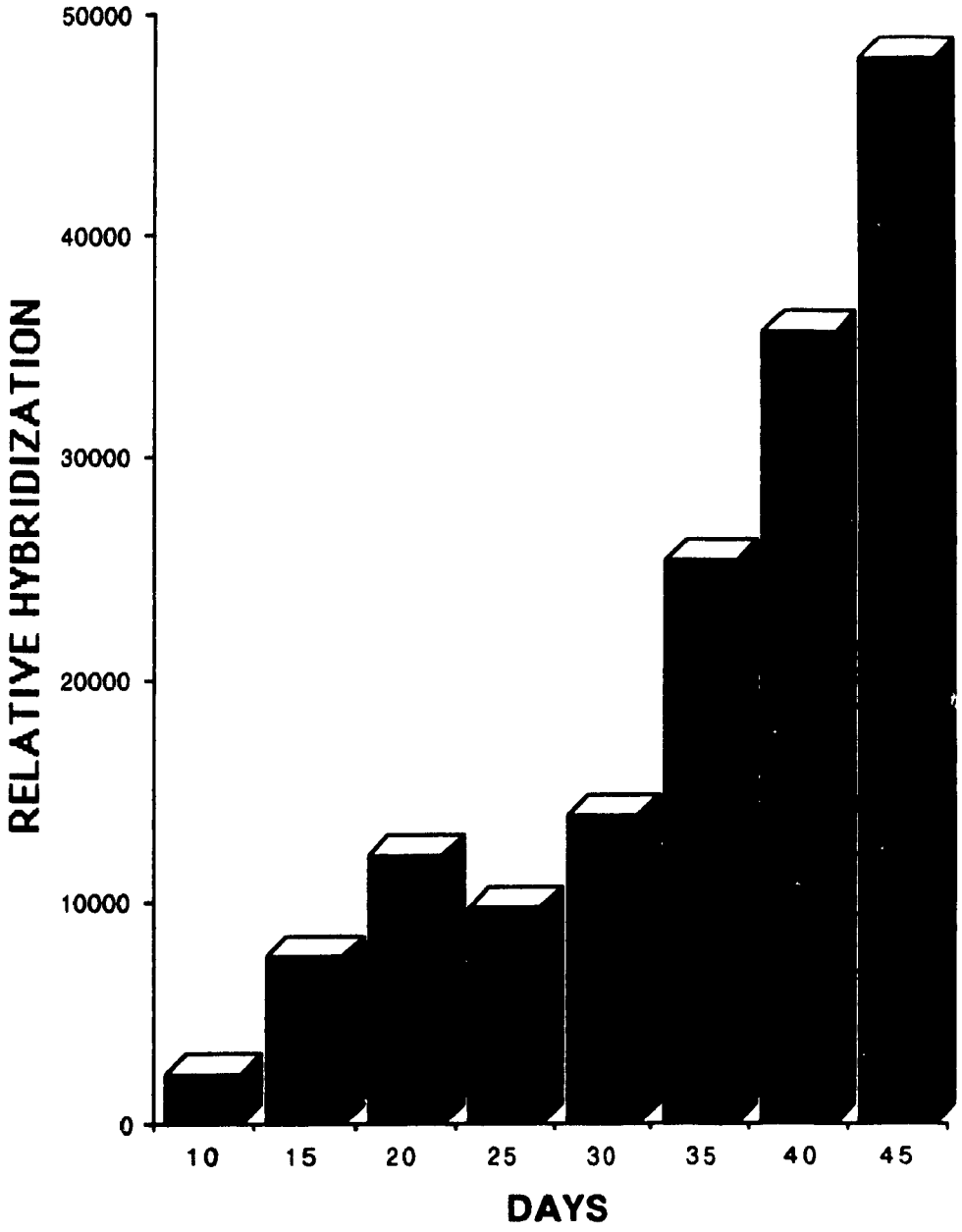

RELATIVE HYBRIDIZATION
50000
40000
30000
20000
10000
0
10
15
20
25
30
35
40
45
DAYS

FIGURE 14

Variations in the levels of ferritin L-chain mRNA per testis of increasing ages expressed as relative hybridization. The columns represent densitometric values obtained by scanning the northern blot radioautograph (Fig.12) with a densitometer. The readings indicate a significant increase in the levels of this mRNA specie as the seminiferous epithelium matures. The increasing values correlate well with the successive appearance of pachytene spermatocytes, round and elongated spermatids.

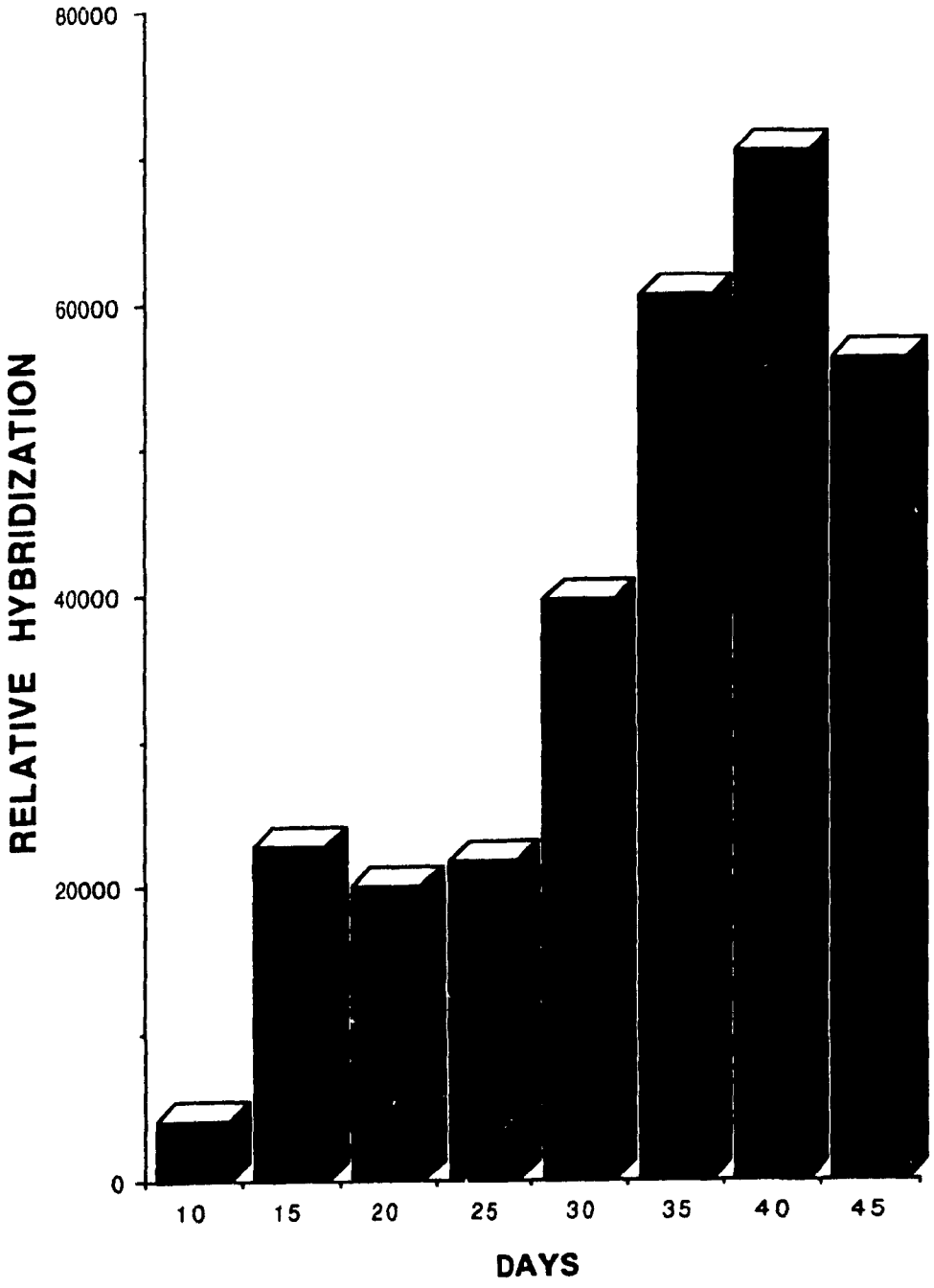
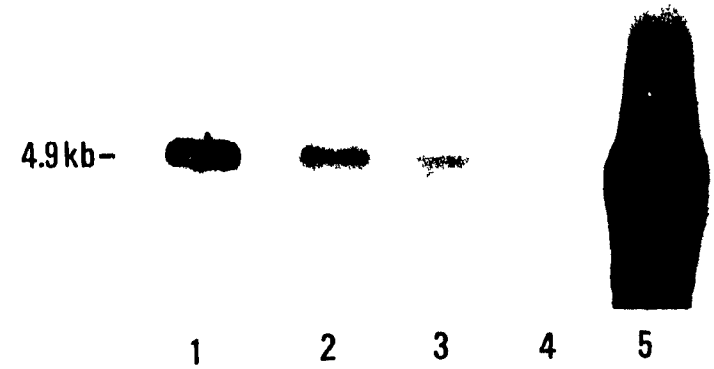

80000
60000
40000
20000
0
RELATIVE HYBRIDIZATION
10
15
20
25
30
35
40
45
DAYS

FIGURE 15

Northern blot analysis of spermatocyte (lane 2), round spermatid (lane 3) and elongated spermatid (lane 4) mRNAs. Each lane represents the total RNA isolated from 10^7 cells probed with a [^{32}P]-labeled nick transcribed transferrin receptor cDNA. Lanes 1 and 5 represent 7 μg of rat liver and whole testis mRNAs run as positive controls.

FIGURE 16

Northern blot analysis of spermatocyte (lane 1), round spermatid (lane 2) and elongated spermatid (lane 3) mRNAs. Each lane represents the total RNA isolated from 10^7 cells hybridized with a cRNA probe transcribed from an SP65 linearized vector containing the L-chain ferritin cDNA. Lane 4 represents 7 μg of rat liver mRNA run as a positive control.



(15)

(16)

FIGURE 17

Densitometric analysis showing the expression profile of transferrin receptor mRNA in adluminal germ cells of the rat seminiferous epithelium. In terms of relative hybridization the analysis indicates that, for equal numbers of cells, the expression of transferrin receptor decreases as the germ cells mature from spermatocytes (Spc) to round spermatids (rSp) and finally to elongated spermatids (eSp).

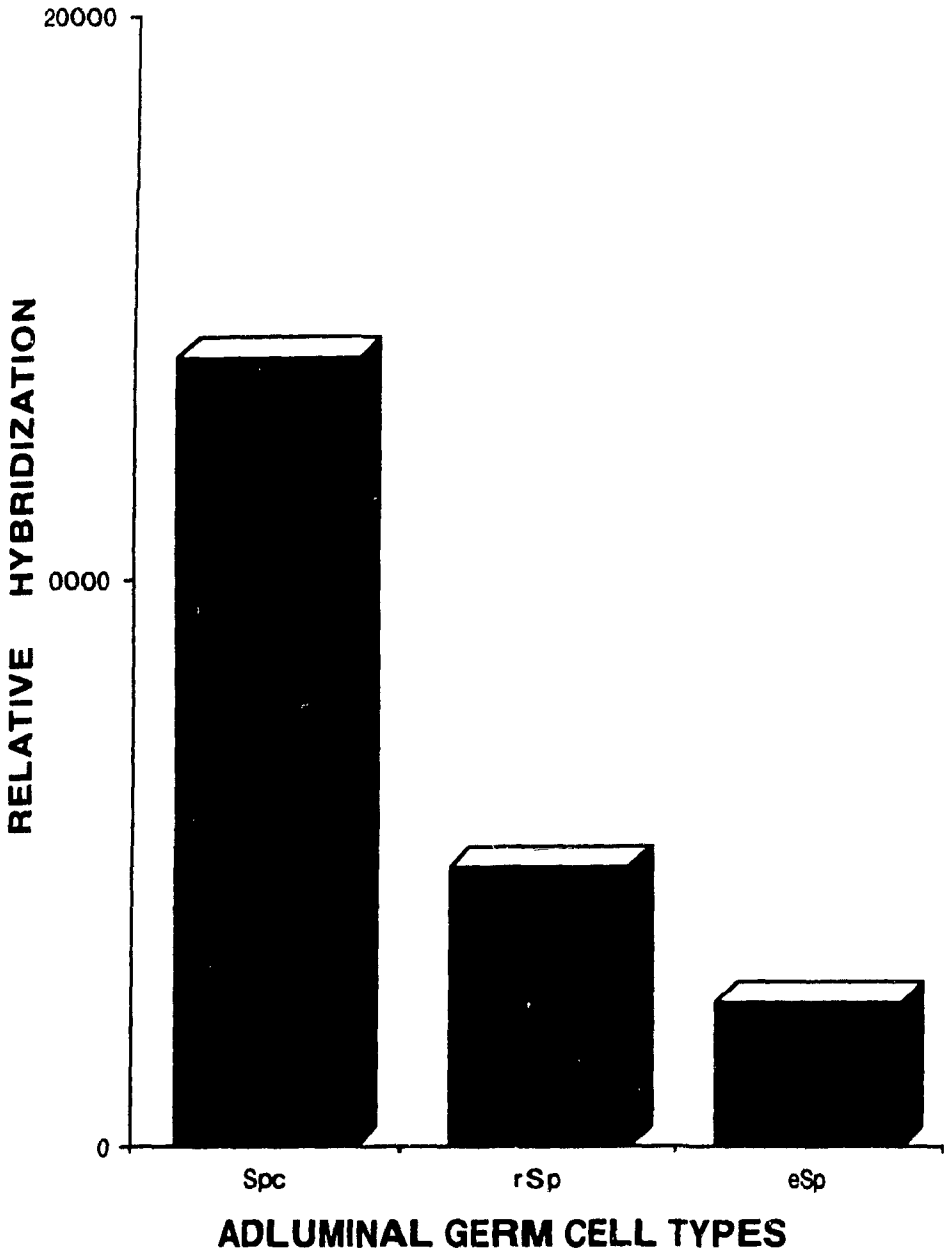

20000
0000
0
RELATIVE HYBRIDIZATION
Spc
rSp
eSp
ADLUMINAL GERM CELL TYPES

FIGURE 18

Densitometric analysis showing the expression profile of ferritin L-chain mRNA in adluminal germ cells of the rat seminiferous epithelium. In terms of relative hybridization the analysis indicates that, for equal numbers of cells, the expression of ferritin L-chain mRNA decreases as the germ cells mature from spermatocytes **(Spc)** to round spermatids **(rSp)** and finally to elongated spermatids **(eSp)**.

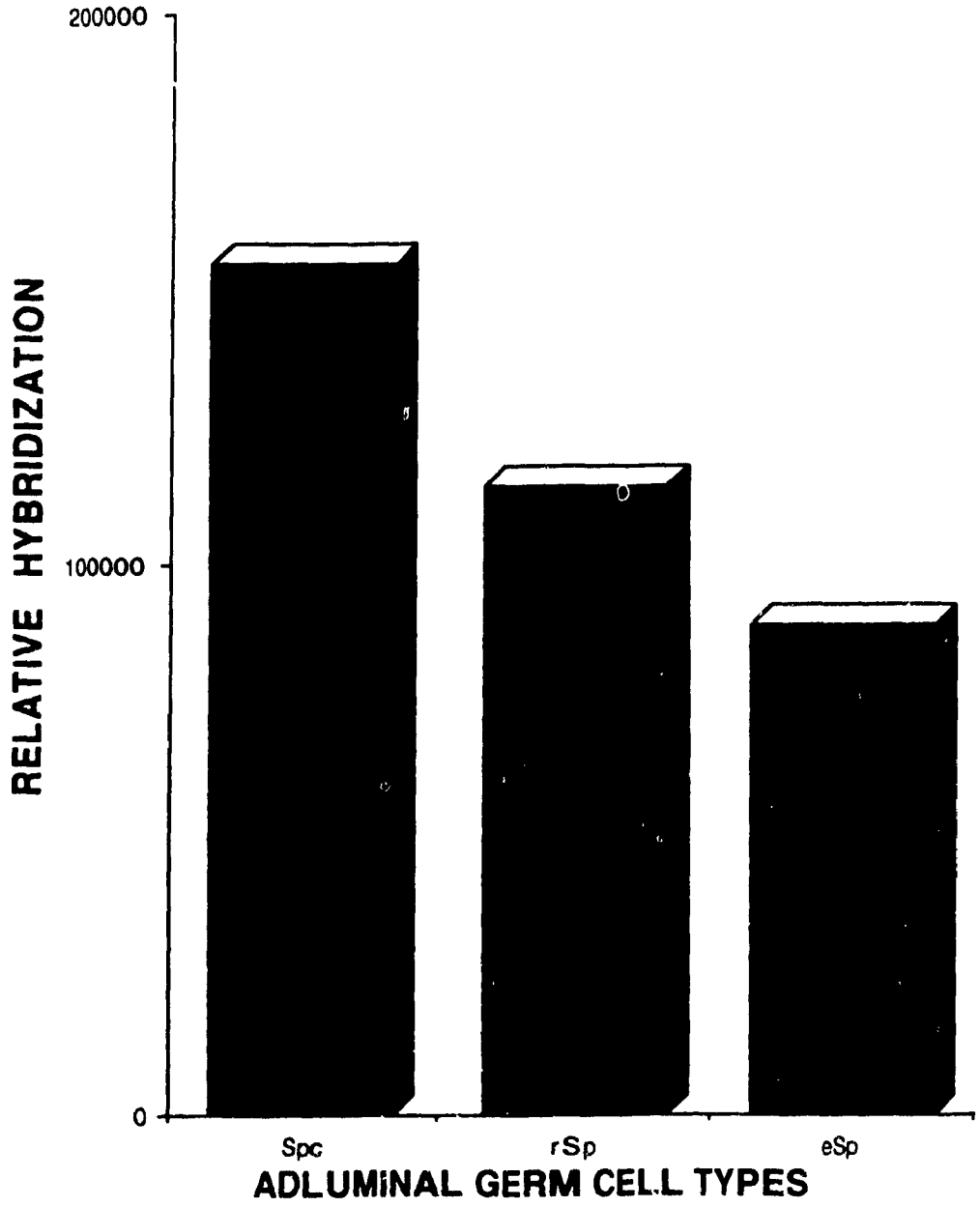

200000
100000
0
RELATIVE HYBRIDIZATION
Spc
rSp
eSp
ADLUMINAL GERM CELL TYPES

FIGURE 19

Transferrin recycling profile in round spermatids. This study shows that testicular transferrin, following binding to cell surface receptors, is recycled back to the extracellular milieu of the adluminal compartment of the rat seminiferous epithelium. Round spermatids were isolated (<90% purity) in the presence of trypsin inhibitor and allowed to coincubate with [^{125}I]-transferrin for 6 hours at 4°C. The incubation medium was then slowly raised to 37 C to permit internalization of the label for 10 minutes. The cells were then treated with trypsin to remove all cell surface protein, both specific and non-specific, and washed 3 times with HBSS at 4°C. Replicates were then allowed to incubate at 37°C for various lengths of time after which they were spun down and samples of the supernatants withdrawn, TCA precipitated and counted.

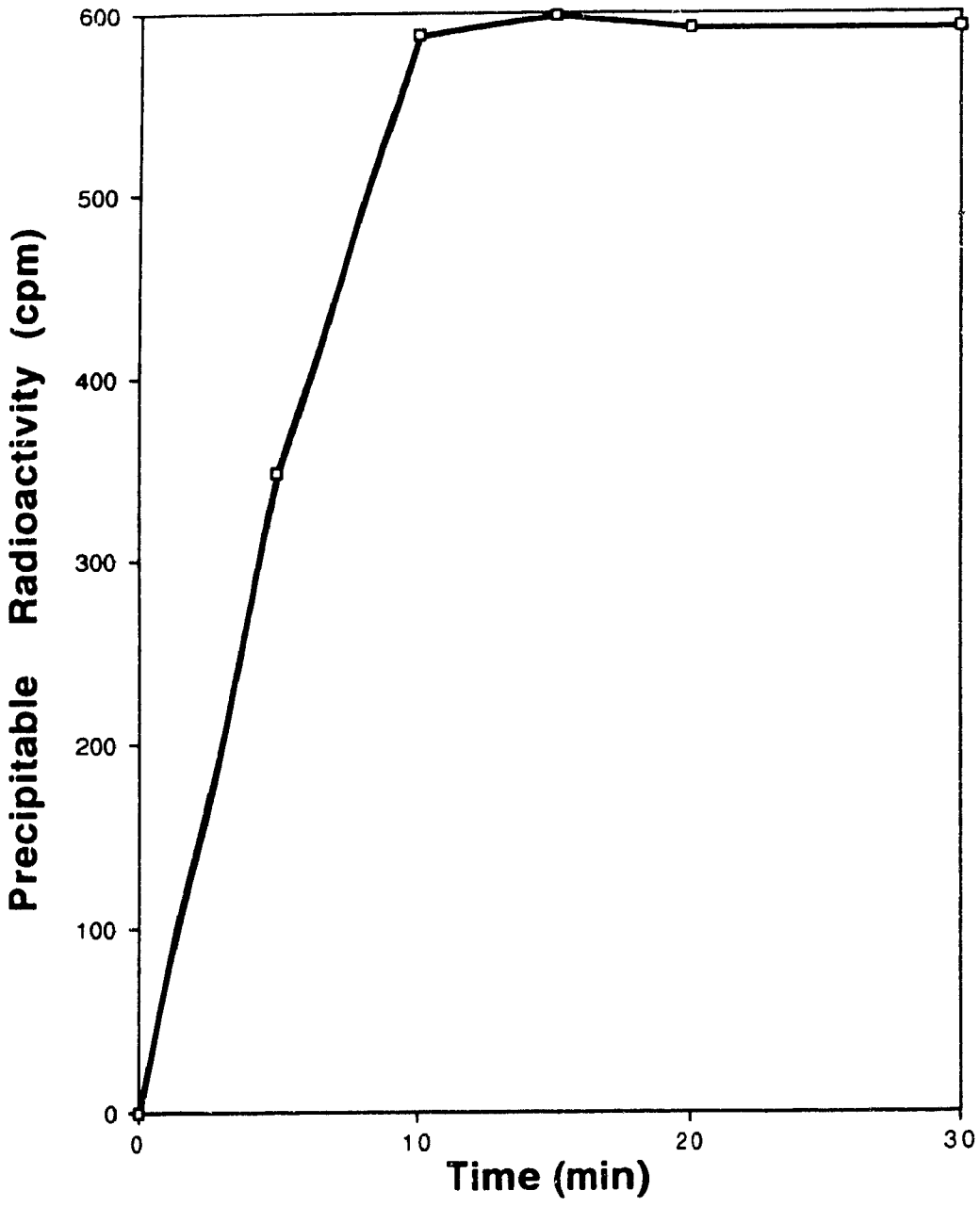
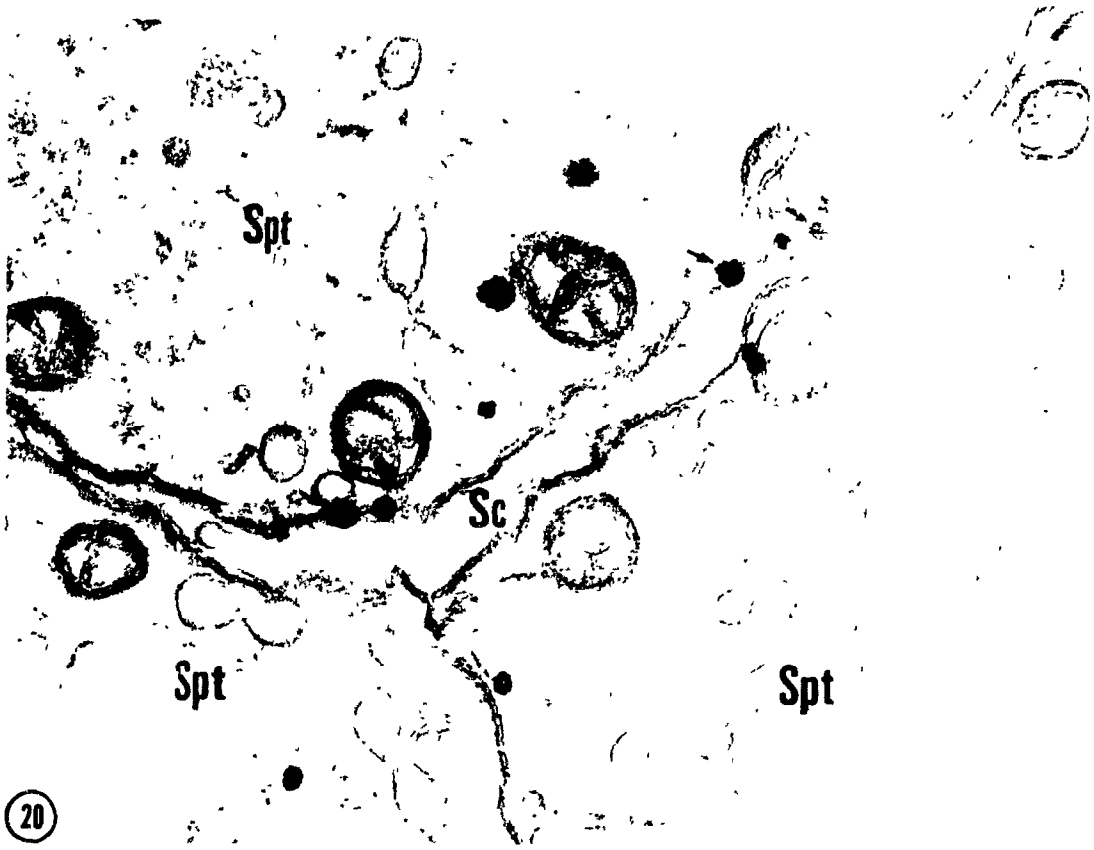

Precipitable Radioactivity (cpm)
600
500
400
300
200
100
0
0
10
20
30
Time (min)

FIGURE 20

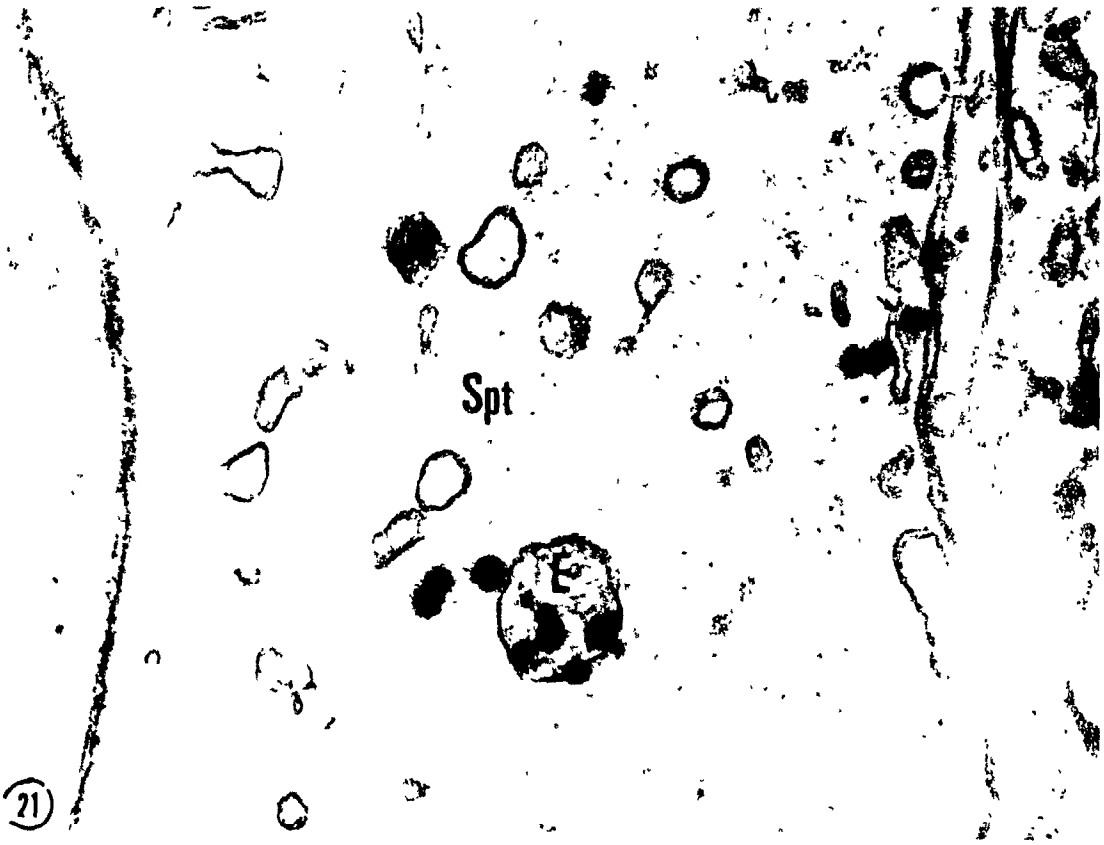
Electron micrograph showing portions of three round spermatids from an animal sacrificed 5 minutes following an intralumenal injection of [^{125}I]-transferrin. Silver grains can be seen overlying or in close opposition to the plasma membrane (arrows). **Spt**, spermatid cytoplasm; **Sc**, Sertoli cell cytoplasm. X42 000.

FIGURE 21

Electron micrograph showing a portion of a round spermatid from an animal sacrificed 15 minutes following an intralumenal injection of [^{125}I]-transferrin. Silver grains can be seen overlying the plasma membrane (arrows) or an endosome (E). Spt, spermatid cytoplasm; Sc, Sertoli cell cytoplasm. X42 000.



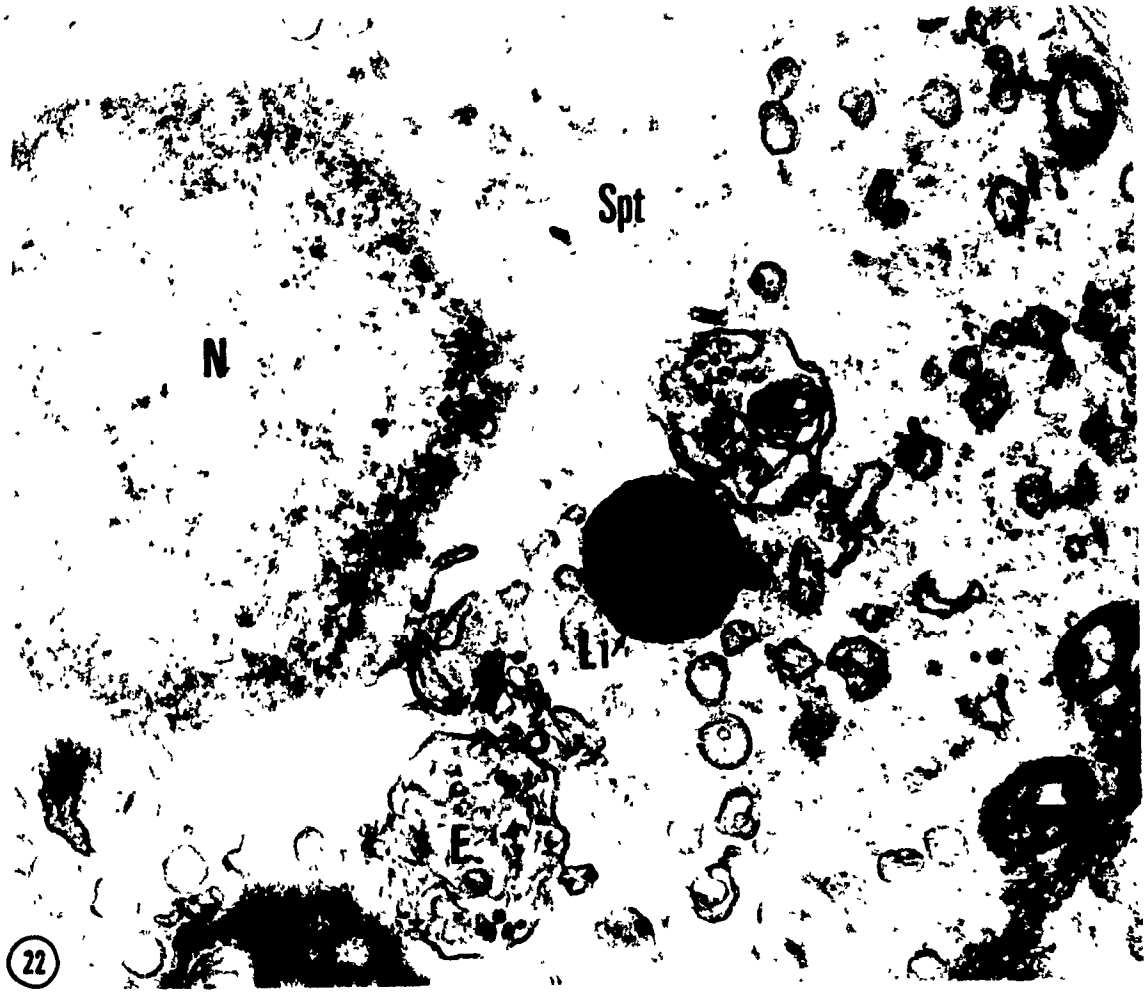
20



21

FIGURE 22

Electron micrograph showing a portion of a round spermatid from an animal sacrificed 30 minutes following an intralumenal injection of [^{125}I]-transferrin. No significant labeling is seen to be remaining in association with the spermatid. E, endosomes; **Spt**, spermatid cytoplasm; **N**, spermatid nucleus; **Cb**, cytoplasmic chromatoid body; Li, cytoplasmic lipid inclusion. X42 000.



(22)

DISCUSSION

Transferrin, being the main iron carrier in mammals, serves to transport vitally required iron to all cells in the organism thus ensuring that critical enzymatic processes can take place. Adluminal germ cells of the rat seminiferous epithelium do not have access to sTf so they depend on Sertoli cells to supply them with iron via tTf. The final steps of the iron delivery pathway, following the secretion of diferric tTf in to the adluminal compartment by Sertoli cells, have been characterized.

One of the objectives of this investigation was to test the hypothesis that haploid germ cells located in the adluminal compartment may possess a novel endocytic route for Tf following binding to the Tf receptor. This hypothesis suggested that the internalized apoTf-Tf receptor complex, rather than being recycled as is generally the case, may be targetted to the lysosomal compartment of adluminal germ cells. The reasoning behind this idea is as follows: 1. That given their physically and environmentally unique disposition, adluminal germ cells may display endocytic function that differs from that of somatic cells, 2. That the presumed recycling of apoTf would seem to be futile since free iron or iron present in a chelatable form is not known to be a constituent of adluminal fluid, 3. That Sertoli cells have

never been observed to internalize apoTf at their apical aspect, which potentially could be a means of apoTf reutilization, 4. That since there has been no function so far ascribed to apoTf in the semen, evolution may have provided for a means of disposal, perhaps via degradation in germ cells, of a carrier that is seemingly without function once released.

In addition, several experiments were carried out to characterize the mechanism of iron sequestration employed by adluminal germ cells. In so doing we expected to elucidate the final events in the seminiferous epithelium iron-delivery pathway following secretion of tTf by the Sertoli cell.

As it is understood to date the model of iron delivery envisaged to take place in Sertoli cells contains the following steps: 1. Diferric sTf binds to Tf receptors at the basal pole of Sertoli cells, in a pH-dependent fashion, and is internalized by receptor-mediated endocytosis, 2. The Tf-Tf receptor complex is routed to the endosomal compartment where acidification to pH 5.5 causes iron to dissociate from the Tf while leaving the complex intact, 3. The apoTf-Tf receptor complex is recycled back to the cell surface where, upon encountering neutral pH, the apoTf quickly dissociates and the receptor binds yet another diferric sTf molecule, 4. The liberated iron is somehow coupled to tTf either before or after packaging at the Golgi stacks, 5. The diferric tTf is

secreted into the adluminal compartment of the seminiferous epithelium (Morales and Clermont 1986b; Morales et al., 1987b) (Fig.5). Events following secretion have not however been fully characterized. Immunohistochemical studies designed to localize tTf and the Tf receptor in the seminiferous epithelium have strongly suggested that Tf receptor is present on differentiating germ cells (Sylvester and Griswold, 1984; Brown, 1985). Recently it has been shown, using northern blot analysis that spermatocyte express Tf receptor mRNA (Roberts and Griswold, 1990). Amongst these reports, however, results differ in the types of germ cell that are proposed to express the Tf receptor.

Studies carried out in this thesis have attempted to clarify this issue and furthermore describe the final step of the iron delivery model: The binding characteristics of tTf to adluminal germ cells and events following the presumed internalization of the tTf-Tf receptor complex.

Polarized Secretion of Testicular Transferrin by Sertoli cells and its internalization by adluminal germ cells

As an important predicate to this study it was necessary to demonstrate de novo synthesis and the polarized secretion of tTf, into the adluminal compartment, by Sertoli cells. In order to accomplish this we used a procedure, involving the metabolic labeling of newly synthesized proteins, previously utilized by Morales et al. (1987b). This experiment was

designed by these investigators to prevent contamination by sTf and allow protein synthesis, labeled with [^{35}S]-methionine, to proceed under as near physiological conditions as possible. Hence the main organ of sTf synthesis, the liver, was removed from the circulation by porta-hepatic ligation and [^{35}S]-methionine incorporation was left to occur in the testes in situ. These measures have been accepted as valid means of ensuring that the reported synthesis of tTf by Sertoli cells (Griswold et al., 1987) is indeed not artifactual as had been suggested by Shabanowitz and Kierszenbaum, 1986; Lee et al., 1986. This experiment clearly verified that: 1. Sertoli cells synthesize immunoreactive Tf, now referred to as tTf, 2. Since no Tf activity was associated with the extratubular fraction (TIF) but only with SNF and the cellular fractions, Sertoli cells therefore only secrete tTf adluminally. Furthermore, given that tTf activity is associated with the cellular fraction which includes total germ cells, this experiment also leaves open the possibility that adluminal germ cells may internalize tTf.

To ascertain whether or not tTf was in fact internalized by adluminal germ cells, a similar experiment was carried out where, under the same conditions, testicular de novo protein synthesis was labeled with [^{35}S]-methionine. In this case, however, the spermatogenic cells were separated by staput velocity sedimentation and done so at low temperature to ensure that any internalized tTf remained within the cells.

Spermatocytes and round spermatids were chosen for use in this experiment as they could be isolated in high purity without contamination of cytoplasts and cellular debris. Since these cells were treated with pronase prior to lysis and immunoprecipitation, the radioactive band seen running at 77 Kd must correspond exclusively to internalized and newly synthesized tTf secreted from the Sertoli cell in vivo (Fig.6). Hence these results show that adluminal germ cells, or at least one of either spermatocytes or round spermatids, do internalize tTf.

Scatchard Analysis of Transferrin Binding to Round Spermatids

If spermatogenic cells internalize tTf, is this process receptor-mediated and therefore is it specific? We decided to investigated the characteristics of the Tf receptor on isolated round spermatids which could be isolated at high number and purity. Under optimized conditions the binding assay was carried out generating a seven-point Scatchard plot (Fig.10). The resulting binding data was analysed using a standard graphics computer program from which a regression line was requested taking into account all datapoints. Because the B=0 datapoint is so far removed from the plot line it is considered to have occured by random error. This conclusion is supported by the calculated correlation coefficient= 0.9 which also takes the B=0 datapoint into account. In terms of r^2 which is equal to 0.8, little

variance was due to random scatter of the datapoints.

The calculation of the apparent Kd from slope data was also performed and a value of 0.6 X 10^{-9} M was obtained. The linearity of the plot and the Kd value indicate a single type of binding site and that it is of very high affinity, respectively. These results are in agreement of those obtained by Holmes et al. (1983) where the Tf receptor on isolated pachytene spermatocytes was found to carry high affinity binding site of a single type with an apparent Kd value of 0.3 X 10^{-9} M (This fraction is being used as a means of comparison since a Kd value for the spermatid fraction was not supplied in that publication (Holmes et al., 1983). Assessment of receptor number on spermatids however was supplied. Whereas these authors reported spermatids to express 551 binding sites/cell, our data suggests that round spermatids carry 1453 high affinity binding sites/cell.

Before carrying out our binding assay, optimal binding conditions for this particular cell type and ligand were carefully established. Dilution experiments to identify optimum cell concentration and binding time for the assay indicated that 8 X 10^6/ml and 6 hours represented the best parameters, respectively. It is however not known, from this authors publication, under which conditions the cited experiments were carried out. The above discrepancy could be explained by the fact that the fractions used in the binding

assays in this study were greater than 90% pure round spermatids while the fractions used in their study were contaminated with elongated spermatids, spermatocytes and other cellular debris to the extent of 30%. This factor could conceivably have caused an underestimation in receptor number.

Transferrin Receptor mRNA Expression in Adluminal Germ Cells

Although transferrin specifically binds to round spermatids and the binding data clearly indicates that internalization of Tf in these cells is a receptor-mediated event, it is still not conclusive proof that that round spermatids specifically express this protein. We therefore turned to molecular means to answer this question. To run whole testicular RNA of a mature rat on northern blot and probe this for Tf receptor mRNA is not sufficient to demonstrate that germ cells express this message. It could be argued that the probe is simply detecting Tf receptor mRNA produced by Sertoli cells, the greatest source of this message in the testis.

In order to eliminate this interpretation, RNA from prepubertal rats of increasing ages was run on northern blot. The rational was that since Sertoli cells stop dividing at 20 days of age in the rat, any differential expression of the Tf receptor among these samples could be attributed to germ cell expression. Because aliquots of each sample were of equal

volume, fractionation by gel electrophoresis revealed the amount of Tf receptor mRNA relative to the stage of testicular development. Densitometric analysis of the resulting autoradiograph depicts this relationship (Fig.13). Our results demonstrate that Tf receptor mRNA levels increase throughout the development of the seminiferous epithelium and moreover that the expression rate positively correlates with the multiplicative increase in germ cell number that accompanies this maturation process.

This correlation was taken to suggest that germ cells express this message. Since Sertoli cells do not increase in number after 20 days, it was reasoned that the observed increase was unlikely due to Sertoli cells. It could be argued however that Sertoli cells, despite their constant numbers, may have up-regulated their own Tf-receptor mRNA production as the epithelium matured thus causing the observed pattern of increase. This interpretation, although unlikely, cannot be dismissed since there exists no studies to our knowledge documenting such a regulatory mode in Sertoli cells.

To conclusively demonstrate that germ cells actually do express Tf receptor mRNA, spermatocytes, round spermatids and elongated spermatids of the adlumenal compartment were isolated. It was also of interest to determine whether Tf receptor mRNA is differentially expressed amongst these cell populations. The mRNA equivalent of 1 X 10^7 cells of each

spermatocytes, round spermatids and elongated spermatids was fractionated by gel electrophoresis. In this way the relative expression rates of this mRNA specie, amongst the three classes of cells, could be visualized. Brown (1986) observed that anti-Tf receptor antibody labeled these cells, where spermatocytes were labeled most of all, spermatids to a lesser extent and elongating spermatids and mature spermatozoa not at all. Our results confirm this decreasing trend in expression as depicted by densitometric analysis. We show however that elongating spermatids express the lowest levels transcripts of all but nevertheless it is expressed in this cell type.

This discrepancy cannot be readily explained with the available data. These results thus show that all three spermatogenic populations of the adluminal compartment express Tf receptor mRNA and moreover that the expression rate progressively decreases as the cells mature.
Our results also confirm northern blot analysis recently carried out which showed that spermatocytes express Tf receptor mRNA (Roberts and Griswold, 1990).

Ferritin L-Chain mRNA Expression in Adluminal Germ Cells

Levels of ferritin L-chain mRNA in relation to seminiferous epithelium maturation and to adluminal germ cell development were evaluated exactly as above. This was done for three reasons: 1. To serve as a positive control for the

Tf receptor experiment above, 2. To determine if ferritin, which is closely involved with the storage of iron, was expressed and how it was expressed in this tissue, and 3. To ascertain how its levels may vary in relation to those of Tf receptor mRNA.

The northern blot analysis shows that this specie of mRNA is also expressed throughout the development of the seminiferous epithelium. The densitometric data reveals moreover that the expression profile of ferritin L-chain mRNA in relation to animal age is very similar to that seen for the Tf receptor. The profile character, which is thus common to both mRNA types, supports the notion that the increase is due to germ cell proliferation and that it is in fact the germ cells which are expressing these two mRNA species.

The northern blot used to determine Tf receptor expression in adluminal germ cells was striped of the cDNA probe for Tf receptor and reutilized in the present experiment. When hybridized with a probe specific for ferritin L-chain mRNA, bands at 1.1kb were detected. In terms of relative hybridization variations were similar to those observed for Tf receptor mRNA. This positive control thus suggests that the result obtained for Tf receptor mRNA was reliable and specific. This finding also demonstrates that a positive correlation exists between the levels of these two mRNA species as a function of germ cell maturation. An

explanation of why this correlation exists can, however at this point, only be a matter of speculation.

The levels of Tf receptor and ferritin mRNA have been shown to be present in an inverse relationship, due to regulation by iron in some cell types (Mullner et al., 1989; Theil, 1990). This observed simultaneous decrease in these two mRNA species may hypothetically be consistent with this model: As the expression of Tf receptor is progressively decreased, by some unknown regulatory mechanism, as germ cells mature, the cytoplasmic IRE (iron regulatory element) becomes activated due to iron depletion; The IRE in turn prevents ferritin mRNA translation and thus, by shifting the intracellular equilibrium to the left, the parallel decrease in mRNA transcription for this specie is observed. The sharper decrease in expression of Tf receptor mRNA, compared to that for ferritin L-chain mRNA (Figs.17,18), may indeed support this hypothetical feedback mechanism.

Alternatively there exists a more probable and perhaps more realistic explanation for these results. It has been shown for a number of proteins that there is a progressive decrease in synthesis as germ cells approach spermiation (Hecht, 1988). At spermiation germ cells are terminally differentiated so therefore maturing adluminal germ cells may display a decreasing need to acquire and store iron. In fact transcription has been shown to be terminated early in

spermiogenesis, previous to or during the initiation of nuclear condensation (Hecht, 1988). It is therefore possible that this observed decrease is due and related only by the fact that transcripts for the two mRNA species, synthesized prior to transcriptional termination, are simply being depleted as adluminal germ cells mature towards spermatozoons.

Recycling of Transferrin in Round Spermatids

As indicated above, the purpose of this investigation was to analyze the fate of tTf once having been internalized by adluminal germ cells. According to our hypothesis, there was good reason to believe that a novel system for the endocytic routing of tTf existed in adluminal germ cells. Again, round spermatids were used since these cells could be isolated in high numbers and purities. The [^{125}I]-Tf-loaded round spermatids were treated with pronase to remove all non-specific activity and replicates were warmed for various lenghts of time to allow activation of the endocytic apparatus. It was found that rather than conforming to our hypothesis, the results showed that measured radioactivity of suspension supernatants localized to the TCA precipitable fraction.

This observation indicated that intact Tf was being recycled out of round spermatids. Had degradation been taking place, free non-precipitable radioactivity was expected to

have appeared in the suspension media. Our results from this experiment also suggest that recycling of the internalized Tf achieved a constant rate at approximately 10 minutes following reactivation of the endocytic apparatus by warming. This period is within the range of known recycling half-times from various cell lines examined in a similar way (Wileman et al. 1985). Attempts to record the earliest time at which recycling occured failed, presumably because levels of radioactivity prior to 5 minutes were beyond our boundaries of detectability. Neverthless an equilibrium recycling rate of approximately 10 minutes could be consistently reproduced.

Since this experiment was repeated a number of times with the same lack of detectable radioactivity prior to 5 minutes, it is possible that this behaviour may be attributable to a "recycling lag" in response to low temperature. In other words it is possible that these cells show a higher sensitivity to temperature-dependent immobilization of their endocytic apparatus, the consequence of which being a longer time to respond to warming. Closer analysis will be required to determine the initial recycling rate of apoTf in these cells.

Therefore, according to the obtained recycling data, the endocytic routing steps for the Tf-Tf receptor complex in round spermatids is similar to that found in other cells. It would also seem that the Tf concentration in tubular fluid, as

measured by Sylvester and Griswold (1984), represents the full compliment of Sertoli cell secretion. Sertoli cells have not been observed to take up radioactive Tf and adluminal germ cells have now been shown not to degrade it. Thus the reported concentration must represent, despite being quite low, all secreted tTf (diferric and apoTf) by Sertoli cells. The function of lumenal apoTf, however, remains an open question. Clinical studies have suggested that up to 80% of Tf present in the ejaculate of human subjects is of Sertoli cell origin and that these levels could be used as markers of Sertoli cell function. Transferrin has been reported to act in a bacteriostatic capacity in some instances (Schade and Caroline, 1946) but with the calculated concentration being so low it was concluded that the purpose of lumenal apoTf must be other than for biological defense (Sylvester and Griswold, 1984). The function, therefore, if one exists, of apoTf itself in the reproductive tract still remains to be illucidated.

Internalization and Subcellular localization of [^{125}I]-Transferrin in Adluminal Germ Cells

As a means of identifying the subcellular compartments involved in the internalization and recycling of Tf, as observed in the above in vitro experiments, we have employed electron microscope radioautography in the following in vivo analysis. Autoradiographs taken at 5, 15 and 30 minutes following injection of [^{125}I]-Tf into the rete testis were

examined. At 5 minutes following injection, the label is seen associated with the plasma membrane of a spermatid. This rapid association time was consistent with that found in other cells carrying high affinity binding sites for Tf. At 15 minutes the label was seen to have been internalized and to have entered the endosomal compartment. Interestingly some label was also seen in close proximity to the plasma membrane. It was not possible however to determine whether this Tf was entering or exiting the cell. At 30 minutes following injection, the corresponding autoradiograph showed that no label was found in endosomes or in the vicinity of the germ cells. Therefore this experiment presents additional support for the observation that tTf is internalized by adluminal germ cells and eventually recycled back, as apotTf, to the adluminal extracellular space. In addition it was observed that the binding and recycling characteristics of the apoTf-Tf receptor complex in these cells are consistent with those generally reported for a number of other cell types (Goldstein et al., 1985).

General Summary and Conclusions

From the experiments carried out herein, the following conclusions can be reached:

1) Adluminal germ cells which are not exposed to interstitial fluids due to the BTB, depend on Sertoli cells to supply them with essential factors necessary for their growth and differentiation. In the case of iron, the Sertoli cell has developed to adequately fulfill this role by directing its secretion of tTf towards the adluminal compartment where the germ cells in question reside. It has become clear that, once secreted, diferric tTf is internalized by spermatocytes, round spermatids and elongated spermatids.

2) Serum Tf is universally known to enter target cells by means of the Tf receptor A like mechanism is also expected to exist between tTf and adluminal germ cell. Indeed a single type of high affinity binding site does reside on the surface of these cells, presumably involved in the process of receptor-mediated endocytosis of tTf.

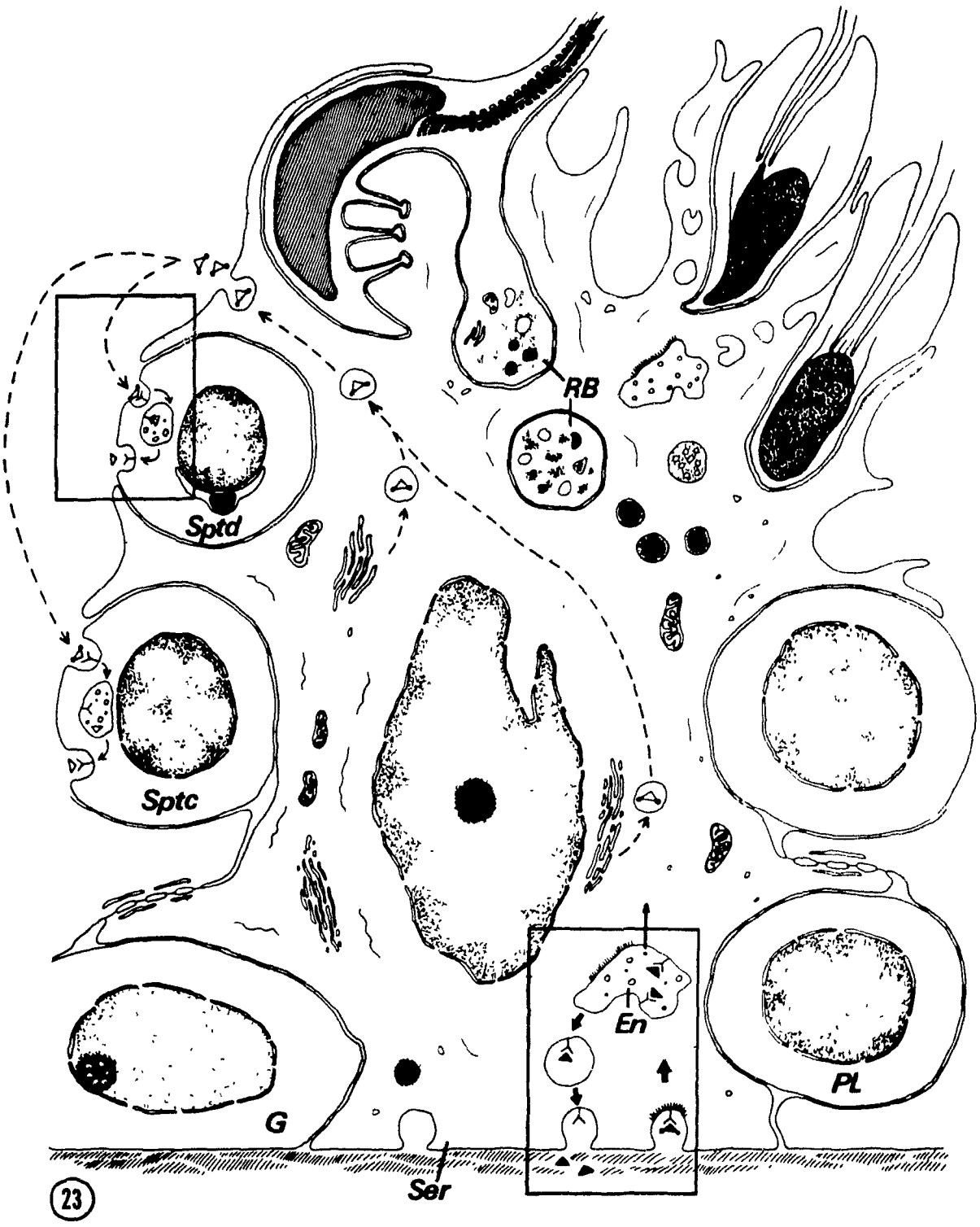
3) Using molecular means it is possible to ascertain with relative certainty if a given protein is expressed by a tissue. Adluminal germ cells which appear to bind tTf with high affinity, do in fact express the mRNA for the Tf receptor. They do so, however, with an inverse

relationship to their level of maturity. Whereas spermatocytes express the most Tf receptor mRNA, elongating spermatids contain the least of the message. Ferritin L-chain mRNA, which is also expressed in these populations of germ cells, shows the same differential pattern of expression.

4) After internalization into the endosomal compartment, the endocytic route employed by the apoTf-Tf receptor complex seems to be highly conserved. Thus, unlike most R-L complexes where the receptor is recycled and the ligand is left behind for degradation, apoTf remains bound to its receptor until the complex is recycled back to the plasma membrane. In round spermatids the tTf-Tf receptor complex appears to lose its complement of iron in the endosomal compartment and subsequently recycle back to the cell surface following kinetics that are consistent with other known systems.

FIGURE 23

Updated schematic representation of the iron delivery pathway across the seminiferous epithelium of the rat. The lower box illustrates the first step of iron delivery: Receptor-mediated endocytosis of diferric serum transferrin (Tf) at the basal pole of the Sertoli cell and recycling of apoTf back to the interstitial space. Acidification of the endosome containing the internalized complex results in the release of the iron atoms from serum Tf. It is then known that these atoms become associated with testicular Tf. The number and nature of the steps between the release of iron and its coupling to tTf still remains to be determined. At the upper aspect of the drawing it is shown that the diferric testicular Tf is secreted by the Sertoli cell into the adlumenal compartment.
The upper box illustrates the subsequent steps which have been characterized in this study. Thus, secreted diferric testicular Tf specifically binds to high affinity transferrin receptors (Tf receptor) on the surface of spermatocytes and spermatids. The newly-formed complex is then internalized by these cells and localizes to the endosomal compartment. Iron is presumably liberated following the same mechanism as above and stored in ferritin. Finally testicular Tf is recycled back to the lumenal fluid. **Ser**, Sertoli cell; **Sptd**, spermatid; **Sptc**, spermatocyte; **G**, spermatogonia; **PL**, preleptotene spermatocyte; **En**, endosome; **RB**, residual body; (▲), serum transferrin; (Δ), testicular transferrin; (), iron.


RB
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ORIGINAL CONTRIBUTIONS

1. In vitro demonstration that adluminal germ cells are capable of binding and internalizing diferric transferrin by receptor-mediated endocytosis.

2. In vivo demonstration that testicular transferrin, secreted by Sertoli cells into the adluminal compartment, is internalized by adluminal germ cells.

3. Demonstration that isolated round spermatids recycle internalized [^{125}I]-transferrin (presumably apotransferrin) back to the adluminal fluid of the seminiferous epithelium.

4. Description of subcellular compartments involved in receptor-mediated endocytosis and recycling of [^{125}I]-transferrin in round spermatids.

5. Demonstration that both transferrin receptor mRNA and ferritin L-chain mRNA are differentially expressed in adluminal germ cells.