Discussion
Earlier studies from this laboratory reported the identification of a rat testicular protein (approximate molecular weight of 24kDa under reducing conditions) with an anti-sperm serum. Antisera raised against this protein recognized immunoreactive epitopes on human spermatozoa and caused agglutination of the same (Shaha et al, 1988). Animals immunized against the 24kDa protein isolated by preparative gel electrophoresis remained infertile as long as antibody titres were maintained up to a certain threshold (Shaha et al, 1990). These antisera were also able to inhibit rodent sperm-oocyte interactions in vitro (Shaha et al, 1989). The above data clearly indicated a role for this protein in fertility-related events. It therefore, became important to characterize it, in terms of its localization and function. Results were expected to contribute towards the understanding of both fertility and infertility. This project was therefore initiated to investigate the characteristics of this protein using rat as the primary model for localization and regulation studies. Since this molecule was shown to be present in sperm of multiple species earlier, a goat sperm in vitro fertilization system was used as an assay for evaluating the efficacy of antibodies against the purified protein for inhibition of fertilization.

The purified 24 kDa protein from rat testis obtained by size exclusion chromatography followed by HPLC showed a significant similarity at the NH$_2$-terminus with rat cytosolic GSTs. GSTs are a family of multifunctional proteins involved in detoxification of electrophilic xenobiotics (Meister and Anderson, 1983) and probably act as intracellular transport proteins (Listowsky, 1993). GSTs have been classified into five major classes which are the alpha, mu, pi, theta and sigma based on their primary structures (Hayes and Pulford, 1995).
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Since the NH$_2$-terminus of the 24kDa protein showed such significant similarity with GST it was of interest to check if the protein possessed other characteristics inherent to the GST molecule. Saturable GST activity was obtained with this protein to the general GST substrate CDNB, which could be inhibited by known GST inhibitors. On SDS-PAGE, it resolved as a thick band in the same region as the mu class GST from rat liver. Upon anion exchange HPLC, the protein eluted at the same time point as that of liver GST. Rat GSTs are invariably dimers consisting of identical or closely related subunits with molecular masses around 24 - 25 kDa (Meister and Anderson, 1983). Since all GSTs are purifiable on GSH affinity columns (Hayes and Pulford, 1995) this protein was tested for its ability to get purified on GSH linked to Sepharose 4B. The purified product showed saturable enzyme activity with CDNB, shared similarity with various subunits of mu class GST at the NH$_2$-terminus and reacted to the antisera originally reported (Shaha et al, 1988). Affinity chromatography with S-hexyl glutathione is known to enrich class mu, pi, and sigma GST while class alpha GST do not display strong affinity for the same (Hayes, 1986). A number of class mu GSTs have been identified in the testis of different animal species. A CDNB-reactive form of rat testis GST designated as GST-MT which resolves between subunits 1 and 2 of class alpha GST on SDS-PAGE and has a pI of 5.7 (Ishikawa et al, 1988) is known. Hamster, mouse and human testicular mu GSTs are also reported (Bogaards et al, 1992, and Awasthi et al, 1993, Campbell et al, 1990). Therefore, with the above studies we established that the 24kDa rat testicular protein clearly belonged to the family of GSTs, as apart from sharing an extensive NH$_2$-terminal similarity, this protein was reactive
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towards GST substrates, reacted to the reference antiserum for GST and showed similar molecular weight on SDS-PAGE as that of GSTs.

The 24kDa protein earlier purified by preparative gel electrophoresis from rat testis was able to generate an immune response in rabbits, rats and mice. Therefore, it was essential to check if the 24kDa proteins, now established as a member of the GST family showed the same properties. Taking advantage of the fact that this protein was conserved through evolution and was present on sperm of multiple species, an established system of goat in vitro fertilization was used to test the effect of these antibodies on sperm-oocyte interaction. Antibodies against proteins purified by both the methods, were able to block fertilization and this block could be reversed if the antibodies were adsorbed with respective proteins. While this observation confirmed earlier data on fertilization obtained with immunization of the 24kDa protein purified by preparative gel electrophoresis, it also established that this protein, which closely resembled GST proteins was a relevant molecule for fertility related events.

Since GSTs are reported to be predominantly cytosolic proteins (Listowsky, 1993), it was curious that antibodies against the rat testicular GST-like protein were able to block a cell surface event like sperm-oocyte interaction. This implied that GSTs may have a very different functional profile in the testis. As a first step towards the understanding of the regulation and function of the testicular GST, testicular fluids were examined for the its presence. It was interesting to find that enzymatically active and immunoreactive GST was present predominantly in the TF as compared to the IF. GST activity of the total testicular homogenate was also three times less than that of TF showing that the primary
concentration of the protein in the testis was confined to the TF. Nature of the immunoreactive and enzymatically active GST from TF was confirmed by GSH affinity chromatography purification followed by NH$_2$-terminal sequencing which revealed similarity with the Yb$_2$ (rGSTM2) subunit over a 30 amino acid overlap. The TF plays a vital role in maintaining the microenvironment for germ cells and Sertoli cells and is rich in diverse types of proteins. A large number of secreted proteins have been identified in this fluid which are released by Sertoli cells (Griswold, 1988). IF-GST activity was 25 - 30 times lower as compared to TF-GST activity and immunoreactive GST was also much less in the IF. The relative abundance of this protein in the TF reflected apical release of the protein as opposed to basal secretion.

Analysis of GST subunits present in TF and whole testis homogenate showed presence of 10 components as compared to 9 in liver. Yb$_2$ form of GST was the most abundant form present in the TF. One fraction which was a relatively minor component in the liver but was a major peak in the testis, was found to have similarity at the NH$_2$-terminus with Yb$_3$ (rGSTM3). In humans GST Yb$_3$ (rGSTM3) has been reported to be a major form in testis and cerebral cortex (Campbell et al, 1990 ). All other fractions were similar between the TF and liver GSTs (Van Ommen et al, 1990). GST subunit Yb$_2$ (rGSTM2) was found to be the most predominant form present in TF as purified by GSH affinity chromatography and hence the sequence obtained was significantly similar to GST Yb$_2$. The predominance of the Yb$_2$ form explains the single sequence data obtained when TF GST was sequenced after GSH affinity purification. TF had to be concentrated several times in order to visualize the other forms.
Proteins present in the TF are deemed to be secretory as TF is primarily a secreted product of the ST. Secretory nature of this protein was indicated by its presence in the TF, however, the actual ability of the cells of the ST to secrete the protein, remained to be confirmed by more direct assays. Time-dependent accumulation of the protein over a period of 8h was observed in cultures of STs of animals of different ages. The relatively low rate of secretion by ST from animals below 18 days of age was consistent with the fact that the seminiferous tubular lumen and the fluid secretion ability develop only after day 18 of age in rats (Vitale et al, 1973). To ensure that germ cell-Sertoli cell combination with the ST remained viable during the period of culture, [3H]-Thymidine was used to label dividing cells and it showed that over the period of culture, the combination of Sertoli-germ cell remained viable, as the germ cells were actively dividing. Germ cells are known to divide in TF cultures upto 72h and therefore can be used to measure various parameters upto that time (Toebosch et al, 1989). Having established that the protein was being secreted into the spent culture media, it remained to be shown that active synthesis was taking place in this system. Radioactive $^{35}$S methionine and $^{35}$S cysteine was incorporated into GSH affinity-purifiable GST from the spent culture media of ST when the radioactive amino acids were supplied during the period of culture indicating that active synthesis and secretion of the protein occurs within the ST. This is the first report of a secretory form of GST occurring in a body fluid. Secretory forms of a closely related enzyme glutathione peroxidase have been found in the epididymis, however none of the GST-related enzymes have been reported to be present in TF. Presence of GST in the TF may have important functional implications as a binding protein for steroids.
(Maruyama and Listowsky, 1984) and as an important detoxification enzyme protecting germ cells from oxidative damage (Yoganathan et al, 1989).

It is interesting to note that all the subunits which were present in the rat liver cytosol were also present in a secretory fluid such as TF. Mammalian secretory proteins, with notable exception of serum albumin, are all glycosylated. When we looked into the glycosylation pattern, the affinity purified protein from TF reacted to Con A, a reactivity which could be displaced by incubating Con A with mannose, showing specificity of the reaction. The affinity purified protein from the liver did not react to Con A. Glycosylation was further checked with lectins specific for galactose, N-acetyl galactosamine and sialic acid. Since the TF was maximally reactive to the mannose-specific lectin, *Galanthus nivalis*, reactivity of different subunits was checked with that lectin and it was found that glycosylation occurred in subunits Yb1 (rGSTM1), Yb2 (rGSTM2) and another subunit which could not be sequenced possibly due to an NH2 terminal block. Glycosylation of pi class GST has been reported (Kuzmich et al, 1991) however, there are no reports on mu class GST being glycosylated. In the literature, most of the reports suggesting post-translational modifications of GST are based on data from *in vitro* experiments. Therefore the *in vivo* biological significance of these events is unclear. GSTs undergo various post-translational modifications which have both activating and inhibiting effects on GST functions. Phosphorylation of rGSTA1 and A2 subunits decreases their affinity for bilirubin, which presumes that affinity of these subunits for other non-substrate ligands will also be reduced by the modification (Taniguchi and Pyerin, 1989). It has been suggested that human LTC4S which has a
protein kinase C phosphorylation site, is phosphoregulated in vivo. Phosphorylation may inhibit production of LTC4 (Nicholson, 1993). Maximal level of methylation achieved in vitro for GSTM6 is only 22%, which resulted in significant reduction in activity toward CDNB (Johnson et al., 1992). Glycosylation has been reported so far only in rat (Tsuchida and Sato, 1992) and human pi GST, while human LTC4 is known to contain a potential N-linked glycosylation site (Hayes and Pulford, 1995). The reported sequences of rGSTM2(Yb2) and rGSTM1(Yb1) from rat liver cytosol do not show any N-glycosylation site. Liver Yb2 gene sequence has not been reported to show any N-glycosylation site (Manoharan et al., 1992). It is possible that O-glycosylation has occurred or that testicular mu GST has N-glycosylation sites and that this TF-GST may be coded for by a different gene. This is the first report of glycosylation of Yb subunis of GST which are found in a secretory fluid.

The two possible sources of GST secretion within the ST are the Sertoli cells and the germ cells. Immunocytochemically this protein was localized to Sertoli cells in testis cross sections and in Sertoli clumps isolated from adult rat testis. In addition, Sertoli cells cultured in vitro from immature, 18 day old rats showed clear localization of the protein, distributed throughout the cytoplasm. Immunoreactive GST identified in immature Sertoli cell culture supernatants showed that Sertoli cells were able to secrete the protein in vitro. Traditionally, all evidences in literature indicate that Sertoli cells are the primary secretory cells in the testis (Hinton and Setchell, 1993). About 100 proteins, including certain enzymes, have been reported to be secreted by these cells (Fritz and Ailenberg, 1991). This is the first report of Sertoli cells secreting GST.
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Proteins in the TF also have the contribution from other testicular cell types which include germ cells at various stages of development. The germ cells have not been known for their ability to secrete, however, factors released from germ cells do affect Sertoli cell functions and a number of proteins are now known to be of germ cell origin which can be recovered from the germ cell conditioned media (Pineau et al, 1993). GST was localized to the acrosome of the developing spermatid and mature spermatozoa in the testis. Saturable GST activity towards CDNB was identified with enriched germ cell fractions and in mature epididymal spermatozoa. Fractionation of enriched germ cell extracts on RP-HPLC revealed presence of only one component with similar retention time as that of rGSTM3(Yb3) in TF GST. The ability of germ cells to synthesize GST was confirmed by two observations, incorporation of radiolabelled 35S methionine and 35S cysteine which was detected in germ cell lysates and presence of two mRNA transcripts as detected by hybridization of germ cell RNA to a probe directed against the conserved region of the Yb gene. Since no radioactively labelled GST was detected in the germ cell culture supernatant it was evident that although germ cells make the protein they do not secrete it out. Germ cells therefore, in all likelihood, express GST as a membrane-bound form, this form can be extracted by detergents only which shows that it is tightly bound to the cells. The expression of GST on the acrosome of germ cells may have important functional implications. Since germ cells are vulnerable to mutations from xenobiotics, presence of GST on their surface may ensure safe elimination of these molecules without affecting the germ cells. From our in vitro fertilization data and evidence on goat sperm localization of GST, it
appears that GSTs may have a role other than detoxification, as the antibodies which affect fertility do not alter enzyme activity.

Sertoli cells and germ cells are known to influence each other by releasing factors (Sharpe, 1993). In order to investigate whether Sertoli cell secretion of GST was being influenced by germ cell factors, several animal models were developed where rats were rendered germ cell-free by either busulfan treatment of foetuses in utero or by irradiation of adult rats or by making the rats cryptorchid. Busulphan-treated rats showed an increase in GST activity if expressed per mg weight of protein or per mg wet weight of the organ which was not the case with cryptorchid or irradiated rats. This data needs to be interpreted with caution and also needs to be verified by in vitro experiments. The testes from busulfan-treated animals contained Sertoli cells which have never been exposed to germ cells and may have not attained full secretory abilities, thereby secreting lesser amount of TF as compared to controls. Therefore the amount of immunoreactive or enzymatically active GST may appear more, the total protein being less. It is however clear that GST secretion by Sertoli cells is not hampered by the absence of germ cells. It is possible that in cryptorchid testes, which were rendered germ cell-free in adulthood, the Sertoli cells may have attained their full secretory capabilities and therefore the total secreted GST appeared to be the same.

GSTs are known to be regulated by hormones (Hatayama, 1986, and Mankowitz et al, 1990). FSH injection into male rats showed an increase of GST activity in the total testicular homogenate, perhaps indicating a gonadotrophic control over testicular GST.

In summary, the 24kDa rat testicular protein was identified to be a member of the GST family by NH₂-terminal sequencing and other
parameters. It is secreted by Sertoli cells within the ST and is also present on germ cell acrosome. While Yb2 form appeared to be the predominant form of GST secreted, germ cells showed the presence of Yb3 GST only. This protein is actively synthesized by cells of the seminiferous tubule and time dependent accumulation could be observed in spent culture media. This protein is glycosylated. Secretion of GST is not negatively affected by the absence of germ cells. TF-GST is glycosylated and antibodies raised against this protein inhibit fertilization of goat oocytes in vitro.