Discussion
Reproductive failure is a common side effect in patients who received low dose MTX therapy for non-neoplastic diseases (van Scott and Reinertson, 1959; Sussman and Leonard, 1980). Clinical studies have shown seminiferous tubular damage, decreased sperm count or azoospermia and infertility (Grunnet et al., 1977; Sussman and Leonard, 1980; Groff et al., 1983). This is because spermatogenesis is more sensitive to DHFR-inhibiting compounds as inferred from the fact that when a deficiency of folic acid is induced in an adult male rat (by injecting a folic acid antagonist, such as aminopterin or pyrimethamine), changes in spermatogenesis are noticeable before they become apparent in bone marrow (Mathur et al., 1977; Awoniyi et al., 1993). Further during spermatogenesis, meiosis is more severely affected than mitosis to DHFR-inhibiting compounds (Mathur et al., 1977; Cosentino et al., 1990). Therefore, in the present investigation, studies on the effect of MTX with and without LCN supplementation on serum hormone profiles (FSH, LH, testosterone and estradiol), testicular histology and biochemical parameters such as testicular lipid classes, enzymes involved in steroidogenesis, HMP-shunt pathway, and pyruvate-malate cycle were undertaken to evaluate the spermatogenic and steroidogenic functions of testis.

4.1. Body weight

The data on body weight in 4 days, 4 and 8 weekly doses of MTX treatment groups suggest that MTX has an adverse effect on general body metabolism. This observation is in agreement with the report of Aarsaether et al. (1988). Nausea, vomiting, ulceration of oral mucosa, gastrointestinal intolerance and diarrhoea are the early signs of low dose MTX toxicity.
(Weinblatt et al., 1988; Morgan et al., 1990), which may likely to alter feeding behaviour of the animal. Gastrointestinal side effects are minor and occur more often with oral administration of MTX as compared to intramuscular route (Djerassi et al., 1967). A number of reports have shown that periods of undernutrition even as short as 1 day lead to a wide variety of metabolic responses in the body which act to decrease energy utilization and mobilise energy stores (Badger et al., 1985; Cameron and Nosbisch, 1991).

In more than one third of rheumatoid patients treated with MTX, intravenously, at the dose of 25 mg/kg body weight, weekly over 3-4 weeks period, stomatitis with painful erosions and/or ulcerations developed within one to five days (Weinblatt et al., 1985; Williams et al., 1985). Other clinical manifestations of chronic low dose MTX therapy include progressive weight loss (Commencing 24 hrs after administration of MTX), alopecia, skin lesions, cirrhosis, anaemia, severe pneumonitis and leukopenia (Wilke and Mackenzie 1986;). Since this drug is excreted by kidneys and liver, hepato-toxicity, abnormal results on liver function tests and renal dysfunction are more marked during low dose MTX therapy (Wilkinson et al., 1978; Woolley, 1983). In the present study, the dose employed was within the range that normally has been used clinically in patients with psoriasis, rheumatoid arthritis, dermatosis etc. Therefore, the prevalence of the aforementioned toxic effects in the MTX treated rats is also a possibility. Rats, mice, dogs and monkeys treated with other DHFR inhibitors such as pyrimethamine, 2,4-diaminopyridines and sulfasalazine showed symptoms similar to those produced by aminopterin and amethopterin administration (Andrade et al., 1976; Swinson et al., 1980; Cosentino et al., 1984).
The toxic side effects of low dose MTX (7.5 to 15 mg/week) often mimics folate deficiency (Blakley, 1969). Several studies have linked folate deficiency with metabolic changes that might result from decreased 5/10 formyl and formamino forms (Stockstad and Koch, 1967; Erbe, 1975). Since the formyl forms are directly involved in purine and pyrimidine biosynthesis (Chabner et al., 1986), the reduction in folate content and alteration of folate coenzyme pattern may alter cell differentiation and tissue growth (Blakley, 1969).

In many species, changes in the nutritional status of the body can have profound influences on the activity of the reproductive system (Badger et al., 1985; Cameron and Nosbisch, 1991; Dong et al., 1994). Specific nutritional or metabolic changes that occur during even brief periods of food restriction provide a signal to the hypothalamus to cause the suppression of pulsatile LH secretion and consequently impair testosterone production (Badger et al., 1985; Cameron and Nosbisch, 1991).

Testosterone and other androgens, being anabolic in nature, have some biological actions on virtually every tissue in the body (Mooradian et al., 1987). The physiological role of testosterone is to regulate the growth and differentiation of the primary and accessory sex organs in male (Kochakian, 1959; Hamilton, 1975; Mooradian et al., 1987). Besides, androgens stimulate or suppress selective proteins in many organs such as liver (Gustaffson et al., 1983), kidney (Kochakian, 1977) and adipose tissue (Wade and Gray, 1979). Testosterone also stimulates an increase in muscle mass and enhances oxygen consumption through the action of normal androgen receptor (Kochakian and Tillotson, 1957). Therefore, in the present study, the observed low level of
circulating testosterone may also be responsible for the decreased body weight. Since, serum androgens can modulate the action of growth hormone (GH) (Martin et al., 1979), it is probable that the decrease in serum testosterone might have modified the growth promoting effects of GH.

Thus, collectively, from the existing evidences, it is suggested that the reduced availability of testosterone accompanying decreased food intake, impaired digestion or absorption of nutrients or altered metabolism of absorbed nutrients and supply of metabolic fuels may have compounded to precipitate a fall in the body weight.

The decreased body weight observed in LCN supplemented MTX treated rats suggests that the toxic effects of MTX are not reversed or prevented by LCN supplementation. This observation is however, in contradiction with the findings of Goldin et al. (1952) who reported that LCN has the ability to protect mice against the toxic effects of aminopterin much more effectively than folic acid.

4.2. Testicular weight

FSH, LH and testosterone are the major regulators of testicular structure and function (Fritz, 1978). The receptors for LH and FSH are located in the plasma membrane of Leydig and Sertoli cells, respectively (Ritzen et al., 1991). While LH is the principal hormone required for the secretion of testosterone, FSH and testosterone act synergistically to maintain spermatogenesis (Steinberger, 1971; Russell et al., 1990). Therefore, in the
present study, the observed low titres of serum FSH, LH and testosterone can be accounted for the reduced testicular weight.

Spermatogenesis is a unique process involving continuous stem cell proliferation, meiotic divisions and remodelling of spermatids into spermatozoa (Steinberger, 1971). It is well-established that the crucial action of MTX is on thymidylate and purine biosynthesis, the inhibition of which leads to the arrest of DNA synthesis and cell proliferation. Therefore, it is possible that testis, a tissue with high mitotic activity, in which cells undergoing divisions constantly are most susceptible to damage leading to cell death. Histological studies furnish evidence for the spermatogenic disruption, desquamation and sloughing of primary spermatocytes and spermatids in rats that had received MTX for 4 days, 4 and 8 weekly doses. Since the seminiferous tubules make up 90% of the wet weight of the normal rat testis (Christensen and Mason, 1965), in the present study, the reduced weight of the testis can be correlated to spermatogenic arrest and regression of seminiferous tubules. Further support for the spermatogenic impairment can be drawn from the decreased sperm counts in caput and caudal epididymidal segments observed in the present study.

Food restriction has a direct suppressive effect upon LH secretion via amplification of the sensitivity of testicular negative feedback together with a direct suppression of GnRH release (Dong et al., 1994). As MTX treatment is known to cause nausea, vomiting, hypophagia and gastrointestinal disorders (Weinblatt et al., 1988; Morgan et al., 1990), it is also possible that restricted
nutritional status would have suppressed the hypothalamic-hypophyseal drive to the reproductive axis.

The non-restoration of testicular weight observed in MTX treated rats supplemented with LCN suggests a further decline of spermatogenic and steroidogenic functions in these rats. Supportive evidence for such a suggestion could be derived from the data on hormones which also displayed a non-reversibility to normal level parallel to testicular weight.

4.3. Accessory sex organ weights

The weight changes in caput, corpus and cauda induced by the MTX treatment was time-bound, transient and specific with respect to the epididymal segments. The decrease in caput epididymal weight observed under 8 weekly doses of MTX treatment and in corpus and caudal segments of 4 days MTX treated rats may partly be due to the decreased sperm count and due to the adverse effect of the drug on epididymal epithelium and luminal composition through alteration in serum testosterone level. An earlier study on the effects of MTX on caput, corpus and cauda histoarchitecture in these segments (Sampathraj, 1994) lends support for this proposal.

Epididymis derives testosterone directly from the circulation (Brooks, 1981) and rete testis fluid entering the proximal end of the ductus epididymis (Gustaffson, 1966). Local production of testosterone would be an alternative source of testosterone (Hamilton, 1975). It is not testosterone itself but rather its 5α-reduced metabolites, dihydrotestosterone (DHT) and 5α-androstan 3α, 17β-diol that may be the primary regulatory hormones controlling the
epididymal structure and function. Takeda et al. (1985) have shown no change in 5α-reductase activity in MTX treated rats. Nevertheless, in the present study, as testosterone production is low as assessed from low serum testosterone titres and steroidogenic enzymes activities, the formation of androgen metabolites viz. DHT and 5α-androstan-3α-17β-diol would also be low which would have eventually decreased the epididymal weight.

In the present study, the weight changes in corpus and caudal segments were more striking than caput. This may be due to the fact that caput region, lying in close proximity to testis receives more testosterone through rete testis fluid and this may probably be sufficient to maintain this region in normal size. Further, in this study, although circulating testosterone titres were found to be lowered, a careful look at the data on the serum testosterone level reveal a close correlation between the magnitude of the effect on caput and the magnitude of the decrease in serum testosterone level.

It is well-established that hormones of the hypothalamo-hypophyseal-testicular axis are the major regulators of growth and functions of accessory sex organs (Orgebin-Crist et al., 1983: Brooks, 1979). Therefore, the observed decrease in the weights of all accessory sex organs may be attributed to low testosterone titres. While the weight changes in coagulating glands, dorsolateral and ventral prostatic lobes were evident even with single dose treatment, the effects on seminal vesicle were manifested only after 8 weekly doses of MTX treatment. This may be attributed to the differential androgen threshold in these organs.
The unaltered weights of the testis, caput, corpus and caudal epididymidal segments, vas deferens and seminal vesicle following one day MTX treatment with and without LCN indicate no apparent adverse effect of MTX on these male reproductive organs, as single dose is of a very short duration.

After LCN supplementation, the observed persistent effects of MTX on organ/tissue weights suggest that the dose employed for LCN may not be sufficient to counteract the adverse effects of MTX.

4.4. Sperm counts and sperm motility

The seminiferous tubule shows different stages of spermatogenesis during any period of time and the tubule, throughout its length does not exhibit the same stage and pattern of this process. When animals are subjected to any drug treatment, the segments of the seminiferous tubule that have already advanced in their spermatogenic cycle may not respond in the same way as those that have just started their germ cell mitotic cycle. So, it necessitates always to determine the epididymal sperm count and also to assess the sperm motility in the epididymal segments in order to understand the effect of the drug on the already formed mature sperms. Hence, in the present study, the sperm count and sperm motility were assessed in the epididymal fluid after MTX administration at different durations.

As expected, the caput and cauda epididymal sperm counts were significantly reduced in rats treated with 4 and 8 weekly doses of MTX, which is in correlation with the spermatogenic disruption observed in this study.
Acquisition of sperm flagellar movement has been shown to be directly associated with accumulation of carnitine in the epididymis (Hinton et al., 1981). Since spermatozoa are dependent on fatty acid metabolism, carnitine is known to play a crucial role in preserving sperm viability as well as determining osmotic pressure within the epididymis (Vogkmayr, 1975). The rate limiting enzyme for the conversion of palmitoyl CoA into palmitoyl carnitine in the liver is palmitoyl transferase. Palmitoyl CoA synthetase catalyses the conversion of palmitate to palmitoyl CoA, which is esterified by glycerophosphate acyl transferase (Mayes, 1990). Aarsaether et al. (1988) have shown MTX administration to induce an increase in the activities of palmitoyl CoA synthetase and glycerophosphate acyl transferase but to have no effect on carnitine palmitoyl CoA transferase, thus suggesting increased palmitoyl CoA formation and its esterification in the liver after MTX therapy and leading to a depletion in carnitine synthesis. Therefore, poor availability of carnitine may be one of the reasons for the low epididymal sperm motility observed after MTX administration in the present study.

Further, it is well known that methionine, choline and folate metabolism are inter-related (Davis, 1986). So, any disturbance in folate metabolism by an antifolate like MTX would perturb methionine synthesis. Infact, Pomfret et al. (1990) have reported MTX to lower hepatic concentrations of betaine, S-adenosyl methionine and methionine, the immediate precursors of carnitine (Zeisel, 1990).

Epididymal carnitine level is 500 fold higher than in blood plasma (Marquis and Fritz, 1965). It is concentrated in the epididymal duct by an
androgen dependent active transport process in the corpus segment (Brooks et al., 1974) and the sperms themselves accumulate carnitine for their energy (Brooks, 1980). In the present study, the loss of motility of sperm under 4 days and 4 and 8 weekly doses of MTX treatment may be due to the hampering of carnitine uptake by low androgen levels also.

Voglmayr (1975) has shown acetylcholine synthesis and release to be directly associated with sperm motility and integrity. It is well established that MTX treatment leads to choline deficiency (Allegra, 1990). When serum choline levels are depleted, there will be a reduction of choline utilization for the biosynthesis of acetylcholine and phosphatidyl choline. Therefore, any change in the normal supply of choline at the epididymal level may likely to hamper the sperm motility.

4.5. Serum gonadotropins

The data on serum gonadotropins along with serum testosterone and estradiol suggest an inhibitory influence of MTX upon hypothalamo-hypophyseal-testicular axis.

It is well-known that hypothalamic factor, gonadotrophin releasing hormone (GnRH) is secreted in a pulsatile fashion to stimulate parallel pulsatile release of LH and FSH (Belchetz et al., 1978; Conn et al., 1987; Haisenleder et al., 1990). GnRH elevates subunit messenger RNA (mRNA) levels, α and β-subunit translation, glycosylation and release of gonadotropins from the anterior pituitary (Starzec et al., 1986; Gharib et al., 1990). GnRH binds to specific receptors on the surface of the gonadotrope cells of the
anterior pituitary gland and stimulate the secretion of FSH and LH (Papavasiliou et al., 1986; Gharib et al., 1990). In the present study, the consistent reduction in both serum gonadotropins, FSH and LH suggest their diminished production from the pituitary. This may be due to the decreased response of gonadotropes to GnRH stimulation or to the reduced production of GnRH from hypothalamus.

The gonadotropins are controlled by a classical negative feedback effect of testosterone and estradiol through effects at the level of hypothalamus (Sherins and Loriaux, 1973; Plant et al., 1978). Negative feedback at the level of pituitary would reduce the sensitivity of the pituitary to stimulation by GnRH, whereas feedback at the hypothalamus would suppress the secretion of GnRH, which in turn would reduce the frequency of LH pulse (Tilbrook et al., 1991). Although receptors for testosterone and estradiol are localised in the hypothalamus and pituitary (Thieulant and Pelletier, 1979), the principal site of negative feedback of the testicular steroids is the hypothalamus and that feedback effects directly at the level of anterior pituitary are minimal (Tilbrook et al., 1991).

In general, when circulating testosterone and estradiol levels are low, serum gonadotropins are expected to be high by a reduced negative feedback. Although this finding has been reported for other DHFR inhibiting compounds (Cosentino et al., 1984; 1990; Awoniyi et al., 1993), such a correlation does not exist in the present study. Rather, along with serum testosterone and estradiol, a concomitant reduction in serum FSH and LH was evident, suggesting an inhibitory effect of MTX on the hypothalamo-hypophyseal-
testicular axis. The discrepancy observed in the present study may be due to the variation in species, dose, duration and mode of treatment.

Administration of a rescue agent, LCN was ineffective in restoring the hormonal levels suggesting a severe impairment of the hypothalamo-hypophyseal axis.

4.6. Serum testosterone

In the present study, the sustained decrease in serum testosterone level observed in all drug treated groups is due to its diminished production and secretion.

LH is the potent stimulator of testosterone production (Eik-Nes, 1975; Wing et al., 1984). In the present study, a parallel decrease in serum testosterone was evident with decreased LH level. This indicates that low LH stimulation has resulted in low testosterone production.

Cholesterol is an obligate precursor for Leydig cell steroidogenesis (Andersen and Dietschy, 1978; Hou et al., 1990). Several studies have shown that rat Leydig cell preferentially utilizes cholesterol derived from plasma lipoproteins for steroidogenesis (Morris and Chaikoff, 1959; Freeman and Ascoli, 1982; Schreiber et al., 1982). High density lipoprotein (HDL) cholesterol has been suggested to be a major source of substrate for testosterone biosynthesis in rodents rather than endogenously synthesised cholesterol (Andersen and Dietschy, 1978). Thus, any alteration in the availability of cholesterol for steroid hormone synthesis or change in the ability of Leydig
cells to utilise cholesterol consequently would have a profound effect on testosterone production and secretion. MTX has been shown to cause accumulation of fat in liver and a general reduction in both total cholesterol and HDL-cholesterol in circulation (Aarsaether et al., 1988). Since testis derives about 40% of cholesterol from circulation (Morris and Chaikoff, 1959), poor availability of cholesterol as precursor may be the cause for low testosterone biosynthesis.

LH plays a major role in the transport of cytoplasmic cholesterol to the inner mitochondrial membrane (Hall et al., 1969). The cholesterol side-chain cleavage that take place in the mitochondria of Leydig cell mediated by cytochrome P₄₅₀ is dependent on LH (Payne et al., 1982; Payne, 1990). The rate-limiting step in steroid biosynthesis is not this enzymatic conversion, but, rather, the transport of cholesterol from the outer region to a specific location in the inner mitochondrial membrane (Hall, 1984). The stimulatory effect of LH on steroidogenesis also involves the mobilization of stored cholesteryl ester, an increase in lipoprotein receptors and stimulation of HMG-CoA reductase, the rate limiting step in de novo cholesterol biosynthesis (Bartke, 1971; Charreau et al., 1981; Scott et al., 1990). Therefore, in the present study, the observed sharp decline in serum level of LH would have interfered with cholesterol transport, side-chain cleavage and ultimately resulted in low testosterone production. These suggestions get supportive evidence from data on testicular esterified cholesterol (vide page 133-134), which accumulated in all MTX treated rats. Nevertheless, as free cholesterol remained unchanged, the poor conversion of cholesterol into pregnenolone or the latter into progesterone may be the plausible reasons for the steroidogenic impairment.
Direct evidence for such a proposal can be drawn from the diminished activities of steroidogenic enzymes observed in this study. Studies on steroidogenic intermediates like pregnenolone, progesterone and androstenedione may help to resolve this problem.

Besides LH, FSH also influences testosterone production by increasing the number of LH receptors on Leydig cells and the steroidogenic response of Leydig cells to LH (Odell et al., 1973; Nazian and Mahesh, 1981; Dalterio et al., 1986). Any change in serum FSH is likely to alter LH binding on Leydig cells. In the present study, decrease in serum FSH level together with serum LH may likely to hamper steroidogenesis. Therefore, the decline in serum FSH may also be another reason for the observed reduction in serum testosterone level.

There appears to be a species-specific pattern of hormone secretions under MTX influence. Koehler et al. (1986a) have reported a decrease in serum FSH, androstenedione, testosterone and unaltered LH levels following repeated (6 mg/kg body weight, once a week for 14 weeks) doses of MTX treatment in rabbits. Sussman and Leonard (1980) reported no significant change in serum LH, FSH and testosterone levels following MTX administration in human subjects. The present study shows that MTX in Wistar strain rats caused a decrease in all the above mentioned hormones, besides estradiol.
4.7. **Serum estradiol**

Although testosterone is recognised as the principal sex steroid in the male, estrogen is produced in small quantities in the testis (Hall, 1988). The formation of estrogens from C21 steroids is catalysed by cytochrome P$_{450}$ aromatase (P$_{450}^{arom}$), which has been detected in Sertoli (Fritz et al., 1976a), Leydig (Valladares and Payne, 1979) and germ cells (Nitta et al., 1993). Nevertheless, the contribution to serum level of estradiol by testis is relatively low (Hall, 1988) as compared to hypothalamic and limbic areas of brain, where the distribution of androgen receptors and aromatase activity are found to be the highest (Roselli and Resko, 1984).

Under the influence of FSH, androgenic precursors are aromatized to estrogens (Roselli and Resko, 1987). The low levels of FSH and testosterone under MTX influence indicate poor stimulation of the aromatase enzyme activity as well as poor substrate availability to be the reasons for low serum estradiol level observed.

In single dose MTX + LCN treated rats, the temporal increase in serum estradiol level may be due to the increased aromatization of serum testosterone to estradiol, as in this group alone, testosterone production may be at a high rate or may not have been affected as inferred from the data on 3β-HSD, which also exhibited a perceptible rise in enzyme activity in this group.
4.8. Testicular histology - Spermatogenesis

In the present study, the spermatogenic arrest discernible in rats treated for 4 days, 4 and 8 weekly doses of MTX administration was time-bound. It is well-established that the crucial target for MTX action is thymidylate synthase, the inhibition of which leads to the arrest of DNA synthesis and cell proliferation (Jackson, 1984). Spermatogenesis is a unique developmental process involving continuous stem cell proliferations, meiotic divisions and remodelling of spermatids into spermatozoa (Russell et al., 1990; Kierszenbaum, 1994). DNA is synthesised in excess in spermatogonia and primary spermatocytes in order to allow and stimulate division and differentiation of these cell types (Söderström and Parvinen, 1976a; Rivarola et al., 1985). Therefore, in the present study, the drug might have had a primary effect on spermatogonia and spermatocytes. The number of primary spermatocytes depends on the size of spermatogonial population, which is a function of the number of stem spermatogonia and rate of proliferation and degeneration of spermatogonia and the amount of degeneration of early or late primary spermatocytes (Vernon et al., 1975). Probably, in the present study, the arrest of spermatogonial proliferation has resulted in the loss of subsequent stages.

In 4 days and 4 weekly dose MTX treated rats, although the animals received equivalent dose regimen, the adverse effects of MTX were comparatively more profound in the latter group. Huckins (1971) reported that every 12.5 days, a new sequence of spermatogonial maturation would be initiated by the A₁ spermatogonia and those already differentiated would
transform into primary spermatocytes. Thus, the quantum of adverse effects produced by MTX over time was comparatively more profound in the latter group.

Chromosomal translocations and/or deletions have also been reported in germ cells of animals treated with low multiple doses of MTX (Siber and Adamson, 1975). Mathur et al. (1977) reported that aminopterin, the parent compound of MTX, besides causing arrest of cell division, resulted in the production of chromosomal abnormalities. The most common was the presence of sticky chromosomes in metaphase. Lagging of chromosomes and anaphase bridges were also observed, and the incidence of these abnormalities was higher in meiosis I than in meiosis II, resulting in the arrest of cell division at metaphase. Darlington and Haque (1962) have suggested that stickiness arose from denaturation and depolymerisation of DNA on the surface of the chromosomes. Therefore, in the present study, it is reasonable to speculate that the adverse effects of MTX might not have been confined to spermatogonia but also extended up to secondary spermatocytes. Premature exfoliation of round and elongating spermatids with residual cytoplasmic bodies may be a generalized response to disorganization of the seminiferous epithelium.

Leucovorin (LCN), an antidote has been reported to ameliorate MTX toxicity (Rosen et al., 1974; Black and Livingston, 1990). LCN administration has been considered to be a valuable approach for the treatment of round cell idiopathic syndrome in which many round cells are common in seminal plasma (Bentivoglio et al., 1993). In the present study also, in 4 weekly dose MTX + LCN treated group, a careful screening of the seminiferous tubules
revealed the differentiation of round and elongated spermatids into spermatozoa, although LCN was unable to counteract the adverse effects of MTX on the early germ cell types viz. spermatogonia and spermatocytes.

The degenerating pachytene spermatocytes, round spermatids and multinucleated giant cells observed in rats that have received 4 weekly doses of MTX had completely disappeared in rats that have received 8 weekly doses of MTX. They were either sloughed off into the seminiferous tubular lumen and washed away to epididymis or phagocytized by the Sertoli cells. As a result, except spermatogonia, all the other germ cell types nearly disappeared in 8 weekly dose MTX treated group. Exfoliation and degeneration of various stages of pachytene spermatocytes and spermatids have caused retraction of Sertoli cell cytoplasm, which lie as strands in the seminiferous tubules. The rare and complete absence of cells with meiotic phase or with mitotic figures suggest that the spermatogonial proliferation and their progression through meiotic division was completely blocked by MTX treatment.

Koehler et al., (1986b) reported germ cell degeneration, tubular damage and infertility in rabbits that have received repeated doses of MTX. Similar effects on spermatogonia and pachytene spermatocytes also have been shown after treatment with other DHFR inhibitors like pyrimethamine (Awoniyi et al., 1993), and sulfasalazine (Levy et al., 1979; Cosentino et al., 1984). Russell et al. (1983a) demonstrated a similar asynchrony of cell types following exposure to procarbazine, another antineoplastic agent.

MTX is known to have effects mainly on cell division (Skipper et al., 1967). The Leydig cells in the testicular interstitium are generally considered
to be a terminally differentiated cell population, in which cell division is rarely, if at all, encountered (Christensen, 1975; Hardy et al., 1989). In the present study, no demonstrable gross changes in Leydig cell number was discernible in both MTX and MTX + LCN treated rats, irrespective of duration. Meyers and Schilsky (1992) reported that Leydig cells are more resistant than the germinal epithelium in men with gonadal failure treated with chemotherapeutic drugs.

Leydig cells require LH to maintain their fully differentiated structure and function (Ewing and Zirkin, 1983). When deprived of LH, Leydig cells atrophy and loose cellular volume (Christensen and Gillium, 1969; Dym and Raj, 1977; Kenney et al., 1968). Chronic LH deprivation causes the slow decline in Leydig cell number only after 16 weeks (Teerds et al., 1989). The sustained decrease in serum LH level observed in the present study may have a definite impact on Leydig cell structure and function. Probably, the defects induced by MTX may be too subtle below the resolution of microscope, perhaps at the level of subcellular organelles.

Further, Riccardi et al. (1982) reported that in the rat, a significant blood-testis barrier to MTX exists at the tubular but probably not at the capillary-interstitial level. According to these authors, MTX levels were 2-4 fold lower in the testicular interstitial fluid and 18-50 fold lower in the seminiferous tubule as compared to plasma level. The junctional complex established in between two adjacent Sertoli cells is permeable to agents with molecular weight less than 600 to 700 (Pelletier and Friend, 1983). If one looks into the molecular weight of MTX, which is only 454 (Liegler et al.,
1969), the hypothesis postulated by Riccardi et al. (1982) may be conflicting. Nevertheless, further studies are necessary for a better understanding of the bioavailability of MTX and its metabolites at the level of seminiferous tubular lumen.

The effects of MTX on spermatogenesis may be direct at the level of testis or indirect through alterations in hypothalamo-hypophyseal-gonadal axis. FSH and LH have been implicated as regulators of mammalian spermatogenesis (Clermont and Harvey, 1967; Russell et al., 1987). FSH plays a role in the development of spermatogonia and early spermatocytes in immature rats as seen by a decrease in the number of these cell types in rats treated with anti-FSH antibodies (Chemes et al., 1979). It has also been reported that FSH plays a role in spermiogenesis (Steinberger, 1971; Weinbauer et al., 1991). In hypophysectomized animals, three cell types viz. stage VII - pachytene spermatocytes, step VII spermatids and step XIX spermatids showed a higher incidence of degeneration (Russell and Clermont, 1977; van Alphen et al., 1988; Weinbauer et al., 1991). Such an effect on these germ cell types may be envisaged in the present study also, as serum FSH levels were consistently low following MTX administration.

The principal action of testosterone is to facilitate the maturation of round to elongated spermatids during spermiogenesis (O'Donnell et al., 1994). Testosterone also acts to stimulate the spermatid binding to Sertoli cells specifically at the transition from stages VII to VIII of the seminiferous epithelium (Cameron et al., 1993). Recent studies have shown that spermatogenic cells including round spermatids internalise testosterone bound
to androgen-binding protein via receptor-mediated endocytosis and this process is maximal at stages VII and VIII (Gerard et al., 1994; O'Donnell et al., 1994).

Although testosterone is capable of maintaining qualitatively normal spermatogenesis, the production of normal range of sperm concentration may not be possible without FSH (Bartlett et al., 1989). Thus, in the present study, it is possible that the diminution of gonadotropins along with testosterone may also partly be responsible for the spermatogenic arrest. The low sperm count in caput and cauda epididymides of MTX treated rats observed in this study support spermatogenic disruption. Morris and Shalet (1990) reported that the withdrawal of gonadotropic support to the testicular tissue before and during the cytotoxic therapy would inhibit cell division in the spermatogenic epithelium.

Nevertheless, one cannot exclude the direct action of MTX on testis as formation of multinucleated giant cells and chromosomal breakages would not take place unless MTX directly interacts with DNA at the level of testis. Similar to the present investigation of MTX-induced seminiferous tubular damage in rats, particularly in tubular membranes with intense odema, hyalinosis and disseminated ruptures, Bentivoglio et al. (1993) have evidenced such changes in the cell structure in the 'plate core', hyalinosis and cytoplasmic basophilia. However, they showed LCN supplementation to increase epithelial mitotic activity and better cellular cohesion in their rats. Unlike the above mentioned observations, in the present study, LCN supplementation was unable to protect spermatogenic process against the toxic
effects of MTX. Probably, the dose employed for LCN supplementation may be too low to counteract the toxic effects of MTX.

Thus, from the present study, and the findings from the existing reports, it is clear that MTX has the potential to arrest spermatogenesis. However, the mechanism by which MTX exerts these effects on each cell type still remains elusive. Abnormalities like the formation of multinucleated giant cells, chromosomal breakage and lysis raise additional questions as to whether MTX caused genotoxicity at the level of spermatogenesis, apart from inhibiting DNA synthesis and cell division through DHFR inhibition.

4.9. Nucleic acids and proteins

In the present study, the diminution in RNA, DNA and protein concentrations observed in rats that have received MTX for 4 consecutive days, 4 and 8 weekly doses is not surprising as it is well-established that MTX is a potent inhibitor of DHFR, which is crucial for the *de novo* synthesis of purine nucleotides and thymidylate synthesis (Goldman and Fyfe, 1974; Bleyer, 1978). During MTX-mediated DHFR inhibition, dihydrofolate accumulates in the cell and the one carbon containing tetrahydrofolate, which is the substrate to *de novo* synthesis of methionine, serine, thymidylate and purines, gets exhausted. This results in the deficiency of purine and pyrimidine pools available for DNA synthesis (Bleyer, 1978).

The reaction most sensitive to folate is the conversion of 2'-deoxy uridylate (dUMP) to thymidylate (dTMP), an essential component of DNA, by the action of thymidylate synthase. The methyl group transferred to the
uracil of dUMP is donated by 5,10-methylene tetrahydrofolate, which is present in decreased amounts after treatment with MTX (Chabner and Young, 1973). Further, depletion of 10-formyl tetrahydrofolate inhibits the biosynthesis of inosinic acid, a purine precursor necessary for DNA and RNA synthesis. During this process, one carbon atom is transferred to pyrimidine ring at the oxidation level of formaldehyde and is reduced to methyl by hydrogen atoms donated from pteridine ring of the folate coenzyme (Blakley, 1969). As a result of these complex interactions, DNA and RNA synthesis is retarded by MTX (Hryniuk, 1972; Goldman and Fyfe, 1974).

Besides, when one-carbon metabolism is poisoned, the only alternative to choline as a source of methyl groups for the regeneration of methionine is lost (Finkelstein, 1990). S-adenosyl methionine and betaine concentrations are also found to be diminished after treatment with MTX (Barak et al., 1984; Pomfret et al., 1990). Therefore, protein synthesis is also inhibited, since reduced folates are cofactors in the conversion of glycine to serine and homocysteine to methionine (Hryniuk et al., 1975; Bleyer, 1978). MTX cytotoxicity thus leads to "purineless" state in cells leading to its death (Hryniuk, 1972; Allegra, 1990).

DNA synthesis is essential for cell division and functional differentiation in testicular germ cells (Söderström and Parvinen, 1976a; Clausen et al., 1982) and occurs in the spermatogonia during the mitotic S phase and for meiosis, in the preleptotene spermatocytes (Monesi, 1962; Rivarola et al., 1985). DNA synthesis in resting cells is minimal as compared to premitotic and mitotic cells. The head region of sperm shows positive
reaction for DNA. The rapid mitotic rate in a tissue correlates well with high
degree of formate incorporation into DNA bases of the tissue (Goldthwait and
Bendich, 1952). These authors have reported that the incorporation of
formate and thymidine into purines was depressed to a greater extent by
aminopterin. Similarly, MTX is also known to suppress the incorporation of
thymidine into DNA, of uridine into RNA, of leucine into protein, and kill cells.
The greater the proliferative rate, the greater are the drug effects (Hryniuk,
1972).

MTX is primarily cytotoxic to cells in S-phase of the cell cycle (Skipper
et al., 1967). MTX is much more effective when cellular population is in
logarithmic phase of growth, rather than in plateau phase, because of its
known inhibitory effect upon RNA and protein synthesis. However, MTX slows
the entry of cells into S-phase and this cytotoxic action has been referred to
as ‘self-limiting’ action (Skipper et al., 1967).

Middle and late pachytene spermatocytes are the most synthetic of all
germs cell types, with RNA synthesis peaking at mid-pachytene and remaining
high thereafter until late pachytene (Söderström and Parvinen, 1976b). The
intense activity of RNA in the cytoplasm of spermatogonia and primary
spermatocytes suggests that these two stages of spermatogenesis are mostly
concerned with protein synthesis. RNA synthesised in the seminiferous tubules
was found to be mostly heterogenous nuclear RNA (HnRNA) which appeared
to have a long life time and (Söderström and Parvinen, 1976b) includes the
precursors of long lived mRNA species needed for the direction of the protein
synthesis during late spermiogenesis, when no nuclear RNA synthesis occurs
(Monesi, 1964). The most likely sources of the stable HnRNA in the rat seminiferous epithelium seems to be the pachytene spermatocytes, especially in stages VI-VIII, where HnRNA and also the rRNA are most actively formed. Other sources are the young spermatids, spermatogonia or peritubular myoid cells, but either the RNA synthesis in these cells or their number is too small to influence markedly the total RNA synthetic pattern (Söderström and Parvinen, 1976b). In the present study, the direct inhibitory effects of MTX on RNA, DNA and protein synthesis in various germ cell types may be the plausible reasons for the reduced testicular RNA, DNA and protein concentrations observed in rats subjected to 4 days, 4 and 8 weekly doses of MTX treatment. Further, histological observations have shown the massive depletion of germ cell types, particularly spermatogonia and spermatocytes. This might be the other cause for the minimal RNA and DNA synthesis.

During the second half of spermiogenesis, the somatic histones are replaced by transitional proteins that are subsequently replaced by arginine-cysteine-rich sperm protamines (Grimes et al., 1977). These proteins may be important for DNA packaging and spermatid maturation (Hecht, 1989). While these proteins are synthesised during or after the elongation of the spermatid nucleus, their mRNA is transcribed during the early steps of spermiogenesis (Hecht, 1989) Thus, any disturbance in RNA and DNA synthesis in germ cells may result in abnormal expression of germ cell proteins.
4.10. Enzymes involved in steroidogenesis

In the present study, MTX administration caused a lowering of testicular 3β- and 17β-hydroxy steroid dehydrogenase enzymes activities in 4 days, 4 and 8 weekly dose MTX treated rats.

Leydig cells are the chief source of most of the androgens secreted by testis (Hall et al., 1969). Biosynthesis of testosterone from cholesterol in Leydig cells involves the action of 4 enzymes. Cholesterol is transported from intracellular stores to the outer mitochondrial membrane and subsequently to the inner membrane, where it is metabolised to pregnenolone. The initial metabolic step in steroid biosynthesis is the conversion of pregnenolone, which occurs in the inner mitochondrial membranes, where the cytochrome P₄₅₀ side-chain cleavage enzyme is located (Hall, 1984; Payne, 1990). This enzyme associated with nicotinamide adenine nucleotide phosphate electron transport system catalyses the cleavage of the side-chain of cholesterol to yield the C21 steroid, pregnenolone (Miller, 1988). This stimulation can be blocked by inhibitors of protein synthesis, suggesting the participation of a steroidogenic protein/peptide, steroidogenic activator polypeptide (SAP) (Pedersen and Brownie, 1987).

Pregnenolone diffuses across the mitochondrial membrane and is further metabolized by enzymes associated with the smooth endoplasmic reticulum. Pregnenolone is metabolized via the Δ⁴-pathway to progesterone by the action of 3β-hydroxy steroid dehydrogenase/Δ⁵-Δ⁴-isomerase (3β-HSD) with NAD⁺ as cofactor (Miller, 1988), which is LH dependent (Shaw et al., 1979). The next reaction catalysed by the
cytochrome $P_{450}$ 17α-hydroxylase involves 17α-hydroxylation of progesterone, followed by cleavage of the C17-20 bond. This step reduces the number of carbon atoms from 21 to 19, yielding androstenedione, the immediate precursor of testosterone (Payne, 1990). Androstenedione and testosterone are interconverted through the action of 17β-hydroxy steroid dehydrogenase (17β-HSD), an NADP-dependent enzyme (Bogovich and Payne, 1980).

In the present study, the diminished activities of both 3β- and 17β-hydroxy steroid dehydrogenase along with low serum testosterone level indicate steroidogenic impairment under MTX influence. LCN supplementation was unable to reverse the adverse effects of MTX on 3β- and 17β-HSD activities. This observation insists the need for a higher dose of LCN supplement to overcome MTX toxicity.

4.11. Enzymes of the HMP-shunt pathway

The striking morphogenetic changes that occur during the post-meiotic phase of spermatocyte development are accompanied by marked changes in the nature of the enzymes utilized in energy metabolism. Testis gets the pentose sugars for nucleotides and nucleic acid synthesis and reducing equivalents for lipogenesis from the HMP-shunt pathway (Free, 1970). Glucose-6-phosphate dehydrogenase (G-6PDH), the first enzyme of the pentose phosphate cycle catalyses the conversion of glucose-6-phosphate into 6-phosphogluconate. During this reaction, one molecule of NADPH is generated (Mayes, 1990). 6-phosphogluconate dehydrogenase (6-PGDH) is a cytoplasmic enzyme, catalysing the second step in the pentose-phosphate
pathway, where dehydrogenation and decarboxylation of 6-phosphogluconate take place to convert it into **D-ribulose 5-phosphate**. During this reaction, a second molecule of **NADPH** is generated (Mayes, 1990).

Glucose converted to glucose-6-phosphate enters the glycolytic and pentose phosphate pathway (Mayes, 1990). In the present study, as the germ cells were found to be markedly depleted, as assessed from histological studies, the glucose metabolism through the glycolytic pathway may be depressed. The decreased metabolism of glucose-6-phosphate through glycolytic pathway might have increased the availability of substrate more for the pentose phosphate pathway, thus resulting in enhanced activities of G-6-PDH and 6-PGDH.

HMP-shunt pathway is to generate NADPH and provide ribulose sugars necessary for nucleic acid synthesis (Mayes, 1990). Spermatogenesis is a continuous process and the germ cells are in the constant phase of division and are in constant need of ribulose sugars for their nucleic acid synthesis. Therefore, in the present study, the increased activities of HMP-shunt pathway in the long term treated groups may be attributed to the demand of ribulose sugars required for nucleic acid synthesis.

### 4.12. Enzymes of the Pyruvate-Malate cycle

Acetyl CoA, the major carbon source for lipogenesis is produced in the mitochondrial matrix by the pyruvate dehydrogenase reaction. Since the inner mitochondrial membrane is impermeable to acetyl CoA, the export of acetyl CoA to the cytoplasm is by the conversion of citrate using citrate synthase, transport across the mitochondrial membrane by a specific carrier, and the
reconversion to acetyl CoA by ATP-citrate lyase (Srere, 1975). ATP-citrate lyase is therefore, a key enzyme in the lipid synthesis. In the present study, the higher activity of ATP-citrate lyase observed after MTX treatment suggests increased cleavage of acetyl CoA and oxaloacetate in the cytoplasm.

**Malate dehydrogenase (MDH)** is involved in the conversion of oxaloacetate to malate, during which NAD is reduced to NADH (Banks et al., 1979). **NADP⁺-isocitrate dehydrogenase (NADP⁺-ICDH)** is vital in controlling lipogenesis, by virtue of providing coenzyme, NADPH and carbon building blocks necessary for lipogenesis (Mayes, 1990). In general, both ICDH and MDH exhibit higher activity, compared to other oxidative enzymes in the testicular cells (Free, 1970). Since ICDH is one of the key enzymes that regulates citrate flow in the cytoplasm, the increased activity favours enhanced utilisation of citrate in the mitochondria. This will lead to shortage in the supply of citrate in the cytoplasm.

Lipid synthesis requires reducing equivalents, primarily as NADPH. The production of this substance is in part accomplished through the activity of **malic enzyme** (promoting the conversion of malate to pyruvate with NADPH as one of the reaction products) and, in part, through the activity of the pentose shunt dehydrogenases. The increase in malic enzyme activity may well be correlated to the increased NAD⁺-malate dehydrogenase. Increased malic enzyme activity may probably be due to the availability of more substrate, malate as a result of increased malate dehydrogenase activity in MTX treated rats.
NADPH thus formed, is essential for lipogenesis, malonyl CoA synthetic pathway in the mitochondria and for mitochondrial and microsomal fatty acid chain elongations (Mayes, 1990). The rate limiting step in cholesterol synthesis i.e., the conversion of 3-hydroxy 3-methyl glutaryl CoA (HMG CoA) to mevalonate by HMG CoA reductase requires NADPH (Miller, 1988). It is also required for the production of testosterone as it is involved in the cholesterol side-chain cleavage i.e C-27 side chain cleavage by cytochrome P_{450} (Hall, 1988) and in the reduction of androstenedione into testosterone (Inano and Tamaoki, 1986; Hall, 1988). The data on the enzymes of pyruvate-malate cycle suggest a duration-dependent stimulatory effect of MTX on these enzymes, suggesting increased NADPH production, thereby accumulating the necessary cofactors for lipid synthesis and steroidogenesis. The enzymes of HMP-shunt also exhibited a similar response to MTX treatment. The unaltered total cholesterol, accumulation of ester cholesterol and the triglycerides in MTX treated rats suggest that the enhanced production of NADPH may be utilised for cholesterol ester synthesis, rather than lipogenesis.

The effects of MTX on enzymes of the pyruvate-malate shuttle were persistent even after LCN supplementation. This may again suggest the need for a higher dose of LCN to counteract the adverse effects of MTX.

4.13. Testicular lipids

Testicular lipids are of functional importance as they serve as precursors of steroid hormones (Perlman, 1950), provide energy for the germ cells (Kingsley-Smith and Lacy, 1959), and maintain seminiferous tubular and germ cell membrane characteristics (Keenan et al., 1972).
An overall increase in the activities of lipogenic enzymes suggests increased production of NADPH and acetyl CoA production in the cytoplasm, suggesting increased lipogenesis. However, a negative correlation exists between lipogenic enzymes and total lipid concentration in the present study. As phospholipids form the major lipid class, constituting 80% of the total lipid, (Oshima and Carpenter, 1968), in the present study, the marked fall in total phospholipid concentration was attributed for the diminution in total lipid concentration.

FSH and LH play an important role in the metabolism of testicular lipids (Nakamura et al., 1968). The above authors have also reported that the testes of hypophysectomised rats atrophied with about 50% reduction in their total lipids. The concentration of most of the lipid classes was decreased, except cholesterol ester, glycercyl ether diester and phosphatidyl serine in hypophysectomized rat. Replacement of LH alone to these hypophysectomized rats maintained the total lipid value at a level lower than the control, while FSH increased the same above the control (Gambal and Ackerman, 1967). A similar decrease in total lipid concentration was seen in cryptorchid testis where a decrease in total lipid content of testis was explained for the loss of weight of the organ and spermatogenic arrest (Davis and Coniglio, 1967). Such a correlation was evident in the present study also. The decrease in total lipid concentration observed after MTX administration in the present study may also contribute for the testicular weight loss and the disruption of spermatogenesis.
4.14. Cholesterol

Any excess cholesterol not utilized for steroidogenesis and cell membrane biogenesis is esterified by acyl coenzyme A:cholesterol acyl transferase (ACAT) and stored in lipid droplets, where it appears to function as a reservoir of free cholesterol (Mori and Christensen, 1980). Hydrolysis of cholesteryl ester(s) is accomplished by neutral cholesteryl esterase. The activities of both ACAT and neutral cholesterol esterase are regulated by trophic hormones (Bartke, 1971). In normal testis, 5-10% of the total cholesterol is esterified and the remaining 90-95% of the total cholesterol content exists as free cholesterol (Oshima and Carpenter, 1968), which forms the base for steroidogenesis (Freeman and Ascoli, 1982).

When there is a sustained decrease in the serum testosterone level, free cholesterol was also expected to be low. However, in the present study, there was no appreciable change in total and free cholesterol in MTX treated rats, despite spermatogenic and steroidogenic impairment. Probably, to meet the demand, de novo cholesterol synthesis may be triggered at the level of testis or the esterified cholesterol may be mobilized to provide the free cholesterol to serve as the substrate for steroidogenesis, since the esterified cholesterol concentrations were high in the drug treated group. Several studies have shown that reduction in substrate activate de novo cholesterol biosynthesis and stimulate HMG-CoA reductase activity (Freeman and Ascoli, 1982; Hou et al., 1990; Payne et al., 1992).

Cholesterol, apart from serving as a precursor for steroidogenesis, form an integral component of plasma membrane of various germ cell types.
(Freeman, 1989). As the serum level of testosterone was consistently low and the spermatogenic disruption and loss of germ cell types were quite extensive in 4 and 8 weekly dose MTX treated groups, there may be an accumulation of ester cholesterol due to non-utilization which may probably have compensated the poor trapping of cholesterol from circulation and/or reduced \textit{de novo} synthesis in Leydig cells. This speculation is substantiated by the observed elevation in esterified cholesterol concentration in rats received 4 consecutive days, 4 and 8 weekly doses of MTX treatment and in rats supplemented with 4 and 8 weekly doses of MTX + LCN supplement.

4.15. Glycerides

Glycerides are the major energy source for testicular germ cells (Oshima and Carpenter, 1968). In the present study, although spermatogenesis was disrupted in rats that have received MTX and MTX + LCN supplementation, the accumulation of glyceride glycerols, especially, di- and triglycerides was more in the latter group. MTX inhibited the secretion of lipoproteins by the liver and markedly lowered the plasma triacyl glycerol and cholesterol concentrations (Tuma \textit{et al.}, 1975; Aarsaether \textit{et al.}, 1988). In the present study, like cholesterol, total glyceride glycerol also showed no alteration by MTX treatment. In 4 and 8 weekly dose MTX and MTX + LCN treated animals, the desquamation of germinal epithelia and the eventual disappearance of germinal elements like spermatozoa, spermatids and most of the spermatocytes as assessed histologically, and the significant fall in sperm concentration observed in caput and caudal epididymal segments furnish evidence for the depletion of germ cell population in the testis. Therefore, it is
possible that there is a decrease in the utilization of glycerides as energy source by the various germ cell types. This might have resulted in the triglyceride accumulation, which might have compensated the poor supply of triacyl glycerol through circulation. This is supported by the data on glyceride glycerol observed in MTX + LCN treated rats which exhibited an increase in di- and triacyl glycerol concentrations. As LCN is known to counteract MTX toxicity (Rosen et al., 1974; Black and Livingston, 1990), the block in the triacyl glycerol secretion by liver may be lifted and normal supply of triacyl glycerol may be reestablished which has accumulated in the absence of germ cell types due to poor utilization.

Diacyl glycerol can be converted into triacyl glycerol or phospholipid via phosphatidic acid (Mayes, 1990). In 4 and 8 weekly dose MTX treated rats, the marginal decrease in diacyl glycerol concentration may be contributed to the corresponding increase in triacyl glycerol concentration. Any change in glyceride glycerol metabolism will be reflected in phospholipid metabolism (Mayes, 1990). In the present study, in MTX and MTX + LCN treated rats, as there is a depletion in total phospholipid and two of its major fractions, phosphatidyl choline and phosphatidyl ethanolamine, diacyl glycerol may be contributed for triacyl glycerol formation, rather than phospholipid synthesis.

4.16. Phospholipids

While cholesterol and glyceride glycerol constitute only 20 % of the total lipids, phospholipids form 80 % of the total lipid in the testis (Oshima and Carpenter, 1968). Therefore, in the present study, in both MTX and MTX + LCN treated rats, the observed decrease in total phospholipid concentration
may account for the decrease in total lipid concentration. The histochemical studies of Wislocki (1949) have shown dense staining for phospholipids in the rat testis during the period of growth associated with rapid differentiation and formation of spermatids. During active spermatogenesis, phospholipids are increased and neutral lipids are decreased in the seminiferous tubules (Kingsley-Smith and Lacy, 1959). Phospholipids are the integral components of the sperm membrane and play an important role in maintaining their structural and functional integrity (Phillips, 1972). In the present study, as there was a heavy loss of germinal elements, particularly spermatocytes, spermatids and spermatozoa as evidenced from the histological studies, it is therefore not surprising to get a decrease in phospholipid concentration.

Gambal and Ackerman (1967) have shown that FSH and LH maintain normal levels of phospholipids in the testis. Hypophysectomy caused marked fall in phosphatidyl choline, ethanolamine and sphingomyelin and an increase in the concentration of phosphatidyl serine (Nakamura et al., 1968). Yokoe and Hall (1970) observed that LH stimulates the incorporation of \(^{32}\)P and choline-\(^{3}\)H into testicular phosphatidyl choline. Therefore, in the present study, the fall in phosphatidyl choline and ethanolamine levels may partly be due to the suppression of gonadotropins.

Choline is the precursor for the biosynthesis of membrane phospholipids, phosphatidyl choline, sphingomyelin and lysophosphatidyl choline. When serum choline levels are depleted, there will be a reduction of choline utilization for the biosynthesis of acetylcholine and phosphatidyl choline. It is well-established that MTX treatment leads to choline deficiency (Jackson,
Therefore, in the present study, the observed decrease in testicular phosphatidyl choline concentration may be due to the poor supply of choline to testis.

There are two distinct pathways for the biosynthesis of phosphatidyl choline - CDP pathway using preformed choline, and the methylation pathway catalysed by the enzyme, phosphatidyl ethanolamine N-methyl transferase (PEMT) (Pascale et al., 1982; Ridgway and Vance, 1987). Choline deficiency by decreasing the absolute contribution by the CDP-pathway would increase the requirement for phosphatidyl choline synthesis via the sequential methylation of phosphatidyl ethanolamine (Hirata and Axelrod, 1980). In this pathway, S-adenosyl methionine donates the methyl group for the enzymatic formation of phosphatidyl choline from phosphatidyl ethanolamine via synthesis of P-N-monomethyl ethanolamine and P-N-N-dimethyl ethanolamine. As it is well-known that choline, methionine and folate metabolism are inter-related (Davis, 1986), any disturbance in the folate metabolism would perturb methionine and choline synthesis. Pomfret et al. (1990) have reported that MTX at the dose of 0.1 mg/kg body weight daily, intraperitoneally, for two weeks lowered hepatic concentrations of betaine (to 55% of control), S-adenosyl methionine (to 75% of control), and methionine (to 70% of control). Therefore, in the present study, S-adenosyl methionine transmethylation may be specifically inhibited, which probably would have resulted in low phosphatidyl choline level. Further, as there was a marked fall in phosphatidyl ethanolamine, there is a possibility for the decreased phosphatidyl choline synthesis.
Phosphatidyl serine is a minor component of the plasma membrane and it has been localised in the inner (cytoplasmic) side of the bilayer (van Deenen, 1966). The synthesis of phosphatidyl serine occurs primarily in the endoplasmic reticulum, but significant amounts have also been found in both Golgi and nuclear membrane. It is then transported to inner mitochondrial membrane where it is decarboxylated to form phosphatidyl ethanolamine. This is formed via the Ca\(^{++}\) stimulated exchange of free serine for the ethanolamine of phosphatidyl ethanolamine (Farese, 1983). In the present study, the consistent decrease observed in the three major phospholipid classes, viz. phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl choline in rats subjected to 4 days, 4 and 8 weekly doses of MTX treatment suggests severe derangement in folate metabolism, resulting in the low provision of precursors.

When phosphatidyl inositol hydrolysis increases, there will be increased phosphatidic acid synthesis (Tyson et al., 1976; Putney et al., 1980), which is dephosphorylated to give the diacyl glycerol, the immediate precursor of both phospholipids and neutral triglycerides (Mayes, 1990). In the present study, there exists a correlation between these two phospholipid fractions and di, triacyl glycerol concentrations. Specific changes observed in phosphatidyl inositol and phosphatidic acid may be due to the shunting of precursors towards di- and triglycerides.

Sphingomyelin is unique among the phospholipid classes, as it is more concerned with the stabilization of membranes (Voglmayr, 1975). Therefore, in
the present study, the decrease in sphingomyelin concentration under MTX influence may affect membrane characteristics of spermatozoa.

The data on phospholipid fractions suggest a differential effect of MTX on individual phospholipid classes. Changes in total phospholipid concentration were mainly due to the diminution in the concentrations of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and sphingomyelin. All the above phospholipid fractions only contribute for the high phospholipid content in rat testis viz. nearly 83%. The other phospholipid fractions like phosphatidyl inositol, phosphatidic acid and cardiolipin showed an increase under drug treatments. These fractions contribute for the balance of 17% only to the total phospholipid content. So an increase in these fractions would not be of much importance in the energy derivation by the testis.

LCN supplement was ineffective in restoring the total phospholipid and its various classes to normalcy. This is anticipated as the adverse effects of MTX on spermatogenesis were still persistent even after LCN supplementation.

From the foregoing study, it is quite evident that MTX has adverse effects on body weight, testicular and accessory sex organ weights, serum gonadotropins, and sex steroids like testosterone and estradiol. Further, the histoarchitecture of the testis has undergone severe negative changes, especially in the long duration groups, indicating an adverse effect of the drug on the structural integrity of the testis. Biochemically, MTX caused adverse effects on nucleic acids, steroidogenic enzymes, pentose-phosphate cycle enzymes, and the testicular energy providing lipids. The increase in the
lipogenic enzymes, esterified cholesterol, di- and triglycerides suggests shunting of substrates from the pyruvate-malate shuttle to storage forms of lipids. LCN is a known supplement for reversing MTX toxicity. But, in the present study, it was not able to restore the histology and only partially could restore a few biochemical responses of the tissue.