

THE MALE ANTI-FERTILITY EFFECTS OF EMBELIN AND SOME OF
ITS CLOSELY RELATED BENZOQUINONES.

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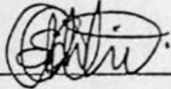
A Thesis submitted in the partial fulfilment for the degree of
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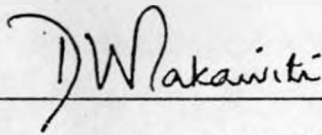
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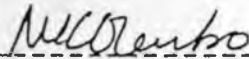
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MATERIALS	CONCENTRATION
Embryonic Testosterone	10^{-10}
Embryonic Testosterone	10^{-11}
Embryonic Testosterone	10^{-12}
Embryonic Testosterone	10^{-13}
Embryonic Testosterone	10^{-14}
Embryonic Testosterone	10^{-15}
Embryonic Testosterone	10^{-16}
Embryonic Testosterone	10^{-17}
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Embryonic Testosterone	10^{-26}
Embryonic Testosterone	10^{-27}
Embryonic Testosterone	10^{-28}
Embryonic Testosterone	10^{-29}
Embryonic Testosterone	10^{-30}

PPO: 2,5-Diphenyloxazole
PSG: Phosphate-Saline Glucose Buffer
RIA: Radio-Immuno Assay
Spp: Species
³**H**T: Tritium labelled Testosterone
WHO: World Health Organisation
n/2n: Haploid (gamete)/diploid (somatic)
Chromosome Constitution.

UNIT ABBREVIATIONS

MULTIPLE

WEIGHT

Kg: Kilogram

10³gs

g: gram

1g

mg: Milligram

10⁻³g

VOLUMES

l: litre

10³mls

ml: Millilitre

1ml

l: Microlitre

10⁻³ml

CONCETRATIONS

Mol: Mole

1 mole

mMol: Milli Mole

10⁻³ mole

mMol: Micromole

10⁻⁶ mole

nMol: Nanomole

10⁻⁹ mole

pMol: Picomole

10⁻¹² mole

fMol: Femtomole

10⁻¹⁵ mole

ABBREVIATIONS

Ab:	Antibody
ABP:	Androgen Binding Protein
Ag.	Antigen
ATP:	Adenosine Triphosphate
BSA:	Bovine Serum Albumin
cAMP:	Cyclic Adenosine Monophosphate
DHT:	Dihydrotestosterone
DMSO:	Dimethylsulphoxide
EDTA:	Ethylenediaminetetra-acetic Acid
FAO:	Food and Agriculture Organisation
FFA:	Free Fatty Acids
FSH:	Follicle Stimulating Hormone
GH:	Growth Hormone
HCG ,hCG:	Human Chorionic Gonadotropin
¹²⁵ I:	Radio Isotope Iodine
IAEA:	International Atomic Energy Agency
ICSH:	Interstitial Cells Stimulating Hormone
LHRF:	Lutenising Hormone
LRF:	Lutenising Hormone Releasing Factor
NAD ⁺ /NADH:	Nicotinamide Adenine Dinucleotides (oxidized/Reduced)
P ₄ :	Progesterone
PBS:	Phosphate Buffer-Saline
PCA:	Perchloric Acid
PCV:	Packed Cell Volume

SYMBOLS

α : alpha

β : beta

γ : gamma

δ : delta/Delta

\bar{x} : Mean

STATISTICS

S.D : Standard deviation

n : Number of Samples

H_0 : Null hypothesis

\succ : Greater than or equal to

\preccurlyeq : less than or equal to

ABSTRACT

There is a broad range of biological activities acclaimed to benzoquinones and their derivatives. Verification of these claims awaits solid scientific evaluation. Embelin, a crystalline benzoquinone is extractable from locally available plants viz Embelia schimperi, Rapanea melanphloes, Maesa lanceolata and Myrsine africana. Other species of this plant e.g. Embelia ribes occurs widely in the tropical climates. Several reports, originally folkloric but now in scientific papers have been presented with regard to the antifertility effect of embelin in both male and female mammals. In the latter this drug has been shown to cause inhibition of implantation and re-absorption of foetus and is said to be neither antizygotic nor blastotoxic. In the males the drug reduces sperm count, motility and weight of testis and prostate gland significantly. Other studies showed that long-term exposure of experimental animals to embelin caused azoospermia with eventual recovery after the termination of the dosage. There is no report as yet of any apparent toxic effect of embelin in the experimental animals, though in this study, a closely related compound, maesaquinone and its acetylated derivative, caused drastic fall in packed cell volume and weight of treated rabbits. No such toxic effects were observed in embelin treated groups.

The rationale for the project was to provide a more detailed study of the antifertility effects acclaimed to embelin, here with regards to the males. Previous reports are largely on gross effects viz sperm counts, testicular size, sperm's fertilising ability etc. The specific site of embelin antifertility action had not been established.

Radioimmunoassay techniques were applied to measure plasma levels of testosterone and progesterone prior, during and after embelin dosage. LH bioassays were carried out by assessing the hormones stimulatory effects on cultured leydig cells. Further, the testicular micro-environment levels of testosterone were determined and compared with those in control animal. Direct effect of embelin on activity of cultured leydig cells was tested by assessing the testosterone biosynthetic ability in presence and absence of embelin in the medium. Preparation of histological slides of testicular tissues from embelin treated and control groups was done to provide a direct visualisation of the antifertility effect of embelin.

The results showed a drastic decline (approx. 90%) in testosterone levels within five days of embelin dosage (40 - 60 mg/kg body wt.). The hormone levels remained very low during embelin treatment but increased sharply upon lifting of the dosage. LH bioassays showed a slow but steady rise of the hormone with continued dosage of embelin. The maximum rise of LH in treated rabbits was 30% ,then

dropped to normal values when dosage was stopped. Embelin, therefore, does not inhibit LH biosynthesis/release. Assay of progesterone (a metabolite in testosterone biosynthetic pathway) showed a concomitant rise in the levels of this hormone with the observed fall in testosterone. This is an indication that embelin antifertility effect is due to its inhibition of testosterone biosynthesis.

In vitro studies using cultured leydig cells support the above observation since embelin at 0.001 mg/ml - 1 mg/ml showed a dose related inhibition of testosterone biosynthesis, with a total inhibition at concentrations above 0.1 mg/ml.

Histological preparations of the rabbits testicular tissue showed a major difference between the embelin treated and control groups. The epididymis of treated rabbits had only a few stored spermatozoa and in some cases, was virtually empty; while the epididymis of the control group was full of spermatozoa. The seminiferous tubules of the treated rabbits were atrophied and the spermatogenic cells showed sloughing-off tendencies. In contrast, the seminiferous tubule of the control group were compact and their spermatogenic cells orderly arranged.

The results, therefore, support the antifertility effects acclaimed to embelin. The accumulation of progesterone during embelin dosage is evidence that the drug inhibition of testosterone biosynthesis

is down pathway after progesterone. There are reports that embelin has anti-estrogenic effect in the females, implying that, the step inhibited by embelin is before androstenedione metabolite in the testosterone biosynthetic pathway, since these two hormones (androstenedione and testosterone) are considered to be the major substrates for estrone and estradiol synthesis.

In conclusion , these results show that embelin is a potential fertility regulating agent in males. It drastically lowers testosterone levels with an eventual recovery after termination of the dosage. The major draw-back of this approach of fertility regulation, is the expected loss of libido and potency, a case whose possible solutions are discussed in the main text.

1. INTRODUCTION

1.1. Contraception and Human Welfare

Research in reproduction aims at improving human welfare, at individual level as well as society in general. Contraception remains a problem, and has been cited as one of the most important of all fields of human understanding and ignorance (Greep, 1976). Moreover, it is a note of concern that contraceptives technology emphasises on the female, leaving the male out of the arena. Yet there is no compelling reason why this should be so and may only be explained as a bandwagon phenomenon. It is argued that the male, being the traditional head of the family and principal breadwinner (not withstanding the present atmosphere of sex equality) should have a compelling interest in keeping his offspring within bounds. Infact male contraceptives can be effective instruments in fertility control, only that the methods presently available are not impressive. Attempts on vasectomy, condoms, coitus interruptus and abstinence have failed to get appreciation, and there is no appropriate hormonal based contraceptives for the males. Fertility as well as libido is a treasured attribute of the male. Approaches that may cause its impairment or loss, whether reversible or irreversible are not likely to gain acceptance on a scale sufficient to bring a notable lessening of birth rate. The need for a suitable male contraceptive keeps expanding exponentially.

Hormonal intervention methods are more subtle and have greater

chances of reversibility of fertility but suffers the possibility of diminished libido. If the latter can be overcome, this approach would then, be a versatile method of fertility regulation. Of interest in this study is the use of plant derived products in fertility control, a case that would have wide applicability since plants are abundant and extraction of the active ingredient would require much simpler technology as compared to drug synthesis.

1.2. Higher Plants as Source of Fertility Regulating Agents

A systematic study has been carried out by World Health Organisation (WHO) to evaluate plants used in various parts of the world as fertility regulators. Some were found to be effective, others toxic. Sufficient evidence has accumulated, however, to allow continuation of research and development of these substances. In a symposium on fertility regulation, Beijing (1980) Norman et al presented a paper entitled "prospects of the higher plants as source of useful fertility regulating agents of human use " in which he made the following observations:

"For centuries, virtually every indigenous culture has utilised plants in one form or another in attempt to limit population. At present, more than 800 scientific articles can be cited that report one or more pharmacological activity exhibited by extracts derived from plants that have some relevance to the topic of fertility regulation".

The field of plant derived substances, however, suffers lack of credibility in its publications. There is also lack of support from industrial sector because prospects of profits are poor in such undertakings (since it would be difficult to have strong patent protection of such products). Lack of historical evidence

of success in such undertakings adds to the disadvantaged position in industrialising these plant extracts. Hope in this field is, therefore, mainly on scientists in the developing countries whose interests are in area of natural products. One can then anticipate that future major discoveries of new drugs from higher plants will be made largely within the developing countries.

1.2.1. Fertility Regulating Plant Products

Review of past work in this field (Bingel and Farnsworth, 1980; Farnsworth and Bingel, 1987 a,b) presents information pertinent to fertility regulating plant products. A number of isoflavones, coumestans, stilbenes, alkaloids and other natural products have been shown to illicit weak estrogenic effects, but for a number of reasons are of little practical significance to human fertility regulation. Large number of alkaloids and other natural products of known structure are reported to have marked uterine stimulatory effect, but most of these studies were based on in vitro activity. These substances also had additional pharmacological effects that preclude their use as human fertility relating agents.

About a dozen plant derived substances of known structure are reported to have anti-implantational and abortifacient activities on basis of animal test results (Bingel and Fransworth, 1980). Some appear to be of sufficient interest to warrant further studies on efficacy and safety. The most promising of these is embelin (Kholkute etal, 1978), Coumaric acid, (Pakrashi and Pakrashi, 1978; 1979) Aristolic acid ester derivative (Pakrashi and Shaha, 1978) and analogues of the latter substance. M-Xylohydroquinone, originally

Figure 1.1

some plant-derived compounds with known fertility regulatory activity (i) Embelin (ii) Coumaric Acid (iii) M-Xylohydroxyquinone (iv) Aristolic Acid Methyl-derivative (v) Gossypol (vi) Spartein (vii) Zoapartanol (viii) Montanol.

isolated from Pisum Sativum, have been studied extensively. However, the studies show that it is only 60% effective as women contraceptive (Sanyal, 1956). Gossypol, a dimeric sesquiterpene aldehyde found in cotton plant, Gossypium spp. is currently under use in the Republic of China as a male contraceptive (Bingel and Fransworth , 1980). Early studies on Gossypol indicated high degree of toxicity (Abou- Donia, 1976; Berardi and Goldblatt, 1969) but its clinical trials as a male contraceptive by Gouzen et al, (1987) showed that it has no adverse effect on libido and that there was no short-term statistical difference in serum potassium levels between Gossypol and placebo groups. However, long term dosage of the drug showed a trend towards reduced serum potassium levels. This phenomenon had been shown to occur in some individuals only and was thought to be associated with nutrition variations in the studied population (Qian, 1979). Other report present two uterine effective compounds, zoapatanol and montanol isolated from zoapatal tree, Montana Tomenosa, (Levine, 1979). These products show abortifacient activity (Jiu, 1966; Levine, 1979). Spartein (Gray and Plentl, 1958) and its optical antipode, pachycarpine (Stubblefield, 1963) have also been used as abortifacient compound , however, had to be administered parentally.

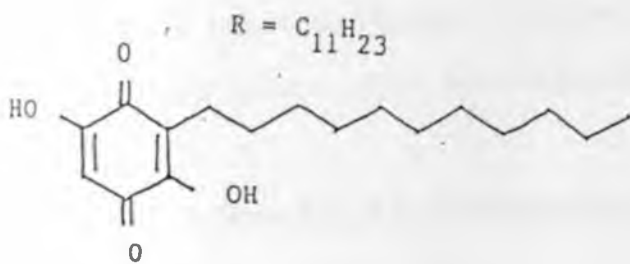
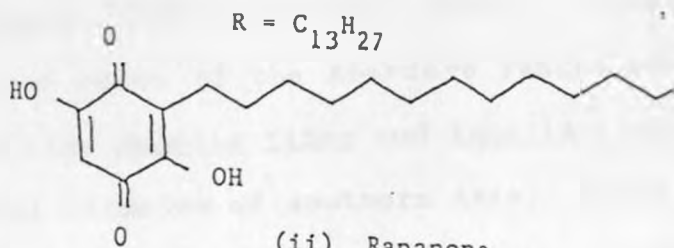
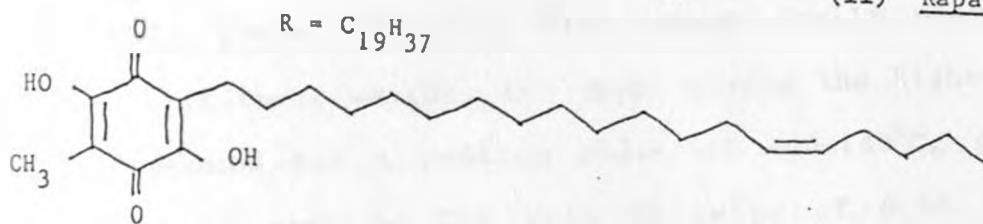
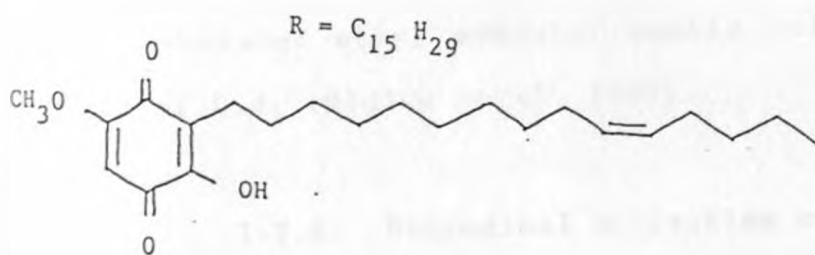
1.2.2. Ethnomedical Drugs:

It is a belief of many scientists even today, that most if not all ethnomedical (folkloric) claims are derived from doctrine of signature i.e. the idea that shape, colour or some other characteristic of the plant, part of plant or exudes, would be

Figure 1.2

Structures of embelin and its closely related benzoquinones

(i) Embelin (ii) Rapanone (iii) Maesaquinone (iv) Maesal

(i) Embelin(ii) Rapanone(iii) Maesaquinone(iv) Maesanin

indicative of their therapeutic use. Hence any claim of ethnomedical value of a plant are often automatically discounted by some investigators. However, given sufficient ethnomedical data concerning how, when and for what purpose(s) a given plant has been used, one may hypothesise its possible mechanism of action and test these hypothesis with experimental animals.

1.2.3. Embelin (2,5-Dihydroxy-3-Undecyl-1,4-Benzoquinone)

Embelin in an orange-brown crystalline extract from locally available plants like Embelia schimperi, Rapanea melanphloes, Maesa lanceolata and Myrsine africana, (Midiwo et al, 1988). These plants mainly grow in the lower zones of the Aberdare ranges and Mt. Kenya while other species like Embelia ribes and Embelia ribes burma, are abundant in tropical climates of southern Asia. Seeds, bark and leaves of these Myrsinaecae family plants have 1-10% embelin of their weight, the seeds giving the highest yield.

The compound has a melting point of 140-143°C, gives a single homogeneous spot on TLC with Rf value of 0.56 in chloroform: methanol (1:1) solvents. (Kaul and Dutt, 1979). Analysis on silica gel plates impregnated with 3% oxalic acid solution eluted with n-hexane: ethyl acetate: acetic acid (17:2:1) gives an Rf value of 0.4, (Midiwo et al, 1988).

1.2.4. Biological Activities of Embelin

There is a broad range of pharmacological activities acclaimed to embelin, (Nanba and Hatori, 1986 ; Chander and Ahmed, 1987 ; Atal et. al , 1984 ; Rao et. al., 1985 ; Prakash and Varshney, 1980 ;

Gupta et. al, 1989). These include:- antiplaque, antibacterial, antifungal, anaesthetic or analgesia, larvicidal in grain storage, effect on energy production (electron transport chain) anticancer effects and also antifertility effects in both male and female animals; the activity that was investigated in this research project.

Embelin has been incorporated in toothpastes to prevent formation of dental plaque and acid in teeth (Nanba and Hatori, 1986; Nanba et al, 1985). Crude extract from Embelia ribes was screened for antiplaque action on Streptococcus mutans. It showed potent inhibition of the bacterial growth at concentration of 62.5 mg/ml and had an inhibitory effect on the enzyme glycosyl transferase.

Benzoquinones have been used in spectrophotometric determination of anti-malarials like amodiaquinine, chloroquine, primaquine, Lremethoprim and pyrimethamine, based on coloured complexes formation, (Sastry et al, 1986).

Effectiveness of natural embelin as a grain protectant was evaluated using some insect pests of wheat grains in storage. There was 50% mortality of Stophilus oryzae and Rhyzopertha dominica at 0.25% concentration of embelin. Some insects showed greater resistance to embelin than others (Chander and Ahmed, 1987).

Embelin has been used as an anti-red flour beetle, Tribolium Casterenum but was found to be mutagenic to the strain when provided in 1 or 2 hydroxy or methyl substituted forms (Chander and Ahmed, 1985). Embelin potassium salts have been tested for analgesic activity. They did not show any effect on serotonin or histamine levels, but the level of adrenalin in cerebral-spinal

fluid increased and the activity of acetylcholinesterase decreased (Dhar et al, 1986). Analgesic studies using mice showed that embelin was effective by oral administration and results compared well with morphine but its mode of action was considered different from that of opiates (Atal et al, 1984; 1977). Embelin and rapanone synthetic derivatives were tested for antihistaminic and antiasthmatic activity and it was concluded that they could be useful as anti-allergy drugs (Iwaki et al, 1985; 1984). This activity was also duplicated by ardisiaquinone derivatives.

There are reports on activity of embelin as an inhibitor of respiration in germinating cowpea seeds. The ubiquinone-like activity of naturally occurring benzoquinone derivatives was shown to restore succinate oxidase activity. Highest effect was shown by rapanone, helicobasidin, dihydromaesaquinone, maesaquinone and embelin. It was concluded that these compounds are structural analogues of ubiquinone. Similar experiments done using rat-liver mitochondria, (Ozawa and Mamose, 1968) had shown respiration related activity. Huang et al, (1980) also reported respiration related inhibition of growth in Mycobacterium tuberculosis. The effective compounds were embelin, barganin and guarcentia. Reports by Otsuka Pharmaceuticals, (1982) cited certain 1,4 benzoquinones as useful anticancer agents.

Other well evidenced activity of embelin anthranilic acid esters and di-o-methyl esters is the local anaesthesia and antibacterial activity (Rao et al, 1985). Modification of their structure was attempted to get different pharmacological profiles. Halo-embelins like 6-Iodoembelin showed better antibacterial activity than embelin while diacetate acid esters of haloembelin had the highest antifungal activity (Tikkanen et al, 1983). Embelin derived

compounds substituted with aromatic primary amines showed good antifungal activity (Rao et al, 1983; Rao et al, 1985). Chloromorphine and rapanone have been shown to be anti-amoebic (Shar et al, 1984).

Some contradictory information to the analgesic and anaesthetic activities are the opposite claims by Schultz et al, (1979) on sensitising activity of methylated benzoquinones. Kubo et al, (1983) also reported activity of maesanin as a host defence stimulant while the other cited reports are more or less on inhibitory activities.

Toxicity studies show only a few adverse effects of embelin but this area has received less attention. Low et al (1985) reported visual deficit and retinotoxicity in Ethiopian chicken population studied. A dose of 1.25-2.5 g/kg per day caused visual defect but doses below 0.25 g/kg did not have any visual effect. Studies by Tikkanen et al, (1983) showed that natural naphthoquinones and benzoquinones were mutagenic to S. typhimurium strain TA2637, causing metabolic activation.

Salient amongst other biological activities, are the reports on antifertility effect of embelin both in male and female animals. Studies on effect of embelin on *Copora lutea* of suckling guinea pigs showed a dose dependent increase in ovarian weight, (Prakash and Varshney, 1980). Powdered berries of Embelia ribes prolonged the diestrous phase of estrous cycle and inhibited fertility in 62% of the studied rats. It affected suckling and prevented pregnancy in 75% of the treated female rats, (Kholkute et al, 1978). When embelin was administered to ovariectomized rats, it altered the activity of various enzymes in the uterine tissue. When

administered to estradiol primed rats, it caused decline in uterine weight. It did not evoke any significant change in the uterine biochemistry of progesterone primed rats, (Prakash et al, 1986). Prakash and Shukla, (1987) studied embelin effect on activity of β -glucosidase in ovariectomized rats and found that it caused marginal increase in the enzyme activity. But when administered to estradiol primed rats, there was significant decrease in activity of the enzyme, which had already been induced by estradiol dipropionate. However, its combined effect with progesterone could not show any significant change as compared with progesterone per se treated groups. Studies by Bhargava et al (1984) had shown that 20-50 mg/kg body wt. of embelin administered to rats at 1-15 days of pregnancy caused inhibition of implantation and re-absorption of foetus. Prakash and Varshney, (1980) had reported that embelin is neither antizygotic nor blastotoxic despite its anti-implantation and abortifacient properties. It showed antiestrogenic and progestational activities but not antiprogestosterone activity. Similar studies have been done on the male antifertility effect of embelin. Seth et al (1983) reported reduction in sperm count and their mobility during experiments with albino rats. When 50-200 mg/kg body wt. of embelin was administered to the animals for fifteen days, the weight of the testicles reduced. Jayaraman et al (1971) had observed reduction in semen volume and sperm motility when powdered berries of Embelia ribes were fed to Bonet Macaque monkeys. He postulated possible intervention of the plant ingredients with testosterone levels of the experimental animals. Agrawal et al (1986) showed that embelin altered the rat testis histology, glycogen levels, sperm counts and weights of accessory sex glands, while Dixit and Bhargava, (1983) had observed that

administration of 80 mg/kg body wt. of embelin orally for 100 days to male dogs caused azoospermia with subsequent recovery 250 days later. In male rats, daily administration of 75-300 mg/kg body wt. of embelin showed a reduction in prostate gland weight and indicated an impairment of metabolic functions of this organ. It was noted that alkaline phosphatase activities were elevated, (Chanhan et al, 1979). Very recently, studies by Gupta et al (1989) reported that subcutaneous administration of 20 mg/kg body wt. of this drug for 15 or 30 days caused reduction of epididymal motile sperms count, pregnancies attained and litter sizes in the albino rats. These effects were reversible within 15-30 days once the drug treatment was withdrawn. Light and scanning electron microscopy showed decapitation of spermatozoal head alteration of outer membraneous sheath of the sperm and the shape of cytoplasmic droplets in the tail region. The drug induced marked inhibition in activities of carbohydrate energy metabolism of the sperm, but the treated rats remained in good health and showed no intoxication symptoms, (Gupta et al, 1989).

Evaluation of these antifertility claims necessitates a sound understanding of the components and mechanisms that govern fertility. Despite the fact that it was not until the 1950s that researchers and clinicians got attracted to the study of structure and function of the male reproduction tract; recent advances made in the concept of testicular fine structure, hormonal control and cellular interrelationships have been quite elaborate, (Greep, 1976).

1.3. The Male Gonads

The male gonads are the testis (equivalent of ovaries in the females). The role of testis in reproductive process was recognised in early antiquity (Aristotle, 1862). Despite this background, our knowledge of reproductive biology remains at relatively backward state and this is an obstacle to development of new methods of birth control. The future may well lie in more subtle way of intervening with spermatogenesis, while leaving uninterrupted the hormonal function that contributes to normal metabolism. Advances in understanding the male reproductive physiology lags behind that in females, (Greep, 1976).

1.3.1. The Male Reproductive Tract

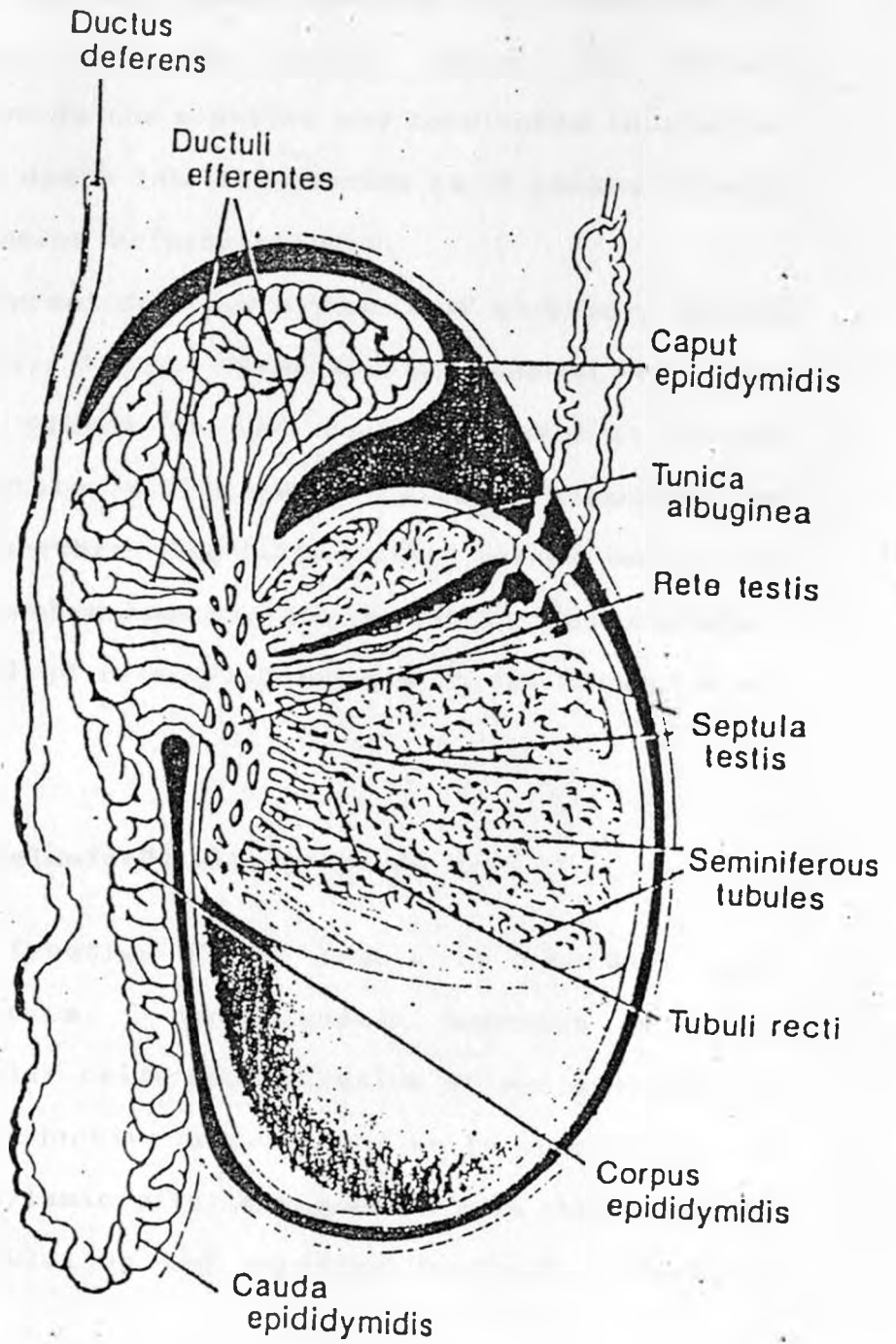
The testicles consist of minute convoluted tubules called seminiferous tubules, within whose lining, germ cells develop into spermatozoa, (La-vallete, 1865). In addition to germ cell line, highly branched sertoli cells occur within the tubules, (Ebner, 1888). In the interstices among the seminiferous tubules are clusters of epitheloidal cells, the Leydig cells, (Leydig, 1850).

The excurrent duct system consist of tubuli recti through which spermatozoa formed in the seminiferous tubules are carried in dilute suspension into the rete testis, a network of thin-walled channels in the axis of the testis or near the posterior surface, depending on the species.

From the rete testis, a dozen or more of ductuli efferentes conduct the sperms and testicular fluid into epididymis, an organ

Figure 1.3.

Structure of the testis and epididymis of man.



consisting of single highly convoluted duct, seven metres long in man. The anterior end is the caput epididymis and the posterior end is called caudad epididymis. The latter straightens out and continues as vas-deference (ductus deference), a slender duct with muscular wall. The duct passes upwards out of the scrotum entering the pelvis in the groin where it courses retroperitoneally towards the mid-line and terminates in slender ejaculatory duct that opens into the urethra as it passes through prostate gland just below urinary bladder.

Opening into the excurrent duct are a number of accessory glands of the male reproductive tract. These include seminal vesicles, a pair of saccular glands on the posterior aspect of the bladder, and the prostate, a firm compact gland surrounding the first portion of the urethra. The bulbourethra glands secrete via short ducts into the membranous urethra just below the prostate. These glands are involved in varying degrees in the formation of seminal plasma.

1.4. Hypothalamic-Pituitary-Gonads Axis

The development and function of the testis is dependent upon neuro-endocrine reflexes. Gonadotrophic hormones promotes maturation of testicular cells and secretion of sex steroids by these cells. The reproductive hormones milieu in circulation is governed by the hypothalamic-pituitary-gonadal axis that operates by feed forward stimulation and negative feedback inhibition mechanisms.

Androgenic molecules appear to exert a very strong inhibitory

effect on LH secretion but have a comparatively low effect on FSH release (Blackwell and Amos, 1971). Testosterone needs to be "activated" to its 5 α - reduced metabolites in order to exert its effect on the hypothalamus and pituitary (Zanisi, 1973). Experiments done by Massa and Martin, (1972) showed that the androgenic potencies in the feedback regulation was best given by 3 α - diols (5 α - androstan - 3 α 17 β - diol) followed by dihydrotestosterone (DHT). 3 β - diols (5 α - androstan - 3 β ,17 β - diol had minimal potency. Thus 3 α - diol is the most likely agent for physiological intercellular mediator of the feedback activity of testosterone on LH secretion. In this connection it is important to recall that, contrary to what happens in the peripheral androgen-sensitive structures, the 3 α - derivatives is the diol formed in large amount by the pituitary and hypothalamus.

With regards to hypothalamic-pituitary-gonadal feedback relationship, the basic issue is the extent to which these mechanisms are responsible for determining hormonal levels in normal physiological circumstances, (Davidson, 1969; MacCann, 1974). This information is crucial in knowing relative importance of feedback, vis a vis other possible determinants of circulating hormone levels for example, environmental stimuli and peripheral mechanisms of distribution, binding and metabolism. In addition, possible importance of developmental changes in parameters of feedback regulation and the relative importance of sites at which the feedback signal is recorded, are essential information, (Davidson et al, 1976).

1.5 The Leydig Cells

They are also called interstitial cells since they are located between seminiferous tubules. These cells synthesize androgen that regulate development and functions of male reproductive tract and secondary sex characteristics, (Hooker et al, 1946; Albert, 1961).

Androgen production is controlled by the action of LH on Leydig cells, as evidenced by in-vivo. (Eik-Nes, 1975) and in-vitro (Cooke et al, 1972) studies. Removal of free LH by dilutions does not halt androgens production, but addition of antisera to LH significantly reduces the biosynthesis of testosterone in Leydig tumour cells, (Moudgal et al, 1971). Direct binding of LH and hCG to Leydig cells has been demonstrated by autoradiography (De Krester, 1974; Dal-lago et al, 1975). Paralleling these observations is biochemical data that show specific binding of ¹²⁵I-labelled LH or hCG to membrane fractions of Leydig cells, (Catt et al, 1972).

Synergism of LH with hCG in stimulating androgen production has been reported, (Hall, 1969; Eik-Nes, 1962). The stimulatory effect of these gonadotropins is almost instantaneous (within 3-6 min.) and is mediated through cyclic AMP, (Pulsinelli and Eik-Nes, 1969). FSH, LH and hCG are similar polypeptide hormones, each approximately 20 amino acid long. They are glycoproteins composed of two sub-units, α and β . The former is identical in all the three. It is the difference in the β - subunit structure that confers their different physiological activities

(Li, 1977).

1.5.1 Foetal and Pubertal Development of Leydig Cells

In most animals, Leydig cells undergo two distinct periods of development, the foetal and pubertal stages. During the foetal stage, the developed Leydig cells secrete testosterone and possibly other hormones that stimulates differentiation of male reproductive tract and cause regression of murellian duct. The Leydig cells then regress and are not active at birth. The second wave of development is at puberty, giving rise to definitive Leydig cells of the adult (Fraser et al, 1969). Mature Leydig cells in adults are virtually never seen undergoing cell divisions, yet their number increases during LH stimulation. This indicates that they differentiate from other type of cells. Many of the interstitial tissues (Fibroblast-like cells and macrophages) have been implicated to be the stem cells, (Fraser et al, 1969).

1.5.2. Testosterone Biosynthesis in Leydig Cells

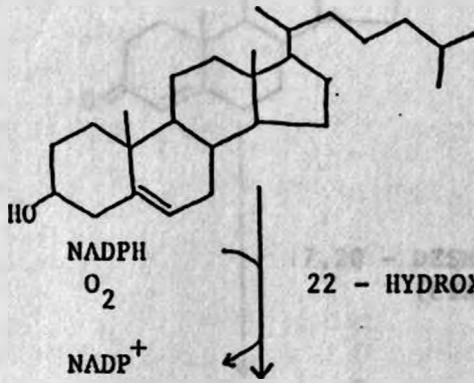
Fatty acids constitutes the main precursors of testosterone biosynthesis in the testis of rats, and possibly in the other animals (Free, 1970; Gomes, 1970). Once inside the cell, the fatty acids are converted to acetyl-CoA by the process of β - oxidation in the mitochondria.

The acetylcoA is then shuttled out to the cytosol and smooth epidoplasmic rectuculum where it is converted to cholesterol (Bucher et al, 1960). Cholesterol destined for steroid biosyntesis re-enters the mitochondria for cleavage of its side

Figure 1.5

Testosterone biosynthetic pathway in the Leydig cells

Cholesterol
(27 carbons)



NADPH
 O_2
NADP⁺

22 - HYDROXYLASE

22 - hydroxycholesterol

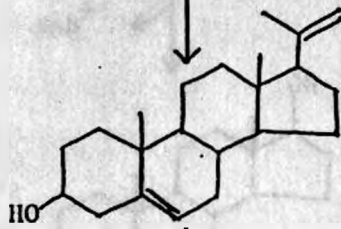
NADPH
 O_2
NADP⁺

20 α - HYDROXYLASE

20, 22 -dihydroxycholesterol

20,22 - DESMOLASE
(LYASE)

Pregnenolone + Isocaproic acid

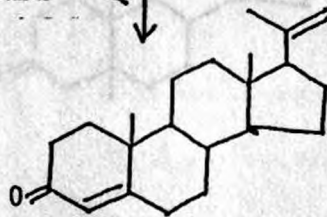


Δ^5 ISOMERASE

NAD⁺
NADH

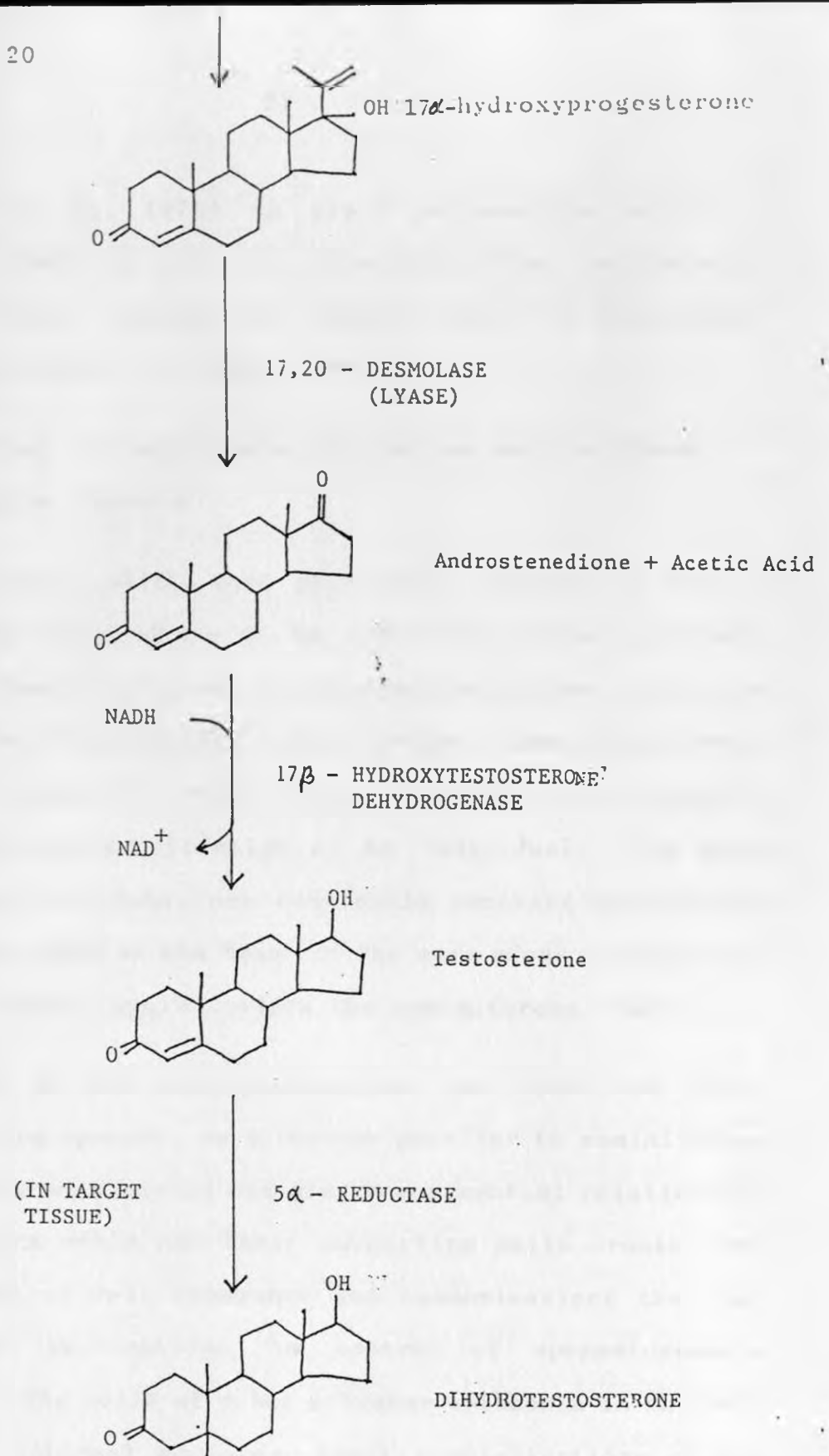
3 β -DEHYDROGENASE

Progesterone



NADPH
 O_2
NADP⁺

17 α - HYDROXYLASE



chain, (Yago et al, 1970) to yield pregnenolone which is subsequently shuttled back to cytoplasm for testosterone biosynthesis whose enzymes are tightly bound to endoplasmic reticulum, (Yanaihara and Troen, 1972).

1.6 Organisation of Seminiferous Epithelium and the Blood-Testis Barrier

The sertoli cells, which were previously thought to form a syncytium, have been shown to be individual cells uniformly spaced on the basal lamina of the seminiferous tubules, with germ cells occupying intercellular spaces between them (Brokelmann, 1963). These supportive cells cease to proliferate at puberty but persist throughout lifetime of an individual. The germ cells, on the other hand, are constantly renewing populations with their stem cells at the base and the more advanced stages at successively higher levels towards the seminiferous lumen.

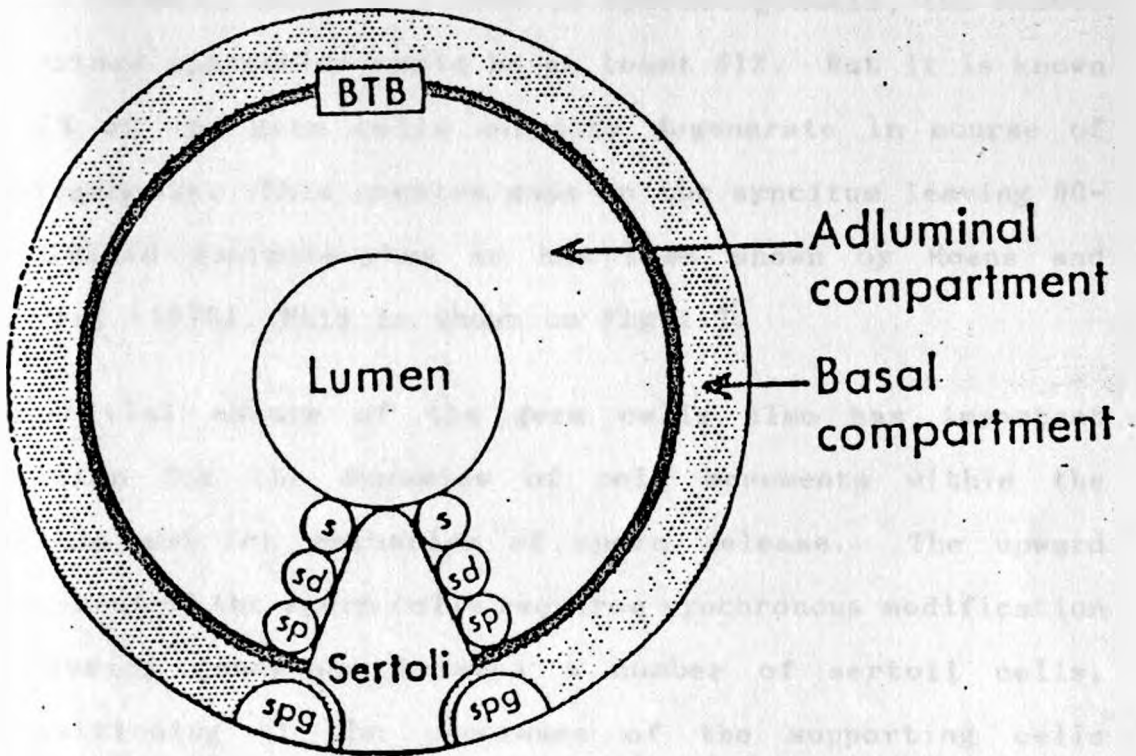
The occurrence of two cell populations, one fixed and other constantly moving upwards, is a feature peculiar to seminiferous epithelium. The continually changing topographical relationship between the germ cells and their supporting cells create some unique problems of cell coherence and communications that may have important implications for control of spermatogenesis (Greep, 1976). The cells of other columnar epithelia in the body have on their internal surfaces, local specialisations of the cell membrane that maintain cohesion and provide for communication between cells. This contributes to mechanical stability of the epithelium as well as to its functional

integration. In the seminiferous epithelium, the necessity of upward motility of developing germ cells with respect to the stationary population of supporting cells does not permit such establishment of enduring surface specialisations for cell attachment.

Seminiferous tubules have neither typical desmosomes nor gap junctions in the interfaces between the sertoli cells and the germ cells in the upper two-thirds of the epithelium, (Fawcett, 1974; 1975). However, a unique type of occluding junctions has been described between adjacent sertoli cells near the base of seminiferous epithelium, (Flickinger and Fawcett, 1967). The finding of these junctions accounts for the observation that blood-borne dyes are excluded from the seminiferous epithelium (Karmano, 1968). Several morphological studies using electron opaque probes of extracellular spaces showed that substances do penetrate the base of seminiferous epithelium and may fill the intercellular cleft surrounding the spermatogonia, but deeper penetration is prevented by occluding junctions between sertoli cells, (Fawcett et al, 1970; Neaves, 1973). It was concluded that these junctions constitute the morphologica basis of blood-testis permeability barrier. This is shown in Figure 1.6. The barrier divides the seminiferous epithelium into a basal compartment containing the stem cells of spermatogenesis and an adluminal compartment (Dym and Fawcett, 1970). The junctions first appear at puberty concurrent with initial establishment of a barrier, the onset of fluid secretion and development of lumen in the seminiferous tubules. Exposure of seminiferous tubule to hypertonic solutions which rapidly open blood-brain barrier, does

Figure 1.6

Compartmentation and organisation of cells in the seminiferous tubule. BTB, blood-testis barrier; s, spermatocytes; sd, spermatids; sp, spermatids; spg, spermatogonia



not open blood testis barrier, proving that this barrier is one of the tightest and most resistant epithelial permeability barriers in the body, (Gilula et al, 1976).

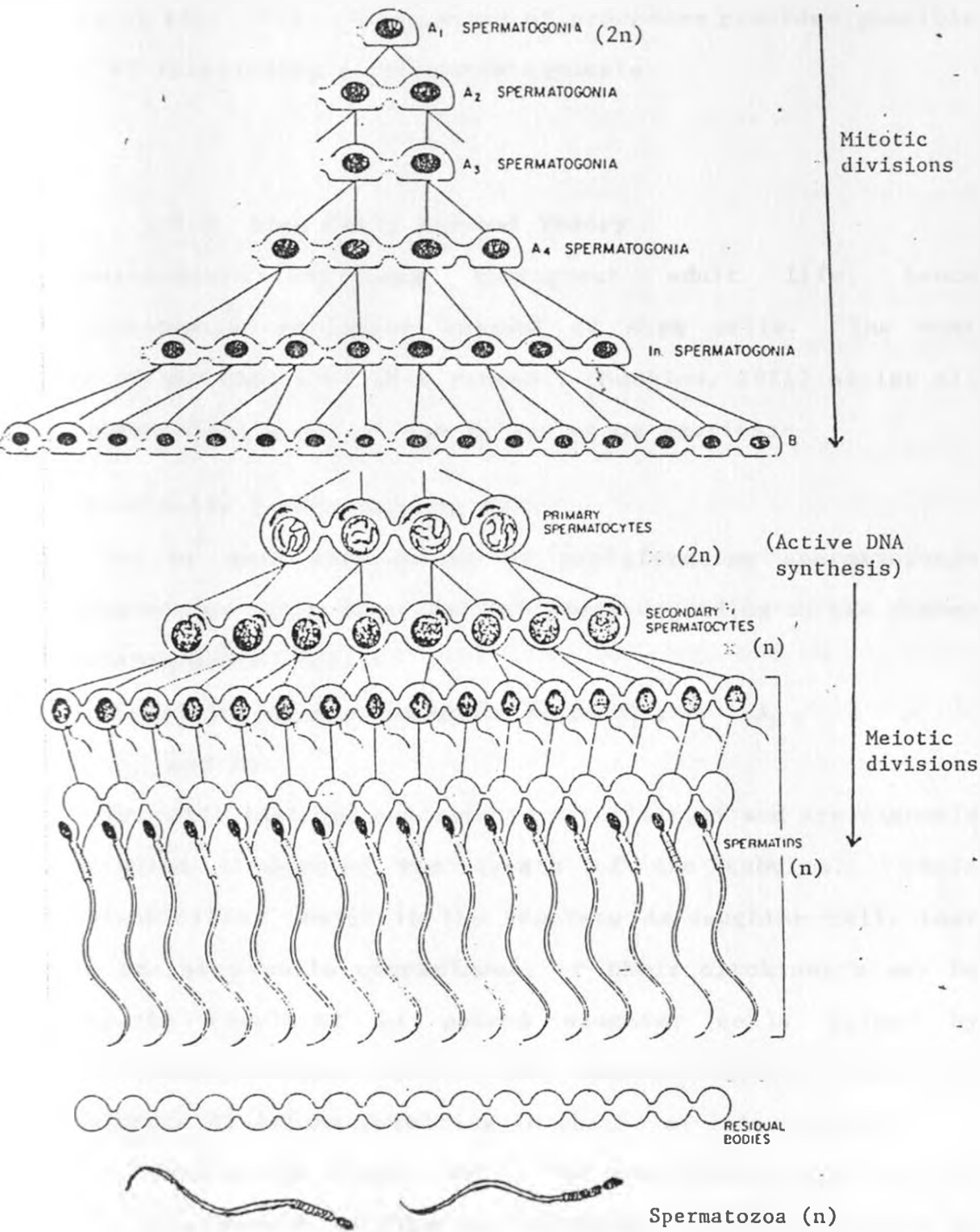
Spermatogenic Cells Syncytia

The groups of developing germ cells remain joined together by intercellular bridges that result from incomplete cytokinesis in the mitotic and meiotic divisions. This syncytial nature of the developing germ cells attributes to the synchrony of the spermatogenic waves, (Fawcett, 1961). From the number of division known to occur in course of spermatogenesis, the number of co-joined spermatids would be at least 512. But it is known that 25% of the germ cells normally degenerate in course of spermatogenesis. This creates gaps in the syncytium leaving 80-128 cojoined spermatocytes as has been shown by Moens and Hugenholtz, (1975). This is shown on Fig 1.7.

The syncytial nature of the germ cells also has important implication for the dynamics of cell movements within the epithelium and for mechanism of sperm release. The upward displacement of the sperm cells requires synchronous modification of occluding junctions between a number of sertoli cells, interpositioning of the processes of the supporting cells between the pre-leptotene spermatocytes and basal lamina and co-ordinated change in shape of the base of sertoli cells. This complex manoeuvre would require cell-to-cell communication among sertoli cells. Similarly, the process of sperm release from a syncytium requires co-ordinated motor activity between the

Figure 1.7

The syncytial nature of mammalian germ cells during spermatogenesis



epitoli cells and the syncitium, (Fawcett and Phillips, 1969; Longos et al, 1973). These array of processes provides possible sites of intervening with spermatogenesis.

1.6.2 Stem Cells Renewal Theory

Spermatogenesis continues throughout adult life, hence necessitates a continuous renewal of stem cells. The most accepted hypothesis of this renewal, (Huckins, 1971) assign all spermatogonia one of the following categories:-

1. Stem cells (spermatogonia A_s)
2. Two or more generations of proliferating spermatogonia designated Apr (Paired) or Aal (aligned) depending on the number of interconnected cells.
3. Differentiating spermatogonia (type A_1 , A_2 , A_3 , A_4 , A_5 and B).

The stem cells (A_s) are believed to occur singly and are randomly distributed throughout the length of the tubules. Their divisions either result in two separate A_s daughter cells that renew the stem cells compartment, or their cytokinesis may be incomplete resulting in paired daughter cells joined by intercellular bridge (Apr). The latter begin a series of synchronous divisions resulting in chains of interconnected cells of increasing length (Aal). The proliferative activity of these cells result in ninefold increase in the population of undifferentiated spermatogonia. The chains of aligned spermatogonia are then transformed to A_1 , which undergo

synchronous divisions at particular stages of spermatogenic cycle, giving rise to A_2 , A_3 , A_4 intermediates and spermatogonia, see figure 1.7.

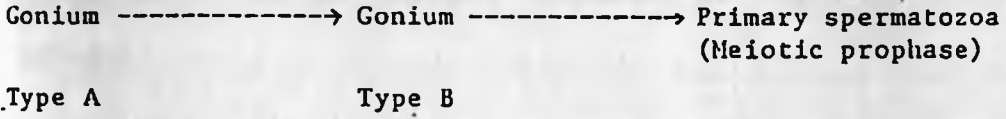
1.6.3. Hormonal Control of Spermatogenesis

Hormones are essential in spermatogenesis from spermatocyte stage to the mature spermatozoa, but for earlier stages (Lacy, 1967). Spermatogonia persist after hypophysectomy, in explanted testicular tubules and do not require exogenous hormone in order to evolve to meiotic prophase (pachytene stage of primary spermatocytes), as shown by experiments done by Lostroh, (1976). Thus from qualitative point of view, type A and B spermatogonia are hormone independent. Quantatively, however, FSH and probably GH as well, increase the yield of viable primary spermatocytes from spermatogonia (Boccabella, 1963). Testosterone regulates transition of primary spermatocytes (prophase, diploid chromosome number) to primary spermatocytes (metaphase). Even in absence of other hormones, spermatocytes complete meiosis and evolve to spermatids (Blaquier et al, 1972; Dayson and Orgebein-Crist, 1973). Testosterone also induces maturational changes, but other hormones must activate supportive function.

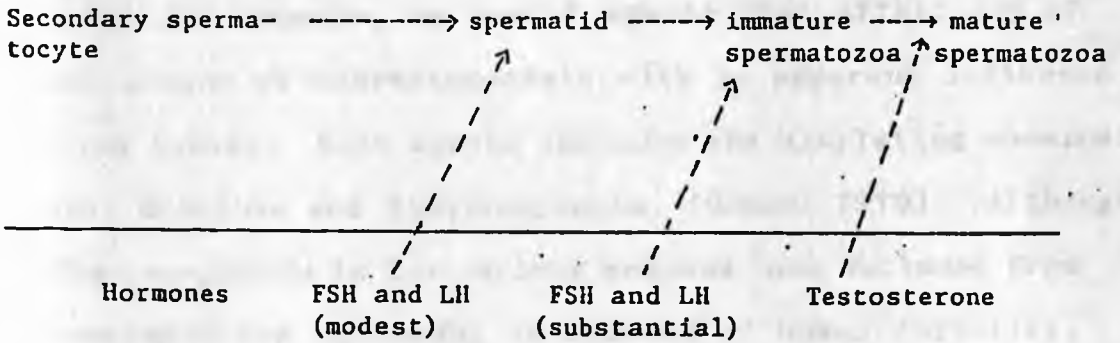
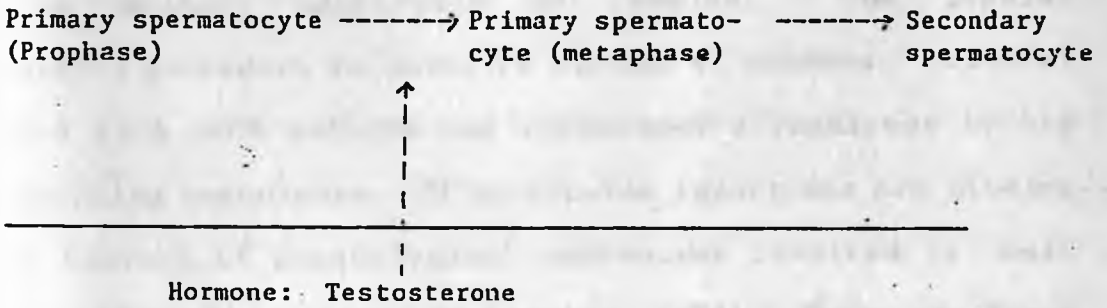
FSH is required in the developing testis for completion of the first wave of spermatogenesis but once full spermatogenesis is in progress, it can be maintained by testosterone alone, (Means 1971). The primary target of FSH are the sertoli cells, whose response to this hormone result in increased ABP synthesis, (Dorrington et al, 1975). ABP binds testosterone and DHT with

Figure 1.8

Hormones that institutes spermatogenesis in immature males.



Hormone: No absolute requirement



high affinity. The complex therefore serve as a storage form of testosterone which can be rapidly released as required (Tindall et al, 1974).

1.7 Methods of Suppressing Male Fertility

Although reports are available of attempts, from rational to magical (self-acclaimed) control of reproductivity in human since very early antiquity, the emphasis has been, and continues to be on methods applicable to females. The popular contraceptive procedure in males is the use of condoms. Cultural discontent with such methods has encouraged a readiness to try other promising techniques. Of particular importance are studies based on control of physiological mechanisms involved in male fertility, (Patanelli, 1968; de Krester, 1974). There are three main areas of approach: -

(a) The disruption of sperm formation either:

(i) directly, for example, by use of agents that affect any of the various stages of spermatogenesis with no apparent influence on the endocrine system. Such agents includes the alkylating chemicals, nitrofurans, diamines and dinitropyrrols, (Gomes, 1970). Although each of these compounds is for various reasons now excluded from serious consideration as useful in control of human fertility, several continue to be widely used in studies of reproductive processes.

OR

(ii) Secondary inhibition by interfering with hormonal milieu necessary for spermatogenesis. These are the agents with effect on gonadotropins e.g. the case of administration of excess dosage

of testosterone or progestagens, (Sesquin et al, 1980; Hardwood et al, 1980).

The capacity of excess testosterone to lower plasma gonadotropin levels has been established, (Lee et al, 1972; Sherins and Loriaux, 1973). Administration of testosterone propionate daily or testosterone ananthate weekly resulted in infertility without suppression of libido or potentia but high dosage of testosterone caused changes in lipoprotein metabolism and blood cells formation. These drawbacks might make long term maintenance on this regime unsafe. Low dose testosterone capsules implanted on rabbits were also shown to have inhibitory effect on spermatogenesis, (Verjan et al, 1975). Testosterone has been administered in conjunction with other steroids, expecting the androgen to maintain accessory glands weight while the concurrently administered estrogens or progestagens depress gonadotropin levels (Kragt et. al., 1975; Frick, 1973). Similar experiments carried out by Briggs and Briggs (1974) led to azoospermia within six weeks.

The use of non-steroid agents to suppress gonadotropins have been ignored. Gomes (1970) and Jackson (1975) showed that hydrazine derivatives (methalibure) had antigonadotropins effect. Cyproterone acetate has antigondatropin and antiadrogenic properties. Experiments by Prasad et al (1970) using cyproterone acetate on rats led to antifertility without loss of libido but Brotherton and Bernard (1974) reported low level of testosterone, sperm count and also lowered libido in men treated with cryproterone acetate. Suppression of adrogen production by this compound apparently involves its competition with progestational substrates for steroidogenic enzymes, (Grants and

Stitch, 1973; Agmo, 1975).

(b). Interference with epididymal maturation of spermatozoa. This epididymal function is dependent upon relatively high concentrations of androgens. It is therefore possible to interfere with the physiology of epididymis at androgen dosage that would have little effect on spermatogenesis, accessory gland function or libido. The discovery of a protein that stimulate forward mobility of spermatozoa, (Acot et al, 1979) is another line of intervention but no compound free of toxic effect has yet been developed to inhibit activity of this protein.

(c). Interrupting sperm transport, for example by interpositioning of a mechanised barrier. Such methods include vasectomy and use of condoms. Recently developed microsurgical techniques in vasectomy gives 80% guarantee reversibility of potency. However, the rate of pregnancies following such reversals is considerably lower, (Anasbacher et al, 1972).

Other lines of approach include interruption of capacitation and fertilisation. It has been shown that acrosomal hyaluronidase is sperm specific, hence the possibility of raising specific antibodies against it (Metz, 1973). Isoenzyme lactate dehydrogenase-x is also sperm specific and antibodies against it have been reported to suppress pregnancies in rabbits following post-coital passive immunisation, (Goldberge, 1973). The existence of zonal reaction and the consequent block of polyspermy is also potential target in controlling fertility. Oikawa et al, (1973) found that certain plant lectins specifically bind to terminal saccharide residues of zona pellucida and were effective in blocking fertilisation in vitro.

But the possibility that the plant lectins could be tolerated in-vivo and that they could be delivered to the site of fertilisation in adequate concentration is quite minimal.

The cited stages of sperm development and fertilization offers a wide scope area of intervention. Thus the potentials of developing a "male antifertility pill" is enormous considering the recent advances in understanding the male reproductive physiology and the biochemistry, specifically spermatozoa formation and maturation.

The use of plant products in conventional drugs is slowly gaining acceptance with both scientists and clinicians communities. In the present study, the potentials of certain benzoquinone plant products, embelin, rapanone and maesaquinone as agents of fertility control are evaluated.

1.8 Rationale for the Research Project

The rationale was to provide a more detailed analysis of the male antifertility effects acclaimed to embelin. Previous reports are largely on gross effects viz changes in sperm count and their motility, reduction in testicular and accessory gland weights and a few citations on changes in certain enzymes activities in the gonads. There are no references with comprehensive details on the effect of this drug on the reproductive hormones concentrations. In broad sense, the study aimed at establishing whether the antifertility effect of embelin could be due to inhibitory action at the gonadotropins level (LH) or at testosterone biosynthesis. If the effect is at the latter stage, possible step inhibited was to be investigated.

Aims and Objectives

I. The effect of embelin on hypothalamic-pituitary-gonadal axis was studied. Specifically, the variations of LH and testosterone levels during embelin regime was compared with the control values. Testicular micro-environment concentration of testosterone during the regime was also noted.

II. Effect of embelin on testosterone biosynthesis *in-vitro* was studied. Mouse and rabbit Leydig cells cultures were incubated with various doses of embelin and the inhibition of testosterone biosynthesis noted. The viability of the cells after exposure to embelin was also studied. The inhibitory potency of embelin was compared with other closely related benzoquinones viz rapanone, maesaquinone and acetyl-maesaquinone.

III. A study on the possible step in steroid biosynthesis that is susceptible to embelin inhibition. This was done by noting the variations of progesterone (metabolite) levels during embelin regime as compared to control values.

IV. Gross morphology studies of embelin effect on testicular tissues was done by comparing histological slides of the testis of the experimental animals with those of the control group.

V. Recovery and toxicity studies: Health of the rabbits was assessed by noting the variations in their packed cell volume (PCV) and body weight during the experimentation. Recovery from

embelin effect was determined by noting the trend of the reproductive hormone (see I above) and the re-acquisition of the ability to sire a progeny after termination of embelin dose.

2. MATERIAL AND METHODS

2.1 Experimental Animals

Sexually mature rabbits of the white New Zealand strain were utilised in this study. All the animals used weighed between 2 - 3.3 kg and each was kept in a spacious cage in the departmental animal house, (lit during daytime/dark at night; and well ventilated, temp. 15-25°C). They were provided with chow pellet food, green vegetables and water ad-libitum. Rabbits obtained elsewhere were acclimatised in the departmental animal house for two weeks before being used in the study.

The experimental rabbits were divided into four groups :

Group A:

To assess the effect of embelin, maesaquinone and acetyl-maesaquinone on the reproductive hormones concentrations and study recovery after terminating the dosages.

Group B:

To assess the effect of embelin dosages on testosterone concentrations in the testicular tissues.

Group C:

To assess the effect of embelin regime on testicular histology.

Group D:

Served as the control rabbits, receiving intramuscular injections of plain corn oil (carrier medium) during the experimentation period.

Group A consisted of 13 animals. Six of them were receiving embelin, four of them maesaquinone and two of them received acetylmaesaquinone (40 - 50 mg/kg body wt.) intramuscular injections at two days interval until the 15th day of experiment. They were then left under animal house conditions with normal feeding for over a month, to study recovery from the drugs effects. Over experimentation period 0.5 ml of blood was collected from the lateral ear-vein and approximately 1 mg EDTA added to prevent clotting. The plasma was separated by centrifugation at 1000 g for five minutes and preserved in a deep freeze (-20°C). The bleeding was done at two days interval during the drug administration and at five days interval during the recovery period. The experiment took five weeks.

Group B animals were four, each receiving 30 mg embelin /kg body wt. The injection were at two days interval up to the 15th day of experimentation. A day after the last injection, the animals were sacrificed under diethyl-ether-sagatal anaesthesia, the testicles excised and preserved in deep freeze in a minimal amount of 0.15M PBS buffer, pH 7.5.

In group C, four of the rabbits received intramuscular injections of embelin (70 mg/kg body wt. in 1.5 mls corn-oil) at two days interval for 16 days. During the period of treatment 0.5 ml of blood was collected from the lateral ear vein and the plasma preserved as described above.

One day after the last injection, the animals were sacrificed under diethylether or sagatal anaesthesia and the testis immediately excised. These were washed in cold physiological saline before fixing them in Bouins solution, according to histological procedures.

Group D: The control rabbits were maintained in similar environmental conditions as the experimental groups. For each experiment there were four control rabbits receiving intramuscular injections of 1.5ml corn-oil (drug carrier medium) during the period of drug regime in their counterparts. Blood was routinely collected at the same intervals as in the drug treated rabbits. In the respective groups the rabbits were sacrificed and the testicular tissue treated similar to the experimental group tissues.

2.2 Reagents and Buffers

Steroids Assay Buffer (0.3M PBS).

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	5.4 g
Na_2HPO_4	23.6 g
NaCl	17.6 g
Sodium Azide	0.2 g
Gelatin	2.0 g

These were made to two litres using distilled water and pH adjusted to 7.4.

Bioassays Buffer (0.08M PBS)

$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	0.296 g
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	2.9 g
NaCl	4.385 g

These were made to one litre using distilled water, pH adjusted to 7.5 and 3 - 4 drops of a bacteriocidal (penicillin) were added before storing the buffer at 4°C.

Buffer for immediate use was made by adding 0.5% BSA to the stock

buffer.

Dextran-Coated Charcoal.

To 0.0625g of dextran in 100 mls of steroid assay buffer was added 0.625g of activated charcoal and the contents mixed thoroughly.

Scintillant.

30g of PPO dissolved in four litres of toluene.

Cell Culture Incubation Medium.

12 mls of BME x 10 or Earle's medium

21 mls of NaHCO_3 (7.5%)

2 mls of double distilled water

5 drops of penicillin/streptopen

Haematoxylin (Lillie Modification, 1968).

Solution A;	$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	2.5g
	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	45g
	HCl	2 ml
	Distilled H_2O	298 mls

Solution B:	Haematoxylin	1.0 g
	95% ethyl alcohol	100 mls

Mix solutions A and B

Eosin.

Eosin Y (C.I. 45380)	1 g
70% ethyl alcohol	1000 mls
Glacial Acetic acid	5.0 ml

Bouins Fluid.

Picric acid (aq. sat.)	75 ml
Formalin	25 ml
Glacial Acetic Acid	5 ml

2.3 Hormone Measurement

Hormone levels were determined by radioimmuno assay techniques (RIA). This is an example of structurally directed assay in that it depends on interaction of a particular shape of the structure of the molecule to be measured (analyte) with another molecule that specifically recognises that shape. Antibody molecules are widely used as specific binding agents in such assays but other binding agents like cellular receptors can be used.

The hormone molecules in the sample and a standard amount of isotope-labelled hormone competes for some limited sites in antisera (antibody), hence a competitive assay system. Only the isotope labelled hormone - antibody complex will be detected in the radio-isotope counter. Thus, the higher the amount of hormone molecule in the sample, the lower the radio-isotope counts and the vice versa.

2.3.1 Testosterone Assay

The assay was done using the conventional RIA that utilised dextran-coated charcoal for separation of free from antibody-bound hormone, Suffi and Jeffcoate, (1988).

Extraction of Steroids from Plasma

To 50 ul of plasma for testosterone assay was added 10 times volume of analytical diethylether and vortex mixed for two minutes. The tube contents were then stored at -20°C for one hour to freeze the aqueous phase, then the diethylether phase (containing steroids) was decanted into the appropriate tubes. The samples were left overnight for diethylether to evaporate and the residue reconstituted in steroid assay buffer.

Rapid assay method was employed in these testosterone assays. Thus 100 ul of the buffer was added to the residue and vortex mixed.

Testosterone Calibration Curve

The stock testosterone sample (supplied) contained 2.2 mol/100 ml (22 pmol/ml). 100 ul of this ethanolic solution of testosterone was allowed to dry then the residue reconstituted with 10 ml of the assay buffer and mixed vigourously.

Five test tubes were appropriately labelled and to each was added 500 ul of the buffer, then serially diluted by transferring 500 ul of the thoroughly mixed testosterone solution to the subsequent tubes. This gave testosterone concentrations:

1100 fmol/tube

550	"	"
275	"	"
138	"	"
69	"	"
34	"	"
0	"	" (Assay buffer only)

Table 2.1

Rapid Assay Method: Summary of Contents in the Assay Tubes

TC	NSB	BO	STDS	SAMPLES	ETHER BLANK	ORDER OF ADDITIONS
200	200	100	-	-	-	BUFFER
-	-	-	100	-	-	STD
-	-	-	-	100	-	SAMPLES
-	-	-	-	-	100	ETHER BLANK
-	-	100	100	100	100	TRACER ³ H TESTO
-	-	100	100	100	100	ANTIBODY
300 μls	300 μls	300 μls	300 μls	300 μls	300 μls	TOTAL VOLUME

2.3.2 Lutenising Hormone Bioassay

LH was assayed by the indirect method of assessing the potency of plasma LH (sample) in stimulating cultured Leydig cells to biosynthesise testosterone. The testosterone secreted in the culture medium was measured by radioimmunoassay. This is the improved in-vitro bioassay method for measuring LH activity using mouse Leydig cells preparations described by Van Damme et al, (1974).

Leydig Cells Culture Preparations

Incubation waterbath was maintained at 34°C in a Dub-noff metabolic shaker incubator.

The culture medium was placed in an ice-tray and gassed slowly for 10 min. with 95% O₂:5% CO₂.

Meanwhile, 6 weeks old laboratory mice were sacrificed by cervical dislocation and 4-6 testis excised. Fat tissue was trimmed off the testis. The latter were then rinsed in 10 ml of chilled incubation Earle's medium in petri-dish 1, then

transferred to petri-dish 2 containing 10 ml of chilled incubation medium where the testis were minced using scissors.

Further disruption of testicular connective tissue was done by squashing with macroset pipette. The minced tissue was transferred to a chilled 50 ml polythene beaker where they were mixed slowly for 10 min. by use of a magnetic stirrer. The loose cells were then filtered through a nylon cloth (mesh) into a chilled 100 ml polythene beaker and transferred to the warmed 95% O₂:5% CO₂ shaker-incubator for 1hr.

After incubation, the cells were sedimented by centrifuging at 24,000 r.p.m., 4°C for 10 min in plastic 1p4 tubes. The sedimented cells were washed with 20 ml of incubation medium and further centrifuged at 24,000 r.p.m., 4°C for 10 minutes before finally suspending them in 10 ml of the incubation medium. To determine cell concentration in the medium, a gently rotating magnetic stirrer was used to resuspend the cells before counting the number of cell/ml of medium using a haemocytometer under phase contrast microscope. A concentration of 200,000 cells per assay tube was used in the bioassay.

Assessment of Various Factors that Affected the Bioassay.

- (i) Presence of embelin in the culture medium.
- (ii) LH levels in the plasma of experimental rabbits.
- (iii) To test direct toxic effect of embelin and the closely related benzoquinones on Leydig cell functions.

For this purpose, the step of one hour incubation of the Leydig cells (see above) was interrupted. Various doses of embelin were added to the incubating cultures (1 hr.). The subsequent step of the cell preparation procedure ensured the washing of these drugs from the cells before stimulating them with LH.

Pre-Assay Preparations:

Concentrations of embelin quality controls viz Q10,20,Q40 dilutions.

The plasma samples were x 20 diluted. Each incubation tube had 100 ul of sample plus 200 ul of Leydig cells.

Summary of the Experiment

The LH standards, quality controls and the test drugs had been diluted accordingly.

LHO - LH6 x 3 (Lutenising hormone standards dilutions).

Q10, Q20, and Q40 x 3 (Quality Controls dilutions).

E₁ - E₃ x 3 Embelin dilutions 0.01-1mg/ml to test direct toxic effect of embelin on Leydig Cell function.

E + LH x 3 effect of 0.1mg/ml embelin on testosterone biosynthesis by LH stimulated Leydig cells.

M + LH x 3 (effect of 0.1mg/ml maesaquinone on LH stimulated Leydig cell function) .

DMSO/Corn oil x 2 (effects of solvents/carrier of benzoquinones on Leydig cell function).

LHO x 2 (Buffer: A measure of basal synthesis of testosterone by unstimulated cells) .

Q10, Q20 and Q40 (2nd set of quality controls).

The tube contents were incubated for 3 hrs at 34°C, and gassed with 95% O₂ : 5% CO₂ environment in the metabolic shaker-incubator. After the incubation, the tube were chilled with ice cubes and one ml of steroid assay buffer containing has Sodium azide added to inhibit further metabolism in the Leydig cells. The cells were then centrifuged (24,000 rpm, 10 min) and the supernatant used for testosterone measurements by the ordinary method:-

To each of the five labelled test-tubes of the standard curve preparations, 2 ml of buffer was added and the standard serially diluted by transferring 2 ml of the stock standard to each of the

subsequent tubes. For each of testosterone dilution 500 μ l solution was transferred in triplicates to 1p₁ assay tubes. Similarly, 500 μ ls of the plasma, ³H testosterone, antisera and buffers were transferred into the appropriate assay tubes.

The tubes were incubated overnight to allow antigen-antibody binding to reach equilibrium then 250 μ l of dextran-coated charcoal was added to all the tubes except TC. The tube contents were then left at 4°C for 20 min before centrifuging at 24,000 r.p.m. to sediment the charcoal and the supernatant decanted into vials. Four ml of the scintillant was added to the vial contents and left to stand overnight before radioactivity was determined.

2.3.3 Progesterone Assay

Progesterone levels were measured using FAO/IAEA RIA kit, Anonymous, (1986) a direct method that does not involve extraction. This method depends on the competition between progesterone in the plasma sample and ¹²⁵I - labelled progesterone for a limited number of number binding sites on progesterone specific antibodies immobilised at the bottom of the assay tubes. Since the separation of free and bound fractions involves only a simple decanting step, no centrifugation is required, nor is scintillant added since these are gamma-ray emissions.

Polypropylene tube coated with antibodies to progesterone were labelled according to experiment requirement. Progesterone standards were reconstituted using 1 ml distilled water, the 100 μ l pippered into the appropriate assay tubes in duplicates. Similarly, quality control samples 'A' (Low) and 'B' (High) and

plasma samples were pippered to the appropriate assay tube. Ordinary plastic tubes were used for TC.

One ml of buffered ^{125}I -progesterone was added to each tube within five minutes after the addition of samples and the standards. The tube contents were incubated for four hrs at room temperature then all tubes except TC were decanted and allowed to drain-dry for 2-3 min. The tubes were finally struck on an absorbent paper to remove any residual droplets on the rim of the tubes before recording the readings of γ - emission using a bench gamma counter.

2.3.4 Testicular Concentrations of Testosterone

Testicles excised from group B rabbits had been preserved for this experiment. The testis were minced with scissors then homogenised using minimum amount o 0.15M PSG buffer. The homogenate was centrifuged at 1000 g for five min. and the supernatant preserved. To the latter, 9 volumes of 3% PCA was added and then precipitated proteins spinned off using bench centrifuge. The acidic solution was neutralised by adding 6M potassium hydroxide using a dropper, until the 1-14 pH indicator paper showed pH 7 colour. The solution was left to stand for KClO_4 crystals sedimentation, then the clear supernatant decanted into a conical flask. The weight of the minced testis and the final volume of extract were noted. All the steps in this procedure were carried out in the cold room (4°C) and the supernatant was preserved in deep freeze, until assayed for testosterone. 100 ul of the supernatant was used in the assay for testosterone done by rapid RIA.

2.4 Histological Procedures

Histological procedures as described by Disbrey and Rack (1970); Drury and Wellington (1967), were followed. The principal involve fixing the excised tissue in as normal state as possible, dehydrating it in order to impregnate it with wax and finally embedding it in a wax block. Tissues for microscopic observation were cut into thin sections using a microtome then stained with dyes. The tissues were preserved by applying DPX-mountant over the slide bearing them before placing the cover slip.

(a) Fixation: This prevents autolysis and bacterial decomposition, coagulates the cell to prevent loss of diffusible substances, prevent osmotic damage during processing, hardens the tissue to prevent distortion by section-cutting blade and modify tissue components to create difference between theory in refractive index, and may also improve staining with dyes. The testicles from the experimental rabbits were excised when the animal was under anaesthesia and immediately immersed in chilled physiological saline (0.85% NaCl). The tissues were cut into pieces of about 1 x 1 x 0.5 mm and transferred to Bouins fluid (fixative) where they remained for 32 hrs before transferring them to 50% alcohol (1 hr) and finally to 70% alcohol where they remained until further processing.

Since tissue contains water, and paraffin wax is water immiscible, the following dehydration procedure was followed:

50% Alcohol - 1 hr

70% Alcohol - 1 hr

(50% and 70% dehydration was ignored since the tissues

were preserved in the 70% alcohol).

80% Alcohol - 1 hr

90% " " - 1 hr

95% " " - 1 hr

Absolute alcohol - 1 - 2 hrs

The tissue were then cleared by transferring them to 50:50 absolute alcohol: xylene for one hr and finally to xylene for two hours. This was followed by impregnating the tissues with molten wax (56 - 60°C) twice, then mounting them to labelled paper moulds (labelled). After orientating the tissue properly, more of the molten wax was poured to fill the mould. The latter was floated in a basin of cold water then submerged to hasten the solidification. This is the process of embedding tissue into a wax block. The tissues were left overnight at room temperature, before the process of sectioning was started. This was done using cambridge rocker rotating microtome with a sharp blade. Thin ribbons of the testicular tissue were sectioned and placed on a grease-free microscope slides smeared with egg albumin adhesive and dried in an incubator (37°C). Warm water (50 - 55°C) was then applied under the ribbon to remove creases, then drained off at one corner of the slide before storing them in an air tight box or in an incubator at 37°C until further processing.

(b) Staining Procedure :

Haematoxylin-eosin dyes combination was used. Haematoxylin is a basic dye and stains basophilic materials such as nucleus, blue. Eosin is an acidic dye and stains eosinophilic materials such as cytoplasm or collagen fibre, pink red. Because these are

aqueous medium, the sectioned tissues were hydrated before the staining.

The following procedure was followed:-

Dewaxing (removing wax with xylene)	5 min
Absolute alcohol	2 - 5 min
95% Alcohol	2 - 5 min
90% "	2 - 5 min
80% "	2 - 5 min
70% "	2 - 5 min

The tissues on the slides were then immersed in haematoxylin (20 - 30 min) and the excess dye washed off with tap water until the tissue appeared blue. They were then differentiated by a quick dip in HCl- alcohol solution, before immersing them into the counterstain, 1% eosin, for 5-7 min. The stained tissues were then re-dehydrated for final preservation. The following procedure was followed:

70% Alcohol	1 min
80% "	1 min
90% "	1 - 2 min
95% "	1 - 2 min
Absolute alcohol	2 min
Abs: xylene (50:50)	2 - 3 min
Xylene 1	5 min
Xylene 2	5 min

In all stages of the staining process, care was taken to avoid any drying of the tissues.

The dehydrated sections were preserved by pouring a drop of DPX-mountant over them before placing a cover slip. Each slide was

identified by a label tag.

2.5 Packed Cell Volume and Body Weight

Packed cell volume (PCV) of the rabbits blood was measured at two or three days interval during the drug treatment period. In the recovery stage, PCV was measured at five days interval.

Blood was obtained by dilating the lateral ear vein with xylene and siphoned through a gauge 21 needle into hyperinised haematocrit tubes. The latter were immediately sealed at one end over a bunsen flame then spinned with a microcentrifuge for four minutes. The hematocrit values were recorded. Weights of the experimental rabbits were measured before the start of each experiment at four-days interval during the first two weeks, and 5 - 7 days interval in the rest of experimentation period.

2.6 Fertility Test

Three of the embelin treated rabbits were tested for fertilising ability three months after stopping embelin dosage. Five embelin treated /control males were mated with reproductively mature female rabbits. Each pair was in separate cage in the animal house for five days before separating the males. Their potency was determined by the successful pregnancies and litter sizes.

3.0 RESULTS

3.1 HORMONAL CHANGES DURING EXPERIMENT

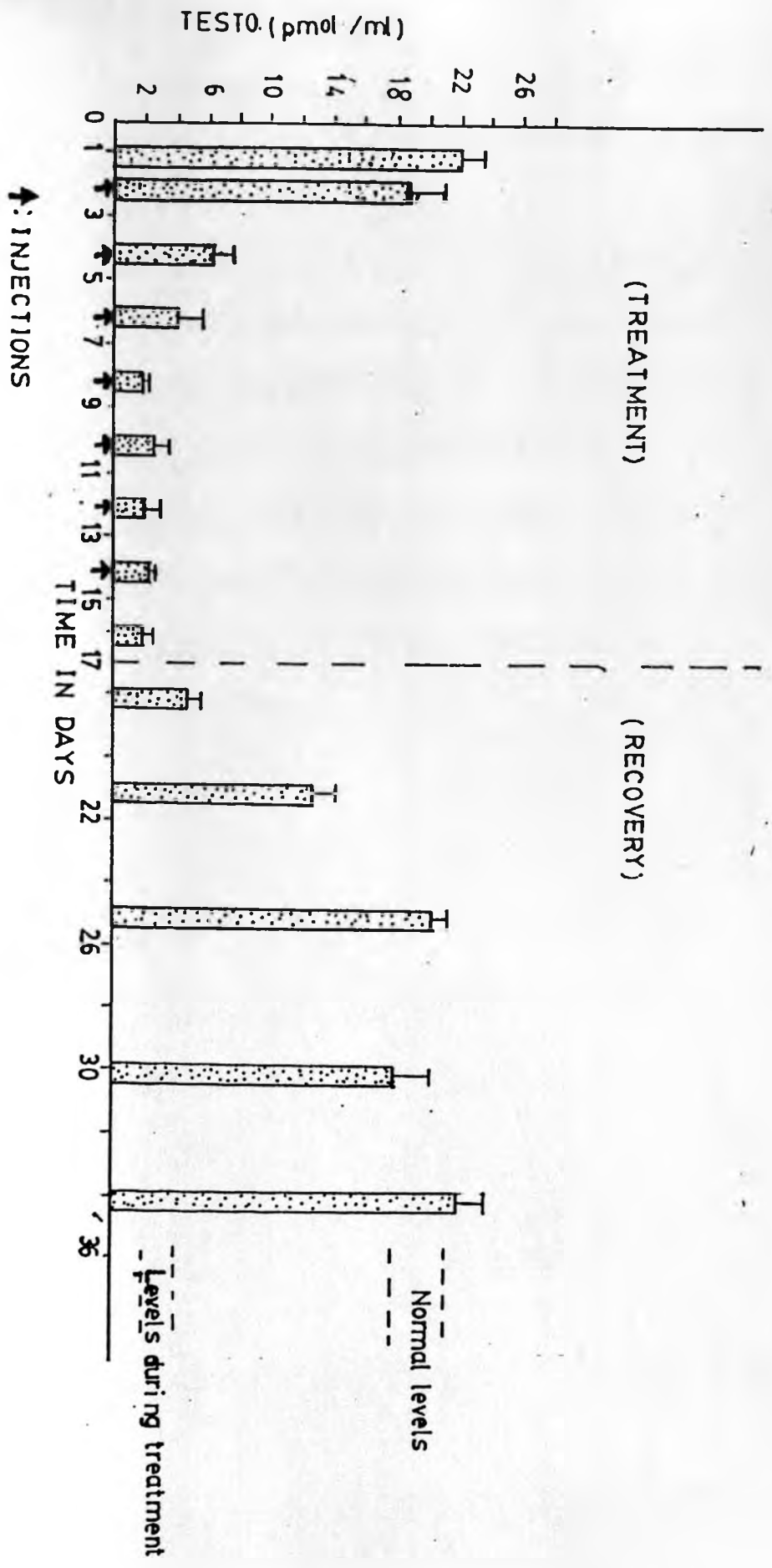
Plasma and testicular microenvironment reproductive hormone levels were measured using radioimmunoassay techniques as described in materials and methods. The data obtained is mainly presented in the form of bar graphs.

3.1.1 Testosterone Concentration

Data collected over the experimental period show that testosterone levels fell with the continued dosage of embelin. When injected with 40 - 80 mg embelin/kg body wt. at two days interval, the effect was already apparent by the third day and had full effect by the end of the first week of continued dosage. It was noted that this inhibitory effect of embelin was removed within three days, upon lifting of the dosage. During embelin administration the levels of testosterone remained diminishingly low, falling from 20 pmols to 2 pmol/ml of plasma. After terminating the dosage the hormone level rose steadily, reaching normal values ten days after the last dosage of embelin. Testicular microenvironment levels of testosterone also dropped with the continued dosage of embelin. When the experimental rabbits were injected with embelin for 18 days, testosterone levels dropped from 240 pmol/ml to 60 pmol/ml of testicular tissue extract.

Figure 3.1

Changes in testosterone (Testo.) levels during the experiment with embelin. Each bar is the mean value \pm s.d. of testosterone levels from six rabbits.



5-1

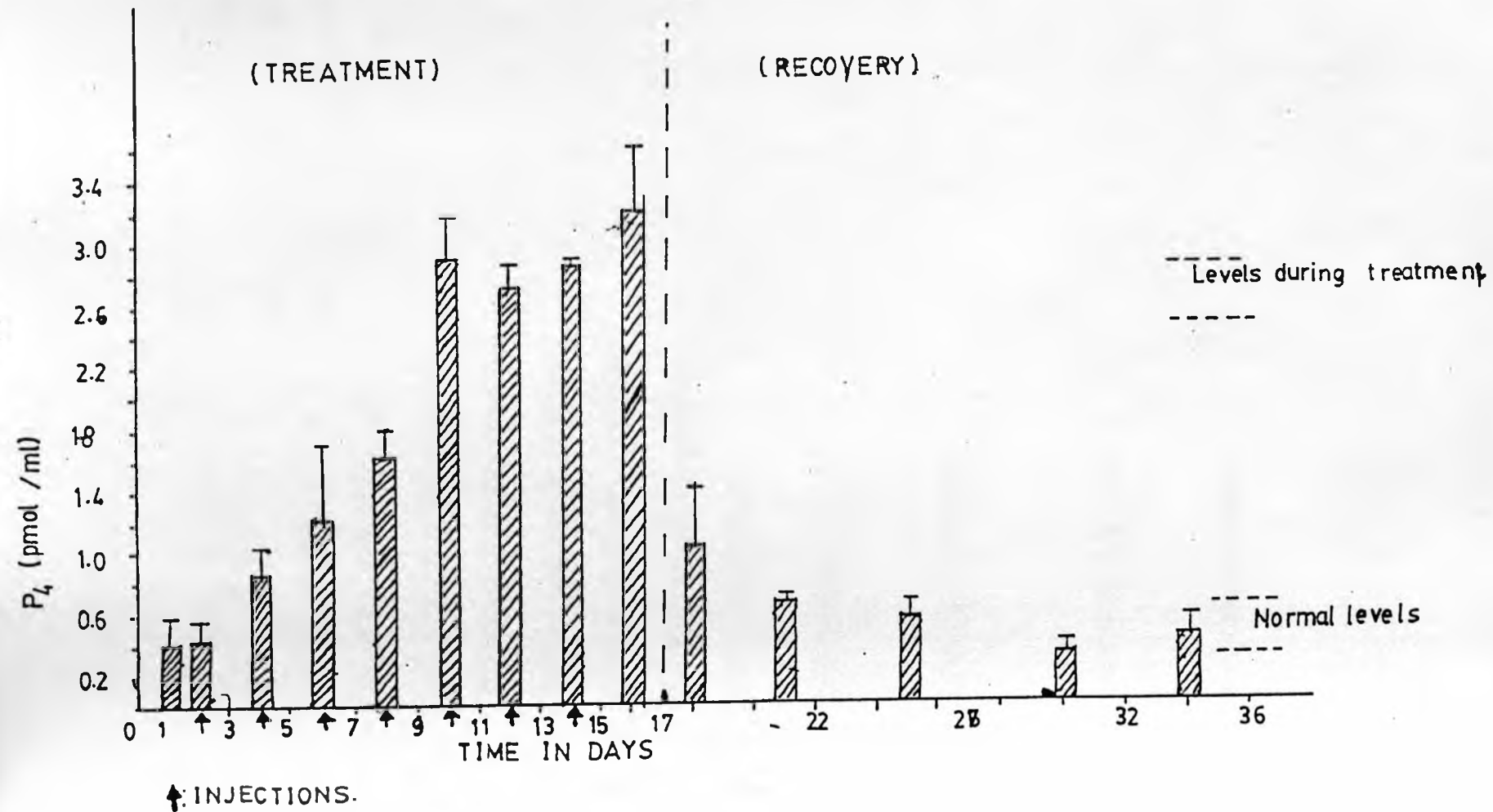


Figure 3.3

Changes in LH levels during the experiment. Each bar is the average value \pm s.d. of hormone level from six rabbits.

3.1.2. Lutenising Hormone Levels

Measurements of LH levels over the experimental period showed a slow but steady rise with continued administration of embelin (Fig. 3.3). The rabbits responded well to the 40 - 80 mg of embelin/kg body wt. injected at two days interval since this hormone rising trend was steady. It rose from 23 uI.U./ml to 33 uI.U./ml. After terminating the embelin treatment, the levels of LH dropped to normal values within ten days.

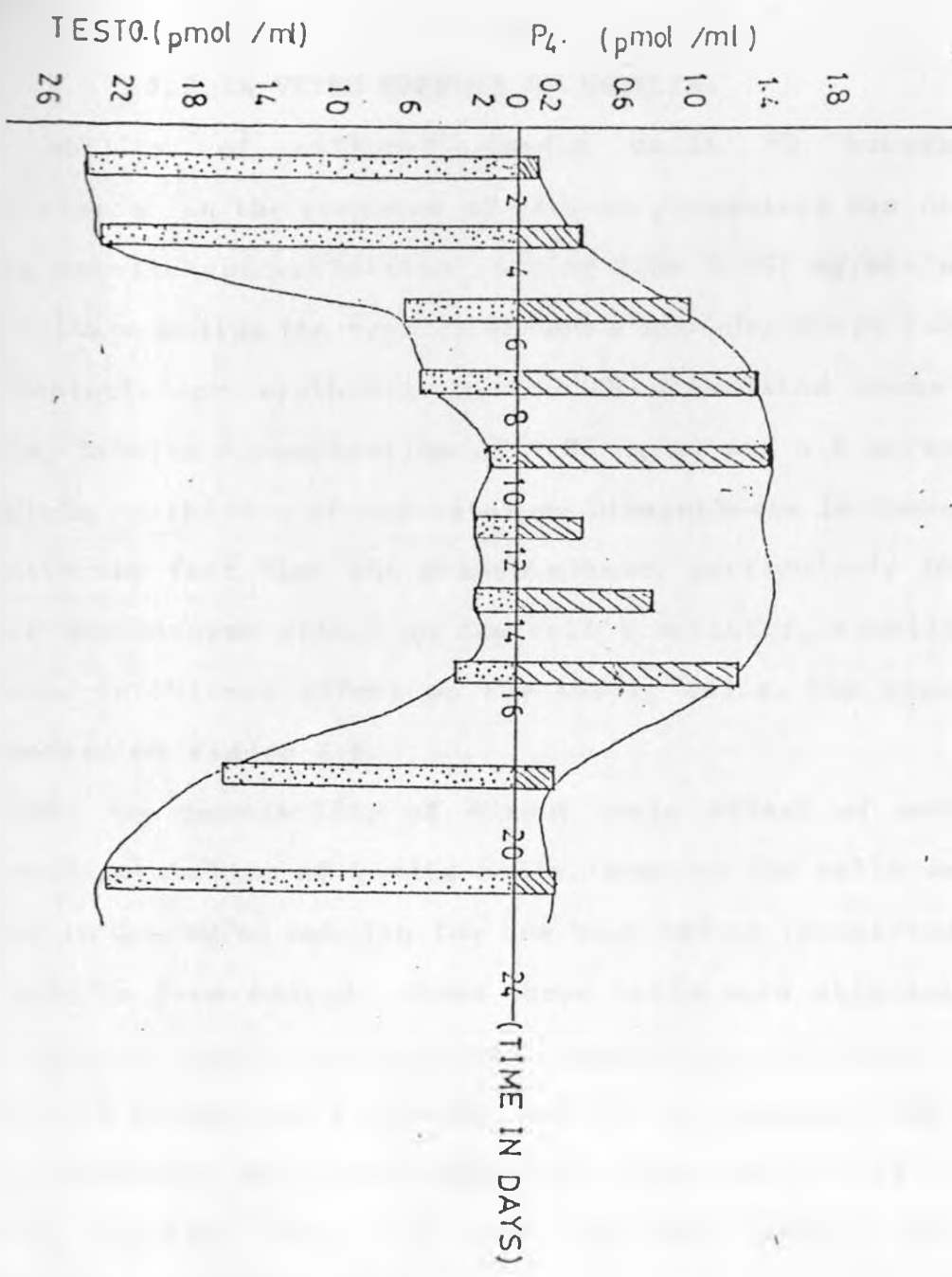
3.1.3 Progesterone Levels

Plasma levels of progesterone were measured over the experimental period (Fig. 2). With 40-80mg/kg embelin dosage at two days interval, there was a marked rise in the levels of this hormone. The effect of the drug was observed by the second day after the beginning of injections, with the maximum rise being realised during the second week of treatment. The rise was from 0.4 pmol/ml to 2.6pmol/ml of plasma. Progesterone elevation was sustained until the dosage was lifted, where upon the levels of this hormone sharply dropped by the third day, and reached normal values a week after the last injection.

There was noted a correlation of rise in progesterone levels with the fall in testosterone levels of the same day samples. In these animals, progesterone rose from 0.2 pmol/ml to 1.4 pmols/ml of plasma while testosterone dropped from 23 pmols to 2 pmols/ml of plasma. The profile is shown in figure 3.4.

Figure 3.4

Correlation of rise in progesterone (P_4) levels with the fall in testosterone (Testo.) levels during the experiment. Each bar is an average value of hormone levels from six rabbits. The line graph is a hypothetical depiction of these changes.



3.2 IN-VITRO EFFECTS OF EMBELIN.

The ability of cultured Leydig cells to biosynthesise testosterone in the presence of various parameters was assessed. Using embelin concentrations ranging from 0.001 mg/ml-1mg/ml in the culture medium the results showed a dose-dependent inhibition of testosterone synthesis in the LH stimulated mouse Leydig cells. Embelin concentration of 0.05 mg/ml and 0.5 mg/ml had complete inhibition of testosterone biosynthesis in these cells. Despite the fact that the solvents used, particularly DMSO, had their own adverse effect on the cell's activity, embelin showed greater inhibitory effect on the Leydig cells. The results are presented on figure 3.5.

To test the possibility of direct toxic effect of embelin on general metabolism of Leydig cells, some of the cells were cultured in 0.1 mg/ml embelin for one hour before transferring them to embelin free medium. When these cells were stimulated with standard LH, their testosterone biosynthetic capability compared well with control cell culture, but for the apparent 25% decline in biosynthetic activity (Table 3.5). This could be direct toxic effect or the result of some residual embelin effect on testosterone biosynthesis.

Previous experiments had been carried out to determine potency of related benzoquinones (Rapanone and Maesaquinone) in inhibition of testosterone synthesis in-vitro. Rapanone compared well with embelin in this activity, while maesaquinone showed greater potency. But test of the latter compound in-vivo caused inflammation at the point of injection, drop in PCV and body weight of the treated rabbits and in some cases resulted in death. These results are summarised in figure 3.9.

Table 3.5

Direct Toxic Effect of Embelin on Testosterone Biosynthetic Activity of Cultured Leydig Cells

The Leydig cells were incubated in a culture medium containing 0.1 mg/ml embelin for 1 hr., then the cells were washed free of embelin before transferring them to an embelin free medium, where they were incubated for 3 hrs to allow testosterone synthesis.

FACTORS	TESTOSTERONE PRODUCED (fmols/ml)	COMMENT(S)
LH ₁ stimulated cells (Embelin free medium)	370	LH ₁ standard has the highest lutenising hormone levels
LH ₆ stimulated cells (Embelin free medium)	170	LH ₆ standard has very low lutenising hormone levels
LH ₀ Case (No stimulation and embelin free)	146	LH ₀ . There is no lutenising hormone. This is basal testosterone production
DMSO environment. Pre-incubation in embelin)LH ₁ stimulation)	125	DMSO has toxic effect on the cells since the level of T are below LH ₀ case
Corn oil environment: Preincubation in embelin (LH ₁ stimulation)	280	Apparently cannot duplicate LH ₁ case for unknown reasons

Drop in testosterone synthesis due to preincubation in corn oil - embelin medium = x 1.3 (25%).

Drop in testosterone synthesis due to preincubation in DMSO - embelin medium = x 3 (70%).

Assuming that corn oil has no toxic effect, nor major error in the bioassay; the effect due to residual embelin or possibly due to its toxicity on Leydig cells activity equals 370 - 280 = 90 fmols testos/ml (30%).

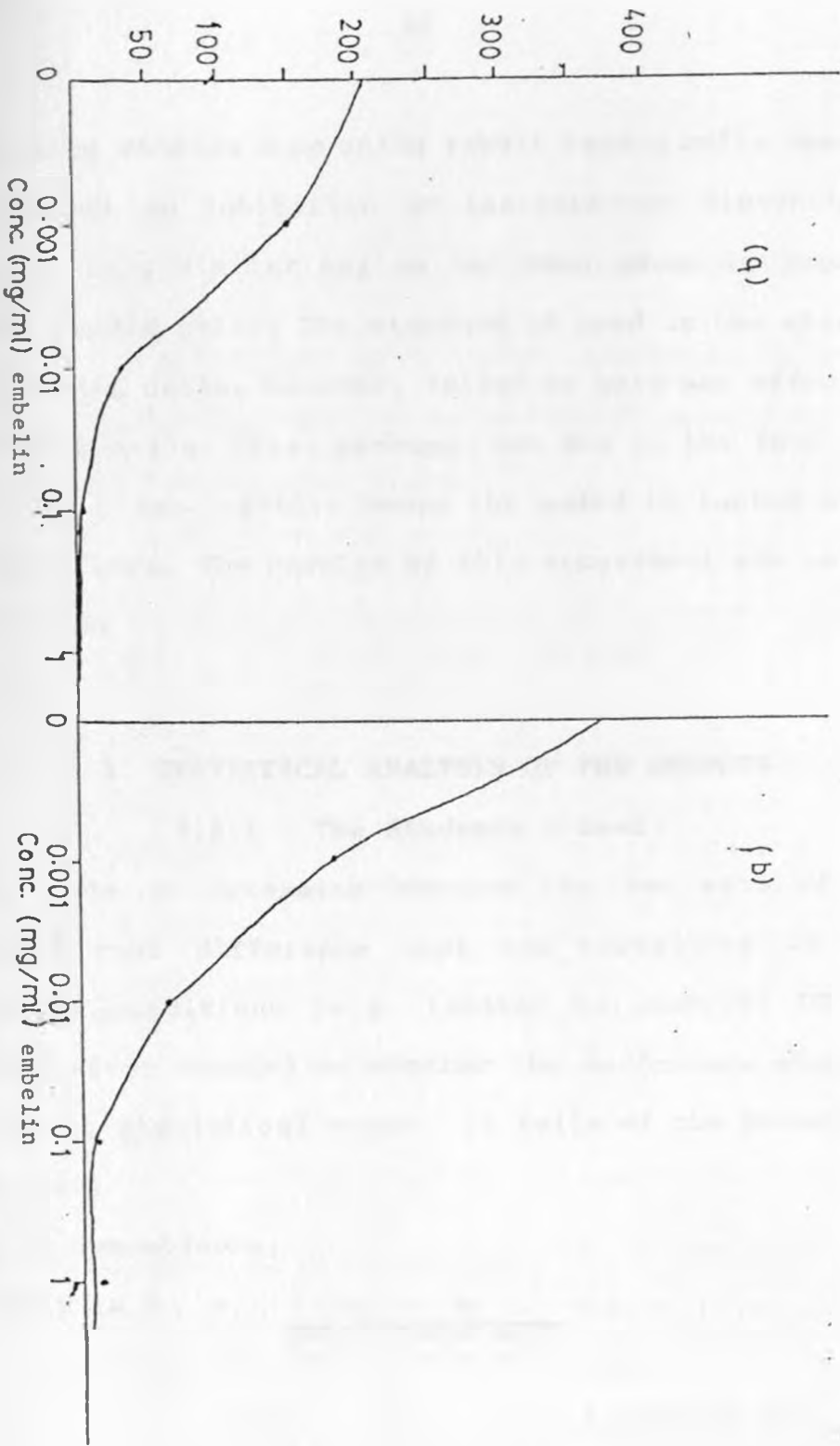
Effect due to DMSO, then = 280 - 90 = 190 fmol testos/ml = 63%.

Figure 3.5

Inhibition of testosterone biosynthetic activity of cultured Leydig cells with the addition of various amounts of embelin.

(a) DMSO environment

(b) Corn-oil environment.



Other in-vitro studies done using rabbit Leydig cells showed that embelin caused an inhibition of testosterone biosynthesis in these cells in a similar way as had been shown in experiments with mouse Leydig cells. The standard LH used in the stimulation of mouse Leydig cells, however, failed to have any effect on the rabbit Leydig cells. This, perhaps, was due to the fact that the latter cells clump together hence the added LH lacked access to the cell surfaces. The results of this experiment are summarised in tabble 3.6.

3.3 STATISTICAL ANALYSIS OF THE RESULTS

3.3.1 The Students t-test:

This was done to determine whether the two sets of results represented real difference that was prevailing in the two experimental conditions (e.g. treated vs control; OR before, during and after dosage) or whether the difference observed was merely due to statistical error. It tells of the probable error of means (xs)

For paired comparisons,

the formula is t =

$$t = \frac{x_1 - x_2}{S \sqrt{\frac{(1 + 1)}{(n_1 + n_2)}}} \quad (\text{pooled s.d.})$$

Table 3.6

LH Stimulation of Rabbits Leydig Cells Testosterone Biosynthesis

Testosterone biosynthesis by the cultured Leydig cell was used as the indicator to assess the implication of various factors incorporated in the culture medium.

EXPERIMENTAL ENVIRONMENT	TESTOSTERONE RELEASED (fmols/tube)	COMMENTS
LH ₀ medium (basal biosynthesis)	90 fmols/tube	LH ₀ , LH ₅ and QCs effects are almost equal i.e the cells did not respond to the stimulation expected from LH ₅ and-QCS plasma. The testosterone biosynthesis remained basal
All LH (L ₁ -L ₆) stimulation	110	
All QCs (Q10, Q20, Q40) stimulation	105	
S10	210	S10, S20 and S40 show a stimulation quite above LHO. There is a good sign of dose dependent response
S20	175	
S40	155	
Embelin Environment	< 20	Embelin inhibition effect is quite marked

NB: LHO medium cells that are not stimulated

LH₁ - LH₆ (dilutions): dose related response (gives straight curve)

QCs - x 10, x 20 and x 40 dilutions of quality control plasma LH

S10, S20, S40 - dilutions of the rabbit plasma LH

S is given by $Sd_1 (n - 1) + Sd_2 (n - 1)$

$$(n_1 - 1) + (n_2 - 1)$$

t is tested at given critical intervals e.g. $t_{0.05}$, 0.01 and 0.001 and at deduced degree of freedom i.e. $(n - 1 + n - 1) =$ degree of freedom. Thus t is given as t; d.f; critical values. When the calculated t is greater than the tabulated value, the null hypothesis, H_0 (which assumes no real difference between the two sets i.e. takes the observed difference to be due to sampling error), is rejected. Conversely, if the tabulated value of t is greater than the calculated value, then the difference observed in the two sets of samples is assumed to result from statistical error and not a sign of real difference. The data in table 3.7 show a good difference in hormone levels of samples collected before those collected during embelin dosage.

3.3.2. INTRA AND INTER-ASSAY COEFFICIENT OF VARIATION.

Intra-Assay Variations

Given by assessing result variations of samples derived from one pool. It gives the analytical error within one assay.

Inter-Assay Variations.

Given by the quality control sets that are incorporated within each of the assay set up. Since the quality control sample is from one pool and will be incorporated in every assay over the whole experimental duration, it is then an indicator of the

Table 3.7

To Assess the Significance of Embelin Effect on Hormone Levels

Statistical analysis of pooled samples of plasma obtained before initiation of embelin dosage and that obtained during its highest effect.

	PARAMETERS			
ASSAY		TESTOSTERONE	PROGESTERONE	LUTENISING HORMONE
BEFORE EMBELIN DOSAGE	n = \bar{x} = SD =	7 23 2.9	5 0.37 0.19	10 16.2 2.59
DURING THE HIGHEST EFFECT OF EMBELIN	n = \bar{x} = SD =	10 2.57 1.23	7 2.06 0.79	10 22.79 1.9
REALIZED CHANGE	MULTIPLE PERCENTAGE	X9 drop 89% drop	X6 rise 82% rise	X1.41 rise 30% rise
CALCULATED t		21.73	5.3	5.68
TABULATED t	Degree of freedom	15 d.f.	10 d.f.	18 d.f.
t 0.05	CRITICAL INTERVALS	2.131	2.228	2.101
t 0.01		2.947	3.169	2.878
t 0.001		4.03	4.587	3.922

Table 3.8

Inter and Intra-Assay Variation Analysis

STATISTICS		n	\bar{x} (pmols/ml)	S.D	C.V (%)
INTRA-ASSAY COEFFICIENT OF VARIATION OF TESTOSTERONE	RABBIT I,	10	9.7	0.94	9.7
	RABBIT II	10	7.3	1.2	16.4
	RABBIT III	10	25.7	3.62	14
INTER-ASSAY COEFFICIENT OF VARIATION	Testoste- rone (Medium)	10	8.46	1.35	15.9
	LH(Q20)	10	20.38	1.83	9
	Progeste- rone (Medium)	6	3.22	0.16	4.97

NB: \bar{x} given as pmol testosterone/ml of plasma

C.V. Coefficient of variation value expresses as percentage

stability of the assay procedures over a wide period of time.

Quality controls provided for testosterone assays were:

T_L , T_M , and T_H . In the assay systems these sets had approximately 4 pmol/ml, 10 pmol/ml and 20 pmols testosterone/ml of sample respectively. Similar quality controls were provided for progesterone assays and LH bioassays. These analysis are represented in Table 3.8.

3.4 HISTOLOGY

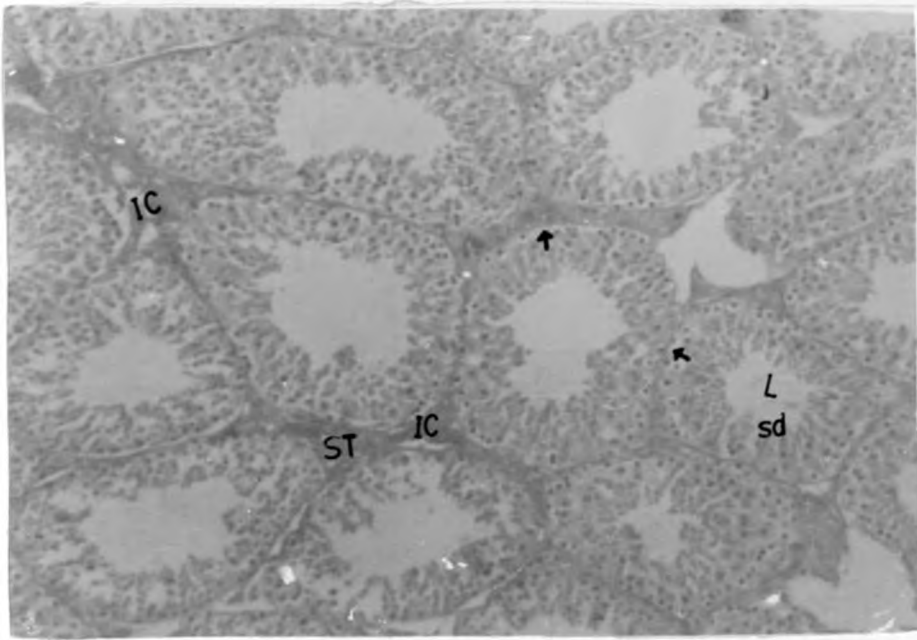
Photomicrographs of histological preparations taken from rabbits that had been under experiment for 18 days, provide more information on effect of embelin on testicular tissue/cells (see the plates).

The general picture of the tissue from embelin treated rabbits was that disorganisation and sloughing off tendency of spermatogenic cells occurred. The epididymis of these rabbits was devoid of any spermatozoa and in some cases was virtually empty. This contrasted with tissues from control rabbits whose seminiferous tubules cells were compactly and orderly arranged. Their epididymis was full of spermatozoa.

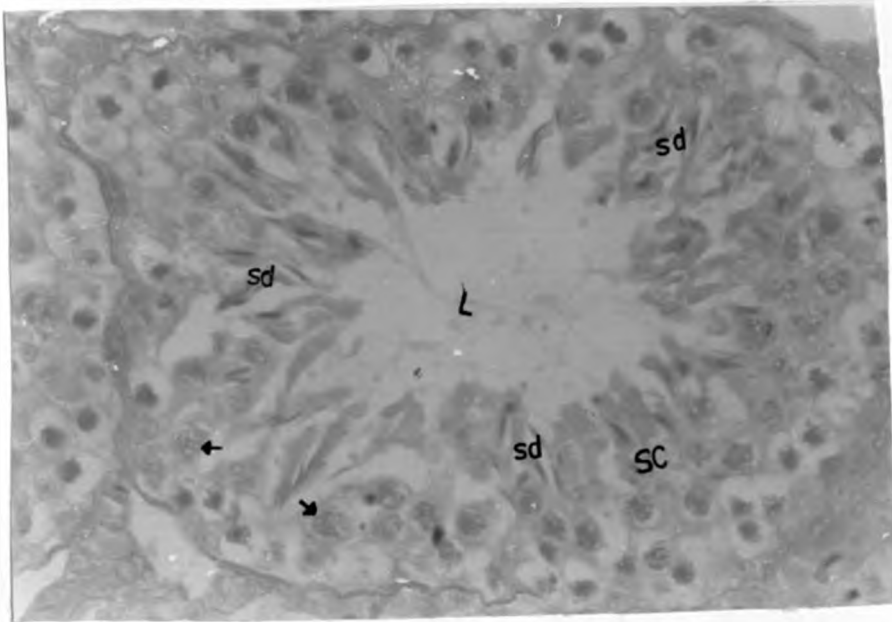
It was observed that the germ cells (spermatogonia) next to the basal epithelia were not affected by embelin treatment, but the cells in the adluminal side became extensively disrupted and vacuolated. The net effect was an atrophied seminiferous tubule with the spermatogenic cells sloughing off towards the lumen. Except vacuolation, sertoli cells remained normal since some of the germ cells still held to the sertoli cell surface (Plate 3a and 3b).

Plates 1 (a) and (b). Seminiferous tubule,ST, from a normal adult rabbit. Note the seminiferous lumen,L, spermatid maturation,sd, interstitial tissue cells,IC (Leydig cells), spermatogonia and spermatocytes (arrows x100 (a), x400 (b)).

(a)

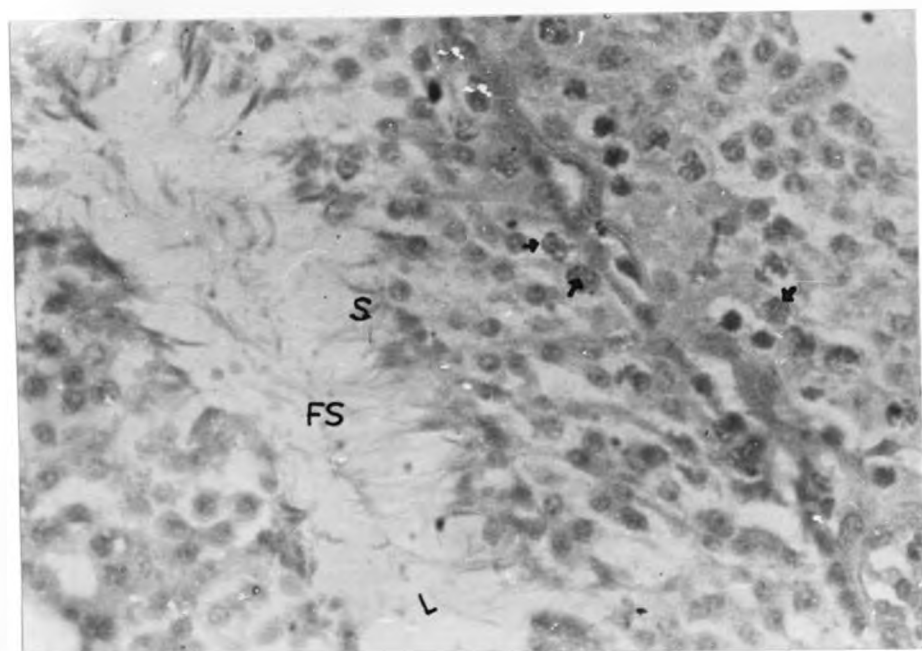
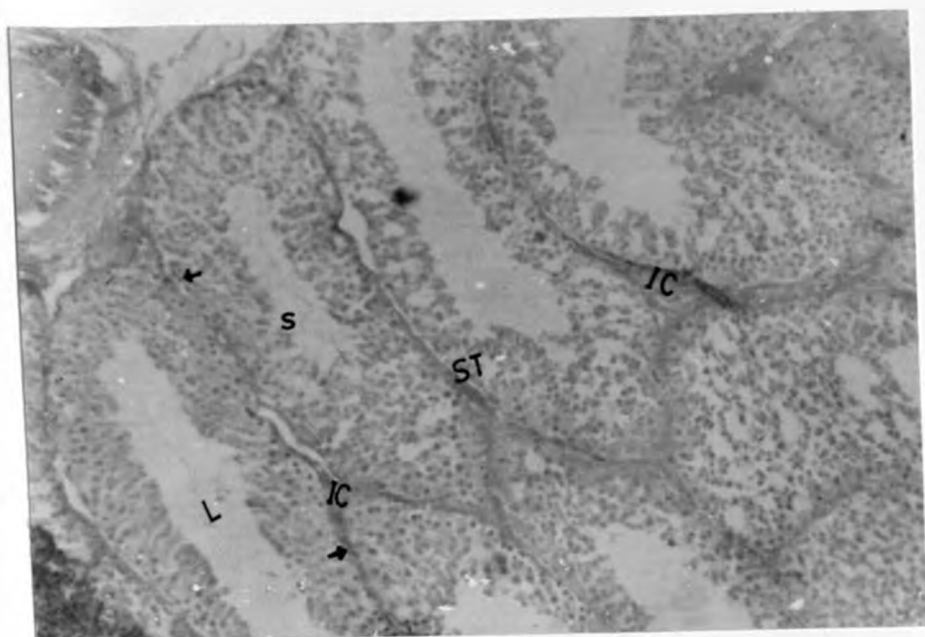


(b)



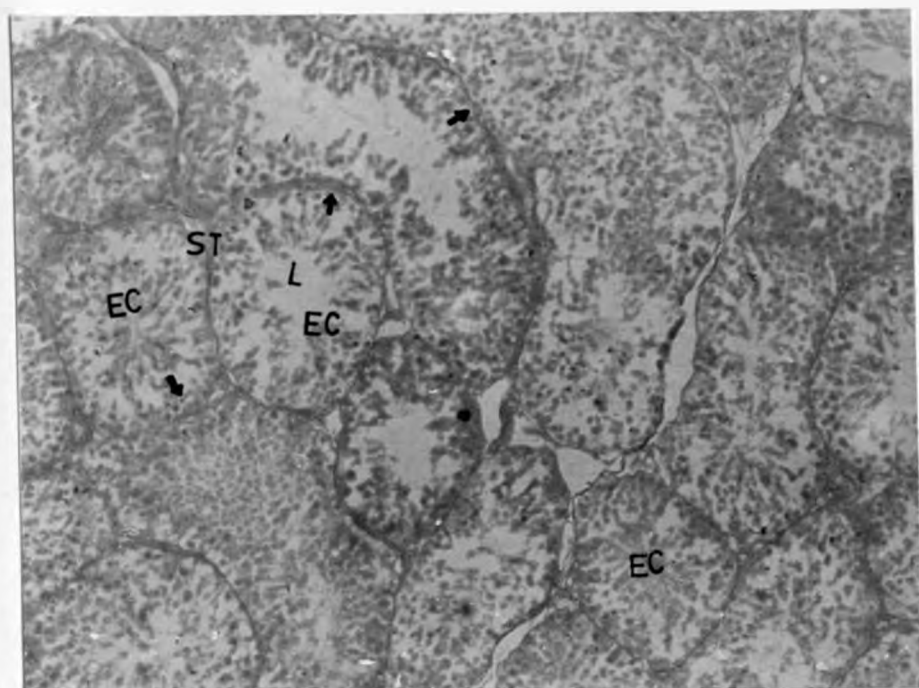
Plates 2 (a) and (b). Seminiferous tubules,ST, from a normal adult rabbit. Note the seminiferous lumen,L, containing free spermatozoa,FS, mature spermatocytes still attached to the sertori cells,S, spermatogonia and spermatocytes (arrows) and the interstitial tissue cells, IC (Leydig cells).

x100 (a) , x400 (b).



Plates 3 (a) and (b). Seminiferous tubule,ST, from
abbits treated with embelin. Note the exfoliation of
spermatogenic cells,EC,into the lumen of the
seminiferous tubule,L. The spermatogonia are still
intact.x100 (a), x400 (b).

(a)



(b)

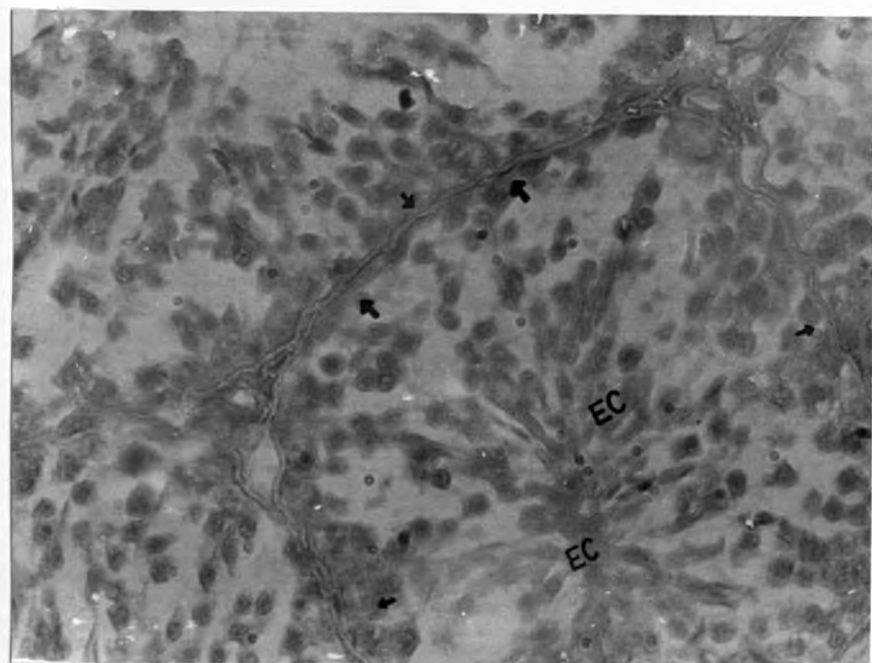
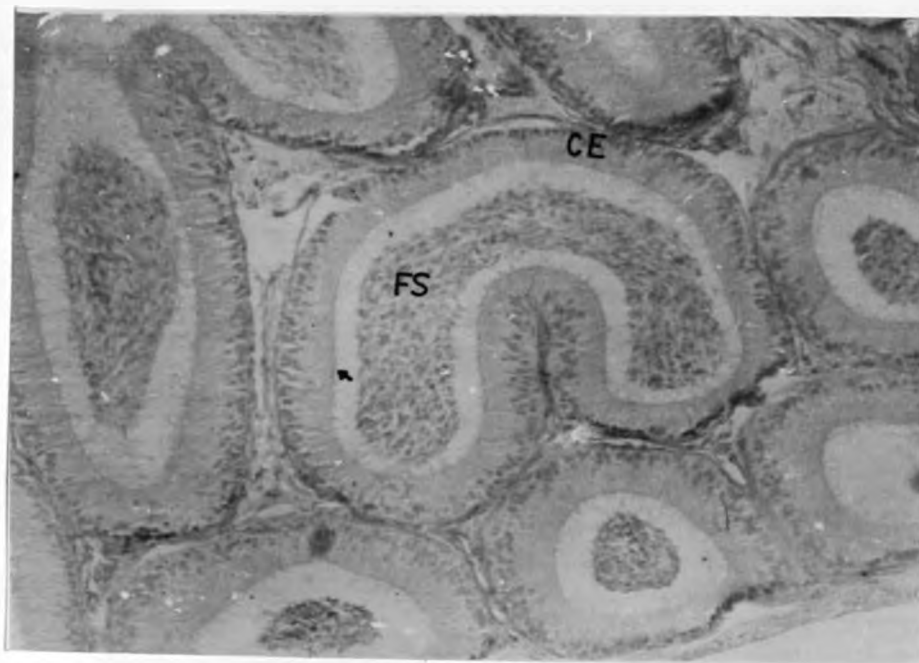


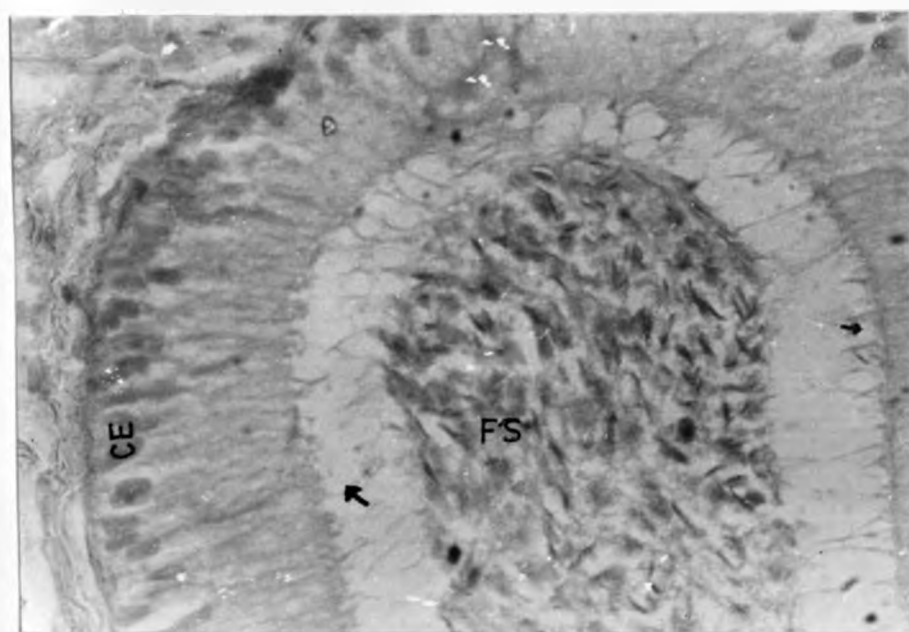
Plate 4 (a) and (b). Epididymis of normal adult rabbit. Note the epididymis lumen full of spermatozoa FS, The columnar epithelia cells,CE, with a ciliated adluminal border (arrows).

x100 (a), x400(b).

(a)

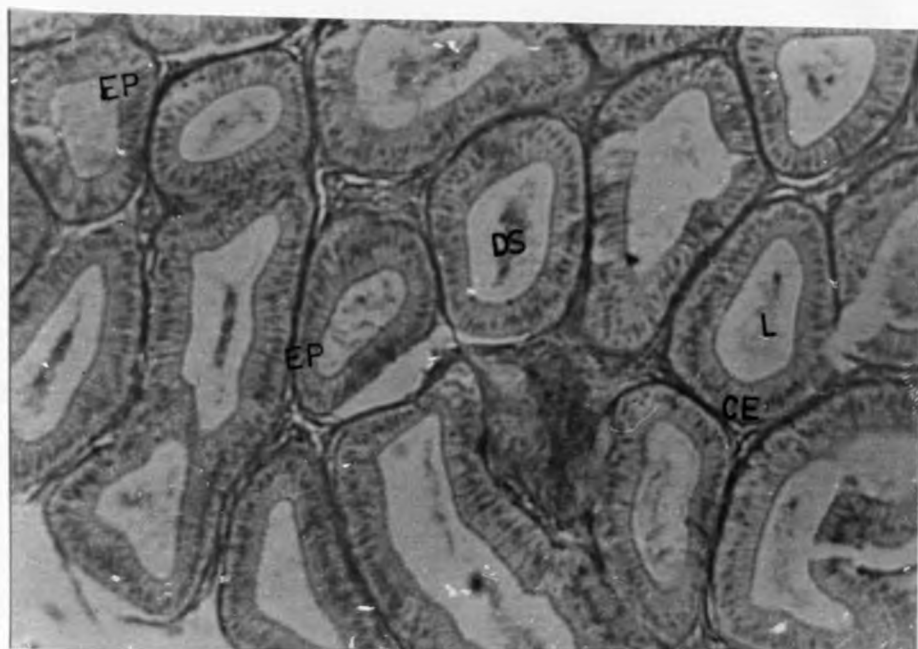


(b)



Plates 5 (a) and (b). Epididymal tissue, EP, of a rabbit treated with embelin. Note the rather empty lumen of the epididymis, L, possibly some decapitated spermatozoa, DS, the columnar epithelial cells, CE, lacking a ciliated adluminal border (arrow).
x100 (a), x400 (b).

[a)



[b)

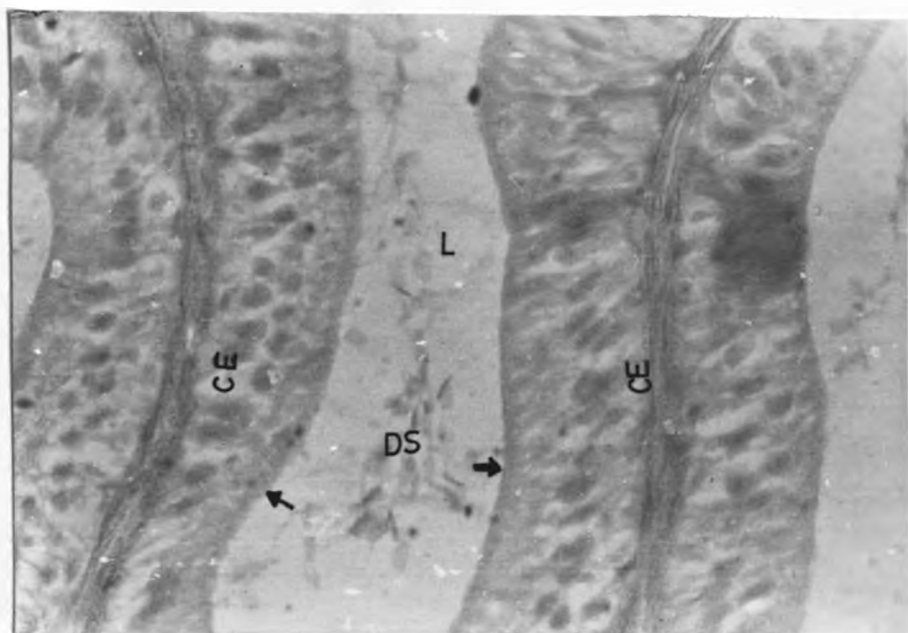
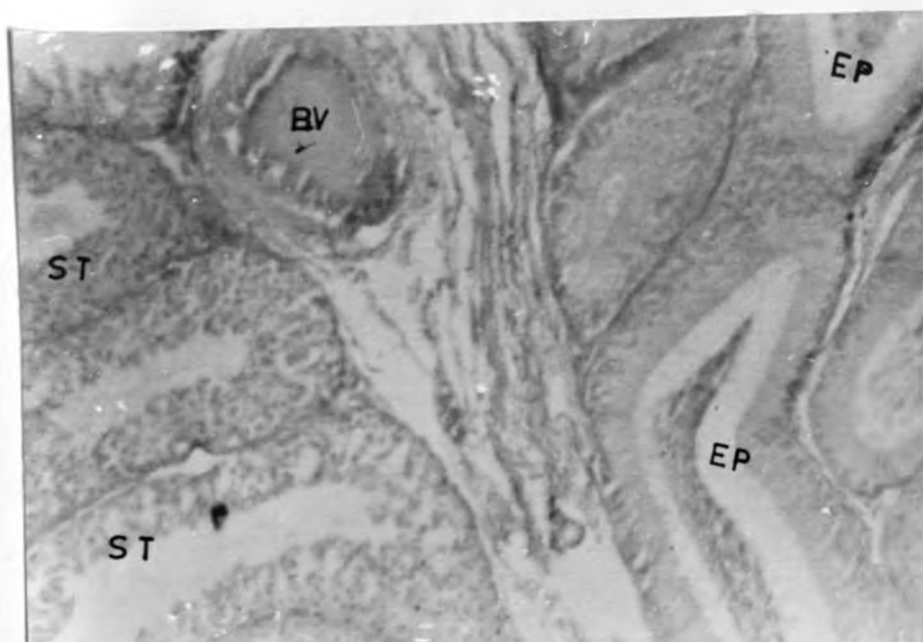
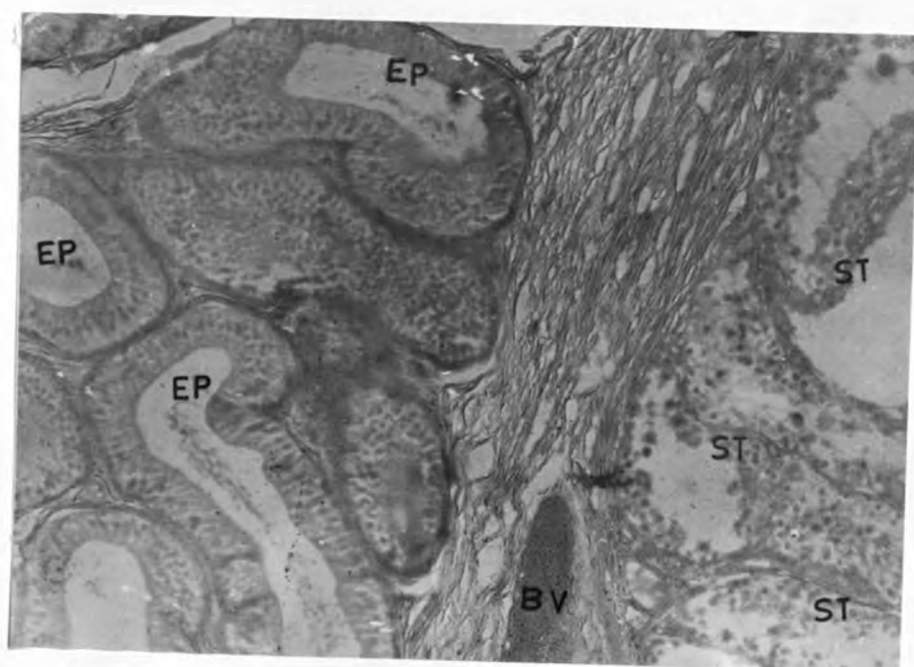


Plate 6. Seminiferous tubule ,ST, and Epididymis,EP,
of a normal adult rabbit. Note the blood vessel,BV,
among the connective tissue dividing the seminiferou-
tubules and epididymis. x100.

Plate 7. Seminiferous tubule ,ST, and epididymis ,EP,
of an adult rabbit treated with embelin. A blood
vessel,BV, lies among the connective tissue dividing
the seminiferous and the epididymal tissue. x100.



(7)



The columnar epithelia of the epididymis of embelin treated group lacked the ciliated inner border that characterise the epithelia from control rabbits. Spermatozoa in the epididymis from control rabbits looked normal, with a dense stained head and a healthy flagellum, unlike those from the treated group whose epididymis was virtually empty, rather, what appeared happened to be a mesh-like dry fibres. These were possibly dead flagella of decapitated spermatozoa, (Plate 5a and 5b).

3.5.1 Health of the Experimental Rabbits

Data obtained from the routine check of packed cell volume and weights of the experimental rabbits (Figure 3.9) showed constancy of weights and the PCV over the experimental period in the rabbits receiving embelin injections. However, the data on maesaquinone and acetylmaesaquinone injected rabbits showed a decline in both weight and PCV over the experimental period. Two of the six rabbits in this group died when there was continued injection of the latter drugs.

3.5.2 Recovery from Embelin Treatment

Data from hormonal studies showed that the levels of the sex hormones normalised after termination of the embelin injections. Further, these males were able to sire a progeny three months after termination of embelin dosage, however, the litter sizes were slightly smaller than those of control rabbits. The average litter size of the control group was 12 young ones while those sired by males that had been exposed to embelin had an average of 8 young ones. Five male and five female rabbits were used in each case.

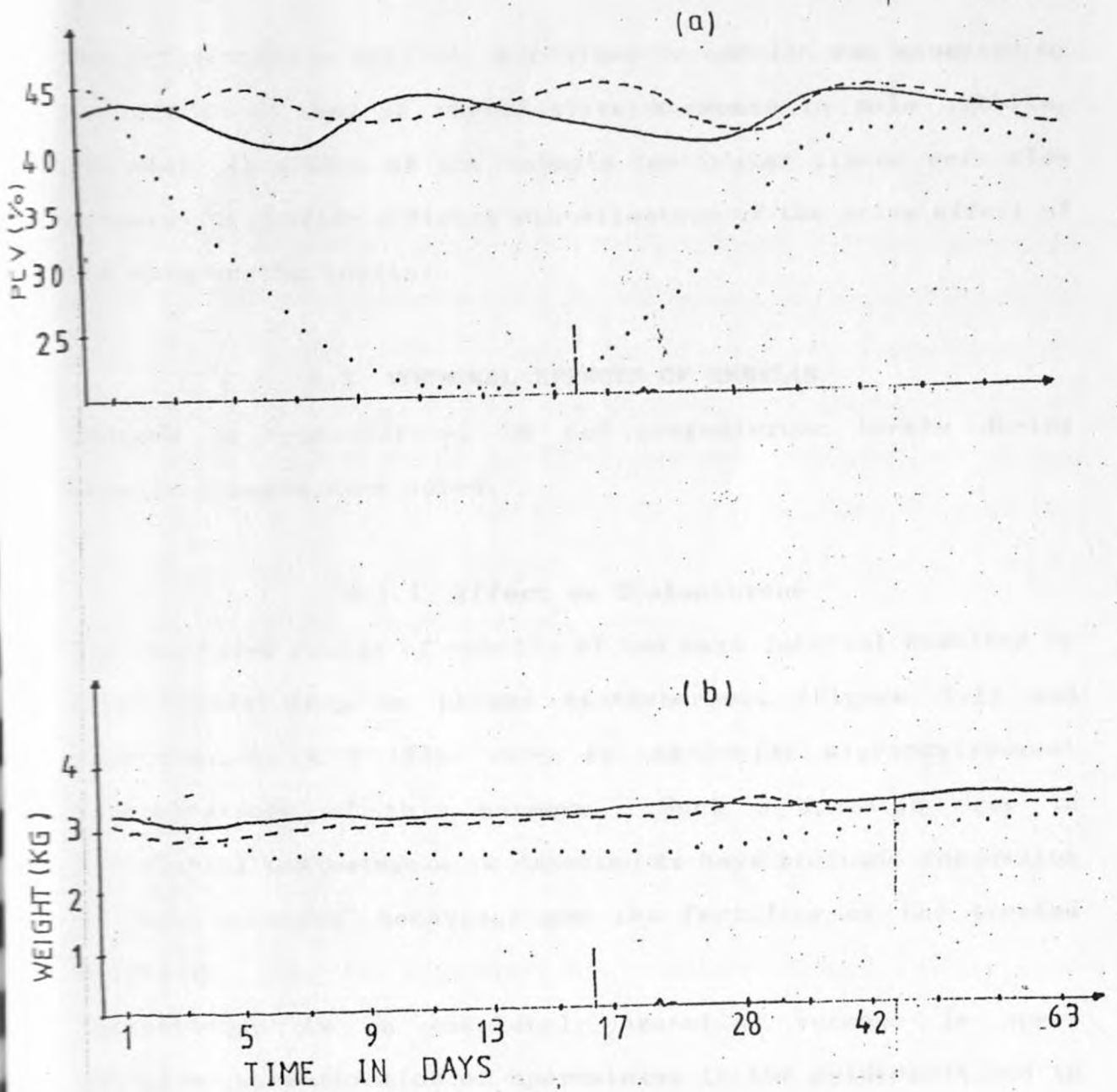
Figure 3.9

Parameters used in monitoring health of experimental rabbits.

(a) Mean variation in rabbits packed cell volume (PCV) during the experiment.

(b) Mean changes in weight of the experimental rabbits during the period.

_____ Embelin treated rabbits (n=8)
..... Maesaquinone treated rabbit (n=4)
----- Control rabbits (n=6)



4.0 DISCUSSION

The antifertility activity acclaimed to embelin was assessed by the effect it had on reproductive hormones in male rabbits. Histological slides of the rabbits testicular tissue were also prepared to provide a direct visualisation of the gross effect of the drug on the testis.

4.1 HORMONAL EFFECTS OF EMBELIN

Changes in testosterone, LH and progesterone levels during embelin dosages were noted.

4.1.1 Effect on Testosterone

The continued dosage of embelin at two days interval resulted in x 10 (90%) drop in plasma testosterone, (Figure 3.1) and approximately x 5 (75%) drop in testicular microenvironment concentrations of this hormone. Such a dramatic drop in circulating testosterone is expected to have profound repression of the "maleness" behaviour and the fertility of the treated animals.

Testosterone is an absolutely essential hormone in spermatogenesis, maturation of spermatozoa in the epididymis and in the maintenance of secondary sexual characteristics in males. Mere administration of testosterone to hypophysectomised rats suffice to maintain spermatogenesis, (Yoshiaki and Hiroshi, 1984) though the initiation of spermatogenesis in young males necessitates participation of FSH (Lostroh, 1976; Steinberger,

1976). Once triggered spermatogenesis can proceed as long as there is adequate supply of testosterone. Lack of this hormone, on the other hand, result in curtailment of germ cell differentiation, especially from meiosis onwards. It is necessary that high concentrations of testosterone (x 20 higher than in the peripheral blood) enter the sertoli cells and stimulate the germ cells (Yoshiaki and Hiroshi, 1984).

The data in the present study also showed higher titres of testosterone in testicular tissue of normal animals compared to the plasma values (240 pmols/ml and 20 pmols/ml respectively). Frankel and Eik-Nes, (1970) had observed x10 higher values of testicular concentration of testosterone compared to plasma values. A range of 8 - 28 pmols testosterone/ml of plasma is common among rodents (Buhal et al, 1982; Eik-Nes; 1975; Bocabella, 1963; Bartlett et al, 1989).

The high microenvironment levels of androgens in the testis is maintained by two types of proteins which specifically bind to adrogens, viz androgen receptor and androgen binding protein. FSH is essential in initiation of synthesis of the latter protein in pre-puberty males and in regressed hypophysectomised rats, but once initiated, the synthesis can proceed as long as there is adequate supply of testosterone; hence the expression "obligatory but transistory" in describing the significance of FSH in spermatogenesis (Fritz et al, 1974; Hiroyuki, 1984).

Hypothesis on Interrelationship of Hormones Action in the Seminiferous Tubule.

Follicle stimulating hormone is bound to specific membrane receptor on the cytoplasmic membrane of sertoli cells where it activates adenylate cyclase. The cAMP formed initiates a cascade of reactions that trigger the synthesis of ABP. The latter is then transported into intercellular spaces where it binds androgen which have diffused into the seminiferous tubule from the Leydig cells. ABP has a high affinity for androgens but the resulting complex has a rapid dissociation constant. The ABP-androgen complex comes into contact with germ cell membrane where it facilitates the transfer of androgen to a postulated cytoplasmic androgen receptor. The formed androgen-receptor complex is then transported to the germ cell nucleus where it initiates spermatogenic processes, (Steinberger et al, 1974).

The ABP-androgen complex may also be secreted into the lumen of seminiferous tubules. This complex is directly transported to the epididymis, where active Δ^4 -steroid 5 α -reductase convert testosterone to 5 α -dihydrotestosterone, (Inano, 1969).

Role of Testosterone in Sperm Maturation

Spermatozoa passing from the testis into the epididymis are incompetent to fertilise. Maturation of their motility and fertilising ability occurs during their epididymal passage. The fertilising ability broadly involves capacity of the spermatozoa for independent motility, the molecular integrity of the surface plasmalemma, structural cross-linking of protein-bound thiol groups in the head and tail organelles, the cytoplasmic droplets;

and in some species (though not in man), the formation of an acrosome. It has amply been demonstrated that testicular androgens are required in high amounts for maintenance of sperm storage function of epididymis and that the process of spermatozoa maturation is itself very androgen dependent, (Bedford, 1976). It is not clear whether androgens act directly on sperms within the epididymal lumen, but it is evident that they do act to maintain optimal activity of the epididymal epithelium. By virtue of its absorptive and secretory roles, the epididymal epithelium ensures an appropriate milieu for the specific subcellular changes occurring in the spermatozoa in the transition from infertile to a fertile state, and in cauda, for storage of mature sperm cells.

The discussion cited above emphasises the absolute requirement of androgen in spermatogenesis and in the maturation of spermatozoa in the epididymis. This implies that if the supply of androgens to the gonads is blocked, formation of mature spermatozoa will cease. The experimental data show that embelin inhibitory effect on testosterone synthesis is markedly affective (90% drop within five days of embelin dosage). This qualifies embelin as a potential male fertility regulating agent. The obvious drawback to this approach of fertility control is the predictable loss of libido and potency due to low levels of circulating testosterone.

Investigation on variations of lutenising hormone and progesterone with the continued dosage of embelin was carried out to ascertain the observed inhibition of testosterone biosynthesis.

4.1.2 Effect of Embelin on LH Levels.

Embelin dosages to the study animals caused a slow but steady rise in levels of lutenising hormone by approximately 30% (Fig. 3.3). This can be explained as compensatory response to the lowered testosterone concentrations cited above.

The homeostasis of reproductive endocrine levels in the blood is maintained by feed-forward stimulation of steroid synthesis and negative-feedback mechanisms encompassing the hypothalamo-pituitary-gonadal axis.

Low levels of circulating androgens triggers production of higher amounts of gonadotropins from the pituitary and the vice versa. Testosterone is believed to undergo reduction reactions to DHT and the corresponding androstanediols (3 α and 3 β - diols) to confer its effect in the target organs. The rate of these reactions vary considerably from tissue to tissue. It is rapidly converted to DHT by anterior pituitary and hypothalamus, though the activity of the latter's 5 α -reductase is lower than that of anterior pituitary, (Martin et al, 1974). Castration considerably activates the transformation of testosterone to DHT in both anterior pituitary and hypothalamus and this activity is depressed by provision of exogenous testosterone, (Yamamoto et al, 1970).

Testicular oestrogens may also play a role in the regulation of the hypothalamic - pituitary - gonadal axis. Once secreted into the systematic circulation oestrogens may, along with testosterone, play a role in the feedback regulation of gonadotropins secretion. There is also possible role within the seminiferous tubule in maintaining an appropriate milieu

necessary for spermatogenesis or that the released oestrogens may exert local regulatory effect on some aspect of metabolisms of the interstitial cells. There are reports siting presence of estradiol receptor protein in the cytoplasm and nuclei of Leydig cells, (Dorrington et al, 1976). This testosterone - estradiol system may constitute an intercellular messenger system operating between Leydig and sertoli cells.

Gonadotropins Releasing Hormone(s)

The generally accepted theory is that gonadotropin releasing hormone(s) are synthesised in specialised hypothalamic neurones, and subsequently transported to median eminence for storage. They are then released into pituitary portal vessels upon stimulation, and transported to anterior pituitary where they express their biological effect (Vale et al, 1973). Administration of LHRH to male animals pre-treated with oestrogen result in liberation of high amounts of LH and rather low amount of FSH, whereas pre-treatment with testosterone markedly reduce ability of anterior pituitary of the male animals to release LH but augments release of FSH. Thus, the hypothalamus manufactures only one gonadotropin releasing hormone for the control of the two pituitary gonadotropins and the activity of such principle is subject to modulation at pituitary level by the circulating sex steroids. The above information brings to notice the relevance of oestrogens and FSH in the hypothalamo-pituitary gonadal axis of males, a case that was not considered in the present study.

Quantitative Parametric Responses of Gonadotropins to Changes in Blood Steroids Concentrations

Important parameters in the study of quantitative characteristics of hormonal feedback mechanism are the steady-state hormone concentrations and temporal relationship between concentration changes. Experiments done using rats, (Julian et al, 1976) showed that testosterone levels of 2 ng/ml suffice to maintain LH at normal levels. It is only when testosterone was varied below 0.5 ng/ml did feed-back response occur. This is a kind of threshold type of responses. Furthermore, decrease in testosterone below the threshold level seemed to have no further influence on the pattern of LH release. When normal or higher levels of testosterone are reinstated in castrated rats, latency for LH suppression is observed to be 6 hrs. Similar latency period is noted in rise of LH with decrease in testosterone levels.

The range of LH response to variations in testosterone levels in circulation is considerably narrow compared to the testosterone range. The characteristic pulsatile secretion of both LH and testosterone, collectively introduce enough "noise" into the system to allow accurate correlation (Julian et al, 1976). There is also the confounding factor of stress in the initial stages of experiments, that may elevate LH levels.

The present study findings are in agreement with the studies by Julian (1976). It was observed that changes in LH levels are relatively small compared to the corresponding variations in testosterone in systematic circulation (Fig. 3.1 and 3.3). The LH values of 15 - 20 uI.U./ml of plasma are within the normal range observed in rodents (Bartlett et al, 1989; Orgebin Crist et

al, 1975).

As a remark the fore cited literature, one notes that both the androgens and oestrogens interplay in the negative feedback regulation of gonadotropins. It is, therefore, important to note the levels of oestrogen vs testosterone and their effect on the gonadotropins, LH and FSH in the experimental animals. This would be an appreciation of quantitative balances of these steroids and the corresponding gonadotropins in the regulation of gonadal functions. In any case, the experimental results agrees with the theoretically expected rise in LH levels as concentration of systemic testosterone drops. This is, therefore, supportive to the observed data of low testosterone levels, implicating embelin to be inhibitory to testosterone biosynthesis. The rise of LH levels with embelin dosage also implies that the drug does not affect synthesis or secretion of LH by the anterior pituitary gland.

4.2 IN-VITRO STUDIES USING CULTURED LEYDIG CELLS

Embelin showed a dose-dependent inhibition of testosterone synthesis in the LH-stiluated mouse Leydig cells. These results ascertain that the effect of embelin inhibitory action is at the Leydig cells, the site of testosterone biosynthesis. Other related benzoquinones,maesaquinone and acetyl-maesaquinon showed similar inhibitory effects. Maesaquinone and its acetyl derivative had greater potency in this regard but in vivo studies showed that they had adverse effect on the experimental rabbits. The point of injection became inflamed, the animal significantly lost weight and the blood PCV drassticaly dropped resulting in death of some of the treated rabbits. These observations could be

result of access of disease germs into the animal's body through the inflamed point or stress due to trauma, otherwise, the direct effect of the drug. This diagnosis is, however, not conclusive on the direct cause of the deaths.

Since the preceding analysis indicates two possibilities of the site of inhibition of testosterone biosynthesis, that is, stimulatory mechanism at the Leydig cells receptors or the result of direct blockage of the testosterone biosynthetic pathway, the effect of embelin on the latter case was investigated.

4.3 INVESTIGATION OF TESTOSTERONE BIOSYNTHESIS PATHWAY

These studies showed that plasma levels of progesterone (an intermediate metabolite in testosterone biosynthesis) increased by $\times 7$ with the continued dosage of embelin. This is a sign that the inhibitory effect of embelin on testosterone synthesis is actually at the biosynthetic pathway and not at the Leydig cells stimulatory stage. It further indicates that the biosynthetic step susceptible to the inhibition is beyond the progesterone metabolite and suggest that the biosynthetic enzymes after progesterone have a good degree of reversibility.

From Fig. 3.4 one notes that the overall amount of steroids levels (testosterone and progesterone) measured was low during greatest inhibition than before embelin administration. In this view one may suggest that it is not progesterone alone that accumulates during the biosynthetic pathway inhibition, rather levels of metabolites up-pathway the site of inhibition also rises. When these metabolites levels rise, there is a high

possibility of stimulation of side pathways resulting in formation of different products. This is demonstrated in Fig.4.1.

4.4 HISTOLOGICAL STUDIES

To provide a direct visualisation of embelin effect on testicular tissue, histological preparations of the tissue were studied. The findings indicate that embelin treatment caused massive degeneration and disorganisation leading to sloughing off tendencies of the spermatogenic cells, (see plates 3a and 3b). This observation is consistent with the observation that spermatogenesis requires quite high levels of testosterone in meiosis and in maturation of spermatids to spermatozoa (Yoshiaki and Hiroshi, 1984).

The syncytial nature of mammalian germ cells undergoing spermatogenesis, (Fawcett, 1975) guarantees the synchrony of the dividing cells resulting in chains of interconnected cells approximately 100 in number (Moens and Hugenholz, 1975). But when such a mass of cells is adversely interfered with, the resultant effect is masses of cells undergoing regression and death leading to their sloughing off into the lumen of seminiferous tubule (compare plates 2 and 3).

Epididymal maturation of spermatozoa also appeared grossly interrupted by the administration of embelin to the experimental rabbits (Plate 4 and 5). This gives an evidence that androgens regulate epididymal morphology and biochemistry, sperm motility and fertilising ability, (Orgebin-Crist et al, 1975). Spermatozoa in the epididymis die rapidly after hypophysectomy. Experiment by Orgebin-Crist (1973) using rabbits showed that

castration diminished fertilising ability of spermatozoa in the distal corpus epididymis within three days and became devoid of spermatozoa five days later. This is in agreement with the observations from the present study that epididymis of the rabbits that had received embelin treatment was devoid of viable spermatozoa and in some cases was virtually empty (Plate 5).

The development and function of the epididymal tissue is also androgens dependent. In mammals, epididymis is formed by the transformation of wolfian duct, which becomes the epididymal duct and mesonephric tubules, which become the ductus efferentes connecting the epididymis to the rete testis. These developments do not occur in foetuses devoid of gonads (by surgery or otherwise). This androgen dependency of the male tract continues during post-natal development (Orgebin-Crist and Davies, 1974). In hypophysectomised rabbits, the epididymal sterio-cilia border and the endoplasmic reticulum were reduced in amounts in three days following hypophysectomy. By the eleventh day, the golgi apparatus was reduced in size and there was marked increase in the number of lysosomes and autophagic vacuoles, (Orgebin-Crist and Davies, 1974). In the present study it was observed that the epididymis of the rabbits treated with embelin lacked the ciliated inner border that characterised the epithelia from the control rabbits.

The overall physiochemical effect of spermatozoal passage through the epididymis is a progressive dehydration, rise in specific gravity and marked alteration in the state of sulphhydryl groups, lipids, proteins, lipoproteins and deoxyribonucleoproteins. The content of DNA as such, does not change significantly, (Mann, 1974).

Metabolism is correlated to motility while changes in acrosomal and nuclear constituents are related to fertilising ability. As spermatozoa age, the integrity of the acrosomal (lysosomal) and plasma membrane is lost leading to increased cellular permeability and loss of vital intracellular components. Formation of organic peroxides is probably another factor which adversely affect the life span of spermatozoa, (Mann, 1974).

Theory of Androgen role in Epididymis

It is clear that sperm maturation and survival depend on high concentration of androgens, but how this comes about is conjectural. The widely accepted theory states that testosterone acts indirectly via epididymal tissue, which in turn, provide factors necessary for sperm maturation to occur.

4.5 RECOVERY STUDIES

Findings of the present investigation showed that reproductive hormone levels normalised a week after termination of embelin dosage. The testicular histology of embelin treated rabbits gave hopeful results since the stem cells at the basal lamina of the seminiferous tubules were not affected by the treatment. The findings that the treated rabbits could sire a progeny after a three month recovery period is an indication that there was ultimate recovery from the embelin treatment. The difference observed between the hormone normalisation period and the formation of fertile spermatozoa can be explained by the time required to re-establish spermatogenesis in the severely disorganised seminiferous tubule cells.

4.6.1 RELATION OF THE PRESENT FINDINGS TO THE PREVIOUS REPORTS PERTINENT TO EMBELIN ANTIFERTILITY EFFECT

The findings obtained from the present studies are supportive to the claimed antifertility effect of embelin. Reports by Seth et al, (1983); Jayaraman et. al., (1971); Gupta et al (1989) indicated a lowering in sperm volume and motility. This is in agreement with the observed empty epididymis, decapitated spermatozoa and atrophied seminiferous tubules in the present study. Gupta et al, (1989) also observed head-tail separation in the epididymal spermatozoal and rapture of outer membraneous droplets. He postulated that the lethal effects observed were the result of arrested glycolytic flux and other energy metabolisms, resulting in sperm death. The present study implicates lack of sufficient androgen levels to be the ultimate cause of the observed lethal effects. Toxicity and recovery studies were in agreement with observations made by Dixit and Bhargava, (1983) and Gupta et al (1989). There was no toxicity reported and the experimental animals actually recovered from the antifertility effect of embelin. In the present study the treated rabbits remained in good health, as determined by their body weights and PCV over the experimental period. The initial decline in body weight was most likely due to stress since the animals regained the lost weight as the experiment proceeded. The slight drop in PCV could be explained on grounds of continued drawing out of blood. This was obviously stressful to haemopoietic tissue and other systems that make the red blood cells. Direct exposure of the Leydig cells to embelin containing

medium and the subsequent transfer of cells to embelin free medium is an evidence that the drug does not significantly affect the physiological activities of the cell since the treatment did not reduce the cells' testosterone biosynthetic ability.

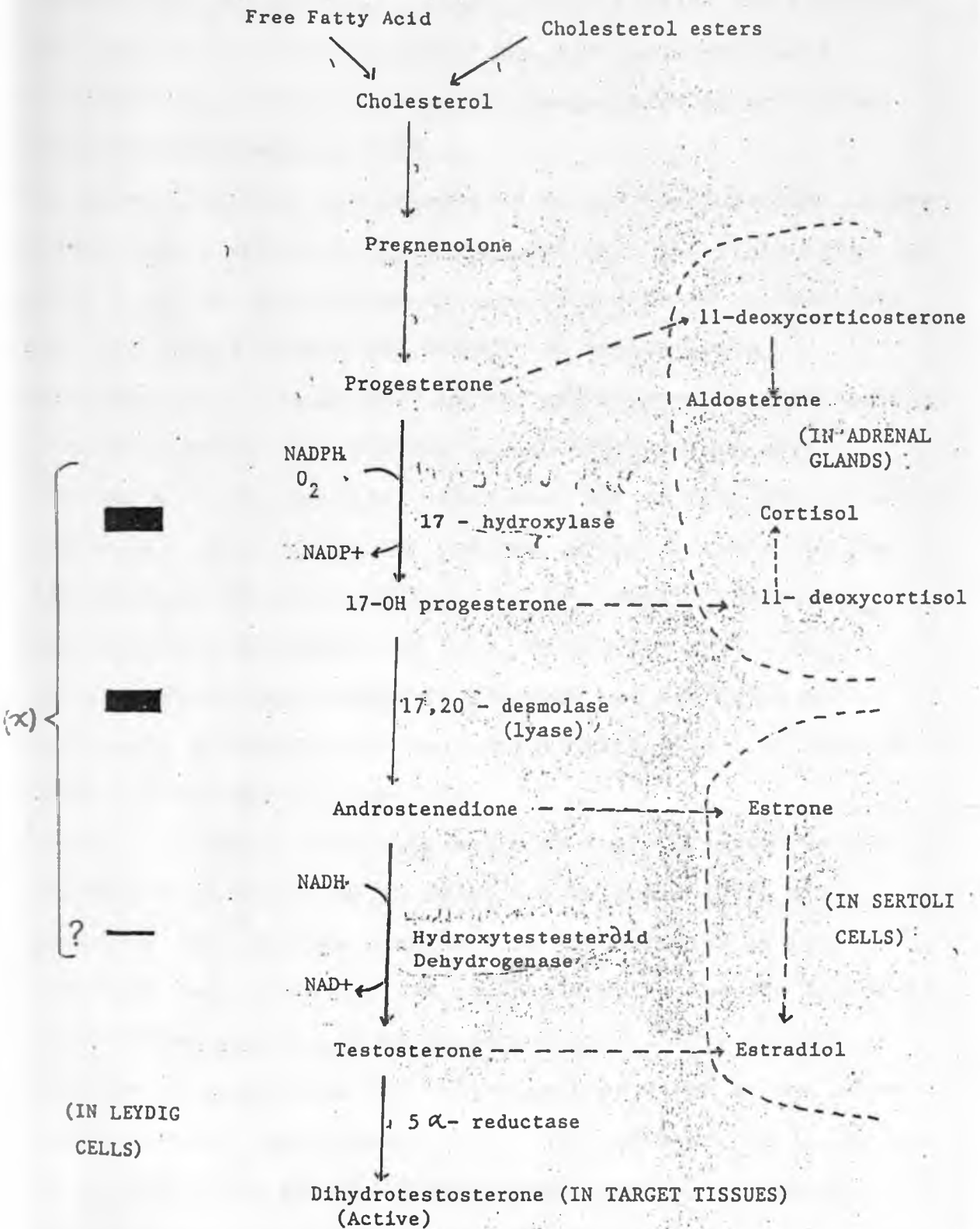
4.6.2 Details on the Site of Embelin Antifertility Action

The present findings rule out any interference of embelin with the LH production or its Leydig cells stimulatory activity. The observation that progesterone level rises during embelin dosage is an evidence that the drug has an inhibitory activity in testosterone biosynthetic pathway and that the enzymatic step(s) affected by this inhibition is/are down-path-way after progesterone (metabolite). The candidates for this inhibition are 17-hydroxylase, 17,20-desmolase or hydroxytestosterone dehydrogenase, the enzymes responsible for the formation of 17-hydroxyprogesterone, androstene-dione and testosterone respectively.

The fact that embelin interferes with the steroids biosynthetic pathway can also provide an explanation for its female antifertility effect. Prakash and Varshney, (1980) experiments on corpora lutea of suckling guinea pigs showed a dose dependent increase in ovarian weight with embelin treatment. This can be explained by the expected rise in peripheral blood levels of progesterone during the embelin dose. The high progesterone levels would cause the growth stimulating effect. Prakash et al, (1986) showed that embelin caused decline in estradiol stimulated uterine growth, but did not evoke any significant change in the uterine biochemistry of progesterone primed rats.

Figure 4.1

Possible site of embelin inhibitory effect.



(x): represents the possible enzymes susceptible to embelin inhibitory effect.

It also caused decline in enzymatic activity in estradiol dipropionate primed rats. Some earlier studies had indicated that embelin is anti-estrogenic but have progestational activities. It did not show anti-progestational activities (Prakash and Varshney, 1980).

The above citations are supportive to our findings that progesterone levels rise during embelin dosage. The claims that embelin could be anti-estrogenic provides a basis to postulate that the drug's inhibitory effect on testosterone biosynthesis, is up-pathway before androstenedione (metabolite) formation, since this metabolite and testosterone are considered to be the major substrates for estrone and estradiol synthesis. This leaves the enzymes, 17-hydroxylase and the desmolase as the most likely sites for embelin inhibitory effects. Such an inhibition then, denies the testis and epididymis, the very necessary steroids viz androgens and estradiol; no wonder the rapid devastating effect of embelin dosage to the males' fertility.

These findings justify some optimism that safe, reversible inhibition of fertility in males can be achieved by this approach. The obvious draw-back in the approach is the resultant loss of libido and potency due to the low levels of testosterone that would be in circulation. This can be overcome by exploiting the environment provided by the blood testis barrier (see Fawcett, et al 1970; Neaves, 1973; Gilula, et al 1976). The aim would be to ensure normal levels of circulating testosterone whereas the testicular microenvironment levels of the hormone are maintained very

low. In this view, testosterone molecules joined to conjugate macro-molecules that are not immunogenic, but big enough to deter entry of the exogenous testosterone past the blood testis barrier into the adluminal face of the seminiferous tubules, would maintain normal circulating levels of testosterone. Meanwhile, embelin that is administered together with the testosterone-conjugate complex, would maintain very low levels of endogenous testosterone, particularly in the testicular environment. However, if the testosterone-conjugate complex is also impassable through the blood-brain barrier, there will be enhanced gonadotropins response to the new environment. The resultant accumulation of metabolites due to testosterone synthesis inhibition, particularly in cases of high levels of circulating gonadotropins, are important factors to consider. This would have effects in all steroid synthesising organs. The alternative that avoids the above complication would be the administration of embelin plus physiological levels of unconjugated testosterone since fertility necessitates relatively higher amounts of the hormone in the spermatogenic tissues. The administered embelin would ensure low levels of the hormone in the said tissues, while the exogenous testosterone maintains the normal systemic values.

4.7 CONCLUSION

The present study provide information that embelin causes lowering of testosterone synthesis to very low levels. The drug does not interfere with LH function, rather, it has direct inhibitory activity on testosterone biosynthetic pathway. Low concentration of testosterone in the body result

in curtailment of spermatogenesis hence the male antifertility claims attributed to embelin.

The findings that embelin affects reproductive hormone milieu could be useful in reproductive physiology research, particularly with regards to interventions on hypothalamo-pituitary-gonadal axis. This has direct implication on fertility regulation in human.

Consideration of clinical significance of the blocked steroids biosynthesis during embelin dosage is also important. The rise of metabolite levels in the body system and the deficient products of the blocked pathway have specific clinical implications.

There is also need for public awareness of benzoquinone producing plants and their effect on human and livestock fertility.

Some physiological defects due to prostate and adrenal glands tumours can be counteracted by use of such a drug that lowers androgen levels.

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