A Thesis submitted by

Keith Arthur Ahlquist

for the Degree of Master of Science

of the University of St. Andrews.

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CERTIFICATE

I hereby certify that Keith A. Ahlquist has spent a total time of four-and-a-half terms on research work under the direct supervision of Dr. H. Jackson, Ph.D., M.B., Ch.B., D.Sc., that he has fulfilled the conditions of Ordinance No. 16 (St.Andrews), and that he is qualified to submit the accompanying thesis in application for the degree of Master of Science.

DECLARATION.

I hereby declare that the following thesis is based on results of experiments carried out by me, that the thesis is my own composition, and that it has not previously been presented for a higher degree.

The research was carried out in the Department of Pathology and the Experimental Chemotherapy Laboratory, Paterson Laboratories, of the Christie Hospital and Holt Radium Institute, under the direct supervision of Dr. H. Jackson, Ph.D., M.B., Ch.B., D.Sc.

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Biochemist , Senior Grade , N.H.S.
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Publication : "Studies in the Pathogenesis of Silicosis by the Membrane Chamber Technique".
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SUMMARY

The distribution and activity of certain enzymes within rat testis has been studied in the normal animal and after the administration of 1) busulphan, and 2) 7,12-dimethylbenz(a)anthracene (DMBA). The arrest of spermatogenesis so effected has been found to be associated with increases in the activity of β-glucuronidase, 5'-nucleotidase, malate dehydrogenase, and glucose-6-phosphate dehydrogenase; and with decreases in the activity of acid phosphatase, alkaline phosphatase, glutamine synthetase, and hyaluronidase.

The activity of malate dehydrogenase has been shown to be primarily confined to the interstitial tissue in the normal animal: a marked change in distribution of this and other dehydrogenase enzymes was found to accompany atrophy of the testis. Experimental evidence suggests that the Sertoli cells exhibit a high activity of β-glucuronidase and of 5'-nucleotidase.

The work of earlier authors in relation to DMBA has been confirmed and extended. It has been shown by fertility trial that in addition to a primary toxic effect upon spermatogonia and early spermatocytes, the administration of DMBA produces a functional impairment of spermatid cells with a subsequent prompt reduction of fertility.

Extension of the established methods for the study of anti-fertility compounds by the development of accompanying enzymological investigations is proposed.

INTRODUCTION

INTRODUCTION

The testis of rat is a valuable experimental tissue since the spermatogenic cells within the seminiferous tubules offer a model of cellular proliferation and differentiation subject to well-defined cyclic development. A number of causes may induce the arrest of normal spermatogenesis. Our understanding of the changes in metabolism and morphological pattern of such effects is far from complete. Aside from the intrinsic scientific interest of the topic, its practical bearing in the context of sub-fertility in domestic animals and its relevance to problems of human population control are of considerable importance. Furthermore, it has been suggested that carcinogenesis and anti-fertility represent metabolic aberrations with certain features in common, and that "selective antifertility effects ... must include some of the pharmacological actions representative of the pre-malignant changes operative in chemical carcinogenesis"[88].

In a recent text by an authority in the field of male reproductive biochemistry[106], it is stated: "little is known about the chemical nature of the developmental factors, possibly hormone-like in nature, which control the successive stages of cellular transformations during spermatogenesis (p. 10); ... so far the biochemical changes accompanying spermatocytogenesis and spermiogenesis have been mainly studied by histochemical methods (p. 10); .... As yet

... these phenomena are poorly understood ... the biochemical mechanisms of antispermatogenic activity have not been studied in detail" (p. 11).

In pointing out that most antifertility substances have been brought to light by serendipity, or otherwise prospectively by the use of time-consuming and tedious fertility or sperm sampling techniques, Jackson[89] observes that "there is a great need for experimental techniques which would enable a more rapid screening of compounds for potential activity".

Particular interest was stimulated by the report from Ford and Huggins of a selective toxic effect upon testis of the potent carcinogen 7,12-dimethylbenz(a)anthracene (DMBA), this being associated, pari passu, with a change in the activity, as measured <u>in vitro</u>, of the enzyme malate dehydrogenase[55]. The present work is concerned with the verification and extension of these findings.

In addition to DMBA, the therapeutic alkylating drug busulphan [Myleran; 1:4-di(methanesulphonyloxy)butane] has been used. It has been shown by Bollag[16] and further investigated by Jackson et al.[85] that busulphan inhibits spermatogenesis. It was thought to be of interest to correlate the response of testis to these two contrasted compounds, particularly in relation to any changes in the activity of malate dehydrogenase and of other selected enzymes.

The experimental approach has been basically exploratory, aspiring to yield a base for more detailed investigation. A review of the known literature relating to the field investigated is presented as no review on this topic appears to be published. This review is not in general comprehensive, but an attempt has been made to include the main body of work on the enzymes under particular discussion.

It was hoped that the investigations might suggest a means of rapid screening of potential antifertility drugs and add to basic knowledge of cell proliferating and differentiating systems.

THE NORMAL RAT TESTIS - ITS STRUCTURE AND FUNCTION.

THE NORMAL RAT TESTIS - ITS STRUCTURE AND FUNCTION

The rat testis is an organ generally of weight about 1.5 g. It consists of an outer coat, the tunica albuginea, bounding an intricate arrangement of convoluted tubules - the seminiferous tubules - some 30 cm. in length and 200μ in diameter. The walls of the tubules are lined by a proliferating cell system - the seminiferous (spermatogenic) epithelium - in which generation of the male fertilising cell, the spermatozoon (pl. spermatozoa) is effected. The space between the tubules is occupied by the interstitial tissue, which is composed of blood vessels, Leydig cells, and supporting connective tissue. The Leydig cells subserve an endocrine function: they are the source of the male sex hormone, testosterone. Mature spermatozoa (sperm) are released from the seminiferous epithelium into the lumen of the tubules and rapidly flushed by a liquid flow into the collection system of the rete testis, and thence pass into the epididymis, a highly extended tubule reservoir in which the spermatozoa are subjected to a 'ripening' process and into which a fluid secretion is made. Spermatozoa are available in this repository for ejaculation.

At intervals of twelve days primordial germ cells (stem cells) at the periphery of the tubules commence a process of division and differentiation, finally producing mature spermatozoa. A sequence of morphologically distinct spermatogenic cell types is involved:

Type A spermatogonia; intermediate type spermatogonia; Type B spermatogonia; primary spermatocytes; secondary spermatocytes; spermatids; and finally spermatozoa. This is a process of mitotic division, except in the unique case of the primary spermatocytes, which undergo meiosis: the derived secondary spermatocytes contain segregated sexual chromatin and half the original number of chromosomes.

The duration of this process has been shown to be forty-eight days in the rat[96]. Spermatozoa are next retained in the epididymis for approximately a further two weeks. The time sequence of this process is outlined in Table 1, p.8 , modified from Jackson[88,89]. At each point along a tubule where histological examination is made after cross-section, a particular association of cell types is exhibited. The course of development has on this basis been divided for descriptive purposes into fourteen distinct stages[33]. Some photographic illustration of the various cell types within the normal spermatogenic epithelium is given in Figure 1, p. 8.

At the periphery of the tubules, adjacent to the basement membrane, lie the Sertoli (or sustentacular) cells, the function of which is not fully understood. A good deal of evidence suggests that they may serve to nourish the developing spermatids. Studies employing electron microscopy appear to show that the Sertoli cell acts as a "bridge cell" between the basal membrane and the

spermatogenic cells. Cytoplasmic processes of the Sertoli cells appear to fill all spaces between the latter. Chemical transfers between the intertubular vessels and the spermatogonia may be effected directly through the tubular wall. Transfers to the other spermatogenic cells must necessarily be via the Sertoli cytoplasm[159]. High activity of many enzymes has been observed in the Sertoli cells[152,159], and both enzymatic and morphological changes of the cells shown to accompany differentiation of the spermatids[152].

The testis and male accessory sex organs are subject to a trophic regulation effected from the pituitary gland. The details of this are not fully understood, but it is believed that both follicle-stimulating hormone (FSH) and interstitial cell-stimulating hormone (ICSH) - the "gonadotrophic hormones" - are required for full development of the tissues, acting synergistically[89]. Furthermore, the anterior pituitary has an indirect influence on the male sex organs by reason of its interaction with the thyroid and adrenal glands[106,p.308]. The classical criterion of androgen output from the Leydig cells has been change in the weight of the ventral prostate, or, better, the measurement of fructose and citric acid in the seminal plasma, a related parameter. Such indirect methods are likely to present a rather insensitive indication of androgen output.

The Leydig cells and the spermatogenic epithelium are morphologically distinct, yet functionally are intimately related.

Certain studies have indicated that the spermatogenic and endocrine functions of testis have a common physiological link[106, p.15]. Normal spermatogenesis demands an adequate balance between gonadotrophic hormones and testosterone. Much evidence supports the concept of a testicular-pituitary feed-back mechanism, but the details of this remain obscure. Recent studies have suggested that this may operate via a substance named "inhibin" which is secreted within the seminiferous tubules at a late stage of spermatogenesis, and which normally suppresses gonadotrophic output. Thus, if the spermatogenic cells are deficient, output of gonadotrophic hormone is increased, this accounting for the hypertrophy of Leydig cells often observed in such conditions[90]. However, it is elsewhere noted that Leydig cells may appear hypertrophied although deficient in androgen, thereby, in accord with more established views, eliciting an increase of gonadotrophic hormone output[142].

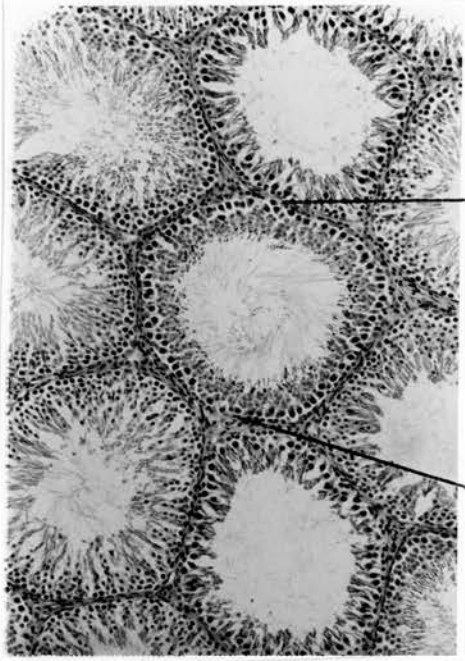
The recent development of methods for the direct measurement of testosterone and its precursor dehydroepiandrosterone (DHA) in plasma do, however, give further evidence of a testicular-pituitary axis[92], and such direct methods of study will no doubt clarify many details of testis metabolism.

TABLE 1

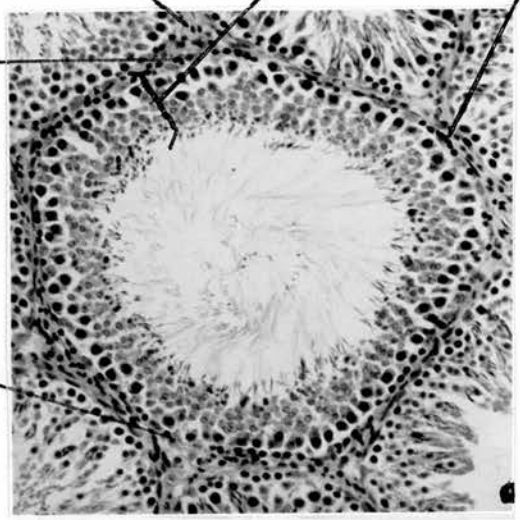
THE DURATION OF SPERMATOGENESIS IN THE RAT

Phase	Days to emission as mature spermatozoa
Stem cells Type A spermatogonia	63+
Type A spermatogonia Intermediate spermatogonia	57 - 63
Type B spermatogonia Resting spermatocytes	50 - 56
Spermatocytes (dividing)	36 - 49
Spermatids	15 - 35
Spermatozoa in epididymis	0 - 14

Fig. 1 : Normal rat testis - cross section.



(Haematoxylin x98)



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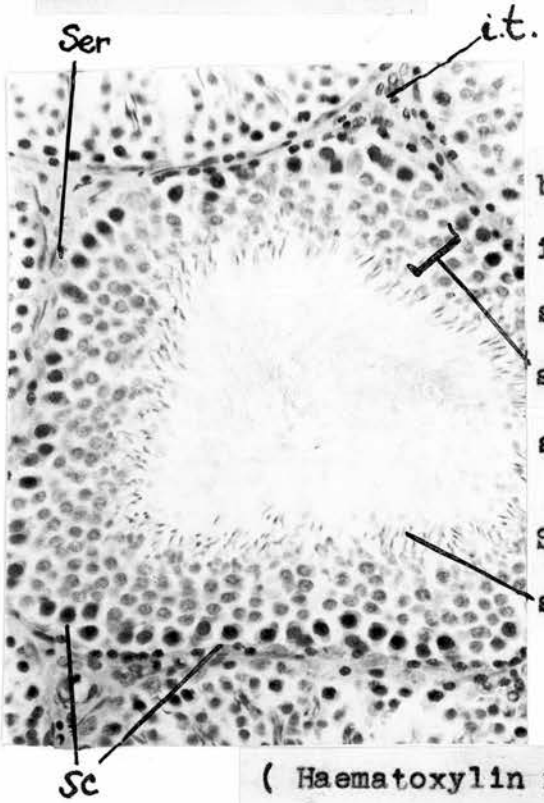

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b.m.	(tubule) boundary membrane
i.t.	interstitial tissue
sc	spermatocytes
sd	spermatids
s.e.	seminiferous epithelium spermatogenic
Ser	Sertoli cell
sz	spermatozoa

REVIEW

THE ENZYME MORPHOLOGY OF RAT TESTIS

REVIEW

INTRODUCTION

The disposition of certain enzymes within the rat testis, as delineated by the techniques of enzyme histochemistry and by homogenisation methods, will be discussed. Attention is restricted to a few only of more than seven hundred enzymes currently recorded[46]. Of these, perhaps three hundred may be considered to be of importance in mammalian physiology[129].

Normal tissue is considered in Section A. Section B is concerned with tissue modified experimentally by the following means: artificial cryptorchidism; X-irradiation; hypophysectomy/hormone administration; selectively toxic chemical agents.

An indication of the general nature of the enzyme discussed precedes each relevant part of Section A. The distribution in testis is next recorded, then an outline is given of any known or suggested specific testicular function. Our conclusions are aggregated in Table 3A, page 24. Trivial names have been used. The classification number and the systematic name suggested by the Commission on Enzymes of the International Union of Biochemistry have also been given (denoted by the letters E.C. in the text)[34].

Where species other than rat have been referred to, this will be made plain. The relevance of such findings to the rat can only be postulated with caution, since species differences are often marked.

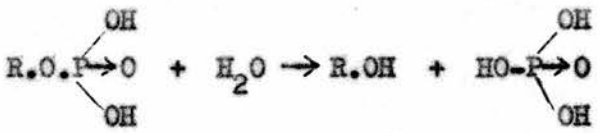
SECTION A : NORMAL TISSUE

i. Acid phosphatase:

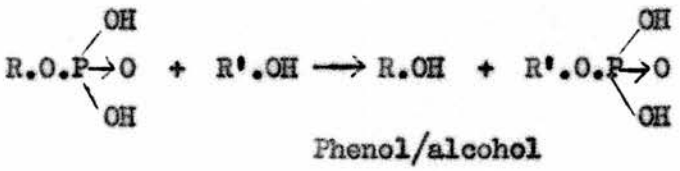
The term "acid phosphatase" refers to non-specific phosphoric monoester hydrolase exhibiting an optimum activity at a pH of about 5.0.

E.C. 3.1.3.2 : Orthophosphoric monoester phosphohydrolase. The enzyme may act as a hydrolase or as a phosphotransferase. Where R/R' represents an aromatic or aliphatic moiety:

(a) As hydrolase -



(b) As phosphotransferase -



It is richly concentrated in the prostate gland. Both acid and alkaline phosphatases are in general of very wide distribution and are often present in high activity, yet their precise function *in vivo* is unknown.

In an early application of the Gomori technique[62] the presence of acid phosphatase was reported in both Sertoli and spermatogenic cells (MOUSE), preponderating in the less mature forms of the latter and generally being absent from spermatozoa, with particular exceptions. The staining was of both nuclei and cytoplasm[165]. Certain defects in the Gomori method later became apparent, but it was found that the method was suitable for testis at the pH employed, 5.0[121].

A phosphatase active at pH 4.9 has been found in extracts of RABBIT testis[119]. Recent workers have found acid phosphatase in the nucleus of younger cell types of the seminiferous epithelium, with negative cytoplasm[154]. Interstitial cells showed a very weak reaction. The enzyme has also been found, by electron microscopy, associated with Sertoli cells, and with all spermatogenic cells[152].

Acid phosphatase is considered histochemically generally useful as an indicator of mitochondrial activity, or, with diffuse reaction, of mitochondrial damage[127].

ii. Alkaline phosphatase:

This is an enzyme of low specificity functioning in a similar manner to acid phosphatase but with an optimum pH of 8-10.

E.C. 3.1.3.1 : Orthophosphoric monoester hydrolase.

Alkaline phosphatase is particularly associated with processes of bone formation.

Within testis the enzyme has been found concentrated in a narrow zone in the basement membrane of seminiferous tubules[31, 44, 152, 154] and associated with capillary walls in the intertubular tissue[44, 154]. Other early spermatogenic cells show a moderate activity[44, 159] as do Leydig cells[44, 154]. Electron microscopy has shown activity in the Sertoli cells[159] and also in pinocytic vesicles in connective tissue cells underlying the tubules[152].

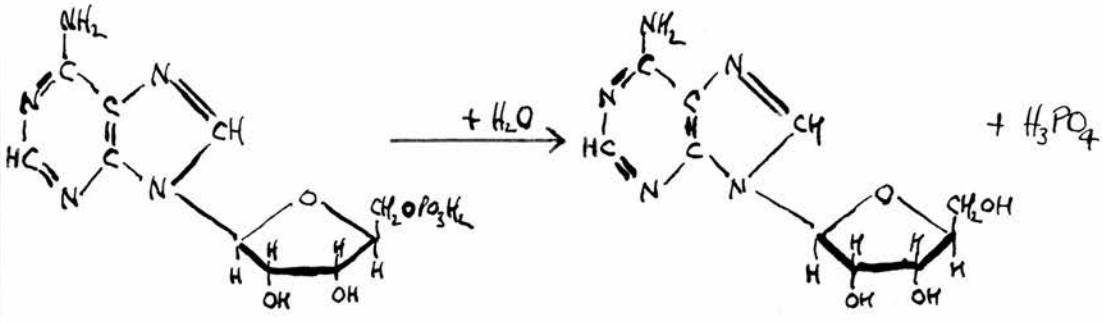
In HUMAN testis intense nuclear staining has been reported in all spermatogenic cells with least in the spermatids, with a slight reaction in immature Leydig cells and in Sertoli cells[102]. Other workers found a negligible reaction in all but the basement membranes and arterial vessels[160].

It has been suggested that the observed strong activity in the boundary tissue of seminiferous tubules indicates the enzyme to be concerned with the transport of sodium and potassium ions across the border tissue, and with the exchange of glucose and fructose between the seminiferous epithelium and the interstitial tissue. In nitrofurantoin intoxication, producing degeneration of the seminiferous epithelium, no change in boundary alkaline phosphatase (or of adenosine triphosphatase) was observed, this being said to indicate unchanged transport across the tubular wall[30].

iii. 5'-Nucleotidase:

This enzyme is a substrate-specific type of phosphoric monoester hydrolase.

E.C. 3.1.3.5 : 5'-Ribonucleotide phosphohydrolase.



Adenosine-5'-phosphate Adenosine

(AMP ; muscle adenylic acid)

A variety of other 5'-ribonucleotides may similarly be hydrolysed by this enzyme, as may 5'-deoxyribonucleotides. Non-specific alkaline phosphatase may also hydrolase these substrates.

AMP is a precursor of the diphosphate (ADP), and this of triphosphate (ATP), and is also required for the synthesis of histidine. ATP is a compound vital to large areas of energy-producing and anabolic metabolism. Adenosine, the nucleoside product of hydrolysis of AMP, can act as a source of ribose phosphates, and may be deaminated to inosine by the enzyme adenosine deaminase, which is known to be present in testis[39]. It is also a component of several nucleotide coenzymes (e.g. aminoacyl adenosine monophosphate, concerned in the activation of amino-acids for protein synthesis).

The enzyme 5'-nucleotidase is concerned generally with the degradation of purine bases, and in nucleotide metabolism. It is said to act primarily as a catabolic enzyme[10, p.20]. It has a weak activity against ADP and ATP. It occurs in several mammalian tissues, also in seminal plasma.

Activity has been detected in the "germinal elements" of rat testis[120]. Localisation has been reported predominantly in the nucleus of these, with a lesser activity in the Leydig cells[160]. An intense activity has been found at the interface between Sertoli cells and late, but not early, spermatids[152].

It has been suggested that the enzyme is concerned with the formation of germ cells rather than their function[160]. HUMAN sperm washed free of seminal fluid will effect the hydrolysis of ATP but they are not active against AMP[101].

iv. Dehydrogenases:

Dehydrogenation is a common means of oxidation in biological systems. The biocatalyst "dehydrogenases" generally require one of the coenzymes nicotinamide-adenine dinucleotide (NAD; DPN; Coenzyme 1) or nicotinamide-adenine dinucleotide phosphate (NADP; TPN; Coenzyme 2) as a hydrogen carrier. Succinate dehydrogenase, a metalloflavoprotein, is unusual in that it incorporates its own coenzyme. The re-oxidation of reduced coenzyme is catalysed by specific flavoprotein dehydrogenases termed "diaphorases".

The general action of dehydrogenases may be indicated thus:

$$AH_2 + \underset{(NADP)}{NAD} \longrightarrow A + \underset{(NADPH_2)}{NADH_2}$$

e.g.

Malic acid Oxaloacetic acid

Since a variety of dehydrogenases will be under discussion, particular reactions will not be detailed. Malate dehydrogenase and glucose-6-phosphate dehydrogenase are, however, considered in the Methods section (pp. 40 and 41). Some areas of metabolism in which particular dehydrogenases are especially concerned are outlined in Table 2, p. 15.

The following dehydrogenases have been found greatly preponderant in the Leydig cells of rat : 3β-hydroxysteroid dehydrogenase (3β-HSD); lactate dehydrogenase (LD); glycerophosphate dehydrogenase; glutamate dehydrogenase; β-hydroxybutyrate dehydrogenase; glucose-6-phosphate dehydrogenase (G6PD); and NAD- / NADP-diaphorases[93,123,154]. A moderate activity was generally found in the spermatogonia, with reaction also in the midpiece of spermatozoa. The most intense reaction was given by LD and by the diaphorases. SD alone showed activity in all layers of the seminiferous

TABLE 2

FUNCTION OF DEHYDROGENASE ENZYMES

Dehydrogenase (D)	Area of metabolism
Malate D Succinate D	Citric acid cycle : final oxidative pathway of C_2 units.
Lactate D	Glycolytic pathway; muscle metabolism.
Glycerophosphate D	Glycolytic pathway.
β-Hydroxybutyrate D	Degradation of fatty acids.
Glutamate D	Oxidative deamination of amino acids.
Glucose-6-phosphate D	Pentose-phosphate pathway: oxidation of carbohydrates formation of ribose (nucleic acid metabolism) generation of $NADPH_2$ (necessary for steroid synthesis)
3β-hydroxysteroid D	Steroid metabolism: e.g. Dehydroepiandrosterone $\xrightarrow{3\beta HSD}$ Androstenedione Δ^5-3-hydroxy Δ^4-keto (Testosterone)

epithelium, with a weak reaction in the Leydig cells[123,154]. Other workers have considered SD to be more active in the interstitial cells than within the tubules[93]. Strong activity of SD has been reported in Sertoli cells[154,159]. Marked activity of G6PD has been observed in the Leydig cells of HUMAN FOETAL testis[166].

3β-HSD has been further studied in the MOUSE Leydig cell[7]. It is postulated that there are three dehydrogenases appropriate to three steroid substrates (17 α-hydroxypregnenolone; pregnenolone; dehydroepiandrosterone [DHA]). In later work it has been found that in addition to the several 3β-HSDs already investigated, distributed solely in the interstitial tissue, substrate DHA sulphate elicits the demonstration of activity in the mature germinal epithelium, with no activity in the interstitial tissue[8]. Additional investigations of 3β-HSDs are reported by other workers[60].

v. Esterase:

This term, as generally employed in histochemical investigation, generally refers to arylesterase.

E.C. 3.1.1.2 : Aryl ester hydrolase.

$$CH_3COO\,Ar + H_2O \longrightarrow CH_3COOH + Ar.OH$$

- where Ar indicates an aryl radical.

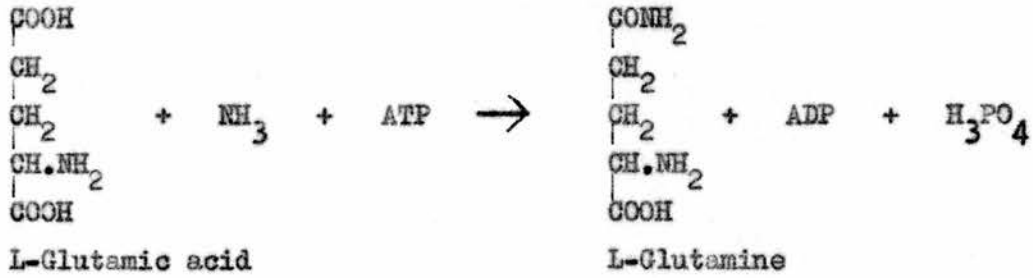
Arylesterase is of wide distribution in mammalian tissues. It has been found in testis particularly associated with the Leydig cells[69,77,122,156], being absent before puberty[156], and is also

reported in the Sertoli cells[122].

A study of interstitial esterase in relation to steroid synthesis indicated that activity was associated with the production of progesterone and testosterone[157]. Investigation of the activity of Sertoli cells revealed a localisation to the cytoplasmic processes wherein the spermatids become embedded just prior to their release, and that the distribution of the enzyme in the tubules underwent cyclic changes corresponding to those seen in the morphology of the Sertoli cells[123].

vi. Glutamine synthetase:

E.C. 6.3.1.2 : L-Glutamate: ammonia ligase (ADP).



This enzyme occurs in many mammalian tissues, where it appears to be the same enzyme as that sometimes designated "glutamyltransferase"[94,98]. The reaction catalysed is thought to proceed via an enzyme-bound activated glutamic acid, ADP having a catalytic function in this binding. The mechanism for glutamine synthesis is thought to be integrated with that for γ-glutamyl transfer[94]. Study of the solubilisation of the microsomal enzyme has suggested that the

synthesis of glutamine is not an intrinsic function of the ribonucleoprotein component, since the activity could very readily be detached[168].

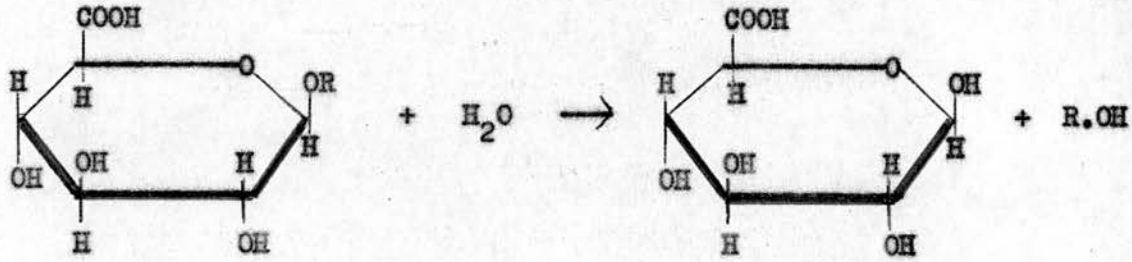
Glutamine synthetase acts generally as an anabolic enzyme [10, p.31]. Its product, glutamine, is a common cell constituent and acts generally as an intermediate carrier of amino groups in processes of amination and amidation. It effects a provision of exchangeable base, particularly in kidney, and serves to fix metabolic ammonia and phenylacetic acid. The N at positions 3 and 9 of purines is derived from glutamine, which thus is a necessary precursor of nucleic acids and of NAD/NADP.

Recent workers have shown that the activity of glutamine synthetase in the neural retina of the chick embryo increases sharply during differentiation of this tissue at a stage when proliferation of cells is minimal. The increase in activity was associated with continuous protein synthesis, and this implies the induction of enzyme aynthesis[117], (see p. 84).

The enzyme has been found present in testis extracts[98]. No histochemical method of demonstration appears to be available.

vii. <u>β-Glucuronidase</u>:

E.C. 3.2.1.31 : β-D-Glucuronide glucuronohydrolase.



β-D-Glucuronide β-D-Glucuronic acid

(R.OH = a phenol or alcohol)

The enzyme occurs in most animal tissues, and is active against a wide variety of β-D-glucosiduronic acids ("glucuronides") and also against β-D-galactosiduronic acids. It does not hydrolyse the α-D-stereoisomers, nor glucosides. Evidence for a transglucuronase function has been given[54].

Fishman describes three general functions of β-glucuronidase: (a) the conjugation of steroid hormones, (b) the hydrolysis of conjugated glucuronides, and (c) participation in cellular proliferation. Conjugation may be either metabolic or detoxicatory, and commonly promotes water-solubility. Experimental evidence of (c) is conflicting and final conclusions are not yet possible[52].

The enzyme is found in crude testicular homogenates[113, p.210]. It has been shown to be present in the seminiferous epithelium, a particularly intense reaction being noted in the primary spermatocytes,

with less activity in spermatids. Only slight activity was found in spermatogonia, Sertoli cells, and spermatozoa. Residual bodies in the lumen of the tubule and interstitial cells were also highly active[68,70]. A marked fall in activity from birth to maturity was noted, after which a steady level was maintained[68].

Evidence has been given indicating a rise in β-glucuronidase activity in tissue (LYMPHOID) which is atrophic, whether after fasting[131] or after local irradiation[132]. In the histochemical investigation of atrophic states both acid phosphatase and β-glucuronidase appear to increase in level since they are common to lysosomes and under such circumstances may be expected to leak from these organelles. It is considered by Hayashi that changes in the content of β-glucuronidase may be taken as a sensitive indicator of the state of the seminiferous tubules[70].

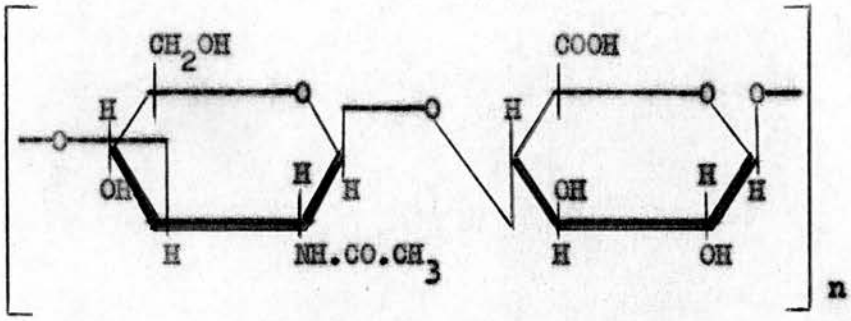
viii. Hyaluronidase:

The only enzyme of this type listed by the Enzyme Commission is said to be of bacterial origin.

E.C. 4.2.99.1 : Hyaluronate lyase.

Mammalian testis has long been known as a source of hyaluronidase. This enzyme effects the hydrolysis of hyaluronic acid, a constituent of connective tissues, and also is active against some chondroitin sulphates. Hyaluronate is considered to be a polymer of a disaccharide unit consisting of glucosiduronic acid

(glucuronic acid) and N-acetylglucosamine.



Hyaluronate

Hyaluronidase acts biphasically: there is first a rapid depolymerisation to a series of oligosaccharides, then a slower hydrolysis during which acetylglucosamine and gluosiduronic acid are released[111]. Some of the oligosaccharides are subject to further attack by β-glucuronidase[53]. Chloride is known to be a necessary activator.

Activity has been reported genuinely present in a number of tissues other than testis, not being derived from bacterial action as previously believed[17]. Its function must be considered as concerned with the catabolic metabolism of connective tissue constituents.

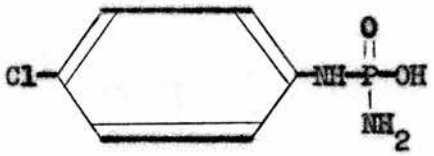
Since methods for the histochemical demonstration of the enzyme do not appear to be available, reported work is based generally upon tissue extract studies. It is considered to be produced within testis solely by the seminiferous epithelium[154], and to be associated only with the more mature sperm cells[113,p.226; 146]

namely secondary spermatocytes, spermatids, and spermatozoa. Recent histo-immunological evidence has directly confirmed this[103].

Testicular hyaluronidase has been extensively studied by Meyer et al.[112, 113]. Its role is not fully understood, but is thought to be probably confined to the dispersion of the cumulus cells in the process of fertilisation, thus facilitating union of the gametes.

ix. Phosphoamidase:

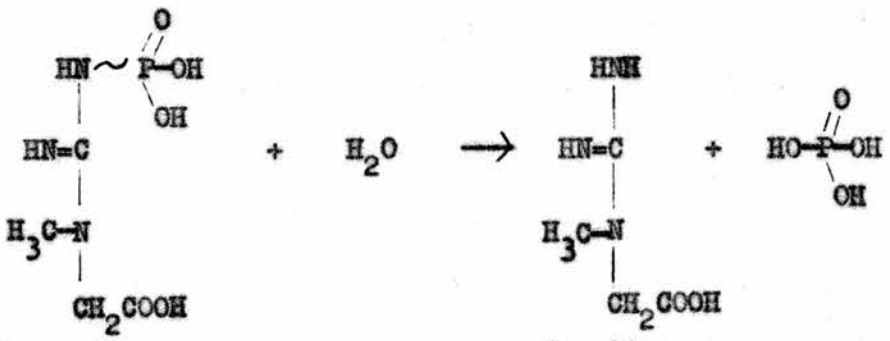
Gomori demonstrated "phosphoamidase" activity against the N-P bonds of the artificial substrate p-chloroanilidophosphonic acid in many normal tissues[63].



p-chloroanilidophosphonic acid.

This would appear to accord with phosphoamide hydrolase, an enzyme active against a number of phosphoamides.

E.C. 3.9.1.1 : Phosphoamide hydrolase.



Creatine phosphate Creatine

(~ represents a high-energy bond)

It has been shown that both acid and alkaline phosphatases may act against the histochemical substrate , as may proteclytic enzymes[73]. Pearse considers that phosphoamidase activity is often confused with that of acid phosphatase[128, p. 449].

It is well established that mammalian testis contains creatine phosphate, the only body tissue of higher content being skeletal muscle [106, p. 216]. It is designated a "phosphagen", representing a readily available source of free energy, in providing which ADP is utilised as an acceptor for the high-energy phosphate grouping, yielding ATP. This reaction is catalysed by the enzyme creatine kinase (E.C. 2.7.3.2 : ATP : creatine phosphotransferase) -

$$\text{Creatine phosphate} + \text{ADP} \longrightarrow \text{creatine} + \text{ATP}$$

Phosphoamidase, by contrast, promotes the simple hydrolysis of the creatine phosphate, with no energy-trapping mechanism, and the free energy is presumably dissipated. Its metabolic role is obscure.

It is to be noted further that the testis of many mammalian species is also rich in arginine, although the distribution of the related phosphagen, arginine phosphate, is uncertain[106, p. 216]. The latter can act as a substrate for phosphoamidase. It has been observed that an arginine-deficient diet in man will effect a great impairment of spermatogenesis[72]. Temporary deficiency of the amino-acid is met by atrophy of the seminiferous tubules, which have a rich content of this compound.

Meyer and Weinmann[110] observed that "the order of phosphoamidase activity in some stages of spermatogenesis was so high as to place the cells of these stages among the most phosphoamidase-active cells in the rat". A detailed study was made. Nil activity was found in undifferentiated spermatogonia but activity increased rapidly with the onset of differentiation, reaching a maximum at the leptotene stage of spermatocyte development, subsequently declining and reaching zero before the reduction divisions. A second phase of activity was noted in concert with spermiogenesis (maturation of spermatids), a peak being reached before the spermatids reached the Sertoli cells, this level being maintained in the released sperm. In an investigation of a HUMAN seminal phosphatase preparation, however, "phosphoamidase" was considered identical with the phosphatase[114].

General study of the distribution of phosphoamidase in rat tissues has indicated that the enzyme does not occur in all cells, but is particularly associated with epithelial cells and is concerned with (i) the performance of physico-chemical work, (ii) processes of non-protein synthesis, and (iii) adult histodifferentiation. A role in energy metabolism is tentatively suggested[109].

Our general conclusions regarding the distribution of enzymes in testis have been summarised in tabular form and are presented in Table 3A, p. 24.

TABLE 3A

TESTIS ENZYMES - SUMMARY

Enzyme	Main Localisation	Suggested Specific Testicular Function
Acid phosphatase	Early germinal cells; Sertoli cells.	-
Alkaline phosphatase	Tubule and arterial walls; Sertoli cells.	Metabolic transport across tubule wall.
5'-Nucleotidase	"Germinal cells" Sertoli cells	Nourishment of late spermatids.
Malate dehydrogenase	Not known	See Table 2, p. 15.
Succinate dehydrogenase	All germinal cells.	See Table 2, p. 15.
Glucose-6-phosphate dehydrogenase	Leydig cells.	See Table 2, p. 15.
Esterase	Leydig cells. Sertoli cells.	Associated with testosterone production. Nourishment of late spermatids.
Glutamine synthetase	Not known	-
β-Glucuronidase	Primary spermatocytes and spermatids. Interstitial cells.	-
Hyaluronidase	Secondary spermatocytes; spermatids spermatozoa.	Mechanism of fertilisation.
Phosphoamidase	Spermatocytes; spermatids.	-

x. The intracellular distribution of enzymes:

Where methods of investigation involving homogenisation and centrifugation have been employed, it is necessary to consider the intracellular distribution of the enzymes. Most available information in this context refers to liver (rat/mouse) and is not necessarily relevant to testis, as observed by Dixon and Webb[46, p.627]. Some details pertaining to certain of the enzymes under discussion are given in Table 3B, p. 25.

xi. Multiple forms of enzymes:

The multiple nature of many mammalian enzymes, within the species, is a topic of great current interest. The term "isoenzyme" is commonly used to designate the individual enzymes. The classification of the Enzyme Commission has disregarded this current development. Furthermore, differences between species are often marked.

Acid phosphatase comprises a group of enzymes variously affected by inhibitory agents[128, p.431], and direct evidence has been given for the occurrence of multiple forms of the enzyme in rat LIVER[64]. Considerable species differences in this enzyme are known to occur[128, p.433]. Alkaline phosphatase has been separated into as many as sixteen varieties by starch gel electrophoresis[18].

Of the dehydrogenases LD is commonly known in five forms and MD in two or more[164, pp.155,171]. Five isoenzymic LDs have been separated, by vertical starch gel electrophoresis, from HUMAN testis

TABLE 3B

THE INTRACELLULAR DISTRIBUTION OF ENZYMES

Enzyme	Primary site	Other sites	Ref.
Acid phosphatase	Lysosomes	Various	46 42
Alkaline phosphatase	Microsomes		46
LD, ICD, G6PD	Supernatant		46
MD	Supernatant	Mitochondria, also microsomes	46
Esterases	Microsomes		144
Glutamine synthetase	Microsomes		168 144
β-Glucuronidase	Mitochondria Lysosomes	Microsomes	46 42
5'-Nucleotidase	Nucleus Microsomes	Mitochondria Supernatant	46

and sperm, plus an additional "Band X" considered to be uniquely characteristic of postpubertal testis[13,136]. The LD of washed HUMAN, RABBIT, and BULL sperm was shown to be 80% of the Band X type, this activity not appearing until the onset of spermatogenesis in RABBIT[172]. Genetic studies of multiple LDs in PIGEON testis are also reported[173].

The separation of HUMAN spermatozoal extracts by acrylamide disc electrophoresis showed five distinct LDs and two MDs[61]. This study is of particular interest since, using as it does a uniform cell type, it is taken to indicate that "molecular heterogeneity of enzymes is characteristic of the individual cell and is not a reflection of heterogeneity of cell types within a tissue". Mitochondrial MD of PIG HEART has been separated into six forms on starch gel[151].

Esterases of rat testis have been separated into eight bands on starch gel[122], and also grouped into classes either unaffected or inhibited by certain organophosphorus compounds such as tetraethyl pyrophosphate (TEPP) [Types A- and B- esterase respectively][2]. Histochemical studies of rat LIVER have suggested that several 5'-nucleotidases may occur[124]. Two types of phosphoamidase have been prepared from a BACTERIAL source[74].

xii <u>Epididymal Enzymes</u>:

The foregoing discussion refers to testis proper. The epididymis, however, is also rich in certain enzymes, β-N-acetylglucosaminidase in particular being very active in the mature rat. In

common with other glycosidases present (α-mannosidase; β-galactosidase) peak epididymal activity is not reached until late maturity. These enzymes are a secretion of the gland itself and not merely a component of sperm: their function is obscure [35]. Appreciable activity of β-glucuronidase and of hyaluronidase has also been observed [36]. In contrast with the esterase of interstitial cells, absent before puberty, epididymal esterase appears twenty-one days after birth in the rat [156].

SECTION B : EXPERIMENTALLY MODIFIED TISSUE

Involution of the spermatogenic elements of testis is a common consequence of a variety of experimental procedures. Findings pertaining to enzyme changes associated with certain of these will be outlined.

i. Artificial cryptorchidism:

Mammalian testis functions optimally at a temperature rather lower than that of the general body temperature, as in the scrotum. Episodes of hyperthermia induce degeneration of the germinal cells and may result in sterility. Such a condition may be experimentally produced by surgically fixing testis within the abdominal cavity (artificial cryptorchidism).

Heat locally applied to testis of rat (44°C, 20 minutes) was shown to produce histologically evident damage to the seminiferous

epithelium twenty-four hours after treatment, but no change in hyaluronidase, SD, acid or alkaline phosphatase was detected at that time[154]. Since marked enzyme changes were evident later, it would appear that damage to the seminiferous epithelium was not a primary consequence of the inactivation of any of the assayed enzymes.

The weight of testis was reduced to about 25% at twenty-one, thirty, and sixty-nine days. Hyaluronidase, whether expressed as units/g. wet weight or as units/whole testis, was markedly reduced at twenty-one and thirty days, with partial recovery at sixty-nine days. Acid phosphatase and SD, normally present mainly in the seminiferous epithelium, were found much increased in the interstitial tissue at twenty-one and thirty days, with slight recovery towards normal at sixty-nine days. The change appeared to be one of distribution rather than one of total activity. Alkaline phosphatase showed little change in activity. No detectable damage to the interstitial tissue resulted. It was considered that the treatment effected a stimulation of the metabolism of the interstitial tissue.

In a study of the unilaterally cryptorchid rat, sampling at times from two to sixty-four days after operation, decreases were noted of interstitial NAD- and NADP-diaphorases, β-hydroxybutyrate dehydrogenase, and 3β-HSD, which became almost undetectable[93]. Interstitial G6PD and LD remained unchanged, whereas SD showed an increase. Of enzymes within the tubules increases were shown in

the diaphorases, LD, G6PD, and β-hydroxybutyrate dehydrogenase. Tubular SD rapidly decreased; this decrease was apparent from four days, and appeared concurrent with the observed interstitial increase. The weight of testis was reduced to about 35% at days twenty-one, thirty-two, and sixty-four.

The respiratory quotient of normal rat testis was found to be 0.93, falling to 0.50 in the cryptorchid state[150]. It was concluded that the germinal epithelium and "other testis cells" have qualitatively different types of metabolism. The hypothesis was advanced that the germinal cells are characterised by a low oxygen uptake: in the adult the high proportion of these cells "dilutes out" the larger contribution made by the other (interstitial) cells. It was also pointed out that the contribution of the Sertoli cells to the total metabolism in the cryptorchid animal should be borne in mind, since cell counts indicate that these may equal in number the interstitial cells.

An investigation of endogenous respiration in testis indicated that the Q_{o2} (rate of respiration) falls to maturity, then is constant, but rises after injury to the testis. It was concluded, by correlating various experimental studies, that the changes in Q_{o2} are related to the levels of pituitary gonadotrophins. No adequate explanation for the rise in Q_{o2} of damaged tissue could be suggested[147]. Short-term studies have been made of the effect of temperature elevation <u>in vivo</u> on subsequent metabolic activity of tissue slices

in vitro[50,51]. It was reported that the Q_{o2} of the tissue showed a transient increase but then a decrease, and that twenty-four hours after hyperthermia the tissue contained 12% less glucose and 27% less lactate than control tissues. It was suggested that the spermatogenic arrest was due to a substrate deficiency, noting that testis is particularly dependent on exogenous sources of metabolic substrate.

The gross weight loss of testis in cryptorchidism is clearly evident, but microscopy also reveals that the proportion of seminiferous tubules drops from about 90% to about 65%[93]. Clegg made quantitative studies of the interstitial cells of rat after artificial cryptorchidism[32]. He comments that the majority view is that "there is probably an actual increase in the amount of interstitial tissue" under these circumstances. A transient increase in the number of interstitial and Leydig cells was found to occur at twenty-one days after operation, the total numbers at other times not being significantly altered. However, the proportion of non-senile Leydig cells was increased over the whole experimental period. It is the general view that it is these cells which are most active in the production of androgen. They may also reasonably be supposed to be particularly active in other metabolic paths, not necessarily under gonadotrophic control. In this context it is of interest to note that a disassociation has been noted between changes in G6PD and 3-βHSD activity of the interstitial tissue in cryptorchidism: the former was unchanged whilst 3-βHSD was reduced to a very low level[93].

An increase in testis esterase (units/g. wet weight) has been found in artificial cryptorchidism, although the total content per testis slowly decreased[77]. β-glucuronidase has been shown to increase in accordance with the severity of the destruction of the seminiferous epithelium[68]. In work using rats of unspecified age there was found a ninefold increase in β-glucuronidase four weeks after bilateral operation, and a fivefold increase after unilateral operation. Only a slight increase in esterase was reported, also an increase in lipase. The β-glucuronidase and lipase activities of the contralateral testes were significantly elevated in the unilaterally cryptorchid animals[67].

A marked decrease in hyaluronidase has been recorded in cryptorchidy, significant on the fourth day after operation, values being nil by the tenth day[146].

ii. X-Irradiation:

The effects of local irradiation upon rat testis are well documented as producing destruction of the germinal epithelium with no evident damage to the interstitial tissue. Recent workers confirmed this and found an associated progressive increase in the testis β-glucuronidase, whether expressed as units/g. wet weight or as units/whole testis[130]. The change was statistically significant at days forty and fifty after treatment. Changes in esterase were poorly defined.

The comparative radioresistance of interstitial and Sertoli cells has been demonstrated by the observation of active mitosis in these cells after irradiation sufficient to effect degeneration of the seminiferous epithelium[148]. Such treatment has been shown to leave the interstitial cells morphologically unchanged and able to respond to exogenous gonadotrophin (HCG) by hyperplasia and hypertrophy[142]. *In vitro*, androgen biosynthesis was much diminished, certain necessary enzymes being reduced in activity (17 α-hydroxylase; 17 β-desmolase; 20 α-hydroxysteroid dehydrogenase). This indicates that the histological appearance and steroidogenic function of interstitial cells are not necessarily correlated.

iii. Hypophysectomy and hormone administration:

Surgical removal of the adult rat pituitary (hypophysectomy) has been shown to effect marked decreases in the activity of testis alkaline phosphatase[44], esterase[77,156], and hyaluronidase[146]. The subsequent administration of gonadotrophin (whole pituitary) restored alkaline phosphatase to normal[44], and part restored esterase[77]. Histochemical studies have shown β-hydroxybutyrate hydrogenase to be completely absent and 3β-HSD to be greatly decreased after this ablative procedure[123], whereas an investigator employing homogenates has noted an increase of 3β-HSD, expressed as units/g. wet weight[140], a further marked increase ensuing upon the administration of chorionic gonadotrophin.

The administration of gonadotrophin (human pregnancy urine) to the mature animal produced a marked increase in testis esterase, with hyperplasia of the interstitial cells[158]. The fifteen-day-old rat was normally histochemically negative for esterase, but after the urine injection activity was found in the interstitial cells.

Homogenate studies in the immature rat revealed significant increases after hypophysectomy of ICD and G6PD, but not of LD. The effect was thought to be organ-specific: liver preparations did not show the effect[143]. Newborn rats treated with chorionic gonadotrophin exhibited a large increase in the histochemical activity of 3β-HSD; β-glucuronidase; sulphatase; 5'-nucleotidase; and esterase[5].

The administration of testosterone has been shown to produce an increase in 5'-nucleotidase activity in the normal adult rat testis[120], and to maintain normal levels of hyaluronidase in hypophysectomised rats, having no effect on this enzyme in the normal animal[146]. It has also been found greatly to depress the activity of β-hydroxybutyrate dehydrogenase and 3β-HSD[123].

Oestradiol administration is said to produce a decrease in hyaluronidase[146] and in esterase[156]. Vasectomy has been shown to produce an increase in esterase[156], and inanition to cause a depression of the activity of β-hydroxybutyrate dehydrogenase and 3β-HSD[123].

iv. Selectively toxic chemical agents:

a) General:

A wide range of compounds has a selective toxic effect upon testis. A number of types of alkylating agent (ethyleneimines; alkane sulphonic esters) produce specific effects of various kinds[89]. The administration of thalidomide to male RABBIT has been shown to result in a deleterious effect upon subsequent progeny[100]. Fluoroacetate produces damage to the seminiferous epithelium[108]. A dietary deficiency of Vitamin E also produces damage[107], and effects changes in testis β-glucuronidase and esterase[130]. Delayed and minimal increases in β-glucuronidase were found in Vitamin E deficiency, in contrast to the marked changes observed in association with the degenerative changes ensuing upon cryptorchidism[68].

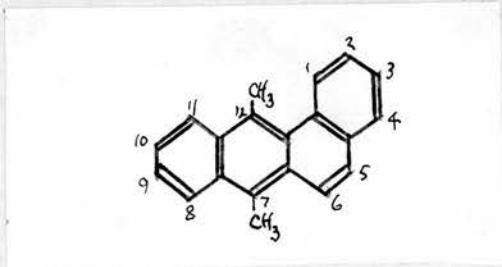
Certain bis-(dichloroacetyl)diamines have a markedly selective effect upon testis, yet Leydig cell morphology and androgen production remain unaffected[12]. The nitro-furan drug Furadroxyl has been shown to cause loss of the ability of seminiferous tubules to oxidise pyruvate *in vitro*[163]. A similar effect may be shown after

X-irradiation or after hypophysectomy. Degeneration of the seminiferous epithelium has been produced by treating rats with toxic doses of nitrofurantoin: therapeutic dosage was ineffective[29]. A diet in which Vitamin A alcohol had been replaced by Vitamin A acid was shown to effect lesions of the germinal epithelium in the rat[75].

Nutritional deficiency of zinc, an element normally present in the prostate gland, is known to cause characteristic injury to the testis. Cadmium, unlike zinc, is very toxic to testis. This toxic effect can be prevented by the simultaneous administration of zinc[104]. It may be noted that certain dehydrogenases contain zinc[46, p.456].

The present work is particularly concerned with the changes in testicular function and enzyme content produced by the chemical agents DMBA and Myleran.

b) 7,12-Dimethylbenz(α)anthracene (DMBA)



7,12 - Dimethylbenz(α)anthracene

A selective ADRENAL necrosis was elicited by Huggins and Morii in both male and female rats by the administration of a single dose of the highly carcinogenic hydrocarbon DMBA [20-30 mg. orally: 1.5-10 mg. intravenous (i.v.)][79]. Homogenate levels of G6PD, ICD, and 6-phosphogluconic dehydrogenase were shown to decline on the second day; to reach a minimum at three days; to be increasing at six days; and to be restored to normal at fourteen days. The effect was not secondary to pituitary stimulation, since the same result followed after hypophysectomy.

Ford and Huggins later produced severe and selective damage to the TESTIS of young adult and of immature rats by a single feeding of similar amounts of DMBA[55]. It was shown that testis MD (as units/g. wet weight) increased steadily in inverse ratio to the weight of testis, reaching twofold levels at thirty-eight to forty days after administration of the carcinogen. Damage was restricted to spermatogenia and spermatocytes: other tubular cells became affected secondarily at a later date. Studies of histology and of prostate weight changes indicated that the Leydig cells were "not compromised by the hydrocarbon".

c) Busulphan [Myleran; 1:4-di(methanesulphonyloxy)butane]:

$$\begin{matrix} CH_2.CH_2.OSO_2CH_3 \\ CH_2.CH_2.OSO_2CH_3 \end{matrix}$$

The induction of temporary infertility in the male rat by

the administration of busulphan (10 mg./kg.) is recorded by Jackson et al.[85,89]. Quantitative histological studies have indicated that damage to Type A spermatogonia and possibly to the stem cells results[87], whereas fertility studies suggest that some interference with other spermatogonial types and even with early spermatocytes is also incurred.

A single dose had no effect on fertility for seven weeks, the germinal cells present at the time of treatment continuing to develop into mature sperm. The toxic effect upon early spermatogenic cell types resulted in a sequential depletion within the tubules of spermatogonia, spermatocytes, spermatids, and finally spermatozoa.

METHODS

METHODS

i. Animals:

Adult white Wistar rats were used throughout. These were fed a commercial ration with water ad libitum. Killing was effected by stunning the animal with a sharp blow to the back of the head, followed by decapitation. The testes were immediately excised, weighed, and sampled as required.

ii. Sampling of tissues:

For the general enzyme studies, immediately after excision and weighing the whole testis was bisected and dropped into 2 ml. of ice-cold 0.15M NaCl containing 0.003M $NaHCO_3$, homogenisation then being effected by fifteen seconds mixing with the Ultra-Turrax TP 18/2 mixer, which has a castellated shearing knife rotating at 24,000 r.p.m. The resulting suspension was poured into a plastic centrifuge tube and the homogenisation tube briefly rinsed and agitated with a further 1 ml. of the saline. The whole suspension was mixed by inversion and then centrifuged for ten minutes at 11,000g in a refrigerated centrifuge ($4^{o}C$). The clear supernatant obtained was removed by Pasteur pipette and stored in ice-water until required: storage after a few hours, if required, was continued at $-18^{o}C$.

Testes for the estimation of hyaluronidase were wrapped in Parafilm immediately after excision and stored in solid CO_2 until assay was made within forty-eight hours. The testis was allowed briefly to thaw, bisected, and dropped into twice its weight of M/10 acetate buffer, pH 3.8. Homogenisation with the Ultra-Turrax was carried out for one minute, with chilling, the resulting suspension then being centrifuged in the cold at 30,000g for ten minutes. The resulting clear supernatant was removed by pipette and stored as above.

iii. Enzyme determinations:

a) General:

The testis extracts were diluted appropriately and used thus in a variety of procedures, most of which normally have been applied to the estimation of serum enzymes.

It was considered necessary to establish that the methods used were adequate. To this end limited activity studies were carried out in each case, and the relevant data are presented in Figures 2-12. Ideally, all the activity curves should be linear in the regions employed. The primary purpose of the investigations was to seek gross changes in enzyme activity and it is believed the methods utilised were suitable. Details of the methods employed are given in Table 4, p. 39. The first four methods are common routine methods in the medical laboratory and will not be discussed further. The last four methods are described in the following text.

TABLE 4

METHODS FOR THE DETERMINATION OF TESTIS ENZYMES

Enzyme	Substrate	Dilution of extract	Method
Acid phosphatase	Phenyl phosphate	1:19, with saline	Gutman and Gutman[65]
Alkaline phosphatase	Phenyl phosphate	1:19, with saline	Kind and King[91]
5'-nucleotidase	Adenosine monophosphate	1:19, with saline	Dixon and Purdom[47]
β-glucuronidase	Phenolphthalein glucuronide	1:2, with saline	Talalay et al.[149]
Malate dehydrogenase (MD)	Pre-formed oxaloacetate	1:79 or 1:99 with phosphate-aspartate	Boehringer Test Combination TC-L[15] (See below)
Glucose-6-phosphate dehydrogenase (G6PD)	Glucose-6-phosphate	0.05 ml. of raw extract diluted to 2.85 ml. with triethanolamine buffer pH 7.6	Boehringer Test Combination TC-W[15] (See below)
Glutamine synthetase	L-Glutamic acid	0.05 ml. of raw extract	See p. 42
Hyaluronidase	Hyaluronate ex tumour (Light's)	0.1 ml. of raw extract	See p. 43

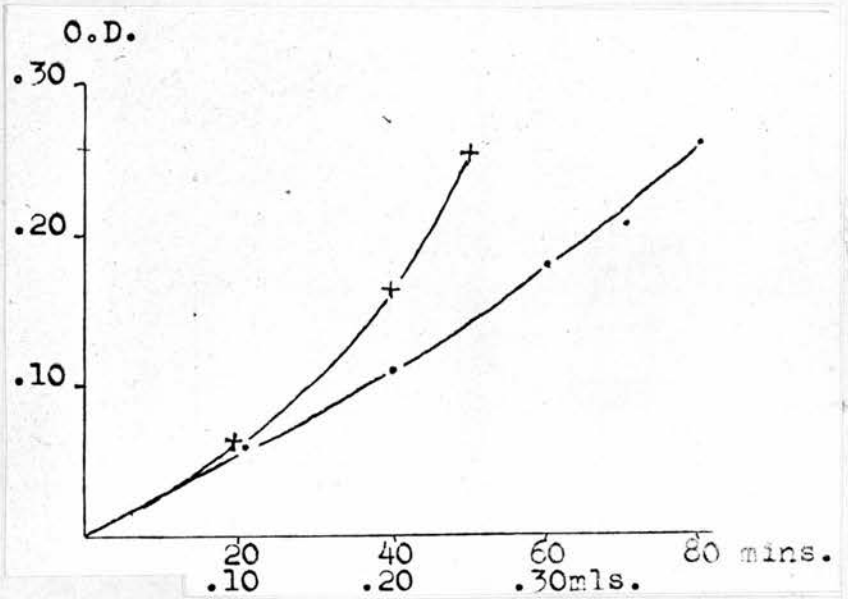


Fig. 2 .

Acid phosphatase method.

• - Abscissa, time of incubation (mins.)
+ - Abscissa, amount of test material (mls.)
Ordinate, O.D., Ilford 608 filter 1 cm. cuvette.

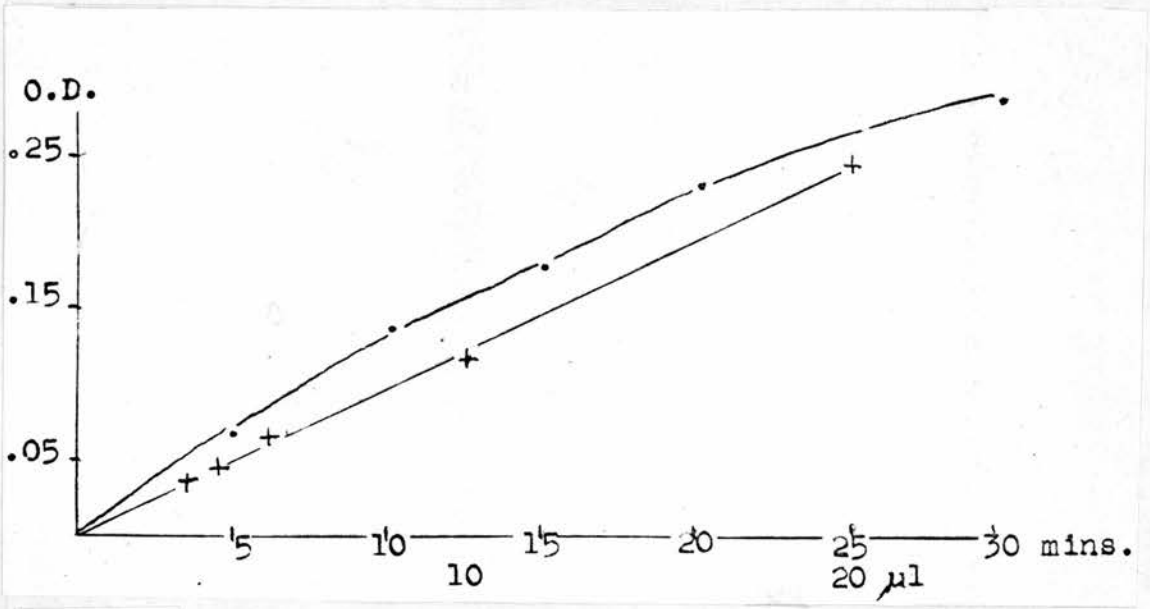


Fig. 3 .

Alkaline phosphatase method.

• - Abscissa, time of incubation (mins.)
+ - Abscissa, amount of test material (µl.)
Ordinate, O.D., Ilford 624 filter, 1 cm. cuvette.

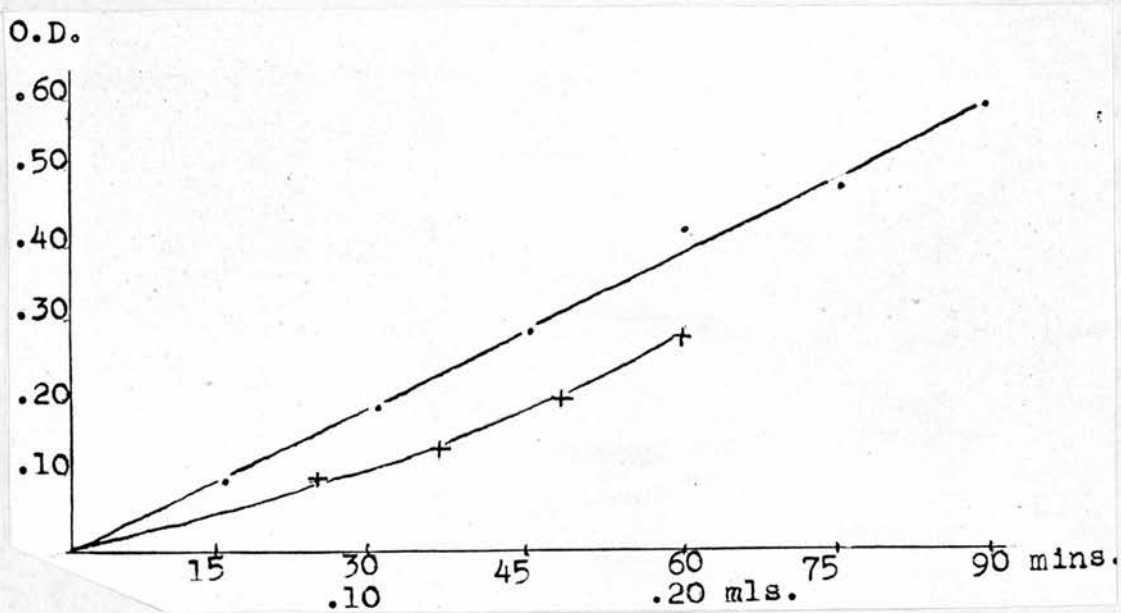


Fig. 4. 5'-Nucleotidase method.

• - Abscissa, time of incubation (mins.) + - Abscissa, amount of test material (mls.) Ordinate, O.D., Ilford filter 608, 1 cm. cuvette.

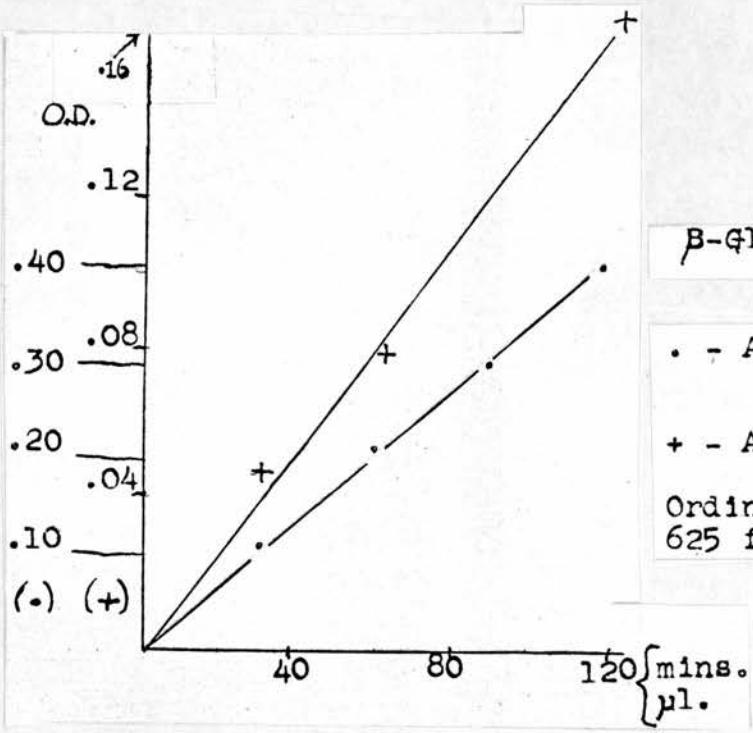


Fig. 5.

β-Glucuronidase method.

• - Abscissa, time of incubation (mins.)

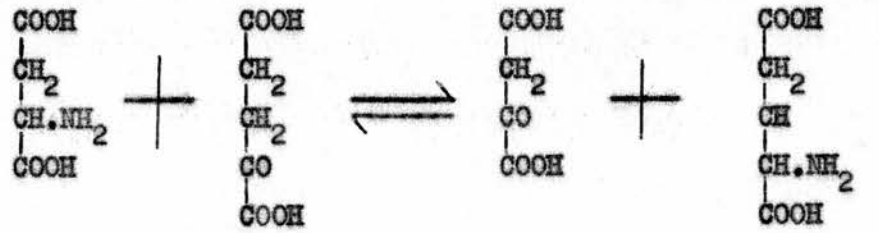
+ - Abscissa, amount of test material (µl.) Ordinate, O.D., Ilford 625 filter, 1 cm. cuvette.

b) Malate dehydrogenase:

The method employed involved the preliminary generation in situ, within the optical cuvette, of the required substrate, oxaloacetate. This is desirable since the compound is rather unstable. Following addition of the test material, the fall in optical density of $NADH_2$, which was associated with the enzymic oxidation, was recorded at 366 mμ. This wavelength was used instead of the preferred wavelength of 340 mμ, where the molar extinction of $NADH_2$ is greater, since an ultraviolet spectrophotometer was not readily available. The optical density was recorded at one minute intervals for eight minutes from completion of the test mixture, and the mean fall per minute used for purposes of calculation.

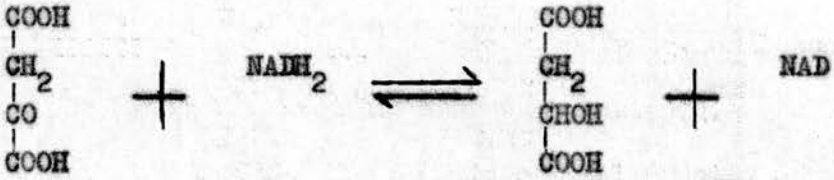
Constant temperature cuvette facilities were not available. Measurements were therefore made at the ambient temperature and correction to 25°C made assuming a temperature coefficient of 1.7[58]. This assumption was checked. Details of the method of calculation are given on page 44, and a representative absorption curve in Figure 6, p. 41.

1. Formation of oxaloacetate: catalysed by glutamic oxaloacetic transaminase (supplied).



Aspartic acid — α-Oxoglutaric acid — Oxaloacetic acid — Glutamic acid

2. Dehydrogenation of malic acid - back reaction; catalysed by malate dehydrogenase.



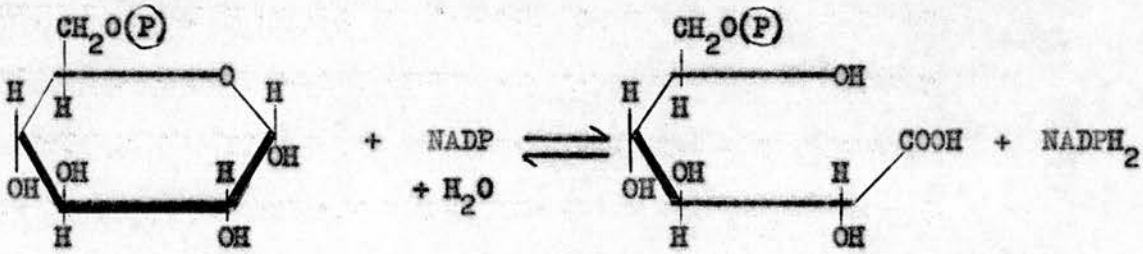
Oxaloacetic acid

Malic acid

c) Glucose-6-phosphate dehydrogenase:

The optical density increase accompanying the reduction of NADP was followed at 366 mμ at one minute intervals from one and a half to six and a half minutes after completion of the test mixture. The mean increase per minute was used for purposes of calculation.

Details of the method of calculation are given on page 44, and a representative absorption curve in Figure 6, p. 41.



Glucose-6-phosphate

Gluconic acid-6-phosphate

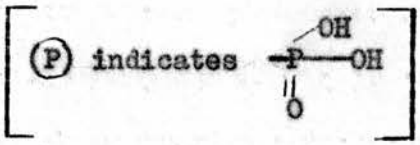
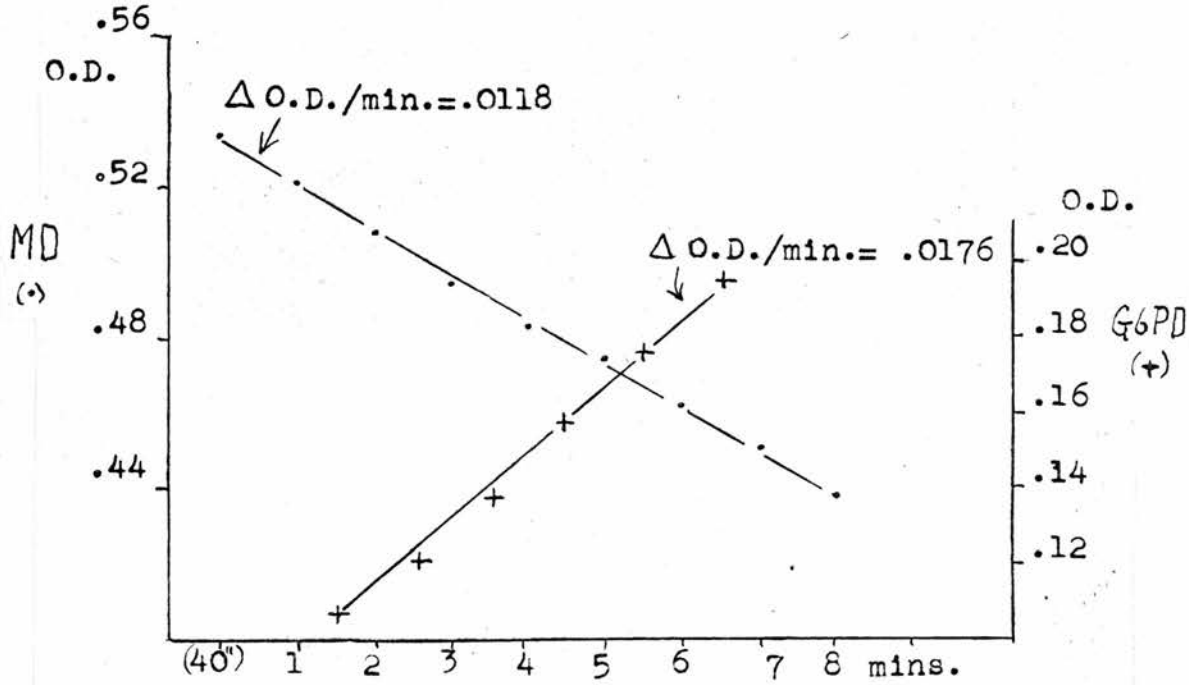


Fig. 6.

Representative absorption curves : MD method - •
G6PD " - +



Abscissa, incremental time (mins.) Ordinate, O.D.,366 mμ
1 cm. cuvette.

MD

Sample dilution - example :

Dilution 1:99, 28.0°C., O.D. change/min. = .0141
Calculated activity 41.5 u./g.

Dilution 1:79, 27.3°C., O.D. change/min. = .0168
Calculated activity 41.9 u./g.

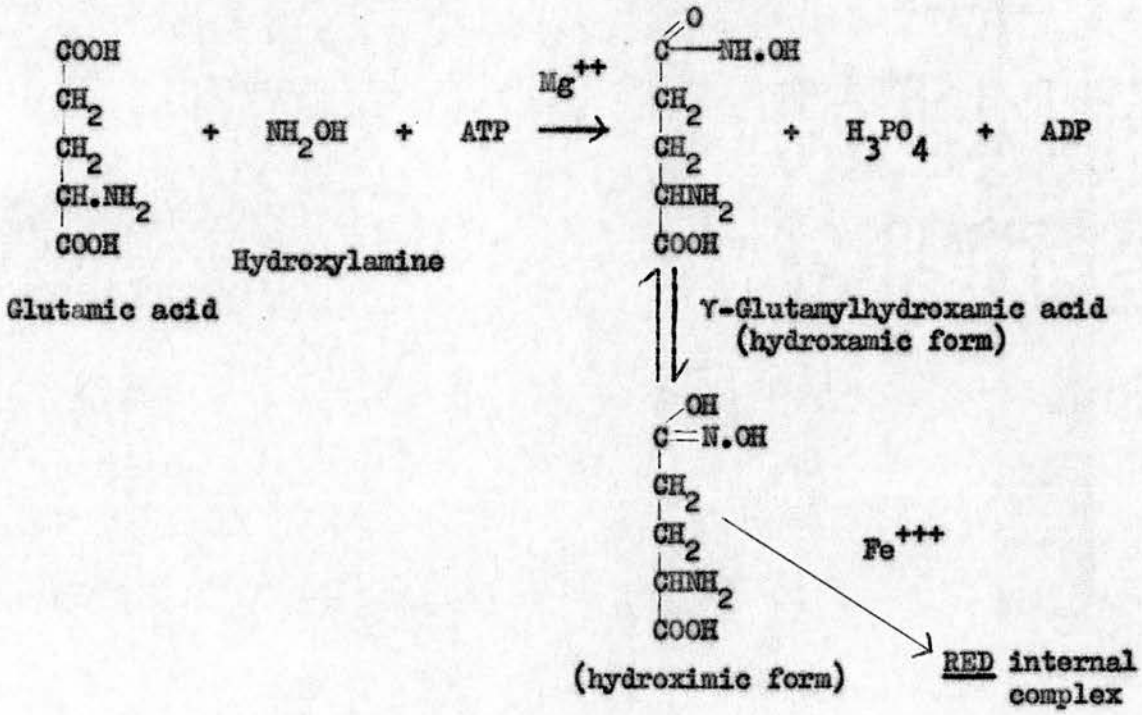
MD method : test mixture with the omission of NADH -

Initial O.D. .603

O.D. at 10 mins. .596 (98.9%)

d) Glutamine synthetase:

The method used was adapted from one employed for nervous tissue by Utley[155]. This utilises the finding that NH_2OH can act as the nitrogen source in place of NH_3 with an unchanged rate of reaction[161], the product then being γ-glutamylhydroxamic acid (GHA), which may be estimated by the colour given with ferric ion.



Mg^{++} and BAL (2:3-dimercapto-propan-1-ol) were used as activators, the energy source being ATP (Sigma). The following two solutions were made up and neutralised to pH 7.2:

1. $MgCl_2$ 407 mg. / $NH_2OH.HCl$ 347 mg. } per 25 ml. TRIS buffer, 0.094M, pH 7.2

2. L-Glutamic acid 587 mg. per 25 ml. TRIS buffer, 0.094M, pH 7.2

The test mixture was composed of:

0.05 ml. testis extract

0.95 ml. TRIS buffer, pH 7.2, 0.094M

0.75 ml. $MgCl_2/NH_2OH$

0.75 ml. glutamic acid

0.5 ml. ATP (22.1 mg., in TRIS buffer)

0.03 ml. BAL (0.18 mg., in ethanol)

The blank mixture was identical with the omission of ATP. After thirty minutes incubation at 37°C, 0.5 ml. of 0.5N HCl containing 10% $FeCl_3$, 8% trichloracetic acid, was added to each tube and mixed in. After centrifugation the optical density of the clear supernatant was read at 500mμ, the point of maximal absorption shown in Figure 9, p. 43. Calibration was against γ-glutamylhydroxamic acid (Sigma) [Figure 7, p.43].

e) Hyaluronidase:

The method used was taken from Bollet et al.[17], employing the colorimetric estimation of enzymically produced N-acetylglucosamine by the method of Reissig et al.[135]. The test mixture was composed of:

Glutamine synthetase method.

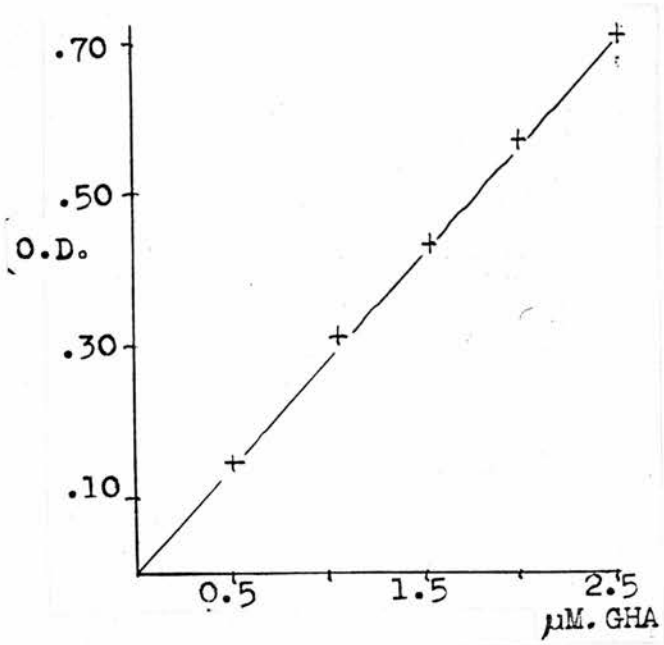


Fig. 7.

Calibration curve. Abscissa, µM. GHA in final test mixture. Ordinate, O.D., 500 mµ, 1 cm. cuvette.

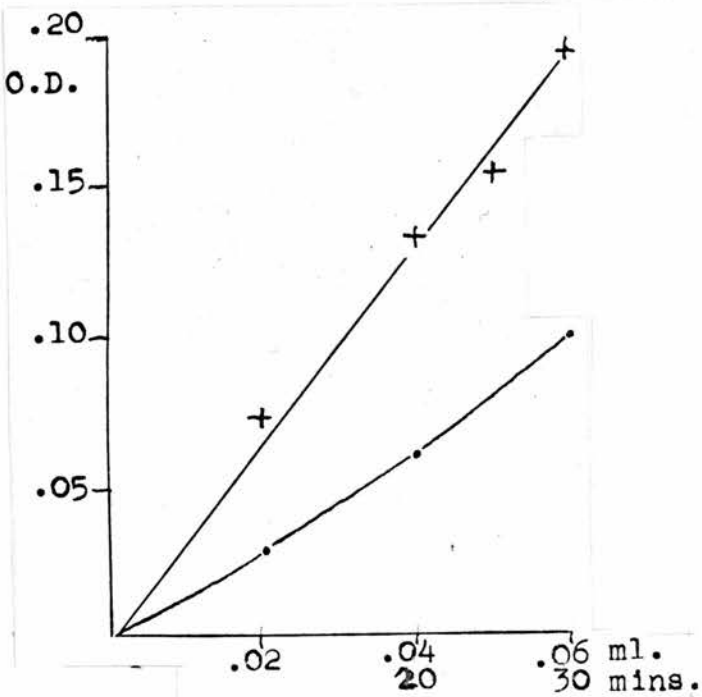


Fig. 8.

• - Abscissa, time of incubation (mins.) + - Abscissa, amount of test material (ml.) Ordinate, O.D., 500 mµ, 1 cm. cuvette.

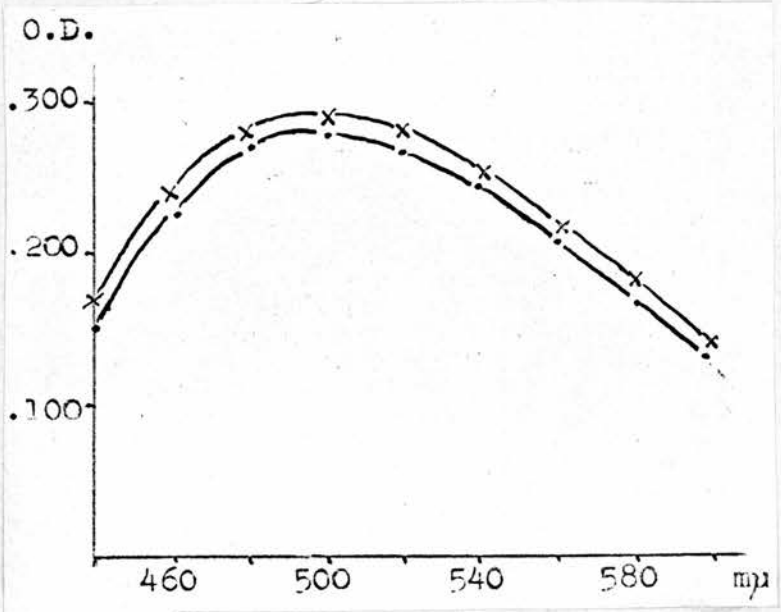


Fig.9 . Glutamine synthetase method - absorption curve.

. - sample test mixture ; + - GHA standard mixture.

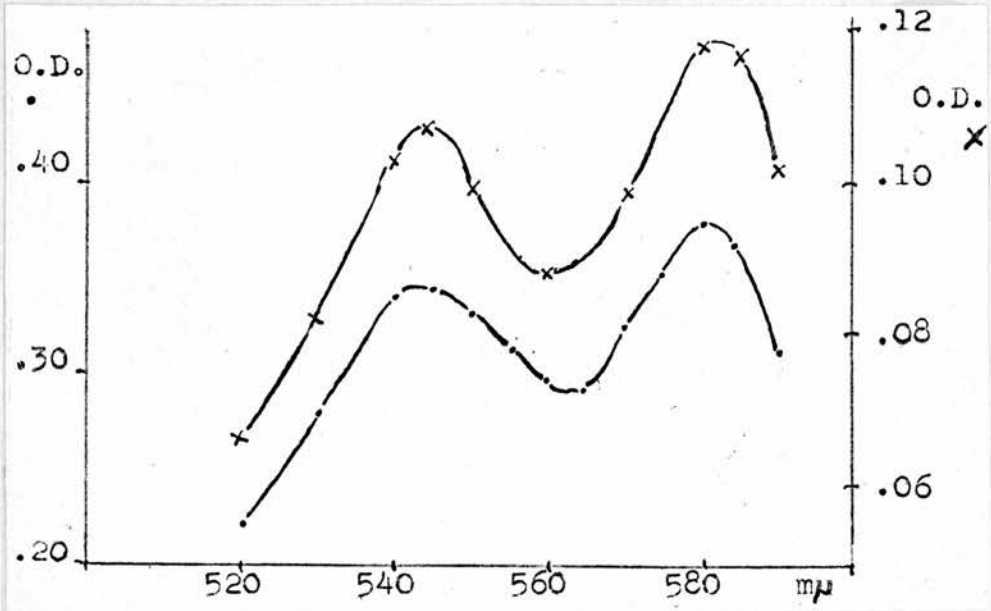


Fig. 10 . Hyaluronidase method - absorption curve.

. - sample test mixture ; + - standard mixture.

Hyaluronidase method.

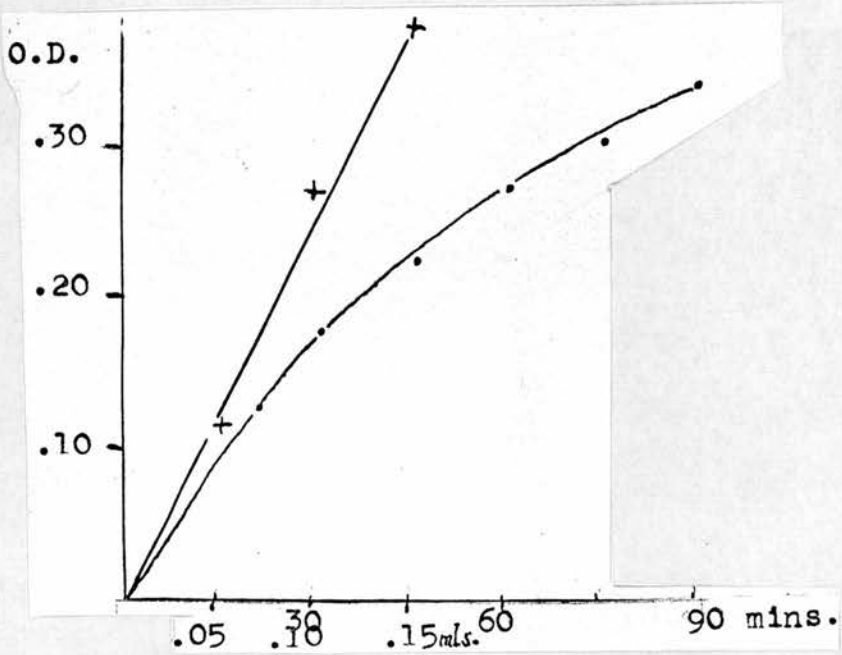


Fig.11

. - Abscissa, time of incubation (mins.) + - Abscissa, amount of test material (mls.) Ordinate, O.D., 580 mμ , 1 cm. cuvette.

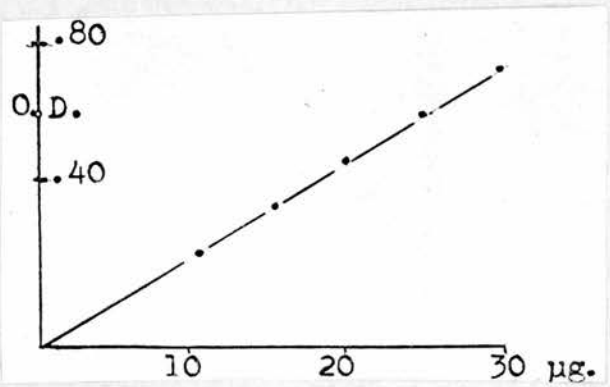
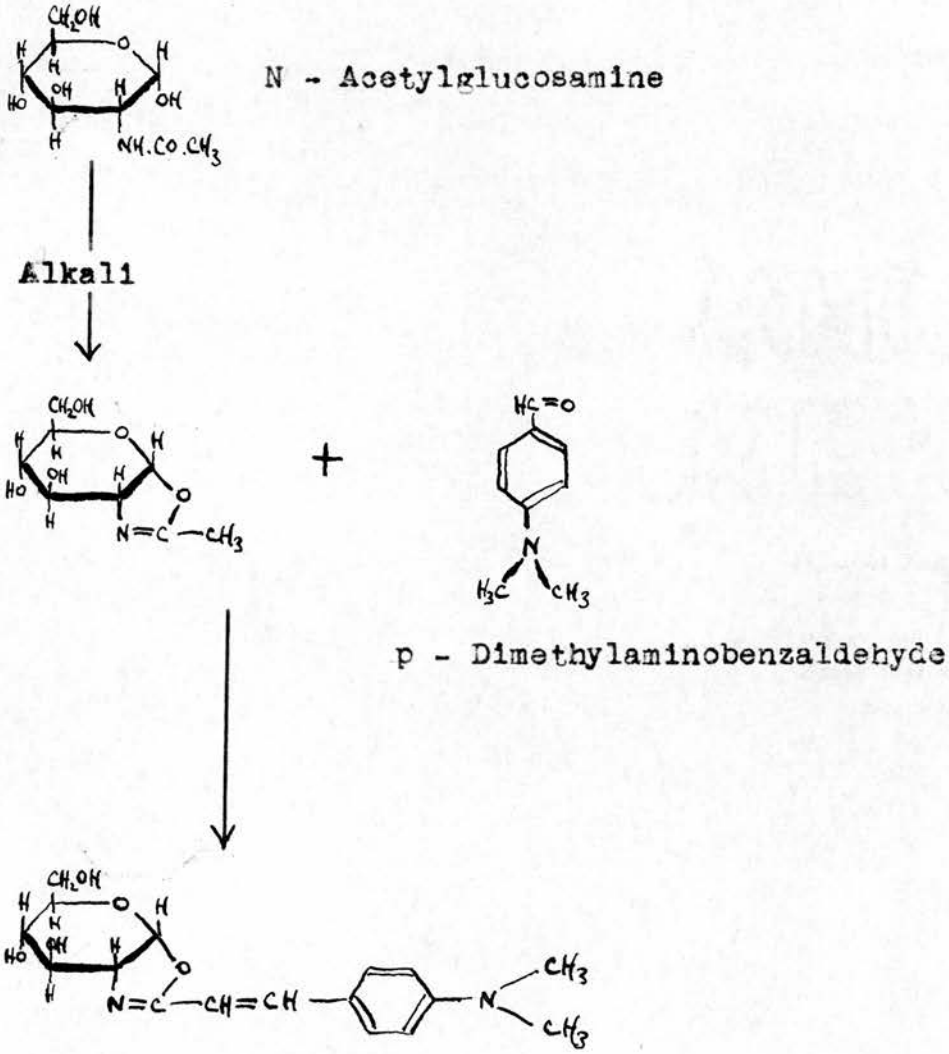


Fig.12.

Calibration curve. Abscissa, μg N-acetyl-glucosamine in final test mixture. Ordinate, O.D., 580mμ , 1 cm. cuvette.

Figure 13 :

Hyaluronidase method - aldehyde reaction.



RED reaction product.

0.1 ml. of testis extract

0.2 ml. M/10 acetate buffer, pH 3.8

0.15 ml. hyaluronate, 1 mg./ml. in buffer.

The mixture was incubated for one hour at 37°C. The two test components were incubated separately to provide a blank, being mixed at one hour. The reaction was stopped with 0.1 ml. of potassium tetraborate (pH 9.1). The tubes were then heated at 100°C for three minutes, and then cooled in water. The intermediate compound formed (? glucoxazoline)[116] (see Figure 13, p. 43) was then allowed to react with 3 ml. of p-dimethylbenzaldehyde reagent (twenty minutes at 37°C) to yield a red colour. After brief centrifugation the optical density was read at 580mμ. Calibration was against N-acetylglucosamine (see Figure 12, p. 43).

f) Calculation and expression of enzyme results:

For purposes of consistency and clarity in presentation all results have been expressed in terms of International Units (I.U.), as recommended by the Clinical Enzyme Sub-Commission of the International Union of Biochemistry. All activities are thus expressed in terms of micromoles of substrate transformed per minute[34]. Details of the unit conversions are given in Table 5, p. 46.

It must be noted that we have expressed hyaluronidase in terms of a unit such that 1 unit produces 1 μmole of N-acetylglucosamine per minute. Activity has actually been expressed in "milli-I.U." on this

basis in order to distinguish our unit from the established International Biological Standard of the W.H.O., which is already denoted the I.U.

Both normal and atrophic testes were desiccated to constant weight. The water content was found to be 86% and 87% respectively. The total volume of saline-homogenate was therefore calculated as (3 + [86/100 x weight testis]) ml. in each case. On this basis the respective units/testis and units/g. testis (wet weight) were calculated for each enzyme.

The dehydrogenase results were calculated as in the examples given below:

1. Calculation of MD results:

From the known molar extinction of NAD:

(Change in O.D./minute at 366 mμ) x 9.11 = International Units per ml.

Testis weight = 1.82 g.

Total volume of extract = (3 + [86/100 x 1.82]) ml.

= 4.56 ml.

Observed change in O.D./min. at 366 mμ = 0.016 (at 26°C)

I.U. per testis = 0.016 x 9.11 x 4.56 x 100 at 26°C

* = 62.1 at 25°C

I.U./g. testis = 62.1/1.82

= 34.1

*Temperature correction:- $V_{t_1} = \frac{V_{t_2}}{1 + (0.07 \times \triangle T)}$

where V_{t_1} = units at lower temperature, t_1 (generally 25°C)

V_{t_2} = units at higher temperature, t_2 (ambient)

$\triangle T = t_2 - t_1$

2. Calculation of G6PD results:

From the known molar extinction of $NADPH_2$:

(Change in O.D./minute at 366 mμ) x .910 = International Units per ml.

Testis weight = 1.55 g.

Total volume of extract = (3 + [86/100 x 1.55]) ml.

Observed change in O.D./min. at 366 mμ = .0104 (at 26.3°C)

I.U. per testis = .0104 x .910 x 4.33 x 1.00/0.05 at 26.3°C

= .751 at 25°C

I.U./g. testis = .751/1.55

= .485

iv. Histology:

Selected testes were fixed in Bouin's fluid or 10% formol-saline and later processed to give paraffin sections (7 mμ) stained with Mayer's haematoxylin. A few sections were further stained with eosin. Sections were examined by light microscopy and illustrative photomicrographs taken.

TABLE 5

CONVERSION OF ENZYME UNITS

Enzyme	Original Unit (u.)	Conversion Factor	Units Employed
Acid phosphatase	King-Armstrong u. → 1 mg. phenol/60 mins.	x .18	I.U.
Alkaline phosphatase	King-Armstrong u. → 1 mg. phenol/15 mins.	x .72	I.U.
5'-Nucleotidase	Reis u. → 1 mg. phosphate P/60 mins.	x .535	I.U.
β-Glucuronidase	Fishman u. → 1 μg. phenolphthalein/60 mins.	x .0524	milli-I.U.
Malate dehydrogenase	-	-	I.U.
Glucose-6-phosphate dehydrogenase	-	-	I.U.
Glutamine synthetase	1 u. → 1 μM γ-glutamo-hydroxamic acid/30 mins.	÷ 30	I.U.
Hyaluronidase	1 u. → 10 μg. N-acetyl-glucosamine/60 mins.	÷ .754	"milli-I.U."

v. Enzyme histochemistry:

The distribution within testis of LD, MD, SD, and ICD was investigated. Selected testes were wrapped in Parafilm after excision and stored in solid CO_2 until required, generally within twenty-four hours. Sections were cut at 10-15 μ in the S.L.E.E. Cryostat, at -20°C. These were taken on to cover slips, stored at -70°C, and stained by the methods of Pearse[128, p.911], generally within a few hours. The coenzyme used throughout was NAD (Boehringer) and the final electron acceptor was Nitro-BT [2,2'-Di-p-nitro-phenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride][153]. The times of incubation were somewhat variable, but in all cases control normal slices were treated in parallel. By illustration, the details of the technique as used for MD are given below, and the chemical basis for the method is shown in Figure 14, p. 47.

Histochemical demonstration of MD activity: Method -

The section mounted on a coverslip was allowed to dry in air for a few moments and then covered with about 0.2 ml. of incubating medium*. After thirty minutes at 37°C the excess stain was gently washed away and the section fixed in 10% formol-saline for ten minutes. After brief washing the coverslip was mounted on to a slide using glycerine-jelly as mountant.

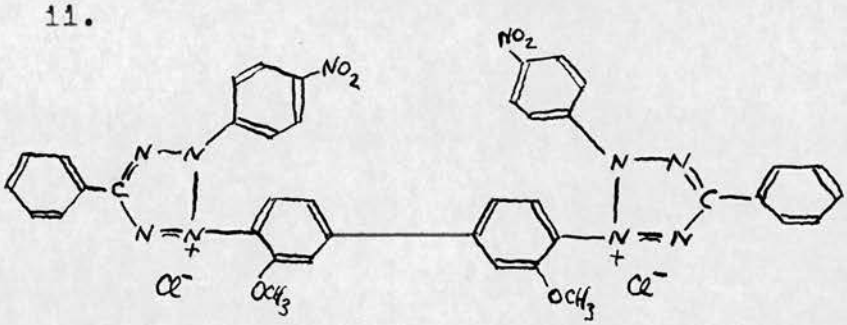
Figure 14 :

Histochemical demonstration of malate dehydrogenase.

i. $2\begin{bmatrix} COOH \\ | \\ CH_2 \\ | \\ CHOH \\ | \\ COOH \end{bmatrix} + 2\ NAD \xrightarrow{Mg^{++}} 2\begin{bmatrix} COOH \\ | \\ CH_2 \\ | \\ CO \\ | \\ COOH \end{bmatrix} + 2\ NADH_2$

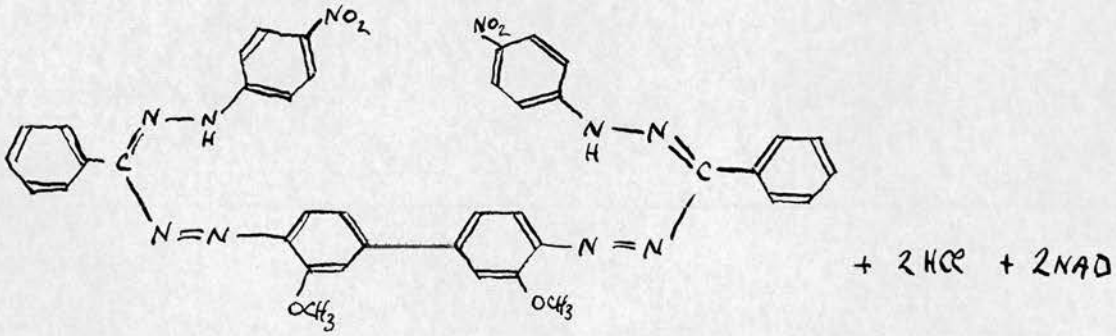
Malic acid — Oxaloacetic acid

ii.



Nitro - BT : ditetrazolium salt ; colourless ; soluble.

$\downarrow + 2\ NADH_2$



Nitro - BT diformazan : deep purple ; insoluble.

* Incubating medium:

This was freshly prepared before use.

† Sodium-L-malate, I.0 M	0.5 ml.
† NAD (Boehringer), 0.2 M	0.5 ml.
† Sodium cyanide, 0.1 M	0.5 ml.
Magnesium chloride, 0.05 M	0.5 ml.
Tris buffer, pH 7.0, 0.2 M	1.25 ml.
Nitro - BT, 1.25 mg. in water	1.25 ml.
Distilled water to make	5.0 ml.
Polyvinylpyrrolidone	375 mg.

† Adjusted to pH 7.0 before use. The pH of the final mixture was checked and adjusted if necessary to 7.0.

vi. Experimental:

The atrophic response of rat testis to the injection of DMBA and of busulphan was investigated. Nine independent groups of animals were employed, allowing the harvesting of testis at selected time intervals after injection (Experiments A-I : Tables 6 and 7, p. 49).

An initial abortive experiment was carried out giving an intraperitoneal injection of DMBA : the solid carcinogen (Kodak) was first purified by column chromatography and the purity checked by thin-layer chromatography. Subsequent presentation of DMBA was intravenous

(caudal vein), using DMBA in Lipomul Emulsion (15% cottonseed oil) kindly made available through the courtesy of Dr. R. M. Smith, The Upjohn Company, Kalamazoo, Michigan. Busulphan was given throughout intraperitoneally dissolved in arachis oil.

Only a few true experimental controls were utilised. Further normal animals were sacrificed throughout the course of the experiments and tissues sampled in parallel with test samples. The enzyme results obtained with these did not appear to differ from those found with the genuine controls.

The results presented are based upon aggregated experimental data.

vii. Fertility trial:

An experimental trial of the effect of DMBA (2.5 mg., i.v.) upon the fertility of male rats was also carried out. This was designed as described previously by Bock and Jackson[14].

Eight treated adult male rats were paired with females of known fertility in separate boxes. The females were changed each week. Vaginal smears were taken on four days of the week and examined for sperm. The occurrence of insemination and the number of resulting offspring were recorded.

TABLE 6

ANIMAL EXPERIMENTS : ADMINISTRATION OF DMBA

Experiment	No. of rats	Dose Administered	Route	Premature Deaths	Sampling	
					No.	day
A	T 20 C 10	10 mg./0.4 ml. olive oil. C - olive oil only.	i.p.	See text		
B	T 8 C 4	5 mg./1 ml. fat emulsion. C - fat emulsion only.	i.v.	T,4	T,4 C,4	34
C	T 6	2.5 mg./0.5 ml. fat emulsion.	i.v.	-	4 2	35 41
D	T 10	5 mg./1 ml. fat emulsion.	i.v.	2	2 3 3	8 20 35
E	T 10	2.5 mg./0.5 ml. fat emulsion.	i.v.	-	3 3 4	35 49 75
F	T 14	5 mg./1 ml. fat emulsion.	i.v.	2	4 4 4	35 49 75

Abbreviations:
T - Test animals
C - Control animals
i.p. - intraperitoneal injection
i.v. - intravenous injection (caudal vein)

TABLE 7

ANIMAL EXPERIMENTS : ADMINISTRATION OF BUSULPHAN

Experiment	No. of rats	Dose Administered	Route	Premature Deaths	Sampling	
					No.	day
G	T 6	10 mg./kg. body weight, in arachis oil.	i.p.	-	3 3	21 29
H	T 12	10 mg./kg. body weight, in arachis oil.	i.p.	-	4 4 4	35 48 56
I	T 12	10 mg./kg. body weight, in arachis oil.	i.p.	-	4 4 4	56 67 75

Abbreviations: See Table 6.

RESULTS

RESULTS

i. Histology:

Cross-sections of testis tubules of control and treated rats were examined by light microscopy in order to define gross changes in the spermatocyte, spermatid, and spermatozoon population of the seminiferous epithelium. Changes in spermatogonia have not been recorded since this is a cell type generally sparse in occurrence and not readily identified.

Our histological observations are given briefly in the following text, and are aggregated in Table 8, p. 53. Illustrative photomicrographs are then displayed (Figures 15 to 42).

a) DMBA, 5 mg./rat (intravenous):

Day Eight:

Some reduction in the number of resting spermatocytes was apparent, the tubules otherwise appearing normal (Figure 15 and 16).

Day Twenty-two:

In the more severely affected testes the majority of tubules contained only Sertoli cells (Figure 17). In those testes in which the gross weight reduction was less marked, most tubules were depleted of spermatocytes and early spermatids, and many contained late spermatids and Sertoli cells only (Figure 18).

Day Thirty-five:

With rare exceptions the tubules were empty of all but Sertoli cells (Figures 19 and 20). The interstitial area was much increased, Leydig cells being planted within a matrix the area of which, relative to the tubules, was often considerable. This matrix stained well with eosin, and was present only in trace amounts in the normal testis. The involuted testis thus presented the appearance of greatly shrunken tubules embedded within this matrix.

Day Forty-nine and Day Seventy-five:

All the tubules examined remained empty of all spermatogenic cells, and showed a prominent intertubular matrix (Figures 21 and 22). The interstitial cells remained unchanged.

b) DMBA, 2.5 mg./rat (intravenous):

Day Thirty-five:

A fair proportion of tubules appeared normal in appearance. Many showed a reduction of spermatogenic cells, particularly of spermatids. Intertubular matrix was insignificant (Figure 23).

Day Forty-one:

Few spermatozoa were observed. Spermatocytes were abundant, yet spermatids were reduced in number (Figure 24). Some large areas of matrix were evident.

Day Forty-nine:

A total depletion of spermatogenic cells was seen in some tubules but most contained spermatocytes, often in division. Spermatids and spermatozoa were generally absent (Figure 25).

Day Seventy-five:

A number of empty tubules remained, but in most early spermatogenic cells were plentiful. Spermatozoa were very limited in number. A few tubules appeared normal. Intertubular matrix was prominent (Figure 26).

c) Busulphan, 10 mg./kg. body weight (intraperitoneal):

Day Twenty-one:

Some reduction of the number of early spermatocytes was evident.

Day Thirty-five:

Many tubules contained only late spermatids and Sertoli cells (Figure 27). In the other tubules spermatocytes were very scarce (Figure 28).

Day Forty-eight:

Most tubules were empty of spermatogenic cells. A few spermatocytes and spermatozoa were observed (Figure 29).

Day Fifty-six:

The tubules were depleted of late spermatogenic cells, but most contained spermatocytes (Figure 30). The intertubular matrix had shown a gradual increase in area and now was abundant. A few spermatids were observed (Figure 31).

Day Seventy-five:

Almost all tubules contained spermatogenic cells, and many had spermatozoa present. The appearance of the seminiferous epithelium had largely returned to normal (Figure 32).

ii. Enzyme Histochemistry:

Normal testis:

Intense purple deposits of diformazan were prominent in the interstitial regions. Around the periphery within the tubules, black granules were seen in some quantity, but only scattered granules appeared to be associated with the later spermatogenic cells. This pattern of distribution appeared similar in regard to LD, MD,

TABLE 8

CHANGES IN THE GROSS CELL POPULATION OF TESTIS
EFFECTED BY THE ADMINISTRATION OF DMBA AND OF BUSULPHAN

Treatment	Day from injection	Effect produced
DMBA	8	r spermatocytes
5 mg.	22	d spermatocytes, spermatids, and spermatozoa
i.v.	35 49 75	- do -
DMBA	35	r spermatids
2.5 mg.	41	d spermatozoa; r spermatids
i.v.	49	d spermatids and spermatozoa r spermatocytes
	75	d spermatozoa; r spermatids
Busulphan	21	r spermatocytes
10 mg./kg.	35	d spermatocytes and spermatids
i.p.	48	d spermatocytes, spermatids, and spermatozoa
	56	d spermatocytes and spermatozoa
	75	r spermatozoa

r - reduction; d - complete depletion

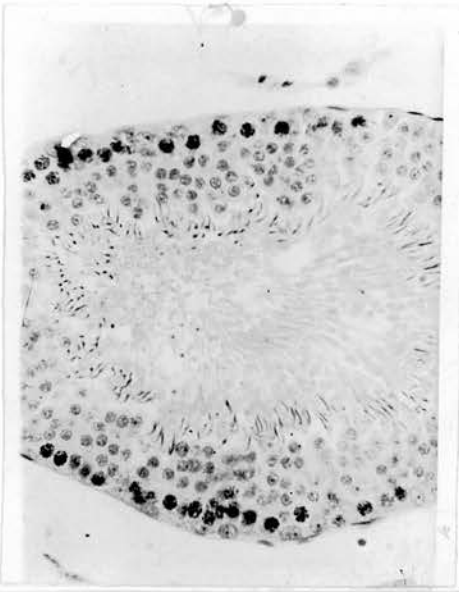


Fig. 15 : DMBA,5 mg., day 8.
Note absence of resting
spermatocytes. (X 225)

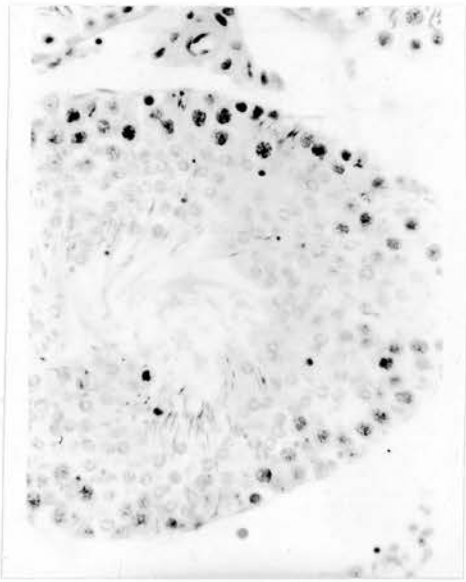


Fig. 16 : DMBA,5 mg., day 8.
Note depletion of resting
spermatocytes. (X 225)

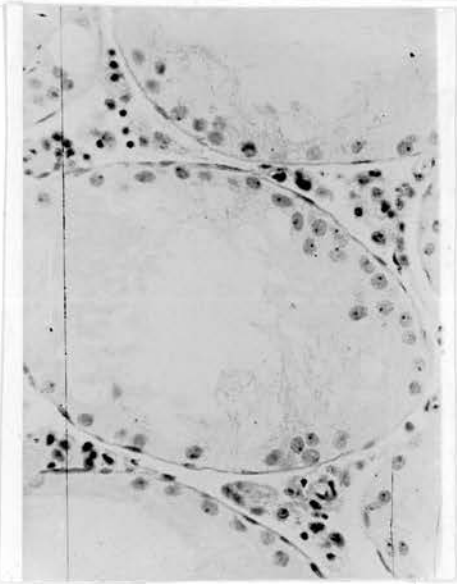


Fig. 17 : DMBA,5 mg., day 22.
Note absence of spermatogenic
cells - Sertoli cells only.
(X 225)

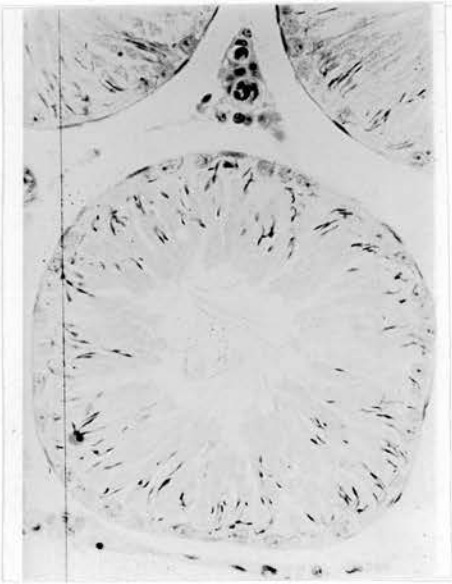


Fig. 18 : DMBA,5 mg., day 22.
Showing Sertoli cells and
late spermatids only.
(X 225)

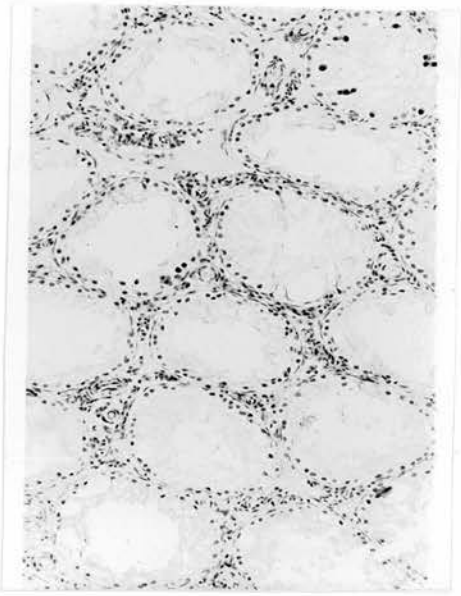


Fig. 19 : DMBA,5 mg., day 35. Sertoli cells only. Note the intertubular matrix. (X 90)

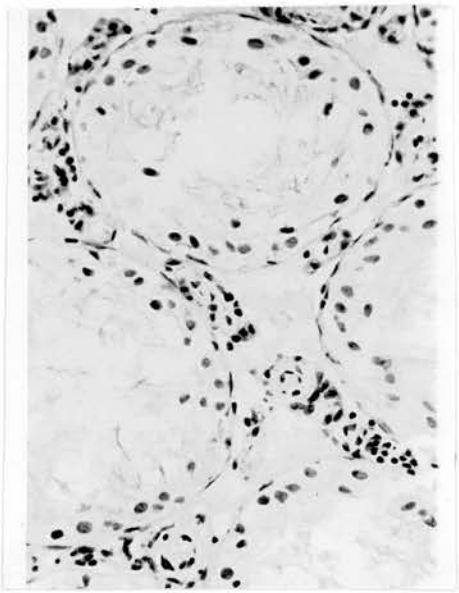


Fig. 20 : DMBA,5 mg., day 35. Detail of tubules with Sertoli cells only. (X 225)

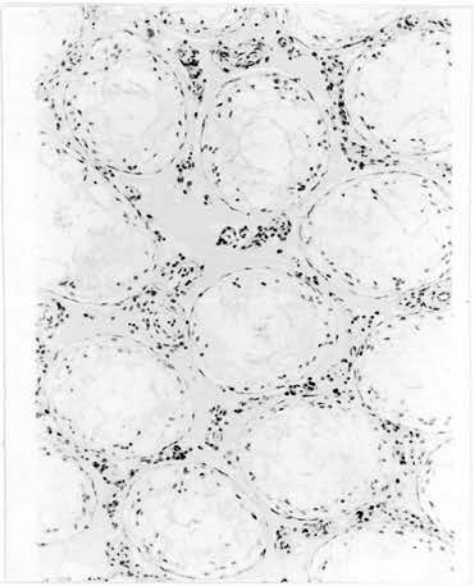


Fig. 21 : DMBA,5 mg., day 75. Sertoli cells only. Note the intertubular matrix. (X 90)

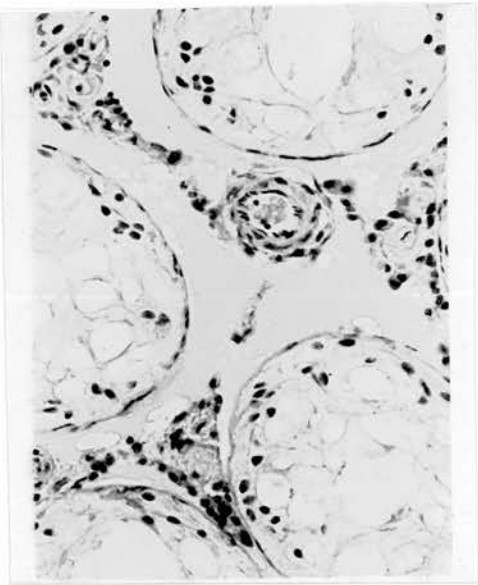


Fig. 22 : DMBA,5 mg., day 75. Detail of intertubular matrix - ? protein exudate. (X 225)

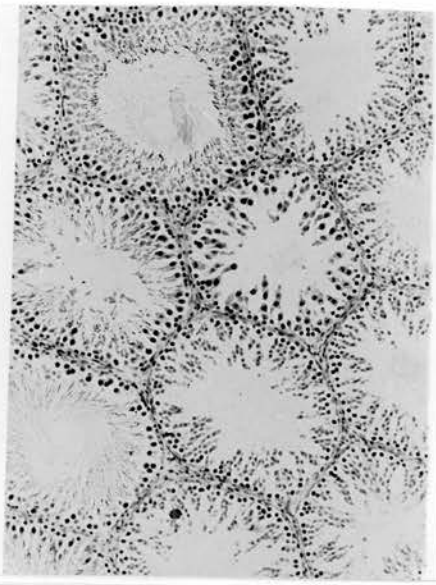


Fig. 23 : DMBA, 2.5 mg., day 35. Some normal tubules, also tubules with reduced number of spermatids. (X 90)

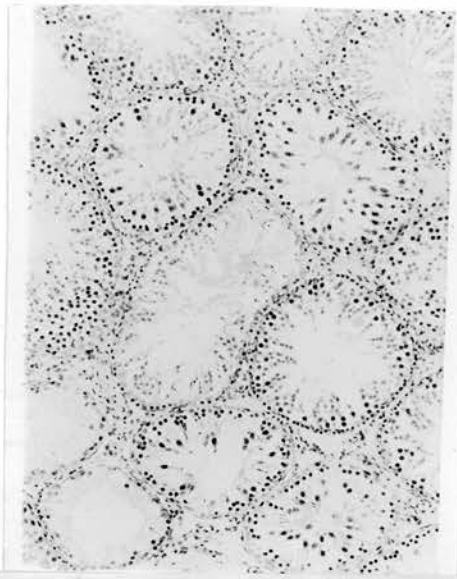


Fig. 24 : DMBA, 2.5 mg., day 41. Note depletion of late spermatids with some reduction of early spermatids. (X 90)

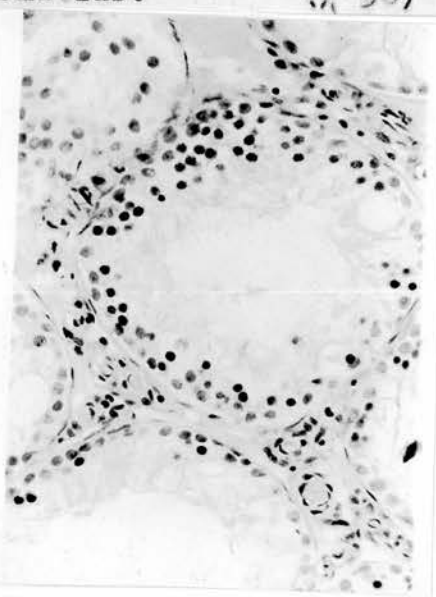


Fig. 25 : DMBA, 2.5 mg., day 49. Note tubule containing Sertoli cells and spermatocytes only. (X 225)

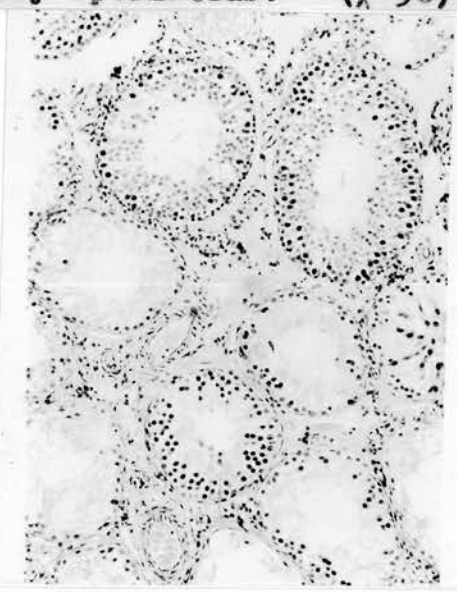


Fig. 26 : DMBA, 2.5 mg., day 75. Some empty tubules, also tubules with regenerating epithelium but no spermatozoa (X 90)

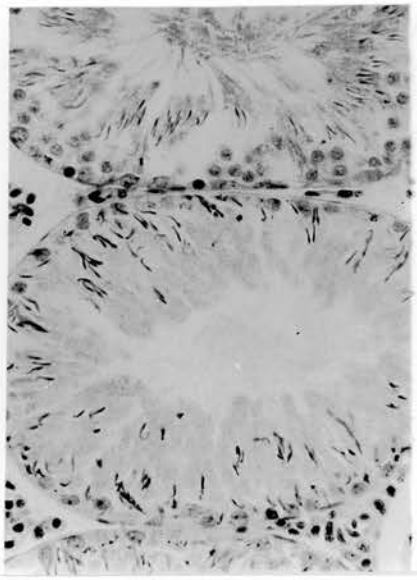


Fig. 27 : Busulphan, day 35. Note tubule containing Sertoli cells and late spermatids only. (X 225)

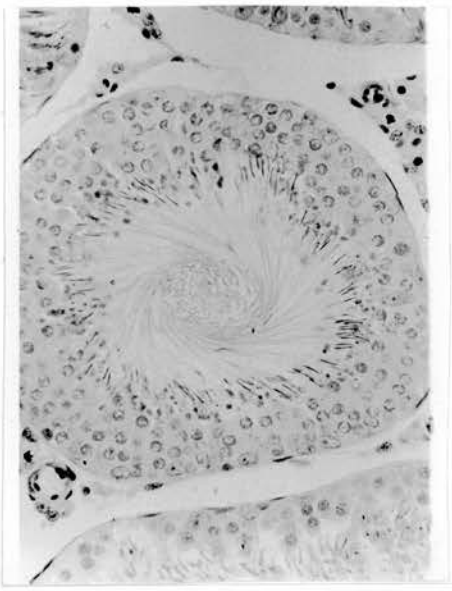


Fig. 28 : Busulphan , day 35. Note tubule deficient of spermatocytes. (X 225)

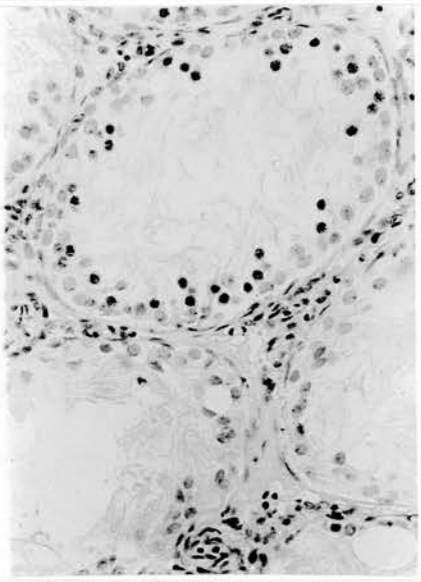


Fig. 29 : Busulphan , day 48. Note empty tubules & tubule with dividing spermatocytes. (X 225)

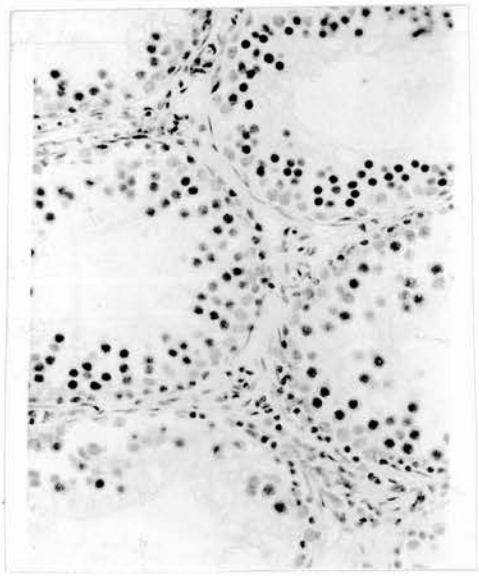


Fig. 30 : Busulphan , day 56. Note regenerating tubules with spermatocytes & no spermatids. (X 225)

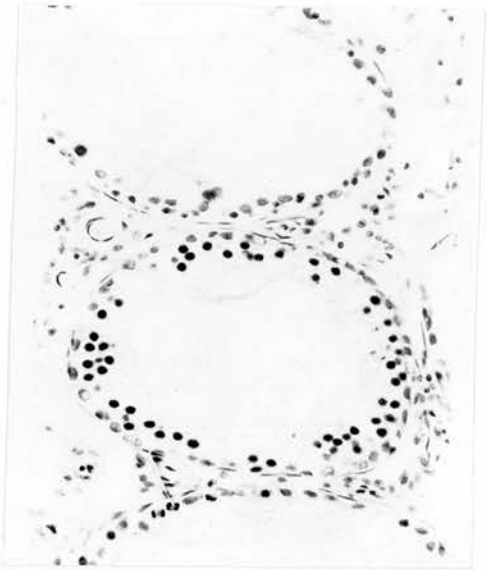


Fig. 31 : Busulphan , day 56. Note tubule with regenerating spermatocytes. (X 225)

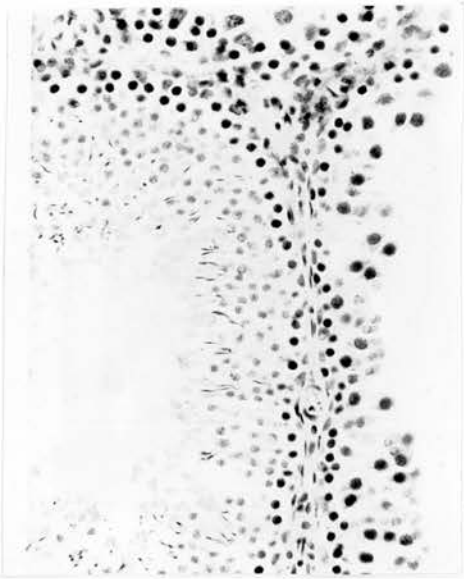


Fig. 32 : Busulphan , day 75. Note, on left, tubule with normal epithelium. (X 225)

Fig. 33 : Normal , MD stain. Note intense interstitial staining. (X 90)

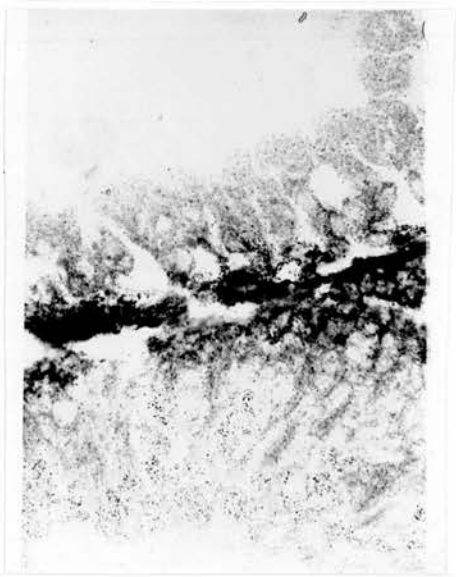


Fig. 34 : Normal , MD stain. Note granular appearance of stain under high power. (X 225)

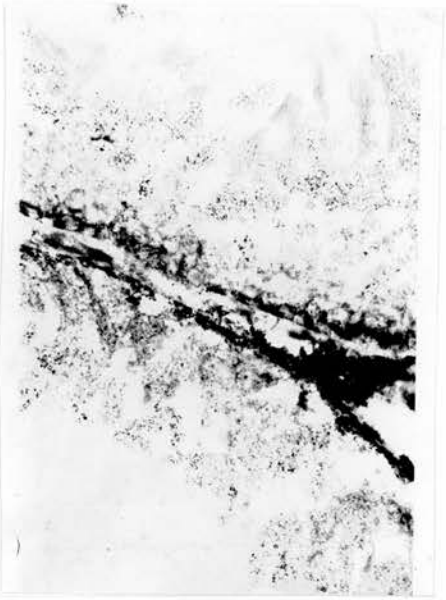


Fig. 35 : Normal , ICD stain. Note the peritubular granules of stain. (X 225)

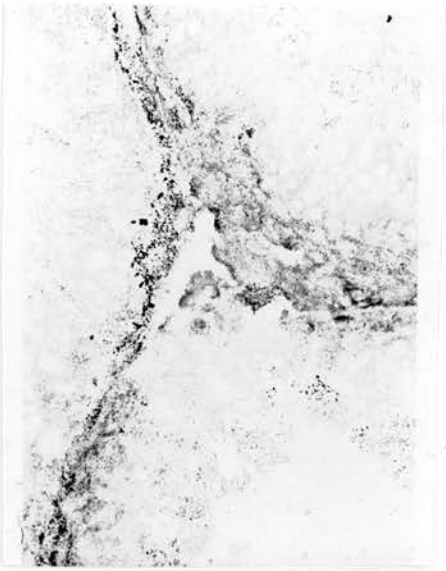


Fig. 36 : Normal , LD stain. Note the peritubular granules of stain. (X225)

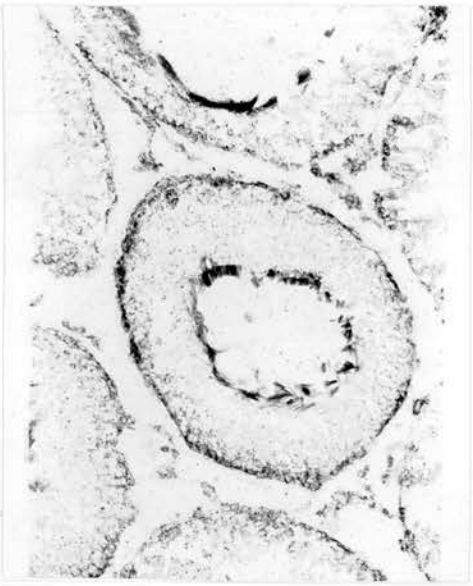


Fig. 37 : Normal , SD stain. Note poor interstitial staining & prominent intratubular staining. (X 90)

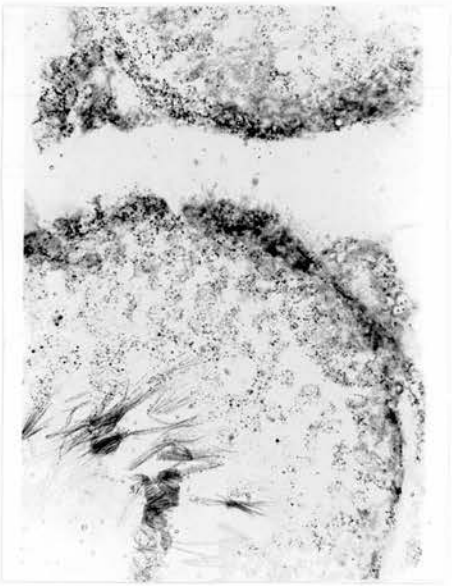


Fig. 38 : Normal , SD stain. Note intense staining of spermatozoa. (X 225)

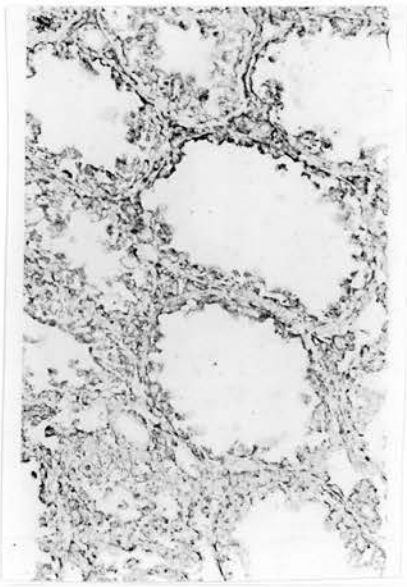


Fig. 39 : Busulphan , MD stain, day 56.
Note the greatly reduced interstitial staining. (X 90)

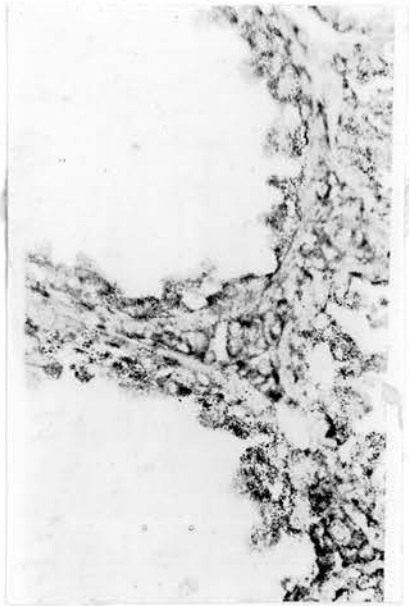


Fig. 40 : Busulphan, MD stain, day 56.
Note granules associated with Sertoli cells and/or early spermatogenic cells. (X 225)

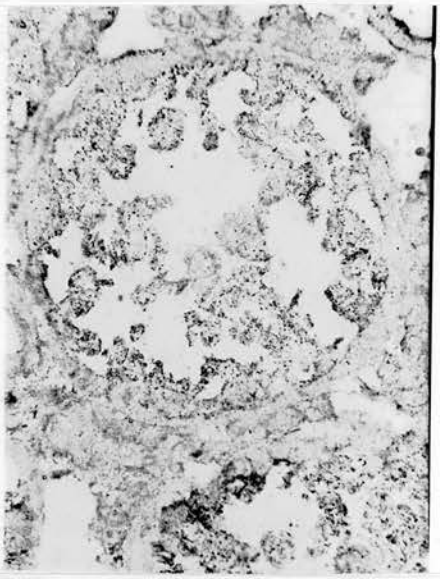


Fig. 41 : DMBA, 5 mg., MD stain, day 49.
Note anomalous staining within a tubule devoid of spermatogenic cells. (X 225)

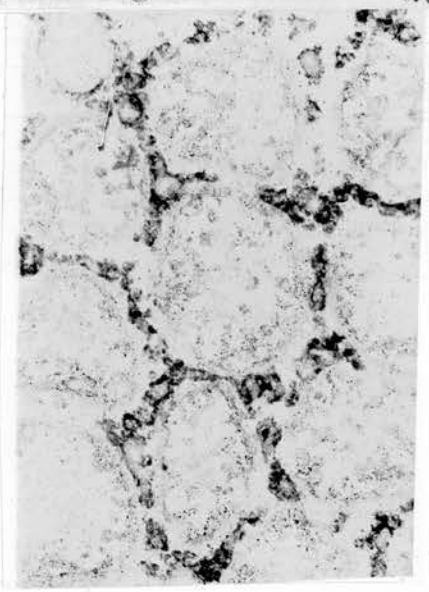


Fig. 42 : DMBA, 5 mg., SD stain, day 49.
Note the intense interstitial staining. (X 90)

and ICD, although LD stained up most readily (Figures 33 to 36). In the case of SD, however, interstitial staining was much less apparent, although spermatogenic cells stained well. Again, peritubular granules were evident, and, in contrast to the other enzymes, intense staining was found associated with the spermatozoa (Figures 37 and 38).

Busulphan, 10 mg./kg. body weight (intraperitoneal), day fifty-six:

Interstitial staining for LD, MD, and ICD was very much less marked than in the normal tissue (Figure 39). Peritubular granules were prominent, as were granules evidently associated with regenerating early spermatogenic cells (Figure 40). In the case of SD, an intense interstitial purple staining was noted, with little staining within the tubules.

DMBA, 5 mg./rat (intravenous), day forty-nine:

A response was given very similar to that observed for busulphan (Figures 41 and 42).

iii. <u>Intraperitoneal injection of DMBA (10 mg./0.4 ml. olive oil per rat)</u>:

All animals appeared normal at seven days. At twenty-six days several had become visibly swollen by an accumulation of ascitic fluid in the peritoneal cavity. Two of four test animals killed at twenty-six days were grossly ascitic, with ascitic fluid total

protein of 3.5 and 2.9 g.%, and with evidence of acute nephritis. A further rat, although not showing ascites, had jaundiced plasma. Within the next few days three further sick test animals were killed, two of which were ascitic, the third being paralysed in the leg. Three animals killed at thirty-seven days had evident pathological changes in tissues within the peritoneal cavity, and one was ascitic. Although a variety of affected tissues were excised from these sick animals subsequent histological examination (Dr. J. P. Smith) revealed no evidence of tumour formation, all changes being inflammatory in nature, suggesting a fibrous reaction to the introduced hydrocarbon.

Of the twenty test animals, thirteen showed evidence of a fibrous reaction within the peritoneal cavity, and six of these were ascitic. In two of the ascitic animals, unilateral testicular atrophy was seen: both animals were grossly ascitic and the atrophy could well be a consequence of this. At day fifty-seven, one of three test animals killed had very small testes, these both being very flabby and of weight only 0.75 g. The MD activity (per g. testis) did not, however, appear to be increased significantly, although the β-glucuronidase was about twofold normal.

iv. Testis weight changes (Figure 43):

After the intraperitoneal administration of busulphan or the intravenous administration of DMBA, marked and consistent decreases of testis weight were apparent. Some recovery toward normal was evident in the busulphan and the half-dose DMBA series, whilst after 5 mg. DMBA, weights remained at minimal levels at seventy-five days.

v. Enzyme activities (Figures 44 to 51):

The changes in enzyme activity are recorded graphically, plotting activity per g. testis (wet weight) and per (whole) testis against the time from administration of 1) busulphan, 10 mg./kg. body weight, i.p. 2) DMBA, 5 mg./rat, i.v., and 3) DMBA, 2.5 mg./rat, i.v. These serial samples are denoted A, B, and C respectively.

Since small numbers only are involved, the results have been expressed in terms of the group mean $\pm$ one standard deviation, as has been recommended appropriate[125B], rather than quoting the standard error.

a) Acid phosphatase (Figure 44):

- per g. testis: A trend to decreased levels was apparent in each case, being most marked in B. In A and C, recovery to within the normal range was evident at day seventy-five. The activities recorded at day thirty-five showed an excessive range. This was due to several values greatly in excess of normal. No experimental

reason for this could be found. However, experimental error would be considered much more likely to result in low levels.

- per testis: Levels of activity were uniformly severely reduced, with some recovery at day seventy-five in A and C, but a continuing fall in B at this time.

b) Alkaline phosphatase (Figure 45):

- per g. testis: Activity was found normal throughout, with the exception of a slight increase at day twenty-one in A.

- per testis: A marked reduction pari passu with fall in testis weight was evident, A and C showing recovery toward normal at day seventy-five.

c) 5'-Nucleotidase (Figure 46):

- per g. testis: Increases were noted in each case, being most pronounced in B, where a 2.2-fold increase was observed at forty-nine and seventy-five days.

- per testis: Activities appeared slightly reduced only, the highest levels per g. testis in B being associated with activities per testis within the normal range.

d) Malate dehydrogenase (Figure 47):

- per g. testis: An increase of activity was noted in each case, rising to a maximum of a little below two-fold levels at the

time of maximum testis weight loss, with later recovery toward normal. The activity at day forty-nine in B was only slightly raised. Since these determinations were carried out together with the C series - these being elevated - it is thought that the apparent discrepancy is genuine and not experimental.

- per testis: A decrease of activity was evident in each case, being least noticeable in A.

e) Glucose-6-Phosphate Dehydrogenase (Figure 48):

A very limited sample only was investigated.

- per g. testis: A marked increase was evident in each case, levels rising about 2.5-fold in B.

- per testis: Little change from normal was apparent.

f) Glutamine synthetase (Figure 49):

- per g. testis: In B, duplicate samples taken at twenty days showed high activity. These determinations were carried out in parallel with other normals and are thus thought to reflect a genuine change. Increased levels were also noted at day forty-nine, similarly with C, and at day seventy-five with A.

- per testis: With the exception of B, day twenty, all activities were reduced. Some recovery toward normal was evident in A and C, but not in B.

g) β-Glucuronidase (Figure 50):

- per g. testis: Marked increases in activity were apparent. With series B, at thirty-five days, the increase was five-fold normal; with C, 1.5-fold. With A at day fifty-six the increase was almost four-fold. At day seventy-five activities appeared to be falling in A and C.

- per testis: The highest levels of activity per g. testis were associated with increases per testis, this being most evident in B.

h) Hyaluronidase (Figure 51):

- per g. testis: Levels of activity were found very greatly reduced, minimal levels being about 12% of normal. In only one case, however, (A, day fifty-seven) was nil activity recorded.

- per testis: Levels were maximally reduced to a level about 6% of normal. Some activity recovery was noted at day seventy-five with A and C.

vi. Fertility trial:

The results obtained in the fertility trial of DMBA are given in Table 9, p. 59, and are discussed on p. 93.

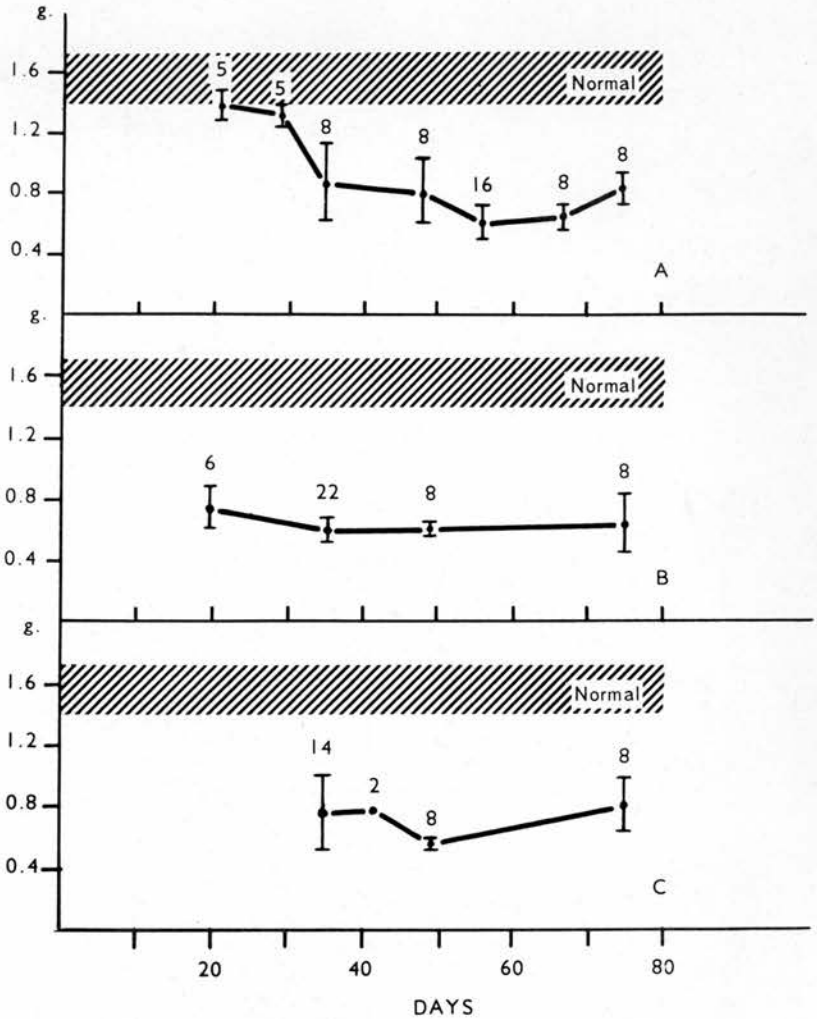


Figure 43 : Changes in Testis Weight.

A : Busulphan , 10 mg. / kg. body weight .

B : DMBA , 5 mg. / rat .

C : DMBA , 2.5 mg. / rat .

Abscissa - days from injection. Ordinate - testis weight (g.)

Range marks indicate mean ± 1 S.D., & numerals number of testes.

Normal range : mean of 58 testes ± 1 S.D.

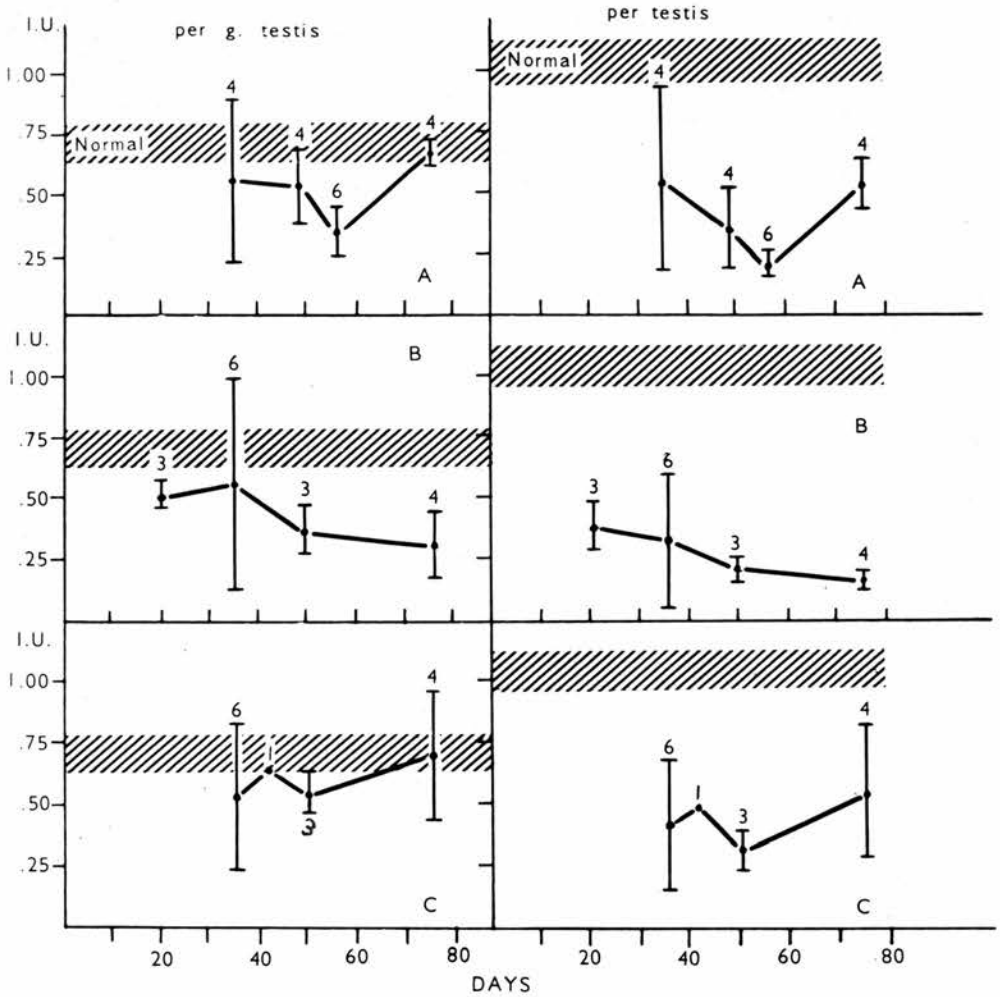


Figure 44 : Changes in Acid Phosphatase .

A : Busulphan , 10 mg. / kg. body weight .

B : DMBA , 5 mg. / rat .

C : DMBA , 2.5 mg. / rat .

Abscissa - days from injection. Ordinate - acid phosphatase (I.U.)

Range marks indicate mean ± 1 S.D., and numerals number of testes.

Normal range : mean of 12 testes ± 1 S.D.

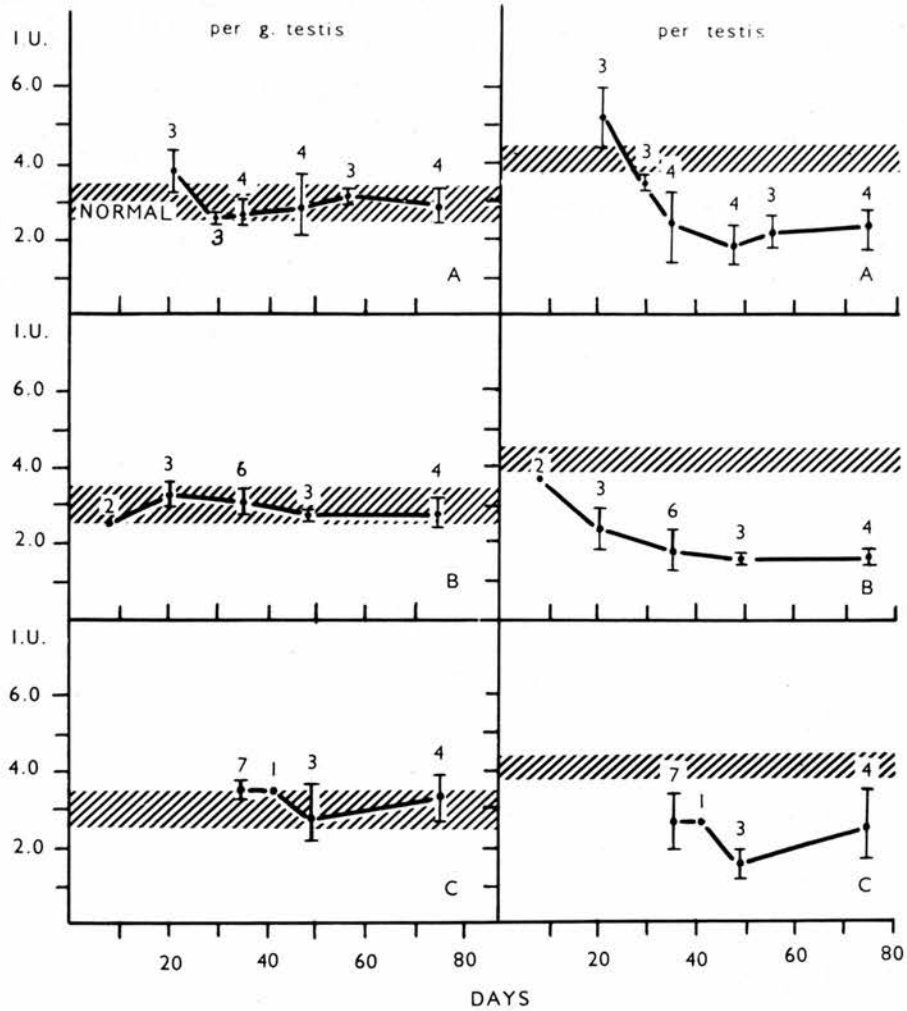


Figure 45 : Changes in Alkaline Phosphatase .

A : Busulphan , 10 mg. / kg. body weight .

B : DMBA , 5 mg. / rat .

C : DMBA , 2.5 mg. / rat .

Abscissa - days from injection. Ordinate - alkaline phosphatase (I.U.)

Range marks indicate mean ± 1 S.D.,and numerals number of testes .

Normal range : mean of 12 testes ± 1 S.D.

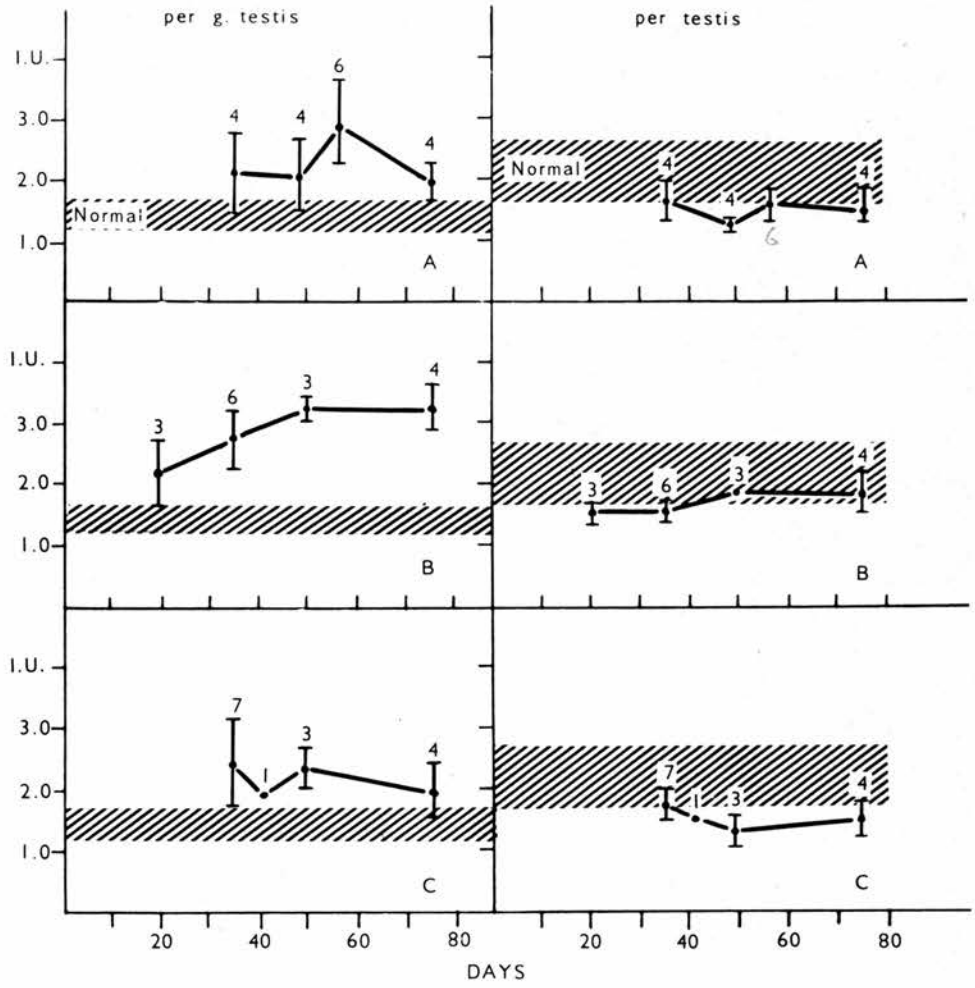


Figure 46 : Changes in 5'-Nucleotidase .

A : Busulphan , 10 mg. / kg. body weight.

B : DMBA , 5 mg. / rat .

C : DMBA , 2.5 mg. / rat .

Abscissa - days from injection. Ordinate - 5'-nucleotidase (I.U.)

Range marks indicate mean ± 1 S.D., and numerals number of testes .

Normal range : mean of 12 testes ± 1 S.D.

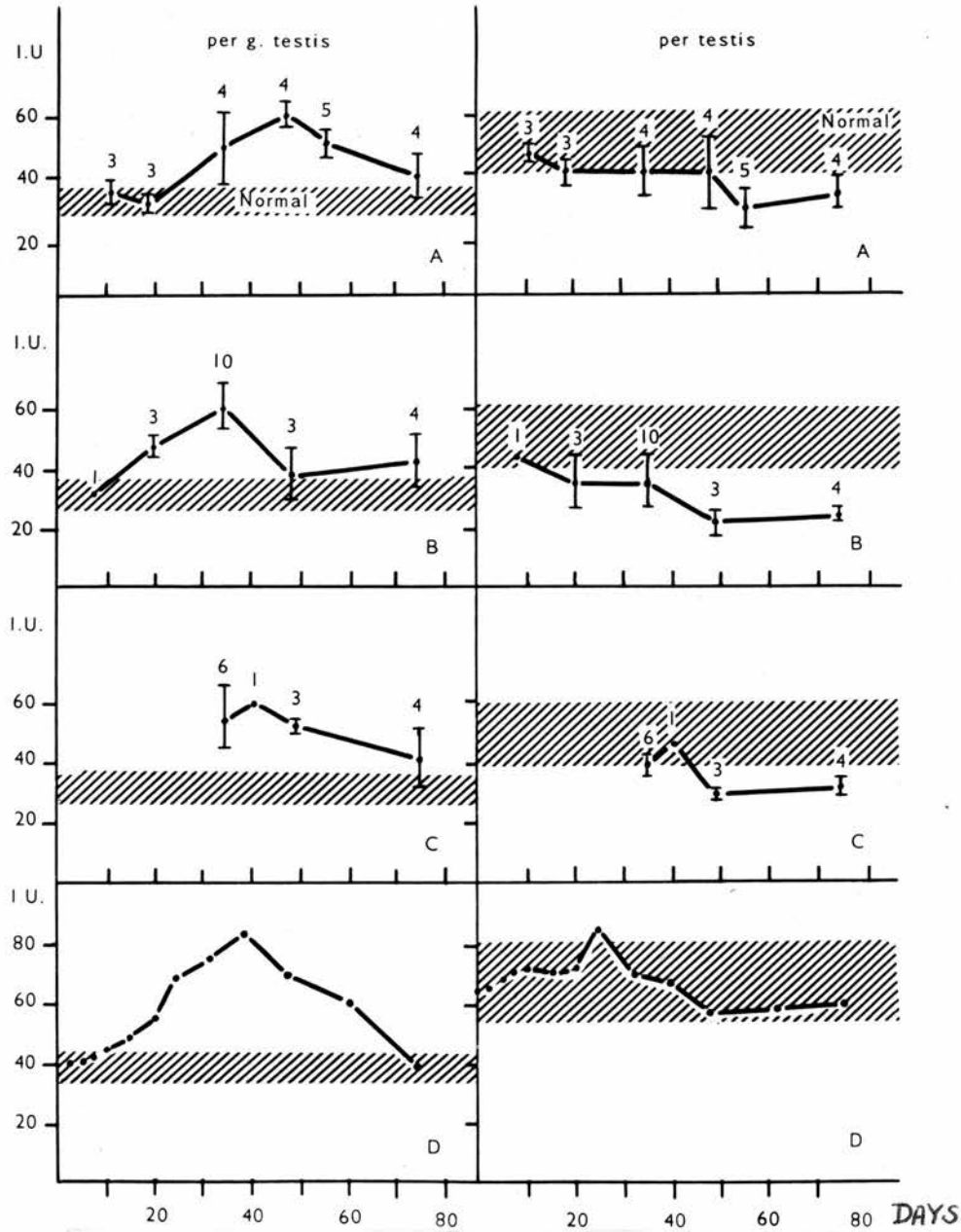


Figure 47 : Changes in Malate Dehydrogenase .

A : Busulphan, 10 mg./kg. body weight ; B : DMBA, 5 mg./rat ;
C : DMBA, 2.5 mg./ rat.

D : DMBA, 5 mg./rat, from Ford and Huggins[55]. Each group consists of four testes. Activity per testis has been calculated.

Abscissa - days from injection. Ordinate - malate dehydrogenase (I.U.)

Range marks indicate mean ± 1 S.D., & numerals number of testes .
Normal range : mean of 25 testes ± 1 S.D.

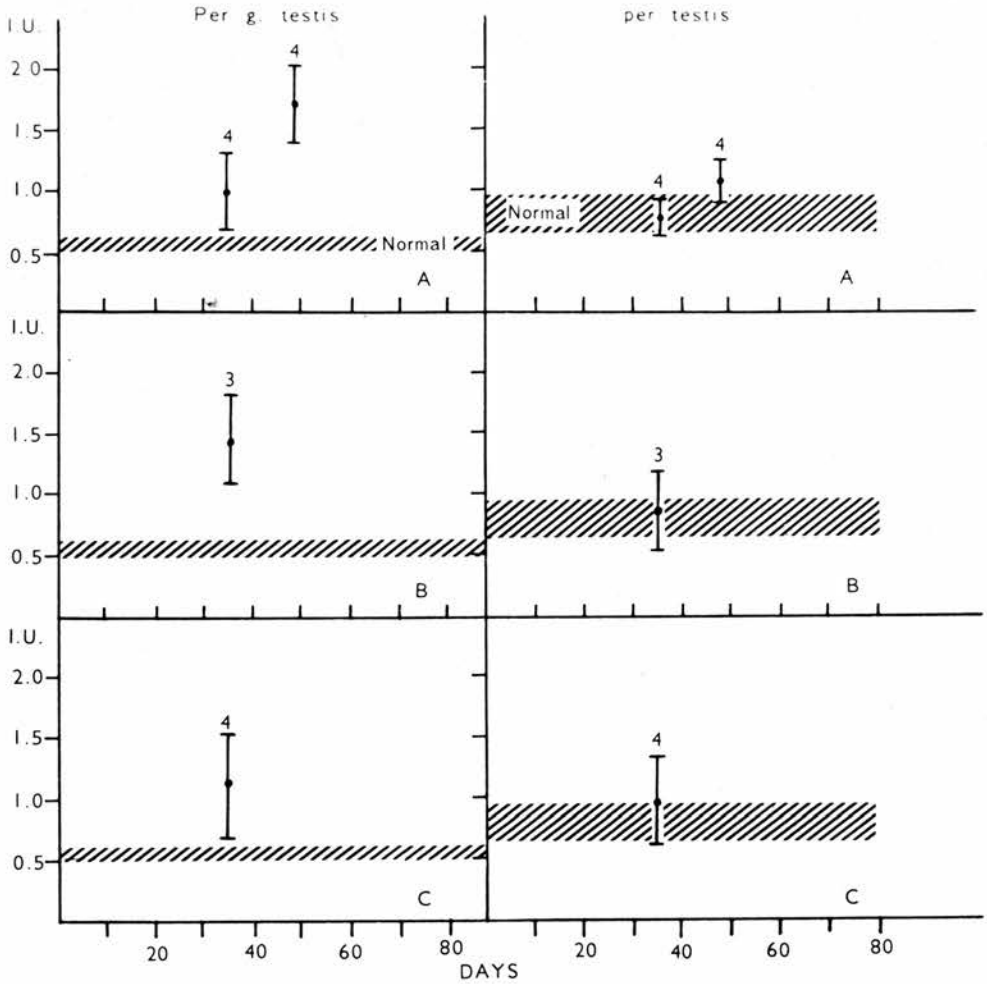


Figure 48 : Changes in Glucose-6-Phosphate Dehydrogenase .

A : Busulphan , 10 mg. / kg. body weight.

B : DMBA , 5 mg. / rat .

C : DMBA , 2.5 mg. / rat .

Abscissa - days from injection. Ordinate - glucose-6-phosphate dehydrogenase (I.U.)

Range marks indicate mean ± 1 S.D., and numerals number of testes .

Normal range : mean of 5 testes ± 1 S.D.

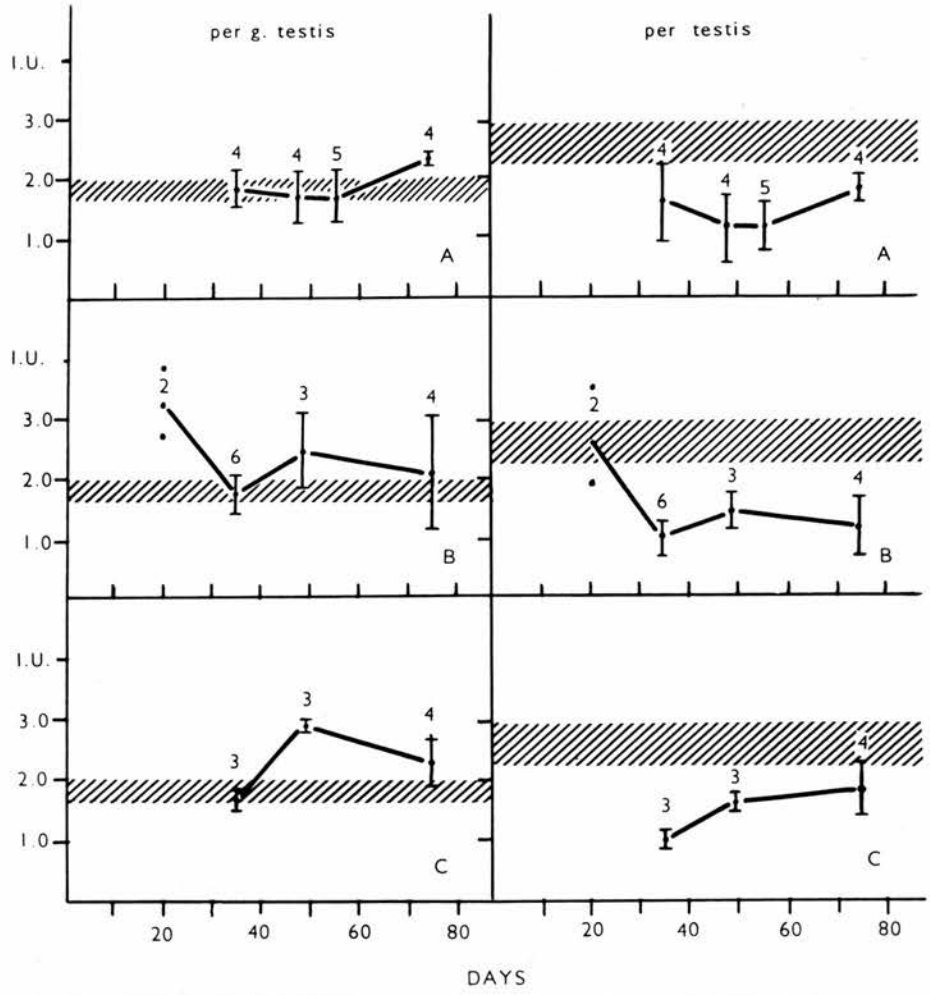


Figure 49 : Changes in Glutamine Synthetase .

A : Busulphan , 10 mg. / kg. body weight.

B : DMBA , 5 mg. / rat .

C : DMBA , 2.5 mg. / rat .

Abscissa - days from injection. Ordinate - glutamine synthetase (I.U.)

Range marks indicate mean ± 1 S.D., and numerals number of testes .

Normal range : mean of 12 testes ± 1 S.D.

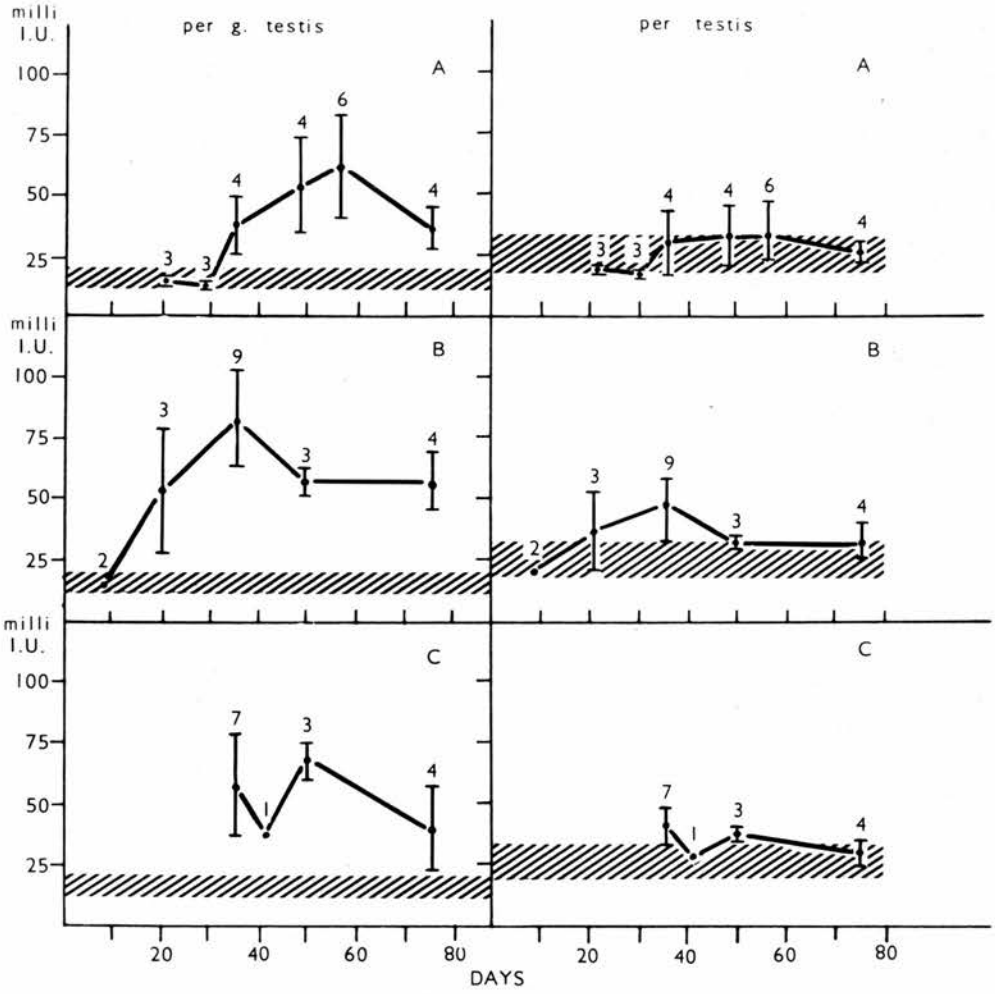


Figure 50 : Changes in β-Glucuronidase .

A : Busulphan , 10 mg. / kg. body weight .

B : DMBA , 5 mg. / rat .

C : DMBA , 2.5 mg. / rat .

Abscissa - days from injection. Ordinate - β-glucuronidase(milli-I.U.)

Range marks indicate mean ± 1 S.D., and numerals number of testes .

Normal range : mean of 24 testes ± 1 S.D.

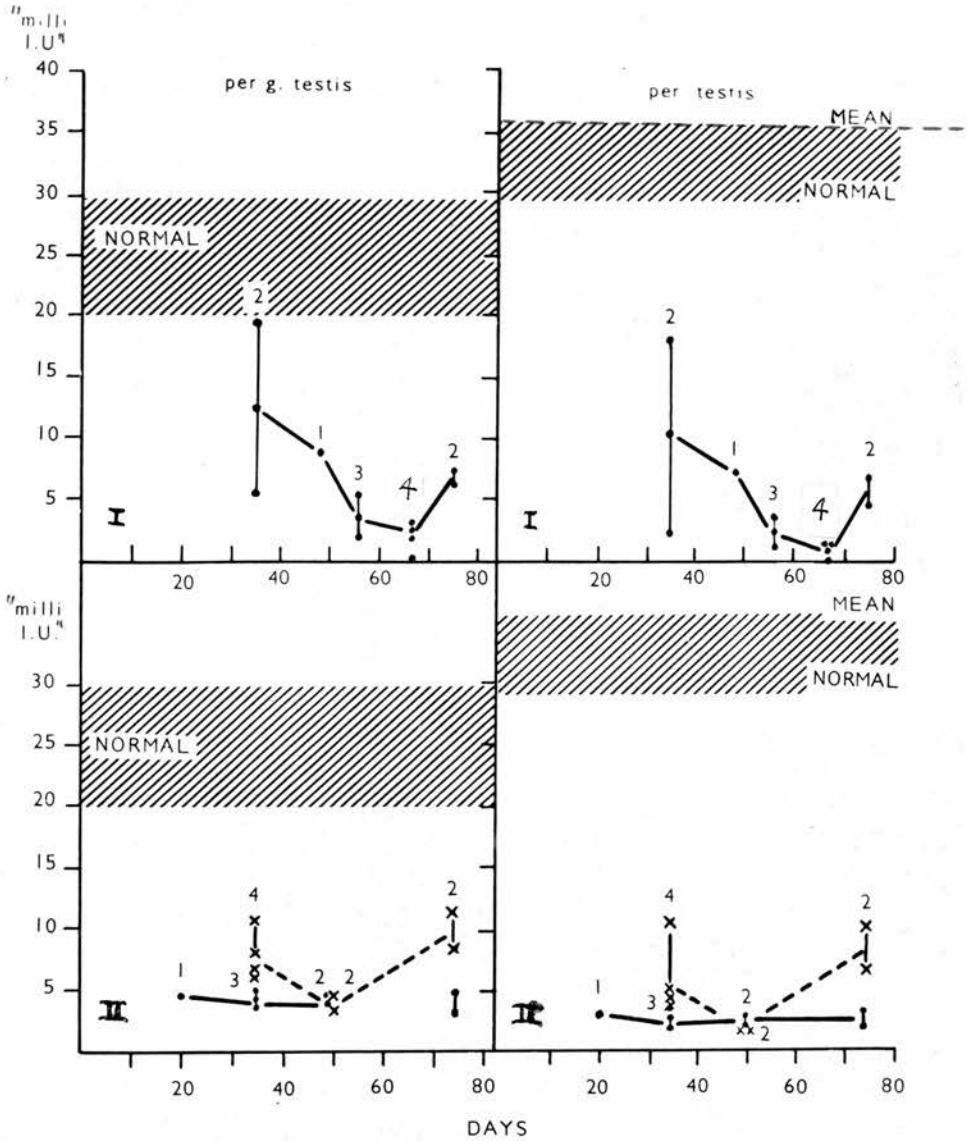


Figure 51 : Changes in Hyaluronidase .

I. Busulphan , 10 mg. / kg. body weight [A]

II. DMBA , .– 5 mg. / rat [B]; x– 2.5 mg. / rat [C]

Each point represents one testis .

Abscissa – days from injection. Ordinate – hyaluronidase("milli-I.U.")

Range of normal – per g. testis , mean of 13 testes ± 1 S.D.

per testis , mean of 13 testes – 1 S.D.

TABLE 9

EFFECT OF SINGLE DOSE OF DMBA (0.25 mg., i.v.) ON THE FERTILITY OF MALE RATS

Mating period		1	2	3	4	5	6	7	8	9
Days from treatment		0-6	7-13	14-20	21-27	28-34	35-41	42-48	49-55	56-62
Rat No.										
1	A	5(1)	0	10	0	0	0	0	0	0
	B	nil	few	abun.	-	few	nil	nil	mod.	nil
2	A	9	0	0	0	(2)	0	0	0	0
	B	nil	nil	nil	-	abun.	nil	nil	abun.	nil
3	A	0	9	0	0	1	12	0	7	0
	B	nil	mod.	nil	-	abun.	abun.	nil	nil	few
4	A	11	5	0	0	0	(2)	0	0	0
	B	few	mod.	nil	-	mod.	abun.	nil	nil	nil
5	A	0	0	8	0	0	0	0	0	0
	B	few	few	few	-	nil	abun.	nil	mod.	nil
6	A	10	6	8	4	0	6	0	0	0
	B	few	mod.	nil	-	mod.	few	mod.	nil	nil
7	A	7	0	6	0	8	4	2	0	0
	B	mod.	nil	nil	-	abun.	abun.	few	mod.	nil
8	A	0	3	0	9(1)	0	0	0	0	0
	B	nil	nil	nil	-	nil	nil	few	few	nil
Average litter size		5.3	2.9	4.0	1.6	1.4	3.0	0.25	0.9	0

A denotes individual litter size (in brackets - born dead)

B denotes number of sperm seen in vaginal smear
mod. - moderate number; abun. - abundant number.

Each vertical column shows the results of pairing eight treated male rats for nine consecutive weeks with females known fertile.

DISCUSSION

DISCUSSION

i. Intraperitoneal administration of DMBA:

The intraperitoneal administration of 7,12-dimethylbenz(a) anthracene (DMBA) produced an unexpected response of a pathological nature. For this reason this part of our experimental work proved abortive, and will be fully discussed before examining our subsequent work. Since this compound is so markedly effective as a carcinogen it was suspected that tumour formation had been induced. However, no histological evidence for this was found.

In work on female rats Huggins et al. obtained 100% (mammary) tumours in seven hundred rats given 20 mg. DMBA orally[80]. Similar results followed injection of DMBA into the lumen of the colon, or intravenous injection[78]. Mammary tumours are not induced in male rats by painting the skin[80] or by the administration of DMBA[81]. Ford and Huggins, after extensive experiments with DMBA given to male rats, commented: "cancer did not arise in the present experiments, and it is inferred that the absence of neoplastic transformation is due to the lethal effects which 7,12-DMBA exerted on cells vulnerable to it"[55].

The intraperitoneal injection of DMBA with Lipomul emulsion as medium did not cause tumours of the peritoneum or abdominal viscera in newborn male, or in female, rats, whereas a compressed pellet of the compound embedded within the peritoneum caused tumour formation

at the site of encapsulation[81]. It is supposed that Lipomul-DMBA disappears rapidly from the peritoneal cavity, before adequate contact with a cell in a state susceptible to the malignant transformation.

It is stated by Huggins that the intraperitoneal injection of concentrated oil solutions of polynuclear aromatic hydrocarbons is unsatisfactory, because "they elicit large amounts of fibrin and fibrous tissue which cover all the abdominal viscera and cause death in many animals"[81]. Reference may be made to Lees[97], who administered intraperitoneally to rats a benzanthracene in sesame oil, and detected many fibrous adhesions throughout the peritoneal cavity at autopsy. Twenty-one of forty-eight animals died within sixty-four days, eleven of twenty-one deaths being at forty to sixty-four days. Other workers have reported severe ascites and death of the test rats after the intraperitoneal injection of DMBA in arachis oil[21].

Boyland[19] and Haddow[66] suggested means of emulsification of hydrocarbons for animal injection, and such preparations are still in use. For example, Bather[9] induced malignant lymphoma in mice by giving 60 μg. DMBA in 0.02 ml. of 1% gelatin, subcutaneously. The lipomul preparation, however, would appear to be a great advance in the convenient presentation of carcinogenic hydrocarbons. It is recommended by Huggins as being "equally effective, less hazardous to laboratory personnel, more convenient, and less costly" as compared with oral presentation[84]. Furthermore, variations in the rate of absorption after oral dosage are obviated.

It is clear that our findings after the intraperitoneal injection of DMBA are in accord generally with the literature discussed above, and no conclusions as to selective testicular effects can be drawn.

ii. Histology:

We have shown the intraperitoneal injection of busulphan to produce histological effects in rat testis similar to those reported by Jackson et al.[86], notably a selective toxic effect upon spermatogonia and possibly primary spermatocytes, with a later regeneration toward normal spermatogenic epithelium. Intravenous DMBA in our hands produced a more severe effect than that reported by Ford and Huggins[55], the same dosage (5 mg.) causing irreversible destruction of the spermatogenic epithelium. However, we have used Wistar rats in contrast to the Sprague-Dawley strain used by the other authors, and a strain difference in susceptibility is quite likely. The half-dose of DMBA (2.5 mg.) did cause the degeneration of testis characterised by Ford and Huggins[55], indicating damage primarily to spermatogonia. By both of these treatments we have therefore effected a subsequent wave of depletion of particular spermatogenic cell types. It was our intention to investigate the enzymic concomitants of this.

A number of premature deaths occurred following the injection of DMBA (5 mg., intravenous) [see Table 6, p. 48]. The production of adrenal necrosis in rat by the administration of DMBA reported by Huggins and Morii[79] has been confirmed by others. A group of seventy-five rats given 20 mg. DMBA orally in sesame oil showed 100% survival

at three days, but 84% had suffered adrenal necrosis[167]. Total destruction of the outer two zones of the adrenal cortex (zona fascicularis, zona reticularis) is produced, the zona glomerulosa and medulla being unaffected. Fatal adrenal insufficiency does not usually occur, and tissue regeneration subsequently takes place[82].

It is suggested that the few premature deaths observed in our present experiments may have been a consequence of adrenal crisis, since death was not immediate but commonly occurred at an interval of about three days from injection, at which time adrenal damage is reported most severe. Animals which survive adrenal necrosis are likely to have a poor adrenal reserve, and this factor may have a bearing upon the changes in spermatogenesis observed later.

It is known that the nutritional status of the animal can have a marked effect upon spermatogenesis[105]. Since our rats were not carefully controlled by recording body weight and food intake, this should be borne in mind, as also the fact that the rat is able to withdraw the testes into the abdominal cavity at will[24].

The changes in testis weight observed in our experimental work (Figure 43, p. 59) appeared to correlate with changes in spermatogenic cell population. The fall for this reason was least abrupt after busulphan, and no recovery of weight loss was observed in the 5 mg. DMBA series, where destruction of the spermatogenic epithelium was evidently irreversible. The minimal weight attained in each case

was about 40% of normal, as compared with Ford and Huggins' 55% normal[55]. It may be emphasised that other workers have observed a fall in testis weight (after hypophysectomy) down to as little as 22% normal yet have considered the histological appearance of the Leydig cells to have remained normal[123].

A feature of the testicular atrophy induced in the present investigations was the clearly evident shrinkage of tubules and the appearance of "intertubular matrix". The latter phenomenom would appear to correlate with the changes observed by others following X-irradiation, when it was reported that irradiation of the upper abdomen of human subjects would frequently occasion a "post-radiation nephritis" in which extensive degeneration of kidney tubules was produced[139]. The most radiation-sensitive tissue component being mesenchymal, arteriolar necrosis so induced effects a profound disturbance of the local blood circulation and hence malfunction of the organ. The associated interstitial oedema "will lead to a state where each tubule is separated from its neighbouring tubules and capillaries by protein exudate, and as time passes this exudate will become the site of collagen precipitation ..." (arteriolar hyaline necrosis). Such a reaction was reported evident in several extra-renal sites. Atrophic changes within testis similar to those observed in the present work were noted. It is suggested therefore that a disturbance of interstitial circulation could therefore be a factor of significance in tubular degeneration effected by DMBA and by busulphan.

iii. Histochemistry:

In interpreting enzymic changes in testis homogenates we must take account of three particular functional areas - a) spermatogenic cells, b) Sertoli cells, and c) Leydig cells, together with other structural tissues such as tubular walls, connective tissues, and blood vessels. Since no study appears to have been made of the distribution of malate dehydrogenase (MD) in rat testis it was thought necessary to investigate this. The related citric acid cycle enzyme isocitrate dehydrogenase (ICD), of unknown distribution, was also studied, together with lactate dehydrogenase (LD) and succinate dehydrogenase (SD), which have been investigated by other workers (see p. 15) and study of which was desirable in order to confirm the methodology. It was also considered of general value to undertake some study and to gain practical experience of enzyme-histochemical methods since enzyme changes within tissues may be assessed both by these means and by homogenate studies. The two approaches are complementary, though both imperfect.

Whereas the enzyme content of tissue extracts may be quantitated, enzyme histochemistry, particularly of the dehydrogenases, offers precise localisation via the distribution of particulate reaction products not susceptible of measurement even by methods of cytophotometry. In interpreting histochemical findings it must be remembered, as observed by Pearse[129], that "we have ... very little evidence in the

case of most histochemically demonstrable enzymes that they are identical with the biochemical enzyme whose name they bear, or that their relative performances can be correlated in any way". Enzyme activity as measured *in vitro*, however, employing homogenates, bears an uncertain relation to the actual concentration of the enzyme, since many variable factors influence the activity, such as coenzymes, activators, and inhibitors. Furthermore, the effective activity of enzymes *in vivo* is largely determined by variable features such as membrane permeability to substrate and spatial organisation within the cell (e.g. endoplasmic reticulum).

About fifty enzymes may be detected histochemically[129]. The functional significance of most of these is obscure. Methods for dehydrogenases are particularly applied since the metabolic function of this group is known to some extent. They are especially associated with the mitochondria, whose integrity is essential to the cell since they comprise the main energy-generating source. Insult to the cell may effect an increase in mitochondrial permeability. Recent dehydrogenase methods can give rise to particles of insoluble coloured formazan of diameter ca. 0.3 μ, several of which may be present within a single mitochondrion (length ca. 3 μ). Damaged mitochondria readily show particularly marked deposits, their permeability to reaction ingredients being increased. On this basis has been developed a technique of "mitochondrial assay" by which tissue damage can be finely localised by microscopic examination at high magnification.

By illustration of this technique we may instance the effects of magnesium deficiency upon kidney. Gross changes may be detected histologically after about nine days' deficiency (calcification), whilst mitochondrial changes are evident upon histochemical examination at the same site within hours[127]. Discussing such methods, Pearse observes "... a further application of these techniques can be foreseen in the field of experimental pharmacology, where it will be possible to demonstrate the earliest effects of drugs upon cells in sections, smears, or tissue cultures ...". Such methods have already given direct evidence that histologically similar cells do not necessarily have the same enzymic composition[127].

Certain discrepancies between histochemical and biochemical findings have been recorded. For example, both ICD and glucose-6-phosphate dehydrogenase (G6PD) have been found intramitochondrially whereas they are recorded in the supernatant after centrifugation following homogenisation of tissue[127]. β-Glucuronidase distribution appears uncertain[128, p.492]. A number of dehydrogenase enzymes have been assayed <u>in vitro</u> both by direct measurement of oxygen uptake by the system and by measurement of the amount of formazan derived during the oxidation. It was found that the latter values were only about one-tenth of the former. It was considered that the formazan reaction mixture inhibited activity of the enzyme[128, p.543].

The Gomori method[63] for the demonstration of phosphoamidase has been severely criticised after comparison with a biochemical method[6]. It was found that only 8% of the original activity of (kidney) sections survived the histological procedures recommended by Gomori. This would appear to account for the notorious difficulty in applying the method, and to lay the quantitative findings of Meyer and Weinmann, in relation to testis (see p. 24), open to criticism. It is to be anticipated that our understanding of the enzymic composition of tissues is likely to be fruitfully enhanced by similar parallel histochemical and biochemical (homogenate) studies.

In the present investigations we have applied standard histochemical methods and examined the resulting stained sections by light microscopy at low magnification. The granules of stain have been considered to originate within the mitochondria, although these could not of course be visualised. In the normal testis MD and ICD were found predominantly in the interstitial tissue, as was LD. The greatly altered staining pattern apparent after busulphan or DMBA treatment was unexpected. Interstitial staining of LD, MD, and ICD was markedly reduced. This altered pattern was repeatedly elicited and contrasted with the normal distribution.

Since the atrophic testis is extremely friable to handle as a frozen section it was thought possible that the interstitial tissue might have dropped away from the shrunken tubules. Confirmation

of the continued presence of the interstitial tissue in our cryostat sections was made by using nuclear stains. The contrasted staining pattern of SD (post-treatment), with intense interstitial staining, also served to confirm the integrity of the sections investigated. Staining for SD was prominent in the spermatozoa of normal testis; the middle-piece of the spermatozoon is known to be rich in mitochondria[106, p.25].

Some staining, particularly with the post-treatment sections, was diffuse and not evidently particulate. It was considered that this could have been due to the solution of diformazan in lipid deposits. The application of lipid stains to cryostat sections yielded negative results however.

It should be noted that intense staining in our dehydrogenase method may indicate a high local concentration of enzyme or a site of particular mitochondrial damage, or both. If we assume minimal mitochondrial damage in the normal testis sections, staining may be taken to indicate the sites of natural occurrence of the enzyme. Thus MD is seen to be primarily distributed in the interstitial tissue. The peri-tubular granules we have observed could be associated with Sertoli cells or with early spermatogenic cells, or with both.

Testis examined for MD forty-nine days after DMBA, 5 mg., still presented this feature of peri-tubular granules, although our histology indicated a complete absence of spermatogenic cells. We therefore conclude that the staining is associated with Sertoli cells.

Staining well within the lumen of the tubules was also noted. This is thought to arise in the cytoplasm of the Sertoli cells, which can form a synctcytium within the tubules. The minor degree of interstitial staining indicates a low concentration of enzyme at this site.

We have demonstrated granular staining within the tubule after busulphan, at fifty-six days. This could be associated with early regenerating spermatogenic cells or with Sertoli cells, or with both. The methods used have not allowed us to clarify this point, and it must be emphasised that granular staining within the lumen of the tubules could be essentially a feature of Sertoli cytoplasm and not of spermatogenic cells.

The general tentative conclusion we may draw from our histochemical studies of the atrophic testes is that the activity of MD appears to remain unchanged in the Sertoli cells and to be much reduced in the Leydig cells.

Our findings may be compared with those of Kormano et al.[93], where histochemical concomitants of unilateral cryptorchidism were investigated. It was found by these authors that interstitial LD and G6PD remained unchanged, whereas interstitial SD appeared increased in activity. We have found a severe reduction of interstitial LD, MD, and ICD, with an increase of SD. Kormano et al., however, observed a marked interstitial decrease in certain other enzymes, and it is

possible that our experimental conditions have provoked a more intense metabolic inhibition than is consequent upon cryptorchidism. Furthermore, adequate quantitative comparisons are not possible, since methods of assessment must necessarily be subjective. For example, Kormano et al. report unchanged interstitial LD and G6PD with an increased tubule activity of these enzymes, although with a shorter time of incubation a report of reduced interstitial activity with unchanged tubule activity could have been offered.

iv. Homogenate studies:

Since a basic purpose of the present work was to confirm the findings of Ford and Huggins[55], enzyme studies were made on an extract of testis obtained likewise with isotonic saline. However, these authors gave no details of the method of homogenisation used.

The ideal "homogenate" is a whole tissue preparation in which cell disruption is as complete as possible whilst the destruction of all cell particulates is minimal[133]; this ideal cannot be realised in practice. The most favoured method of homogenisation utilises the Potter-Elvejehm rotating-piston type of homogeniser: the speed of rotation of the piston must be low, its fit within the sample tube loose, and the period of homogenisation brief[141]. Variable results are perhaps inevitable. In illustration of the importance of the technique of obtaining homogenates, we may quote the conclusion of Bucher et al.[25] after investigating the biosynthesis of cholesterol from acetate by rat liver:

"the principal factor in obtaining homogenates capable of synthesising cholesterol appeared to be the kind of mechanical force employed to disrupt the tissue".

For reasons of convenience homogenisation was effected in the present work using the Ultra-Turrax TP 18/2 mixer (Janke-Künkel, Staufen), a stainless steel shearing device, which has a particular advantage in that the shearing gap is known and constant. Such devices may be expected to yield reproducible results[138]. The period of mixing was arbitrarily fixed at fifteen seconds.

Adequate investigation of the resulting homogenate demands the use of electron microscopy, a facility not at hand. The evidence available indicates that the end result compares with that obtained after prolonged ultrasonic disintegration. It is therefore likely that enzymes were extracted from most intracellular organelles. No real attempt at cellular fractionation was made, although the homogenate was subjected to centrifugation at 11,000 g for ten minutes to simulate the conditions of Ford and Huggins. Our investigations thus have an empirical basis. However, as will be seen, agreement with the results of Ford and Huggins is quite close. It may be observed that details of centrifugation techniques are in the best current practice defined in terms of "g-minutes", a method of expressing an integrated complete schedule of centrifugation, as suggested by de Duve[41].

The normal level of activity of testicular MD (per g. testis) reported in the present work [32.3 ± 5 International Units (I.U.): x 24] is in substantial agreement with that given by Ford and Huggins[55] [39.8 ± 5 I.U. : x 42]. The latter workers, however, employed a different strain of rat and give inadequate detail regarding their method of estimating MD. Moreover their method of homogenisation of testis was not defined. Strain differences in regard to DMBA response are known to be marked[170].

Comparison of our experimental results, however, with those of Ford and Huggins reveals a close similarity. The latter reported an increase of MD (per g. testis) in inverse relation to the loss of testis weight, attaining to a maximum of about two-fold at day thirty-eight, when testis weight was minimal. The spermatogenic epithelium was almost restored to normal by day seventy-five, with normal testis weight and MD activity.

We have ourselves found a similar pattern of initial response in regard to both our administered doses of DMBA and to busulphan, all levels of MD rising two-fold at day thirty-five. However, regeneration of the spermatogenic epithelium was less complete at day seventy-five, and testis weight still clearly subnormal. The severe effect we have elicited with 5 mg. DMBA appears anomalous in that testis weight remained at minimal levels at day seventy-five, with no regeneration of spermatogenic epithelium, and yet MD activity appeared to decrease as in the experiments where regeneration did occur. It is suggested that this indicates a toxic inhibition of MD in this particular case.

It is clear that our 5 mg. DMBA dose produced total destruction of the spermatogenic epithelium with no subsequent recovery. The 2.5 mg. dose appeared to give an effect similar but rather more severe to that observed by Ford and Huggins with a 5 mg. dose. The changes effected by the administration of busulphan lagged behind those produced by DMBA. This correlates with the lag in testis weight loss and would appear to indicate that the main toxic effect was upon a spermatogenic cell type rather earlier than that affected by DMBA. It may be noted that Ford and Huggins did not undertake spermatogonial cell type counts and thus were not in a position to specify with precision the cell type initially affected in their experiments.

Ford and Huggins offer no explanation for their observed changes in MD, but note a similar two-fold increase of activity, expressed per g. testis, in a) early infancy, b) post-hypophysectomy, c) unilateral cryptorchidy, and d) after DMBA. They conclude that "estimation of MD is a simple and useful measure of damage and subsequent repair in testis"[55]. It will be observed that every category listed here entails an abnormally low proportion of spermatogenic tissue in relation to interstitial tissue, which generally is considered to remain unchanged.

Ford and Huggins report values solely in terms of activity per g. testis. For purposes of comparison with our own presented results we have calculated and charted their values also in terms of activity per whole testis (Figure 47, p. 59). It is then clear that despite a

marked reduction of testis weight, which was minimal at forty days (50% of normal), activity per whole testis remained almost constant, being decreased only slightly in the regenerating testis.

The rats used in the present investigations appeared to be more sensitive to DMBA. The mean activity per testis recorded by Ford and Huggins is calculated to be 68.5 I.U. This level was unchanged at thirty-five days and maximally reduced to 56 I.U. (82%) at fifty-six days. The strain of rat used by these authors was evidently of rather less body weight than those used in the present experiments (mean weights 260 g. and 340 g. respectively). Our dosage of 5 mg. per rat thus is equivalent to 14.7 mg./kg. body weight as compared with Ford and Huggins' 19.2 mg./kg. Nevertheless, the effect upon testis was much more severe. Our normal activity was 51 I.U. per testis. This level was reduced to 36 I.U. (70%) at day twenty and to 22 I.U. (43%) at day forty-nine.

Our histochemical findings would appear at first sight to be in contradiction with these homogenate results. The former appear to indicate a reduction in interstitial MD activity, the latter the maintenance of high MD activity at the same site, if we minimise the contribution of the Sertoli cells. Some clarification of the apparent discrepancy may be made if we attempt calculations based upon certain reasonable assumptions:

a) that the number of Leydig cells remains constant, as has been shown in cryptorchidism[32];

b) that Leydig cells comprise the bulk of interstitial tissue, and represent the predominant enzymic component of this;

c) that the number of Sertoli cells per testis remains constant;

d) that normal testis is composed of 90% tubule tissue and 10% interstitial tissue, by weight, whereas the atrophic testes are 66% tubule and 33% interstitial tissue[93];

e) that in the normal testis 4/5 of the MD activity is associated with the interstitial tissue, whilst only 1/3 of the MD is so disposed in the atrophic testis. This is a crude approximation based upon our histochemical assessment.

Allowed these basic premises we may relate values thus:

Normal testis : MD activity = 32 I.U./g. whole testis

= 32 x 4/5 x 100/10 I.U./g. interstitial tissue

= 260 I.U./g. interstitial tissue

Atrophic testis : MD activity = 61 I.U./g. whole testis
(DMBA, 5 mg., day thirty-five)

= 61 x 1/3 x 100/33 I.U./g. interstitial tissue

= 61 I.U./g. interstitial tissue

Thus our observed values are seen to be consistent with a four-fold decrease in MD activity of interstitial tissue, although the activity per g. whole testis has increased two-fold. However, such calculations are no more than illustrative, for we have no adequate information relating volume and weight of particular functional areas of testis, and the cellular composition of the atrophic testis is very different from that of the normal.

We have undertaken a very limited study of changes in G6PD. Our results (Figure 48, p. 59) would appear, however, clearly to follow the same pattern observed for MD. The enzyme has been reported distributed predominantly in the interstitial tissue[93,166], and our considerations regarding MD may be applied to changes in G6PD. Our normal value of G6PD was found to be 0.59 I.U. ± 0.2 (x 5) [per g. testis] as compared with a value given by Ford and Huggins[55] of 0.63 ± 0.2 I.U. (x 42). The latter authors did not investigate serial changes in G6PD following DMBA administration, but they assayed both MD and LD in many experiments and reported that their respective levels were "not dissociated but ran parallel to each other"[55].

If we review our discussions regarding dehydrogenase enzyme changes (MD, G6PD, LD) we can conclude that these are secondary to destruction of the spermatogenic epithelium and give no direct information about the spermatogenic cells.

Our findings in relation to changes in β-glucuronidase may be compared with those of Pecora and Arata[130]. Although our normal levels were only 70% of those reported by the other authors, who used a different strain of rat (Long-Evans), the experimental response was strikingly similar. The Italian workers found a five-fold increase per g. testis and 1.8-fold increase per testis forty days after X-irradiation of the rat testis: these increases accord exactly with those observed in our experiments thirty-five days after DMBA, 5 mg.

Tissue β-glucuronidase can show an increase due to the presence of invading macrophages rich in this enzyme[430]. No evidence of such invasion was evident in the present experiments. Moreover, if degenerating spermatogenic cells were subjected to digestion by such phagocytic cells, other associated acid hydrolases would be expected to increase, whereas no such increase was evident in acid phosphatase in our experiments.

The observed intense increase in the activity of β-glucuronidase could be supposed the result of either a) activation of existing enzyme or b) induction of enzyme synthesis. Little activity has generally been found associated with the Sertoli cells (see p. 20), and it is therefore suggested that the observed increase in whole testis activity derives from increased interstitial activity. Histochemical studies of the changes observed would be of interest, since it is feasible that the Sertoli cell activity may be increased. It

may be noted that other workers have pointed out that this enzyme is an unusual enzyme in that its activity in the cancer cell exceeds that observed in the normal cell, whereas most enzymes show a "decreased activity concomitant with the neoplastic transformation"[26].

We have observed a marked decrease in acid phosphatase in the course of tubular degeneration. The findings would appear to suggest a primary association with the earlier spermatogenic cells. During the recovery phase after busulphan and after 2.5 mg. DMBA some recovery toward normal was apparent. Since activities did not fall to zero it is evident that some activity is associated with another site. This may be held to correlate with the report of a marked change in distribution of acid phosphatase after heat treatment of the rat testis. Activity was found within the tubules in the normal testis and preponderantly in the interstitial tissue after heat treatment, although no definite change in total activity was reported[154].

Our findings in relation to changes in 5'-nucleotidase are distinct, namely a marked increase per g. testis associated with a small decrease in the activity per testis. Our limited histochemical information indicates this enzyme to be primarily localised in the nucleus of spermatogenic cells (see p. 14). Such an observation does not agree with our present findings, since after DMBA, 5 mg., when spermatogenic cells are absent, the activity per testis is little changed. Our findings are consistent with a localisation in the Sertoli cells or in

the interstitial tissue. If the conclusions of Wachstein and Meisel[160] be questioned, activity in reality being detected in the Sertoli cell processes, we arrive at a more acceptable correlation with electron microscope studies[152] and our own results. We may thus suggest that 5'-nucleotidase is particularly associated with the Sertoli cells.

Interpretation of our results concerning glutamine synthetase is not easy in the absence of any information regarding distribution in the testis. No obvious correlation between the increased activities expressed per g. testis is evident. Activities per testis would appear to follow a more coherent plan, and to suggest that a large part of the glutamine synthetase activity is associated with the spermatogenic cells. This observation should be considered in relation to the association of glutamine synthetase activity with cellular differentiation reported by others (see p. 18).

The markedly decreased levels of hyaluronidase observed after tubular degeneration are in accord with the known association of the enzyme with the later spermatogenic cells. On only one occasion, however, was a nil level recorded (post-busulphan, fifty-seven days). It is to be noted that our method of estimation of hyaluronidase has been selected largely on the grounds of convenience, since all present methods are subject to criticism[106, p.134]. The purity and origin of the substrate employed have great influence upon the activity recorded[112], and the pH optimum is variable and dependent

further upon the method of estimation used[128, p.612; 70]. It has also been shown that testicular hyaluronidase is subject to reversible deactivation at high dilutions, as also is β-glucuronidase[11].

Our findings in relation to alkaline phosphatase appear to correlate with a predominant localisation within the tubule wall, activities per testis tending to parallel the tubule shrinkage and regeneration.

Reviewing the foregoing discussion, we may conclude that the most significent changes we have detected during degeneration of the spermatogenic epithelium are in relation to β-glucuronidase, 5'-nucleotidase, and hyaluronidase. The effects following administration of busulphan and of DMBA were similar.

The virtual disappearance of hyaluronidase was quite to be expected and has been reported by others in a different context (p. 31). Our results indicate that in the case of β-glucuronidase and possibly 5'-nucleotidase the testicular degeneration produced was associated with an actual increase in the measured activity of enzyme. Our methods do not reveal whether or not the actual amount of enzyme was increased.

v. Lysosomes:

Discussion of changes in β-glucuronidase and acid phosphatase prompts a consideration of lysosomal factors. Having refined with

particular care methods for the fractionation of rat liver tissue by differential centrifugation, de Duve et al. described in 1955 a new intracellular organelle associated with the "light mitochondrial" fraction. This was denoted the "lysosome"[42]. It was found to occur in several different forms, and to be intimately associated with processes of intracellular digestion. Lysosomes may now be identified by electron microscopy.

Lysosomes were shown to consist of a group of acid hydrolase enzymes contained within a lipoprotein bounding membrane. The enzyme group included β-glucuronidase, acid phosphatase, ribonuclease, deoxyribonuclease, and cathepsin. The activity of these enzymes in tissue is said to exhibit a "structure-linked latency". As investigated in vitro, sedimented light mitochondrial fraction contains lysosomes, with a proportion of "free" acid hydrolase in the supernatant. Rupture of the lysosomal membrane by means of surface-active agents effects release of the organelle enzymes with a consequent increase in activity of the apparent free component.

The study of lysosomes in tissues undergoing regressive or necrotic changes has indicated an early release of the associated enzymes (transference to the unsedimentable fraction) and a selective retention of these, the other cell constituents disappearing more rapidly[43b]. The lysosomal hydrolases are thus considered the main agents of catabolic changes taking place in regressing tissues.

It is believed that lysosomes are probably present in all animal cells, although they do not appear to have been directly demonstrated in testis[43a]. The usual histochemical "marker" for lysosomes is the acid phosphatase stain. However, the presence of any other of the group of associated enzymes serves as an indicator of the entire complement.

It may be observed that "membranes of various dimensions and different organisation appear to represent a common and basic principle of organisation in the cytoplasm"[145]. The lysosomal membrane is thus only one of many within the cell. Lipoprotein is an inherent structural part of all such biological membranes.

Among "membrane-active" compounds concerned in cell metabolism are Vitamins A and E. It has been suggested that Vitamin A is associated with a control mechanism operating with lipid-soluble substances such as steroid hormones and other fat-soluble vitamins[99]. Vitamin A (alcohol) is known to have surface-active effects, and may cause rupture of lysosomal membranes[45]. Vitamin E is thought to inhibit the peroxidation of unsaturated lipid. Muscular dystrophy induced in rabbits by deficiency of Vitamin E has been found to be associated with an increase in the (free) lysosomal activity of the affected tissue[171]. Mention has earlier been made of the connection of both these vitamins with spermatogenic arrest (p.34).

Since lysosomes are particularly fragile organelles it is certain that the homogenates in our present experiments contained no intact lysosomes, and clear that the associated hydrolases will have been within our extract fraction. The marked difference in acid phosphatase and β-glucuronidase response is therefore unexpected. However, acid hydrolases are not solely to be found in lysosomes. In comparing histochemical and biochemical findings pertaining to acid hydrolases it is evident that discrepancies could arise due to their normal localisation within lysosomes, for most methods of homogenisation would rupture these. It is perhaps for this reason that studies on β-glucuronidase distribution in tissues are confused, as noted above (p.67).

vi. Enzyme synthesis:

The synthesis within the cell of any enzyme demands the necessary gene. In many cases this acts only permissively and enzyme is not produced unless a particular catabolic substance is either present (induction) or absent (repression)[46, p.498]. The apparent increase in activity of an enzyme may be due to de novo synthesis and/or removal of inhibitor and/or unmasking of activator. Induction/repression must be carefully distinguished from activation/inhibition: the former terms are used only in connection with the actual synthesis of enzyme.

The primary site of protein synthesis within the cell is located within the microsomes (ribosomes). We may therefore assume enzyme synthesis to take place within these organelles. It is known that many carcinogenic polycyclic hydrocarbons can produce a rapid increase in the ability of rat liver microsomes enzymically to metabolise certain foreign compounds. Microsome-bound enzymes which respectively demethylate and reduce synthetic methylated aminoazo dyes (aminoazo demethylase and reductase) have been shown to increase in activity markedly after the administration of 3-methylcholanthrene and other compounds. This is necessarily an *in vivo* phenomenon and it is believed that genuine induction of synthesis in the liver is effected[37]. Boyland has postulated that the induction of enzyme synthesis by carcinogens may be due to the inactivation of suppressor factors (repressors) which normally control production of the enzyme[20].

DMBA is reported anomalous in that it produces only a slight effect[37], but Huggins et al.[84] reported significant results in regard to DMBA when investigating menadione reductase (E.C. 1.6.5.2) a soluble liver enzyme which catalyses the oxidation of reduced pyridine nucleotides by Vitamins K, or their synthetic analogue, menadione:

$$NAD(P)H_2 + \text{2-methyl-1,4-naphthoquinone (Menadione)}$$

$$= NAD(P) + \text{2-methyl-naphthohydroquinone}$$

Administration of 30 mg./kg. of DMBA to rat had no effect on dehydrogenase activity of liver, but menadione reductase showed an increase of 142%[84].

Protection against the toxic effect of DMBA upon testis can be afforded by prior administration of certain aromatic hydrocarbons, including small dosage with DMBA itself[83]. A dose of 6 mg. i.v. DMBA was uniformly lethal to the twenty-five-day-old rat. When 2 mg. i.v. of 3-methylcholanthrene was given twenty-four hours before the DMBA, selective damage to the testis was prevented and levels of LD and MD failed to rise. This protective action was eliminated when dl-ethionine (37.5 mg. i.p.) was given four hours before (but <u>not</u> after) administration of the 3-methylcholanthrene[56].

It has been shown that induction of menadione reductase synthesis in the liver is associated with the protection against DMBA toxicity afforded by aromatic hydrocarbons in relation to adrenal necrosis[82], fatal toxicity[83], and testicular degeneration[83].

Tritium-labelled thymidine injected into the rat is taken up exclusively at cell sites devoted to the synthesis of deoxyribonucleic acid (DNA), thus providing an experimental indicator of such synthesis - an index of tissue proliferation. A depression of DNA synthesis in liver after administration of DMBA has been found by this technique, the change being concurrent with the increase in menadione reductase[84].

It has been shown that the spermatogonia and resting primary spermatocytes of MOUSE incorporate tritiated thymidine[49,115]. It is in these cell types that DNA synthesis is occurring at a rapid rate, and they are the spermatogenic cells which are damaged by the administration of DMBA. It is therefore reasonable to propose that we draw some parallels with liver enzyme studies, and suggest that inhibited DNA synthesis in testis may be accompanied by the induction of enzyme synthesis.

Aminoazo dye reductase and demethylase are detoxicating enzymes likely to be characteristic of liver, the major detoxicating organ. However, induction of menadione reductase has been demonstrated, apart from liver, in mammary gland, mammary cancer, lung, adrenal, and adipose tissue[82]. It is therefore suggested that testis may also be found to exhibit activity of this enzyme subject to similar changes.

The immediate relevance of these considerations to our own studies is questionable. β-Glucuronidase exhibits detoxicating properties, although its function as such is obscure. It is associated particularly with lysosomes, although it is likely that it is actually synthesised in the microsomes. Most importantly, the various published studies on the induction of liver enzyme synthesis are based upon short-term experiments, in contrast to our present work. It may in addition be noted that 3-methylcholanthrene

and certain other hydrocarbons, in common with many other drugs, are also known to stimulate the biosynthesis of ascorbic acid in rat liver[38,48].

It is suggested that these indications of metabolic adaptation have a general relevance to all animal tissues, including testis. The enzymes which have currently been investigated appear to be somewhat esoteric, and it would be of great interest if such short-term experiments could be extended to more physiological enzymes, particularly those especially associated with the microsomes.

vii. DMBA and busulphan - mode of action:

Although the end-effects of DMBA and of busulphan treatment are similar, it is unlikely that the primary effect is the same in each case. It is therefore of interest to attempt some consideration of possible primary effects.

It has been shown that crystalline polybenzenoid hydrocarbon carcinogens are soluble to a slight extent in aqueous systems. *In vitro* investigations have revealed that crystalline hydrocarbons after introduction to aqueous incubation media will penetrate into and accumulate within the cytoplasm of cells within seconds, reaching concentrations which may be greatly in excess of even saturated aqueous levels in the extracellular media. Spermatogonia and spermatocytes of MOUSE were among the tissues in which this phenomenon was observed, also interstitial cells[137].

Evidence has elsewhere been given[3] that hydrocarbon carcinogens, including DMBA, are taken up by cells and selectively concentrated in lysosomes. Hydrocarbon dissolved in serum was incorporated into primary lysosomes (the preformed intracellular organelles), crystalline hydrocarbon into phagosomes (phagocytic lysosomes). It was found that the non-carcinogenic hydrocarbon anthracene, although taken into lysosomes, differed from the carcinogens in that it could subsequently readily be leached out, or, possibly, metabolically degraded.

The binding of the carcinogens, and particularly of DMBA, may be related to their molecular configuration. It has been suggested that polynuclear aromatic hydrocarbons to be effective as carcinogens must bear a steric resemblance to steroids[169]. DMBA has been shown to be structurally very similar to cortisone, and its ability to effect adrenal necrosis interpreted on this basis[79]. It is known that cortisone and related steroids stabilise lysosomal membranes against induced permeability changes[162], and there is evidence that DMBA may decrease their stability[3]. It may thus tentatively be suggested that the cytotoxic effect of DMBA is due to increase of lysosomal membrane permeability, this being induced by the competitive exclusion from an essential site of a steroid stabilising factor.

Recent suggestions have been made that a rearrangement and loss of lysosomes precedes cell division and that they may be in some way concerned with the initiation of cell division[4]. The experimental basis for these conclusions has, however, been criticised[57]. These considerations must be related to the conclusion of Ford and Huggins that the selective toxic effect of DMBA upon rat testis was not a sudden event, but "a genetic death occurring after some hours"[55]. The complex mechanism of cellular injury is little understood, but we could reasonably anticipate various primary toxic effects. It has been observed that in the case of liver injury lysosomal damage is a late effect, preceded by mitochondrial injury, even this being a late effect, the primary effect being unknown[134]. This does not necessarily invalidate our propositions regarding damage to testis.

The introduction of methyl groups into certain portions of polynuclear hydrocarbons produces greatly enhanced carcinogenicity, most markedly in DMBA. This compound is metabolised by rat-liver homogenates mainly by oxidation of one or other of the methyl groups, yielding the 7- or 12-hydroxymethyl derivative. It has been suggested that the great increase in carcinogenicity is connected with the latent introduction of such groups[22]. Ford and Huggins found a selective toxic effect upon testis to be a unique property of DMBA not shared by 3-methylcholanthrene and other tested hydrocarbons[55]. This may have a connection with the previous observation.

The especial vulnerability of spermatogonia within the testis may be due to their close apposition to the tubule wall and hence ready accessibility to DMBA conveyed to the site. If the Sertoli cell is rightly considered as a "bridge cell" (see p. 6) then we may postulate that DMBA is either excluded from this cell or taken in and neutralised in some way, therefore not reaching the later spermatogenic cells.

In a study of the uptake of tritiated busulphan in MOUSE testis, after intraperitoneal administration, it was shown that the compound entered the testis and reached its maximum level therein within thirty minutes. The elimination pattern of the busulphan then followed the loss of weight of the testes up to about fifteen days, during which testicular degeneration was observed similar to that recorded after irradiation[118].

Busulphan is a difunctional alkylating agent. It is believed that its cytotoxic effect is due to the induction of cross-linkage of the twin strands of the DNA macromolecule by alkylation. The duplication of DNA is thus frustrated[23B]. Recent experimental work with bacteria supports this view[95].

A feature accompanying spermatogenesis is the progressive disappearance of ribonucleic acid (RNA) from the spermatogenic cell and the accumulation of DNA: the spermatogonia are rich in RNA whereas the spermatozoa contain predominantly DNA[106, p.10]. We should

therefore expect that interference with the integrity of the DNA template would result in an inhibition of spermatogenesis.

The cause of the inhibition of spermatogenesis consequent upon hyperthermia is not known. Experimental work with RABBIT testis slices has shown that the biological effect appears to correlate with an induced lack of metabolic substrate. After twenty-four hours hyperthermia of testis the tissue was found to contain 12% less glucose and 27% less lactic acid than control tissue, and to be of significantly reduced metabolic activity[50,51]. Since the spermatogenic cells are very dependent on exogenous substrate supplies, this metabolic exhaustion may feasibly be the cause of an arrest of normal development. One may speculate that the Sertoli cell - the "nurse" cell - may have an essential importance in this context.

The chronic feeding of nitrofurans to rats has been shown to produce a loss of the ability of seminiferous tubules to oxidise pyruvate <u>in vitro</u>. This effect parallels the development of spermatogenic arrest at the primary spermatocyte stage, and has also been observed after X-irradiation and after hypophysectomy. The oxidation of pyruvate is a particular function of the later spermatogenic cells. It is suggested that "consequent to the metabolic defect in the processes leading to sperm maturation, the primary spermatocytes are unable to secure the requisite energy to complete their meiotic division and as a result remain in a state of arrest"[163].

viii. DMBA Fertility Trial:

The results of a fertility trial subsequent to the administration of DMBA (2.5 mg., i.v.) to male rats are presented in Table 9, p. 59. The average litter size per weekly mating period here recorded may be compared with a calculated normal mean of 8.7 reported elsewhere using the same technique[14]. It is clear that there is a marked reduction in fertility over the course of the experiment. This becomes most extreme from week seven, when matings are, with rare exceptions, sterile. At this interval of time from treatment, sterile matings indicate an initial toxic effect upon spermatogonia and spermatocytes, as may be seen from Table 1, p. 8.

The effect of DMBA may be compared with that of busulphan. After administration of the latter compound, no effect was observed on fertility until week eight, when a phase of sterility commenced[85]. In contrast, we have found DMBA to produce a variable although definite reduction in fertility in the earlier weeks. This could be due to some systemic toxic effect of the compound giving rise to loss of libido. However, insemination has frequently been observed and a number of matings proven fertile. It is therefore suggested that DMBA has some toxic effect upon spermatid cells and even upon epididymal spermatozoa. This effect must be presumed functional since in neither the present work nor in that of Ford and Huggins[55] has any immediate depletion of these cell types been histologically apparent. Examination of the uterine contents of the inseminated

rats, if carried out, may have given direct evidence of genetic damage resulting in embryo deaths in our seemingly infertile matings during these early weeks.

Investigations of the effect of busulphan and other related compounds upon testis have similarly revealed discrepancies between findings elicited from histological study and from fertility trial[88]. These two experimental approaches are complementary and we suggest they could profitably be extended by the addition of biochemical, i.e. enzymological, investigations.

CONCLUSIONS

CONCLUSIONS

We have been able to correlate certain enzyme changes with the gross changes in testis cell population produced experimentally. We have not, however, with one exception, shown any particular enzyme of the group investigated to be solely associated with a specific spermatogenic cell type. Hyaluronidase has been found to be restricted to the later stages of spermatogenesis; this has already been well documented by others. We have given evidence for the suggestion that the Sertoli cells may contain a high activity of β-glucuronidase and of 5'-nucleotidase.

Interpretation of the enzyme changes observed is complicated by the mixed cellular composition of the testis. Increase in the activity of dehydrogenase enzymes has been found to be secondary to the involution of the spermatogenic tissue and to bear no direct relation to the cells of the spermatogenic epithelium. Clarification by histochemical methods of the distribution in normal and atrophic testis of β-glucuronidase and of glutamine synthetase would usefully supplement our homogenate studies. A histochemical method for the demonstration of glutamine synthetase activity is, however, lacking.

It is suggested that attention could most profitably be given to enzymes especially concerned with energy metabolism, such as adenosinetriphosphatase and creatine kinase, which may be found to be

particularly associated with the characteristic proliferation and differentiation of spermatogenic cells. The role of phosphoamidase needs further exploration.

It would be of interest to find whether testis exhibits menadione reductase activity, and to establish whether toxic inhibition of spermatogenesis is accompanied by the induction of synthesis of this or of other microsomal enzymes. A short-term effect is feasible, and the monitoring of anti-fertility compounds could most usefully be accomplished by such rapid studies.

REFERENCES.

1. Abul-Fadl,M.A.M., King,E.J., (1949) Biochem. J. 45 51.

2. Aldridge,W.N., (1953) Biochem. J. 53 110.

3. Allison,A.C.,Mallucci,L., (1964) Nature (London) 203 1024.

4. Allison,A.C.,Mallucci,L., (1964) Lancet 2 1371.

5. Arvy,L., (1962) C.R. Acad. Sci. (Paris) 255 1532 (abstracted in Chem. Abstr. (1963) 58 718f)

6. Atkinson,W.B.,Herbener,G.H., (1961) Ann. Histochim. 6 481.

7. Baillie,A.H.,Griffiths,K., (1964) J. Endocrin. 29 9.

8. Baillie,A.H.,Griffiths,K., (1965) J. Endocrin. 31 307.

9. Bather,R.,Cushing,Jacqueline, (1963) Cancer Res. 23 707.

10. Bergel,F., (1961) "Chemistry of Enzymes in Cancer" Charles C. Thomas, Springfield, Illinois.

11. Bernfeld,P.,Tuttle,C.P.,Hubbard,R.W., (1961) Arch. Biochem. Biophys. 92 232.

12. Beyler,A.L.,Potts,G.O.,Coulston,F.,Surrey,A.R., (1961) Endocrinology 69 819.

13. Blanco,A.,Zinkham,W.H., (1963) Science 139 601.

14. Bock,Marion,Jackson,H., (1957) Brit. J. Pharmacol. 12 1.

15. Boehringer Corporation (London) Ltd., 54-58 Uxbridge Rd., London W.5.

16. Bollag,W., (1953) Experientia 9 268.

17. Bollet,A.J.,Bonner,W.M.,Nance,Joanne L., (1963) J. Biol. Chem. 238 3522.

18. Boyer,S.H., (1961) Science 134 1002.

19. Boyland,E., (1932) Lancet 2 1108.

20. Boyland,E., (1962) "Cancer and Hormones", Univ. of Chicago, p.135.

21. Boyland,E.,Sydnor,K.L., (1962) Brit. J. Cancer 16 731.

22. Boyland,E.,Sims,P.,Williams,K., (1965) Biochem. J. 94(2) 24p.

23A. Brodie,B.B.,Gillette,J.R.,La Du,B.N., (1958) Ann. Rev. Biochem. 27 438.

23B. Brookes,P.,Lawley,P.D., (1961) 80 496, Biochem. J.

24. Brown,J.H.U.,Barker,S.B., (1961) "Basic Endocrinology" p. 96, Blackwell, Oxford.

25. Bucher,Nancy L.R.,McGovern,Naomi H.,Kingston,Ruth, Kennedy,Margaret H., (1953) Fed. Proc. 12 184.

26. Burstone,M.S., (1962) "Enzyme Histochemistry and its Application in the Study of Neoplasms", Academic Press, New York, p. 359.

27. Cameron,R.G., (1961) "The Chemistry of the Injured Cell" Charles C. Thomas, Illinois.

28. Campbell,Diana,King,E.J., (1961) Proc. Assoc. clin. Biochem. 1 56.

29. Cieciura,L., (1962) Endokrynologia Polska 13(2) 181 (abstracted in Biol. Abstr. (1962) 40 .

30. Cieciura,L., (1962) Endokrynologia Polska 13(3) 295 (abstracted in Biol. Abstr. (1963) 41 5893.

31. Cieciura,L., (1962) Folia Morphol. (Warsaw) 13 225 (abstracted in Biol. Abstr. (1963) 41 10046.

32. Clegg,E.J., (1961) J. Endocrin. 21 433.

33. Clermont,Y.,Leblond,C.P., (1953) Amer. J. Anat. 100 241.

34. Commission on Enzymes of the International Union of Biochemistry - Report, Pergamon Press, Oxford (1961).

35. Conchie,J.,Findlay,J.,Levvy,G.A., (1959) Biochem. J. 71 318.

36. Conchie,J.,Findlay,J., (1959) J. Endocrin. 18 132.

37. Conney,A.H.,Miller,E.C.,Miller,J.A., (1956) Cancer Res. 16 450.

38. Conney,A.H.,Burns,J.J., (1959) Nature (London) 184 363.

39. Conway,E.J.,Cooke,R., (1939) Biochem. J. 33 479.

40. Craig,A.W.,Fox,B.W.,Jackson,H., (1961) J. Reprod. Fertil. 2 466.

41. De Duve,C.,Berthet,J., (1953) Nature (London) 172 1142.

42. De Duve,C.,Pressman,B.I.,Gianetto,R.,Wattiaux,R., Appelmans,F., (1955) Biochem. J. 60 604.

43a. De Duve,C., (1963) in Ciba Foundation Symposium "Lysosomes" ed.de Rueck,A.V.S.,Cameron,M.P., p. 5.

43b. De Duve,C., ibid p.23.

43c. De Duve,C., ibid p.24.

44. Dempsey,E.W.,Greep,R.O.,Deane,H.W., (1949) Endocrinology 44 88.

45. Dingle,J.T., (1963) in Ciba Foundation Symposium "Lysosomes" ed. de Rueck,A.V.S.,Cameron,M.P., p.384.

46. Dixon,M.,Webb,E.C., (1964) "Enzymes" 2nd. edn. Longmans,London.

47. Dixon,T.F.,Purdom,M., (1954) J. Clin. Path. 7 341.

48. Drayton,P.G.,Vrindten,P.,Percel,J.M., (1964) Biochem. Pharmacol. 13 143.

49. Edwards,J.L.,Klein,R.E., (1961) Am. J. Path. 38 437.

50. Ewing,L.L.,Vandermark,N.L., (1963) J. Reprod. Fertil. 6 9.

51. Ewing,L.L.,Vandermark,N.L., (1963) J. Reprod. Fertil. 6 17.

52. Fishman,W.H. (1950) in "The Enzymes", eds. Boyer,P.D., Lardy,H.,Myrbäck,K. 1(1) 635.

53. Fishman,W.H., (1951) in "The Enzymes", eds. Boyer,P.D., Lardy,H.,Myrbäck,K., 1(2) 769.

54. Fishman,W.H.,Green,S., (1957) J. Biol. Chem. 225 435.

55. Ford,Elizabeth,Huggins,C., (1963) J. Exp. Med. 118 27.

56. Ford,Elizabeth,Huggins,C., (1964) Proc. Amer. Assoc. for Cancer Res. 5 Abstr. 73.

57. Gahan,P.B., (1965) Lancet 1 323.

58. Glock,G.,McLean,P., (1953) Biochem. J. 55 400.

59. Glock,G.,McLean,P., (1955) Biochem. J. 61 388.

60. Goldberg,B.,Jones,G.E.S.,Borkowf,H.I., (1964) J. Histochem. Cytochem. 12 880.

61. Goldberg,G., (1963) Science 139 602.

62. Gomori,G., (1941) Arch. Path. 32 189.

63. Gomori,G., (1948) Proc. Soc. exptl. Biol. Med. 69 407.

64. Goodlad,G.A.J.,Mills,G.T., (1957) Biochem. J. 66 346.

65. Gutman,E.B.,Gutman,A.B., (1940) J. Biol Chem. 136 201.

66. Haddow,A., (1935) Nature (London) 136 868.

67. Hayashi,M.,Ogata,K.,Shiraogawa,T.,Kawose,O., (1958) Nature (London) 181 186.

68. Hayashi,M.,Murakami,Z.,Koga,Y.,Satake,T., (1958) Trans. Soc. path. Jap. 47 588 (abstracted in Abstr. Jap. Med. 1 113.)

69. Hayashi,M.,Ogata,K.,Shiraogawa,T.,Murakami,Z.,Tuneishi,S., (1958) Acta Path. Jap. 8 113 ;(abstracted in Excerpta Medica Sect. 5 12 2340.)

70. Hayashi,M.,Fishman,W.H. (1961) J. Histochem. Cytochem. 9 615.

71. Hess,R.,Scarpelli,D.G.,Pearse,A.G.E., (1958) Nature (London) 181 1531.

72. Holt,L.E.,Jnr.,Albanese,A.A.,Shetles,L.B.,Kajdi,C., Wangerin,D.M., (1942) Fed. Proc. 1 116.

73. Holter,H.,Li,Si-Oh, (1950) Acta Chem. Scand. 4 1321.

74. Holzer,M.E.,Burrow,D.J.,Smith,R.A., (1962) Biochim. Biophys. Acta 56 491.

75. Howell,J.M.C.,Thompson,J.N.,Pitt,G.A.J., (1963) J. Reprod. Fertil. 5 159.

76. Huggins,C.B.,Moulder,P.V., (1945) Cancer Res. 5 610.

77. Huggins,C.B.,Moulton,S.H., (1948) J. Exp. Med. 88 169.

78. Huggins,C.B.,Morii,S.,Grand,L.C., (1961) Ann. Surg. 154 No. 6 Suppl. 315

79. Huggins,C.B.,Morii,S., (1961) J. Exp. Med. 114 741.

80. Huggins,C.B.,Yang,N.C., (1962) Science 137 257.

81. Huggins,C.B.,Fukunishi,R., (1963) Cancer Res. 23 785.

82. Huggins,C.B.,Fukunishi,R., (1964) J. Exp. Med. 119 923.

83. Huggins,C.B.,Ford,Elizabeth,Fukunishi,R.,Jensen,E.V., (1964) J. Exp. Med. 119 943.

84. Huggins,C.B.,Ford,Elizabeth,Jensen,E.V., (1965) Science 147 1153

85. Jackson,H.,Fox,B.W.,Craig,A.W., (1959) Brit. J. Pharmacol. 14 149.

86. Jackson,H.,Fox,B.W.,Craig,A.W., (1961) J. Reprod. Fertil. 2 447.

87. Jackson,H.,Partington,Mgt.,Fox,B.W., (1962) Nature(London) 194 1184.

88. Jackson,H., (1964) Brit. Med. Bull. 20 107.

89. Jackson,H., (1964) Fortschritte der Arzneimittelforschung 7 133.

90. Johnsen,S.G., (1964) Acta Endocr.,Suppl. 90, p.99.

91. Kind,P.R.N.,King,E.J., (1954) J. clin. Path. 7 322.

92. Kirshner,M.A.,Lipsett,M.B.,Collins,D.R., (1965) J. Clin Invest. 44 657 .

93. Kormano,M.,Harkonen,M.,Kontinen,E., (1964) Endocrinology 74 44.

94. Krishnaswamy,P.R.,Paminjans,V.,Mester,E., (1964) J. Biol. Chem. 237 2932.

95. Lawley,P.D.,Brookes,P., (1965) Nature (London) 206 480.

96. Leblond,C.P.,Clermont,Y., (1952) Ann. N.Y. Acad. Sci. 55 548.

97. Lees,J.C., (1937) Quart. J. Exp. Physiol. 27 168.

98. Levintow,L., (1954) J. Natl. Cancer Inst. 15 347.

99. Lucy,J.A., (1964) in "Metabolic Control Mechanisms in Animal Cells", Natl. Cancer Inst. Monograph,p. 93.

100. Lutwak-Mann,Cecilia, (1964) Brit. med. J. 1 1090.

101. Macleod,J.,Summerson,W., (1946) J. Biol. Chem. 165 533.

102. Mancini,R.E.,Nolazzo,J.,de la Balze,F.A., (1952) Anat. Rec. 114 127.

103. Mancini,R.E.,Alonso,A.,Barquet,J.,Alvarez,B.,Nemirovsky,M., (1964) J. Reprod. Fertil. 8 325.

104. Mann,T., (1958) Ann. Rev. Physiol. 20 275.

105. Mann,T., (1960) Proc. Nutr. Soc. 19 15.

106. Mann,T., (1964) "The Biochemistry of Semen & of the Male Reproductive Tract", Methuen, London.

107. Mason,K.E.A., (1925) Proc. natl. Acad. Sci.,Wash. 11 377.

108. Mazzanti,L.,Lopez,M.,Berti,M.G., (1964) Experientia 20 592.

109. Meyer,J.,Weinmann,J.P., (1957) J. Histochem. Cytochem. 5 354.

110. Meyer,J.,Weinmann,J.P., (1957) Amer. J. Anat. 101 461.

111. Meyer,K.,Chaffee,E.,Hobby,G.L.,Dawson,M.H., (1941) J. Exp. Med. 73 309.

112. Meyer,K., (1947) Physiol. Rev. 27 335.

113. Meyer,K.,Rapport,M.M., (1952) in Advances in Enzymology, ed. Nord,E.F. 13 199-236.

114. Møller,K.M., (1955) Biochim. Biophys. Acta 16 162.

115. Monesi,V., (1962) J. Biol. Chem. 14 1.

116. Morgan,W.T.J., (1938) J. Soc. Chem. Indust. 57 1191.

117. Moscona,A.A.,KirkD.L., (1965) Science 148 519.

118. Moutschen,J.,Ezell,H.K.,Jnr., (1960) Rad. Res. 13 898.

119. Mukherjee,A.C.,Ghosh,B.N., (1962) Ann. Biochem. Exptl. Med. (Calcutta); (abstracted in Chem. Abstr. (1963) 57 12876b.)

120. Negri,L.Weber,G., (1953) Arch. "De Vecchi" per L'Anatomia Pat. E. Med. Clin. 19 379 ; (abstracted in Excerpta Medica,Sect. 5, 8 1939 (1955).)

121. Newman,W.,Kabat,E.A.,Wolf,A., (1950) Amer. J Path. 26 489.

122. Niemi,M.,Härkönen,M.,Kokko,A., (1962) J. Histochem. Cytochem. 10 186.

123. Niemi,M.,Ikonen,M., (1962) Endocrinology 70 167.

124. Novikoff,A.B., (1958) J. Histochem. Cytochem. 6 251.

125A. Palade,G.E., (1951) J. Exp. Med. 94 535.

125B. Pantony,D.A., (1961) "A Chemist's Introduction to Statistics,Theory of Error and Design of Experiments" Lecture Series No. 2,R.I.C.

126. Pearse,A.G.E.,Reis,J.L., (1952) Biochem. J. 50 534.

127. Pearse,A.G.E., (1958) J. clin. Path. 11 520.

128. Pearse,A.G.E., (1960) "Histochemistry,Theoretical and Applied" 2nd. edn. Churchill, London.

129. Pearse,A.G.E., (1962) Ann. Rev. Scientific Basis of Med., p. 215.

130. Pecora,P.,Arata,L., (1964) J. Reprod. Fertil. 8 281.

131. Pellegrino,C.,Villani,G., (1956) Biochem. J. 62 235.

132. Pellegrino,C.,Villani,G., (1957) Biochem. J. 65 599.

133. Potter,van R., (1955) in "Methods in Enzymology", eds. Colowick,S.P.,Kaplan,N.O., 1 10.

134. Rees,K.R., (1964) in Ciba Foundation Symp. "Cellular Injury",ed. de Rueck,A.V.S.,Knight,Julie, p. 56.

135. Reissig,J.L.,Strominger,J.L.,Leloir,L.F., (1955) J. Biol. Chem. 217 959.

136. Ressler,N.,Olivero,E.,Joseph,R.R., (1965) Nature (London) 206 829.

137. Richter,K.M.,Saini,V.K., (1960) Amer. J. Anat.107 209.

138. Roodyn,D.B., (1959) Intern. Rev. Cytol. 8 279.

139. Russell,H., (1953) Edin. Med. J. 60 474.

140. Samuels,L.T., Hemreich,M.L., (1956) Endocrinology 58 435.

141. Schneider,W.C., (1961) in "Biochemist's Handbook", eds. Long,C.,King,E.J.,Sperry,W.; Spon, London, p.810.

142. Scheon,E.J., (1964) Endocrinology 75 56.

143. Schor,N.A.,Cara,J.,Perez,A., (1963) Nature (London) 198 1310.

144. Sellinger,O.Z.,Verster,F. de B., (1962) J. Biol. Chem. 237 2838.

145. Sjöstrand,F.S., (1959) in "Biophysical Science", ed. Oncley,J.L., Wiley, New York, p. 30.

146. Steinberger,E.,Nelson,W.O., (1955) Endocrinology 56 429.

147. Steinberger,E.,Wagner,C., (1961) Endocrinology 69 305.

148. Srivastava,P.N.,Mathur,R.S., (1963) Experientia 19 476.

149. Talalay,P.,Fishman,W.H.,Huggins,C.B., (1946) J. Biol. Chem. 166 757.

150. Tepperman,J.,Tepperman,H.M.,Dick,H.J., (1949) Endocrinology 45 491.

151. Thorne,C.J.R.,Grossman,L.I.,Kaplan,N.O., (1963) Biochim. Biophys. Acta 73 193.

152. Tice,L.W.,Barnett,R.J., (1963) Anat. Rec. 147 43.

153. Tsou,K.-C.,Cheng,C.-S.,Nachlas,M.M.,Seligman,A.M., (1956) J. Amer. Chem . Soc. 78 6139.

154. Turpeinen,P.,Turpeinen,O.,Talanti,S., (1962) Endocrinology 70 731.

155. Utley,J.D., (1964) Biochem. J. 13 1383.

156. Verne,J.,Hébert,S., (1952) Ann. Endocr. (Paris)13 924 (abstracted in Biol. Abstr. (1955) 29 11163.)

157. Verne,J.,Hébert,S., (1952) Ann. Endocr. (Paris)13 659 (abstracted in Biol. Anstr. (1954) 28 10806.)

158. Verne,J., (1953) Ann. Endocr. (Paris) 14 747 ; (abstracted in Chem. Abstr. (1954) 48 8362d.)

159. Vilar,O.,Perez Del Cerro,M.I.,Mancini,R.E., (1962) Exp. Cell Res. 27 158.

160. Wachstein,M.,Meisel,E., (1954) J. Histochem. Cytochem. 2 137.

161. Waelsch,H., (1952) in Advances in Enzymology, ed. Nord,E.F., 13 277.

162. Weissmann,G., (1964) Lancet 2 1373.

163. Welden,F.,Marberger,E.,Boccabella,A.,Boccabella,R., (1955) Endocrinology 56 727.

164. Wilkinson,J.H., (1962) "An Introduction to Diagnostic Enzymology" , E. Arnold, London.

165. Wolf,A.,Kabat,E.A.,Newman,W., (1943) Amer.J. Path. 19 423.

166. Wolfe,H.J.,Cohen,R.B., (1964) J. Clin. Endocr. & Metab. 24 616.

167. Wong,T.-W.,Earner,N.E., (1964) Endocrinology 74 284.

168. Wu,C., (1961) Fed. Proc. 20 218.

169. Yang,N.C.,Castro,A.J., (1961) Science 134 386.

170. Young,S.,Cowan,Dorothy M.,Sutherland,Lucy, (1963) J. Path. Bact. 85 331.

171. Zalkin,W.H.,Tappel,A.L.,Caldwell,K.A.,Shibko,S.,Desai, I.D., Holloday,T.A., (1962) J. Biol. Chem. 237 2678

172. Zinkham,W.H.,Blanco,A.,Kupchyk,C., (1963) Science 142 1303.

173. Zinkham,W.H., (1964) Science 144 1353.

174.

ABBREVIATIONS.

ADP	Adenosine diphosphate.
AMP	Adenosine-5'-phosphate.
ATP	Adenosine triphosphate.
BAL	2:3-Dimercapto-propan-1-ol.
C	Control.
DMBA	7,12-Dimethylbenz(α)anthracene.
E.C.	Commission on Enzymes of the International Union of Biochemistry
G6PD	Glucose-6-phosphate dehydrogenase.
GHA	γ-Glutamylhydroxamic acid.
3βHSD	3β-Hydroxysteroid dehydrogenase.
ICD	Isocitrate dehydrogenase.
i.p.	Intraperitoneal.
I.U.	International Unit.
i.v.	Intravenous.
LD	Lactate dehydrogenase.
MD	Malate dehydrogenase.
NAD	Nicotinamide-adenine dinucleotide.
NADP	Nicotinamide-adenine dinucleotide phosphate.
O.D.	Optical density.
SD	Succinate dehydrogenase.
T	Test.