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BIOCHEMICAL ASPECTS OF THE ACTIONS OF DRUGS ON SPERMATOGENESIS

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I. INTRODUCTION

Spermatogenesis is a unique system in which the timing and direction of the stages of development from a primordial cell, probably identical with a stem cell, is known with a considerable degree of certainty. Owing to the organization and structure of the testis it has been possible to unravel the neatly co-ordinated "cycles" into discreet stages which can be readily identified by relatively simple histological procedures. With these techniques, a detailed analysis of the timing of cellular events involved has been made in an amphipod (*Orchestia gammarella*) (186), a grasshopper (*Melanoplus differentialis*) (202) and the fruit fly, *Drosophila melanogaster* (43), in the ram (227), rat (53,167), mouse (167,225), hamster (167), guinea pig (49, 167) and rabbit (282, 283), and in man (126).

Having established the nature of the cell kinetics involved in spermatogenesis, the logical next phase is to understand the biochemical changes which accompany these kinetic changes, controlling the changing cell in its complex maturation in the testis. Much of this latter information may be derived from a study of the mode of action of numerous chemicals on spermatogenesis, some of which produce known biochemical lesions in other differentiating systems. In a complementary manner, an understanding of these changes would also throw some light on the mode of action of new drugs and at the same time provide some information on extra-, intra-, and intercellular control required to maintain normal spermatogenesis.

The object of this review is to summarize our knowledge of testicular biochemistry to provide some basis for the interpretation of the mode of action of some of the many drugs that act on this cell system.

A drug could affect spermatogenesis in a number of different ways: 1) Inhibition of the normal overall rate and sequence of development (kinetic damage); 2) Direct action on a cell type resulting in its subsequent death and depletion from the system (cellular damage); 3) Interference with cell morphology resulting in a change appearing in either testicular or epididymal phases (morphological damage); 4) No interference with the kinetics of the system but a

change manifest during subsequent cleavage of an egg fertilized by an affected spermatozoon. For the purpose of this review, the resulting death of embryos during gestation is included (early genetic damage); 5) No interference with the kinetics of spermatogenesis or with embryonic development but nevertheless an action resulting in genetic changes in progeny (late genetic damage); 6) An effect on the structural matrix of the testicular tissue including Leydig and Sertoli cells (matrix damage). Although it is recognized that this section should be further subdivided, it was considered unnecessary for the scope of this review. The above descriptions of types of damage which have been observed in spermatogenesis are intended only as an approximate guide to the rationalizing of these events. Overlap occurs and many types of damage may be included in one or more of these categories.

Certain aspects of the normal cell kinetics of spermatogenesis have been reviewed (255) as well as the general antifertility action of groups of agents in both sexes (138–141).

II. CELLULAR KINETICS AND STRUCTURE

It has been shown from histological and autoradiographic studies in the rat, that the earliest recognizable cell in the testis tubule, spermatogonia Type A, undergoes five mitotic and one meiotic (reduction) division, resulting in young spermatozoa in the lumen of the testis in 48 days (53). The spermatozoa then pass into a collecting vessel (rete testis), through a long, single, and highly convoluted tube of varying transverse dimensions (epididymis), and are finally ejaculated about 15 days after leaving the testis. The total time from the stem cell to its ejaculated progeny is about 63 days (9 weeks) (Fig. 1).

The testicular phase of this process has been divided into four 12-day periods or "cycles," that is, any given area of the germinal epithelium becomes the starting point for a new generation of spermatogenic cells every 12 days. Since the whole process takes 48 days, any transverse section of the tubule will include four groups of cells of 12 days' difference in age, belonging to the progeny of four separate "stem cell" populations that appeared at 12-day intervals. Cells lying along the basal membrane are those which are passing through their first 12 days, whereas those which occupy the second layer are from 12 to 24 days of age. The third layer consists of cells between 24 and 36 days old, whereas those which occupy the central lumen consist of developing spermatids 36 to 48 days from the stem cell. In the rat, these waves of stem cell renewal pass along the tubules and the topography of the areas of regeneration at any given time have been skillfully mapped in certain regions of the tubule (238).

Fourteen characteristic patterns of cell associations or "stages" have been described in transverse sections of the rat testis tubules (167). These stages are clearly identified by the detailed morphology of the spermatids in the innermost layer of cells. Since the progression of these stages also represent different points of time along the 12-day cycle, it would be expected that any longitudinal section may also show this progression corresponding to a wave of maturation passing along the tubule. This, however, is only approximately the case and the nature of the anomalies observed have been discussed in detail (238).

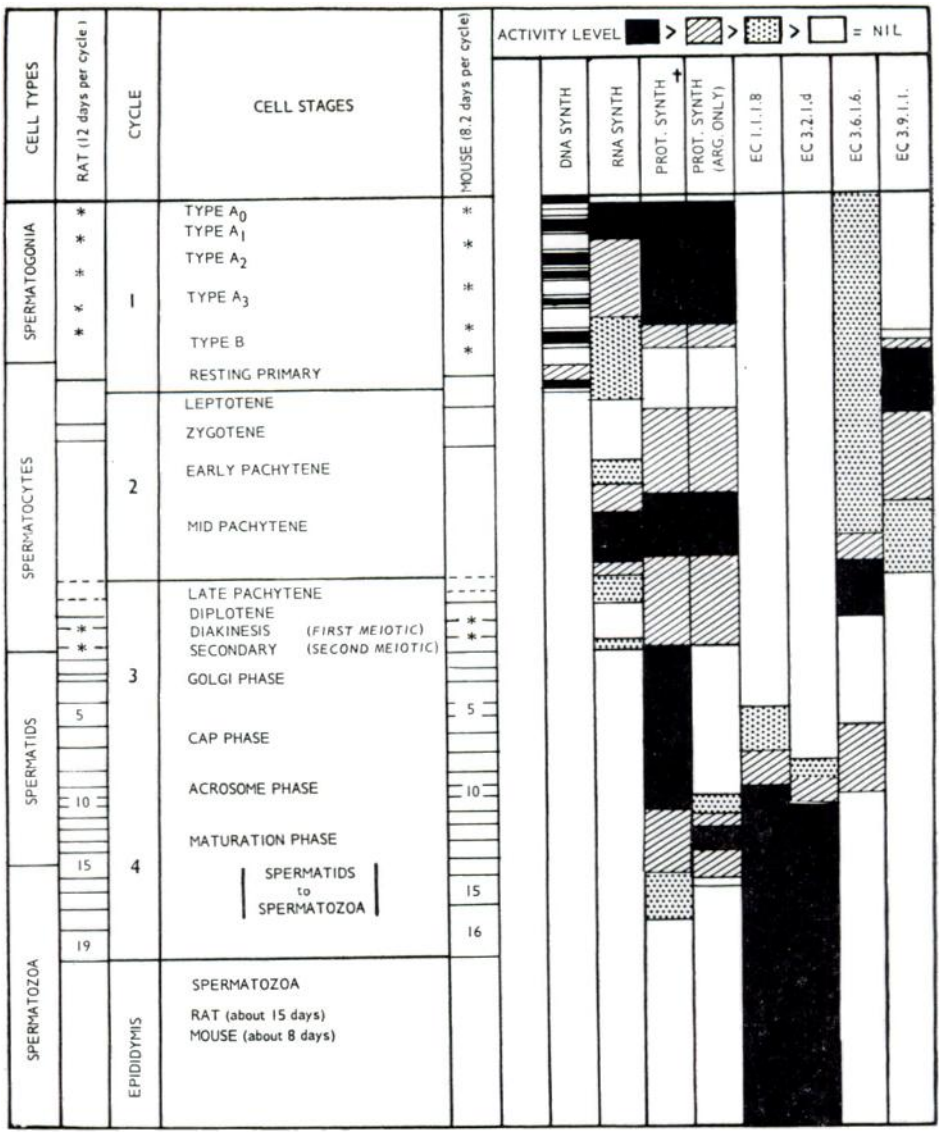

FIG. 1. Stages of spermatogenesis and spermiogenesis in the rat and mouse are shown. Each cycle of the testicular phase of the rat corresponds to 12 days, and of the mouse, 8.2 days. The regions of nucleic acid and protein synthetic activity are shown as well as the regions of activity of four enzyme systems. The spermatid stages used in classifying the transverse sections are shown. (†, protein synthesis using lysine, tyrosine, histidine, leucine, tryptophane, and phenylalanine; *, divisions. Enzymes EC 1.1.1.8, glycerophosphate dehydrogenase; EC 3.2.1.d, hyaluronidase; EC 3.6.1.6., nucleosidediphosphatase; EC 3.9.1.1., phosphoamidase.)

By extension of this topographical analysis to the human testis, a complex mosaic of stages bearing less obvious relation to one another was identified (126). It is clearly desirable to determine the configuration of these areas of cellular activity at any given time in order to understand the nature of the com-

plex process of extratubular control. Even in the relatively more uniform situation in the rat, it is difficult to envisage how this control would be exercised. One may suppose that in this species, an annulus of stem-cell-triggering activity would be required to move along the tubule to set off new generations of spermatogonia from successive stem cells lying along the basal membrane. The human type of testis would require a more random triggering of scattered stem cells.

Renewal of spermatogonia every 12 days in the rat testis requires that at the equivalent of the third mitotic division, one of the progeny should recommence a new cycle as a spermatogonia Type A (50, 52). Since the concept of such a heterogenous division, with the possible exception of early egg development, is unacceptable in current cell kinetic theories (99), it would be logical to consider that a stem cell compartment exists which continues to divide into further stem cells until some environmental influences convert it into a basic cell to begin the differentiating system of spermatogenesis proper, which then becomes the main system observable in the germinal epithelium. In each tissue, the balance of stem and differentiating compartments is probably determined at any given time by some controlling factor, either hormonal or environmental. Evidence for this type of control in bone marrow (162, 163), small intestine (119), regenerating liver (163), and vicia root meristem (119) is now recognized and that for a similar control in spermatogenesis is accumulating (126).

The synchronous development of spermatogenesis in certain insects has some advantages in basic kinetic and structural studies. The developing cyst in the testicular follicle of the grasshopper (*Melanoplus differentialis*) possesses cells all in the same stage of the spermatogenic cycle at any given time and offers a means of examining the associated synchronous biochemical activity (202). In studies with tritium-labelled DNA precursors, the spermatogonial cell cycle has been shown to be of 28 hours duration, of which the synthetic period is 12 hours. The total timing of spermatogenesis in this species is 28 days, of which 7 spermatogonial divisions take 8 to 9 days, premeiotic DNA synthesis and meiosis take 9 to 10 days, and spermiogenesis 10 days. The timing in the fruit fly, (*Drosophila melanogaster*), has also been partially elucidated (43).

Much significant additional information about the cellular mechanisms involved in spermatogenesis has been obtained from electron microscopic investigation of structure. In particular, studies on the ultrastructure of Sertoli cells (295) and their secretory activity (33) suggest a unique and important role for this cell. They have been recognized as "nurse" or sustentacular cells for spermatids and spermatozoa (36, 256), and more recent evidence (295) suggests that they act as "bridge cells" to assist in the transfer of important nutrient material from the basal membrane to the developing germinal cells. Microtubules possibly associated with such transfer activity have been described in the Sertoli cells in the guinea pig testis (46). Sertoli cells are arranged in an ordered fashion along the basal membrane and are known to have long trunk-like projections which lie radially through the tubule. Each cell becomes associated with six primary spermatocytes through these connections until the spermatogenic cells leave the

testis lumen as spermatozoa after which the Sertoli cells commence the process again (73). They are furthermore known to be very rich in glycogen, and it is conceivable that spermatocytes and spermatids, whose glycogen reserves fall rapidly after the primary spermatocyte stage as they leave the basement membrane (198), derive some of their carbohydrate from the Sertoli cells with which they are in contact. Sertoli cells are also known to contain a variety of enzyme systems (205, 221, 289) and are rich in ribonucleic acid (31, 199).

In *Asellus*, an isopod (199), surrounding the germ cells there is a layer of large polyploid nuclei. These follicle cells convey RNA to the germ cells in two secretory waves, at the beginning (leptotene to zygogene) and the end (diakinesis to anaphase) of the meiotic prophase. There appears to be some functional similarity between these follicle cells and Sertoli cells, but it would be presumptuous to extend this analogy further. The Sertoli cell in mammalian tissue, however, deserves a more rigorous and detailed examination from a biochemical point of view as it is likely to yield important information with regard to mechanisms of organization in a differentiating cell system. A study of the fine structure of developing spermatozoa in *Asellus militans* has also been undertaken (246).

Of particular biochemical interest is the detailed electron microscopic study of the transition of the spermatogonial mitochondria into the specialized organelle associated with the spermatozoa midpiece (6).

Further detailed fine structure studies of spermatogenesis have been described in the albino rat (6, 33, 297, 298), mouse and other rodents (42, 262), in the cat (36), crab (309), toad (37), chicken (206, 207), pigeon (209), Swiss mouse (98) and bat (89), in man (132), and in the grain mite (193), the fire-brat insect (*Thermobia domestica*) (18) and a pulmonate snail (214).

By a polarization optical technique, the fine structure of the DNA in the head of spermatozoa from the cave cricket (*Ceuthophilus nigricans*) has been studied (146). It is suggested from this work that deoxyribonucleoprotein, in molecules about 20–30 Å thick, is arranged in bundles of several hundred molecules in the form of a long helix about 2000 Å thick. This, in turn, is wound to form another elongated helix, 8000 Å in diameter. Two such helices, together with a matrix protein, make up a sperm chromosome. Clearly much useful information on fine structure changes after hormone and drug treatment could be obtained, but interpretation of these events is only now becoming possible.

III. BIOCHEMISTRY AND ENZYME ACTIVITY

A. Nucleic acids

The conversion of a single diploid cell into 96 haploid spermatozoa requires a 48-fold increase in the amount of deoxyribonucleic acid by replication. From autoradiographic evidence, all of this synthetic activity occurs within the first quarter of the testicular phase of spermatogenesis and in the rat and mouse is confined to those cells of the spermatogenic series which lie on or near the basement membrane. The 5 mitotic divisions in the rat testis are accompanied by intense DNA synthesis as shown by thymidine-H^3 uptake studies (53). Similar intense activity has been seen in the testis of the C_3H mouse (194), where the

spermatogonial intermitotic synthetic periods have been clearly demonstrated as peaks of increased thymidine-H^3 uptake. No DNA synthesis has been observed to occur during meiotic prophase of the rat or mouse; the oldest cells to show activity are the resting primary spermatocytes (53, 284) before the meiotic prophase commences. DNA synthesis is also complete before meiosis in the locust (*Schistocerca gregaria*) (64). It was further shown in grasshopper (*M. differentialis*) (172) that DNA synthesis occurs at different stages of the synthetic (S) period in different chromosomes, the last to synthesize DNA being the sex chromosome. Late labelling appears to be a characteristic feature of certain heterochromatic regions.

Ribonucleic acid synthesis has also been shown by incorporation of uridine-H^3 or cytidine-H^3 in the mouse testis (195, 197). It occurred at a high rate during early spermatogonial stages decreasing to a very low rate in resting prespermatocytes, the whole of the period that DNA is being synthesized (194), and fell to an even lower level in the early meiotic stages. It increased rapidly in midmeiotic prophase (midpachytene), but declined again during later stages just preceding the meiotic division. No synthesis occurred during metaphase and anaphase stages of the division itself, and no labelling in spermatid stages later than Stage 8 were observed. Further studies also revealed that during the spermatid stages, labelled RNA was transferred from the nucleus to the cytoplasm. In the later stages of spermatid development the prelabelled RNA is concentrated in the "residual bodies" and extruded from the cells shortly before their release into the lumen of the tubule. It would appear from these observations therefore that the mature spermatozoa are unlikely to contain much RNA. Sertoli cells also incorporated RNA precursors into their nuclei, and particularly concentrated them in the nucleoli. This supports Brachet's observations that this cell type contains a high level of ribonucleic acid (31). A detailed time study (197) has shown that RNA synthesis was continuous throughout spermatogonial development except for short periods of about 1 hour during metaphase and anaphase. After brief exposure to the same precursors in *Schistocerca gregaria* (64) all spermatocytes with the exception of those in the first and second metaphase, anaphase, and early telophase stages of meiotic prophase, were labelled. Further detailed cytological analysis in this species, together with *Cyrtacanthacris tartarica* and the grasshopper (*Chorthippus brunneus*) showed that the X univalent, the unpaired chromosome which characterizes the male insect, is completely inactive in RNA and DNA synthesis during the whole of meiosis (129, 130). As the chromosome condensation occurs during spermatid metamorphosis, both RNA and protein synthesis decrease. The depression of RNA synthesis is not directly related to chromosome condensation, since the latter commences after the RNA synthesis has fallen to low levels, but occurs at a time when the DNA-protein complex is undergoing rearrangement whereby the lysine-rich histone is being replaced by, or supplemented with, an arginine-rich protein. In *Orchestia gammaralla* (187), cytoplasmic RNA is increased in spermatogonia but is less in "primary" than "secondary" spermatogonia. In the rat testis (62, 63) it was shown by means of a histochemical method using pyronine, that cytoplasmic RNA in-

creased gradually during the early pachytene stage and then decreased until just before cell division. No obvious changes in total RNA content was observed by these workers. Within the otherwise positively stained large nucleolus at pachytene, an RNA-negative zone appeared; this gradually increased in size and filled the entire nucleus. From these data, and from data derived from a measurement of nucleolar diameter changes, it would appear that RNA is synthesized in the nuclei during late meiotic prophase and is suddenly released to the cytoplasm as the cell enters metaphase (195). These results are reminiscent of the observations of Jacobsen (145) in another mitotically dividing system, the leukemic leucocyte. A progressive decrease in the ribonucleic acid content in the cytoplasm during spermatogenesis in grasshopper, mouse, rabbit, and planaria has also been reported (31). In a number of families of insects, Verson's or apical cells have been described; they line the spermatogenic follicles and elaborate a basophilic granular material (chondriome) which "is fed to" the germ cells. These "nurse" cells are often seen in close association with spermatogonia. In the Pteropode (*Hyalocylus striata*), a similar role of the apical cell has been described (quoted in 199).

In the isopod, *Asellus* (199), the synchronized spermatogenic cells are surrounded by a secretory layer of large cells, with large polyploid nuclei. The secretion from these cells occurs in waves during the leptotene-zygotene stages, and again during diakinesis-anaphase I, and has been shown, by histochemical means, to be transferred to and absorbed by germ cells. The secretion appears to be an RNA that is considered by these authors to be essential for the completion of meiosis. In the plant kingdom (228) a similar diminution of RNA occurs during meiotic prophase.

B. Proteins

Histochemical and autoradiographic evidence for the localization of areas of protein synthesis in the mouse testis has been described (196, 197). Although the regions of protein synthesis may be determined by uptake of labelled amino acids, there is the problem of relating the change in amino acid uptake to changes in enzyme or other protein levels in the tissue. This correlation appears more clear-cut when arginine-H^3 is used to determine such synthesis in the mouse testis (196, 197). It was earlier shown (1, 25, 26, 27) that protein is synthesized during spermiogenesis. Furthermore, a protein extractable from bovine spermatozoa (32) was shown to be 35 % arginine, from which polypeptides consisting of 80 % arginine have been separated. A basic keratin-like protein has also been isolated from mammalian spermatozoa (131). From autoradiographic data (196), it would appear that the arginine-rich protein is synthesized predominantly in the late, elongated spermatids in Stages 11 to 14 in the mouse, during which time the synthesis with other amino acids appears to be either low or absent. Furthermore, the labelling appears to occur predominantly over the nucleus. The rate of protein synthesis, as measured by grain counting, was found to be closely related to RNA synthesis in the rest of spermatogenesis. The arginine-rich protein synthesis was a true synthesis and not a transition from other

existing proteins (196). The effect of glucose on the uptake of L-lysine by spermatogenic cells has also been studied (65).

Sertoli cells also synthesize protein in their nucleoplasm and nucleoli. Histochemical evidence for leucine amino-peptidase (EC 3.4.1.1.) has also been obtained (295).

C. Respiratory and energy systems

Glucose is an essential requirement for the proper functioning of the testicular tissue (264) and its depletion in the blood supply together with a decrease in the availability of oxygen has been suggested as one of the main causes of testicular failure when the testis is exposed to elevated temperatures. Only a few of the enzymes of the glycolytic system have been studied in the testis by histochemical methods on intact tissue or by gross biochemical methods on homogenates. The distribution of glycogen, which forms a relatively conspicuous particle visible in electron microscopy as well as in light microscopy, has been examined in the testis. There appears to be no glycogen in spermatogonia of the mouse (218), very little in this cell type in other rodents and dogs, and more in the ram and in spermatogonia Type B of stallions and cats. Bull and human (198) spermatogonia, however, are rich in glycogen. In human tissue, the glycogen disappears in the later stages of primary spermatocyte (198), and it has been suggested that this rise and fall in the glycogen level is consistent with the carbohydrate stage in the synthesis of amino acids and proteins. Carbohydrate is further required for the synthesis of mucopolysaccharides present in the acrosome stages of spermatid, and has also been demonstrated in Sertoli cells of adult rat, guinea pig and man (295).

Glycerol phosphate dehydrogenase (L-glycerol-3-phosphate: NAD oxidoreductase, EC 1.1.1.8) appears coincidentally with the appearance of mature spermatozoa. The increase of the enzyme activity of the testis observed by gross biochemical assay is due mainly to the increasing numbers of spermatids in Stages 18 to 19 of spermiogenesis, and that observed in epididymis is due almost entirely to spermatozoa (263). The level of the enzyme activity also appears to be directly related to the capacity of the spermatozoa for aerobic metabolism. It was further shown that the size of the mitochondrial midpiece of the spermatozoa was directly related to the activity of this enzyme.

Since the discovery of the isoenzyme composition of *Lactate dehydrogenase* (L-lactate:NAD oxidoreductase, EC 1.1.1.27) and its relative ease of resolution by starch and polyacrylamide gel electrophoresis, many tissues have been examined for variation in isoenzyme composition. Normally five isoenzymes of lactate dehydrogenase may be identified, possessing distinct electrophoretic mobilities qualitatively similar for most of the tisues examined. The testis, however, possesses an extra isoenzyme; this has been found in the spermatozoa of the bull, rabbit, mouse, rat and guinea pig (103, 311, 312) and in man (24, 101, 248). In the mouse testis (3) the enzyme has a greater affinity for DL-α-hydroxyvalerate than do other forms of the enzyme. It has been suggested from more detailed work with human testicular preparations that two genetic loci

(102) may be involved in the biosynthesis of this enzyme and that the degree of activity of this unique enzyme may be a useful index of male fertility (24). Pigeon testis shows three distinct groups of lactate dehydrogenase enzyme patterns (313) suggesting that in this species, the isoenzyme system may be under the control of three separate genes. The roles of the individual isoenzymes are of course unknown. If the crystallization of the unique testis isoenzyme can be achieved, a better understanding of its role in spermatozoa metabolism is likely.

The respiratory enzyme systems in spermatozoa have been reviewed (14) and a cytochemical technique for the localization of oxidative enzyme systems in spermatozoa in semen smears has been described (14).

Of the enzymes in the citric acid cycle, only two have been examined in any detail in the testis. *Malate dehydrogenase* (L-malate:NAD oxidoreductase, EC 1.1.1.37) assists in the oxidation of malate to oxaloacetate. This enzyme is clearly important in the oxidation of acetyl CoA arising from carbohydrate, fat, or other sources. A similar importance would also be attached to *Succinate dehydrogenase* (Succinate: acceptor oxidoreductase EC 1.3.99.1) an iron-containing flavoprotein, which assists in the conversion of succinate to fumarate. The distribution of malate dehydrogenase in the testis is unknown but succinate dehydrogenase has been shown to occur in most of the cells of the germinal epithelium. The latter enzyme is somewhat more active in interstitial cells (220, 292) and Sertoli cells (195, 292), than in spermatogonia and spermatocytes. The histochemical localization of this enzyme in bull spermatozoa has been demonstrated by light and electron microscopic methods (210, 211).

Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) is active in Sertoli cells. This may be related to the sustentacular activity of this cell, and to its activity in maintaining glucose levels for the developing spermatid, which would not have any access to glucose otherwise. *Glucose-6-phosphate dehydrogenase* (D-glucose-6-phosphate : NADP oxidoreductase, EC 1.1.1.49), the first step of the glucose-phosphate shunt, has been shown to be active in the Leydig cells especially of the human foetal testis (307). *Glutamine synthetase* [L-glutamate: ammonia ligase (ADP), EC 6.3.1.2] an enzyme activated by Mg^{++} or Mn^{++} and inhibited by Ca^{++}, catalyzes the conversion of L-glutamic acid to glutamine. The conversion involves the dephosphorylation of ATP to ADP, the latter assisting enzyme-complex formation. The enzyme is involved in a number of transamination reactions, as well as anabolically in the synthesis of purines. It has been identified in testis extracts (171) but has not been demonstrated histochemically.

β-Glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31) catalyzes the hydrolysis of β-D-glucuronides to D-glucuronic acids. The enzyme is also responsible for the conjugation of steroids and takes some part in the mechanisms involved in cellular proliferation. It has been demonstrated in testicular homogenates (189) and appears to be particularly associated with primary spermatocytes and less so with spermatids. The remaining germinal elements as well as Sertoli cells showed only very weak activity, or none. The residual bodies shed

into the lumen during spermiogenesis showed a high degree of activity (124). The enzyme is also usually associated with degeneration of tissue, and is present in testis undergoing atrophy. The activity of the enzyme after X-irradiation of rat testis has also been measured (237). A related hydrolase, *hyaluronidase* (hyaluronate glycanohydrolase, EC 3.2.1.d) is a well known testis constituent, and catalyzes the hydrolysis of hyaluronate and chondroitin and mucoitin sulphates, as well as certain transglycosylation reactions. The activity increases as the testis matures and is highest in the mature testis (271). It appears to be particularly associated with the spermatozoon, since the enzyme activity is directly related to the sperm count (20, 265). From fluorescent antibody studies, it was originally suggested that both primary and secondary spermatocytes produce the enzyme (250, 291), but a more recent investigation (180), with a rabbit antiserum to a highly purified enzyme preparation from bull testis, has shown that both spermatids and spermatozoa were fluorescent and, in particular, that a concentration of activity was associated with the acrosome. This supports an earlier contention that the acrosome is the carrier of this enzyme in mammals (170). Since agents that normally remove the periodic acid-Schiff stain in spermatozoa, namely digitonin (9) or weak alkali (51), also remove the fluoresence, a close association perhaps exists between the mucopolysaccharide of the acrosome and hyaluronidase. Cock spermatozoa appear to lack this enzyme (181), but the acrosome structure of this species is known to be unusual. The ability to hydrolyze the β,1–3 links between glucuronate and 2-acetylamino-2-deoxy-D-glucose in hyaluronate suggests that the function of hyaluronidase is in some way related to the penetration of the egg by the spermatozoa before fertilization (9). The enzyme appears to act in two steps, an initial reaction converting the polysaccharide to oligosaccharide components, followed by a combined action of the hyaluronidase and β-glucuronidase to convert the oligosaccharide to monomeric sugar derivatives.

Nonspecific phosphatases, with both acid and alkaline pH optima, have been demonstrated in the testis. A detailed comparison of the histochemical localization of these in the testes of the Indian Desert gerbil (*Meriones hurrianae*) and the house shrew (*Suncus murianus sindensis*) has been undertaken (183). *Acid phosphatase* (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was demonstrated in both Sertoli cells and early spermatogenic cells (308) of mouse at a pH optima of 5.0, and in the rabbit testis at pH 4.9 (203). In rat testis (223) cyclical changes have been observed. More recently, electron microscopy has revealed activity in Golgi apparatus of spermatogenic and Sertoli cells (289) which appears to be associated with the state of the mitochondrion. *Alkaline phosphatase* (orthophosphoric ester phosphohydrolase, EC 3.1.3.2) with a pH optimum of 8 to 10 has been found to be associated with the basement membrane (67a, 289, 292), capillaries (67a) and Leydig cells (292), and electron microscope studies have shown activity associated with Sertoli cells (289). *Nucleoside diphosphatase* (nucleosidediphosphate phosphohydrolase, EC 3.6.1.6), which catalyzes the hydrolysis of nucleoside diphosphates, has been studied with particular reference to thiamine pyrophosphate phosphohydrolase activity, and

is localized in the large Golgi zone in both spermatogonia and spermatocytes (221, 289). In particular, the spermatocytes of Stages 10 to 13 were especially rich. In Stage 14, however, the activity disappeared at metaphase. During the spermatid phase, a small active area next to the nucleus was present from Stage 1 to Stage 7; during the acrosome phase, this active area decreased in intensity and moved away from the nucleus into the cytoplasm. During the final stages of spermatid development (17 to 18), however, the Golgi apparatus began to fragment, and the activity then spread throughout the Sertoli cells. It has been suggested (4) that the Golgi zone may contain several nucleoside phosphatases and the same zone of both spermatogenic cells and Sertoli cells has been shown to exhibit di- and trinucleosidephosphatase activity (289). Another phosphoric monoester hydrolase, *viz.*, *5-nucleotidase* (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5), an essentially catabolic enzyme, has been detected in the nuclei of cells of the male germinal epithelium of the rat, an intense zone of nucleotidase activity existing between the Sertoli cell and the late spermatids (289).

An interesting suggestion was put forward (287, 288) based on work with spermatozoal tails of the perch (*Perca fluviatilis*). Since hydrolysis of ATP by sperm tails was accompanied by a swelling due to an intake of water, it was suggested that under normal circumstances, the two processes may be inversely related, the energy being transferred from one to the other.

The histochemical localization of *phosphoamidase* (phosphoamide hydrolase, EC 3.9.1.1.), an enzyme which assists in the hydrolysis of phosphocreatine to creatine and of phosphoarginine to arginine, has been studied (188). Early spermatocytes (leptotene stage of meiotic prophase) showed the highest levels of activity, with decline towards the beginning of the later pachytene stage. No activity was observed in Type A spermatogonia.

D. Lipid metabolism

Of eight species studied, guinea pig had the highest total lipid content (3.4 %) in the testis and man had the lowest (1.6 %) (22). The lipid content appears to be a characteristic feature of the species and is independent of the linoleic content of the diet. The extent of accumulation of the C_{22} acids may be due to the relative efficiency of the enzymes that convert linoleic acid to these forms. The comparative neutral fat content of rat, guinea pig, and rabbit testes have been independently investigated (158, 243) and also the lipid content of the testis of the seasonally variable cotton rat (*Sigmadon hispidus*) (81). A detailed histochemical analysis of spermatogenesis and spermiogenesis in the Indian pangolin (*Manis crassicaudata*) has been described (182) using the Sudan B technique, and of particular interest was the intense activity of the Golgi apparatus of the spermatocyte and spermatid and in the mitochondria of the spermatid.

On complete separation of spermatozoa from environmental fluids by centrifugation, it was demonstrated (67) that phospholipid synthesis was complete before the spermatozoa leave the rat testis tubule and enter the epididymal phase; but in isolated spermatozoa of rainbow trout, biosynthesis of fatty acids, glycerides and phosphatides continues (286).

E. Ester hydrolysis

Several hydrolases have been detected in testicular tissue. *C-esterase* (acetic ester acetyl-hydrolase, EC 3.1.1.6) is normally responsible for assisting the hydrolysis of acetate esters to alcohol and acetic acid. The histochemical localization of such an enzyme has been demonstrated in the seminiferous epithelium, notably in Sertoli cells (222), the strongest activity being present in Stages 3, 4, and 5 at the period when the developing spermatids are grouped and surrounded by the Sertoli cell cytoplasm. The activity diminishes as the spermatozoa are released and increases again in the next few stages. These authors also described a small quantity of enzyme activity visible as the sperm is released from the seminiferous epithelium in Stage 8. *Arylesterase* (aryl ester hydrolase, EC 3.1.1.2) generally of wide distribution in mammalian tissue, has been found in the Leydig cell and to a certain extent in the Sertoli cell of the postpuberal testis (125, 134, 219, 294). The enzyme activity appears to be confined to the cytoplasmic processes of the Sertoli cell and undergoes cyclical changes in level of activity parallel with the morphological changes of the cell (220). Its activity in the blood plasma of dogs is influenced by castration and by testosterone (8).

Pseudo cholinesterase (acyl choline acyl-hydrolase, EC 3.1.1.8), an enzyme characteristic of smooth musculature in general, does not show any distinct activity in the testis, rete testis, ductuli efferentes, and upper caput epididymis, but occurs with varying degrees of activity along the distal part of the epididymis (251). It was concluded from this observation that the regulation of smooth muscle in this organ is normally inhibited and is released from inhibition during ejaculation of spermatozoa (252). It is of interest also that spermatozoa of the bull also contain the enzyme (212).

F. Steroids

The action of drugs on the spermatogenic epithelium may be a direct one in many instances, but it is important to recognize the changes that occur through the normal hormonal control systems involved in testis biochemistry. The object of this section is to review some of these changes and to attempt to interpret the action of those substances which appear to influence the testis by interference with one or more of these mechanisms.

A major function of the testis is to synthesize androgens. These are normally synthesized by the Leydig cells of the testis and by the adrenals and are responsible for maintaining the proper functioning of sexual accessory organs as well as secondary male sex characteristics. Very low levels of testosterone ensure the proper functioning of the testis, exerting a direct action on the spermatogenesis and by a feed-back mechanism inhibiting the pituitary secretion of gonadotrophic hormone. The latter, in turn, controls the functioning of the testis and accessory tissues. Treatment of male rats with testosterone propionate (0.1 mg per day) inhibits the gonadotrophin secretion by the pituitary, and consequently testis function is also inhibited. Higher doses overshadow the inhibition of gonadotrophin secretion and maintains the testis directly.

One of the characteristic features of the depletion of gonadotrophin activity

on the testis is an arrest of the spermatogenic cells at the level of the secondary spermatocyte and early spermatid.

Although the main site of androgen synthesis has been considered to be the interstitial tissue, direct evidence has only recently been obtained by histochemical (169, 301) and biochemical (47) methods. Recent work (47) with rat testis suggests that both interstitial and seminal tubular tissue can synthesize testosterone, the former contributing some 90 % of the total testicular secretion of androgen. The steroid metabolism of an interstitial cell tumour has also been described (269). Testis slices accumulate an intermediate in testosterone synthesis, namely androst-4-en-3,17-dione, but *in vivo* the synthesis goes to completion and testosterone is formed (285). Noteworthy in this respect is the observation (96) that androst-4-en-3,17-dione is converted to testosterone by human blood *in vitro*. Testosterone-^{14}C has been isolated as a product of incubation of rabbit testis slices with acetate-^{14}C (120, 121) and also with glucose-^{14}C, and the importance of cholesterol in this system has been stressed (185). In the glucose-^{14}C experiment, testicular protein was also labelled. The results indicate that the adult testis, as well as the fetal rabbit gonad, is capable of a complete biosynthesis (173) of testosterone through progesterone and dehydroepiandrosterone, in much the same way as liver. A number of attempts have been made to investigate the routes by which the testis synthesizes androgens. It has been shown that mevalonic acid (260, 261) is converted rapidly into 5-phosphomevalonic acid by a 700 $\times$ *g* supernatant from rat testis homogenate, but if this is further centrifuged, at 110,000 $\times$ *g*, the supernatant is then capable of synthesizing 5-pyrophosphomevalonic acid, isopentenyl pyrophoshpate, and allyl pyrophosphate. On comparing slices with homogenate (120), it was suggested that a homogenate behaved like a slice that was stimulated with interstitial cell stimulating hormone (ICSH). Subcellular particles have also been isolated from rat testis which can form testosterone (266).

An important enzyme system in the final conversion of pregnenolone to progesterone is *3-β-hydroxysteroid dehydrogenase* [3-β-hydroxysteroid:NAD(P) oxidoreductase, EC 1.1.1.51]. The Leydig cell is by far the richest source of this enzyme except in certain primates (177). It has been shown that more than one enzyme of this type occurs in Leydig cells (11, 104) and in the foetal mouse (10, 12) and human foetal testis (13) and these appear at different stages of development. Whether these isoenzymes have specific substrates remains to be determined, but it is theoretically possible for at least four to exist: 1) converting pregnenolone to progesterone; 2) converting the corresponding 17-hydroxy pregnenolone to 17-hydroxy progesterone; 3) converting 3-β-hydroxyandrost-5en-17-one to the corresponding dione; and finally 4) converting androstene itself to testosterone. Most of the enzymes responsible for these conversions have now been found in the testis (176) and some have been partially purified (175). By using double isotope labelling techniques the relative contribution of 5-pregnenolone and progesterone to the biosynthesis of testosterone has been determined in mouse testis (75). Changes in their relative contributions after X-rays have also been measured (21, 74). The catabolism of testosterone-^{3}H in

men (2) and testosterone-^{14}C in rats (273) has been described. The back-conversion of progesterone to pregnenolone has also been observed in rabbit testis slices (257).

An ingenious use of double isotope labelling to determine the relative contribution of two alternative catabolic pathways has been described (160). Catabolism of Ring A of testosterone is possible by two alternative routes, reduction *via* an allylic alcohol or *via* the saturated ketone. By using a 3-β(^{3}H), 4(^{14}C)-labelled 4-androstene-3α, 17β-diol and 3α(^{3}H), 4(^{14}C)-labelled 4-androstene-3β,17β-diol, the two alternative routes were compared (482). In normal subjects, the allylic alcohol must be oxidized to testosterone almost exclusively before reduction. The double labelling technique provides a potentially valuable tool in measuring the relative changes in the contribution of two pathways brought about by the action of a drug. The conversion of pregnenolone to progesterone has also been described in dog (72) and in rat (208).

When testosterone is given continuously from birth at levels suppressing pituitary gonadotrophins, initiation of spermatogenesis at puberty is unaffected but subsequent spermatogenesis is blocked (38, 275, 281). It appears, therefore, that the initial wave of spermatogenesis at puberty requires a different stimulus from that which maintains it in the adult. The difference in the timing of spermatogenesis in the pre- and postpuberal testis may be a reflection of this different initial stimulus (133).

An adequate androgen level is necessary for the full development of the spermatogonial process and is capable alone of maintaining spermatogenesis in hypophysectomized rats (123, 174) even though testicular weight is not fully maintained. The fact that this weight difference is apparently due to a 30% reduction in the spermatogonia Type A population (122) suggests that other factors, such as follicle stimulating hormone (FSH) (242), may be required to maintain the full complement of these spermatogonia (*cf.* 161). In man, dehydroepiandrosterone, but not testosterone, exhibits a diurnal rhythm in plasma levels (156). The possibility thus exists of a diurnal susceptibility to antiandrogenic drugs that interfere with this pathway, but, as far as the authors are aware, this has not been observed. There are many similarities between the Leydig cell-anterior pituitary axis and the adrenal cortical-anterior pituitary axis and these have been discussed (156). Prednisone was shown in this work to cause a marked depletion in the dehydroepiandrosterone levels with much less marked action on the testosterone levels in the plasma.

The effect of androgens on the phosphorylase (a) and phosphorylase (t) activity of the ductus deferens of the rat has been investigated (168). Testosterone appears to reverse the effects of castration on this activity, although glycogen levels appear to remain unchanged. Animals that normally exhibit a seasonal variation in sexual activity also show a seasonal susceptibility to the effect of implantation of testosterone pellets (17).

An interesting feature of the action of testosterone at the molecular level, which has excited the interest of a large number of investigators, is that it appears to reduce the binding between histone and DNA in the prostate (268) which in turn

alters the gene activity in this gland (306). It would be interesting to correlate this activity with the changes observed in erythropoietin activity that follow administration of testosterone (192). Changes in the nucleic acid composition of the seminal vesicles of mice after castration can be reversed with testosterone (153), reversal being accompanied by an increase in the cellularity of the gland. This appears to be one system where gene-controlling activity brought about by steroids may be studied in greater detail.

IV. INTERFERENCE WITH HORMONAL CONTROL BY STEROIDS

In an attempt to develop agents useful for inducing male sterility, various steroidal derivatives closely related to the androgens have been made, in the hope that some competitive action with naturally occurring androgens may suppress spermatogenesis.

The action of one of these agents, namely MCHPA (1,2-α-methylene-6-chloro-Δ^6-17α-hydroxyprogesterone-17-acetate) on the testes of adult and prepuberal rats has been investigated. Its action on the adult testis appears to be typical of that of an antiandrogen in that the later stages of spermatogenesis are the most sensitive to the drug and the spermatogonia the least sensitive. Its mode of action is considered to be a "competitive blocking of androgen receptors within the target organs" (215). In hypophysectomized animals, testosterone is capable of maintaining spermatogenesis and the normal functioning of the accessory organs. However, MCHPA will interfere with this restorative action of testosterone (215). Some further clues with regard to the mechanism of action of this steroid may be obtained by treatment of female rats during the last part of pregnancy and litters during the first 3 weeks postpartum. The male offspring from such treatments developed vaginae and other female characteristics; the result indicates that interference with androgen activity is at a very early stage in the development of sex organs (216, 217).

Several other steroidal antiandrogens have been tried in the male rat, including 2α-hydroxymethyl-17-hydroxy-5α-androstan-3-one, 2-hydroxy-5α-androst-2-en-17-ol, 2,17α-dimethyl-5α-androst-2-en-17-ol, and 2-formyl-5α-androst-2-en-17-ol. All decreased the weight of the testis, ventral prostate and seminal vesicle and arrested spermatogenesis (155). 19-Norprogesterone on the other hand did not cause testicular damage but affected ventral prostate and seminal vesicle weights. These results are consistent with the view that the compounds have different degrees of antiandrogenic action.

The effect of continuous application of *oestrogenic hormone* to the male rat (161) by absorption from an implanted pellet has been examined, 1 to 6 months after subcutaneous implantation. The pellet in this study consisted of hexoestrol (7.5 mg) and stilboestrol (7.5 mg). An initial obvious decrease in spermatids was followed by a more gradual fall in the level of spermatocytes, until finally only spermatogonia and a few primary spermatocytes were left. Spermatogonia proved to be the most resistant and spermatocytes the most sensitive to the action of this steroid. ICSH administered simultaneously had no effect but FSH effected a dramatic return to normal spermatogenesis. During the estrogenic action,

degenerating cells were phagocytosed by Sertoli cells, and the latter showed an increase in lipid droplets. Administration of FSH caused a marked disappearance of these droplets.

The action of *progesterone* and biologically related compounds have been extensively investigated in the female (241). It is considered that its main action is mediated *via* the pituitary by inhibiting the release of gonadotrophic hormone (19, 71). It has further been shown that progestens can inhibit spermatogenesis in man (127) and in rabbits (80). Both progesterone and 6α-methyl-17-α-hydroxyprogesterone acetate (MAP) (79), inhibit spermatogenesis and accessory gland function in the ram testis. Histological changes include atrophy of Leydig cells, disorganization of the germinal epithelium and sloughing of the immature elements, giving rise to a considerable amount of debris in the lumen. It is not clear from this work if any particular cell type is more sensitive than others. A fall of sperm count to minimal values between the 10th and 14th weeks after treatment suggested that early spermatogonial populations had been affected.

Testosterone production may be inhibited by low doses of 2α-methyl dihydrotestosterone propionate (66), but it is not clear whether this is an action mediated *via* the pituitary or directly on the testosterone synthesizing cells in the testis.

V. NATURE OF DRUG ACTION

A. Antimetabolites

The biochemical lesions induced by antimetabolites have been the subject of detailed and extensive studies in many biological systems and a great deal is now known with regard to their site of activity. It is clear that in this field in particular, there is potentially a considerable amount of information to be gained, not only to confirm the site of action of the agent itself but also to throw some light on the phase of spermatogenesis when such a biochemical change is of most importance. Surprisingly, the limited number of studies in spermatogenesis have produced little that is useful to either study. One characteristic feature amongst the few agents that have been tried in rats is that a general depression of fertility throughout the major part of the spermatogenic cycle occurs, without any of the distinct sterile periods such as those induced by the alkylating agents. This subfertility could be the result of a disturbance of the uniform production of spermatozoa due to kinetic damage of the spermatogonial population or by short-lived cellular damage resulting in only a small fraction of cells in each cell cycle being killed. After treatment the rat would thus be wholly sterile, intermittently sterile, or still fully fertile. The detailed examination of the mean litter sizes, which tend to show the kind of variation expected, supported this possibility (92).

6-Azauracil inhibits orotidylic acid decarboxylase after conversion to 6-azauridylic acid *in vivo* (303). After treatment of male rats with a dose of 200 mg/kg daily for 20 days, the mitotic counts in the testis fell to 30 % of the control values (148). When *6-azauridine* was given at higher dose levels (five doses of 500 mg/kg) and shorter time intervals, namely every 8 hours, a mixed group of intermittently and fully fertile animals was produced (92). The main period of subfertility occurred over the period in which nucleic acid synthesis is known to occur.

6-Azacytidine is deaminated *in vivo* to 6-azauridine. After five daily treatments with 600 mg/kg to a group of male rats (92), essentially the same kind of result as that obtained with 6-azauridine was observed, with the exception that a reduced fertility, as a result of the action of the compound on spermatozoa and late spermatids, was more apparent. The possibility that this additional activity could be due to the presence of deaminase activity in spermatozoa and late spermatids was not determined. *5-Iododeoxyuridine* (five daily doses of 100 mg/kg) produced a similar depression of fertility corresponding to an action on the DNA-synthesizing cells of the rat, whereas *6-thioguanine* (2-amino-6-mercaptopurine, 80 mg/kg given intraperitoneally) from fertility data appeared to exert a greater action on the late spermatids and spermatozoa, but the nature of the damage was not determined (92). *Amethopterin* (1.5 mg/kg) failed to produce any significant action (92) on the germinal epithelium of the rat as determined by routine mating experiments.

With regard to these somewhat sparse data, it would be necessary, in order to gain some insight into the mode of action of this group of agents on the spermatogenic epithelium to 1) determine by quantitative histological methods if depletion of the spermatogonial and primary spermatocyte populations (partial cellular damage) had occurred, and 2) estimate if loss of function had occurred without loss of cells (early genetic damage), and 3) ascertain whether, owing to the known synchronizing activity of these antimetabolites, some kinetic damage had been effected, which would result in an upset of the normal balanced phase frequency amongst the tubules and result in intermittent fertility.

Ethionine inhibits the incorporation of methionine and glycine into liver proteins (267) and is also recognized as an inhibitor of xanthine oxidase (EC 1.2.3.2.) (68) as well as of several other enzyme systems (for review see 277a). Furthermore, sex differences in its action have been noted (88) especially with regard to its tumour-inhibitory activity (204), which is greater in females than in males. The basic mode of action of this agent is clearly very complex and many sites appear to be involved. There is also good evidence (70, 88) that the hormonal system plays a part in many of its actions, since castration markedly enhances the toxicity and tumour-inhibitory activity of ethionine in male rats, an effect which is reversed by testosterone. It is suspected that the mechanism of action is in some unknown way mediated through the androgens (70). The order of disappearance of the cell elements after administration of ethionine to the male rat (154) closely resembles that produced by oestrogenic hormone (161) (Section IV); this finding further supports the view that the endocrine system may be involved.

B. Alkylating agents

The basic lesion resulting in a biological action by an alkylating agent is still not understood. Many suggestions have been made based either on the observation of labelled macromolecules, isolated from a number of systems after treatment with an isotopically labelled drug *in vivo*, or by direct treatment of a macromolecule *in vitro*. It is clear that there are potentially several sites of attack by these agents, and they have been summarized in several recent publications (258,

304). Alkylating agents are known to react with thiols (229), phosphate esters (247), ribonucleic acid components (178, 179), deoxyribonucleic acid components (34, 272, 279) and proteins (178). The biologically significant lesion may be the result of different extents of action with these sites at different cellular levels. Spermatogenesis offers a variety of biochemically important processes which these agents may attack and thus gives a means whereby the important activities of these agents may be selectively illustrated.

Nitrogen mustard derivatives have been extensively investigated from the point of view of their antitumour action (258, 304). In work associated with the screening of agents likely to induce sterility in the screw-worm fly (*Cochliomyia hominivorax, Coqueral*) (58), *2,2'-dichloro-N-methyldiethylamine hydrochloride* (mechlorethamine, HN_2) showed no effect on the male insect. Work in Drosophila, however (23), showed that certain nitrogen mustard derivatives, notably the *aryl-2-halogenoalkylamines* can induce mutations causing complete sterility in the male. In this species these nitrogen mustards exert their maximal activity on mature spermatozoa, in which the chromatin is known not to be anabolically active (83, 84). It would thus appear that genetic damage in male screw-worm flies cannot be the result of metabolic antagonism during the synthesis of nucleoproteins. Another action of nitrogen mustard in Insecta is to prevent egg hatching, and this indicates that it exerts its action on intact nucleic acid within the spermatozoa. In the wasp, *Habrobracon*, spermatozoa were more readily affected at similar dose levels than the larger, yolk-laden eggs of the female (305). Perhaps part of this difference in sensitivity was due to the accessibility of the chromosome to the alkylating agent.

In higher organisms, HN_2 has been reported to cause prolonged sterility (105) in rats and testicular lesions in the mouse (164), but more recent work has failed to repeat this result (140). *Nitromin* (2,2'-dichloro-N-methyldiethylamine hydrochloride-N-oxide) is less toxic than HN_2, and there is some evidence that maturing spermatozoa were affected, but no other testicular lesion was obvious (140). Certain of the aryl-substituted mustards have also been tried in male rats and in general showed little activity. *Melphelan* [*p*-(N,N-di-2-chloroethyl)amino-L-phenylalanine, CB 3025] and *chlorambucil* [*p*-(N,N-di-2-chloroethyl)amino-phenylbutyric acid, CB 1348] did not affect the fertility of male rats (142), and this lack of activity strongly suggests, in support of the work in insects, that the influence of nitrogen mustard derivatives is not directed towards the synthetic phases of nucleic acid but, if at all, towards the fully synthesized and condensed form of DNA. The extent of action of these agents would then be greatly dependent on the accessibility of the DNA in the chromatin.

The intermediate in the biological action of nitrogen mustard derivatives is the ethyleneimmonium ring (258). TEM (triethylene melamine, Tretamine) exerts an action on the fertility of male rats (28). In mice this effect was shown to be a composite action on spermatogonia Type A (227) and on spermatids (56). It was then demonstrated that the action on spermatids resulted in the development of dominant lethals (15). Some animals littered from spermatozoa treated in the spermatid stage were semisterile (40, 41) because of the presence of chromosomal abnormalities.

At lower doses of TEM, the sterile period resulting from an action on spermatid development is not reflected by a fall in spermatozoal count in the rabbit (94) four to five weeks after treatment, but the action on spermatogonia results in an aspermia about 10 weeks after treatment. This would indicate that the action on spermatids is a functional impairment. Treatment of sea urchin spermatozoa *in vitro* (95), although not strictly comparable, suggest that this functional impairment may be due to the greater frequency of polyspermic fertilization. It needs to be determined, however, whether this is a major cause of the embryonic breakdown that results from dominant lethal mutations in mammalian studies with TEM (early genetic damage) (15). The spermatogonial action results in an impaired cell incapable of development through the complex series of processes required in spermatogenesis and could thus be described as cellular damage. The more drastic actions of *ethylene urea* and *ethylene urethane* (142), which can chemically empty the tubules of the rat testes of all spermatogenic cells, could thus be due to an extreme combination of both forms of damage.

On treating Drosophila spermatozoa with triethylene melamine, and allowing them to remain in the female for varying lengths of time after treatment, the number of translocations relative to the number of sex-linked lethals was found to increase 8- to 10-fold (300). This was not the case with *ethyleneimine* itself, and it was assumed in this case that cross-linking due to the action of triethylene melamine had occurred during storage and that during this period potential breaks would become true breaks. The nature of the potential break after ethyleneimine is different, however, and the break may even be capable of being repaired during the storage period, since no increase in frequency of true breaks occurred.

Several other derivatives of ethyleneimine have been tested on spermatogenesis in the rat (142). These include *ThioTEPA* (triethylenethiophosphoramide), *1,3-di- (ethylenesulphamoyl) propane*, *6-chloro- 2,4-diethyleneiminopyrimidine*, *diethyleneurea*, and *diethyleneiminosulphoxide*. All of these derivatives produce actions on spermatogenesis qualitatively similar to that of TEM and many of them have also been examined as insect sterilants (58–61).

The nature of the biochemical processes resulting in the cellular and early genetic damage produced by these agents is unknown, but the two types of damage could represent different degrees of damage to the DNA itself (159) or to some of the transcription and translation processes involved in the production of essential proteins from the DNA. Work with certain ethyleneimine derivatives in Neurospora mutants indicates that the biological effects seen in that system are the result of a nuclear action (147) as distinct from an extranuclear one.

The action of these agents appears to be directed in part toward cells that are undergoing rapid DNA synthesis as well as toward the developing spermatids. In certain cases, as with ethylene urea and ethylene urethane, the tubules become emptied of all germinal elements; this suggests that the stem cell compartment itself had been so seriously depleted as to allow only a minimal recovery. The testis wall structure and Sertoli cells remain intact, as also occurs after a very high dose of X-radiation (57).

It is noteworthy that from viscosity and electron microscopic observations

(159), DNA strand synthesis appears to develop a bifurcate branching activity after treatment with ethyleneimine; this suggests that a basic change in synthetic activity has occurred. The final stage of the ethyleneimine action is a complete disruption of synthesis and the development of short, fragmented strands. Many of the observed effects of this class of chemical may well represent various degrees of this type of damage.

Alkyl esters of alkane sulphonic acids have the useful property of delivering the alkyl group to an active nucleophilic centre within biological material. The nature of the biochemical lesion produced as a result is dependent on the structure of the alkyl group, and since this may be varied in an infinite number of ways, the spectrum of activity is potentially very great. The mutagenic action of the simple esters of methane sulphonic acid, notably the methyl and ethyl esters, have been studied in viruses (35), bacteria (280), barley (Hordeum sps) (191), rats (234), mice (144, 232), and Drosophila (44, 82, 86). *Busulphan* (Myleran, 1,4-dimethanesulphonoxybutane) a substance with two potentially alkylating groups, used in the treatment of chronic myeloid leukeamia (97), has been shown to interfere with the early developmental processes of the myeloid (77), intestinal (278), and spermatogenic (29, 142, 143) cell systems.

In rodents, the short, straight-chain alkyl esters of methane, ethane, *n*-propane, and iso-propane sulphonic acids produce qualitatively identical patterns of activity on the testis, the most efficient in terms of dose given being the simplest of the series, namely *MMS* (methyl methane sulphonate). When given parenterally, intravenously, or orally to rats (143), this agent exerts a characteristic action on maturing spermatozoa in the early and middle stages of their development in the epididymis and the lumen of the testis. Repeated administration of low doses has a highly cumulative action and causes sterility in male rats for as long as treatment is continued, but fertility returns within 3 weeks after the treatment is ended (141). The sterility in this case is of the early genetic type and has been shown, after a single dose, to be due to the induction of dominant lethals in rats (234) and mice (232). In rats, spermatozoal penetration of the ovum has been shown to occur (234) and two degrees of dominant lethal damage have been demonstrated, one in which the eggs undergo a limited number of cleavages but fail to implant and another in which implantation occurs but is followed soon after by death of the egg. Treatment of the spermatozoa of sea urchins (95) caused a dose-dependent delay of the first cleavage of the egg, and it was suggested that a similar cleavage delay in mammalian eggs would upset the delicate hormonal balance of the developing egg and the endometrium and would in all probability lead to its death and rejection.

^{14}C-Methyl methane sulphonate has also been used. Its mode of detoxification in rats and mice has been resolved (240), and it has been shown to react directly with deoxyribonucleic acid and ribonucleic acid (166). It would appear that the most susceptible base in the nucleic acid structure is guanine, which is preferentially attacked in the 7 position (166). However, the manner in which the 7-substituted guanine induces the genetic and other changes observed is still an open question. It is not understood why, if the ester alkylates DNA as a primary

lesion, the highly condensed form in the sperm head should be the more vulnerable.

Both isopropyl methane sulphonate and isopropyl ethane sulphonate have an action on the spermatogenic epithelium qualitatively different from those of the straight-chain esters. In this case, all cells undergoing DNA synthesis, *viz.*, spermatogonia and primary spermatocytes, are affected by the drug, and cellular damage is the main cause of sterility (233). These esters appear to be equally effective when given orally or parenterally, and, in view of their relatively short half-life in aqueous solution (about 13 min at 37°C), this suggests that an active intermediate is formed *in vivo* which may be more stable than the parent ester.

The bifunctional alkane sulphonate, Busulphan, produces sterility in male rats about 9 weeks after treatment with a single dose (143). This sterility is associated with aspermia, and sections of testis taken at intervals for 9 weeks after treatment reveal that only the first division of the spermatogonia is affected by the drug. A detailed quantitative analysis of cellular changes (233) showed that the first intermitotic interval is in some way prolonged, and that this prevents the appearance of new spermatogonial cells to replace the maturing, unaffected cells, whose dynamics through spermatogenesis appear to be unaltered by the treatment. A similar phenomenon has been described for the action of this alkylating agent on the cells of the myeloid system (77). Using ^{14}C-busulphan, labelled in the butane portion of the molecule, it has been shown that the main excretory product is a tetrahydrothiophene derivative (229, 253) in which the sulphur does not come from the original busulphan but from sulphydryl groups *in vivo*. This product, however, could simply represent the main detoxification system and may not be related to the event which is associated with the biological activity. The mutagenic action of busulphan has been examined in Vicia and Hordeum (201) as well as on the primary spermatocytes of rat (234).

Busulphan belongs to a series of bifunctional methane sulphonates of straight chain alkane diols. The simplest member of this series is *methylene dimethanesulphonate*. This ester (93), besides exerting an action on spermatogonia, also affects spermatozoa within the epididymis. Thus both early genetic damage, as well as cellular damage, is produced by this agent. The diester of ethyleneglycol (*1,2-ethylene dimethanesulphonate*), however, exerts its maximal effect on the spermatocyte and spermatid stages of development (141).

The diversity of the biological effects of the lower members of this series suggests that their reaction with different nucleophilic centres is dependent on the special relationship of the two alkylating regions of the molecule. The effects of *2-chloroethyl methane sulphonate* on spermatogenesis in rat (142) and Drosophila (85) has also been examined. The pattern of activity in the rat resembled that of other simple alkyl esters in producing sterility due to early genetic damage in late spermatids and maturing spermatozoa. In Drosophila, however, it has been reported to affect spermatogonia after having been transformed into a bifunctional agent. The production of visible mutations (7) and translocations in spermatogonia (299) has also been described.

It is noteworthy that *dimethyl busulphan* (2,5-dimethanesulphonyl hexane)

induced cellular damage in spermatogonia, and early genetic damage in spermatozoa in the proximal parts of the epididymis and testis lumen (143).

C. Bis(dichloroacetyl)diamines

N,N'-bis-(dichloroacetyl)-N,N'-diethyl-1,4-xylenediamine (WIN 13099), *N,N'-bis-(dichloroacetyl)-N,N'-diethyl-1,6-hexamethylenediamine* (WIN 17416) and *N,N'-bis-(dichloroacetyl)-1,8-octamethylenediamine* (WIN 18446) suppress spermatogenesis in the monkey, rat, dog, mouse, and guinea pig without affecting gonadotrophin secretion (55, 69). These studies have led to trials in men (128) and aspermia has been reported without loss of libido when volunteers were administered WIN 13,099 in doses of 0.5 to 1.5 g daily for periods of 9 to 54 weeks. The sperm count dropped slowly over 9 weeks until very low counts were obtained from the 10th week onwards. On cessation of treatment, sperm counts began to recover about 8 to 9 weeks and were fully recovered by 12 to 14 weeks after cessation.

Histological examination of animal testes after high doses showed the tubules to be depleted of cells other than the Sertoli cells. This would indicate that the earliest spermatogenic cells had been affected by the drug. The depletion however extends from the spermatids, through spermatocytes to spermatogonia and markedly resembles the action of temperature elevation of the testis (see Section VI). A similar deduction may be made from human data, since recovery from medication is known to take upwards of 10 weeks after cessation of treatment and the spermatozoal count is normal by 20 weeks.

D. Dinitropyrrole derivatives

1-(N,N)diethylcarbamylmethyl)-2,4-dinitropyrrole (ORF 1616), studied in the rat (235) at dose levels of 50 to 500 mg/kg, inhibited spermatogenesis at the primary spermatocyte stage. The cytoplasm of pachytene spermatocytes exhibited increased reactivity to periodic acid-Schiff staining within a few hours, and by 24 hours, this cell type was missing. "Drastic cytological changes" were also observed in spermatids, mainly in Stages 1 to 14, the remaining spermatid stages, 15 to 19, being unaffected. By extrapolation, it would appear that about 40% of the spermatogonia Type A had also been affected, since degeneration of cells entering the meiotic prophase 15 days later was observed. Recovery of fertility by 47 to 48 days after treatment, however, indicated that the effect on spermatogonia Type A did not depress the sperm count enough to induce subsequent sterility. Unless the human spermatogenic epithelium is more sensitive than that of the rat to this compound, it is unlikely that it will be a useful antifertility agent, as doses of 10 to 30 g would be required.

The biochemical basis for the effect is unknown, but in view of the early histochemical changes observed with the periodic acid-Schiff staining and the involvement of the developing spermatid, it could be inferred that mucopolysaccharide metabolism had in some way been affected. The possibility that more general changes in glucose metabolism in the testis occur cannot be overlooked, however,

since the primary spermatocyte changes observed resemble those which are produced by induced glucose and oxygen lack and by heating experiments (see Section VI).

E. Nitrofuran derivatives

Furadroxyl [5-nitro-2-furfuraldehyde-2-(2-hydroxyethyl)semicarbazone] causes a reversible spermatogenic arrest (213, 244) without affecting spermatogonia, Sertoli cells, or interstitial tissue in the rat testis. Two other related compounds, *nitrofurazone* (Furacin, 5-nitro-2-furfuraldehyde semicarbazone) and *nitrofurantoin* [Furadantin, n-(5-nitro-2-furfurylidene)-1-amino-hydantoin] produce similar effects, namely, a block of spermatogenesis at the primary spermatocyte stage (214). On the other hand, nitrofurazone also causes (224) degeneration of the seminiferous tubules including spermatogonia, but not of Sertoli cells and interstitial cells. These agents inhibit glucose utilization (236) and it seems likely that they act in the same way as the dinitropyrroles described above. Certain thiophene derivatives, notably *5-chloro-2-acetylthiophene*, show a similar pattern of action on the germinal epithelium and their basic mode of action is likely to be similar. In the case of Furadroxyl, the effects on the testis are prevented by simultaneous administration of cysteine (76). Ten days after treatment with Furadroxyl, hyaluronidase activity and the number of spermatids were similarly decreased; this observation supports the idea that the enzyme is associated with this stage of spermatid development (276).

F. Methylhydrazine derivatives

Natulan [1-methyl-2-*p*-(isopropylcarbamoyl)-benzyl-hydrazine hydrochloride, Ro 4-6467] is a tumour inhibitor, and causes marked depression of spermatogenesis with atrophy of the testis (30). Male rats mated after treatment were sterile from the second week, and fertility did not recover (92). ^{14}C-Labelled Natulan is converted into terephthalic acid isopropylamide. It is considered that the drug acts by forming hydrogen peroxide and hence hydroxyl radicals (302), which indirectly or directly affect DNA.

G. Carcinogenic hydrocarbons

DMBA (dimethylbenzanthracene) produced a spermatogonial depletion which was reported to be accompanied by an increase in malate dehydrogenase activity (90, 91). This work has more recently been repeated, however, and although considerable spermatogonial depletion occurred the apparent rise in malate dehydrogenase activity was found to be due to loss of testis weight, since the total enzyme activity per testis did not change appreciably (1a). There appears to be a true increase in β-glucuronidase activity, but this enzyme increase may be a reflection of the increasing atrophy of the tissue. α-Glycerophosphate dehydrogenase activity decreased as the spermatozoa left the testis and were not replaced by new cells (263). *Benz(α)pyrene*, when administered to young mice in food did not affect their fertility (249).

H. Deuterium oxide

Deuterium oxide induces sterility in male mice (135, 136). This sterility results from a failure of spermatozoa to fertilize the egg (137) rather than from breakdown of the egg in early cleavage. It would appear from this work that the action is due to an abnormality in acrosome formation. Cytopathic effects of deuterium oxide on the testes of dogs and mice have also been recorded (5).

I. Cadmium and zinc

Cadmium was shown (149–151, 230, 231) to have a destructive effect on the testis of the rat when administered parenterally as cadmium chloride, and similar activity has been described in the mouse (184) and monkey (*Macacus irus*) (100). Intratesticular (150) and subcutaneous (39) injections in rabbits were also effective. Cadmium apparently interferes with the normal uptake of zinc by the testis and dorsolateral prostate, but not with its uptake by other tissues studied (114). Male chicks (78) did not respond to 1 mg/100 g body weight of cadmium chloride given subcutaneously.

The biochemical interpretation of the mode of action of cadmium salt interference is limited by lack of knowledge of the biochemistry of zinc in the testis and accessory glands (293), but zinc-deficient diets in rats are known to result in irreversible testicular atrophy (190). It has been suggested (45, 116) that vascular factors are of great importance in the regional destruction of the testis rather than specific sensitivity of the seminiferous tubules. Endothelial changes precede tubular changes, and it is possible (116) that there is an enzyme system peculiar to the panpiniform plexus, the vascular network which maintains the testis several degrees below body temperature, which is sensitive to cadmium and possibly dependent on zinc. It would appear that the enzyme concerned is normally involved in thermal regulation of the testis, since cadmium damage does not occur in nonscrotum animals studied.

Of considerable interest is the observation that weekly subcutaneous injections of 0.5 mg cadmium sulphate to 6- to 7-week-old rats over a 10-week period induced sarcomata at the site of injection. Also, both Leydig cell hyperplasia and neoplasia occurred together with the expected testicular atrophy (118).

^{65}Zinc is selectively incorporated into the dorsolateral prostate (117). Its uptake is believed to be influenced by both androgen and estrogen levels (106), since a striking fall in ^{65}Zn uptake may be reversed by concomitant application of chorionic gonadotrophin or testosterone (107). The ^{65}Zn uptake has even been used as a sensitive indicator of endocrine function in animals in which there is no apparent seasonal variation such as rats under constant laboratory conditions (108, 115). This residual or "archaic" reproductive cycle has furthermore been associated with a cyclic sensitivity of the rat to administered sex hormones (109), to adrenal secretions (110), and to psychosexual stimuli (111). A technique has been devised to use ^{65}Zn uptake as a sensitive assay for interstitial cell stimulating hormone activity (112). A further use of ^{65}Zn uptake (113) by the dorsolateral prostate was to determine the nature of the observed changes after total body irradiation of rats (470 r) which produced a depletion of spermatogonia in

the testis. The depletion was considered to evoke a pituitary response similar to those described after castration, which resulted in a temporary reduction in the capacity of the dorsolateral prostate to accumulate ^{65}Zn. Administration of testosterone or chorionic gonadotrophin reversed both the inhibition of ^{65}Zn uptake and the depletion of spermatogonia.

VI. EFFECTS OF ELEVATION OF TESTICULAR TEMPERATURE

When the scrotal temperature is raised to normal body temperatures, cellular damage occurs amongst the primary spermatocytes as well as to a lesser extent amongst the spermatids and spermatogonia. The inclusion of the results of some work on the effects of elevated temperatures on the histology and function of the testis is relevant to the action of certain drugs. This aspect has been investigated by a number of workers but with some conflicting interpretations. In guinea pig (310), rat (200, 274), and bull (157, 165) primary spermatocytes are considered to be the cells most sensitive to elevation of temperature, but there are no reliable quantitative studies. Such studies would be most useful as a basis of comparison with the effects of certain drugs (nitrofuranes, dichloroacetyl diamines, thiophenes, and dinitropyrrole compounds) that are known to exert an action on primary spermatocytes.

In rams, an immediate effect of raising the temperature of the testis some 7°C higher than normal was a dramatic increase in the uptake of oxygen and utilization of glucose (296). The authors argued that the chief cause of the resultant spermatogenic arrest was lack of oxygen and glucose to satisfy the sudden increased requirement by the testicular tissue (see also 264). The events seen after such local heating closely resemble those produced by circulatory arrest produced by ligation of the rat testis (226).

VII. ASSESSMENT OF GENETIC CHANGES

One of the inevitable consequences of the use of new drugs in the human male, in particular in connection with population control, is the possibility of induction of genetic changes in the offspring that may result from altered spermatozoa. The timing of the spermatogenic cycle in man is only partially known (126) and no studies of timing are available for the epididymal phase. Assuming this to be approximately 3 weeks, human spermatozoa might carry genetic damage for at least 4 months after treatment with a drug.

In animals, the simplest method of detecting genetic damage induced by drugs on the male germinal epithelium is by scoring the "dominant lethals" (pre- and postimplantation deaths). Preimplantation deaths are estimated by difference between the number of eggs ovulated and the total number of implantations and postimplantation deaths by the number of deciduomata. This gives some indication of the mutagenic properties of drugs, but since embryos are not allowed to come to term, one cannot estimate other potential damage (late genetic damage) apart from gross malformations. Because of the simplicity of the test, it is the one that has been used most frequently (232, 234, 254).

An alternative method of assessment of mutagenic action is by measuring the

amount of heritable semisterility induced by a drug. This method has been used for X-rays (270), triethylene melamine (40), HN_2 (87), and methyl methane sulphonate (144) in mice. The technique is protracted and involves large numbers of animals. In an ideal experiment, the mutagenic action of a drug at all stages of spermatogenesis should be measured. The minimum number of samples would be 9 in the case of the rat, 7 in the mouse, taken at weekly intervals after treatment and covering the whole of spermatogenesis including the epididymal phase. In order to assess mutagenic action during each of these time periods, at least 60 males should be treated and the first and second generations fathered by them tested for semisterility and eventual chromosomal aberrations. The treated males should be mated with females of proved fertility, males of the first generation (approximately 240 animals) selected and remated with at least three times as many females (approximately 720 animals). Those males which consistantly father litters with 50 % of normal numbers or less would be selected and remated to females which would then be autopsied on the 12th day of gestation. Approximately equal numbers of live embryos and postimplantation deaths at this stage is a good indication of heritable semi-sterility, and this can be confirmed by cytological examination of the fathers of such litters. The whole examination would take 4 to 5 months and involve over a thousand animals. Seven to nine of these examinations would be necessary to cover the whole of spermatogenesis for one drug at one dose level and the data so obtained would only give an indication of a limited type of mutagenic action, namely the ability to cause chromosome breaks which remained unrepaired for sufficient time to allow the fragment to be transformed from its original position to another chromosome. A drug would have to be of considerable therapeutic potential to merit such a tedious and expensive examination.

The measurement of dominant lethal mutations, therefore, albeit crude, is still the simplest primary screening test for mutagenicity of a drug in male mammals (16).

A considerable literature exists on the determination of mutagenicity of drugs in *Drosophila melanogaster* (see 86a). But since there are clearly major differences of biochemistry involved between insects and mammals, the results in this system may be of only academic interest with regard to their potential hazard in man.

VIII. CONCLUSIONS

Although it is perhaps still premature to attempt to assign the different reactivities of the numerous cytotoxic agents that have been tried in spermatogenesis to the different categories of damage they produce, as outlined in the introduction to this review, a tentative grouping may nevertheless be considered worthwhile, if only to be criticized and proven invalid by further work.

Kinetic damage. This is difficult to recognize, since it requires a tedious examination of the distribution and proportion of the phases of the testis present in any transverse section of the tubule at different times after treatment with the drug. The only satisfactory demonstration of true kinetic damage is probably the observation of the changing pattern of mitoses observed after treatment of mice

with 6-azauracil. From inadequate data, it may be inferred that this type of damage may be present after treatment with antiandrogens, bis(dichloroacetyl)-diamines (WIN compounds), progestens, antimetabolites, dinitropyrrole, nitrofurans, thiophene derivatives, and cadmium salts and after elevation of testicular temperature. In none of these cases however has this type of damage been satisfactorily proven.

Cellular damage. Its presence is more clearly defined, and most of the drugs examined, especially when the effects are noted by histological examination, have shown varying degrees of this form of damage. Spermatogonial damage has been observed with certain antiandrogens, although these cells may not be the most sensitive to these agents. Certain ethyleneimine derivatives, notably ethylene urea and ethylene urethane, show gross cell destruction in the testis. Of the alkane sulphonates, Busulphan, dimethyl busulphan, methylene dimethane sulphonate, and isopropyl methane sulphonate show spermatogonial damage. Methylhydrazine derivatives as well as cadmium salts also show gross damage to this cell type. Cellular damage to primary spermatocytes seems to be a characteristic feature of temperature elevation of the testis, and several drugs which are thought to be acting on the testis by inducing this mechanism, such as the dinitropyrrole, nitrofuran, thiophene, bis(dichloroacetyl)diamine derivatives as well as cadmium salts, also exert a major action on this cell type. Bis(dichloroacetyl)diamines also exert some action on spermatids at an early stage. The final proof of cellular damage is the quantitative measurement of the extent of pycnosis and fragmentation, but this is not often observed, either because of the rapid removal of damaged cells or owing to the failure of damaged cells to develop into the normal complement of more mature forms.

Morphological damage. It is a point for discussion as to whether there should be a distinction between "cellular" and "morphological" damage, but if the cell survives for a period and leaves the testis with the spermatozoon, the damage is regarded as more persistent and classified as "morphological." Such changes in spermatid development have been noted after treatment with the alkane sulphonate derivatives, and have been described in detail in human studies after treatment with some of the bis(dichloroacetyl)diamines. Abnormal and bizarre forms of spermatozoal structure have also been described. The possibility exists that minor deformities of spermatozoa may not interfere with their viability but early or late genetic damage could not be ruled out. The most commonly observed form of morphological damage in the testis is the production of large polyploid cells during the late spermatocyte and early spermatid phases of development. A clear-cut example of morphological damage results from treatment with deuterium oxide, which is considered to induce a morphological change in the structure of the acrosome, so that the normal process of fertilization is impaired.

Early genetic damage. The most obvious form of genetic damage sustained by the germinal system is that resulting in deaths during early embryonic life. This may be due either to failure of fertilized eggs to implant or to death after implantation. Many instances of reported sterility after treatment of the male may be the result of this type of damage, and for potential antifertility agents it would

be essential to know if this is so, since lower doses may induce late genetic damage. Measurement of dominant lethal action may be made in early embryos. Most of the alkylating agents produce some degree of early genetic damage. In the particular case of the simple alkyl alkane sulphonates such as methyl, ethyl, and *n*-propyl methane sulphonate, the main type of damage produced is directed towards the adult spermatozoa in such a way that the resulting fertilized egg fails to cleave normally, and both pre- and postimplantation deaths occur. The bis-(dichloroacetyl)diamines and all antimetabolites should be suspected of causing some degree of early genetic damage, but except for some work in insect spermatogenesis, there is no satisfactory proof of such damage in the higher animals.

Late genetic damage. Damage to offspring of treated males has been conclusively demonstrated with triethyleneimine, nitrogen mustard, and methyl methane sulphonate. Owing to the very tedious nature of the experiments, very few drugs have been rigorously studied from this point of view. In general, those drugs which produce changes in DNA structure, such as the alkylating agents and some antimetabolites, as well as those which have already shown some early genetic damage at higher dose levels, may be suspected of producing late genetic damage.

Matrix damage. This occurs in some instances in association with extensive damage to the germinal epithelium, as with ethylene urea and ethylene urethane. The progestens, methylhydrazine derivatives, and cadmium salts also show extensive damage to the matrix, and in the case of the last mentioned agent, the testicular structural tissue may be the primary site of action.

An interpretation of the action of any new group of cytotoxic substances must take into account the possibilities that the effects observed may be the result of a direct action of the agent on the cell or an indirect one mediated through some of the many complex hormonal and other regulatory pathways in the whole organism. To unravel this complex question is probably easier in the testis than in any other differentiating system in the mammal, since the cellular kinetics of the spermatogenic epithelium, at least in the rodent, is now well known. The interrelationships of this system with the pituitary-gonadal axis is becoming clearer, and a way is opening to understand the mechanism by which the whole organism may control the functioning of one of its component systems. It is clear that the final definition of the mode of action of a drug must involve basic biophysical and chemical mechanisms at the level of a single molecule or a small group of molecules that are essential biochemical foci of action, whether they be at gene or cytoplasmic level.

An important technical advance in this field would be the separation of the component cells of the testis by some physical method of centrifugation, countercurrent, or filtration, destroying as little as possible of the enzyme activity, so that detailed biochemical investigations involving cell component separations may be effected. Continued study of the spectrum of drug action on spermatogenesis is desirable, however, since apart from recognizing new drug specificities of action, the potential interest to the emergency problem of population control cannot be overstressed.

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