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CHEMISTRY AND PHYSIOLOGY OF THE GAMETES

Report of a WHO Scientific Group

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CHEMISTRY AND PHYSIOLOGY OF THE GAMETES

Report of a WHO Scientific Group

1. INTRODUCTION

1.1 A WHO Scientific Group on the Chemistry and Physiology of the Gametes was convened in Geneva from 2-8 November 1965 to advise the Director-General on the state of knowledge and current research in this field. It was the eighth of a series of Scientific Groups giving detailed consideration to the biology of reproduction. The meeting was opened by Dr L. Kaprio, Director, Public Health Services, who welcomed the members on behalf of the Director-General. Professor J. J. Pasteels was elected Chairman and Dr R. Ortavant Vice-Chairman; Professor A. Monroy and Dr C. Polge agreed to act as Rapporteurs.

1.2 Knowledge of the biology of animal germ cells and their roles in fertilization and early development was considerable even before the end of the nineteenth century and has increased steadily ever since. During the past two decades this growth of knowledge has received great impetus from important practical problems, namely the need to increase productivity in food-supplying animals and the treatment of sterility and the control of fertility in human beings. In addition, there has been a great resurgence of interest in radiation biology and the fundamental mechanisms of genetic systems.

1.3 The Group reviewed the several aspects of the origin of germ cells in the embryo, their development to the definitive gametes of the adult, and the interactions of the gametes in fertilization and early development. Particular attention was given to mammals, where it was noted that there is, in general, a relative paucity of information on the primates. The basic similarities between different orders of mammals, and indeed between different phyla of the animal kingdom, are none the less very striking, and the value of comparative studies over a wide range of species was once again affirmed. The importance of pioneer basic studies on marine invertebrates was most evident.

1.4 This report summarizes the present state of knowledge of the biology of the germ cells with particular reference to the mammals. It ends with a list of items which the Group considers to be particularly worthy of further research.

2. ORIGIN OF THE GERM CELLS

The primordial germ cells

2.1 It is now virtually certain that the gametes of both sexes arise from the primordial germ cells which differentiate early in embryonic development. In several species of mammal they can first be recognized in the region of the primitive streak and root of the allantois. They then migrate through the dorsal mesentery to the gonadal folds from which the gonads develop.

2.2 At first, the primordial germ cells of the two sexes are indistinguishable, although it is known that they are genetically distinct, but the subsequent development of the gonadal fold and of the germ cells within it is very different in the two sexes. In the male, the medulla of the gonadal fold plays the most prominent part in the differentiation of the testis, and the primordial germ cells migrate to this region. In the female, the cortex of the gonadal fold plays the leading role in the differentiation of the ovary, and the germ cells remain there.

2.3 The primordial germ cells divide by mitosis and eventually give rise to the distinctive germ cells of the two sexes. This sexual differentiation appears to be imposed by environmental influences from the soma, which are themselves genetically determined. In the male, primordial germ cells are still present in the testis at birth: the most primitive class of adult germ cell, the spermatogonium, may be present before birth in some species, but in others is not formed until some time later. The spermatogonia give rise to spermatozoa by a complex process of cell division and differentiation. At the same time they form new spermatogonia through which the stock of germ cells is continually replaced. In the female the primordial germ cells conclude their development earlier and give rise to primary oocytes before or soon after birth. Subsequently, primary oocytes in diplotene and later stages of development exist in the ovary throughout life: no other class of germ cell is present and there is no mechanism whereby the stock of oocytes can be renewed. Complete development of the oocyte, followed by ovulation, does not occur until puberty.

2.4 The gonad is derived from three origins: (1) the primordial germ cells give rise to spermatogonia in the male and oogonia in the female; (2) the epithelium of the gonadal fold gives rise to the Sertoli cells of the testis and the follicle cells of the ovary; and (3) the interstitial cells of the gonad, together with the stroma and blood supply, arise from the mesenchyme. The germ cells of the testis, with their supporting Sertoli cells, become organized into a definitive germinal or spermatogenic epithelium. There is no similar epithelium in the female. The term "germinal epi-

thelium" as conventionally applied to the epithelial covering of the ovary is a misnomer derived from an erroneous conception of its function.

2.5 Noxious agents and genetic factors that affect the primordial germ cells during foetal life can lead to subsequent sterility or reduced fertility.

3. SPERMATOGENESIS AND THE SPERMATOZOON

The spermatogenic epithelium of mammals

3.1 The definitive spermatogenic epithelium of mammals begins its development well before puberty. Spermatozoa first appear at puberty, but spermatogenic activity increases until adulthood. The factors that control this development, and the maintenance of the epithelium in the adult, are not well understood, although it is clear that to a large extent they operate through the adenohypophysis and the hypothalamus.

3.2 The most primitive type of spermatogonium is the stem-cell spermatogonium. Through the process of "stem-cell renewal", the stem-cells not only maintain their numbers, but give rise to spermatogonia that are committed to initiate the cell divisions and irreversible morphogenetic changes that culminate in the formation of spermatozoa. The stem-cell spermatogonia ensure the continual renewal of the spermatogenic epithelium, but what determines whether the progeny of a stem-cell shall be a new stem-cell or a committed spermatogonium is not known. The spermatogonia ultimately give rise to primary spermatocytes. These divide meiotically to give spermatids which differentiate to spermatozoa without further division.

3.3 The interval between the first division of a stem-cell and the release of its descendent spermatozoa from the testis, "the duration of spermatogenesis", has been determined in a number of species by acute exposure to X-rays and by the use of radioactive precursors of deoxyribonucleic acid (DNA). This interval is about 35 days in the mouse, 48 days in the rat, 40 days in the ram, 54 days in the bull, and 72 days in man. This period, though it is different in different species, is remarkably constant within species, and experimental attempts to alter it, for example by the administration of gonadotrophins, have been unsuccessful. It follows that all spermatozoa leaving the testis at any given time are of the same age, whether measured from the formation of the parent stem-cell or from any other event in the period designated "the duration of spermatogenesis". At any given time in any given part of the spermatogenic epithelium, the germ cells exist together in well defined associations of cell type. In most mammals, these associations are arranged in sequence along the length of the tubule in a wave-like pattern: this sequence within the tubule is

not recognizable in man. Normally, four or five generations of spermatogenic cells are present in each cell association, and the cell divisions and morphogenetic changes within and between these generations are highly co-ordinated. The temporal spacing between the generations is initially determined by the divisions of the stem-cells, but how this is done, and how the co-ordination between the various cells is maintained, is not known.

3.4 The prophase of the first meiotic division of the primary spermatocyte occupies a large part—about three-eighths—of the duration of spermatogenesis. During this time, changes occur in the nucleus that are roughly analogous to those described for the primary oocyte (paragraphs 4.3 and 4.4), including the formation of chromosomes of “lampbrush” type which synthesize ribonucleic acid (RNA), but the significance of these changes is far from clear. The second meiotic division of the secondary spermatocytes to form the spermatids proceeds rapidly. Microspectrophotometric analyses show that the prophase primary spermatocytes contain a tetraploid amount of DNA, the secondary spermatocytes a diploid amount, and the spermatids a haploid amount. A further important consequence of meiosis is that the secondary spermatocytes and the spermatids each have individual genotypes.

3.5 The morphogenetic changes in the spermatid that culminate in the formation of spermatozoa also occupy about three eighths of the duration of spermatogenesis. These changes, collectively known as spermiogenesis, involve a profound rearrangement of both the nuclear and cytoplasmic elements of the spermatid. At first, the nucleus assumes an interphase appearance with a nucleolus. It may be assumed that this nucleus is metabolically active though its genes exist in a segregated haploid condition. Subsequently, the nucleus condenses as a whole and not into individual chromosomes, the nuclear protein changes to a histone with a very high arginine content, and the nucleus becomes unusually resistant to deoxyribonuclease. The spermatozoon nucleus is, in effect, a resistant condensed interphase nucleus, and claims that individual condensed chromosomes have been distinguished within its structure may be discounted.

3.6 One pole of the spermatid nucleus becomes covered by a characteristic cap, the acrosome, which is formed by the Golgi apparatus. The acrosome contains a labile periodic acid-Schiff (PAS) reactive substance. It is believed to contain hyaluronidase and an enzyme called “zona lysin” that enables the spermatozoon to enter the egg. Hyaluronidase can be readily extracted from mammalian spermatozoa, but attempts to extract “zona lysins” have generally been unsuccessful. Recently, however, a lipoglycoprotein, which dissolves the zona pellucida and disperses the corona cells of rabbit eggs, has been isolated from the acrosomes of ram,

bull and rabbit spermatozoa. In some species, the nuclear membrane beneath the acrosome becomes modified to form an organelle, which has been called the "perforatorium"; the function of this is not clear.

3.7 Centriolar elements of the spermatid give rise to the tail filaments of the spermatozoon. The filaments become attached to the nuclear membrane at the opposite pole to that covered by the acrosome. They are believed to be composed of actomyosin-like material. Mitochondria of the spermatid become rearranged around the part of the tail filaments that is nearest to the nucleus. They form the "midpiece" region of the tail and are associated with the respiratory metabolism of the spermatozoon.

3.8 During spermiogenesis, the spermatids become deeply embedded in pockets in the Sertoli cells. The bulk of the spermatid cytoplasm, with its contained RNA, is released towards the end of spermiogenesis and taken up by the Sertoli cells. As a result, the spermatozoon is devoid of RNA, and therefore likely to be particularly subject to senescence changes. The mechanism whereby spermatids are released from the Sertoli cells is not known. A small droplet of residual spermatid cytoplasm, "the cytoplasmic droplet", remains attached to the neck of the spermatozoon. It contains fat and remnants of the Golgi apparatus, and is somehow related to the life of the spermatozoon within the epididymis. The droplet usually becomes detached from the spermatozoon before, or at the time of, ejaculation.

3.9 It is clear that the Sertoli cells play an important role in the maintenance of the germ cells and undergo changes in association with spermatogenic events. Normally, the Sertoli cells of the adult do not divide: they may therefore be subject to important senescence changes. Sertoli-cell tumours in the dog can produce large amounts of oestrogen, and in some animals, notably the stallion, oestrogen secretion from the normal testis is known to be considerable. The significance of testis oestrogen is not known.

3.10 The stem-cells may be subject to inherent senescence changes as well as to influences from senescence changes in the body generally (adenohypophysis, etc.). In the bull, there is clear evidence that the fertility of semen used in artificial insemination declines slowly with increasing age of the donor, although there are no obvious indications of this in the seminal characteristics.

3.11 Considerable loss among spermatogenic cells of all classes appears to be a normal feature of the spermatogenic epithelium. It has been suggested that this loss represents selection against serious genetic aberrations that arise during cell division and differentiation. In some interspecific hybrids, such as the mule, interference in pairing of the paternal and maternal homologous chromosomes during the prophase of the first

meiotic division leads to complete cessation of spermatogenesis, and hence to sterility.

3.12 Spontaneous and induced disturbances of spermatogenesis often give rise to malformed spermatozoa. It is usually believed that morphologically abnormal spermatozoa cannot fertilize eggs, and in some instances there is good evidence that this is so. It is possible, however, that some forms of morphologically abnormal spermatozoa are capable of fertilization and are associated with death of embryos. In man and the bull, it has been shown that semen containing spermatozoa that appear to be morphologically normal, but which contain abnormal amounts of DNA, is associated with infertility and probably with embryonic death. It is also known that embryonic death can be caused by X-ray induced damage to spermatozoa that is not apparent in their structure and behaviour. The treatment of spontaneously occurring abnormal spermatogenesis appears to have met with little success.

3.13 In many mammals, environmental factors associated with season, such as light and temperature, exert a profound effect on spermatogenesis. Certain nutritional factors, such as vitamins A and E, certain amino and fatty acids, and certain trace elements, such as zinc, are essential for the maintenance of the spermatogenic epithelium. In addition, it is known that normal spermatogenesis can be profoundly disturbed by a variety of noxious factors, such as hyperthermia, high scrotal temperature, stress, hypoxaemia, oestrogens, progestogens, arsenic, cadmium, nitrogen mustard, colchicine, nitrofurans, various alkylating agents, ionizing radiations, and testis antigens. The effects of elevated temperature are not understood, but the most readily affected cell is the pachytene primary spermatocyte. Arsenic may replace phosphorus in the synthesis of DNA. Colchicine causes the arrest of cell division at metaphase. Alkylating agents are of especial interest since they can operate very specifically against particular classes of spermatogenic cells. Very little is known about the metabolism of the spermatogenic epithelium.

Spermatozoa in the ductus epididymis

3.14 Within the ductus epididymis spermatozoa undergo a process of maturation during which they complete their development and acquire full functional competence. Maturation is associated with the migration of the cytoplasmic droplet from the neck of the spermatozoon to the distal end of the midpiece, with morphological changes in the acrosome and possibly also in the mitochondria, and with an increased capacity for motility and fertility. Spermatozoa from the testis are believed to be sterile. The maturation changes appear to occur mainly in the caput and corpus epididymis. The mature spermatozoa are stored prior to ejaculation in the cauda

epididymis and vas deferens, and the vast majority of spermatozoa are found in these regions.

3.15 Spermatozoa live within the ductus epididymis for a very much longer time than they do in the female reproductive tract (see paragraph 5.5), or *in vitro* at a similar temperature. Sperm longevity in the epididymis has been observed to be as much as 30 to 70 days in the rat, rabbit, guinea-pig and bull and several months in certain bats. It is clear, however, that senescence changes occur before death. The spermatozoon's ability to contribute to viable embryos is lost before it loses its ability to fertilize eggs, and fertilizing capacity is lost before the loss of motility. These senescence changes manifest themselves in the spermatozoon by variability in the Feulgen-reactivity of the nucleus and by an increased eosinophilia. After prolonged periods of abstinence from sexual intercourse, the ejaculate often contains a high proportion of dead and senescent spermatozoa, and it is possible that such ejaculates could be a cause of embryonic death. There is doubt as to how unejaculated spermatozoa are removed from the epididymis: some workers believe that they are digested, or undergo pycnotic degeneration, and are absorbed, while others believe that they are merely forced out of the duct by normal peristalsis. Spermatozoa have been found in the urine of some species and this condition may be considered normal.

3.16 The survival of spermatozoa in the epididymis is dependent upon the endocrine activity of the testis and is greatly decreased if the testis or hypophysis is removed. Little is known about the way in which the epididymis influences spermatozoon maturation and survival, or about factors which may influence this.

3.17 Spermatozoa remain inactive in the epididymis and are transported through the duct by peristalsis aided by the increasing diameter of the lumen. At ejaculation, spermatozoa are expelled from the vas deferens and the cauda epididymis by spastic contractions of the duct. The time required for the passage of spermatozoa through the epididymis appears to be about one to three weeks and is influenced by the frequency of ejaculation. The age of spermatozoa in the ejaculate, in terms of time elapsing from their release from the testis, can therefore vary with the frequency of ejaculation. There is also evidence that some mixing of spermatozoa occurs within the epididymis and, in consequence, the spermatozoa of a given ejaculate are not all of the same age.

3.18 Nothing is known about the metabolism of spermatozoa within the epididymis, but their quiescence suggests that the rate of metabolism remains at a low basic level. Some workers believe that metabolism in the epididymis is controlled principally through a low availability of oxygen and glycolysable substrate, others that metabolic inhibitors are involved. In the trout, there is evidence that epididymal spermatozoa are inhibited by potas-

sium, and, in mammals, there is evidence that the ratio of potassium to sodium is high in the epididymis.

3.19 Occasionally spermatozoa penetrate the walls of the ductus epididymis and invade the peritubular tissue. The onset of this condition may be associated with inflammation of the epididymis, but its etiology is not clear. It may perhaps give rise to the phenomenon of auto-immunization against spermatozoa that has been observed in some sterile men. In these men, spermatozoa may be ejaculated in an agglutinated condition.

3.20 Sperm-filled sacs, called spermatocoels, occasionally form in various parts of the epididymis and are often associated with obstruction of the duct. Spermatozoa from spermatocoels often appear to be normal but of impaired fertility.

Spermatozoa in vitro

3.21 At ejaculation, spermatozoa are mixed with the secretions from the accessory glands which form the bulk of the seminal plasma. Although much is known of the chemistry of seminal plasma, and of its effects on spermatozoa *in vitro*, its physiological importance *in vivo* is not well understood.

3.22 At ejaculation, or on removal from the vas deferens or cauda epididymis, spermatozoa respond to their changed environment by an outburst of motility and metabolic activity which leads to relatively rapid exhaustion and death. The nature of this activation is not well understood, but, among other factors, increased availability of oxygen and metabolizable substrate appears to be important.

3.23 There have been many studies on the effects of a wide variety of physical and chemical agents on the metabolism, motility and survival of spermatozoa *in vitro*, but very few of these studies have related observations *in vitro* to measures of fertility.

3.24 The life span of spermatozoa *in vitro* varies according to the species and depends on the nature of the suspending medium. It is comparatively short at room and body temperatures, but can be prolonged by refrigeration, and in some cases by increased carbon dioxide tension. Bull spermatozoa can retain fertility for several days if stored at $+4^{\circ}\text{C}$ after dilution with protective media, but the fertility of stored samples falls progressively with time of storage, and, in practice, samples are not generally used for commercial artificial insemination after three days of storage. There is evidence that this decline in fertility is associated with an increase in the incidence of embryonic death, and that the rate of decline differs between bulls. After treatment with glycerol, spermatozoa of the bull have been successfully stored for many years at temperatures of -79°C (solid carbon dioxide) or -196°C (liquid nitrogen) and this technique is now

extensively used for the storage of spermatozoa for artificial insemination in cattle. At extreme low temperatures, senescence changes appear to be very slow, but the possibility of effects from free radical activity should not be overlooked. Attempts to store the spermatozoa of other species at low temperatures have met with less success, although human spermatozoa are particularly resistant to freezing and deep-frozen human semen has been used successfully for artificial insemination. Claims to have preserved bull spermatozoa by freeze-drying have not been substantiated.

3.25 The resistance of spermatozoa to low temperatures depends in part on the rate of cooling. If bull spermatozoa are cooled too quickly, many die of "cold shock". The susceptibility of spermatozoa to cold shock varies among species, and among individuals within species. It increases with age of spermatozoa, and can be prevented by protective substances such as egg yolk. Spermatozoa are also damaged by dilution: this effect can be lessened by the same agents that protect against cold shock.

3.26 Mammalian spermatozoa *in vitro* obtain energy for their physiological activities by three processes: (1) the glycolysis of certain exogenous hexose sugars through the Embden-Meyerhof glycolytic system, (2) the respiratory oxidation of the terminal products of glycolysis and of a variety of exogenous substrates through the Krebs' tricarboxylic acid cycle, and (3) the respiratory oxidation of intracellular lipids through the Krebs' cycle. In the presence of oxygen, processes (1) and (2) can operate simultaneously, but the latter is about fifteen times more productive of adenosine triphosphate (ATP) per mole of sugar utilized than the former. Under anaerobic conditions, only glycolysis can occur: this may be an important source of energy for spermatozoa in the epididymis, although there is evidence that epididymal spermatozoa can also degrade hexoses through the hexose-monophosphate shunt. It is probable that process (3) occurs only when extracellular metabolic substrate is not readily available. The relative importance of glycolytic and respiratory metabolism appears to vary somewhat among species. It is dependent upon the availability of oxygen, and upon other less clearly defined factors. There is evidence that spermatozoon survival is longer when respiration is restricted.

4. OOGENESIS AND THE EGG

The oocyte pool

4.1 Primary oocytes are formed in mammals by the time of birth, or very shortly thereafter. The adult ovary contains no germ cell equivalent to the stem-cell spermatogonium of the testis, and does not renew its stock of oocytes. It follows that any circumstance that reduces the number of

oocytes, or affects their ability to complete normal development, can have a permanent detrimental effect on the fertility of the female. It also follows that oocytes ovulated towards the end of reproductive life are considerably older than those ovulated at the beginning of reproductive life. In the human female, this difference will be 25 to 30 years. It is known that certain congenital abnormalities (such as mongolism) are commoner among children of older women than among those of younger women, and it is likely that this is associated with aging changes in the oocyte.

4.2 The oocyte pool decreases as a result of massive early degeneration, followed by continuous atresia with age, and to a much less degree by periodic ovulation. Losses from atresia can be greatly accelerated by various noxious agents, particularly by ionizing radiations, but the causes and significance of spontaneous atresia are unknown. Losses from ovulation can also be speeded somewhat by the administration of gonadotrophin, but why particular follicles develop and ovulate while others either develop and regress or remain quiescent is also unknown.

Development of the oocyte

4.3 The primary oocytes of mammals quickly enter the prophase of the first meiotic division and reach the diplotene stage during late foetal development, or shortly after birth: nuclear development is then delayed. There is considerable species variation in the appearance of the diplotene nuclei of arrested oocytes (e.g., the "typical" diplotene of the human oocyte, the condensed diplotene of the guinea-pig, and the diffuse, interphase-like, dictyate oocyte of the mouse and rat). In all species the oocyte enlarges rapidly as the follicle begins to grow, the chromosomes display an oxyphil staining reaction and become "lampbrush" in character, but characteristic species differences in nuclear structure remain. During the late stages of follicle development the oocyte chromosomes condense and enter diakinesis. Shortly before ovulation the first meiotic division is completed and the secondary oocyte and first polar body are formed. The chromosomes of the secondary oocyte quickly enter the second meiotic division, but this becomes arrested at metaphase, and in most mammals the oocyte is ovulated at this stage of its development. The second meiotic division does not normally proceed to completion until after activation of the oocyte by a spermatozoon, and haploid female germ cells, equivalent to the spermatid and the spermatozoon of the male, do not, therefore, exist in mammals. In the dog and the fox, the egg is ovulated as a primary oocyte and the formation of both polar bodies occurs in the oviduct.

4.4 Observations on echinoderms and amphibia have shown that during the course of oogenesis there is active synthesis of ribosome and messenger RNA. This ceases towards the end of oocyte development. Studies with

radioactive precursors of RNA show that labelled molecules first appear over the nucleolus, then in the nuclear sap, and finally in the cytoplasm. They remain in the cytoplasm and are associated with ribosomes that remain inactive until after fertilization. A similar pattern of labelling occurs in the oocytes of the mouse, where there is rapid incorporation of uridine into the oocytes of growing follicles, but none into the oocytes of follicles with well developed antra. In the oocyte of the rat and the mouse, the distribution of RNA in the cytoplasm is unilateral and corresponds with an accumulation of RNA in adjacent follicle cells.

Development of the follicle

4.5 An accurate chronology of follicle development is not available for any mammal. Observations on successive stages of development in young females are open to criticism because of the high rate of follicle atresia which always prevails, and because of the possibility that the rate of development in juvenile animals is different from that in adults. Observations based on procedures that destroy specific developmental stages of follicles in adults are also equivocal because these procedures may affect the rate of development of other follicles. It is clear, however, that the time required for a primary follicle to develop to maturity is much longer than a single oestrus (menstrual) cycle, as was formerly believed. In the adult female mouse, an acute exposure to 50R of X-rays kills all the early oocytes, but fertility is maintained for 60 to 70 days with an average of 4 litters per female. These pregnancies must utilize oocytes that had several layers of follicle cells at the time of irradiation. In woman, moderate irradiation may be followed by a few ovulations, then an anovulatory period, and eventually by recovery. Several months are required for the development of mature follicles from survivors among the earliest follicles present at the time of irradiation. If complete development of the human follicle could occur in one menstrual cycle, there would be no anovulatory period after moderate irradiation. In the rat, there is evidence that the number of follicles capable of responding to exogenous gonadotrophin is correlated with the number of primordial follicles present in the ovary.

4.6 The factors that control follicle growth and ovulation are inadequately understood, and *inability to foretell the time of ovulation remains a serious gap in knowledge of human reproduction.*

The ovulated egg

4.7 The ovulated mammalian egg is surrounded by a thick covering, the zona pellucida. Formation of the zona begins in follicles with a single cuboidal layer of cells and first becomes apparent as an interrupted inter-

cellular structure related to individual follicle cells. It is likely that the zona is a product of the follicle cells, rather than of the oocyte. Microvilli extend into the zona from both the follicle cells and the oocyte, and these doubtless facilitate the exchange of metabolites between the oocyte and its environment. Late in follicle development, the microvilli become much reduced in size and the passage of substances through the zona is probably affected. The cessation of RNA metabolism in the oocyte may be associated with the disappearance of the microvilli from the zona.

4.8 Initially the zona lies in close apposition to the oocyte, but a perivitelline space becomes evident at the time of the formation of the first polar body. The zona is mucoprotein in character, and, in many species, can be dissolved by acid and by proteolytic enzymes, but is resistant to hyaluronidase. Recent evidence indicates that sialic acid forms an integral part of its structure.

4.9 The zona pellucida is in turn surrounded by follicle cells comprising a regular tightly arranged inner layer, the corona radiata, and an irregular loose outer mass, the cumulus oophorus. The intercell matrix of the cumulus oophorus is composed principally of hyaluronic acid and is readily dispersed by hyaluronidase and by extracts of spermatozoa. The cumulus and corona cells disperse as the egg passes through the oviduct, but the time required varies greatly among species. It is short in the mare, ewe and cow, and long in the rabbit, rodents and carnivores.

4.10 The fertile life of the ovulated egg, which may be defined as the period during which it is competent to react to spermatozoon penetration to produce a normal embryo, is short. Observations in the rat, hamster, guinea-pig, rabbit, pig, ferret, ewe and cow indicate that this period is 10 to 20 hours, and it is likely that this applies to mammals generally. Anomalies of fertilization and development are frequent in older eggs — in particular, the frequency of polyspermy and that of retention of the second polar body both increase with increasing age of the egg. The time relations between insemination and ovulation are regulated in most mammals by limited periods of oestrus activity, or by ovulation induced by mating, but in man and in certain other primates this is not so and fertilization of senescent eggs may be a cause of considerable embryonic loss. Changing conditions in the oviduct associated with changes in ovarian activity may have a profound effect on the fertile life of the egg.

The egg *in vitro*

4.11 Mammalian oocytes artificially released from follicles resume their development under suitable culture conditions *in vitro*, where maturation changes follow the same time sequence as those observed *in vivo*. Oocytes

matured *in vitro* are capable of apparently normal fertilization when transferred to mated recipient females.

4.12 Ovulated oocytes of the rabbit have been kept *in vitro* for three to four days at temperatures between 0°C and 10°C, but the incidence of embryonic mortality, after transplantation to mated recipients, increases with the length of storage *in vitro*. Thus far, attempts to preserve ovulated eggs at very low temperatures have been unsuccessful. After treatment with glycerol, oocytes in slices of ovary may, however, survive prolonged storage at very low temperatures (−79°C) and normal young have been obtained from orthotopic grafts of ovary treated in this way.

5. FERTILIZATION AND EARLY DEVELOPMENT

Preliminaries to fertilization

5.1 In several mammalian species spermatozoa can reach the site of fertilization in the oviducts within fifteen minutes of insemination. This rapid transport is assisted by muscular activity of the female reproductive tract, but may be influenced by the site of insemination and by other factors such as stress and the stage of oestrus. The supply of spermatozoa in the oviduct is maintained for some time by replenishment from lower regions of the tract.

5.2 Spermatozoon motility may assist transport through the cervix (in species with intravaginal insemination) and through the uterotubal junction, and is probably essential in enabling spermatozoa to contact the egg and penetrate the zona pellucida.

5.3 Despite the large numbers of spermatozoa normally ejaculated, only a few hundreds or thousands are found in the oviducts at any one time. This drastic reduction of spermatozoon numbers at the site of fertilization is probably important in reducing the frequency of polyspermy, i.e., the presence of more than one spermatozoon within the cytoplasm of the egg.

5.4 In some primitive plants, spermatozoids are attracted towards the ova by substances that diffuse from the ovum—a form of behaviour referred to as “chemotaxis”. There is no unequivocal evidence for chemotaxis of spermatozoa in the animal kingdom. It is possible that in some species the mass of cumulus cells surrounding the egg after ovulation may increase the chances of sperm-egg collisions.

5.5 The life span of spermatozoa within the female reproductive tract is generally quite short—about 6 hours in the mouse, 14 hours in the rat, 22 hours in the guinea-pig, 30 hours in the rabbit, 2 days in man, sheep

and cow, 3 days in the pig, 5 days in the ferret, and 6 days in the horse. A notable exception to the general rule is provided by certain bats in which fertile spermatozoa may survive in the uterus for several months: the conditions that make this extraordinary longevity possible are not known. Observations on laboratory and farm animals suggest that the functional life of spermatozoa varies in different regions of the female tract, and with the phases of the oestrus cycle. In the chicken, where the spermatozoa of each mating are normally stored within the female tract for many days and utilized to fertilize successive eggs as they pass through the oviduct, there is clear evidence that fertilization by older spermatozoa is associated with teratogenic development of embryos. There have been no similar observations in mammals, where the principal effect of aging of spermatozoa in the female tract appears to be loss of fertilizing ability.

Entry of the spermatozoon into the egg

5.6 It is now known that removal of the cumulus oophorus and corona radiata is not a prerequisite of fertilization in mammals. Spermatozoa are able to make their way between the cells of the cumulus, presumably by means of the hyaluronidase that they carry. Penetration of the zona pellucida is attributed to the "zona lysin", which, like hyaluronidase, is believed to be located in the acrosome. Spermatozoa pass rapidly through the zona and leave behind them discrete channels through which they have passed. The spermatozoon head quickly traverses the perivitelline space and becomes attached to the plasma membrane of the egg. The nature of this attachment is not clear. The spermatozoon then slowly enters the cytoplasm: in some species the whole spermatozoon invariably passes into the cytoplasm, in others, only the spermatozoon head enters and the tail remains in the perivitelline space, while in other species the tail sometimes enters the cytoplasm and sometimes does not. It is clear that entry of the tail is not universally necessary for successful fertilization in mammals, but whether or not elements of the tail, such as mitochondria and centriolar structures, make a significant contribution to the embryo in some species is not clear. Recent observations with the electron microscope suggest that mitochondria from the spermatozoon remain inactive after passage into the egg cytoplasm.

5.7 In various invertebrates the acrosome discharges a tubular filament when the spermatozoa come into the vicinity of the egg. This phenomenon is referred to as the "acrosome reaction". The acrosome filament makes contact with the egg and plays an essential role in the entry of the spermatozoon. The acrosome reaction is elicited by substances called "fertilizins" which are contained in the egg envelopes: fertilizins in solution

agglutinate spermatozoa. There is evidence that a spermatozoon-agglutinating substance may diffuse from rabbit eggs.

5.8 It is known that in several mammals spermatozoa cannot penetrate the zona pellucida until they have resided for a period of an hour or so within the female genital tract, and it is likely that this is universal for all mammals. Evidently some change occurs in the spermatozoon which enables it to penetrate the zona: this change is referred to as "capacitation". The nature of capacitation is unknown, but it probably involves the acrosome. Changes in the acrosome may well liberate or expose the enzymes necessary for penetration of the zona. Spermatozoa observed within the zona pellucida or the perivitelline space display profound alterations in acrosome structure and it is likely that these are associated with capacitation and are analogous to the "acrosome reaction" already referred to.

5.9 The importance of the acrosome in mammalian fertilization is illustrated by the observation that certain bulls and boars with a genetically determined malformation of the acrosome are totally sterile. Apart from their acrosome defect, the spermatozoa of these animals appear to be normal, but are unable to fertilize eggs.

5.10 The nature of the attachment between the spermatozoon head and the plasma membrane of the egg is not known. It was formerly believed that the spermatozoon entered the cytoplasm by a process analogous to phagocytosis. The spermatozoon within the egg cytoplasm is not, however, contained within a vesicle such as would result from a phagocytic process, and recent observations leave little doubt that entry is achieved by a process of membrane fusion. The bounding membrane of the spermatozoon becomes continuous with the plasma membrane of the egg, and the spermatozoon moves slowly into the cytoplasm.

5.11 The success of fertilization in most species depends upon restricting entry into the egg cytoplasm to one spermatozoon only, and polyspermy, when it does occur, is associated with failure of normal development. The mechanism of ensuring monospermy is complex and varies in different species. In the sea urchin, the entry of the fertilizing spermatozoon is quickly followed by the elevation of a "fertilization membrane", which prevents the entry of further spermatozoa. The formation of this membrane is preceded by the rupture of granules in the cortex of the egg cytoplasm. In mammals, contact between the spermatozoon and the egg plasma membrane is followed by a rapid reaction in the membrane which is associated with the release of granules from the cortex in a manner strikingly similar to that observed in the sea urchin. It is supposed that substances released from the cortical granules diffuse through the perivitelline fluid and alter the structure of the zona. In the mouse, rat and rabbit, the zona is more easily digested by trypsin before penetration by

spermatozoa than after it. In some species, the egg plasma membrane quickly becomes refractive to other spermatozoa, in others a rapid change induced in the zona prevents entry of further spermatozoa. The rabbit, pika and mole are unusual in that this "zona reaction" does not occur. In these species, numerous spermatozoa may penetrate the zona and collect in the perivitelline space, but they are excluded from the cytoplasm by the prior changes in its surface. The speed of the zona reaction evidently varies between species: in some, spermatozoa are very rarely found in the perivitelline space, whereas in others, a few such spermatozoa are not uncommon.

5.12 In rats, guinea pigs, ferrets, pigs, sheep and cows, the efficiency of the mechanisms that ensure monospermy decrease with increasing age of the egg after ovulation, and, in the rat and the mouse, they have been shown to be adversely affected by hyperthermia.

5.13 Ovulated eggs of the rabbit and hamster have been successfully fertilized *in vitro* when cultured with spermatozoa taken from the female reproductive tract, and living offspring have been obtained after transfer of these eggs to suitable recipients. Many other attempts to achieve fertilization *in vitro* have, however, been unsuccessful, particularly those made by workers who have not appreciated the necessity of using "capacitated" spermatozoa. Fertilized rabbit eggs have been successfully cultured *in vitro* up to the early blastocyst stage, when the embryos collapse and become disorganized. Less success has been achieved with the eggs of other species. Single-cell mouse eggs have so far proved to be refractory to culture, but 4-cell and 8-cell eggs will develop to blastocysts. Very little is known about the requirements of mammalian eggs *in vitro*, but improvements in culture techniques hold great promise for advancing the study of many problems.

Activation of the egg

5.14 An essential feature of fertilization is the activation of the egg to develop into an embryo. Although the rupture of cortical granules does not appear to be a universal phenomenon throughout the animal kingdom, it is in many species the first sign of activation. Probably this rupture of granules is directly associated not only with the changes that prevent polyspermy, but also with the removal of systems that inhibit development within the cytoplasm. This development quickly manifests itself in the formation of pronuclei from the nuclear elements of the egg and the spermatozoon.

5.15 In mammals, the egg rapidly completes its second meiotic division after the entry of the spermatozoon and formation of the second polar body. The cytoplasm becomes slightly reduced in volume, there is some

rearrangement of nuclear and cytoplasmic elements, and the male and female pronuclei are formed. The pronuclei develop rapidly and synchronously: interference with one affects development in the other. They come together but remain intact until condensation of the chromosomes prior to syngamy. Their structure, particularly the formation and behaviour of the nucleoli, suggests that these pronuclei are metabolically active from a very early stage of development.

5.16 It was formerly believed that the unfertilized egg was in a state of depressed respiratory metabolism, and that the primary effect of activation was the restoration of a normal respiratory level. While this appears to be true in the sea urchin and some other species, it does not hold for the majority of animals examined. The eggs of fishes and amphibia do not show any change in rate of respiration as a result of fertilization, and oxygen consumption does not increase until the blastula stage of development. In some species there is a transient decrease in respiration after fertilization. Measurements of oxygen consumption are not, however, very instructive and more useful information is likely to emerge from direct studies of the energy-yielding systems. Recent observations in the sea urchin suggest that a block in carbohydrate metabolism and its associated synthesis of ATP is established during oogenesis, and that this block is removed at fertilization.

5.17 Studies with radioactive compounds have shown that the egg membranes of certain marine invertebrates have a low permeability to amino acids, carbohydrates and phosphates before fertilization and a very much higher permeability to these substances after fertilization. In the case of phosphate, there is evidence that fertilization activates a specific phosphate transport system. Nothing is known about changes in the rate of penetration of substances into the eggs of mammals after fertilization. However, the development of 8-cell mouse eggs *in vitro* is supported by glucose and malate, whereas the development of 2-cell eggs is not. This difference may depend upon the activation of enzyme systems within the embryo, or it may reflect a difference in the ease with which substrates enter the embryo, or both conditions may prevail. New and improved methods for the culture of mammalian eggs *in vitro* should greatly help to elucidate problems of this kind.

5.18 There is some evidence that enzymes operating during early embryogenesis are either present in the unfertilized egg in inactive or partially active forms, or that the programmes for their synthesis are present in inactive forms. In the sea urchin, glucose-6-phosphate dehydrogenase appears to become soluble within five minutes of the entry of the spermatozoon. Possibly, this change is brought about by changes in the distribution of ions in the cytoplasm. It has been shown that potassium exists mainly in a non-exchangeable state before fertilization and in an exchan-

geable state after fertilization. The hatching enzyme of sea urchins, however, appears to be absent in the unfertilized egg, although its messenger RNA is present.

5.19 A peculiar feature of the unfertilized eggs of many animals is an accumulation of DNA in the cytoplasm. The amount of this DNA exceeds by several times the DNA content of the nucleus. Recent studies suggest that the cytoplasmic DNA has similar characteristics to nuclear DNA, but its precise localization and function are not known. Possibly it serves as a source of primary genetic information during the early stages of development, or as a store of nucleotides for the synthesis of nuclear DNA. In the sea urchin there is no net increase in the DNA content of the embryo during the early cleavage stages. There is no evidence for an accumulation of DNA in the cytoplasm of mammalian eggs.

5.20 An important characteristic of the unfertilized egg is its inability to carry out protein synthesis, and there is evidence that this is imposed by a restraining influence on the ribosomes. Ribosomes from newly fertilized sea-urchin eggs can incorporate amino acids into protein *in vitro*, whereas ribosomes from unfertilized eggs cannot do so. Recent observations suggest that messenger RNA is attached to the ribosomes in the unfertilized egg, but that this complex is rendered inactive by a protein coating. Removal of this coating by trypsin releases the activity of the ribosomes *in vitro*, and it is suggested that a similar process operates at fertilization. A transient activation of proteases at fertilization has indeed been observed in the sea urchin egg.

5.21 In fish, amphibia and echinoderms, the RNA (messenger and ribosome) synthesized during oogenesis is used by the embryo until approximately the gastrula stage of development. This means that during this period, protein synthesis, or a large part of it, is controlled by RNA that was synthesized during oogenesis. It follows that the success of early development will be influenced, among other things, by events that affect RNA synthesis during oogenesis. The newly fertilized egg appears to be fully equipped with the protein-synthesizing machinery needed to support its early development.

5.22 In lethal hybrid embryos of echinoderms and amphibia, development proceeds more or less normally during the early stages, but is invariably arrested at the onset of gastrulation. Similar observations have been made on embryos subjected to heavy X-irradiation, and to inhibition of nuclear activity by actinomycin-D. In the mouse, however, actinomycin-D causes arrest of development before the 8-cell stage. This suggests that, in mammals, the messenger RNA synthesized during oogenesis directs development during the first two or three cleavages only.

5.23 Sooner or later, the primary genetic information of the zygote nucleus must be made available for development, and this involves the metabolic

activity of new messenger RNA and new ribosomes. Observations on echinoderm and amphibian embryos indicate that new messenger RNA begins to be synthesized very soon after fertilization, but is not used until much later in development. How this RNA is conserved and made available for metabolism at the right time is not known, but the "switch" mechanisms that control processes of this kind are likely to be key factors in the control of differentiation. The accumulating evidence that genetic transcription occurs some considerable time before the gene products are actually used is of great interest. Its significance is not understood.

5.24 In fish eggs irradiated with X-rays immediately after fertilization, synthesis of rapidly-labelled RNA (probably messenger RNA) continues although DNA synthesis ceases, and a similar observation has been made in the lethal echinoderm hybrids. It is suggested that this RNA is a "mis-sense" form which subsequently leads to the arrest of development by directing the synthesis of "mis-sense" protein or by being incapable of being "translated" at all.

5.25 There is good evidence that the nucleolus is involved in the synthesis of ribosome RNA. In both amphibia and echinoderms, the onset of ribosome RNA synthesis coincides with the appearance of a large nucleolus in the cells of the embryo. Anucleolate mutants of *Xenopus* fail to synthesize ribosome RNA, although the synthesis of messenger RNA and transfer RNA proceeds normally. In the mouse there are indications that nucleoli become active in the synthesis of RNA as early as the four-cell stage: this RNA is assumed to be ribosomal because of its associations with the nucleolus and because of the evidence that zygote RNA is metabolically active early in development (see paragraph 5.22).

5.26 Clearly, factors that affect the formation and activity of DNA codes, their transcription to RNA codes, and the translation of RNA codes in the synthesis of specific proteins, will have a profound effect on the development of the embryo. Much work is needed, during both gametogenesis and embryogenesis, before these basic processes can be understood and the hazards to normal development can be evaluated.

6. PROBLEMS REQUIRING FURTHER INVESTIGATION

The Scientific Group considers that the problems listed below are among those most urgently in need of further investigation. These items are arranged in the order in which they arise in the body of the report, and the references in parentheses indicate the relevant paragraphs.

1. The morphogenesis of the gonads, especially in primates, with particular reference to the primordial germ cells (2.1; 2.2; 2.3; 2.4; 2.5).

2. The role of nervous and hormonal and other influences on the development and maintenance of the spermatogenic epithelium (3.1; 3.13).
3. The cytology of spermatogenesis, with particular reference to the stem-cells (3.2), the long meiotic prophase of primary spermatocytes (3.4), the nucleus of the spermatids (3.5), the co-ordination of the spermatogenic epithelium (3.3), and the degeneration of germ cells (3.11).
4. The biology of the Sertoli cells, with special reference to their role in spermiogenesis, the release of spermatids from the spermatogenic epithelium, and their possible oestrogen-secreting activity (3.8; 3.9).
5. The effects of aging on the germ cells and consequent effects on fertility (3.10; 3.15; 3.24; 4.1; 4.10; 4.12).
6. The genesis of abnormal spermatozoa, the evaluation of their fertilizing capacity, and methods whereby abnormal spermiogenesis can be treated (3.12).
7. The biology of the epididymis, with particular reference to the maturation, metabolism, survival and elimination of spermatozoa and the study of agents that may affect these events (3.14; 3.15; 3.16; 3.17; 3.18).
8. Mechanisms that lead to autoimmunization against spermatozoa (3.19).
9. The biological significance of seminal plasma with special reference to the survival, transport, capacitation and fertility of spermatozoa (3.21; 3.22).
10. Changes in spermatozoa *in vitro* that are related to changes in fertility and species differences in the resistance of spermatozoa to low-temperature storage (3.23; 3.24; 3.25).
11. The arrest of oocyte development at the diplotene stage, follicle growth and atresia, ovulation, and methods of foretelling the time of ovulation in human beings (4.2; 4.3; 4.4; 4.5; 4.6; 4.11).
12. The relationship between the oocyte and the follicle cells, the formation and structure of the zona pellucida, and the significance of the "microvilli" (4.7; 4.8; 4.9).
13. The culture of mammalian eggs *in vitro*, and the *in vitro* study of maturation, fertilization and early development (4.11; 4.12; 5.13; 5.17).
14. Factors influencing the transport, storage and viability of spermatozoa in the female reproductive tract (5.1; 5.2; 5.3; 5.5); the possibility of chemotactic behaviour in spermatozoa and other factors that may influence sperm-egg collisions (5.4; 5.7).

15. Capacitation and the acrosome reaction in mammals (5.8), the mechanisms that ensure monospermy (5.3; 5.11; 5.12), and the significance of the perforatorium (3.6).

16. Activation of the egg at fertilization, with special reference to the cortical granules (5.11; 5.14), the block to polar body formation (4.3; 5.14), the pronuclei (5.15), the energy-yielding systems (5.16), changes in the permeability of membranes (5.17), activation of enzymes (5.18), protein synthesis (5.20), RNA synthesis (5.21—5.26), and DNA synthesis (5.19; 5.24).

17. The significance of cytoplasmic DNA (5.19).

18. The induction of abnormal embryogenesis by interference with nucleic acid metabolism in germ cells, zygotes and early embryos (5.26).

19. The sensitivity of germ cells to ionizing radiation.

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