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Isolation and Characterization of Autosomal Male Sterile Mutants in *Drosophila melanogaster*

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ISOLATION AND CHARACTERIZATION OF AUTOSOMAL MALE STERILE MUTANTS IN *DROSOPHILA MELANOGASTER*

by

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Lynn J. Romrell

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ABSTRACT

Isolation and Characterization of Autosomal Male Sterile Mutants in *Drosophila melanogaster*

by

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Utah State University, 1971

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In order to study the genetic control of spermiogenesis, recessive, male-sterile, autosomal mutants of *Drosophila melanogaster* were induced with ethyl methanesulfonate. A total of 31 mutants were recovered, 15 of which were located on the second chromosome and 16 on the third chromosome. Eight second- and 6 third-chromosome mutants demonstrating sterility in all homozygous males were used for further analysis.

Complementation tests showed that 2 of the 8 second chromosome mutants (and none of the 6 third chromosome mutants) were noncomplementing, indicating that two of the mutants produced were alleles of the same locus. Mapping of the second chromosome mutants indicated a clustering near the heterochromatin in the left arm.

In 2 of the mutants, spermiogenesis was studied with the light and electron microscopes. Mutant C_2-3 has an anomaly associated with cytokinesis accompanying meiosis. The primary spermatocyte undergoes nuclear division, but a failure of cytokinesis leaves 4 spermatids to develop within a common cytoplasm. The mitochondria fuse, usually forming a single large nebenkern, which then divides into two approximately equal parts, as a normal nebenkern does. In the mutant these two

mitochondrial derivatives usually undergo further division generally giving rise to 8 or fewer mitochondrial derivatives. Multiple paracrystalline bodies are often observed in the primary mitochondrial derivatives. Up to 4 paracrystalline bodies may form, one at each contact point between the membranes of the primary mitochondrial derivative and the membranes around the four axonemes contained in the common cytoplasmic unit. The groups of 4 spermatids almost complete maturation before the bundles degenerate.

Mutant C_2-10 is characterized by two anomalies: (1) disruption of the axonemal complex, and (2) formation of multiple paracrystalline bodies within the primary mitochondrial derivative. This mutant undergoes limited elongation with some variation between bundles of maturing spermatids. Axonemal complexes apparently complete differentiation even though disrupted and scattered in the cytoplasm. Mitochondrial derivatives are often very large and contain several paracrystalline bodies. The paracrystalline bodies form within the primary mitochondrial derivative at contact points between the membranes of the derivative and the cytoplasmic membranes. Abnormally large numbers of microtubules are observed within spermatids containing large mitochondrial derivatives and appear to be rather uniformily distributed around the derivatives. The large derivative size is presumed to be due to failure of normal elongation. Spermatids degenerate rather late in the maturation process.

(77 pages)

INTRODUCTION

Cell differentiation is almost certainly regulated by gene function. Cells often progress from "unspecialized" states to unique states with specialized functions. The mechanisms controlling differentiation remain major mysteries of cell biology.

There are several possible approaches to the study of differentiation. One may observe egg development from the time of fertilization through the development of a sexually mature organism. Alternatively, one may examine a specialized tissue and trace its development to its embryonic origin. Differentiation of golgi, mitochondria, or other cell organelles may be studied. Each of the above systems has particular advantages and disadvantages as a system for the study of differentiation.

As a system for the study of differentiation, spermiogenesis has several advantages: (1) it occurs in adults with the process being relatively short in duration, (2) it has well defined initial and terminal points, (3) it results in an end product that is unique in structure and function, and (4) it has been studied in sufficient detail that many steps in the process are well defined.

Using Drosophila as an experimental organism for the study of genetic control of spermiogenesis adds abundance of genetic information to the above list. One can use special balancer stocks to facilitate the isolation of mutants expressing male sterility as their only obvious phenotype. These mutants, presumably defective in spermiogenesis, can be easily mapped using standard techniques.

Using phase and electron microscopes, one can determine the stage at which development stops or becomes aberrant. Therefore, specific anomalies may be correlated with particular male sterile mutants. Through the study of many male sterile mutants, it may be possible to determine gene activities during spermiogenesis and to define some effect of gene activity at the ultrastructural level.

Although the Y chromosome and its fertility factors have been studied in some detail, relatively little is known of the role of autosomal genes in spermiogenesis. This investigation was conducted in an attempt to establish the role of autosomal genes in spermiogenesis and to increase the overall understanding of the genetic control of differentiation.

REVIEW OF LITERATURE

Spermatogenesis in Drosophila

Spermatogenesis in Drosophila has been of interest since the early observation of the absence of crossing over in Drosophila males (Morgan, 1912, 1914). Through microscopic study, some insight has been gained into the meiotic process in the two sexes. It has been observed that Drosophila males lack the synaptimemal complexes found in females (Moses, 1968, 1969). The interest in this problem, as well as interest in the cytogenetics of this well-studied organism has resulted in several early investigations of spermatogenesis.

Cooper (1950) reviewed early investigations on normal spermatogenesis in Drosophila. More recently, details of spermatocyte and spermatid ultrastructure have been supplied by Yasuzumi, Jujimura, and Ishida, 1958; Deams, Perijn, and Yates, 1963; Meyer, 1964; Bacetti and Bairati, 1964; Bairati and Baccetti, 1965; Leik, 1965; Bairati, 1967; Anderson, 1967; Shoup, 1967; Meyer, 1968; Hess and Meyer, 1968; Bairati, 1968; and Perotti, 1969. Phillips (1970) has recently reviewed the structure and morphogenesis of insect sperm.

Two types of spermatogonial cells occur in Drosophila testis. Primary spermatogonia maintain a supply of gonial cells and also produce secondary spermatogonia. The secondary spermatogonia are the precursors of primary spermatocytes. Each primary spermatogonium divides mitotically to give rise to primary and a secondary spermatogonium. The production of a primary spermatogonium at each division maintains a constant number of these gonial cells (Cooper, 1950).

Secondary spermatogonia undergo four mitotic divisions giving rise to cysts containing sixteen primary spermatocytes. There is near-absolute mitotic synchrony during the secondary gonial divisions. "Somatic pairing" of homologues occurs during these and virtually all mitotic divisions (Cooper, 1950).

Detailed descriptions of the primary spermatocytes and of the meiotic divisions are lacking. Primary spermatocytes increase in size and the nucleus becomes spherical again. The "lampbrush" Y chromosomes (Hess and Meyer, 1968) are seen during chromosome condensation at prophase. The two divisions follow in quick succession. Nuclei of the secondary spermatocytes are much smaller than those of the primary spermatocytes. In secondary gonial cells and early spermatocytes, mitochondria are generally grouped to one side of the nucleus (Cooper, 1950). During prophase this group breaks up and the mitochondria are scattered in the cytoplasm around the nucleus. During the meiotic divisions, the mitochondria line up along the spindle, parallel to individual astral rays. Incomplete cytokinesis in the meiotic and gonial division makes each clone a syncytium of sixty-four cells.

In the young spermatids the nucleus increases in size. The nebenkern is formed by the fusion of the mitochondria. At the same time, the acrosome is formed by the Golgi complex. The acrosome assumes an apical position during initial elongation. The flagellum, which was established prior to the attachment of the primary centriole to the posterior nuclear surface, continues elongation. The nebenkern divides in half giving rise to the two mitochondrial derivatives. Only one, the primary mitochondrial derivative, becomes filled with a paracrystalline material; the secondary derivative is shown as a much reduced structure

in mature sperm. Microtubules extend anteriorly and posteriorly from the dense mass at the base of the nucleus. These microtubules are thought to function in spermatid elongation and nuclear condensation (Kessel, 1966; Anderson, 1967; Shoup, 1967).

Acrosome formation during spermiogenesis has been studied by Anderson (1967), who also described nebenkern formation and morphogenesis. Pratt (1968) has given an excellent description of nebenkern formation and differentiation in Murgantia. Details of head and acrosome elongation have been supplied by Shoup (1967). Meyer (1964, 1966) studied the paracrystalline structure of the mitochondrial derivatives. Flagellar structure and formation have been described by several investigators (Bairati and Baccetti, 1965; Anderson, 1967; Shoup, 1967; Phillips, 1970). Perotti (1969) has given a detailed description of mature sperm ultrastructure. A detailed description of the spermatogenetic stages in Drosophila melanogaster is now available (Stanley, Reed, Romrell, and Bowman, in manuscript).

Genetic Control of Spermiogenesis

Bridges (1916) demonstrated that factors carried on the Y chromosome function in spermiogenesis. X/O males, males lacking the Y chromosome, although phenotypically normal are completely sterile. Thus the formation of normal sperm is dependent upon fertility factors carried on Y chromosome. Stern (1929, as cited by Hess and Meyer, 1968) studied males deficient for parts of the Y chromosome and found that flies deficient for either the long or the short arm of the Y chromosome are sterile. He concluded that there are two complexes carried on the Y chromosome that are necessary for sperm development. Brosseau (1960)

isolated a series of male sterile Y chromosome mutants and found five fertility factors in the long arm and two in the short arm of the Y chromosome.

Many other sex-linked and autosomal mutants of Drosophila which result in male sterility are known (Lindsley and Grell, 1967). Also, male sterility often is associated with chromosomal aberrations. Such mutants may be sterile due to: (1) changes in genes which control functions common to all or a great many genes, (2) changes in normal abdominal development which may affect normal sexual development, (3) changes in male genitalia which may prevent or interfere with normal copulation, and (4) changes in other structures which may be of importance in normal breeding. A total of 13 non-Y-linked-mutants which express male sterility as their only phenotype have been reported (Edmondson, 1951; Lindsley and Grell, 1967). Only 3 of these stocks are currently in existence. Edmondson (1951) used ultraviolet light to induce male and female steriles on chromosome II. She found ten complementing male-sterile mutants. Unfortunately, these stocks have been discarded. Apparently there has been no systematic check for sterile mutants on chromosome III. Geer (personal communication) has isolated several male sterile mutants on the X chromosome. It appears that there are genes, other than the fertility factors located on the Y chromosome, which function in sperm development. Hess and Meyer (1968) have recently reviewed the activity of the Y chromosome in several Drosophila species. Structures, not found in other tissues, occur in the primary spermatocyte nuclei. DNA unfolding from chromomeres in the Y chromosome results in structures similar to the lateral loops of lampbrush chromosomes.

Hess (1967, 1968) has shown that a complete set of Y chromosomal loops is necessary for normal sperm development. Deficiencies of loop-forming loci results in sterility. Normal spermiogenesis requires the normal unfolding of all loops during the spermatocyte stage (Hess and Meyer, 1968; Hess, 1967, 1968, 1970; Meyer, 1969). Hennig (1968) demonstrated the existance of a rapidly labelled RNA species in the D. hydei testis that is complementary to the Y chromosome DNA.

Several observations have demonstrated that sperm differentiation and function are dependent upon the diploid genotype. During meiosis the X and Y chromosomes segregate, and only one half of the spermatids possess a Y chromosome. Both X- and Y-bearing spermatids differentiate normally. In the case of attached X-Y compound chromosomes, one half of the spermatids receive the compound X-Y chromosome and the other half receive neither X nor Y chromosome. Both types of spermatids function in egg fertilization. Muller and Settles (1927, as cited by Lindsley and Grell, 1969) questioned whether genes must act in mature sperm in order for the sperm to remain functional. No shift in sex ratio was observed following insemination of Drosophila females (i.e., 0-7 versus 7-14 days). They concluded that the X chromosome carries no genes which must act in the haploid phase for the maintenance of sperm function. Using a translocation heterozygote, Muller and Settles demonstrated that flies deficient for a segment of the second chromosome comprising approximately 3.1% of the haploid autosomal complement, are recovered at the same frequency after different storage periods. They concluded that the entire autosomal complement is devoid of genes that must function in the mature spermatozoa.

McCloskey (1966) examined the consequence of deleting, one at a time, the majority of each major autosomal chormosome and all of

chromosome IV. He compared relative survival of reciprocal duplication-deficiency-bearing gametic types and found no shift with increasing time of sperm storage within the female. He concluded that maintenance of sperm viability does not require gene function in mature sperm. This also demonstrates that spermatid differentiation is normal in the absence of each chromosome.

Baldwin and Chovnick (1967) demonstrated that sperm nullosomic for chromosome III can function in fertilization. Lindsley and Grell (1969) reported the recovery of progeny resulting from the fertilization of disomic eggs by both X- and Y-bearing sperm of the following autosomal constitutions: (1) nullo-two, (2) nullo-three, and (3) nullo-two, nullo-three. Although it has been demonstrated that spermatids can mature in the absence of certain chromosomes, it has not been shown that maturation can occur in the absence of all chromosomes.

The above evidence does not demonstrate synthetic inactivity in the haploid phase. Apparently this is not the case. Incorporation of labeled amino acid in developing spermatids has been shown in Drosophila (Das, Kaufman, and Gay, 1964; Hennig, 1967; Brink, 1968) and other species (Block and Brach, 1964; Das, Siegel, and Alfert, 1965; Monesi, 1964). Uridine uptake in developing spermatids has not been shown in Drosophila (Olivieri and Olivieri, 1965) but has been shown in early grasshipper (Block and Brach, 1964; Henderson, 1964; Muckenthaler, 1964; Das, et al., 1965) and in mouse spermatids (Monesi, 1965). Olivieri and Olivieri (1965) and Hennig (1967) have found uridine incorporation in early spermatocytes but not in more mature stages. These results suggest that messenger RNA may be synthesized in early stages and held until it functions in later sperm development.

Lindsley and Grell (1969) cite two possible explanations for the normal development of nullosomic sperm:

> (1) Stable messenger RNA molecules are transcribed in the primary spermatocyte and distributed at meiosis for the subsequent direction of spermiogenesis.
> (2) The 64-spermatids in a cyst, which are connected by intercellular bridges, represent a 64-nucleus, 64-ploid heterokaryon, which forms 64 spermatozoa as a unit under the control of the combined genotype of the 64 nuclei.

In conclusion, the studies described have shown that genetic information carried on the X and Y chromosomes and the autosomes of Drosophila is necessary for normal sperm development. This information functions in such a way that the genetic content of individual spermatids does not restrict their development. The diploid genetic content determines sperm development.

Analysis of Male Sterile Mutants

The observation that X/O males are sterile lead to several early investigations of abnormal sperm development (Morgan, Bridges, and Sturtevant, 1925; Safir, 1920; Schultz, 1947; Stern and Hadorn, 1938). The results reported were contradictory. Only gross abnormalities can be resolved with the light microscope.

Fine structure analysis of X/O males (Kiefer, 1966; Meyer, 1968) has uncovered abnormalities usually limited to the mitochondrial derivatives and axonemal complex. The mitochondrial derivatives sometimes are found free in the cytoplasm rather than in contact with the axonemal complex. Sometimes two or three paracrystalline bodies form at random positions within the mitochondrial derivatives. Normally the paracrystalline body formation begins within the primary derivative

at the point of contact with the axonemal complex. Kiefer (1966) reported that all nebenkern derivatives appear defective.

A low frequency of axonemal complexes exhibit missing components or disruption of the normal spatial pattern. Kiefer (1966) interprets abnormal complexes as results of irregular development rather than degeneration, since normal and abnormal complexes within the same bundle have reached the same point of development. Flagellar fibers are completely missing in some spermatids. Only 30 to 40 developing spermatids were found in bundles in X/O males.

Kiefer (1968, 1969, 1970) has described abnormalities associated with Y deficiencies rather than complete absence of the Y chromosome. In all cases, sperm in Y deficient flies develop faster than those in X/O males. One mutant was found which produces motile sperm. At least some ultrastructurally mature sperm were reported in each mutant. Although no masses of degenerating sperm were observed in the testes of X/O males, such masses were seen in all Y deficiency mutants. Differences between various Y-mutants were reported, in general, to be quantitative rather than qualitative. Hess and Meyer (1968) postulated that the Y fertility factors function in the organization of sperm organelles since disorientation of these elements seemed to be the major effect of Y-chromosome deficiencies.

Spermiogenesis in a male sterile translocation heterozygote has been studied (Shoup, 1967). In the translocation heterozygote a portion of the left arm of the X chromosome was translocated to a heterochromatic region of chromosome II (T(1;2H)25(20)y125). The sperm head failed to elongate in the mutant, and the change from lysine-rich to arginine-

rich histones in the nucleus failed to occur. Microtubules, normally associated with the head during condensation and elongation, were not observed.

Lindsley (1968) studied the genetic control of sperm development. One hundred and twenty gamma-ray-induced sex-linked male sterile mutants were produced in which 80% had X chromosome translocations to either the second or third chromosome. These mutants demonstrated failure of head elongation. The remaining sex-linked mutants and 50 autosomal male steriles collected from natural populations demonstrated abortive sperm differentiation. Apparently, there has been no ultrastructure study of sperm development in autosomal male sterile mutants.

MATERIALS AND METHODS

General Procedures

All stocks were cultured on standard corn meal-sucrose-yeast-agar medium with propionic acid added as a mold inhibitor. Flies were cultured at 25°±1°C in temperature control rooms or at room temperature (approximately 25°) in the laboratory. Canton-S is the laboratory wild type stock. The mutants of *Drosophila melanogaster* used in this study are listed in Table 1.

Isolation of Mutants

Male sterile mutations were induced with ethyl methanesulfonate (EMS), a monofunctional alkylating agent. Wild type males were aged to three days after eclosion in the absence of females. The males were placed in half pint milk bottles containing folded Kimwipe paper tissue saturated either with a solution of EMS (0.02M), acetate (0.7%) and sucrose (2%) at pH 6.8 or a solution of EMS (0.02M) and sucrose (2%).

Figure 1 gives the details of the corsses used in the isolation and identification of male sterile mutations. The males used in Cross 2 were collected during the first five days after eclosion. This eliminated the recovery of premeiotic clusters of mutants. Cross 3 is a repeat of Cross 2. This is necessary since monofunctional alkylating agents characteristically induce mosaic mutants. Both mutated and unmutated copies of the same treated chromosome might be produced by a female in Cross 2. The repeat of Cross 2 allows mosaics to

Table 1. Description of stocks and mutants used in the isolation and mapping of male sterile mutants. Additional data found in Lindsley and Grell (1967).

Symbol	Name	Location
Canton-S		wild type
SM5	Second Multiple 5	inversions, chromosome 2 marked with Cy
TM3	Third Multiple 3	inversions, chromosome 3 marked with Sb and Ser
Cy	Curly	inversions, chromosome 2
Ser	Serrate	inversions, chromosome 3
Sb	Stubble	inversions, chromosome 3
ca^{nd}	Claret non-disjunctional	3-100.7, recessive mutant
Bl	Bristle	2-54.8, dominant mutant
D	Dichaete	inversions, chromosome 3
J	Jammed	2-41.0, dominant mutant
Pm	Plum	inversions, chromosome 2
Sp	Sternopleural	2-22.0, dominant mutant
S	Star	2-1.3, dominant mutant
Tft	Tuft	2-53.2, dominant mutant
L	Lobe	2-72.0, dominant mutant
Pin	Pin	2-107.3, dominant mutant

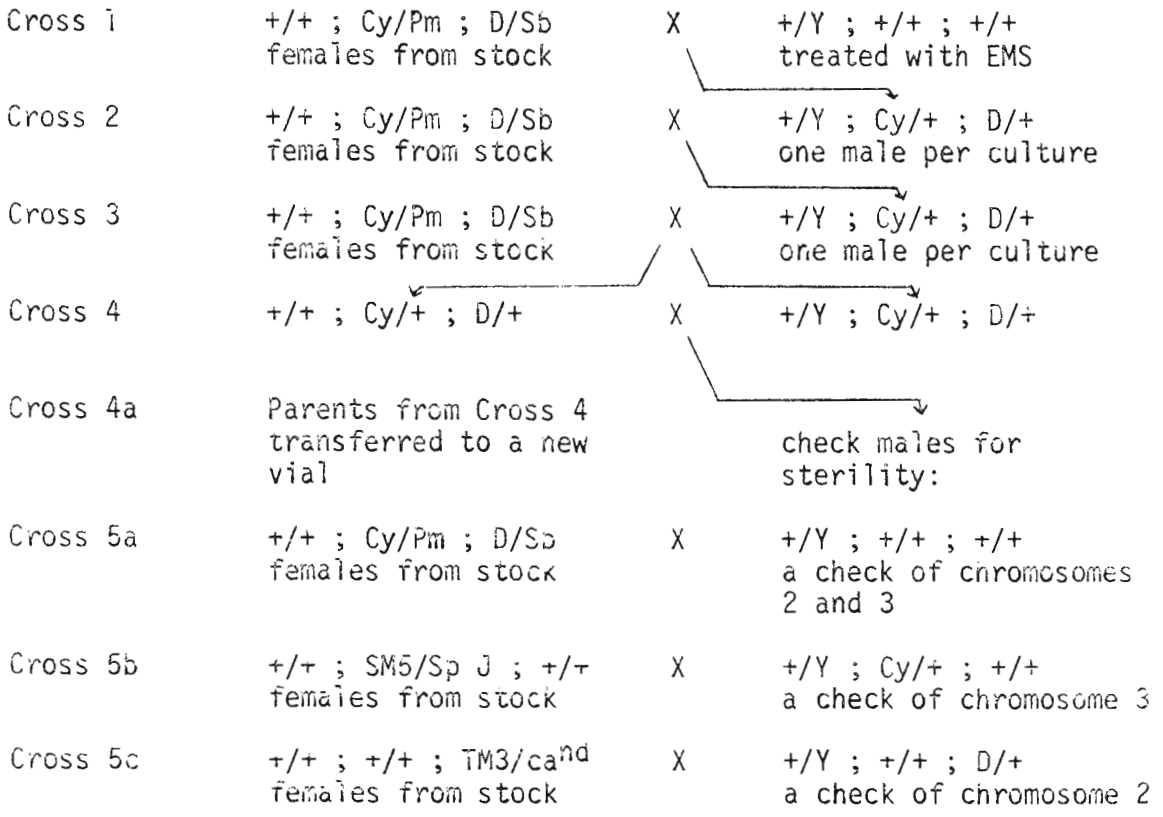


Figure 1. Induction and identification of male sterile mutants in chromosomes II and III.

segregate before the final identification of the mutant. Sterile mutants on chromosomes two and three have been maintained in balanced stock as heterozygotes over SM5 and TM3 respectively.

Mapping of Mutants

Mutants were mapped using standard techniques (Fig. 2). Since male sterility must be scored in the F_2, only non-Curly males were scored. Loci were determined by recombination analysis and complementation tests.

Cytology and Fine Structure

For general light microscopic observation, testes of adult males were dissected in Drosophila Ringer's solution (Butterworth, Bodenstein, and King, 1965). Using phase contrast microscopy, testes were checked for elongation of bundles, motility of sperm, general stages of cells within the testes, and general morphology of the reproductive tract. Alternatively, testes were stained with aceto-carmine-fast green (Dippell, 1955) and examined with the phase contrast microscope for the occurrence of elongate mature sperm heads. Female reproductive systems were dissected and examined following mating to male sterile mutants to ascertain sperm transfer.

For fine structure analysis, testes of male sterile mutants and wild type controls were dissected in fixative, either within 5 hours of eclosion or after being mated and tested for sterility. Testes were fixed 1-2 hours in (1)3% glutaraldehyde in cacodylate buffer at room temperature followed 1% OsO_4 for 1 hour in the same buffer or (2) Karnovsky's fixative (Karnovsky, 1965) followed by post fixation

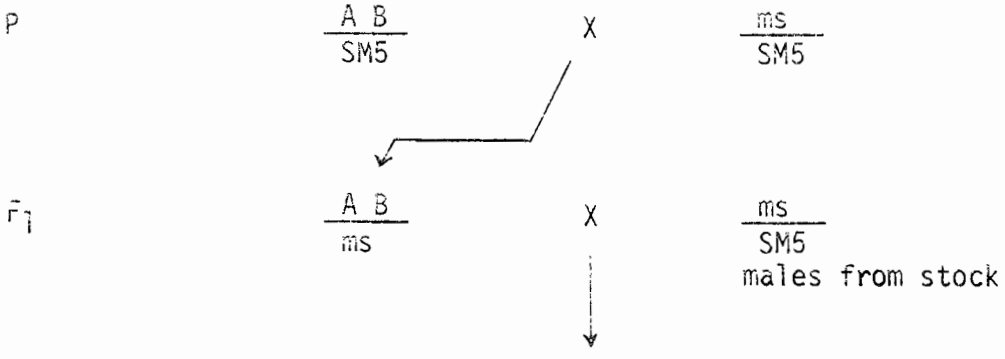


Figure 2. Design of a typical 3-point cross to determine locations of male sterile mutants. The symbols A and B represent linked dominant markers; ms represents any male sterile mutant.

with OsO_4. Tissues were dehydrated in ethyl alcohol, cleared in propylene oxide and embedded in Epon 812 (Luft, 1961). For detailed light microscopy, one micron sections were cut with glass knives and stained with Richardson's stain (Richardson, Jarett, Finke, 1960). Thin sections were cut with a diamond, double stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), and examined with a Zeiss EM9-S electron microscope.

RESULTS

Genetic Analysis

Thirty-one male-sterile mutations were induced with ethyl methanesulfonate. Fifteen mutations were in the second chromosome; 16 in the third (Tables 2 and 3). Seven mutant stocks have been lost. A check of female fertility following isolation revealed a high incidence of concomitant female sterility. Of the third-chromosome mutants tested all have some degree of female sterility and four demonstrate sterility in all homozygous females. One of these, C_3-11, is only semisterile in homozygous males. Two second chromosome mutants that demonstrate complete male sterility are completely sterile in homozygous females.

Complementation tests on the complete steriles uncovered only one presumed allelic pair on the second chromosome (Figure 3). Mutations C_2-1 and C_2-2 were found to be noncomplementing, i.e. are sterile in heterozygous combination with each other. This is true of none of the third chromosome mutants tested.

The approximate map location of the second-chromosome complete steriles are given in Figure 4. Also listed are the locations of the other previously isolated male-sterile mutants (Lindsley and Grell, 1967). There appears to be a clustering of mutations in the left arm near the heterochromatin. Three of the mutants map within the four map units 51 to 55.

Table 2. Second chromosome recessive male sterile mutants

Mutant	Fertility*	
	male	female
C_2-1	sterile	fertile
C_2-2	sterile	fertile
C_2-3	sterile	sterile
C_2-4	semisterile	-
C_2-5**	sterile	-
C_2-6	semisterile	-
C_2-7	semisterile	fertile
C_2-8	semisterile	-
C_2-9	semisterile	-
C2-10	sterile	fertile
C_2-11	sterile	fertile
C_2-12	sterile	fertile
C_2-13	sterile	fertile
C_2-14**	sterile	-
C_2-15	sterile	sterile

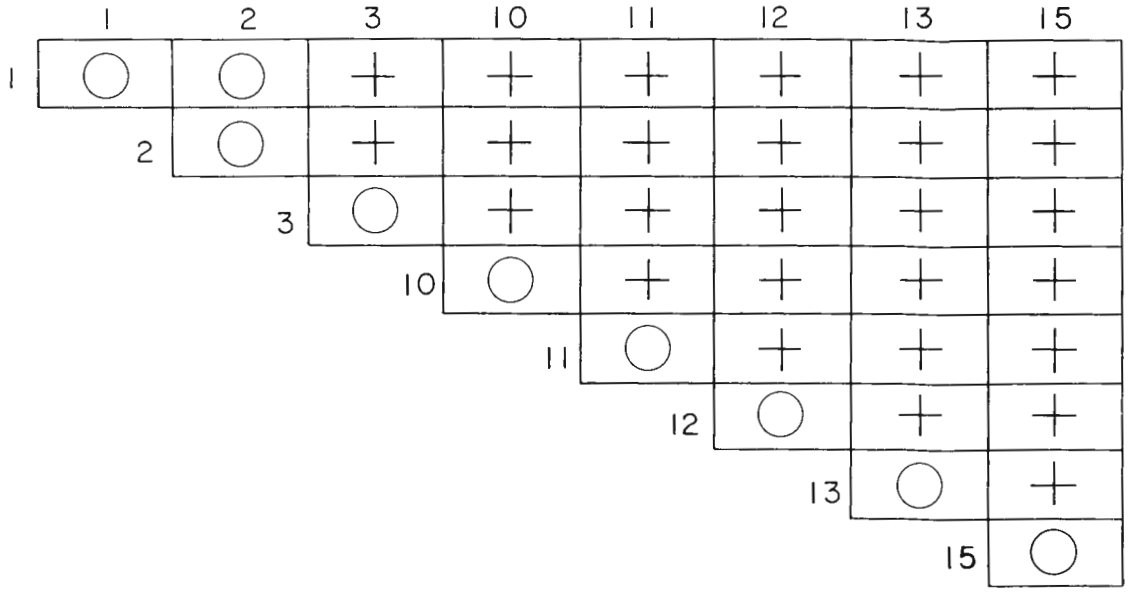
*Checked in homozygotes.
**Mutant stocks have been lost.
C_2 refers to chromosome II; the number following to the order in which the mutants were recovered. Sterile means that all homozygous flies are sterile; semisterile means that some homozygous flies are sterile.

Table 3. Third chromosome recessive male sterile mutants

Mutant	Fertility*	
	male	female
C_3-1	sterile	sterile
C_3-2**	sterile	-
C_3-3	sterile	sterile
C_3-4	sterile	semisterile
C_3-5	semisterile	semisterile
C_3-6	semisterile	semisterile
C_3-7	semisterile	-
C_3-8	semisterile	-
C_3-9**	sterile	-
C_3-10	sterile	sterile
C_3-11	semisterile	sterile
C_3-12**	sterile	-
C_3-13**	sterile	-
C_3-14	sterile	semisterile
C_3-15**	sterile	-
C_3-16	sterile	-

*Checked in homozygotes.
**Mutant stocks have been lost.
Same system of nomenclature as in Table 2. C_3 refers to chromosome III.

Second chromosome mutants



Third chromosome mutants

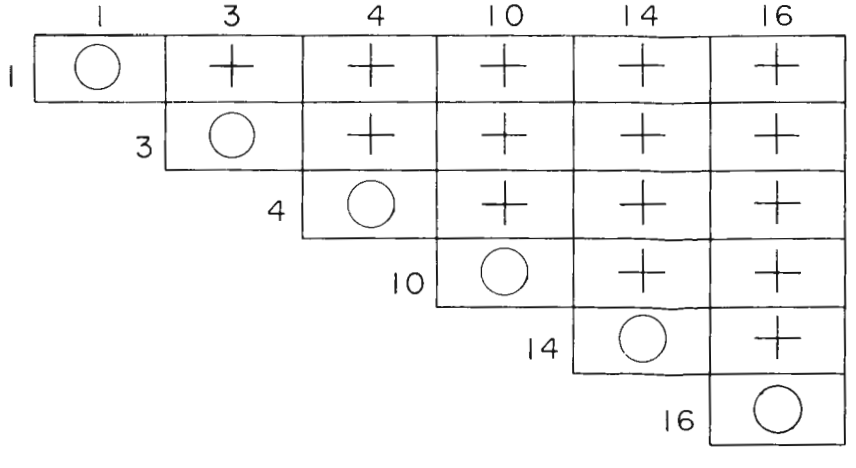


Figure 3. Complementation Test. The symbol "+" means heterozygotes are fertile and "○" means they are sterile.

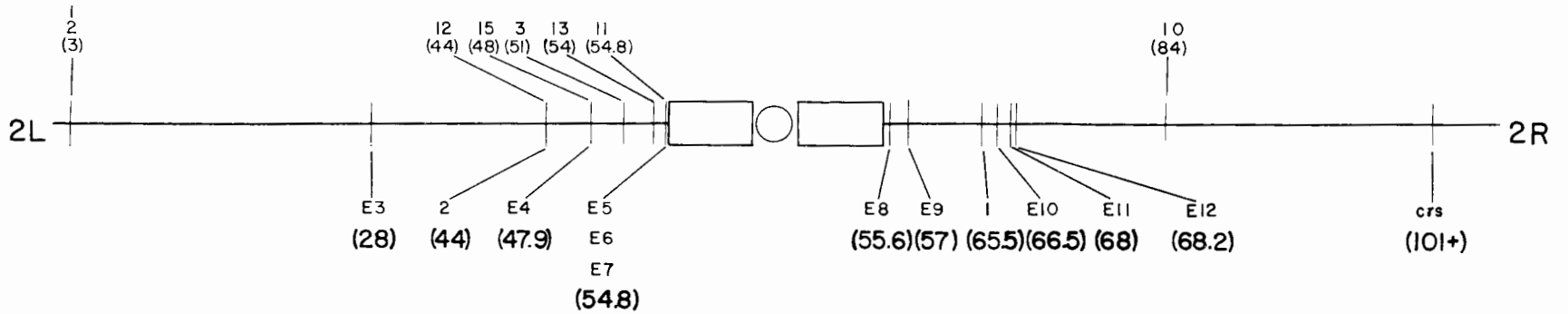


Figure 4. Approximate map locations of second chromosome male sterile mutants. The upper number refers to the mutant; the lower number, in parentheses, to the map locations. Locations in bold print indicate mutants isoloted by other investigators; only numbers 1 and 2 and crs are in existence.

Microscopic Analysis of Two Male Sterile Mutants

Light microscope observations

Spermiogenesis was studied in mutants C_2-3 and C_2-10 with the light and electron microscopes. Phase microscopy of C_2-3 male homozygotes revealed: (1) the presence of an apparently normal male reproductive system, (2) the presence of elongate bundles of maturing spermatids, (3) the presence of apparently mature elongate heads in stained squash preparations, and (4) the absence of motile sperm in the reproductive tract. No attempt was made to measure bundles of spermatids, since it would be extremely difficult to isolate bundles and determine their stage of development, which must be done in order to make proper length comparisons with wild type controls. Female reproductive tracts were examined immediately following and 24 hours after copulation with sterile males. All regions of the reproductive tract appeared to be devoid of sperm.

Phase microscope observations of mutant C_2-10 male homozygotes revealed: (1) the presence of an apparently normal reproductive system, (2) the presence of large numbers of primary spermatocytes and other early developmental stages in the testes of males as old as 10 days, (3) the presence of few elongate bundles approaching wild type length, and (4) the absence of elongate heads in stained squash preparations. Again, no attempt was made to measure the spermatid bundles. No spermatozoa were found in female reproductive tracts following copulation with sterile males.

Electron microscope observations

The earliest noted deviation in spermiogenesis in mutant C_2-3 is a failure of cytokinesis following meiosis. The primary spermatocyte

undergoes nuclear division producing four nuclei. Cytokinesis fails resulting in four spermatids developing within a common cytoplasm. The mitochondria fuse usually forming a single large nebenkern rather than the four smaller nebenkernen that would form in wildtype spermatids, one to each of the four spermatids separated by meiotic cytokinesis. The "giant" nebenkern of mutant C_2-3 first divides into two approximately equal bodies which have irregular shapes and show contact with membrane sheaths surrounding the four axonomal complexes (Figure 6). In this mutant, the derivatives may divide further giving rise to as many as 8 mitochondrial derivatives in a common cytoplasm (Figure 8). In normal spermiogenesis the nebenkern also divides into two approximately equal masses but there is no further division.

As elongation begins in the mutant (Figure 7), differentiation appears rather normal except that the components of four spermatids are observed in each cytoplasmic unit. The acrosomes and centrioles establish apparently normal contact with each nucleus. Although the mitochondrial derivatives are unusually large, they appear to undergo normal elongation. Microtubules, in contact with the dense material around each centriole, extend anteriorly around each nucleus as is observed in normal spermatids. Microtubules also extend posteriorly, as they normally do, around the axonemal complex and the mitochondrial derivatives.

The regular occurrence of four axonemes and their associated mitochondrial derivatives within a single cytoplasmic unit is characteristic of this mutant (Figure 9). Normally within a developing bundle of 64 spermatids, one observes a single axoneme and a primary and a secondary mitochondrial derivative in definable cytoplasmic units (Figure 10). Figures 9 and 10 demonstrate these contrasts between normal and

mutant spermatids at the stage in which the paracrystalline body begins to form. In the normal spermatids, the derivatives are approximately circular in cross-section with a paracrystalline body just beginning formation in the primary derivative. The secondary derivative is slightly smaller in cross-section and does not demonstrate the formation of a paracrystalline body. In the mutant, the derivatives are of various sizes and are rather irregular in shape. The paracrystalline bodies form at each point of contact between the primary derivative and the membrane around the axoneme. In normal spermatids there is but one axoneme and associated membrane complex available to establish such a contact point. Since there may be four axonemal complexes within each cytoplasmic unit in the mutant, a single primary derivative may establish up to four contacts. Also, a single axoneme may establish contact with more than one primary mitochondrial derivative. Therefore, multiple paracrystalline bodies form within mitochondrial derivatives of the mutant dependent upon the number of contact points with the membranes around the axoneme. Microtubules are seen in the cytoplasm around the mitochondrial derivatives in both mutant and normal spermatids.

Later in development, at the stage at which the satellite tubules are established, there is occasional, more complete demarkation of single spermatids (Figure 11). Most spermatids, however, remain in common cytoplasmic units in groups of four. Nuclei at this stage appear fairly normal, although their shape may be slightly irregular due to the close association of other nuclei within the same unit. Many mitochondrial derivatives, particularly those that have established contact with more than one axoneme, are larger and more irregular in shape than those at the same stage in normal spermatids.

Later in development (Figure 12) the more complete demarkation of single spermatids is no longer observed. Cytoplasmic units usually contain four spermatids although some contain larger numbers probably due to the fusion of cytoplasmic units. At most stages a few cytoplasmic units are observed which contain components of only two or three spermatids. This may be due to the incomplete development of some spermatids or the level of the section may not include all four spermatids. Development is relatively normal with the paracrystalline bodies increasing in size and the axoneme undergoing further differentiation. Spokes and secondary fibers are now prominent within the axoneme. The first indications of degeneration are also now apparent in the mutant with the appearance of large myelin figures around the groups of spermatids (Figure 12). Degeneration progresses rapdily and numerous large myelin figures occur within bundles at a slightly later developmental stage (Figures 13 and 14).

Some nuclei apparently undergo complete elongation and condensation, for normal-appearing, fully mature nculei have been observed with the light microscope in stained squash preparations. No fully mature heads have been observed with the electron microscope in thin sectioned material. Nuclei show somewhat abnormal condensation (Figure 13) in that condensed chromatin is oriented more to the peripheral edge of the nucleus than normal. This may reflect the somewhat abnormal shape of the nuclei due to the close association with the other nuclei in the same cytoplasm. Nuclei in normal spermatids at this stage are generally more circular in cross-section.

Many degenerating bundles in which spermatids show nearly complete differentiation are abserved within the testes of sterile mutants

(Figure 15). The regular occurrence of four spermatids in cytoplasmic masses, however, is still apparent. The axonemal complexes exhibit apparently normal maturation. All of the axonemal components, such as cored central and satellite tubules, spokes, and secondary fibers, are present and are in apparently normal arrangement. The mitochondrial derivatives are almost completely filled with the paracrystalline material and are approximately the size of normal, fully mature derivatives. Spermatids undergo no further differentiation, and no fully mature individual spermatids have been observed.

Mutant C_2-10 is characterized by two anomalies: (1) disruption of the anonemal complex and (2) multiple paracrystalline bodies within the primary mitochondrial derivative. Sections of spermatid bundles at the tail level, at all stages after the establishment of the axoneme, generally demonstrate the presence of both normal and disrupted axonemes (Figures 17, 18, and 22). Axonemes in which the basic 9 + 2 tubule pattern is not disrupted (Figure 17), appear normal. The mitochondrial derivatives often appear normal and lie in their normal positions on either side and in close association with the membranes around the axonemes. In spermatids in which the axonemes are disrupted (Figure 18), the mitochondrial derivatives are generally observed more widely separated in the cytoplasm.

The scattering of the axonemal doublets is random, but the doublets are always found associated with cytoplasmic membranes (Figures 18, 19, 20, 22, and 27). Physical links between the doublets and the membranes around them have been observed in mutant (Figure 19) and in normal spermatids. At slightly later stage, characterized by beginning of paracrystalline body formation, the doublets are often even more widely

scattered. Paracrystalline bodies form within the primary mitochondrial derivatives at contact points between the cytoplasmic membranes and the outer membrane of the mitochondrial derivative (Figures 19 and 20). The primary derivatives are often of very large cross-sectional area and are of irregular shape. The secondary derivatives also show association with the cytoplasmic membranes and are also of abnormally large cross-sectional area. Mutant spermatids often contain several secondary derivatives (Figure 20), whereas normal spermatids contain only one.

The matrix within the secondary derivatives is much less dense than within the primary derivatives (Figure 22). At the stage in which the satellite tubules have been established many anomalies are readily apparent. In general, mutant spermatids (Figure 22) demonstrate greater cross-sectional area than do normal spermatids (Figure 21). Although there is some variability in size of the mitochondrial derivatives, they are generally of much greater size in the mutant spermatids. The primary derivatives are of about normal density, but many of them contain multiple paracrystalline bodies. Although some spermatids contain an apparently normal axonemal complex, others show disrupted complexes or the absence of complexes at the level of the section. The observation of normal axonemes at this level does not establish that axonemes are normal through their entire length. Axonemes which are normal at this level probably disrupted at more distal levels. Since axonemes apparently elongate from normal centrioles, greater numbers of normal axonemes would be expected in cross-sections nearer to the centrioles. It is also apparent that the spermatids within the bundle are not in perfect register. Sections often pass through heads at various levels and through tails within the same bundle. This

makes it difficult to establish the level of section of individual spermatids within a single bundle. Normally bundles are rather closely registered such that cross-sections pass through all spermatids within a bundle at approximately the same level.

Nuclei undergo some condensation and elongation (Figures 23 and 24), but, apparently, development stops prior to complete head maturation. Centrioles have been observed in cross-section at most developmental stages. No disrupted centrioles were observed among those sectioned.

The axonemes complete their differentiation even though they may be scattered in the cytoplasm (Figure 27). Spokes, secondary fibers, dense and tubular satellite bodies, and the cores within the central tubules and the satellite tubules develop. The large mitochondrial derivatives usually contain multiple paracrystalline bodies. Bundles degenerate (Figure 28) without further spermatid development.

There is a variation in the degree of abnormal development between spermatids within a single bundle and between bundles. No bundles have been observed which demonstrate completely normal development nor have fully mature individual spermatids been observed.

Random counts have been made of the number of microtubules within the cross-sections of spermatid tails. Counts were made in bundles which had completed the formation of the satellite tubules (same stage as Figures 21 and 22). Although small cytoplasmic masses, containing a few microtubules, are present, microtubule counts were limited to bundles containing mitochondrial derivatives. One of the mutant bundles from which counts were made contained 63 cytoplasmic units in which mitochondrial derivatives were observed. This would indicate that restricting counts to such masses would give a reasonable estimate of

the total microtubules present. Random counts of mutant spermatids within one bundle ranged from 17 to 107 with a mean of 56. The count of 17 microtubules was made in a cytoplasmic unit which included only a single, rather small, primary mitochondrial derivative. The count of 107 microtubules was made in a spermatid that was large in cross-section and contained a very large and irregularly shaped primary mitochondrial derivative and two secondary mitochondrial derivatives. Counts of microtubules within spermatids from a bundle demonstrating larger mitochondrial derivatives ranged from 109 to 211 with a mean of 174. Bundles which have more normal mitochondrial derivatives appear to have correspondingly fewer microtubules. Counts of microtubules within normal spermatids at the same stage varies from 20 to 32 with a mean of 27. Apparently there is a correlation between the size and number of the mitochondrial derivatives and the number of microtubules within the cytoplasm of the developing spermatid. Although the microtubules appear to be rather uniformly distributed around the mitochondrial derivatives, in mutant spermatids the microtubules are in closer association with neighboring microtubules than they are in normal spermatids. Cross-links are often observed between neighboring microtubules around the mitochondrial derivatives in the mutant. Similar cross-links have been observed between the microtubules around the nuclei of normal spermatids, but such links usually are not observed between the microtubules around the mitochondrial derivatives.

Figures 5-16. Spermiogenesis in male sterile mutant C_2-3.

Figure 5. A developing nebenkern in an early spermatid. The nebenkern (nb) is composed of interlocking mitochondria. One nucleus (n) is shown in the upper right. X 9200.

Figure 6. An early spermatid sectioned through the tail shortly after division of the nebenkern. The two mitochondrial derivatives (m) have areas of cytoplasm (cyt) within them. Four axonemes (a) are present within a single cytoplasmic unit. X 28,000.

Figure 7. Spermatids in early stage of elongation. Three, of presumably four, nuclei (n) are seen in cross-section. Acrosomes (ac) are located adjacent to two nuclei and the centriole (c), surrounded by dense material, is in contact with the third nucleus. A longitudinal-section through an axoneme is shown lying between the mitochondrial derivatives (m). X 13,400.

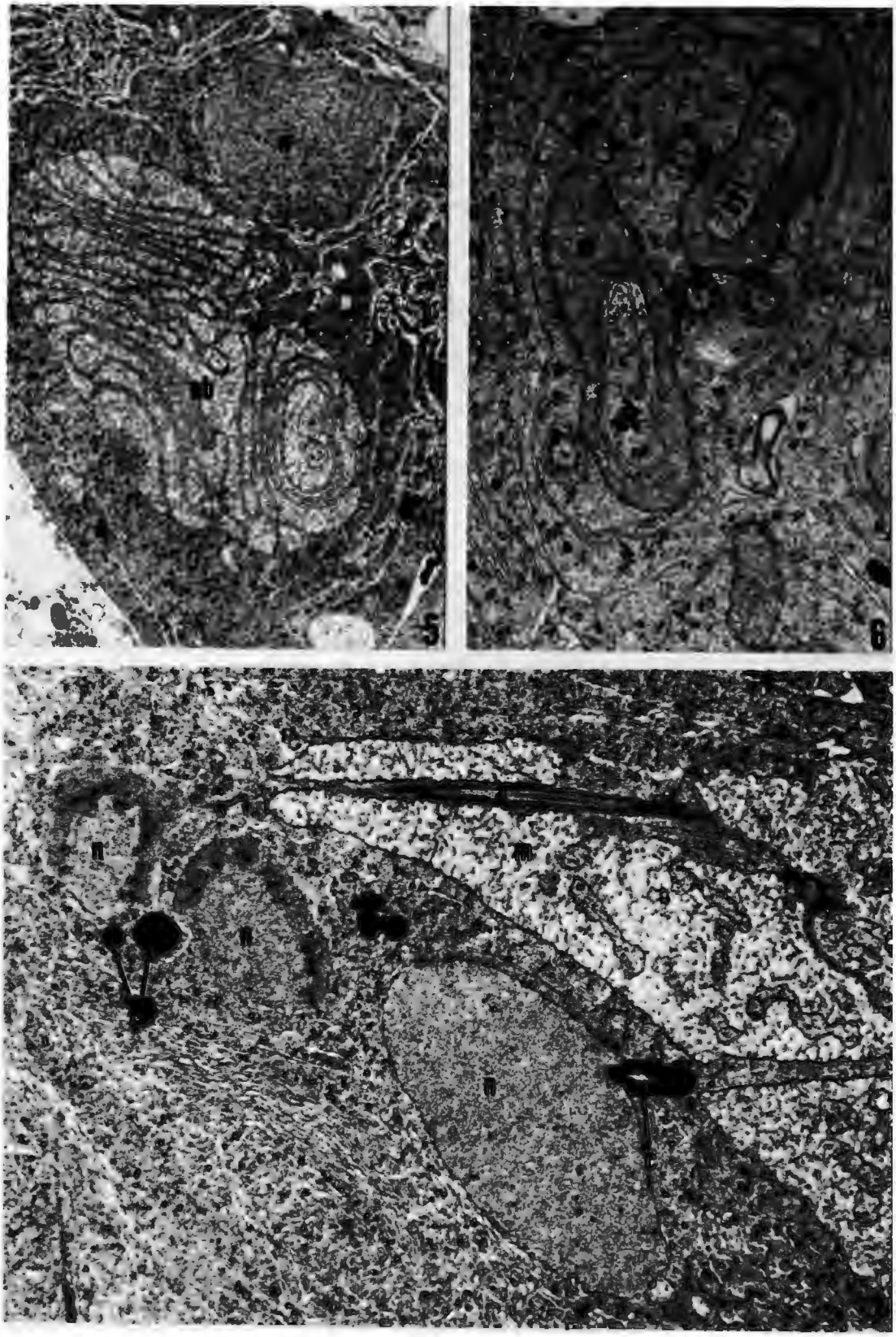
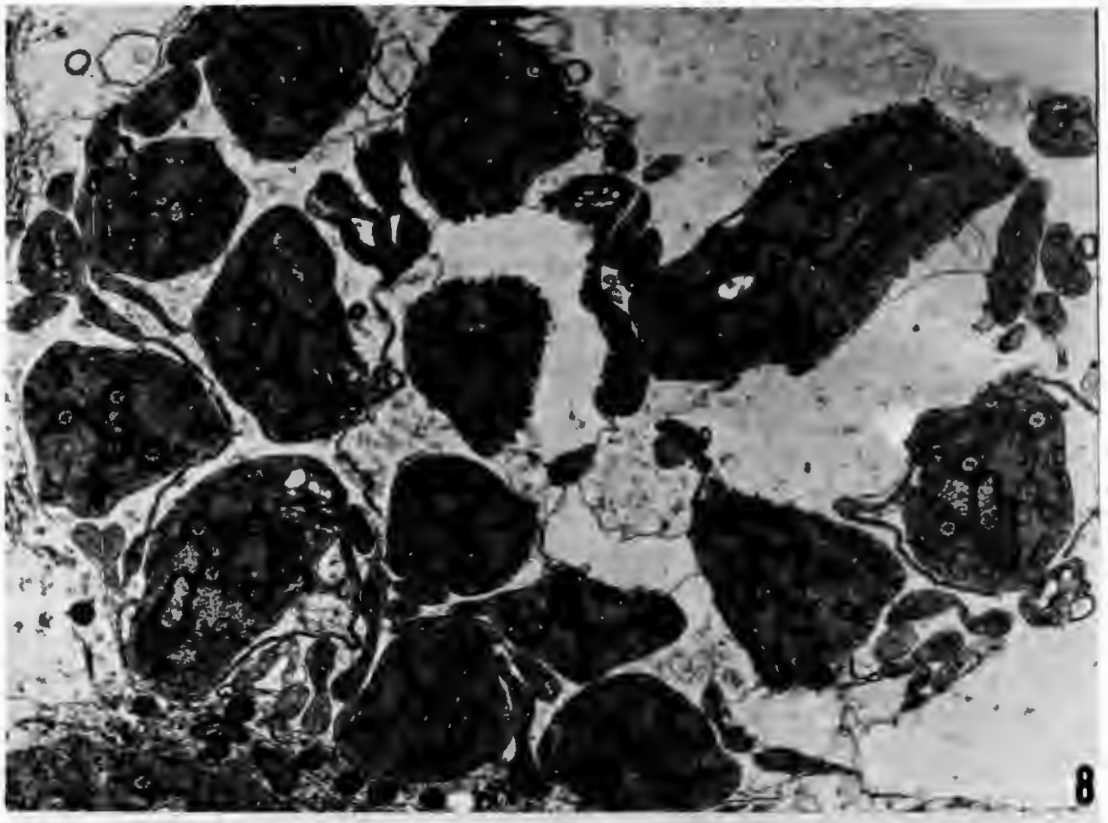


Figure 8. Section through the tails of a bundle of spermatids. Discrete cytoplasmic masses usually contain four axonemes and various numbers of mitochondrial derivatives. X 13,800.

Figure 9. One of the cytoplasmic unit as seen in Figure 8. The outer membranes around the four axonemes are in contact with the mitochondrial derivatives. Paracrystalline material is beginning to form at the contact points between the primary derivative (m_1) and the membranes around the axoneme (see arrows). Microtubules (mt) occur in the cytoplasm around the derivatives. X 52,400.

Figure 10. A normal spermatid at the same stage of development as those in Figures 8 and 9. Only one axoneme and a primary (m_1) and a secondary (m_2) mitochondrial derivative are seen in each cytoplasmic unit. Paracrystalline material (cry) is present within the primary mitochondrial derivative. X 52,400.



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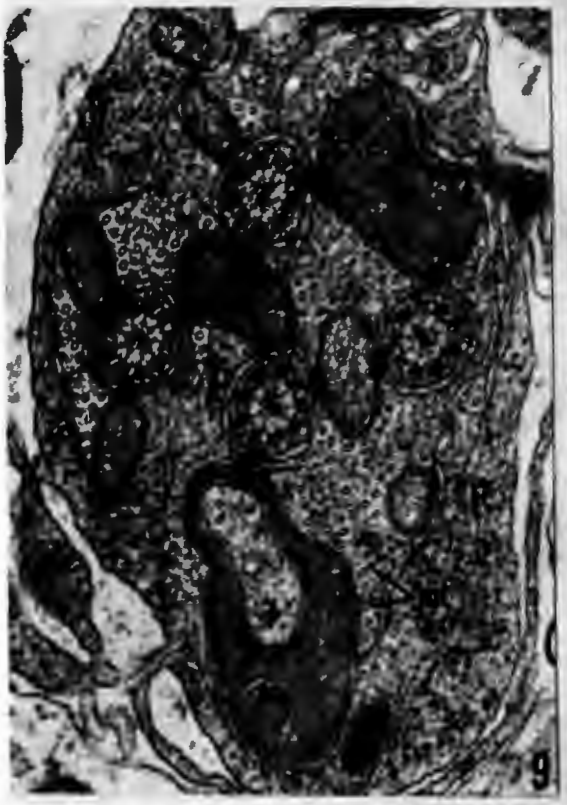

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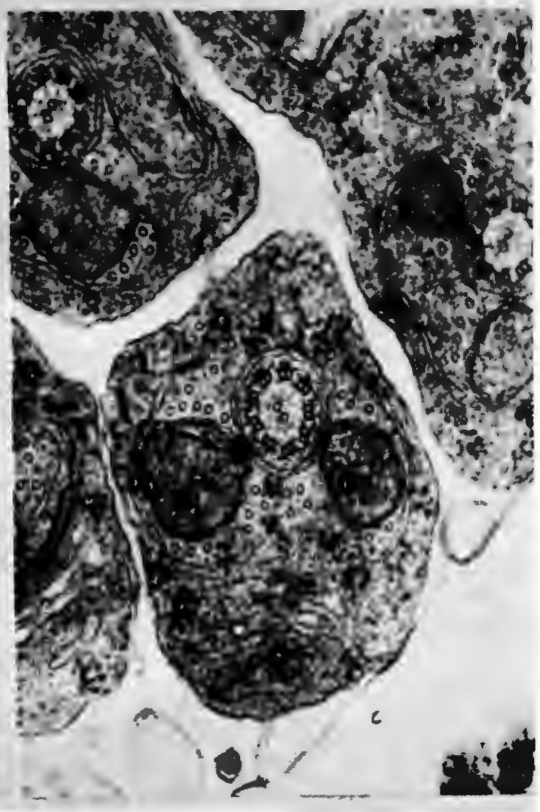


Figure 11. Slightly later stage in development of spermatids in mutant C_2-3 in which there is a limited separation of a few individual spermatids (arrows). Two nuclei (n), in an early stage of chromatin condensation, are observed in cross-section within a cytoplasmic unit. A single row of microtubules (mt) if juxtaposed on the convex side of each nucleus. Satellite tubules (st), as well as, spokes and secondary fibers, are now present in the axonemal complex. Large, irregular-shaped, primary derivatives (m_1) are often found. Paracrystalline material (cry) forms at each contact point with the membranes around the axoneme. X 32,000.

Figure 12. Spermatids in groups of four within discrete cytoplasmic units following incomplete separation. Large myelin figures (mf) are present. X 32,000.

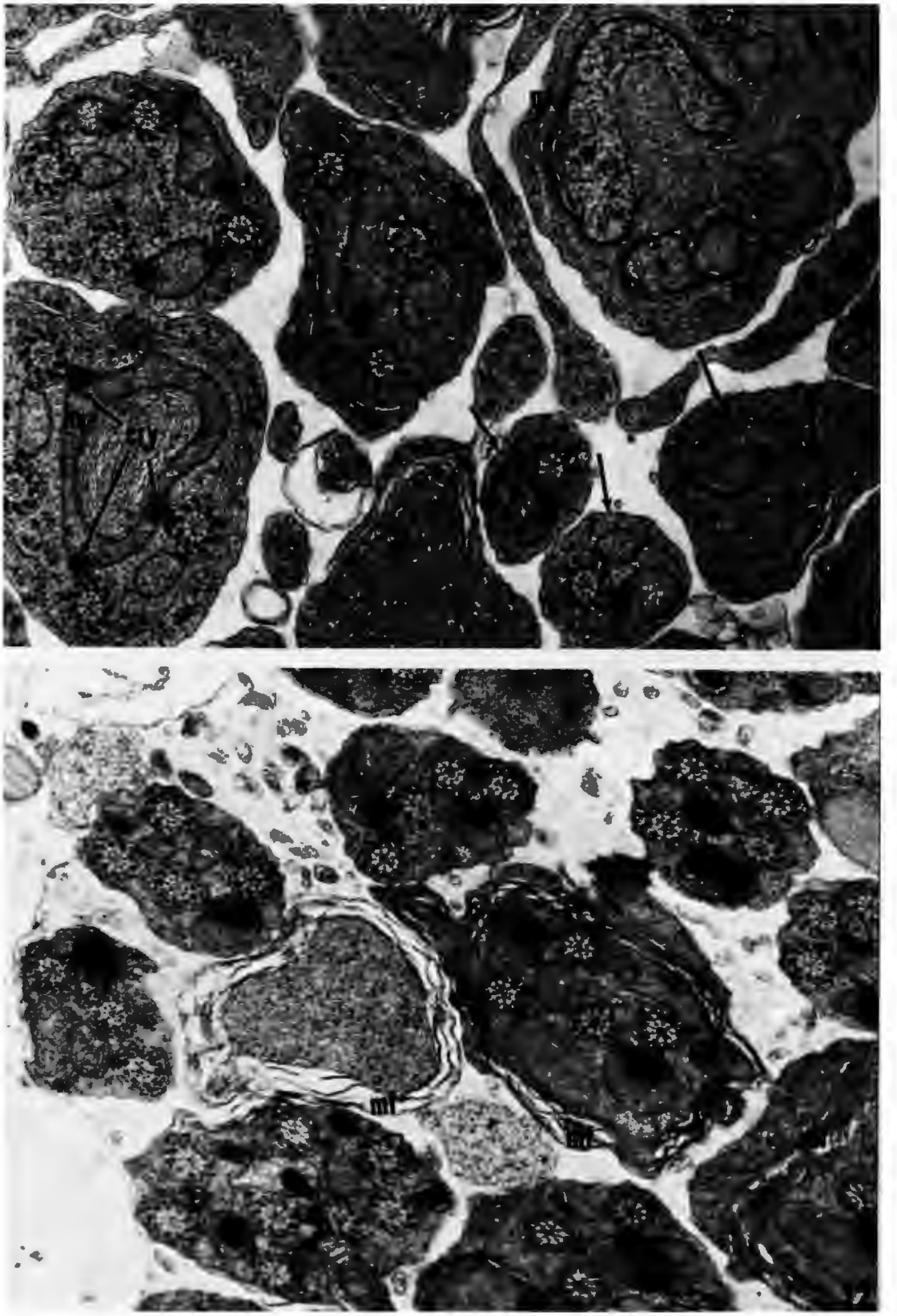
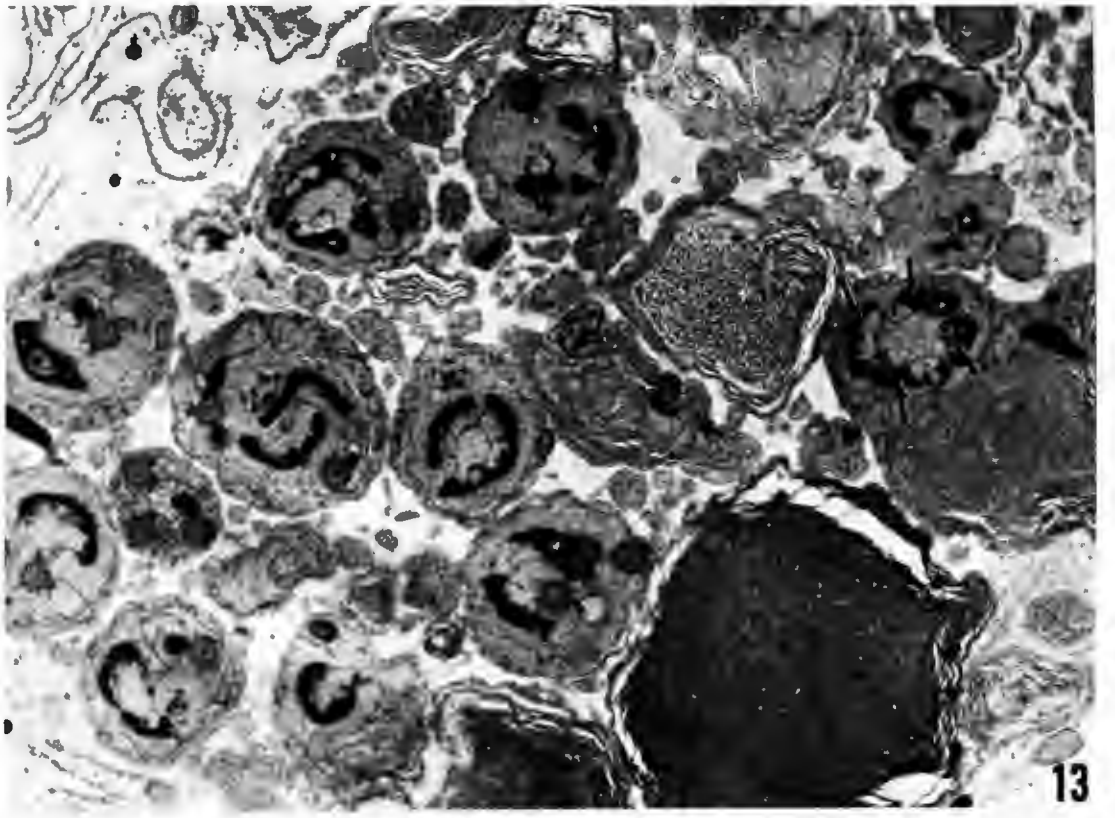


Figure 13. Section at the level of spermatid heads. In one cytoplasmic mass all four nuclei are in the cross-section (arrows). Many large myelin figures are seen in the bundles of spermatids. X 24,000.

Figure 14. Section at the level of spermatid tails (at approximately the same developmental stage as in Figure 13). Axonemes are often seen in groups of four in distinct cytoplasmic units. Very large myelin figures (mf) occur within the bundle. X 24,000.



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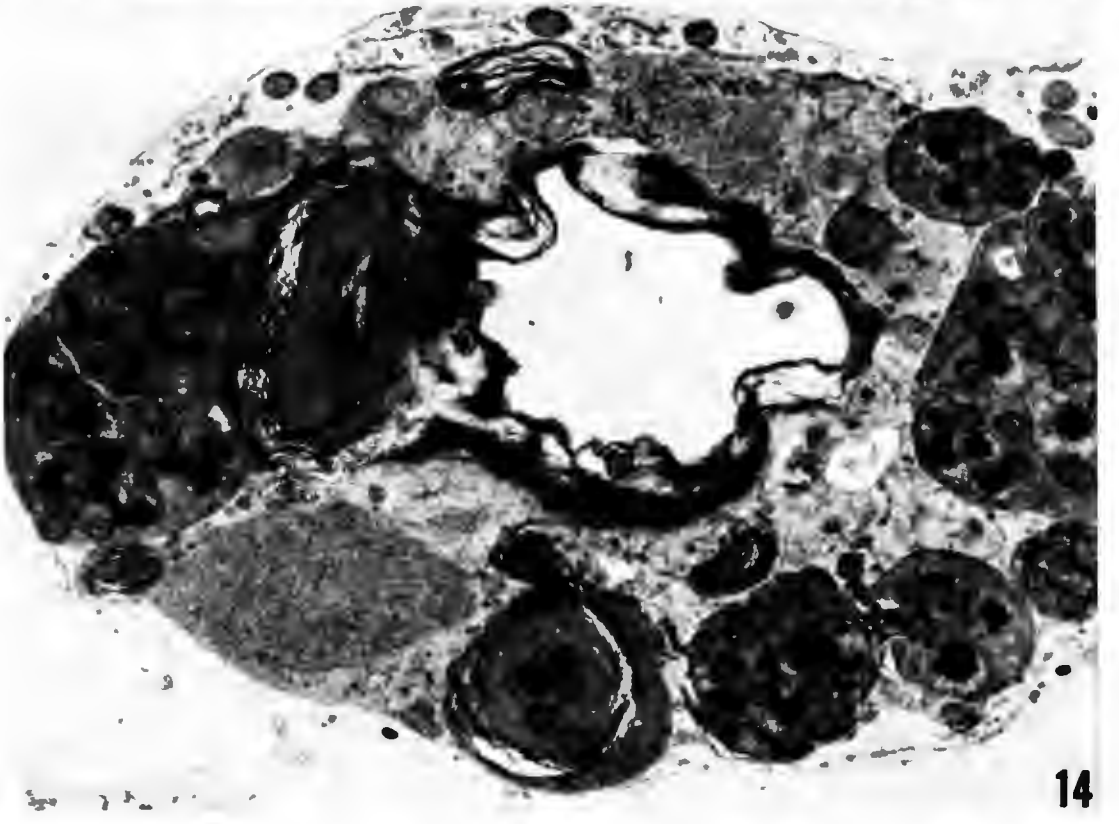

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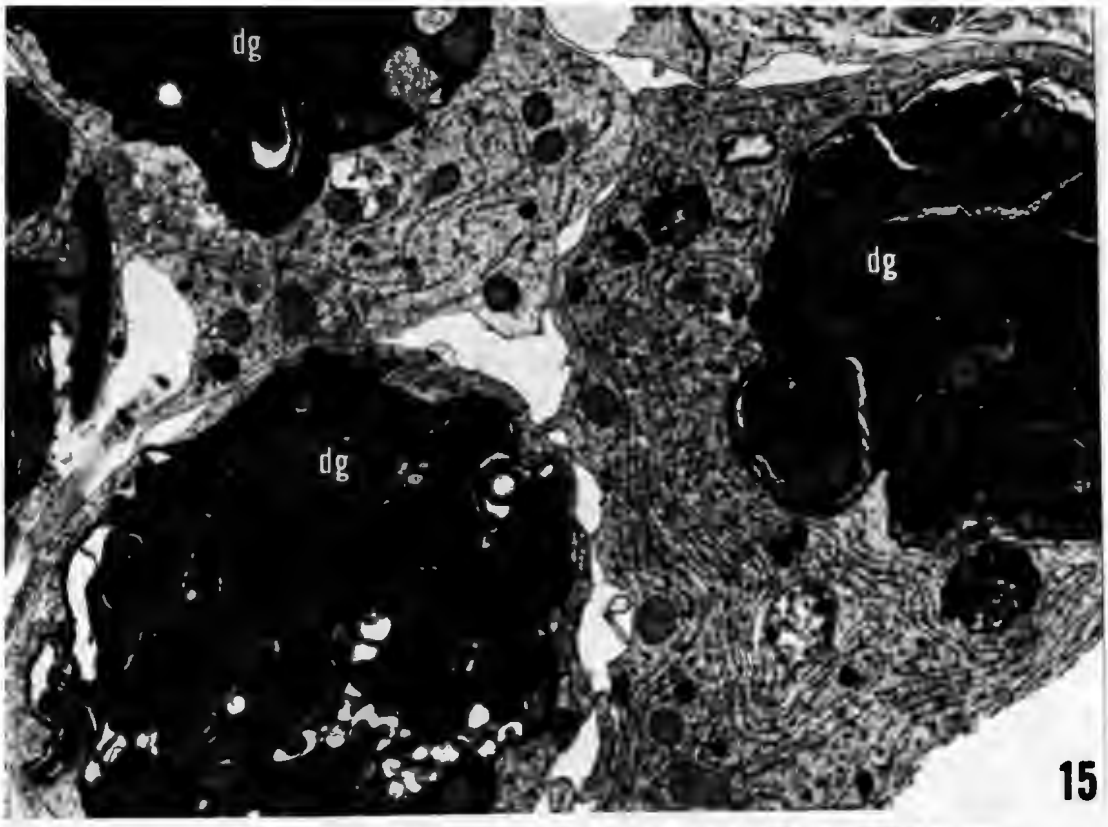
Figure 15. Degenerating bundles (dg) demonstrating the regular occurrence of groups of four spermatids. X 13,800.

Figure 16. Degenerating spermatids (dg) next to a unit of four spermatids. Only the two, cored central (ct) and the nine, cored satellite (st) tubules remain as remenants of the axonemal complex in the degenerating spermatids. The axoneme show apparently complete differentiation and the primary derivatives are almost completely filled with the paracrystalline material (cry). X 78,600.



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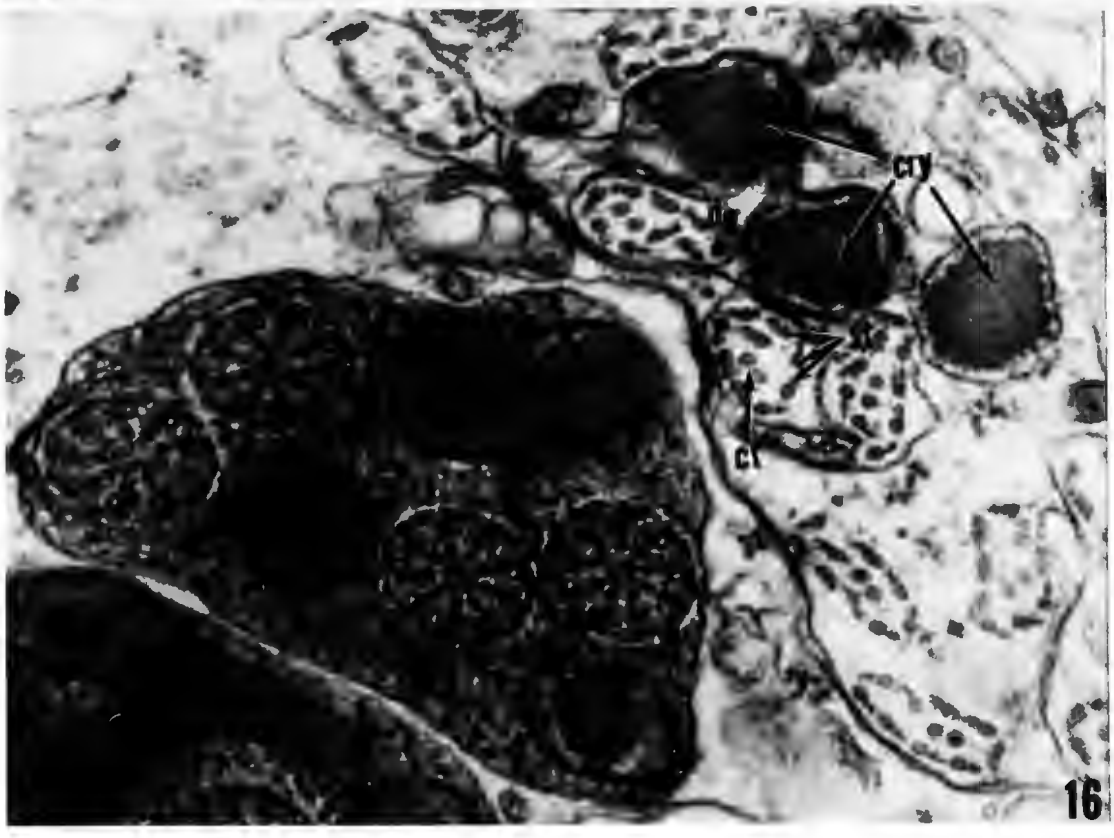

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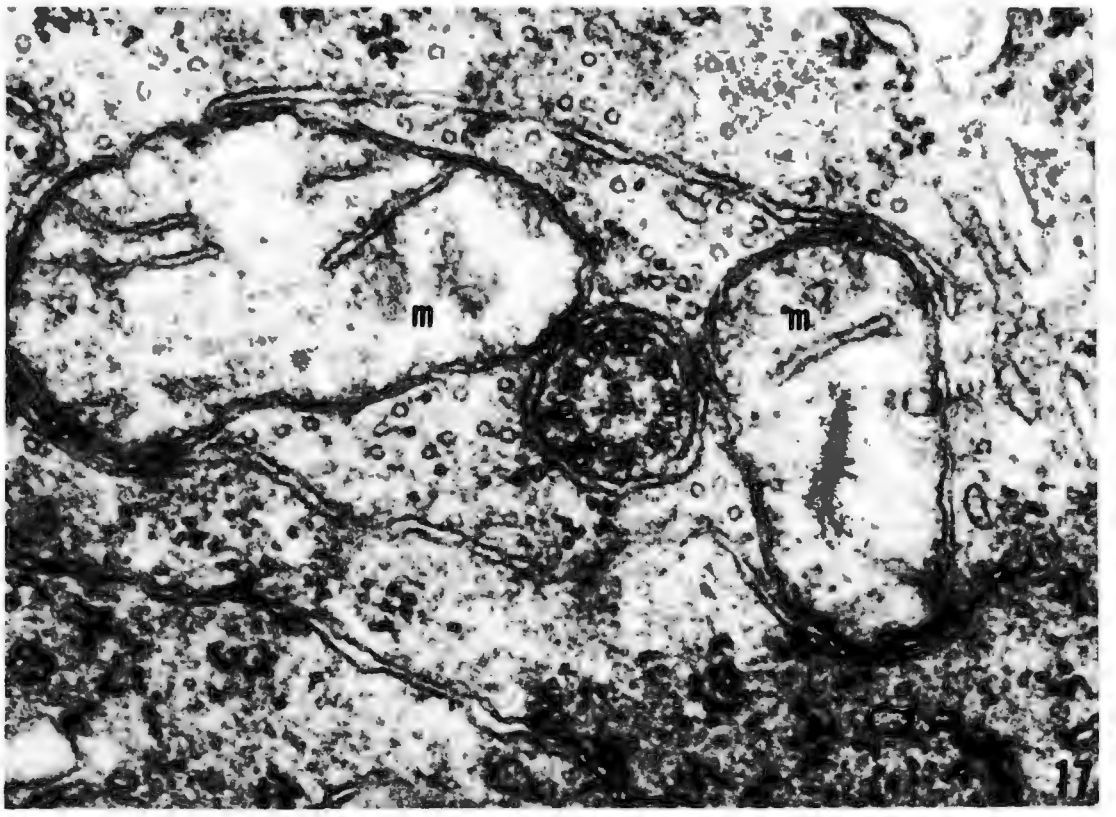
Figures 17-28. Spermiogenesis in male sterile mutant C_2-10.

Figures 17-18. Normal and abonormal spermatids from the same bundle in mutant C_2-10. A normal axoneme (a) with the 9 + 2 tubule pattern is shown in Figure 17. The two mitochondrial derivatives (m) lie on either side of the axoneme. The axoneme is disrupted in Figure 18 with the pair of central tubules (ct) and the nine peripheral doublets (pd) scattered in the cytoplasm adjacent to cytoplasmic membranes. Only one of the two derivatives lies adjacent to the disrupted axoneme. X 78,600.



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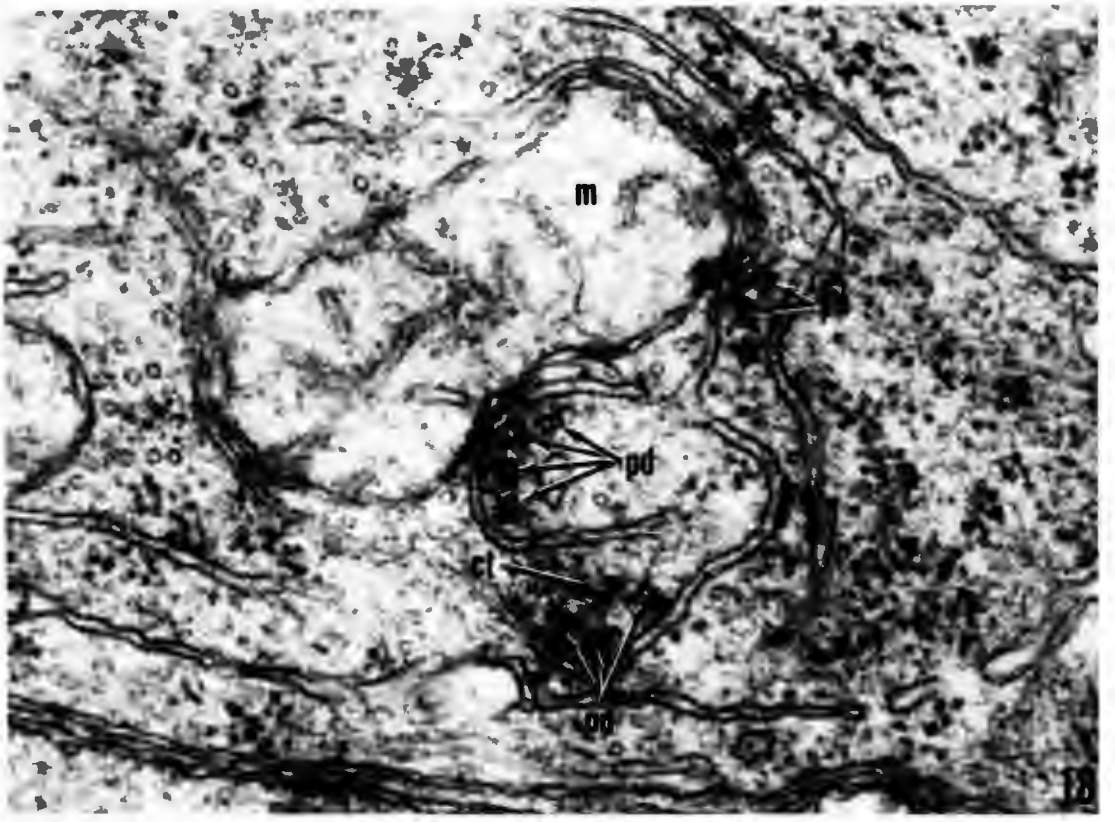

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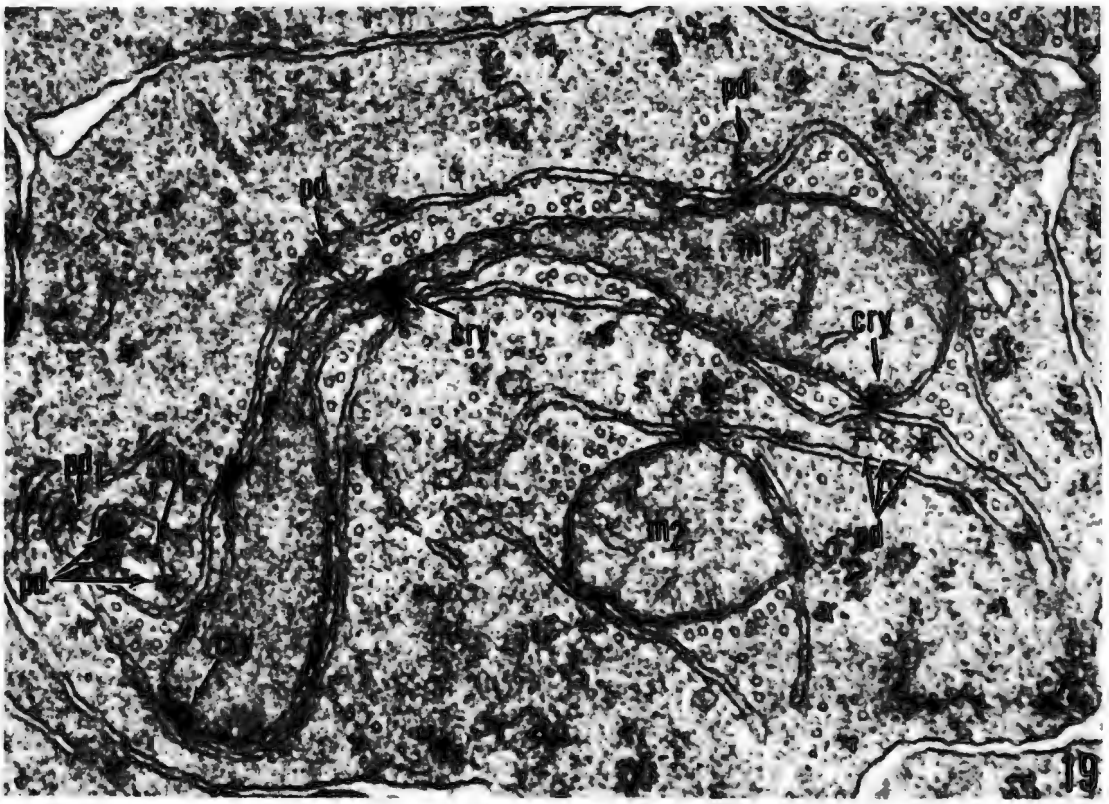
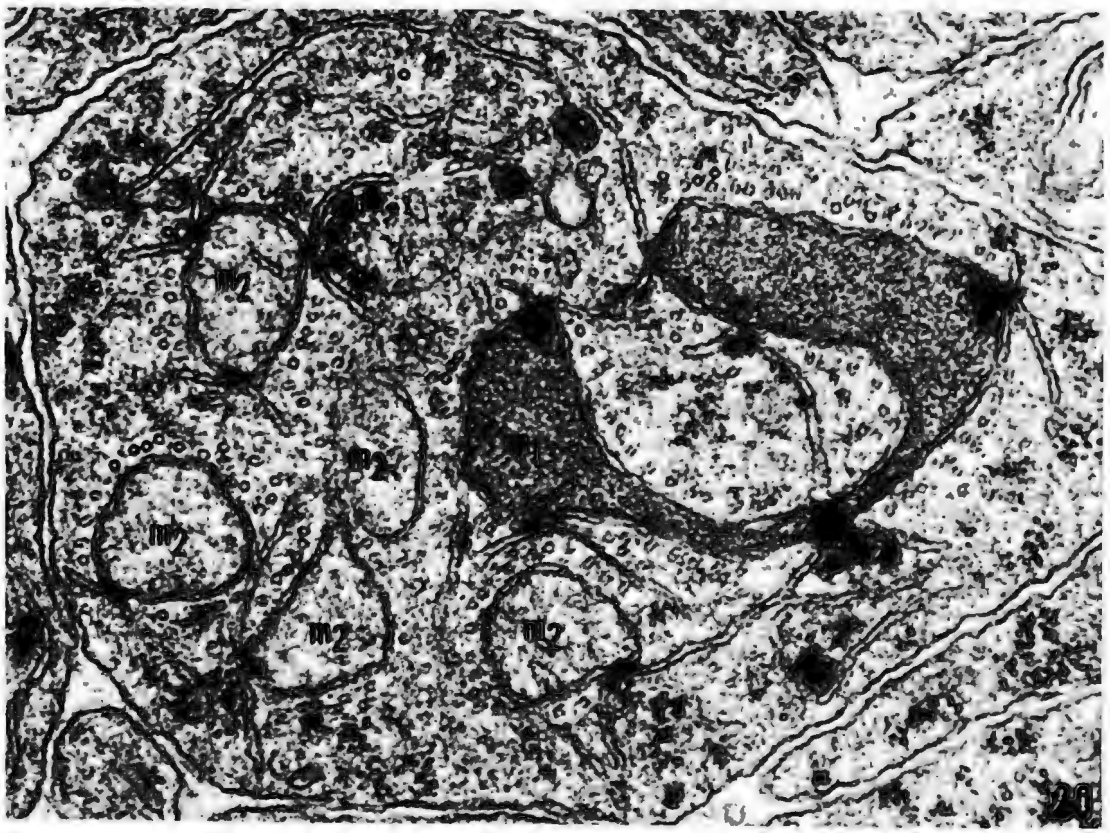
Figure 19. Spermatid at the beginning of the paracrystalline body formation. The nine peripheral doublets (pd) and central pair of tubules (ct) are scattered in the cytoplasm but remain associated with cytoplasmic membranes. One doublet (pd_1) shows a link connecting it with the cytoplasmic membrane. Paracrystalline material (cry) forms at the points of contact between the membrane of the mitochondrial derivative and the cytoplasmic membranes. X 56,000.

Figure 20. Spermatid containing several secondary mitochondrial derivatives. The density of the secondary derivative (m_2) is markedly less than that of the primary derivative (m_1). X 56,000.


pd
pd
m1
cry
cry
pd
pd
pd
m2
19


m2
m2
m2
m2
m2
20

Figures 21-22. Normal and mutant spermatids at the same developmental stage. Mutant spermatids (Figure 22) have a larger cross-sectional area, much larger primary and secondary derivatives, and a much less dense matrix within the secondary derivative. The section includes the tip of an acrosome (ac) and a nucleus (n), as well as, tails. Axonemal complexes within the mutant spermatids vary from normal configuration to the complete absence of axonemal complexes. Both figures X 40,000.

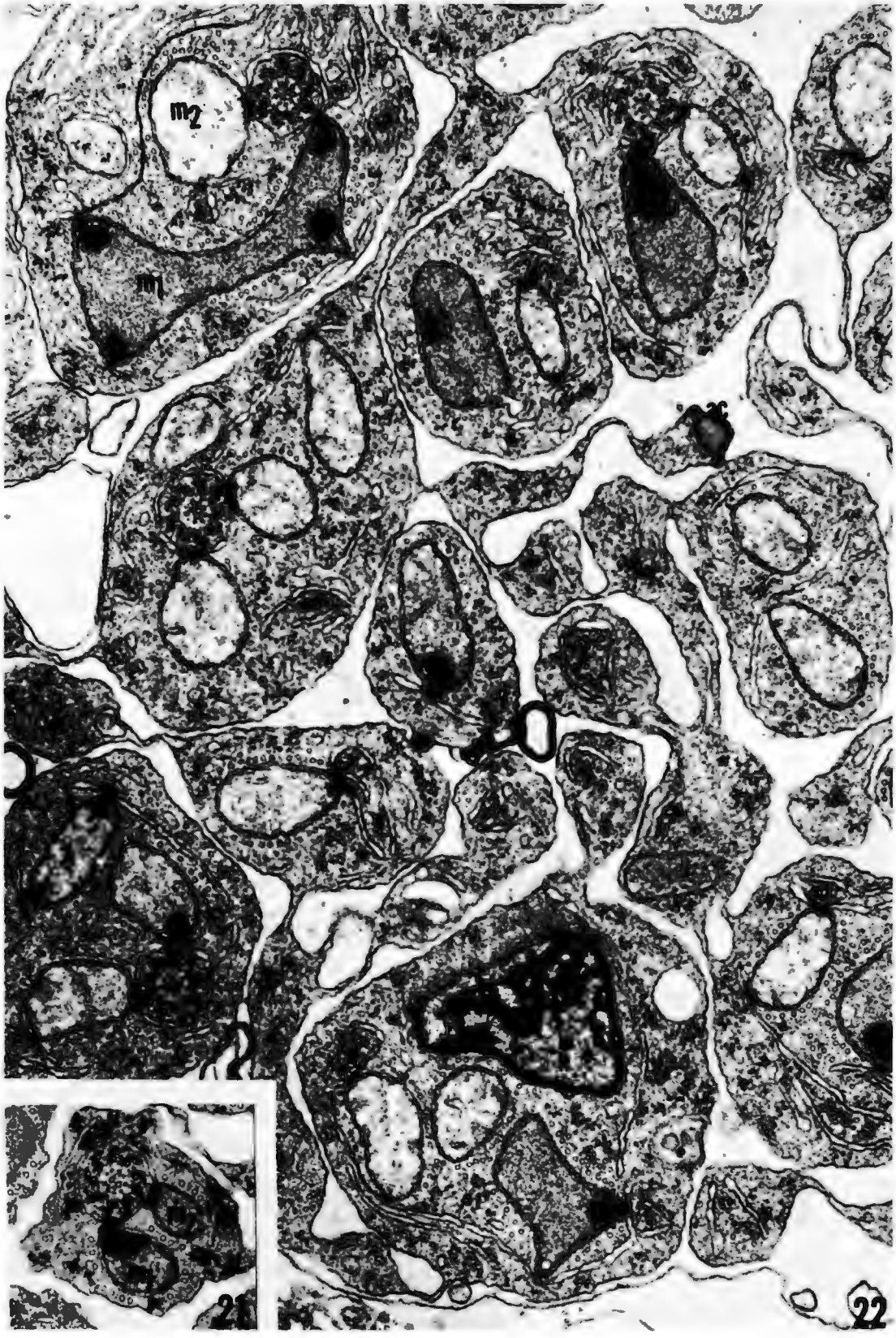
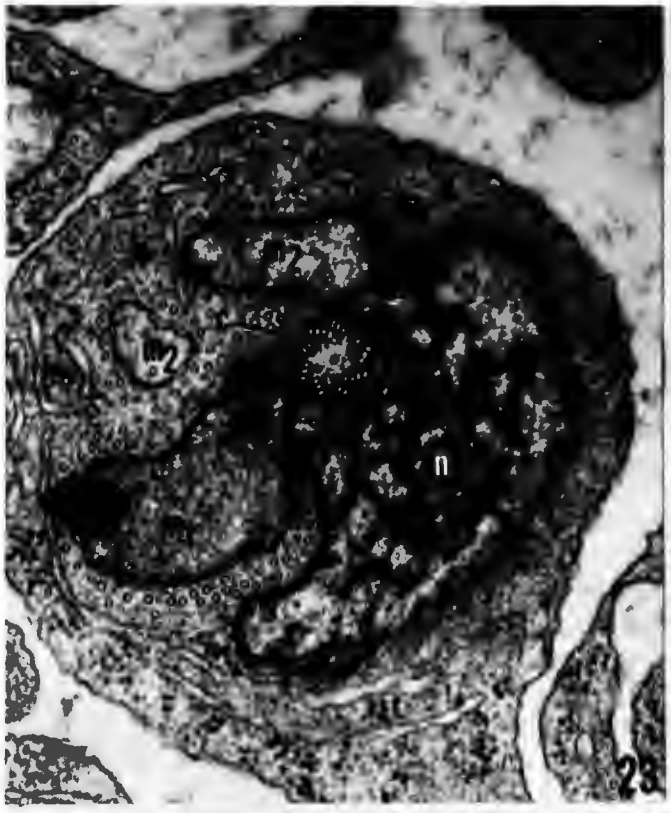

m2
m1
21
22

Figure 23. Cross-section of a C_2-10 spermatid at the level of the flagellar base. Section shows an apparently normal centriole (c), a nucleus with partially condensed chromatin (n), two secondary derivatives (m_2), and a primary derivative containing two paracrystalline bodies (m_1). X 52,400.

Figure 24. Longitudinal-section of a C_2-10 spermatid at the nucleus, tail junction site. The spermatid is at approximately the same developmental stage as the one shown in Figure 23. The nucleus (n) contains partially condensed chromatin and is not fully elongate. X 16,000.

Figure 25. Spermatid tail with an abnormal axoneme. The axoneme shows two positions in which the normal peripheral doublets and satellite tubules are aberrant (arrows). There are extra tubules and a breakdown of the normal tubule pattern. The spermatid also sontains two secondary derivatives and a primary derivative with two paracrystalline bodies. X 52,400.

Figure 26. Spermatid tail lacking an axoneme. Next to the aberrant spermatid is the acrosome (ac) of an adjacent spermatid. X 52,400.



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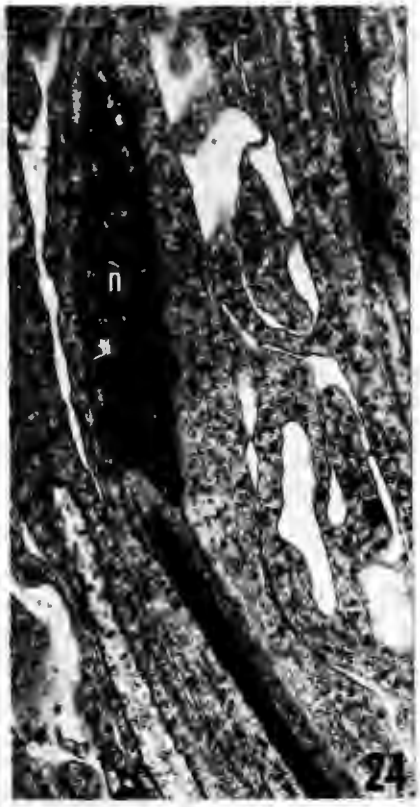

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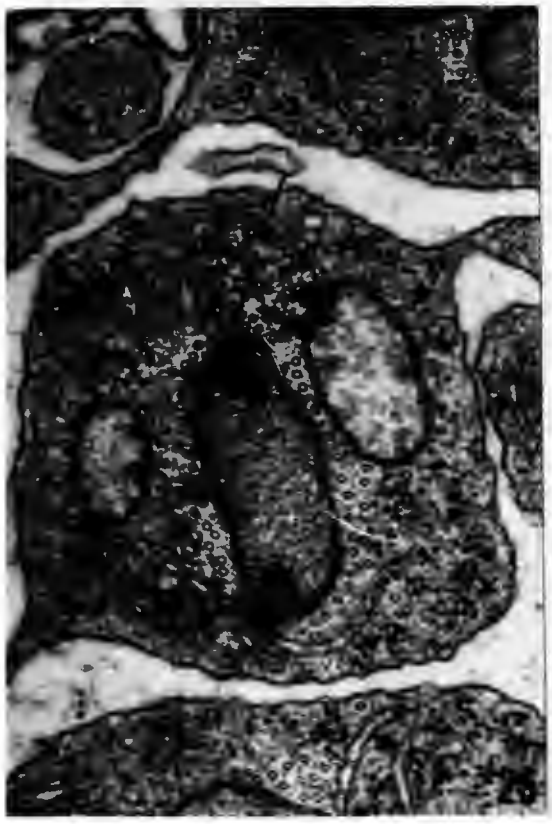

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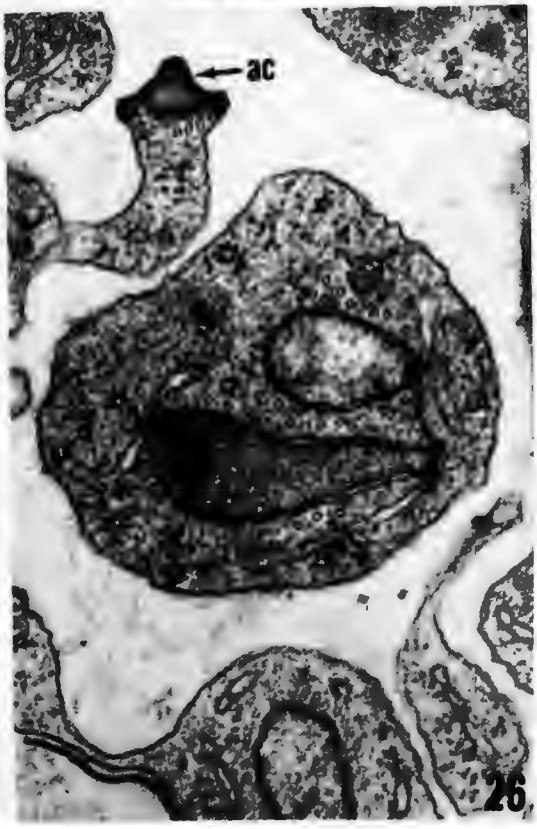

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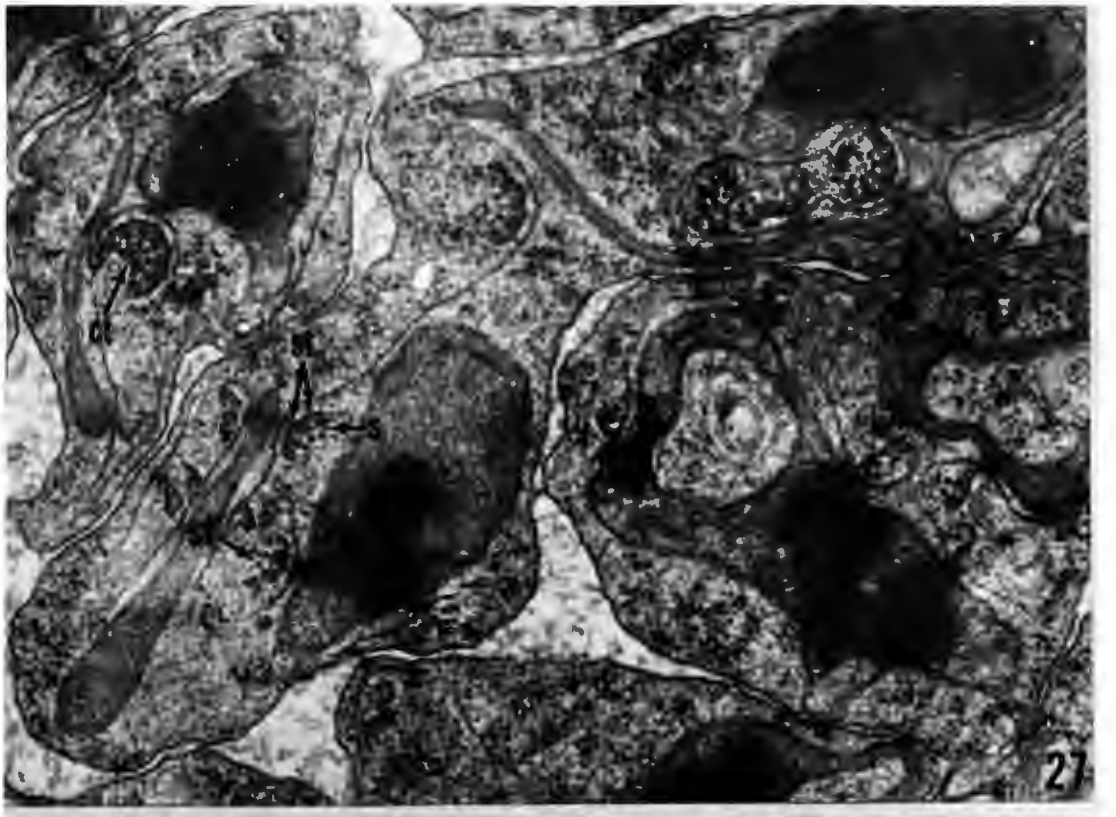
Figure 27. Late spermatids containing disrupted axonemes which have completed their differentiation. Spokes (s) project from disrupted peripheral doublets. Cores are present in central (ct) and satellite (st) tubules and the paired dense secondary fibers (sf) are present. Very large and irregularly shaped mitochondrial derivatives are also shown. Multiple paracrystalline bodies are seen within the primary derivatives. X 40,000.

Figure 28. A section through degenerating spermatids at a late developmental stage. Disrupted axonemes (a) lie near degenerating primary derivatives containing multiple paracrystalline bodies (m_1). X 40,000.



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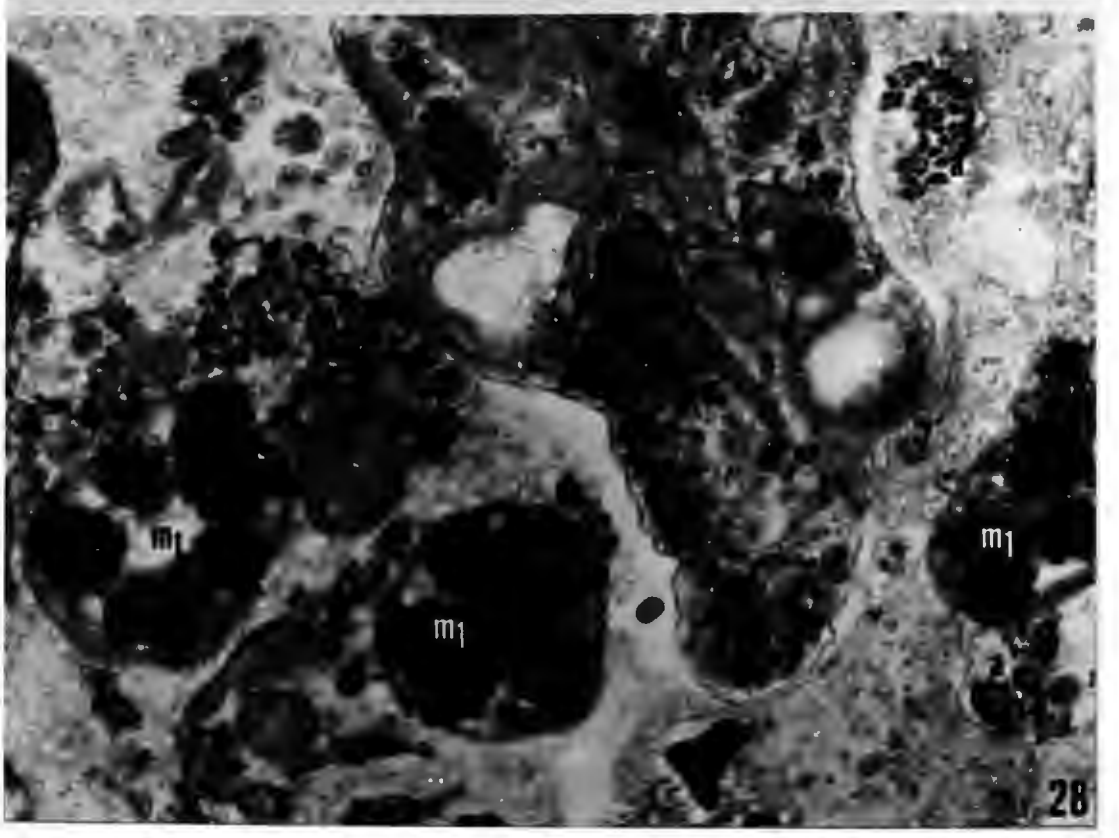

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DISCUSSION

The recovery of 31 autosomal male sterile mutants provides circumstantial evidence that autosomal genes function in spermiogenesis. However, since any mutation preventing sperm transfer would result in sterility, such mutants would not in themselves support the contention that autosomal genes function in sperm development. Establishing this hypothesis requires the demonstration either of sterility in mutants with a normal transfer system or of mutants in which development is blocked prior to complete maturation.

Detailed analyses of mutants C_2-3 and C_2-10 has revealed that no fully mature sperm are formed. Spermiogenesis in both mutants progresses to a rather late stage in development before the developing bundles undergo degeneration. In mutants C_2-1, C_2-2, and C_2-11, preliminary studies indicate that spermiogenesis is blocked rather early. The development of spermatids in mutants C_2-1 and C_2-2 is blocked after initial elongation and in mutant C_2-11, spermatogenesis appears to be blocked at the primary spermatocyte stage. Clearly, therefore, there are autosomal genes whose function is necessary for the production of normally differentiated, fully functional spermatozoa.

Complementation analysis of 14 autosomal male-sterile mutants has uncovered only one allelic pair among 8 mutants located on chromosome II. The frequency of such repeat mutation can be used to estimate the total number of genes in which mutation may cause male sterility. Since the number of repeats is low, only one, the estimate obtained is subject to large error but may be used in the absence of a more precise

determination. The estimate is based on two assumptions: (1) that the induction of mutation by EMS is random, and (2) that all genes capable of mutating to male sterility do so with equal frequency. Under these conditions, the frequency of genes that mutate to give male sterility may be estimated using the Poisson distribution. This gives an estimate of 25 second chromosome genes whose products are necessary for the production of fully functional sperm. An estimate of third chromosome genes cannot be made since there are no repeats among the 6 third chromosome mutants. If autosomal mutants are considered as a group, a total of 85 such genes is estimated. Although the estimate must be considered with some reservation, it is apparent that a reasonably large number of autosomal gene products are required in the production of sperm.

Concomitant female sterility was found in several of the male sterile mutants. A high correlation between male and female sterility was found among the third-chromosome mutants; all of those tested show some degree of female sterility. Only 2 of 9 second-chromosome mutants exhibit female sterility. One would expect male and female sterility to result from mutations in genes whose functions are common to both reproductive processes. Changes in abdominal structure or size are known to result in both male and female sterility in a few mutants (Lindsley and Grell, 1967). Although most of our mutants appear to be morphologically normal, this possibility cannot be ruled out in all cases.

The high correlation between male and female sterility found in third-chromosome mutants is further supported by other studies. Gill (1963) recovered 5 female sterile mutants in chromosome III. One

of these shows complete male sterility, and another shows partial male sterility. Eight other third- and 4 second-chromosome mutants (Lindsley and Grell, 1967) have been shown to exhibit both male and female sterility along with other mutant phenotypes. Therefore a total of 18 third-chromosome mutants, but only 6 second-chromosome mutants, have now been shown to demonstrate concomitant male and female sterility. Based on these observations it appears that the third chromosome contains a number of loci which provide functions common to both reproductive processes whereas the second chromosome contains considerably fewer such loci.

Mutant C_2-3, which is characterized by the failure of meitoic cytokinesis, is also female sterile. Since it is generally felt that meiosis and the formation of polar bodies in Drosophila (Cooper, 1950) does not require cytoplasmic separation, female sterility was unexpected. According to Fahmy (1952), in Drosophila subobscura the three polar bodies fuse and persist until late cleavage, sometimes as late as the blastoderm stage before they degenerate. Fahmy used Feulgen stain, and therefore, would not have been able to identify cell membranes. In early cleavage and during formation of the blastoderm in Drosophila and other insects, nuclear division is not accompanied by cell division (Mahowald, 1963a, 1963b). Nuclei accompanied by a halo of clear cytoplasm migrate to the periplasm where folds of plasma membrane extending from the egg surface eventually separate the layer of nuclei from the undivided mass of yolk. It is generally felt that this is the first cytoplasmic division although there apparently has been no detailed description of the formation of the polar bodies at the ultrastructural level. The recovery of a mutant in which male sterility results from failure of

meiotic cytokinesis suggests a need for further study of the formation and degeneration of the polar bodies, with particular attention to the question of cytoplasmic separation.

It should be noted that mutant C_2-10 does not show female sterility. The anomalies characteristic of this mutant appear to be sperm specific. This supports the hypothesis that some gene loci control structures or processes affecting the production of both types of gamates, whereas other loci may affect only one sex.

The earliest defect observed in mutant C_2-3 is the failure of the cytokinetic divisions which normally accompany meiosis. This failure leaves the components of 4 spermatids to develop in a common cytoplasmic unit. This unusual spatial relationship leads to the following series of events. Each post-meiotic cytoplasmic unit contains four times the number of mitochondria found in normal spermatids. Although aggregation of mitochondria has not been observed it appears that all of the mitochondria fuse forming a single large nebenkern. A single exception to this has been noted, one cell in which two nebenkernen were forming. The "giant" nebenkern routinely divides into two approximately equal parts. Following this first division there may be secondary divisions resulting in the formation of usually 8 or fewer mitochondrial derivatives, in contrast to normal spermiogenesis where only one division of the nebenkern is observed. From the frequent observation of odd numbers of mitochondrial derivatives it appears that there is not a synchronized series of division within the cytoplasmic unit following the initial division of the nebenkern.

Although the properties of mutant C_2-3 do not permit a critical test, two types of explanations of the above phenomena may be tenta-

tively offered. First, division of nebenkernen may be conditioned by the size of the structure, i.e. even in absence of external influences bodies larger than a critical size may have an inherent tendency to divide. Structures of normal derivative size would not be expected to undergo further division. This means that the maximum number of derivatives should be about eight in single cytoplasmic masses of mutant C_2-3. Second, the secondary divisions of the mitochondrial derivatives may be influenced by attachment to axonemal complexes and by the spatial relationships of these structures within the cytoplasmic units. According to this hypothesis stresses generated during elongation cause fragmentation of the derivatives.

As in normal spermatids, the paracrystalline body forms within the primary mitochondrial derivative at the point of contact between its membranes and the membranes around the axoneme. This paracrystalline body eventually nearly fills the mitochondrial derivative in normal spermatids. In the C_2-3 mutant, several paracrystalline bodies may form in a single mitochondrial derivative, one at a point of contact with the membranes around each axoneme. In addition, more than one primary derivative may be found in each cytoplasmic unit. The paracrystalline bodies generally reach the size of those found in normal spermatids, but no larger, even if the derivatives are of large cross-sectional area. This appears to be true of all developmental stages. This observation may be interpreted to mean that following its initiation, growth of the paracrystalline body occurs at a constant rate.

Although spermatids progress through almost complete maturation, there is no general cytoplasmic separation into single spermatozoa. Occasionally, in some of the intermediate stages of spermiogenesis,

individual spermatids are observed. This may be due to separation in only limited regions along the longitudinal axes. Since nuclei and axonemes complete differentiation, it is apparent that normal cytoplasmic division is not a requisite occurrence for normal differentiation of cellular organelles except possibly the nebenkern.

Mutant C_2-10 does not show normal elongation of the spermatids and their organelles. The large cross-sectional areas of the spermatids and the mitochondrial derivatives would be expected if they are of normal volume but fail to elongate. The question now arises as to what may be responsible for the failure of elongation. Microtubules, which may function in the elongation of spermatids (Anderson, 1967; Anderson, Weissman, and Ellis, 1967; Phillips, 1970), are found in the cytoplasm around the mitochondrial derivatives. If it is assumed that microtubules are the functional determiners of spermatid length, and that the amount of microtubule material available in each spermatid is constant, then the length of spermatids should be inversely correlated with the number of microtubules seen in cross-sections. In mutant C_2-10 microtubules are rather uniformily distributed around the mitochondrial derivatives. Large numbers of microtubules are present in the cytoplasm around the large primary and secondary derivatives, and smaller numbers are present around small mitochondrial derivatives. The number of microtubules appears to vary directly with the size and number of mitochondrial derivatives. The mean number of microtubules per spermatid in one mutant bundle was 56. In some cases the large number of microtubules may be due to sections passing through the caudal end of the head sheath. The general occurrence of the observation, however, suggests that this does not account for increased numbers of

microtubules in all sections. Wild type spermatids at the same developmental stage had a mean of 27 microtubules. Assuming that all microtubules reach equal length and that both mutant and wild type bundles produce the same amount of microtubular protein, the mutant spermatids would reach only half the length of the normal spermatids. Light microscopic observations on testis squash preparations indicate that many spermatid bundles are indeed shorter than normal, but a critical evaluation would require isolating individual bundles of spermatids, measuring the bundles using the light microscope, preparing the same bundles for electron microscopy, and counting the number of microtubules present in each bundle. Furthermore, one would have to obtain wild type controls at the same developmental stages.

Another very interesting finding in mutant C_2-10 was that disrupted axonemes, which develop from an apparently normal centriole, complete differentiation. At all stages following the establishment of the flagellum, disrupted axonemes are observed. Some sections show normal axonemes, although even these may be disrupted at more distal levels. Since axonemes develop from apparently normal centrioles, sections near the centriole should exhibit a greater number of normal axonemes. In this mutant, spermatids within individual bundles are out of register; and, therefore, it is difficult to determine the level of the section with respect to the head. In cross-sections of bundles which include heads, there appears to be a greater number of normal axonemes, especially in the spermatids neighboring those sectioned through the head region.

Based on the observations that disrupted axonemes are found in all developmental stages and that development proceeds to near completion

before spermatids degenerate, it is concluded that the subunits of the disrupted axonemes complete development, rather than disruption occurring after normal development has been completed. Thus, normal development of axonemal components does not depend upon their retention in the normal 9 + 2 orientation. Kiefer (1970) has demonstrated that satellites, doublets, spokes, and secondary fibers form an interconnected unit. He has further observed that these structures remain together as a unit even when the axonemal complexes are completely disorganized, either during abnormal development or during degeneration. The present observations are consistent with those of Kiefer. Structural components of the axoneme are normally assembled even though the axoneme is disrupted prior to the development of some of the components of the unit. The interconnected units, as described by Kiefer, are apparently, therefore, units of assembly.

The disrupted axonemes in mutant C_2-10 appear to contain all of the components of normal axonemes. What, then, causes the disruption of the axonemal complex? At least two possibilities can be suggested: (1) the axoneme lacks some stabilizing structure or bond, or (2) the membrane around the axoneme is unstable, which leads to the disruption of the axonemal complex. In all spermatids of the mutant C_2-10, scattered doublets are associated with cytoplasmic membranes. Once the axonemal membrane is broken, it is indistinguishable from other cytoplasmic membranes. Physical links between the scattered doublets and the membranes have been observed in early developmental stages. Such links have also been observed in normal spermatids. Ringo (1967) and Allen (1968) have described similar links in flagella and cilia of several organisms. The physical links between axonemal doublet complexes and

the membranes around the axoneme are maintained in the mutant whether the disruption is caused by the lack of some stabilizing structure within the axoneme or by instability of the membrane around the axoneme. It has not yet proven possible to distinguish between these two possibilities at the ultrasturctural level. Even though the axonemal doublet complexes appear normal, minute structures not yet detected or intermolecular bonds that cannot be resolved by the electron microscope may be responsible for the failure of the units to maintain the normal "9 + 2" configuration.

The following scheme is proposed as an explanation of the several anomalies associated with mutant C_2-10. Shortly after initiation of flagellar differentiation from a normal centriole, the 9 + 2 axonemal pattern is disrupted. The axonemal doublets, in contact with the axonemal membranes, are dispersed through the cytoplasm. The primary mitochondrial derivative then forms contacts with the cytoplasmic membranes, and paracrystalline bodies begin to form. The axoneme, which possibly serves as the central axis for elongation of the tail of the spermatid, is not present as a functional unit due to its disruption. In the absence of this organized structure, the mitochondrial derivatives form multiple attachments to the cytoplasmic membranes. The attachment to a number of membranes outside the central axis presumably prevents normal elongation and reduction of the cross-sectional area of the mitochondrial derivatives. This further prevents normal elongation of the spermatid. More microtubules form around the derivatives, their number varying with the size of the derivatives. The spermatids then undergo limited elongation but degenerate prior to complete maturation. The axonemal complex apparently completes differentiation even though its components are disrupted.

Formation of multiple paracrystalline bodies occurs in both C_2-3 and C_2-10. In normal spermatids, paracrystalline body formation begins within the primary mitochondrial derivative at each contact point between the membranes of the mitochondrial derivative and the membranes around the axoneme. In mutant C_2-3 a single primary mitochondrial derivative may be seen in contact with up to four axonemal points. It should also be noted that more than one primary derivative may establish contact with a single axonemal complex and that paracrystalline bodies form within each mitochondrial derivative. In large irregular primary mitochondrial derivatives of mutant C_2-10, paracrystalline bodies form at contact points between the membranes of the mitochondrial derivatives and the cytoplasmic membranes, even where no axonemal components are present. Hess and Meyer (1968) state "that the formation of paracrystalline bodies always starts at the point of contact of the nebenkern derivative with the axial fiber complex". The demonstration of paracrystalline body formation at the points of contact of the primary mitochondrial derivative with the cytoplasmic membranes strongly suggests that the important factor is contact with the membrane rather than orientation of the axoneme itself. It is also obvious that contact is necessary for the initiation of paracrystalline body formation, but that a specific site of contact, relative to the axoneme, is not important. Any contact with cytoplasmic membranes whether they surround the axoneme or not may initiate the formation of paracrystalline bodies. The above statement must be qualified in that under normal conditions mitochondrial derivatives attach at a specific site on the axonemal complex and, therefore, paracrystalline body formation begins at a specific site relative to the axonemal complex.

In both mutants there is disruption of the normal spatial relationship of spermatid organelles. In spite of this alteration, all organelles are present. These mutants allow one to change the spatial relationship of organelles without direct chemical or physical treatment. Therefore, the observation that initiation of paracrystalline body formation is dependent upon contact between the membranes of the primary mitochondrial derivative and the membranes around the axoneme seems to be a valid generalization for normal spermatids. The morphology and differentiation of mitochondrial derivatives is highly variable in insect species (Phillips, 1970). The generalization made here obviously may not be applicable to other insect species. For example, the observations on the flour moth Ephestia (Smith, 1968) are contradictory to the above generalizations. In Ephestia, paracrystalline material forms in both mitochondrial derivatives at the side opposite the axonemal complex. Apparently there is no contact with cytoplasmic membranes at the site of paracrystalline body formation.

Obviously many phenotypic anomalies may be secondary effects of mutational change. As has been shown above, it is possible to use these secondary effects to investigate the developmental interrelationships of developing sperm organelles. It is anticipated that the analyses of additional mutants will further elucidate the nature of ultrastructural changes during cellular differentiation.

SUMMARY

In order to study the genetic control of spermiogenesis, recessive, male-sterile, autosomal mutants of Drosophila melanogaster were induced with ethyl methanesulfonate. In 2 of the mutants, spermiogenesis was studied with the light and electron microscopes in an attempt to correlate genetic mutation with abnormal sperm development. Mutant C_2-3 has an anomaly associated with cytokinesis following meiosis. The primary spermatocyte undergoes nuclear division, but cytokinesis fails to follow, thus leaving the 4 spermatids to develop within a common cytoplasm. The mitochondria fuse, usually forming a single large nebenkern, which then divides into two approximately equal parts, as a normal nebenkern does. In the mutant these two mitochondrial derivatives usually undergo further division generally giving rise to 8 or fewer mitochondrial derivatives. Multiple paracrystalline bodies are often observed in the primary mitochondrial derivatives. Up to 4 paracrystalline bodies may form, one at each contact point between the membranes of the primary mitochondrial derivative and the membranes around the four axonemes contained in the common cytoplasmic unit. The groups of 4 spermatids almost complete maturation before the bundles degenerate.

Mutant C_2-10 is characterized by two anomalies: (1) disruption of the axonemal complex, and (2) formation of multiple paracrystalline bodies within the primary mitochondrial derivative. This mutant undergoes limited elongation with some variation between bundles of maturing

spermatids. Axonemal complexes apparently complete differentiation even though disrupted and scattered in the cytoplasm. Mitochondrial derivatives are often very large and contain several paracrystalline bodies. The paracrystalline bodies form within the primary mitochondrial derivative at contact points between the membranes of the derivative and the cytoplasmic membranes. Abnormally large numbers of microtubules are observed within spermatids containing large mitochondrial derivatives and appear to be rather uniformily distributed around the derivatives. The large derivative size is presumed to be due to failure of normal elongation. Spermatids degenerate rather late in the maturation process.

The mutants studied in this investigation demonstrate the following important developmental relationships: (1) Membrane contact is necessary for the formation of the paracrystalline body. Although paracrystalline bodies may form within the primary mitochondrial derivative, they do not form in the absence of membrane contact. Furthermore, contact with membrane, not necessarily contact with the axonemal complex, appears to be the factor necessary for paracrystalline body formation. (2) Normal configuration of the axonemal components is not necessary for the differentiation of the axonemal complex. Disrupted axonemal complexed complete differentiation even though components are scattered in the cytoplasm. (3) Apparently cytoplasmic separation is not a prerequisite for the normal differentiation of most spermatid organelles with the exception of the nebenkern and its derivatives.

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