DNA DISTRIBUTION IN SPERMATID NUCLEI OF NORMAL AND SEGREGATION DISTORTER MALES OF *DROSOPHILA MELANOGASTER**

E. HAUSCHTECK-JUNGEN

Strahlenbiologisches Institut der Univ. Zurich August Forel-Str. 7 8029 Zurich, Switzerland

AND

D. L. HARTL

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

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ABSTRACT

Using the DNA-specific dye BAO [2,5-bis-(4'-aminophenyl-(1')]-1,3,4-oxadiazol), we have examined spermiogenesis in wild-type males of *Drosophila melanogaster* and in males carrying various combinations of the *Sd* and *Rsp* mutations involved in segregation distortion. Wild-type strains, even those newly collected from nature, are heterogeneous with respect to the incidence of spermiogenic abnormalities, principally in having a variable number of spermatid nuclei per cyst that fail to undergo complete elongation. Among segregation distorter males, *Rsp/Rsp* homozygotes have the greatest incidence of nuclear nonelongation or incomplete elongation, *Rsp/Rsp+* heterozygotes are intermediate, while *Rsp+/Rsp+* homozygotes have the least amount of abnormality. Indeed, *Sd Rsp+/Sd+Rsp+* males have significantly fewer spermiogenic aberrations than do wild-type strains.

NATURAL populations of *Drosophila melanogaster* carry at low frequency a certain type of second chromosome designated *SD* (segregation distorter) (SANDLER, HIRAIZUMI and SANDLER 1959). *SD/SD+* heterozygous males produce a gross excess of *SD*-bearing offspring; *SD/SD* homozygous males are sterile or nearly sterile (SANDLER, HIRAIZUMI and SANDLER 1959; HARTL 1973). The mechanism of meiotic drive in heterozygous males is the dysfunction of nearly all the *SD+*-bearing sperm; the sterility of homozygous males is caused by dysfunction of nearly all the sperm (TOKUYASU, PEACOCK and HARDY 1972a; PEACOCK, TOKUYASU and HARDY 1972; ERICKSON and HARTL 1976; see review by HARTL and HIRAIZUMI 1976). Electron microscopic observations of testes of *SD/SD+* males have revealed abnormalities in spermatogenesis (NICOLETTI 1968; TOKUYASU, PEACOCK and HARDY 1972a, 1977; PEACOCK, TOKUYASU and

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HARDY 1972). Indeed, a one-to-one ratio of normal to abnormal sperm has been reported, which agrees well with the ratio expected based on the meiotic drive (TOKUYASU, PEACOCK and HARDY 1972a; PEACOCK, TOKUYASU and HARDY 1972). Specific abnormalities in spermatogenesis include defective sperm head morphogenesis and, in some cases, absence of tail individualization (TOKUYASU, PEACOCK and HARDY 1972a, 1977; PEACOCK, TOKUYASU and HARDY 1972).

Genetically, *SD* chromosomes carry a mutation at each of two closely linked loci straddling the centromere. The allele on the left arm of the chromosome is called *Sd* (segregation distorter); the allele on the right arm of the chromosome is called *Rsp* (responder) (SANDLER and HIRAIZUMI 1960; HARTL 1974). The wild-type alleles of *Sd* and *Rsp* are designated Sd^+ and Rsp^+, and the symbol *SD* refers to a chromosome that is genetically *Sd Rsp*.

The *Sd* and *Rsp* loci interact as follows: meiotic drive occurs at the *Rsp* locus, but Sd^+ behaves as a recessive suppressor of drive. That is to say, Rsp/Rsp^+ males will produce an excess of *Rsp*-bearing functional sperm unless the males are homozygous Sd^+/Sd^+. Segregation in *Rsp/Rsp* or Rsp^+/Rsp^+ homozygotes is normal.

Males that exhibit meiotic drive (*i.e.*, *Sd/—*; Rsp/Rsp^+) produce only about half as many functional sperm as do normal males (HARTL, HIRAIZUMI, and CROW 1967; NICOLETTI, TRIPPA and DEMARCO 1967). As mentioned above, homozygous *Sd Rsp/Sd Rsp* males produce almost no functional sperm (HARTL 1973; ERICKSON and HARTL 1976). Moreover, *Sd Rsp/+ Rsp* males produce about 50% dysfunctional sperm, despite the absence of meiotic drive (HARTL 1969, HIHARA 1974).

In this paper, we report a study of spermatogenesis in males carrying various combinations of *Sd* and *Rsp* and their wild-type alleles. Special attention was paid to nuclear morphology during the processes of elongation and individualization.

MATERIALS AND METHODS

(A) *Normal spermatogenesis*

It will be necessary to preface the results of our study with a brief account of normal spermiogenesis and to follow this with a summary of relevant observations on segregation distorter. Classical light microscopic observations of spermatogenesis have been well summarized by COOPER (1950) and ÅBRO (1964). However, current interpretations of spermatogenesis have been strongly influenced by the electron microscopic work of ANDERSON (1967), BAIRATI (1967), TATES (1971), STANLEY *et al.* (1972), and especially TOKUYASU, PEACOCK and HARDY (1972a,b; 1977), PEACOCK, TOKUYASU and HARDY (1972), HARDY (1975) and TOKUYASU (1974a,b; 1975a,b). For our purposes, the descriptive terminology introduced by TOKUYASU, PEACOCK and HARDY (see above references) is most useful.

Spermatogenesis in *D. melanogaster* occurs synchronously in bundles of 64 spermatids held together by two cyst cells. Developing cysts move along the inner testicular wall from the blind end of the coiled, tubular, 1.8 mm-long testis into the middle level of the testis and then into the testicular lumen. Early spermatids are roughly spherical cells, some 13 μm in diameter (ÅBRO 1964); their nuclei are oval, and the mitochondria are loosely aggregated toward one side of each cell (ÅBRO 1964, TATES 1971, STANLEY *et al.* 1972).

Key sequential processes in spermiogenesis are defined by PEACOCK, TOKUYASU and HARDY (1972) as elongation, individualization, entrapment, coiling and release, although HARDY (1975)

chooses to define an additional distinct phase, maturation, between elongation and individualization. These processes must be described briefly in those aspects relevant to the present study.

(1) *Elongation*: In this process the spermatids elongate approximately symmetrically with respect to the middle level of the testis, heads oriented toward the base, reaching a final length of about 1.7 mm; 99.4% of this length is tail (PEACOCK, TOKUYASU and HARDY 1972). The tails of the spermatids are first interconnected by cytoplasmic bridges and then they become syncytial, although not all 64 spermatids in a bundle may be interconnected at any one transverse level; however, spermatid heads are not interconnected (TOKUYASU, PEACOCK and HARDY 1972a).

Nuclear transformation begins approximately simultaneously with tail elongation and has been described in detail by STANLEY *et al.* (1972) and TOKUYASU (1974b). The beginning of nuclear elongation coincides in time with an inward deformation of part of the nuclear envelope. The nucleus as a whole is concave in both transverse and longitudinal profile: TOKUYASU (1974b) has aptly dubbed it "canoe-shaped." Electron-dense chromatin is visible first along the nuclear envelope, especially at the concave side, and as nuclear transformation proceeds the chromatin becomes more condensed. When tail elongation is essentially completed, the nucleus also attains nearly its final length, but it is still canoe shaped. Nuclear transformation continues on into the post-elongation (TOKUYASU 1974b) or maturation (HARDY 1975) phase. The coarse chromatin network begins to compact, first along the convex portion of the nuclear envelope, but in small clumps elsewhere; further development and compaction of the chromatin network results in the final aggregation. The canoe shape gradually disappears and the nucleus becomes, approximately, an elongated, tapered cylindrical structure of length roughly 9–11 μm (TOKUYASU 1974b; HARDY 1975) and maximum width of about 0.30–0.35 μm (STANLEY *et al.* 1972; TOKUYASU 1974b).

(2) *Individualization*: This process begins in the head region and involves the movement of the cyst membrane over the whole length of the spermatids. Excess cytoplasm and the cytoplasmic bridges between the syncytial spermatid tails (PEACOCK, TOKUYASU and HARDY 1972; TOKUYASU, PEACOCK and HARDY 1972a,b) form a characteristic fusiform swelling, the cystic bulge in the basal region of the testis. The cystic bulge is about 100μm long and some 15–18 μm in diameter, but its diameter increases to 30–35 μm near the apex of the testis due to the accumulation of cellular debris (TOKUYASU, PEACOCK and HARDY 1972a). The bulbous cystic bulge at the apex of the bundle is referred to as the waste bag (TOKUYASU, PEACOCK and HARDY 1972b).

(3) *Entrapment*: In the entrapment process, interconnections are formed between the head cyst cell and a cell of the terminal epithelium in the base of the testis. Entrapment evidently serves to anchor the sperm bundle in the terminal testicular zone (PEACOCK, TOKUYASU and HARDY 1972; TOKUYASU, PEACOCK and HARDY 1972b). During elongation and individualization, sperm heads in a single bundle may normally become longitudinally displaced by 20 μm or more (TOKUYASU, PEACOCK and HARDY 1977), but entrapment can also lead to displacement, particularly of abnormal heads. PEACOCK, TOKUYASU and HARDY (1972) have noted that "abnormal heads are frequently excluded from the head trap and appear to be pushed back along the cyst; this may be well be a result of their large size and irregular shape."

(4) *Coiling*: After entrapment, the sperm tails in the cyst gradually coil into a circinate structure at the base of the testis. As coiling proceeds, the waste bag, still associated with the tail cyst cell, is pulled through practically the entire length of the testicular lumen and comes to reside in the basal portion of the testis along with the coiled sperm.

Sperm tails in a bundle that have not undergone individualization remain syncytial and normally do not coil. As coiling of the normal sperm occurs and the cyst is pulled through the testicular lumen, the nonindividualized sperm remain behind and accumulate in a random coil within the confines of the tail cyst cell, forming a separate lumen of abnormal tails, but this lumen is in fact an extension of the waste bag. By the time the normal sperm are fully coiled, the abnormal sperm are completely segregated from them and are bunched together along with other cellular debris in the waste bag. During the coiling process, the head of a sperm, particularly if morphologically abnormal, may be severed from the tail and remain entrapped along with the other heads while its tail is relegated to the waste bag (PEACOCK, TOKUYASU and HARDY 1972; TOKUYASU, PEACOCK and HARDY 1972b).

(5) *Release*: In this process, which includes release of the coiled sperm from the cyst cell and storage of sperm in the seminal vesicle, the sperm heads are freed and the mature sperm move individually into the seminal vesicle. The remaining packet, which includes the waste bag and any nonindividualized sperm, is slowly degraded. Abnormal sperm that have not been eliminated by the coiling process, or individualized sperm with grossly abnormal heads, may be unable to move to the seminal vesicle and are degraded in the base of the testis along with other debris (PEACOCK, TOKUYASU and HARDY 1972; TOKUYASU, PEACOCK and HARDY 1972b).

(B) *Segregation distortion*

The genetic basis of segregation distortion has been described above. Here we focus on recent histochemical and electron microscopic data.

KETTANEH and HARTL (1976) have studied the transition from relatively lysine-rich somatic histones to relatively arginine-rich sperm histones that normally occurs in spermiogenesis during the nuclear transformation period. Male-sterile *Sd Rsp/Sd Rsp* genotypes fail to undergo this histone transition, a result suggesting that the loci involved in segregation distortion may be related to the structural genes for sperm histones or to regulatory genes affecting the histone transition.

The ultrastructure of spermiogenesis in segregation distorter males has been studied by NICOLETTI (1968), PEACOCK, TOKUYASU and HARDY (1972) and TOKUYASU, PEACOCK and HARDY (1977). Their results support the hypothesis that the major developmental lesion in segregation distortion involves nuclear transformation. In addition, most segregation distorter males exhibit variable amounts of failure in individualization.

TOKUYASU, PEACOCK and HARDY (1977) report that spermiogenesis in cysts of *Sd Rsp*/+ + males is completely normal until the end of the elongation process. In the latter half of the post-elongation or maturation phase, two populations of nuclei can be distinguished in transverse section. One population, accounting for almost exactly half of the number of nuclei, shows a distinctly less dense aggregation of chromatin than is observed in the other population. Such differences can in fact be observed in early post-elongation stages, but they are extremely subtle (TOKUYASU, PEACOCK and HARDY 1977). Essentially the same result has been found in homozygous *Sd Rsp* males (PEACOCK, TOKUYASU and HARDY 1972), but in this case virtually all the nuclei are affected. The time at which these abnormalities first appear during nuclear transformation may coincide with the time of the histone transition (TOKUYASU, PEACOCK and HARDY 1977).

Another spermiogenic abnormality that frequently occurs in segregation distorter males is failure in the individualization process. The extent of this abnormality varies between lines carrying different segregation distorter chromosomes and even between cysts within a single testis. In heterozygotes carrying *SD* (Canberra), for example, an average of only 33.5 ± 3.2 sperm per cyst undergo individualization; in *SD* (Madison), by contrast, an average of 47.2 ± 9.3 sperm per cyst undergo individualization (TOKUYASU, PEACOCK and HARDY 1977). In the homozygous *Sd Rsp/Sd Rsp* studied by PEACOCK *et al.* (1972), as few as three sperm per cyst may be individualized.

The nonindividualized sperm, which are syncytial, become associated with the waste bag in the base of the testis and are subsequently degraded. For this reason, segregation distorter males typically exhibit a greater amount of cellular debris, particularly nonindividualized sperm, in the lumen at the base of the testis than do normal males (PEACOCK, TOKUYASU and HARDY 1972).

Although failure in individualization certainly accounts for the dysfunction of some of the sperm in heterozygous segregation distorter males, a considerable number of sperm destined to be dysfunctional do undergo normal individualization, entrapment, coiling, and release, particularly in strains such as *SD* (Madison). What processes may be operative between the storage of sperm in the seminal vesicle and fertilization of eggs in females that discriminate normal from abnormal sperm more critically than do the individualization and coiling processes are as yet unknown (TOKUYASU, PEACOCK and HARDY 1977).

(C) *Experimental material*

Chromosomes and strains used in the study are listed in Table 1. Males of seven genotypic classes were examined:

TABLE 1

Chromosomes and strains used in the study

Second chromosome (or strain)	*Sd Rsp* Genotype	Notes and references
Tokyo	+ +	Wild type control; standard laboratory strain.
Spartenburg	+ +	Wild type control; founded from a large number of flies trapped in South Carolina in the summer of 1975.
Blattenbach	+ +	Wild type control; founded from a large number of flies trapped near Blattenbach, Switzerland, in the summer of 1976.
Eierbrechtstrasse	+ +	Wild type control; founded from a large number of flies trapped in a wooded area near Zurich, Switzerland, in the summer of 1976.
cn bw	+ +	Standard laboratory strain; *cn* = cinnabar eyes, *bw* = brown eyes.
R(Cy)-34	+ +	Recombinant from *R*-1 (see below); *Cy* = Curly wings.
R (cn)-10	*Sd* +	Recombinant from *R*-1 (HARTL, 1974).
Cy R(cn)-10	*Sd* +	Recombinant from *R(cn)-10.*
R(Cy)-40	+ *Rsp*	Recombinant from *R*-1 (HARTL, 1974).
In(2L + 2R)Cy	+ *Rsp*	HARTL (1975).
R-1	*Sd Rsp*	HIRAIZUMI and NAKAZIMA (1967).
SD-72	*Sd Rsp*	SANDLER, HIRAIZUMI and SANDLER (1959).
SD(NH)-2	*Sd Rsp*	HIRAIZUMI and NAKAZIMA (1965).

(1) + +/+ +: Tokyo, Spartenburg, Blattenbach, and Eierbrechtstrasse
(2) *Sd* +/+ +: *R(cn)-10/cn bw*
(3) + *Rsp*/+ +: *R(Cy)-40/cn bw*
(4) *Sd* +/*Sd Rsp*: *Cy R(cn)-10/SD(NH)-2*
(5) + *Rsp*/*Sd Rsp*: *R(Cy)-40/SD(NH)-2* and *In(2L + 2R) Cy/SD(NH)-2*
(6) *Sd Rsp*/+ +: *SD(NH)-2/cn bw*, *SD-72/cn bw*, *R-1/cn bw*, and *SD(NH)-2/R(Cy)-34*
(7) *Sd Rsp*/*Sd Rsp*: *SD-72/SD(NH)-2*

Flies were raised on Lewis' medium (LEWIS 1960) at 25° and 70% relative humidity, except for Blattenbach and Eierbrechtstrasse, which were maintained at room temperature.

(D) *Cytology*

Cytological methods followed those of HAUSCHTECK-JUNGEN and MAUER (1976). Slides made of nonfluorescing glass were washed in a 1:1 solution of ether and 70% ethanol. Males were dissected in sterile Ringer solution and the testes placed in a flat drop of Ringer solution. The sheath of each testis was removed to allow the cysts to spread in the fluid, and the slide was air dried. The material was fixed with a drop of 3:1 solution of ethanol (100%) and acetic acid; the fixation procedure was performed three times. Then the slides were washed, hydrolyzed for eight minutes in 6N HCl at room temperature, washed again, and strained for three hours in the fluorescent dye BAO (2,5-bis-[4'-aminophenyl-(1')]-1,3,4-oxadiazol), obtained from Fluka AG, Chemische Fabrik, 9470 Buchs, Switzerland) after the method of RUCH (1970), followed by washing in SO_2 water and tap water. The slides were first dehydrated in 50% and 70% ethanol, then air dried and stored in glycerol at 4° for six months. Slides were later made

permanent by removing the glycerol with 50% ethanol and transferring the slides to 100% ethanol, then xylene, followed by fluormount (Gurr).

Photographs were taken either with Leitz objective 40 × 0.65 Achromat and darkground condenser D1.2–1.4 (bulb: HBO 200 Osram; excitation filter: BG12; barrier filter: K530), or with Zeiss objective 40 × 0.63 Plan and darkground condenser D1.2–1.4 (bulb: HBO 100W; excitation filter UG1; barrier filter 530 + 410). The film used was Tri-X Pan Kodak.

Measurements of spermatid nuclear lengths were obtained by the method of HAUSCHTECK-JUNGEN and MEILI (1967). A small, toothed measuring wheel was pushed smoothly along the nuclear contour in prints in which the nucleus was in sharp focus along its entire length. The gaps between adjacent teeth served as the initial unit of measurement, and this was later converted to μm. Since the measured nuclei were air dried flat onto the slide, the error of measurement is small relative to the scale of measurement. Our measured nuclear lengths in wild-type males are consistent with measurements made by other authors using different light microscopic or electron microscopic procedures (see references below).

BAO was chosen for this study because of the extensive experience of HAUSCHTECK-JUNGEN and MAURER (1976) in using the technique to study spermatogenesis in sex-ratio males of *D. subobscura*. BAO gives better resolution of DNA structures than does aceto-orcein, Feulgen, quinacrine, acridine orange, Mithramycine, or Giemsa (HAUSCHTECK-JUNGEN, unpublished). In spermatids, BAO fluoresces for several minutes before fading, and it is sensitive to such small amounts of DNA as are present in mitochondria in the nebenkern. Air-dried preparations have proven to be less disruptive to cysts than are squash or smear techniques (HAUSCHTECK-JUNGEN, unpublished). Preparation artifacts involving the number of individualized spermatids per cyst and the number of cysts per testes are not introduced by these procedures, as evidenced by the fact that results obtained in *D. subobscura* using fixed, paraffin-embedded, and sectioned material are identical to those obtained using air-dried preparations stained with BAO (HAUSCHTECK-JUNGEN and BURKARD, unpublished).

An important question is whether, using our procedures, parts of two bundles of different developmental stages can be brought into juxtaposition and fused so intimately that they are identified as a single bundle. This does not happen, at least to any appreciable extent. In the first place, our procedures are sufficiently gentle that spermatid tails and heads in a single bundle tend to stay together and are air dried to the slide *in situ*; only in a minority of bundles are the spermatid heads well spread. Bundles of different developmental stages do sometimes lie close together, or one may lie across the other, but such instances are easily recognized as the tails are usually of different lengths and are never perfectly aligned all along their length. Our examination includes only spermatid bundles that are well separated from nearby bundles and have continguous tails. If bundles could fuse and so appear as one, we would on occasion observe an apparently normal bundle with more than 64 spermatid heads. In thousands of bundles examined, this has never been observed.

RESULTS AND DISCUSSION

General observations: A number of key processes in spermiogenesis are particularly easy to identify using BAO. Figure 1A, for example, is a pre-elongation cyst. Note the distinctive coarse chromatin granules in the spermatid nuclei. "Granules" have been observed with the electron microscope by STANLEY *et al.* (1972) and TOKUYASU (1974b) as well.

Figures 1B, 1C, and 1D show spermatid nuclei in various stages of nuclear transformation. The nuclei in Figure 1B are early in nuclear transformation; the nuclei are elongating and assuming their characteristic lanceolate shapes, which may correspond to the "canoe" shapes in three dimensions (TOKUYASU 1974b); the chromatin is condensed into small clumps, but no definite compaction has yet

occurred. In Figure 1C some of the DNA appears as a brightly fluorescing longitudinally oriented rod, representing an area of greater chromatin compaction at one side of the nucleus. We know from the work of STANLEY *et al.* (1972) and TOKUYASU (1974b, plates 3b and 4b) that at one stage chromatin is electron dense mainly along the convex surface of the nuclear envelope; this chromatin may be represented by the fluorescing rod in the nuclei of Figures 1C and 1D.

Note also in Figure 1C that the chromatin is somewhat more compacted at the posterior (rounded) end of the nucleus than at the anterior (pointed) end. This distribution of chromatin has apparently not been noted before, but it can also be observed in plate 7a of TOKUYASU (1974b).

Figure 1D shows nuclei still in nuclear transformation, as evidenced by the small clumps of chromatin that are condensed, but not compacted. The nuclei are now more elongated, much of their width has been lost, and a greater degree of chromatin compaction is evident.

The process of individualization is also readily identified using BAO. Figure 1E shows a bundle early in individualization, with the cystic bulge in evidence just behind the heads. A bundle later in individualization is shown in Figure 1F. Here the remnants of the cystic bulge are seen at the base of the photograph. Spermatid bundles containing unelongated heads occasionally undergo individualization, as seen in Figure 1G. The individualized portions of the tails are separated and sharp, but the nonindividualized portions remain together and appear as a cord of fluorescent material; the cystic bulge marks the junction between the individualized and nonindividualized parts of the bundle.

It occasionally requires careful study to determine whether a bundle has undergone individualization. Late in the elongation period particularly, spermatids can be spread even though their tails remain syncytial, though in this case considerable cytoplasmic material is found between the spermatids. On the other hand, in some individualized bundles, a small amount of cytoplasmic material may also remain between the individualized spermatids. We have circumvented this problem by considering as "individualized" only those bundles in which a cystic bulge as in Figures 1E, 1F, and 1G or a waste bag was clearly evident.

PEACOCK, TOKUYASU and HARDY (1972) and TOKUYASU, PEACOCK and HARDY (1972b) have reported that sperm with grossly abnormal heads sometimes fail in individualization. The results of such a failure are apparent in Figure 1H, in which the tails of the morphologically normal sperm are individualized, but the tails of the abnormal, club-shaped heads remain syncytial, forming several cords of nonindividualized tails amidst the normal ones.

Measurements of length of nuclei in mature sperm: Figure 2 depicts the distribution of lengths of spermatid nuclei found among 100 individualized sperm from each of the seven genotypes examined. Units on the abscissa are 0.71 μm; thus the interval labeled "10" denotes nuclear lengths of 7.1 $\pm$ 0.36 μm. Only individualized sperm from the testes are included in the histograms, not those in the seminal vesicle. We chose to measure testicular sperm because sperm with grossly abnormal heads may proceed abnormally through coiling or release and

thereby be excluded from the vesicle (PEACOCK, TOKUYASU and HARDY 1972; TOKUYASU, PEACOCK and HARDY 1972b, 1977).

Mean nuclear lengths in μm and standard deviations are summarized in the first column of Table 2. Our measurements of nuclear length in controls (++/++) are consistent with those of other authors using different techniques. Although there is apparently considerable variation in mean nuclear lengths among different strains, mean nuclear lengths measured with the light microscope using aceto-orcein almost all fall within the range 8–11 μm, more typically in the range 9–10 μm. (See BEATTY and BURGOYNE (1971) for a recent study and Sidhu (1963) for an extensive tabulation of his and earlier results.) Sidhu's (1963) measurements give a mean nuclear length of 9.09 μm, very closely to our value of 9.07 μm.

HARDY (1975), measuring lengths of sperm nuclei in various strains with the electron microscope, gives values ranging from 9.6 to 11.0 μm, and STANLEY *et al.* (1972) estimate nuclear length at 13 μm. These values are considerably

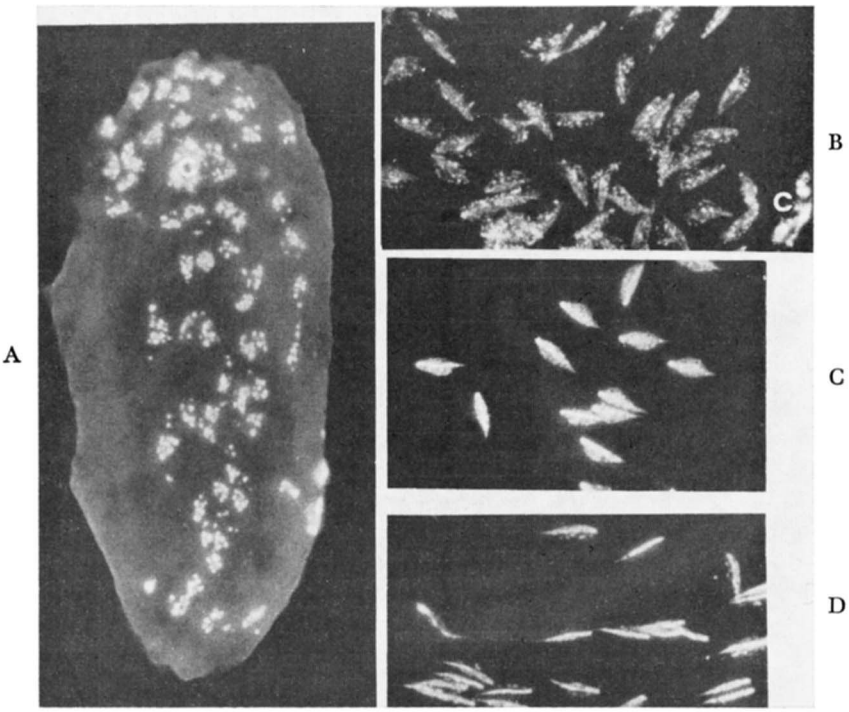
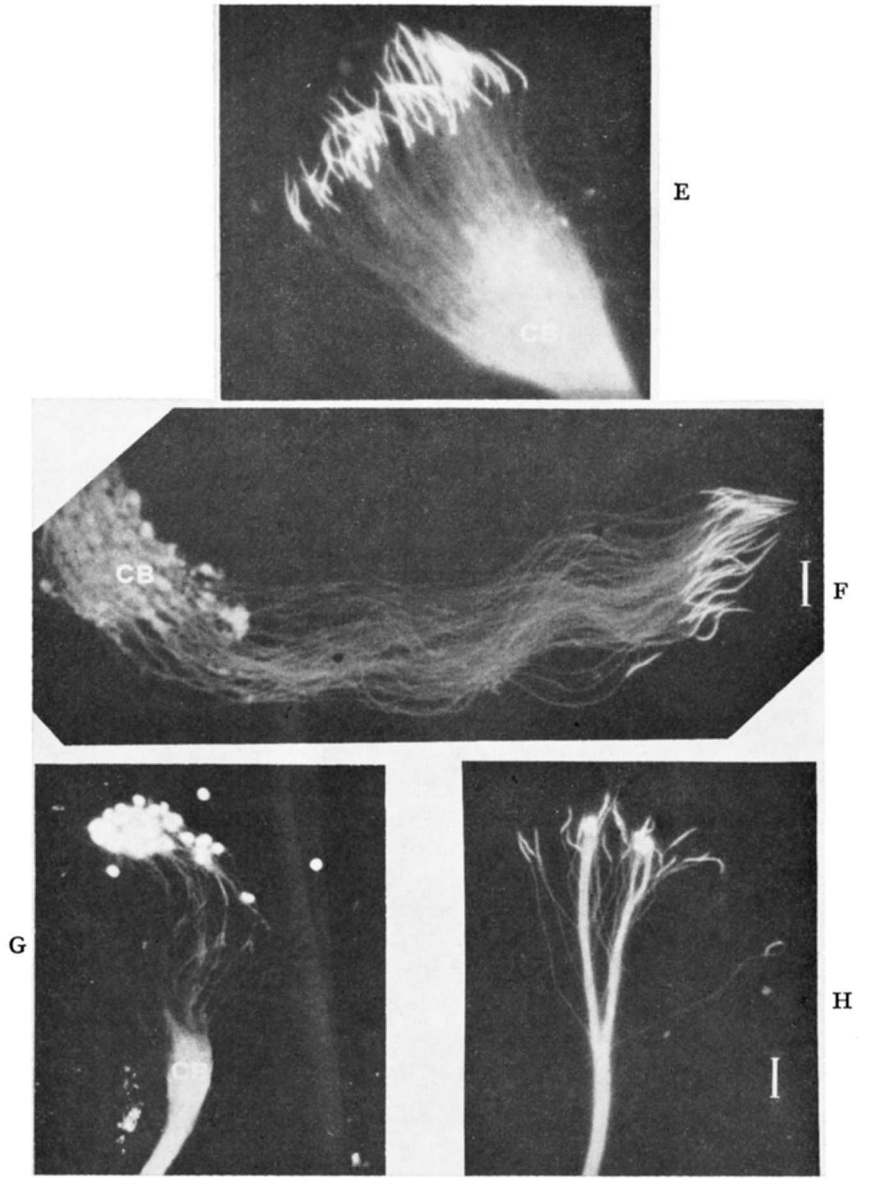


FIGURE 1.—A-F is interpreted as a series of successive stages in spermiogenesis. (A) A preelongation cyst from + *Rsp/Sd Rsp*. At this stage, controls and segregation distorter males cannot be distinguished. Note the distinctive coarse chromatin granules in the spermatid nuclei. (B) Nuclei from *Sd Rsp*/+ +. Some chromatin is still in granules, but the nuclei are lanceolate; note the different appearance of the cyst cell nucleus. (C) From Spartenburg; Some chromatin compacts to form a dense, longitudinally oriented rod, showing advanced compaction (bright fluorescence) at the posterior end of the nucleus. (D) From Spartenburg; nuclei have few granules and the major amount of chromatin is in the rodlike structure. (There is a tail region



of another bundle underneath the one shown.) (E) A bundle early in individualization showing the characteristics cystic bulge just behind the heads, from Blattenbach. (F) Individualization in *R-1/cn bw*, showing collapsed cystic bulge at the left of the photograph and partly individualized tails. (G) A partly individualized bundle from *Sd Rsp/Sd Rsp* showing that individualization of spermatids with unelongated nuclei can sometimes occur. Note the cystic bulge at the boundary between the individualized and nonindividualized regions of the tails. (The nucleus of the rightmost tail is missing.) (H) A bundle from *Cy R(cn)-10/SD(NH)-2* showing spermatids with unelongated nuclei that have failed to be individualized. (No cystic bulge is present.) Figures A to E and G are to the same enlargement as Figure 3A; the bars on A, F and H denote 10 μm. (C = cyst-cell nucleus; CB = cystic bulge.)

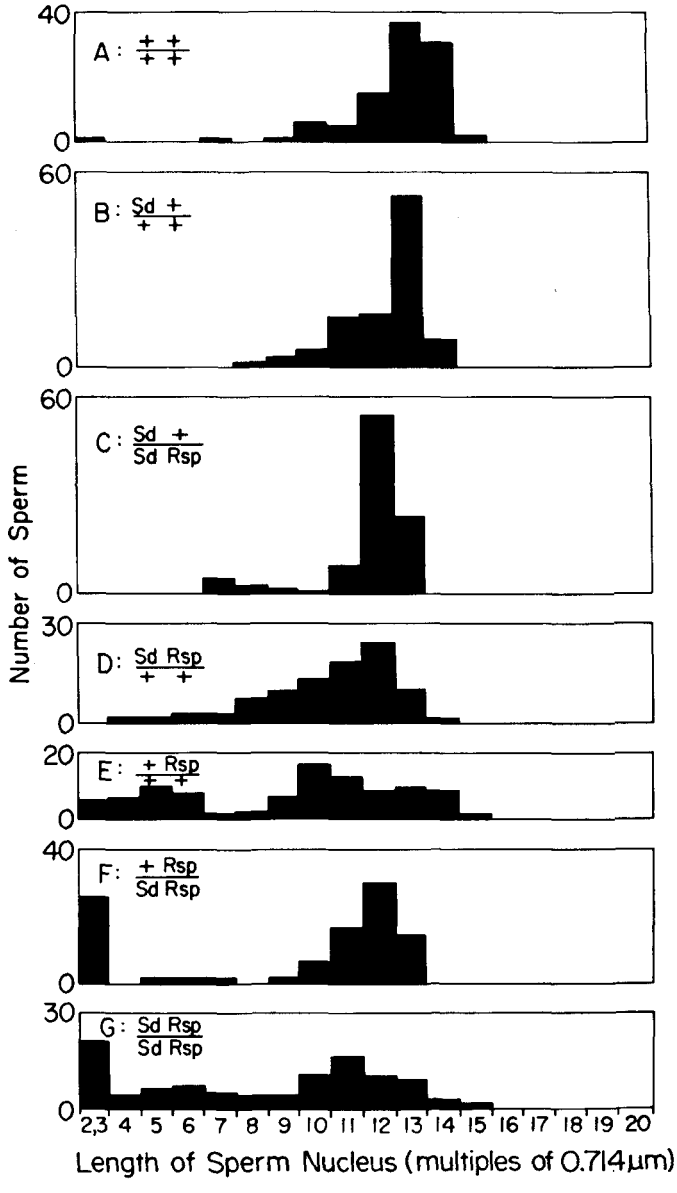


FIGURE 2.—Distribution of nuclear lengths of 100 individualized sperm from males of each of the indicated genotypes. Units on the abscissa are 0.71 μm. Due to incomplete spreading of sperm bundles, not all nuclei in any cyst could be measured. The number of cysts in which nuclei were measured and the number of males from which these were obtained is given in Table 2.

TABLE 2

Nuclear length of individualized spermatids ± standard deviation (μm)

Genotype	All nuclei	Nuclei > 6.43 μm	Number of cysts	Number of males
+ +/+ + *(A)*	9.07 ± 1.21	9.19 ± 0.865	11	6
Sd +/+ + *(B)*	8.78 ± 0.87	8.81 ± 0.832	7	3
Sd +/*Sd Rsp (C)*	8.35 ± 1.05	8.68 ± 0.615	8	5
Sd Rsp/+ + *(D)*	7.42 ± 1.56	8.00 ± 0.906	8	5
+ *Rsp*/+ + *(E)*	6.57 ± 2.50	8.17 ± 1.17	10	5
+ *Rsp*/*Sd Rsp (F)*	6.49 ± 2.99	8.37 ± 0.682	8	4
Sd Rsp/*Sd Rsp (G)*	5.78 ± 2.78	8.17 ± 1.01	6	5

Mean nuclear lengths in μm and standard deviations of 100 individualized, testicular sperm. The distributions themselves are shown in Figure 2; the letter near each genotype above identifies the corresponding histogram in Figure 2. The second column provides the same statistics for those parts of the distributions in Figure 2 that are within the normal range of nuclear lengths, *i.e.*, above the interval labeled 9. The two right-hand columns give the number of cysts and the number of males from which the 100 sperm were sampled.

larger than those obtained by us and others using light microscopy. However, TOKUYASU (1974b, Figure 8b) shows a nearly perfect longitudinal section of a mature sperm head having a nuclear length of 9.2 μm, which is rather close to the value we have obtained using BAO. (Indeed, mean nuclear length of the sperm in Table 2A, excluding the two sperm with abnormally short nuclei, is 9.19 μm.)

As seen in Table 2, the greatest mean nuclear length was found in controls (Figure 2A; the two sperm with abnormally short nuclei are both from Tokyo males). The distribution of sperm nuclear lengths in *Sd* +/++ males (Figure 2B) is not markedly different from that in controls, although the difference in mean nuclear length is of borderline significance ($t = 1.95$, $d.f. = 198$, $p \simeq 0.05$). Mean nuclear length of sperm from all other genotypes is highly significantly smaller than that in controls.

Figure 2C shows the distribution of nuclear lengths in sperm from *Sd* +/*Sd Rsp* males; Figure 2D shows the distribution of nuclear lengths in sperm from *Sd Rsp*/++ males. Although half the sperm produced by both types of males are dysfunctional, there is no clear-cut bimodality of the distributions. Therefore, although the mean nuclear length of sperm from these males is smaller than normal, it is not so much smaller that nuclear length can be used at a criterion for identifying dysfunctional sperm. This is consistent with the findings of HARDY (1975), who found that, despite variation in sperm nuclear length among different strains, nuclear length is not markedly affected even by gross aneuploidy of the nuclei, although nuclear volume is affected.

Our findings are complementary to those of TOKUYASU *et al.* (1977), who report the finding of a one-to-one bimodal distribution in the extent of chromatin compaction in cysts from *Sd Rsp*/++ males in the latter half of nuclear transformation. (See earlier discussion in MATERIALS AND METHODS.) The differences in chromatin compaction observed by TOKUYASU, PEACOCK and HARDY

(1977) are very subtle and could be detected in the light microscope only with great difficulty, if at all. What our above results imply is that the one-to-one distribution in extent of chromatin compaction reported by TOKUYASU, PEACOCK and HARDY (1977) is not accompanied by any obviously bimodal distribution of nuclear lengths.

Figure 2E shows the distribution of sperm nuclear lengths in + *Rsp*/++ males; it is clearly bimodal. Although there is no distortion of the segregation ratio in males of this genotype, a significant fraction of sperm have abnormally short nuclei, *i.e.*, they are unelongated or incompletely elongated. Thus the *Rsp* allele, by itself, seems to have major effects on nuclear morphology that are quite distinct from those effects associated with segregation distortion found by TOKUYASU, PEACOCK and HARDY (1977). Note in comparing Figures 2E, 2D, and 2C that the abnormal distribution of nuclear lengths induced by heterozygous *Rsp* (2E) is more normal when *Sd* is also heterozygous (2D) and still more normal when *Sd* is homozygous (2C).

The effects of homozygosity for *Rsp* on nuclear morphology are shown in Figures 2F and 2G. Sperm nuclei from + *Rsp/Sd Rsp* males produce the distribution shown in Figure 2F. Judged on the basis of male fertility, half the sperm from these males are dysfunctional, although the segregation ratio produced by the males is normal (HARTL 1969; HIHARA 1974). Figure 2G is the distribution of sperm nuclear lengths from *Sd Rsp/Sd Rsp* males, which are sterile due to the dysfunction of virtually all their sperm. Although the distribution of nuclear lengths is clearly abnormal, it is not much more abnormal than the distribution found in + *Rsp/Sd Rsp* males (Figure 2F); about 50% of the sperm have nuclear lengths within the normal range.

A pertinent question about the mean nuclear lengths in Table 2 is whether they differ by virtue of only those nuclei that are unusually short, due to failure of elongation or incomplete elongation; that is, whether the nuclei within the normal range of lengths have a normal mean length. The proper statistical approach to this question is to truncate the distributions in Figure 2 to the range of lengths found in controls. Accordingly, we have truncated each distribution in Figure 2 at the interval labeled number nine and recalculated the mean nuclear length of sperm in the upper part of the distribution. As can be seen in the second column of Table 2, the means are still smaller than in controls (all differences from controls are statistically significant), but the absolute differences are not nearly as large as obtained previously and there is no discernible pattern.

Altogether, then, the most prevalent nuclear abnormality we have found in the various genotypes is failure of nuclear elongation or incomplete elongation. (Again we emphasize that such differences in chromatin compaction as described by TOKUYASU, PEACOCK and HARDY (1977) are probably too small to be conveniently resolved with the light microscope.) Abnormal nuclear elongation is most strongly correlated with the genotype at the *Rsp* locus: nuclear lengths are most normal in sperm from Rsp^{+}/Rsp^{+} males (Figure 2B), intermediate in sperm from Rsp/Rsp^{+} heterozygotes (Figures 2C, D, and E), and most abnormal in sperm from *Rsp/Rsp* homozygotes (Figures 2F and G).

It should be emphasized that the measurments in Figure 2 were made on sperm whose tails had undergone the individualization process, excluding all possibly ambiguous bundles of the type described previously. The flagellum showed elongation in sperm from all genotypes. Figures 2F and G show that, at least sometimes, individualization can occur in the absence of elongation of the spermatid nucleus. Figure 1G is an example from *Sd Rsp/Sd Rsp*; Figure 3A is an example from + *Rsp/Sd Rsp*. Since nuclei in the "2, 3" class of Figure 2 are spherical and of the same diameter as nuclei about to undergo elongation, we interpret the abnormal nuclei as having failed in nuclear elongation rather than as having elongated and then degenerated. In Figure 2E it can be seen that spermatids with only slightly elongated nuclei can sometimes undergo individualization.

Number of abnormal spermatid nuclei per cyst: The previous section considered nuclear lengths of single, individualized sperm. In this section we report gross morphology of the nuclei in 3,034 cysts, including pre-individualization, during individualization, and post-individualization, from 114 males. Some of the types of cysts encountered are shown in Figure 3. (The tails of the sperm shown in each plate of Figure 3 are completely contiguous along virtually all their length, so that the spermatids in each plate all belong to the same cyst; the photos have been cropped to the region of the heads.)

Figure 3B shows a pre-individualization cyst from *Sd Rsp/++* with 35 unelongated and 29 at least partially elongated nuclei. This cyst, it is to be noted, is unusual for a number of reasons. First, as noted in MATERIALS AND METHODS, the nuclei in a cyst rarely spread sufficiently for accurate counting as this one did. Second, it is extremely rare to find a distribution of elongated to unelongated nuclei so close to one-to-one. Third, it is highly unusual to have the unelongated nuclei segregated in one part of the field as they are here; usually the elongated and unelongated nuclei are interspersed. Finally, we note the extremely long nucleus at five o'clock in Figure 3B. HARDY (1975) has shown that aneuploidy leads to changes in nuclear volume, but nuclear length is quite insensitive to aneuploidy. Thus, the long nucleus is probably not aneuploid. Then, too, the quantity of DNA in the unusually long nucleus cannot be judged subjectively by its "brightness" without microfluorometry.

Figure 3C shows a pre-individualization cyst from Spartenburg. It contains 64 nuclei. Not only is this cyst unusual in being well spread, it is unusual to find 64 nuclei in a cyst. KIEFER (1966) has found that only 18.5% of mature bundles contain the expected 64 sperm. In his study, the range of sperm number per bundle was 57–64, and the mean number of sperm per bundle was 61.

Figure 3D shows a cyst containing 60 unelongated nuclei from Spartenburg. Again, the nuclei are unusually well spread. We must emphasis that these nuclei are truly abnormal and are not merely at some early stage of development, as the tails of the spermatids are elongated.

Data on the gross nuclear morphology in 3,034 cysts are presented in Table 3. Because cysts rarely spread sufficiently well to permit accurate counting, we have classified them into three categories: those in which all of the nuclei are

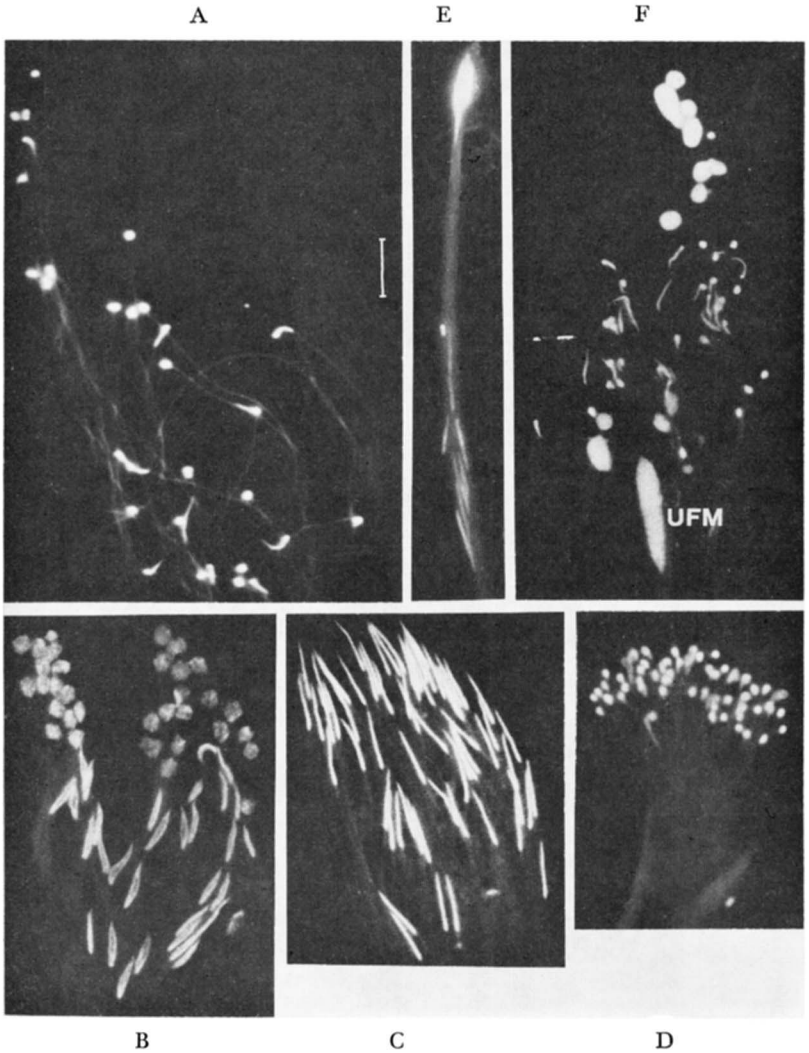


FIGURE 3.—(A) Individualized sperm with abnormal heads in + *Rsp/Sd Rsp*. (B) 35 unelongated and 29 at least partially elongated spermatid nuclei in a cyst from *Sd Rsp*/+ +. The ratio of normal to abnormal nuclei is rarely as close to one-to-one, and the abnormal nuclei are not usually segregated in one part of the field. This bundle is not individualized, and the sperm tails are contiguous along their length. (C) 64 normal nuclei in a pre-individualization cyst from Spartenburg. (D) 60 unelongated nuclei in a pre-individualization cyst from Spartenburg. (E) Abnormally positioned but elongated nuclei in a cyst from *Sd Rsp/Sd Rsp*. Note that one of the abnormally positioned nuclei is unelongated. (F) Blebs of unknown fluorescent material (UFM) in a cyst from *Sd Rsp/Sd Rsp*. (All Figures are to the same enlargement; the bar on A denotes 10 μm.)

TABLE 3

Genotype	Percent cysts with all nuclei elongated	Percent cysts with some nuclei unelongated or incompletely elongated	Percent cysts with all nuclei unelongated or incompletely elongated	Number of cysts	Number of males
+ +/+ +: Spartenburg	76.8	13.8	9.2	173	5
Blattenbach	82.9	12.1	5.0	397	17
Eierbrechtstrasse	88.7	7.8	3.4	319	17
Sd +/+ +	96.0	3.9	0.0	482	11
Sd +/*Sd Rsp*	86.2	11.7	2.0	341	15
Sd Rsp/+ +: *SD(NH)-2/cn bw*	83.3	7.1	9.5	42	5
SD-72/cn bw	62.9	28.6	8.4	178	6
R-1/cn bw	73.1	23.4	3.4	145	10
+ *Rsp*/+ +	73.8	21.0	5.0	237	10
+ *Rsp*/*Sd Rsp*	60.1	31.6	8.1	354	8
Sd Rsp/*Sd Rsp*	67.7	12.0	20.2	366	10

Frequencies of cysts containing all elongated nuclei (first column), a mixture of elongated and unelongated or incompletely elongated nuclei (second column), and all unelongated or incompletely elongated nuclei (third column). These data were obtained from examination of the number of cysts given in the fourth column derived from the number of males given in the last column. Further breakdown of the second column is not possible due to difficulties in accurately counting nuclei, but among 299 "mixed" cysts with nuclei sufficiently well spread to allow counting, at least 65% had fewer than 32 abnormal nuclei. The three wild-type strains are significantly heterogeneous, as are the three *Sd Rsp*/+ + genotypes.

elongated (as in Figure 3C), those in which all of the nuclei are unelongated or incompletely elongated (as in Figure 3D), and those in which some elongated and some unelongated or incompletely elongated nuclei are found. More detailed breakdown of the third category is not possible due to difficulties in counting, but we have actually been able to count nuclei in 299 "mixed" cysts. In this sample of 299 cysts, an average of 65% contained fewer than 32 spermatids with incompletely elongated nuclei. (The range was 45%–85% and depends somewhat on genotype.) The figure 65% probably underestimates the true fraction of cysts that have only a small number of abnormal nuclei, as it appears that cysts with a large number of abnormal nuclei are more likely to spread and thus be included in our sample of "countable" cysts.

A number of items in Table 3 are to be noted. First, the data are not easily compared with those in Figure 2 because many of the abnormal nuclei reported here would fail to be individualized and thus would not be included in the measurements in Figure 2. Secondly, there is significant heterogeneity among strains of the same genotype, both in controls ($\chi^2_4 = 14.4$, $p \simeq 0.01$) and in *Sd Rsp*/++ ($\chi^2_4 = 11.8$, $p \simeq 0.02$); thus one genotope cannot easily be compared with another. Third, the ratios of bundles with nuclei that are all normal to "mixed" to all abnormal are nowhere near those expected of a binomial expansion to the power 64; this is merely to say that the spermatid nuclei in a cyst do not appear to behave independently. Indeed, there appears to be no particular correlation between the fraction of "mixed" bundles in a genotype and the fraction of bundles containing exclusively unenlongated or incompletely elongated nuclei. This is

particularly apparent when comparing + *Rsp/Sd Rsp* with *Sd Rsp/Sd Rsp*. Among bundles containing one or more abnormal nuclei, + *Rsp/Sd Rsp* has a preponderance of "mixed" bundles, whereas *Sd Rsp/Sd Rsp* has a preponderance of bundles with exclusively unelongated nuclei.

The genotype with the least amount of nuclear nonelongation or incomplete elongation in Table 3 is *Sd* +/++. Indeed, this genotype serves as a convenient internal control on the experimental procedures. In particular, the bundles classified as having all of their nuclei unelongated or incompletely elongated cannot be due to misidentification of a normal developmental stage, as no such bundles were found in *Sd* +/++ males. Similarly, bundles classified as having a mixture of normal and abnormal nuclei cannot be due to fusion of parts of bundles of different developmental stages, as the frequency of such bundles found in *Sd* +/++ males in much smaller than that found in other genotypes.

Despite the within-genotype heterogeneity in Table 3, a number of general patterns do emerge, and these are consistent with the results of the previous section. *Sd* +/++ is the genotype with the smallest number of abnormalities. In fact, in this case, *Sd* +/++ has fewer abnormalities than the "best" wild-type strain. ($\chi^2_2 = 22.3$, $p << 0.01$ for *Sd* +/++ *vs.* Eierbrechtstrasse.) Then, too, although + *Rsp*/++ does not show significantly more abnormalities than Spartenburg ($\chi^2_2 = 5.2$, $p \simeq 0.08$), Spartenberg is a very poor strain in this respect; + *Rsp*/++ does show more abnormalities than does the median control ($\chi^2_2 = 8.8$, $p \simeq 0.01$ for + *Rsp*/++ *vs.* Blattenbach). Also, again in line with the conclusions of the previous section, the number of nuclear abnormalities in spermiogenesis tends to be greatest in *Rsp/Rsp* homozygotes, intermediate in *Rsp/Rsp*$^+$ heterozygotes, and least in *Rsp*$^+$/*Rsp*$^+$ homozygotes.

Location of spermatid heads in cysts: During the nuclear transformation processes, the spermatid nuclei are normally located in the basal region of the cyst. Spermatid heads in normal cysts lie parallel, though not always at exactly the same level (TOKUYASU, PEACOCK and HARDY 1977). However, in every one of the seven analyzed genotypes, we occasionally found nuclei in abnormal positions. The abnormally situated nuclei were usually found in the basal half of the tail region, and most but not all of these nuclei were elongated. Figure 3E shows a number of such nuclei in the tail region of a nonindividualized group of spermatids from an *Sd Rsp/Sd Rsp* male. Although nuclei can become displaced during the individualization process (PEACOCK, TOKUYASU and HARDY 1972, TOKUYASU, PEACOCK and HARDY 1972b), abnormally positioned nuclei can undergo nuclear transformation in abnormal positions.

Many of the abnormally positioned nuclei, such as those in Figure 3E, evidently end up in waste bags, at least in homozygous *Sd Rsp*. In this genotype, we found a number of waste bags containing spermatids with elongated nuclei. It should be mentioned here that misplaced spermatid heads is a type of aberration that is relatively common in *D. subobscura* (HAUSCHTECK-JUNGEN and MAURER 1976). but rarer in *D. melanogaster*.

Fluorescing material of unknown origin: From the beginning of elongation up to the beginning of individualization, peculiar fluorescing material can some-

times be found in the head and tail regions of the cysts. Figure 3F shows this material in a cyst from an *Sd Rsp/Sd Rsp* male. The fluorescing material is common in cysts from *Sd Rsp/Sd Rsp* males (90–100%of cysts), but rare in other genotypes (1–5% of cysts). Single portions of the substance appear spherical, or like stretched-out drops. The diameter of the material can be as large as the diameter of the sperm bundle. We did not see the material in one *Sd Rsp/Sd Rsp* male, but in this male nearly all the sperm bundles were undergoing complete degeneration. The origin and composition of this material is unknown, though BAO, like Feulgen, binds to aldehyde groups.

After individualization, some waste bags were found to contain the same unknown substance. Because of the strong fluorescence of this material we were unable to ascertain whether spermatid nuclei were also present in these waste bags.

Contents of seminal vesicles: In all but homozygous *Sd Rsp/Sd Rsp* males, only sperm with normal nuclei were present in the seminal vesicle; because the seminal vesicle was not disrupted, a small number of aberrant nuclei may have escaped our notice. In the seminal vesicle of *Sd Rsp/Sd Rsp* males the number of sperm was small, favoring the recognition of abnormal sperm, and some sperm nuclei were found to be unelongated. However, many of the sperm nuclei appeared to be morphologically normal, although they may possess subtle nuclear abnormalities of the type reported in heterozygotes by TOKUYASU, PEACOCK and HARDY (1977).

The above findings confirm what has been emphasized by TOKUYSAU, PEACOCK and HARDY (1972a, b), that the processes of individualization, coiling, and release are quite efficient in excluding from the seminal vesicle sperm with grossly abnormal heads. On the other hand, individualized sperm with minor nuclear abnormalities do apparently gain entry into the seminal vesicle (TOKUYASU, PEACOCK and HARDY 1977).

CONCLUDING REMARKS

BAO has proven to be a useful fluorescent stain for the study of spermatogenesis in *D. melanogaster*. Because it stains DNA, it permits detailed study of nuclear morphology and even reveals DNA distribution during various stages of nuclear transformation. Moreover, such processes as tail elongation and individualization are easy to recognize using BAO. Like light microscopy in general, the technique allows relatively rapid analysis of a large number of specimens. While we have been unable to detect such subtle abnormalities in the extent of chromatin compaction as reported in *Sd Rsp/++* males by TOKUYASU, PEACOCK and HARDY (1977), we have observed a number of spermiogenic aberrations that have so far been undetected with electron microscopy, individualization of sperm with unelongated nuclei, for example.

We have found abnormalities in spermiogenesis associated with one of the mutations involved in segregation distortion. Specifically, the *Rsp* allele leads to an enhanced frequency of nuclear nonelongation or incomplete nuclear elongation, and the effect is more pronounced in *Rsp* homozygotes than in heterozygotes.

The effect of the *Sd* allele, by contrast, is minimal. Our sample of *Sd* +/++ males has significantly fewer nuclear aberrations in spermiogenesis than do controls, although the mean sperm nuclear length is somewhat smaller than in controls. It is at this point unclear whether the effects of *Rsp* on spermiogenesis are related to the sperm dysfunction associated with distortion of the segregation ratio. The fact that such abnormalities are found in + *Rsp*/++ males, in which distortion of the segregation ratio does not occur, suggests that the spermiogenic aberrations may be secondary or pleiotropic effects of the locus.

The finding of a significant amount of spermiogenic abnormality associated with *Rsp* receives support from an improbable source—theoretical population genetics. CHARLESWORTH and HARTL (1978) have developed a model of segregation distortion that takes into account all know features of the system in respect of non-Mendelian segregation and male fertility. They could account for the observed frequencies of ++, + *Rsp*, and *Sd Rsp* in natural and artificial populations only by postulating a somewhat reduced fertility of + *Rsp*/++ and + *Rsp*/+ *Rsp* males. The fertility reduction required is small, of the order of a few percent, but it is certainly of the magnitude to be expected from the frequency of abnormalities actually observed in the present study. Therefore, while the abnormalities we have observed may not be directly related to the mechanism of segregation distortion, they may well be crucial in maintaining the segregation distorter polymorphism in natural populations.

It is of some interest that wild-type strains, even those freshly collected from the wild, are significantly heterogeneous in the frequency of nuclear aberrations during spermiogenesis. (Although spermatids with unenlongated or incompletely elongated nuclei are found in pre-individualization cysts, such abnormalities are not usually found in sperm in the seminal vesicle; the processes of individualization, coiling, and release are normally quite efficient in excluding grossly abnormal sperm from the seminal vesicle.) How the heterogeneity among wild-type strains is to be explained is unclear. The Spartenburg, North Carolina, strain, for example, has as many nuclear aberrations in spermiogenesis as does + *Rsp*/++. Perhaps some of the aberrations are due to the *Rsp* allele segregating in natural populations. HARTL and HARTUNG (1975) have found that the frequency of the *Rsp* allele in a natural population from Raleigh, North Carolina, is 45%. Among males in this population, therefore, about 30% are Rsp^{+}/Rsp^{+}, 50% are Rsp^{+}/Rsp, and 20% are *Rsp*/*Rsp*. A rather high incidence of *Rsp*-induced spermiogenic abnormalities in this population would therefore be expected. Since natural populations do vary in the frequency of *Rsp* (HARTL 1977), one would expect heterogeneity in amount of spermiogenic abnormalities from this source alone.

Our results are to be compared with those of HAUSCHTECK-JUNGEN and MAURER (1976), who studied spermiogenesis in sex-ratio males of *D. subobscura*. HAUSCHTECK-JUNGEN and MAURER (1976), using BAO and fluorescent microscopy to study nuclear morphology of spermatids, found a wide variety of aberrations in spermiogenesis that were later confirmed with electron miscroscopy (DUBLER-HÄNGGI 1977). Among these abnormalities were spermatid nuclei mis-

positioned in the cyst, unelongated or incompletely elongated nuclei, elongated but abnormally shaped nuclei, and degenerating nuclei. In sex ratio, however, the most striking aberration was the frequent abnormal position of spermatid nuclei in the cyst, often associated with incomplete elongation of these misplaced nuclei. In the case of segregation distortion, abnormally positioned nuclei are relatively rare.

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Corresponding Editor: A. CHOVNICK