DEFECTIVE HISTONE TRANSITION DURING SPERMIOGENESIS IN HETEROZYGOUS *SEGREGATION DISTORTER* MALES OF *DROSOPHILA MELANOGASTER**

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ABSTRACT

Males of *Drosophila melanogaster* that are heterozygous for the segregation distorter (*SD*) chromosome produce a gross excess of *SD*-bearing offspring because most of the non-*SD*-bearing sperm are dysfunctional. These dysfunctional sperm exhibit abnormalities in chromatin condensation and compaction during spermiogenesis. Use of the fluorescent dye sulfoflavine, which is specific for basic proteins, has now revealed that the dysfunctional sperm are also defective in the normal transition from somatic to spermatid-specific histones.

WE have studied a mutation in *Drosophila melanogaster* in which about half the sperm become dysfunctional owing to defective chromatin condensation or compaction during spermiogenesis. Use of the fluorescent dye sulfoflavine, which is specific for basic proteins (LEEMAN and RUCH 1972), has revealed that the normal transition between somatic and spermatid-specific nuclear histones (DAS, KAUFMANN and GAY 1964) occurs in the post-elongation stage of spermiogenesis. In the mutant, the dysfunctional spermatids that are defective in chromatin condensation or compaction are also defective in the histone transition.

The "mutation" in question is actually a type of second chromosome designated *SD* (stands for segregation distorter), which is found in most natural populations throughout the world (SANDLER, HIRAIZUMI and SANDLER 1959; HARTL 1975; HARTL and HIRAIZUMI 1976; CROW 1979). Males that are heterozygous for *SD* and a normal chromosome (*SD*/+) produce 95% or more *SD*-bearing offspring rather than the expected 50% because most of the non-*SD*-bearing sperm fail to function (HARTL, HIRAIZUMI and CROW 1967; NICOLETTI, TRIPPA and DEMARCO 1967). TOKUYASU, PEACOCK and HARDY (1972, 1977) have noted ultrastructural abnormalities in chromatin condensation and compaction in about half the developing spermatids in *SD*/+ males.

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Males that are homozygous for *SD* are often sterile or nearly sterile owing to the dysfunction of virtually all their sperm (HARTL 1973). Ultrastructural abnormalities in chromatin condensation and compaction also occur in such males (PEACOCK, TOKUYASU and HARDY 1972; KETTANEH and HARTL 1980). Cytochemical studies using the dye fast green, which is specific for basic proteins (ALFERT and GESHWIND 1953) have indicated that spermatids in *SD/SD* males fail to undergo the normal transition from somatic to spermatid-specific histones (KETTANEH and HARTL 1976). On the other hand, it is not certain whether the molecular mechanism of sperm dysfunction in homozygous males is the same as it is in heterozygous males. LINDSLEY (1981) estimates that about 2000 loci are involved in male fertility. Thus, recessive alleles at many loci can lead to male sterility when homozygous, and some unknown fraction of these might involve a defective histone transition totally unrelated to the effects of *SD*. A case in point is a male-sterile *X*-autosome translocation, which SHOUP (1967) found to be defective in the histone transition during spermiogenesis. Homozygosity for *SD* might therefore bring about a defective histone transition and male sterility for reasons unrelated to the mechanism of sperm dysfunction and segregation distortion in heterozygotes. On the other hand, were sperm dysfunction related to a defective histone transition in heterozygotes, then one would expect heterozygotes to exhibit an approximately one-to-one distribution of spermatids having a normal or a defective histone transition. This consideration encouraged us to undertake the present study of the histone transition in heterozygous *SD/+* males.

MATERIALS AND METHODS

Experimental animals were of genotype *SD(NH)-2/cn bw*, denoted here as *SD/+*. *SD(NH)-2* is an *SD* chromosome extracted from a Japanese population (HIRAIZUMI and NAKAZIMA 1965), and *cn bw* is a chromosome from a laboratory strain used routinely in studies of segregation distortion. The proportion of *SD*-bearing progeny from *SD(NH)-2/cn bw* males is nearly 100%. Control males were from the wild-type Eierbrechtstrasse strain. We studied 157 cysts from 31 *SD/+* males and 134 cysts from 34 control males.

Males one to three days old were dissected in sterile Ringer solution and the testes placed on a slide of nonfluorescing glass that had previously been washed in a 1:1 ether-ethanol solution. The sheath of the testis was removed to allow the contents to spread, and the tissue was air dried for 10–30 min followed immediately by transfer to 70% ethanol. Fixation was in 4% formaldehyde in SORENSEN buffer (pH 7) at room temperature for at least four hours or at 6° for 20 hours. Then the slides were washed in running tap water for 10 min (HAUSCHTECK-JUNGEN and MAURER 1976).

After removal of most of the DNA by hydrolysis for 3 hr in 5% TCA at 60°, slides were washed three times in 70% ethanol, once in CLARK and LUBS buffer (pH 8.2), stained in 0.1% solution of Brilliant Sulfoflavine (ChromaGesellschaft Schmid und Co., Stuttgart-Untertürkheim, Germany) in the same buffer (LEEMAN and RUCH 1972), dehydrated in ethanol and xylene, and mounted in Fluormount (Gurr). The structural formula of sulfoflavine is shown in Figure 1. According to LEEMAN and RUCH (1972), basic amino acids are responsible for sulfoflavine staining. Consequently, any kind of basic protein, wherever it may be located in the cell, will elicit fluorescence of sulfoflavine. In most types of cells, the nucleus contains the highest concentration of basic proteins, the histone proteins. One exception to this rule will be reported in the present paper: in primary spermatocytes, the cytoplasm fluoresces more strongly than the

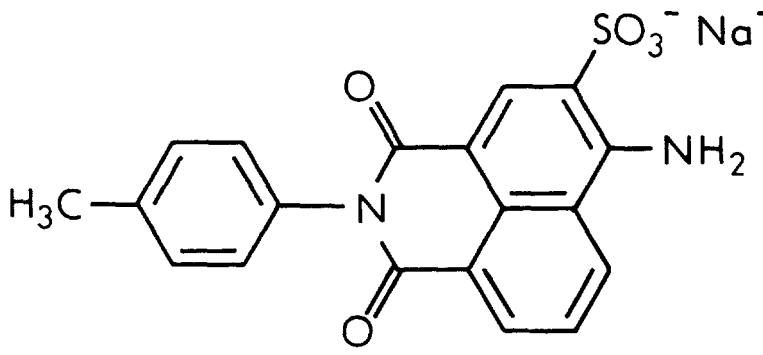

FIGURE 1.—Chemical structure of sulfoflavine. (From LEEMANN and RUCH 1972).

nucleus. A similar situation has been reported in butterfly spermiogenesis by FRIEDLÄNDER and HAUSCHTECK-JUNGEN (1982), but in neither case is the fluorescing cytoplasmic substance thought to correspond to histone. That histone does bind to sulfoflavine is supported by the observations of PORTMANN and HAUSCHTECK-JUNGEN (in preparation), which indicate that isolated somatic histones from rat are stained with sulfoflavine. Staining of lysine residues can be blocked by deamination (BLOCH 1966), which was carried out after hydrolysis and the alcohol washes by treatment with a solution of 5% sodium nitrite and 5% acetic acid three times for 15 min each. After deamination, slides were washed in running tap water for ten minutes.

As a check on the concordance of sulfoflavine and fast green staining, a control series of slides using 0.1% fast green was prepared as above, except for the replacement of CLARK and LUBS buffer with distilled water. Fast green and sulfoflavine have previously been compared in a series of experiments by LEEMANN and RUCH (1972). As a rule, fluorescent dyes are several orders of magnitude more sensitive than comparable nonfluorescent dyes, which merely change the amplitude of the light passing through the biological material. Spermatid nuclei can be stained with fast green both immediately after meiosis and as mature sperm. However, the process of decreasing fluorescence of spermatid nuclei due to loss of somatic histones and the later increasing fluorescence due to the gain of sperm-specific histones is impossible to document with fast green because of its relatively low sensitivity.

RESULTS

LINDSLEY and TOKUYASU (1980) have recently published a comprehensive review of spermatogenesis in *Drosophila melanogaster*, and we will use their descriptive terminology in presenting our results. The testes of *D. melanogaster* are a pair of coiled tubules approximately 0.1 mm in diameter and 2.0 mm in length. The testes are closed at the apical end but open basally into the testicular duct. Primary spermatogonial cells are formed near the apex of the testis by division of a small number of stem cells (HARDY *et al.* 1979). As each primary spermatogonium is formed, it becomes surrounded by two cyst cells creating an envelope within which subsequent events occur. While the cyst is still in the apical region of the testis, the primary spermatogonium undergoes four successive and synchronous mitotic divisions with incomplete cytokinesis, resulting in 16 syncytial primary spermatocytes. These primary spermatocytes undergo a growth period lasting approximately 90 hr, during which they increase in volume approximately 25 fold; concomitantly with spermatocyte growth the cyst is dis-

placed from its initially subapical position to a position near the midpoint of the testis. In sulfoflavine preparations, spermatocytes exhibit bright fluorescence of the cytoplasm and dull fluorescence of the nucleus (Figure 2a), but it is not clear whether the nuclear fluorescence is due to nucleoplasm or to overlying cytoplasm. In any case, the fluorescent material in the cytoplasm is unlikely to be histone. Certainly the arginine-rich sperm histones are not involved, as the fluorescence is eliminated by deamination. Some other lysine-rich basic cytoplasmic protein is evidently responsible for the cytoplasmic fluorescence, and it is worth noting here that this material can also be stained with fast green.

After spermatocyte growth, the two meiotic divisions take place in rapid succession. Cells undergoing meiosis have a reduced, though variable, amount of cytoplasmic fluorescence when stained with sulfoflavine, but the condensed chromosomes fluoresce brilliantly (Figure 2b). Following meiosis the cyst undergoes a brief pre-elongation period during which the mitochondria coalesce into the nebenkern; cytoplasmic fluorescence continues, except in the region occupied by the nebenkern (not illustrated).

Spermatid elongation follows the assembly of the nebenkern. During elongation, which lasts approximately 35 hours, the cyst of spermatids grows to a length of approximately 1.8 mm and is oriented with the nuclei of the spermatids near the base of the testis and their caudal ends near the apex. The nucleus undergoes a transformation in shape, from roughly spherical to a tapered cylinder flattened on one side to a canoe shape as the flattened area becomes concave. At the end of elongation the nucleus is approximately 9 μm long and 1.2 μm wide. In sulfoflavine preparations, the nuclei fluoresce very weakly during elongation. How-

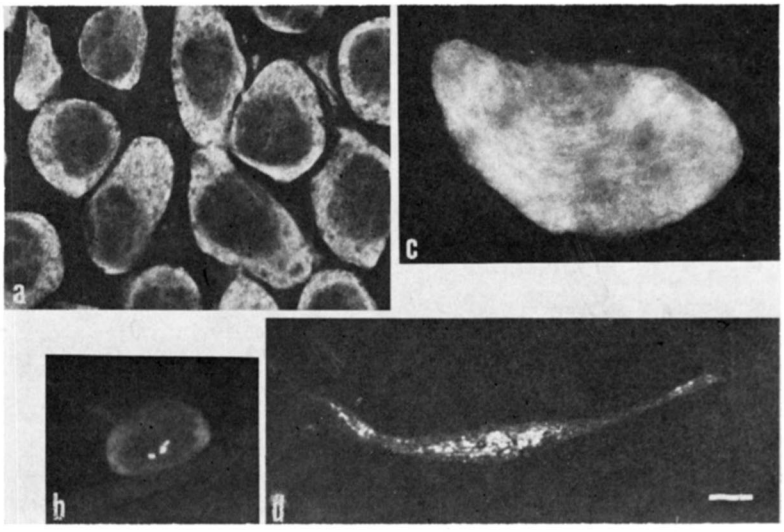

FIGURE 2.—Sulfoflavine fluorescence in (a) primary spermatocytes, (b) a cell in meiosis, (c) a cyst early in elongation, (d) a single spermatid later in elongation. The magnification bar in (d) represents 10 μm, and all photographs are to the same magnification. Parts (a)–(c) from Eierbrechtstrasse, part (d) from *SD*/+, which is undistinguishable from controls at this stage.

ever, the cytoplasm of cysts early in elongation does fluoresce (Figure 2c), and later in elongation this fluorescence can be observed as scattered spots along each spermatid (Figure 2d). The fluorescent cytoplasmic material is evidently not an arginine-rich protein, as the fluorescence does not appear in deaminated preparations.

Figure 3a shows the nuclear region of cyst late in elongation; the nuclei fluoresce slightly more than in the tail region. After a brief transition period, during which microtubules are redistributed around the nucleus and the proper head-tail alignment of the spermatid is established, the spermatids enter the post-elongation period. Chromatin condensation and compaction occurs at this time (TOKUYASU 1974; HARDY 1975). The chromatin first begins to condense near the convex side of the nuclear envelope, forming a layer of peripheral chromatin. As the layer of peripheral chromatin becomes thicker, the area of chromatin-associated nuclear envelope becomes smaller. Within the nucleus a network of condensing chromatin is formed, and this network is connected to the peripheral chromatin. Nuclear transformation continues by the compaction of this network and the elimination of excess nuclear envelope, finally forming an essentially mature nucleus having the shape of a tapered cylinder approximately 10 μm long and 0.3 μm wide. Overall, there is a slight posterior-to-anterior gradient in

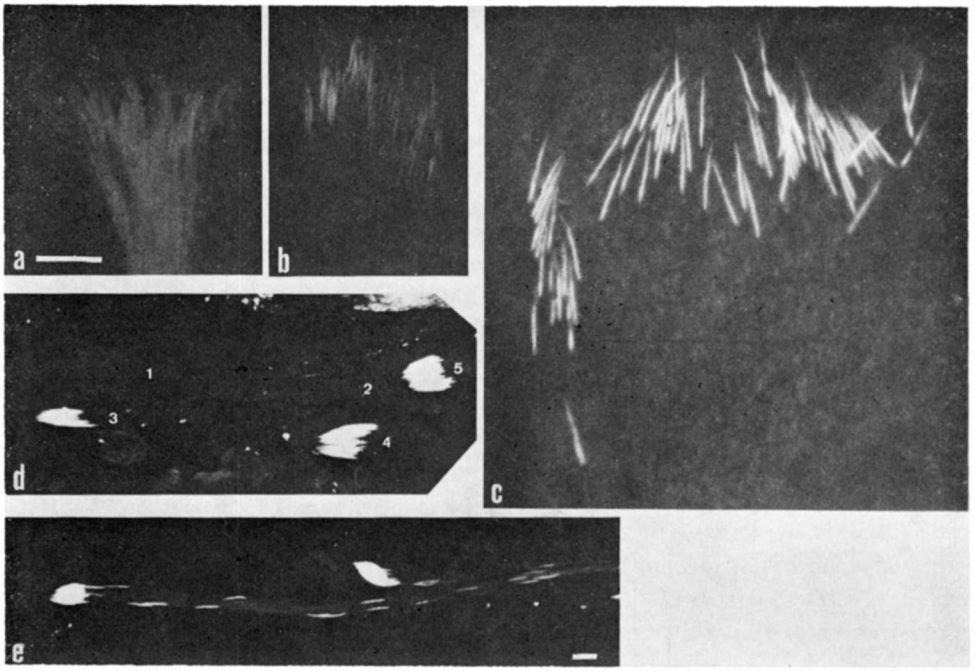

FIGURE 3.—Sulfoflavine fluorescence in cysts from Eierbrechtstrasse (a) nearing end of elongation, (b) early post-elongation, (c) later post-elongation, (d) showing various stages in histone transition, (e) showing histone transition in spermatid nuclei misplaced in the cyst. Magnification bars in (a) and (e) represent 10 μm. Parts (b) and (c) magnified as in (a); part (d) magnified as in (e).

chromatin condensation and compaction (TOKUYASU 1974; HAUSCHTECK-JUNGEN and HARTL 1978).

Dramatic results are observed in post-elongation in sulfoflavine-stained preparations. Figure 3b shows the nuclear region of a cyst during post-elongation. Nuclear fluorescence initially appears at the posterior end of the nucleus, then in patches throughout the nucleus, and finally as illustrated in Figure 3b. The fluorescence of the nuclei gradually becomes more intense during post-elongation and eventually appears as in Figure 3c. Note in Figure 3b and 3c that there is no obvious heterogeneity in degree of fluorescence among the spermatid nuclei of a cyst. This fluorescence is evidently due to arginine-rich proteins, as the same pattern of fluorescence is obtained after deamination. These arginine-rich proteins are thought to be the sperm-specific histones that replace the somatic histones during spermiogenesis.

Figure 3d shows the nuclear regions of five cysts in post-elongation. The nuclei of cyst number 1 are almost nonfluorescent, which we interpret as indicating that the histone transition has not begun. The nuclei in cyst number 2 exhibit dull fluorescence, which we interpret as the beginning of the histone transition. The brilliant fluorescence of the nuclei in cysts 3–5 characterizes completion of the histone transition. In earlier studies of the Eierbrechtstrasse strain (HAUSCHTECK-JUNGEN and HARTL 1978), we have observed a few aberrant spermatid bundles in which some of the spermatids had not elongated completely, so their nuclei are misplaced in the cyst. Nuclear transformation does occur in such misplaced nuclei, and Figure 3e shows that the misplaced nuclei also undergo the histone transition. Also, on occasion, a nucleus will fail to elongate properly and will appear ball-shaped or club-shaped. Arginine-rich nuclear proteins are also found in these abnormally shaped nuclei (not illustrated). In the Eierbrechtsstrasse strain, 33 out of 134 cysts examined (25%) had at least one such abnormal nucleus.

After post-elongation, each spermatid becomes invested in its own membrane during the individualization process in which a spindle-shaped enlargement called the cystic bulge traverses the entire length of the spermatid bundle from the region of the heads to the tips of the tails. As the cystic bulge progresses, it accumulates excess nuclear envelope and cytoplasmic organelles, including the fluorescent cytoplasmic material (Figure 4a). When the cystic bulge finally reaches the apical end of the cyst some 20 hours after the onset of individualization, it is called the waste bag, and the fluorescent cytoplasmic material it contains is still in evidence (Figure 4b). This fluorescent cytoplasmic material is not found in deaminated preparations, which implies that it is not an arginine-rich protein. Abnormal spermatids often fail to be individualized and remain syncytial in strings that lie parallel in the cyst to the succcessfully individualized spermatids.

Individualization is followed by the coiling process during which the sperm bundle is coiled into the base of the testis. As coiling occurs, the enveloping cyst, including the waste bag, is gradually drawn through the lumen of the testis and ultimately collapses into a disc-like configuration in the base of the testis. Ab-

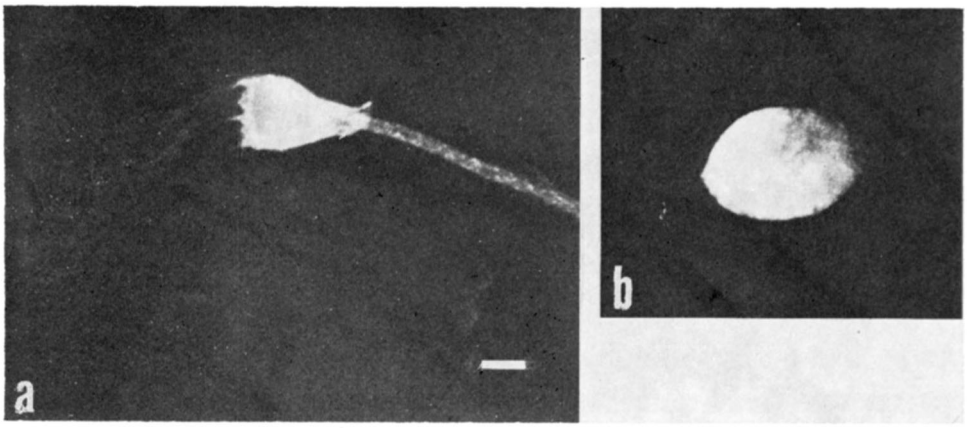

FIGURE 4.—Sulfoflavine fluorescence of (a) the cystic bulge, which is moving from left to right, and (b) the waste bag. Both photographs are from the Eierbrechtstrasse strain, and the magnification bars represent 10 μm.

normal sperm that have failed to be individualized are unable to coil and are pushed caudally into the waste bag during the coiling process; eventually these abnormal sperm are completely segregated from the normal ones. Subsequently, the normal sperm are liberated from the cyst into the testicular lumen, and the waste bag and defunct cyst cells are phagocytosed by terminal epithelial cells and degraded.

In heterozygous *SD*/+ males [*SD(NH)-2/cn bw*], spermatogenesis appears normal in sulfoflavine preparations until post-elongation. In some cysts one or more nuclei fail to elongate, but the proportion of such cysts is the same as occurs in controls (34 out of 157 cysts, or 22%). The first discernible difference between *SD*/+ and control males appears in post-elongation at the onset of the histone transition, when *SD*/+ spermatid bundles exhibit a heterogeneity in the amount of fluorescence among spermatid nuclei. Approximately half of the elongated nuclei exhibit some fluorescence and half do not (compare Figure 5a with Figure 3b). In most cases the nuclei of spermatid bundles are not spread out on the slide but remain clumped together; the combined nuclear fluorescence of the nuclear region of such unspread bundles is less in *SD*/+ than in controls. However, when individualized cysts are sufficiently spread, it is apparent that two types of nuclei are present (Figure 6). One type fluoresces brilliantly and is not distinguishable from nuclei in Eierbrechtstrasse controls; the other type fluoresces very weakly (Figure 6, arrows). In a few cases, all 64 nuclei in a cyst fail to elongate normally, and chromatin condensation in the postelongation period is missing. Nevertheless, in *SD(NH)-2/cn bw*, about half of such abnormal nuclei become fluorescent and half do not.

In some cases the nuclei of a spermatid bundle are sufficiently spread to be counted, and Figure 7 gives the distribution of fluorescent nuclei per cyst in *SD*/+ and control males. The difference is striking: a substantial fraction of nuclei in *SD*/+ males fails to undergo the histone transition. The mean number

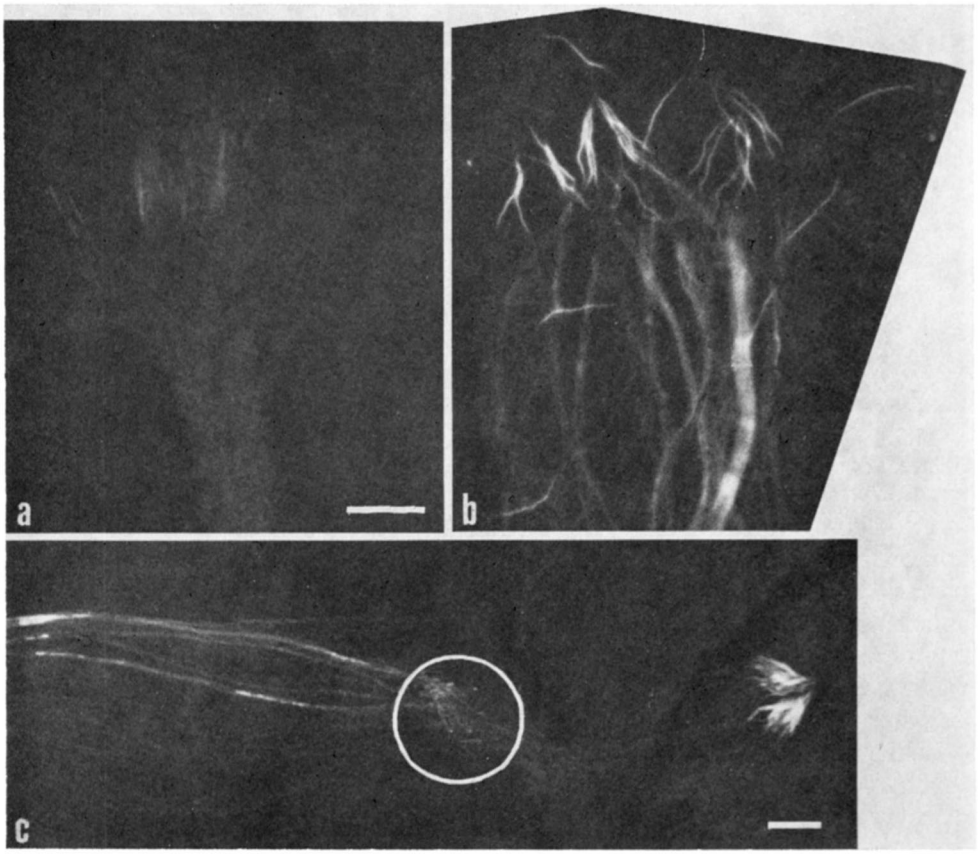

FIGURE 5.—Sulfoflavine fluorescence in cysts from *SD*/+ (a) early in post-elongation, showing the nonfluorescence of about half the spermatids, (b) after individualization, showing the cordlike strings of nonindividualized tails which terminate in nonfluorescent nuclei, (c) end of individualization, showing a group of weakly fluorescent nuclei (circle) that are being eliminated from the bundle. The magnification bars represent 10 μm. Part (b) is magnified the same as (a).

of fluorescent nuclei per cyst in *SD*/+ is 27.9 ± 5.2 as compared to 62.7 ± 2.2 in controls. Moreover, some of the fluorescent nuclei in *SD*/+ are aberrant in the distribution of fluorescence; they may have fluorescence only at the proximal end, only at the distal end, only in the middle, or in several distinct places along the nucleus, in contrast to the invariably homogeneous distribution of fluorescence in mature nuclei in controls. On the other hand, the number of fluorescent nuclei in *SD*/+ cysts may underestimate the number of nuclei that are functionally normal because nuclei below a certain subjective threshold of fluorescence may be scored as nonfluorescing even though they may have sufficient arginine-rich histones to complete maturation.

In *SD*/+ males, most of the spermatids with cytologically abnormal nuclei fail to be individualized but remain in syncytial groups that can easily be recog-

FIGURE 6.—Sulfoflavine fluorescence in portion of a cyst having individualized head region in *SD/+*. Some nuclei fluoresce brilliantly, others fluoresce weakly (arrows). The weak fluorescence corresponds approximately to that of the transitional period in Figure 3a.

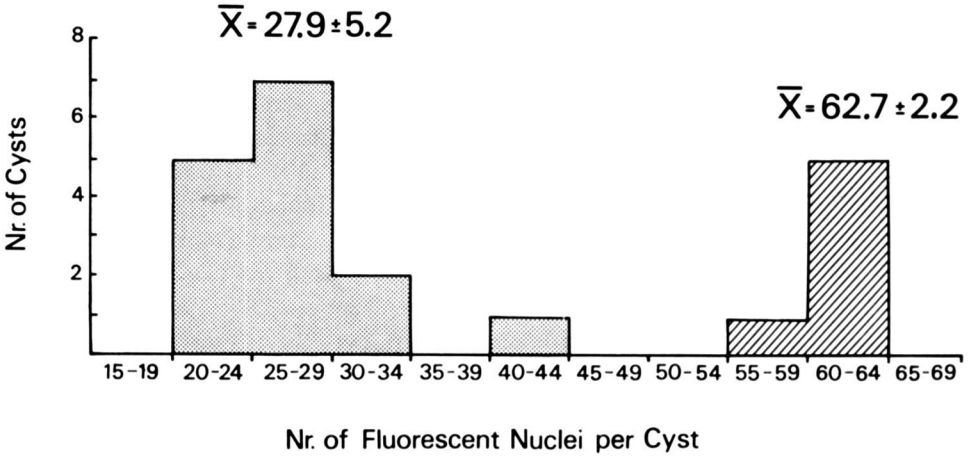

FIGURE 7.—Distribution of number of fluorescent nuclei in post-elongation cysts in *SD/+* (stippled) and Eierbrechstrasse (shaded).

nized because of the fluorescence of the cytoplasm (Figure 5b). During the coiling process these nonindividualized spermatids are pushed caudally along the cyst, and the weak fluorescence of the displaced nuclei is apparent in the encircled region in Figure 5c.

Thus, in *SD/+*, approximately half of the spermatid nuclei are defective in the histone transition as evidenced by their lack of fluorescence with sulfoflavine or their aberrant distribution of fluorescence. Moreover, these abnormal spermatids fail to be individualized and are relegated to the waste bag and thereby become unable to participate in fertilization. Inasmuch as the fertility of *SD/+* males is approximately one-half that of normal males (HARTL, HIRAIZUMI and CROW 1967), it seems reasonable to infer that the spermatids with a defective histone transition are the ones that carry the non-*SD* chromosome.

DISCUSSION

A number of items related to the interpretation of our results should be mentioned. First, we do not know how many arginine-rich nuclear proteins are involved in the histone transition. Fluorescence due to sulfoflavine could be due to one protein species or to several (KAYE and MCMASTER-KAYE 1975; MCMASTER-KAYE and KAYE 1976). Second, lack of fluorescence in late postelongation nuclei could indicate *in vivo* absence of the arginine-rich species, which we believe it does, or it could indicate an abnormal form of the protein that is destroyed or eliminated during tissue preparation.

A possible objection to our interpretation is to imagine that the nonfluorescent nuclei in *SD/+* males fail to fluoresce because the fluorescence may depend on the concentration of chromatin per unit nuclear volume, and TOKUYASU, PEACOCK and HARDY (1977) have shown that the cross-sectional nuclear diameter among dysfunctional spermatids is greater than that among functional spermatids. We reject this hypothesis for two reasons. First, in our control strain, a significant fraction of nuclei fail to elongate and have incomplete or absent chromatin condensation (HAUSCHTECK-JUNGEN and HARTL 1978). Nevertheless, in the present study, such nuclei do fluoresce with sulfoflavine in spite of their abnormally large nuclear volume and incompletely condensed chromatin. Secondly, among cysts in *SD/+* males in which all nuclei fail to elongate, approximately half exhibit fluorescence with sulfoflavine and half do not. Consequently, even gross changes in nuclear volume and chromatin condensation fail to inhibit fluorescence, and these unelongated nuclei are far more abnormal than the subtle abnormalities in dysfunctional spermatids reported by TOKUYASU, PEACOCK and HARDY (1977).

The function of spermatid-specific nuclear histones remains unproven, but they are widely believed to be involved in chromatin condensation and/or compaction during spermiogenesis (BLOCH 1969; KAYE and MCMASTER-KAYE 1975; SUBIRANA 1975). The appearance of spermatid-specific nuclear histones seems to coincide in time with nuclear transformation in postelongation spermatids, which certainly supports the hypothesis. The defective histone transition in the nuclei

of non-*SD*-bearing spermatids in *SD*/+ males also supports the hypothesis because, as TOKUYASU, PEACOCK and HARDY (1977) have found, such nuclei are defective in chromatin condensation and also often fail to realize their normal cross-sectional diameter. On the other hand, nuclear elongation does not depend on spermatid-specific histones, as elongated nuclei in *SD*/+ males often lack these histones whereas unelongated nuclei can possess them; TOKUYASU suggests that the final shape of the nucleus depends in large part but not exclusively upon the perinuclear microtubules (TOKUYASU 1974; LINDSLEY and TOKUYASU 1980).

The site of synthesis of the spermatid-specific histones is unknown, as neither we nor other investigators have been able to identify them in the cytoplasm (BLOCH 1966). Theoretically, they could be synthesized by the head cyst cell, as appears to be the case in Lepidopteran spermiogenesis (FRIEDLÄNDER and HAUSCHTECK-JUNGEN, in press), or they could be synthesized in the tail region of the syncytial spermatids, or near the nuclei themselves. Since nuclei that are mispositioned in the cyst successfully undergo the histone transition, it seems reasonable to postulate that the histones are synthesized near the perinuclear endoplasmic reticulum.

The ultimate molecular basis of segregation distortion remains unknown. The temperature-sensitive period of segregation distortion is in early meiosis (MANGE 1968), perhaps in the primary spermatocytes which suggests that some critical event occurs at about that time. The first demonstrable molecular and ultrastructural abnormalities occur much later, in post-elongation. However, spermiogenesis is a preprogrammed developmental process, a "unidirectional cascade of events" (TOKUYASU 1975), so genetically caused abnormalities in the process, whenever they are first expressed, will always be traceable to events in the primary spermatocyte or even earlier.

The genetic interactions leading to segregation distortion are also unclear. The oddest aspect of the phenomenon is that the genetically normal sperm are rendered dysfunctional. A number of hypotheses for the genetic interactions have been proposed (HARTL 1973; GANETZKY 1977; HIRAIZUMI, MARTIN and ECKSTRAND 1980), but all share the assumption that the non-*SD* chromosome affected by *SD* becomes genetically active when normally it should be suppressed. Segregation distortion is due primarily to the interactions of two loci (SANDLER and HIRAIZUMI 1960; HARTL 1974), but at least three other major loci are also involved (GENETZKY 1977; HIRAIZUMI, MARTIN and ECKSTRAND 1980; HARTL 1980). How these loci function in normal cells is unknown, but it is a provocative working hypothesis that they somehow regulate the histone transition during spermiogenesis.

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