SPERMIOGENESIS IN THE CRAYFISH (*PROCAMBARUS CLARKII*).

I. Structural Characterization of the Mature Sperm

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Aflagellate sperm were first described in detail in 1878 by Grobben (6), but they have remained poorly understood despite efforts to homologize them with flagellate types. It would appear from the confusion in the literature that these unique forms are not to be forced into a mold. The weakness of such attempts rests mainly in the uncertain specificity of staining methods for identifying mitochondria, Golgi elements, centrioles, etc., and their derivatives. Indeed, even the nucleus was not positively identified until the Feulgen reaction was applied to crayfish sperm by McCroan in 1938 (9). The electron microscope, especially when carefully combined with closely correlated light microscopy and cytochemistry, provides a fresh and more certain approach to the kind of problem in cellular differentiation presented by the aflagellate sperm.

Utilizing this approach, we have investigated the morphology and genesis of several types of aflagellate sperm, a study which is still in progress. Some of our results, particularly concerning the sperm of the crayfish, have already been presented at meetings and have been published in brief (10–12). This is the first report depicting and characterizing the morphology of the mature

sperm of *Procambarus clarkii* and is one of several describing our observations in detail (13).

Sperm from *Procambarus clarkii* males, obtained during the months of April through June,[1] were examined fresh, in methacrylate sections of testis and vas deferens fixed in 1 per cent buffered OsO_4 (Palade), in Carnoy-fixed Feulgen-stained squashes and in paraffin sections of similar material fixed in Helly's fluid and stained in various ways. Thin sections of osmium-fixed material were examined with RCA EMU 2 and Philips EM 100B electron microscopes.

The most mature spermatozoon of *P. clarkii* found *in situ*[2] is a highly refractile, non-motile, flattened spheroid uniformly about 7 μ in diameter and 5 μ thick. It is extremely sensitive to the tonicity of the environment; in a hypotonic medium, *e.g.* spring or distilled water, the cell swells considerably and the arms, which were wrapped around the body of the sperm, unwind and tend to stretch out, giving the impression of being under internal pressure. A phase micrograph of two such living sperm is shown in Fig. 1. The cell seen in frontal view has four arms (three of which are in focus). This the characteristic number for this species, although five are occasionally found (*cf.* Fasten's (3) description of *Cambarus virilis* where six is the common number and five and seven are often encountered). The globate acrosome complex (1, 10) ("capsule" (*e.g.* 5) or "vesicle" (*e.g.* 3) of early observers) appears as a series of concentric rings, and is seen in profile in the lateral view. This structure appears essentially as diagrammed for the European crayfish *Astacus fluviatilis* by Herrmann in 1890 (7). In that work, the main portion of the sperm—namely, the flattened crown-like body continuous with the arms and in which the capsule sits, is identified as the nucleus on the basis of its derivation. This view was disputed (*e.g.* 3) until eventually proved correct by Feulgen staining (9). Even subsequently, the radial processes have been considered to be other than nuclear in origin (14). Our observations on *Homarus* and *Uca* (12) have shown that, as in *P. clarkii*, the Feulgen-positive material extends along the arms until the processes become so attenuated that any stain present would not be visible (see also 16). Fig. 2 is a Feulgen-stained preparation of whole sperm. The nuclei, seen in frontal and lateral view, obviously extend into the arms and the processes can be followed for considerable distance. Electron micrographic evidence supporting this is discussed below. The pale spot in the center of the stellate nucleus represents a thin region in which the acrosome sits. We have found no evidence in our electron micrographs of a hole through the nucleus at this point (*cf.* 9).

Figs. 3 and 4 are electron micrographs of *P. clarkii* sperm. Fig. 3 is a lateral section through a nearly mature spermatozoon, still surrounded by the sustentacular cells whose membranes fused earlier with the spermatid cell membrane and thus engulfed a large amount of discarded cytoplasmic material, including the mitochondria (11). The sperm is now surrounded by a periodic acid–Schiff positive (PAS) casing of finely granular material, about 0.15 μ thick, presumably laid down by the sustentacular cells. It is probably this shell that ruptures or is dissolved in water, releasing the arms.

The nucleus has already been identified in electron micrographs by means of adjacent Feulgen stained thick sections (10). Its characteristic attenuated and clumped appearance is not peculiar to osmium fixation, but occurs after other fixatives as well. The arms, wrapped around the body of the sperm, are cut transversely several times. These profiles, together with the appearance of the material they contain, suggest their continuity with the body of the nucleus.

In Fig. 4, a frontal section through the nucleus of a free mature sperm, the continuity between the body of the nucleus and the arms is obvious. The internum consists of two phases: a homogeneous structureless material of low density to electrons, in which are embedded irregular clumps of dense material 50 to 150 mμ in width. From these radiate branching and anastomosing fibrils 70 to 200 A in width. Frequently clusters of these fibrils can be seen passing into the arms and along

[1] Obtained from Carolina Biological Supply Co., Elon College, North Carolina; other species were collected fresh from nature in North Carolina. In previous reports, these crayfish were designated as *Cambarus clarkii* (10–13), according to the early classification of Hagen (*Illus. Cat. Mus. Comp. Zool.*, Harvard College **3**, 1–109, 1870). They are more correctly designated as *Procambarus clarkii* (Girard) according to the more recent scheme of Hobbs (*Am. Mid. Nat.*, **28**, 334–357, 1942).

[2] The ripe sperm are free and not contained in spermatophores, as they are, for example, in the fiddler crab, *Uca pugnax*.

their length. Large bundles of such clusters may account for the axial processes described by other workers with the light microscope (*e.g.* 14). The structural emptiness and lack of apparent order is in marked contrast to the high degree of order found in many sperm of the flagellate type (*e.g.* 4) and indicates that the latter is not a necessary concomitant of sperm differentiation.

The tegument of the sperm, which contains both the nucleus and acrosome, is actually a complex of at least two membranes. These originated in the spermatid as elaborations of the nuclear envelope which were formed in conjunction with, and at the expense of a vesicular component of the cytoplasm (11). This mass of convoluted membranes, continuous with the envelope in many places, occupied almost half the volume of the spermatid at one time and fused peripherally to separate the spermatid from the discarded cytoplasmic material, thus forming a new cell envelope. This process is described in detail elsewhere (13).

The membranes are probably largely utilized in covering the growing arms of the nucleus, but bits of the residual membrane are left as indicated in Figs. 3 and 4, where they can be seen to be continuous with the envelope. In most respects, then, this nucleus stands unique to anything so far described and apparently has no parallels among the flagellate sperm.

Apart from the nucleus and associated membranes which occupy much of the sperm volume, there are only two other components of the sperm: the centrioles and the acrosomal complex. Of these, the centrioles appear to play little or no role in sperm development. A pair of typical centrioles can be followed from the early spermatid, where they are found together among the

FIGURE 1

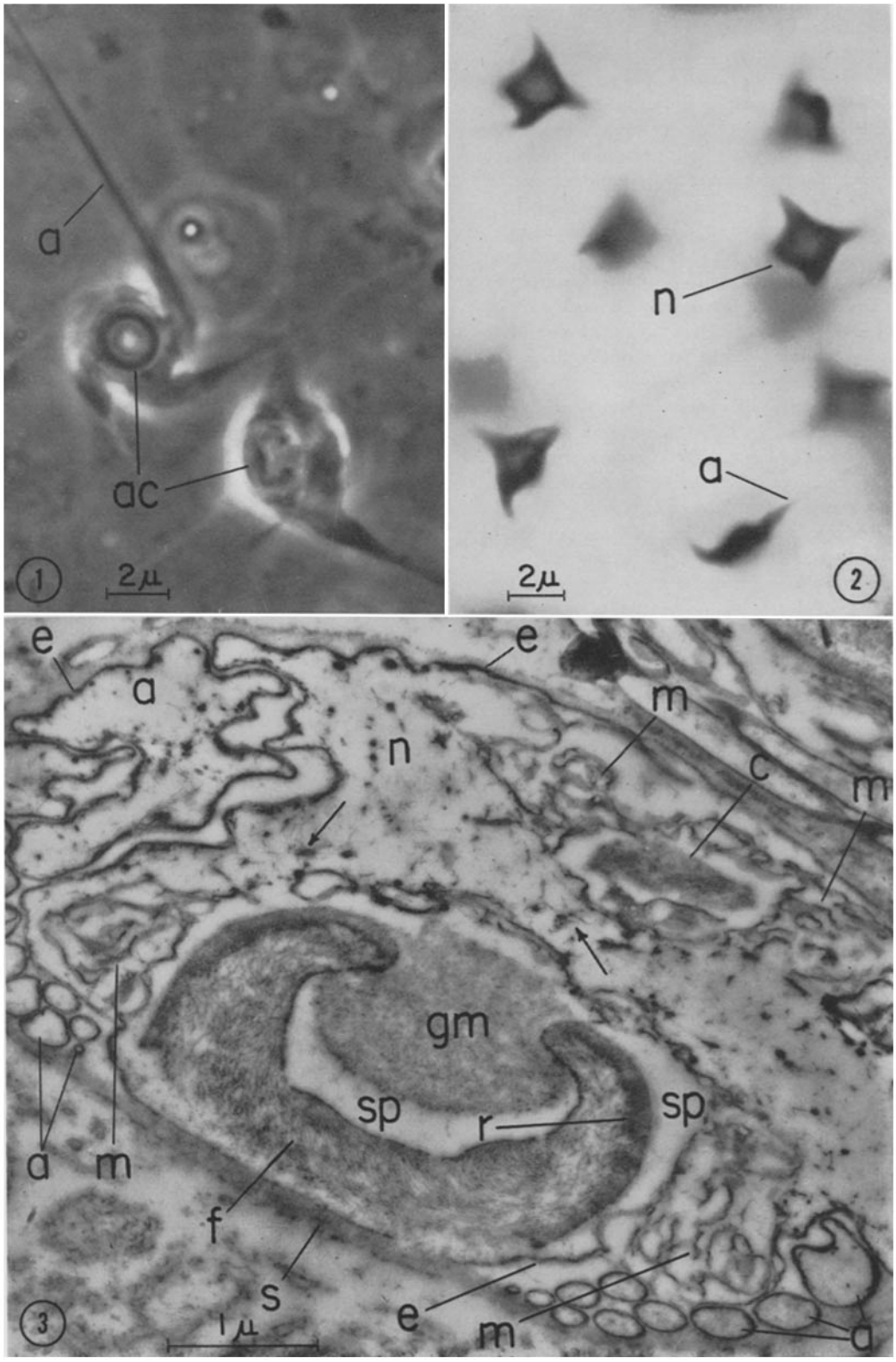
Phase micrograph of two crayfish (*Procambarus clarkii*) sperm in modified Ringer's fluid. The four arms of which one is shown at (*a*) which are normally tightly wound about the body of the sperm have opened out as a result of an osmotic effect which also causes the cells to swell. The acrosomal capsule (*ac*) appears in top view as a refractile annulus and in side view as a cup. Magnification approximately 4700.

FIGURE 2

Photomicrograph taken through a Wratten 74 filter of several whole mature sperm in a squash of Carnoy-fixed testis, stained by the Feulgen reaction. The nucleus (*n*) is seen to extend along the arms (*a*) until they become too attenuated for the stain to be seen. The pale area in the center of each nucleus is the depression in which the acrosomal capsule rests. Apparently the preparative method caused the arms to unfurl. Magnification approximately 4400.

FIGURE 3

Electron micrograph of a section through a mature sperm (chrome-osmium (Dalton) fixation, methacrylate embedding). The section is median and in a plane perpendicular to the plane of radial symmetry of the sperm. The profile of the nucleus (*n*) is butterfly-shaped, a reflection of its biconcave nature. Scattered clumps of dense material (arrows) and fine filaments are embedded in a homogeneous material of low density to electrons. The arms (*a*), wrapped around the sperm, are cut transversely several times. Their contents resemble that of the nucleus. Small masses of membrane material (*m*) are continuous with the dense membrane complex (*e*) that envelops the nucleus and the acrosomal complex. The granular residuum of a centriole is indicated (*c*). The acrosomal complex consists of three parts: a dense rim (*r*) that is PAS-positive and acidophilic; filaments (*f*) (some of which can be seen to be tubules at higher resolution) that are continuous with the rim and fill the region that is palely PAS-positive; and granular material (*gm*), that fills the cup-like cavity and plugs its opening. The spaces (*sp*) appear to be due to shrinkage. A finely granular PAS-positive shell invests the entire sperm (*s*). Magnification approximately 23,000.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membranes on the opposite side of the nucleus from the acrosome, to later stages where they are apt to be found on either side and never in association with any particular structure or in predictable juxtaposition with each other. In later stages, they appear to degenerate and become elongate masses of fine granules encapsulated by the membrane complex (Fig. 3). In no case are the centrioles associated with the radial processes and other structural elaborations with which they are identified by previous workers (*e.g.* 3, 9, 14). In fact, the older work was undoubtedly largely speculative since the centriole has not been positively identified in aflagellate sperm until now.

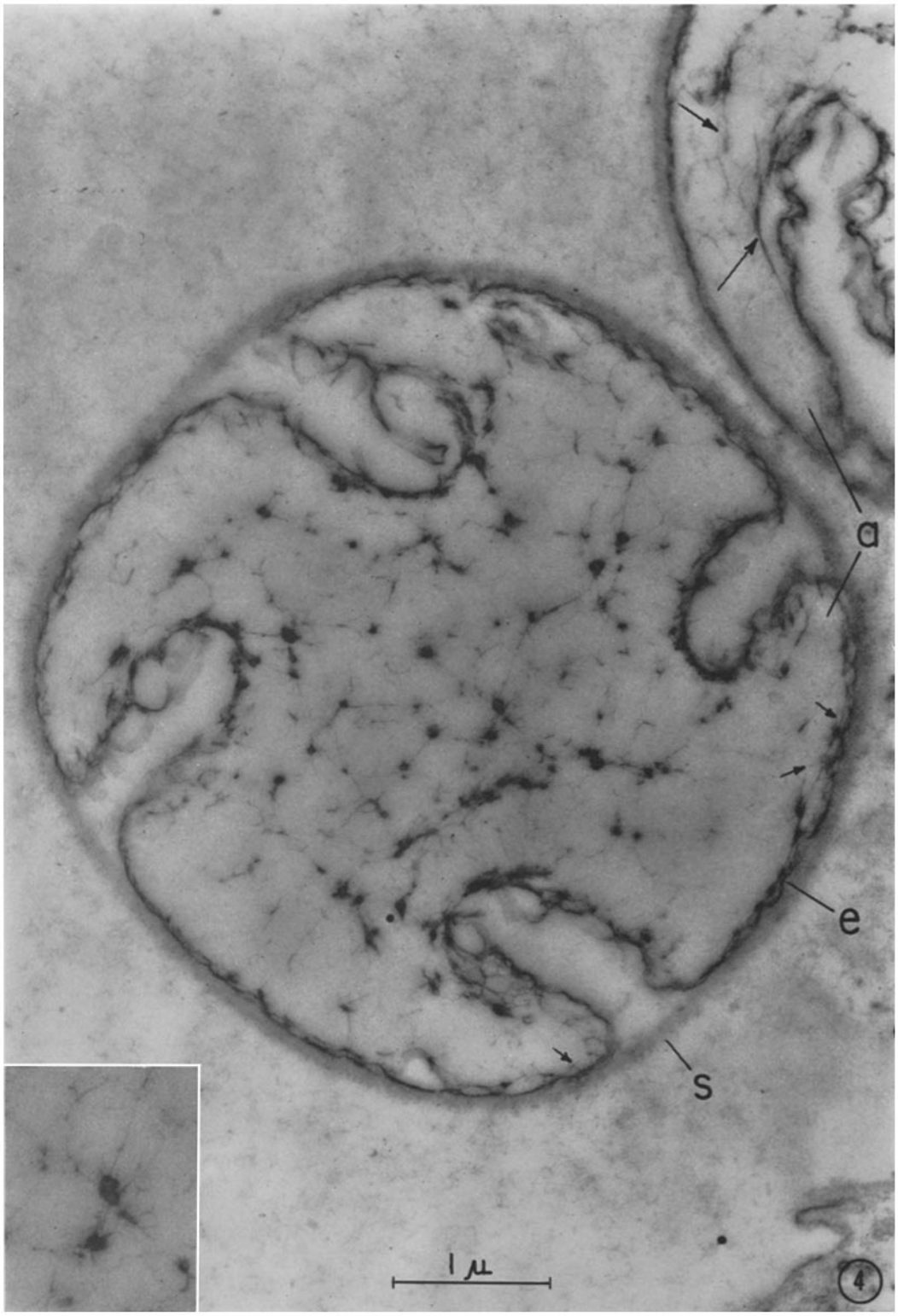
In *P. clarkii*, the complex, PAS-positive structure identified by us as the acrosome (10, 11) has the following history: in the early spermatid, a large cytoplasmic area is set off near the periphery of the cell in the neighborhood of the interzonal spindle material, and becomes surrounded by a membrane. It is not associated with either an organized Golgi element or mitochondrial hyperplasia. However, large masses of small vesicles (0.1 μ) with and without associated dense 120 A granules, are found in the vicinity, as they are elsewhere in the cell. Occasionally, small clumps of "annulate lamellae" (15) are associated with the vacuole in whose internum accumulations of dense 200 A particles appear. These aggregate to form a granule, about 2 μ in diameter, which is acidophilic and strongly PAS-positive, suggesting the presence of both protein and polysaccharide. This granule spreads out to form a thick lining on the side of the vacuole proximal to the nucleus. From this lining, filaments (100 to 200 A in width) extend into the vacuole which in turn fills with fine granular material and becomes PAS-positive (but not acidophilic). The new spermatid membrane joins with the rim of the lining, forming a closed structure. A deep invagination of the lining proximal to the nucleus (which has now become biconcave) forms a double-walled cup whose lumen facing the nucleus becomes filled with a cloudy pale fast green staining material. The inner space of the cup becomes filled with fine tubules which are preceded by masses of vesicles on the base of the vacuole (11). The structure is not basophilic.

Although the term acrosome was originally applied to the apical piece, or perforatorium of flagellate forms, it has since come to have more restricted significance. Bowen (1) used the term to describe that apical structure in the sperm formed by (or under the influence of) the Golgi apparatus, or idiosomal complex. Accordingly, the acrosome generally develops from a dense chromophilic granule within a clear vesicle associated with the Golgi complex. More recently, the acrosome in various stages has also been shown to be PAS-positive (2). Thus, because of its origins and its PAS-positive character, we have termed the entire structure in *P. clarkii* (Fig. 3) the acrosome. This must be considered provisional, however, since the role or fate of the components in fertilization are not yet known.

The final point characterizing this sperm is the conspicuous absence of mitochondria or mitochondrial derivatives (11, 12). In the spermatid mitochondria are small, sparse, and distributed

FIGURE 4

Electron micrograph of a frontal section (*i.e.* in the plane of radial symmetry) through the nucleus of an osmium-fixed mature sperm of *Procambarus clarkii*. The internum is continuous with the arms (*a*). The dense clumps and fibrils scattered through the nucleus can be seen in detail in the inset. At the upper right of the micrograph, fibrils and clusters of fibrils (arrows) can be seen extending into an arm (*a*). Masses of such material may account for the filament-like processes extending into the arms that are sometimes seen with the light microscope. The membrane surrounding the nucleus (*e*) is complex and dense; the number of layers of which it is composed is uncertain. In places (small arrows) fine fibrils appear to be associated with the complex envelope. Where the arms join the main body, shreds of membrane are seen looping out from the envelope; they appear to be the remains of the membrane elaborations continuous with the nuclear envelope found in earlier stages. The entire sperm is invested with a granular, PAS-positive shell (*s*) which contains the furled arms. Magnification approximately 24,000. Inset, 41,000.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marginally, in the region of the cytoplasm that is cast off. Very rarely a mitochondrion may be entrapped in the elaborate membrane system, but it either disappears or loses its identity, since no trace of it is found in the late sperm. It is unlikely that the membrane elaborations associated with the nuclear envelope are mitochondrial equivalents since they neither arise in association with mitochondria, nor do they in any way resemble them. Furthermore, neither fresh nor immature sperm are observed to accumulate or reduce Janus green. Since mitochondria in flagellate sperm presumably serve as energy sources for motility (8) it is not surprising that they are absent in aflagellate, non-motile forms.

The aflagellate sperm of *P. clarkii* described here can be homologized to flagellate forms only to the extent that both contain a modified nucleus with which to transmit genetic information and an acrosome presumably for effecting fertilization. The structure of both in *P. clarkii* is, however, not precisely paralleled in flagellate forms. The defunct centrioles and lack of mitochondria accompanying the absence of flagella constitute homology in a negative sense. The membrane elaborations whose function is unknown apart from providing integument, have no counterpart. Bizarre as this non-motile sperm may be, its effectiveness is nonetheless eloquently attested to by the abundance of crayfish in the world.

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