phocytes in greater numbers in 11-d tumor sponges may be the result of a continuing inflammatory response similar to that occurring in blank sponges. Alternatively host cell recruitment into the tumor sponge may be the result of a specific host recognitive event to the transplanted tumorigenic cells[7].

These observations are in agreement with the earlier findings of Hayry and Roberts[5] who demonstrated, by histological examination, a prominent inflammatory response to tumor-bearing implanted polyurethane sponges that vastly exceeded that which occurred in blank sponges. Our study employing pre-implanted gelatin sponge matrices has identified and quantitatively determined the numbers of host and tumor cells in a tumor system following the digestion of the sponge matrix in collagenase. The earlier documentation in our laboratory of conspicuous vascularization of whole retrieved blank gelatin sponges pre-implanted in nude mice[10], we believe, justifies complete solubilization of the gelatin matrix to ensure total cell recovery. In earlier studies[5-7, 11], employing polyurethane or viscous cellulose sponges, gentle compression of recovered sponges was thought to be sufficient for complete cell retrieval. This method was considered adequate since histological examination revealed the localization of granulation tissue only to the superficial layers of the sponge[5].

The often stated advantage of the 'compression' technique is that the recovered cells are spared any trauma due to enzyme digestion. Collagenese digestion is a routinely used method of tumor disaggregation and has been demonstrated not to affect the expression of surface immunoglobulin (SIg) and brain associated thymus antigen (BATA) on B and T lymphocytes respectively[21].

More recently[14] it has been reported that limited exposure to collagenase does not alter the expression of Fcγ receptors on 5-day cultured murine bone marrow macrophages. We have also shown in our laboratory (unpublished data) that collagenase digestion to recover CHO cells cultured in vitro in gelatin sponges neither diminishes the recovery nor the plating efficiency of these cells. This study has quantitated some of the host cells infiltrating a tumor sponge as well as determined the fate and clonogenic capacity of surviving tumor cells. By providing anchorage for injected cells as well as acting as a 'trap' for infiltrating host cells with potential effector functions, the pre-implanted sponge model furnishes a retrievable arena for studying in vivo host-tumor cell interactions. The delineation of host cells and the fate of tumor cells from the tumor mass can be studied in this model and will allow future studies in which the injection of tumor or leukocyte-derived factors into pre-implanted sponges would make it possible to elucidate the mechanism(s) of host cell recruitment into a tumor.

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Short Communications

Physiological role of apyrene spermatozoa of *Bombyx mori*

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Summary. Observation by electron and phase-contrast microscopy demonstrated that in *Bombyx mori* the dissociation of eupyrene bundles, apparently through digestion of the prostatic secretion or an endopeptidase, was promoted by the vigorously flagellating movement of apyrene spermatozoa in the spermatophore.

Key words. Apyrene spermatozoa; eupyrene spermatozoa; spermatophore; sperm maturation; glandula prostatica; endopeptidase; *Bombyx mori.*

Some invertebrates have two types of spermatozoa, apyrenes without nuclei, and eupyrenes which fertilize eggs[1,2]. The former are small and slender, while the latter are bigger and longer. The physiological role of the apyrene spermatozoa is not yet known[3]. In the silkworm, *Bombyx mori,* these two types of spermatozoa are clearly distinguishable in the testicular follicles[4,5]; in the testis the apyrene bundles divide into individual spermatozoa, while the 256 eupyrenes formed by division of primary spermatogonia remain as bundles in a cyst envelope[5]. Apyrenes and bundles of eupyrenes in the vesicula (v.) seminalis remain unchanged and immotile until they are transferred to the female bursa (b.) copulatrix during mating[4,5]. In the female, seminal fluid from the v. seminalis and the secretions from most exocrine glands of the male reproductive system become enclosed in a spermatophore formed inside the b. copulatrix[6,7]. In the silkworm, eupyrene spermatozoa mature in the spermatophore[8]; apyrene spermatozoa acquire motility[9,10] and then the matrix of the eupyrene cyst disappears and the individual eupyrene spermatozoa migrate into the receptaculum seminis[11]. Active apyrenes have been shown to be necessary for separation of eupyrenes from their bundles[10].

We found that the spermatophore of the silkworm is a metabolic reactor, in which the various processes necessary for sperm maturation occur. These include, at least, a specific pathway coupled with glycolysis for conversion of arginine to 2-oxoglutarate[12,13], which acts as a preferred substrate for spermatozoa and as a regulator of their respiration[12,14], and also a specific arginine-supply system composed of an endopeptidase[15] that cleaves proteins specifically at the C-sides of arginine residues, and exopeptidases[16]. The secretions of the various glands (g.) of the male reproductive system have been found to contain the following components necessary for eupyrene maturation separately: the secretion of the g. lacteola contains glycogen[8,17]; the seminal fluid in the v. seminalis contains arginase[18], which determines the irreversible reaction series of the 2-oxoglutarate-producing pathway, and exopeptidase; the secretion of its distal part contains glycogen granules[8,17]; the secretion of the g. prostatica contains the strong endopeptidase[15] and exopeptidase[16].

High viscosity of the contents of male reproductive glands and spermatophore was confirmed after each isolation from these tissues. The viscous contents of these glands caused their partial mixing during ejaculation, because they are transferred serially, resulting in heterogeneity of the spermatophore contents[19].

In the male v. seminalis, the individual apyrene spermatozoa are immotile, while eupyrene bundles containing immotile spermatozoa surrounded by a slightly electron-dense matrix are each wrapped in a thin electron-dense membranous cyst envelope, as shown in figure 1 A. After transfer by ejaculation to the spermatophore, the apyrene spermatozoa are activated by the prostatic fluid and begin to move, then both the envelope and the matrix of the eupyrene cyst soon disappear, leaving only the naked sperm bundles, and finally the bundles begin to dissociate (fig. 1 B). There were more apyrene spermatozoa than eupyrene spermatozoa in seminal fluid; they constitute over 65% of the total spermatozoa. Dissociation of eupyrene bundles starts from their ends, at the heads and tails of the eupyrenes, and some apyrenes are present among individual eupyrenes in these partially digested and loosened bundles (fig. 1 C).

It was difficult to observe the relation between active apyrene movement and dissociation of eupyrene bundles continuously in the spermatophore. Therefore, we studied changes in the spermatophore. The contents were isolated at certain times after mating, and diluted with silkworm-physiological saline solution. Apyrene spermatozoa in the seminal fluid in male v. seminalis were immotile (fig. 2 A). But soon after the beginning of spermatophore formation inside the female b. copulatrix, they moved vigorously (fig. 2 B), the envelope of the eupyrene cyst disappeared and the bundle increased 2.5 times in width. Dissociation of the bundle began from both ends. It was always observed that the activated apyrene spermatozoa could hardly advance despite their vigorous flagellar movement. The apyrene movement resulted in stirring the medium. These findings were consistent with electron microscopic findings in the spermatophore (fig. 1).

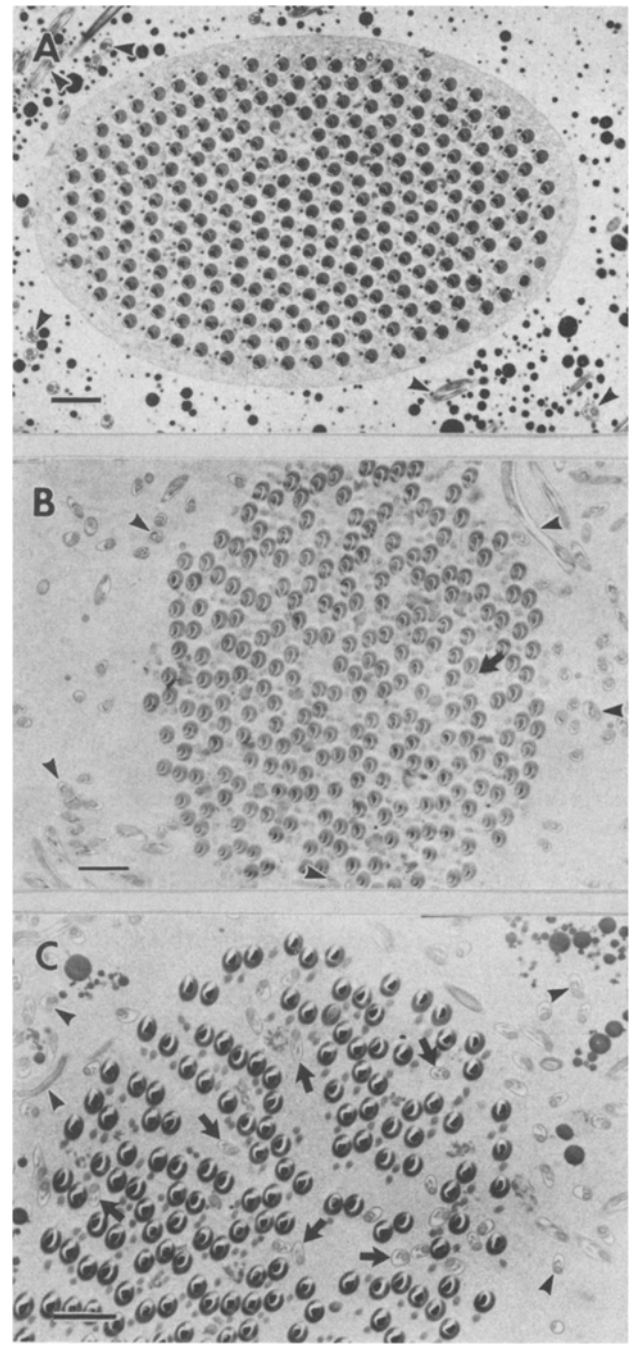

Figure 1. Ultrastructural changes in apyrene spermatozoa and eupyrene cysts of *Bombyx mori.* Scale shows 2.0 μm. *A* Seminal fluid in the male vesicula seminalis. Many individual apyrene spermatozoa (arrowheads) and a eupyrene cyst composed of an eupyrene bundle and a slightly electron-dense matrix wrapped in a thin envelope can be seen. There are also numerous electron-dense granules of various sizes. *B* Spermatophore contents 30 min after mating. The eupyrene bundle has begun to loosen and become disordered. The cyst envelope and matrix have disappeared. Apyrene spermatozoa (arrowheads) are seen around the bundles. Some of them come near the bundle, and one (arrow) has already penetrated into it. *C* Spermatophore contents 60 min after mating. The eupyrene bundle has become more disordered. Matrix in the eupyrene cyst has disappeared for the most part. Some apyrene spermatozoa (large arrows) are seen among eupyrene spermatozoa in the digested cyst matrix.

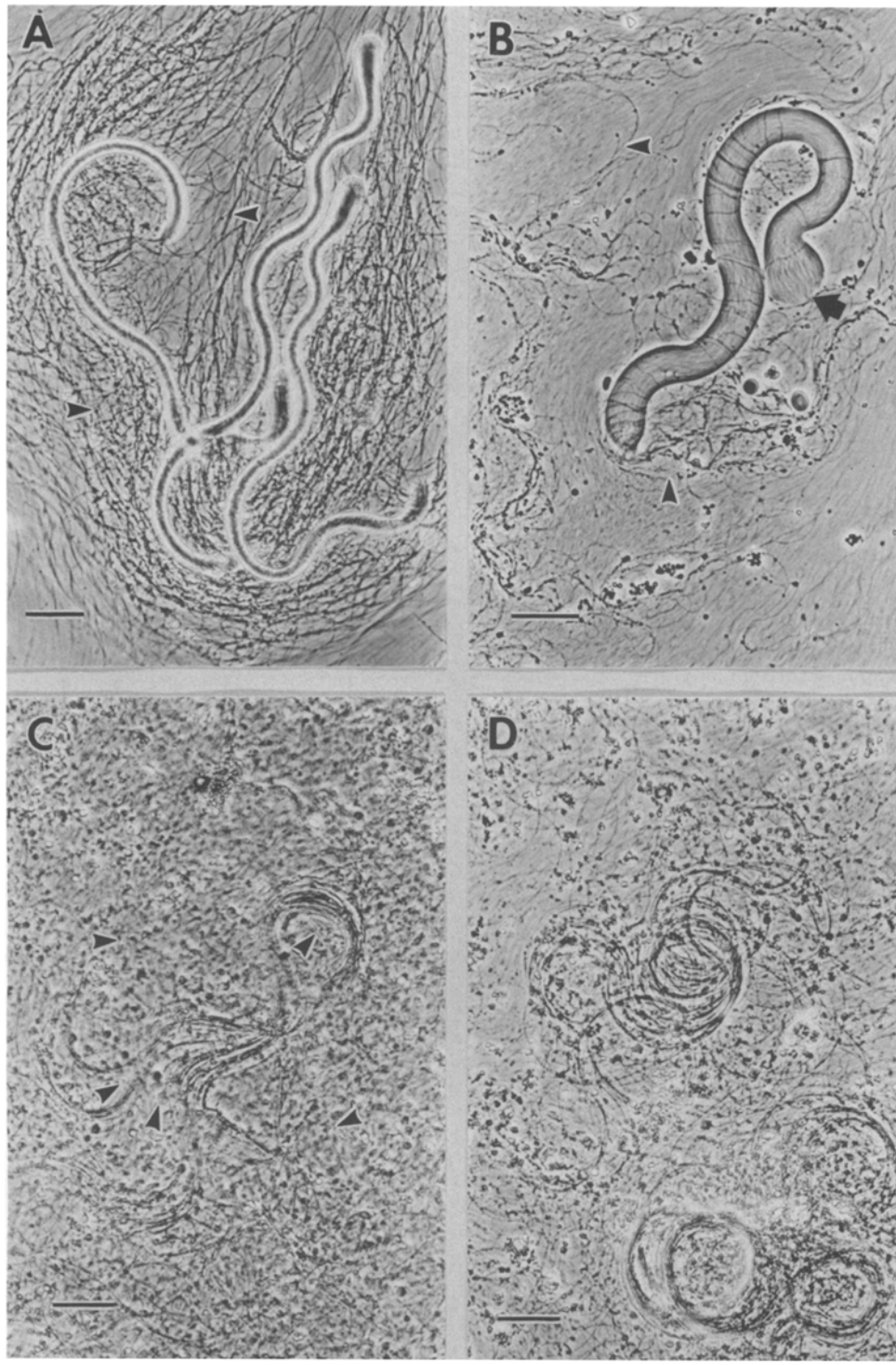

Figure 2. Morphological changes in apyrene spermatozoa and eupyrene bundles in the spermatophore (phase-contrast microscopy); *B–D* spermatophore contents diluted four times with silkworm-saline solution. Scale = 0.1 mm. *A* Seminal fluid in the male vesicula seminalis. The length and width of a eupyrene cyst are about 890 and 25 μm, respectively. The head of the cyst is about 1.6 times wider than the tail. Many immotile apyrene spermatozoa (arrowheads) surround the eupyrene cysts. *B* Spermatophore contents at 20 min after mating. Apyrene spermatozoa (arrowheads) have acquired motility, and some of them are seen round a eupyrene cyst that is about 2.5 times wider than an intact cyst and has begun to loosen, especially at its head (arrow) and tail. *C* Spermatophore contents at 80 min after mating. Some actively flagellating apyrene spermatozoa (arrowheads) have squeezed among loosened eupyrene bundles. The eupyrene cyst matrix has disappeared completely and eupyrene bundles have become loose, with separation of individual spermatozoa. *D* Spermatophore contents at 180 min after mating. Individual eupyrene spermatozoa have acquired motility.

We also found that amorphous masses from the v. seminalis disappeared in regions where the apyrene sperm showed a churning motion, but remained in regions where apyrenes were not present. Thus, activated apyrene spermatozoa promoted digestion of this material which was probably necessary for sperm maturation. Vigorously flagellating apyrenes squeezed between individual eupyrenes which became partially loosened, though still largely remaining bound together. At 80 min after mating, dissociation of the bundle in the spermatophore, which had begun from the ends, was almost complete (fig. 2C). Thus, the individual eupyrene spermatozoa became separated from each other, acquired motility and began to flagellate (fig. 2D). However, they still moved with flagellar undulations of much longer wave length and lower frequency than those of apyrenes. Since addition of either 5 µl of the g. prostatica suspension (one gland homogenized in 50 µl silkworm Ringer) or a purified endopeptidase, trypsin from bovine pancreas (Type I, Sigma Chemical Co., St. Louis, USA) or endoproteinase Arg-C from g. submaxillaris of mouse (Boehringer Mannheim GmbH, Mannheim, West-Germany) to 5 µl of the seminal fluid (content of one v. seminalis diluted in 200 µl silkworm Ringer) had always a very similar effect in causing digestion of cysts and dissociation of bundles, the active factor in the prostatic secretion was presumably an endopeptidase. These in vitro experiments were repeated at least five times to confirm the results.

In the silkmoth, the separation of enzymes[15, 16, 18] from their substrates[8, 19] in the male reproductive glands must be an effective biological mechanism for the supply of fresh nucleated spermatozoa at fertilization, since both the enzymes and substrates remain intact before ejaculation, but react together after ejaculation, resulting in sperm maturation.

However, the high viscosity and heterogeneity of the contents of spermatophore[19] would retard these reactions. Therefore, apyrene spermatozoa must be activated in the spermatophore to stir the contents and promote dissociation of eupyrene bundles and separation of each individual eupyrene spermatozoon both mechanically and by biochemical reactions. The flagellating apyrenes also cause digestion of the soft plug, a proximal part of the spermatophore, resulting in opening to the ductus seminalis. These functions of apyrenes are similar to those of apyrenes in some molluscs[20, 21], which help in the migration of eupyrenes.

Recently, four possible functions of apyrene spermatozoa were suggested[3]. The possibility that they are a source of nutrition for eupyrenes[22] is unlikely, because they are present in such a small quantity. The other three possibilities; that they facilitate escape of eupyrenes from the testis[23, 24], that they promote eupyrene movement in females[25], and that they play a role in competition between rival sperms deposited by different males[3], are also unlikely.

The last possibility appears to be true in some Lepidoptera which can mate several times. In *Bombyx mori*, remating is often possible[26]. However, the first-inseminated spermatozoa are not always dominant over the later, reinseminated ones in fertilization[27]. In either case, apyrene spermatozoa do not have any relation to sperm competition.

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Evidence of a direct action of triiodothyronine (T_3) on the cell membrane of GH_3 cells: an electrophysiological approach

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Summary. Electrophysiological experiments demonstrate that triiodothyronine (T_3) exerts a direct effect on the membrane of a strain of cultured rat pituitary tumor cells, GH_3/B_6. These cells respond to pressure application of T_3 (2–5 nl, concentration $1 \cdot 10^{-10}$ M) with an increase in the membrane resistance (R_m) and a hyperpolarization. Spontaneously firing cells become silent.

Key words. Triiodothyronine; GH_3 cells; membrane potential; membrane resistance; action potentials.