

THE LOCALIZATION OF GLYCOGEN IN THE SPERMATOOA OF VARIOUS INVERTEBRATE AND VERTEBRATE SPECIES

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ABSTRACT

With the periodic acid-thiosemicarbazide-silver proteinate procedure for the detection of polysaccharides in thin sections, glycogen is localized in the cavities of centrioles and basal bodies, within the axoneme (and surrounding it), in mitochondria, and in the "packing" cytoplasm of the middle piece of spermatozoa of several invertebrate and vertebrate species. The cytochemical localization of glycogen is verified by extraction with α -amylase solution. These findings establish the existence of stored glycogen in sperm. The polysaccharide presumably serves as an endogenous source of energy in the absence of extracellular metabolites, under either aerobic or anaerobic conditions. Other hypotheses on the physiological significance of intracellular glycogen stores in sperm are discussed. Sperm that store glycogen contain some enzymes of glycogen metabolism. In the presence of glucose-1-phosphate, ATP, and Mg^{++} ions, an amylophosphorylase catalyzes the *in vivo* synthesis of glycogen. The newly formed product resembles γ -particles, and is digestible with α -amylase.

INTRODUCTION

Spermatozoa are highly specialized for motility and the other activities essential to fertilization of ova. To reduce its volume and protect the genome from damage during exposure to various environments encountered in transit to the ovum, the sperm nucleus becomes highly condensed and metabolically inert. To favor its motility, the developing spermatozoan divests itself of most of its cytoplasm. There remains seemingly very little space for the intracellular storage of substrates which could provide the energy essential for their motility. In some species, the spermatozoa are released into a specialized internal environment rich in exogenous nutrients. For example, mammalian spermatozoa normally metabolize carbohydrates added to the seminal plasma by the

accessory glands (Mann, 1964). However, under aerobic conditions and in the absence of glycolyzable material, these sperm utilize endogenous lipids as their main source of energy. Thus, mammalian sperm have the enzyme systems capable of utilizing either environmental carbohydrate or endogenous lipids depending upon the availability of exogenous substrate and oxygen.

On the other hand, some species discharge their spermatozoa into an aqueous environment that is devoid of metabolizable compounds. These sperm must, therefore, depend exclusively upon endogenous substrates. According to Afzelius and Mohri (1966), the motility of sea urchin spermatozoa, for example, depends upon energy obtained by the aerobic oxidation of phospho-

lipids in their middle piece. Other work, however, indicates that some invertebrate sperm may use endogenous carbohydrate such as glycogen as an energy source (Spikes, 1949; Personne and André, 1964; Lanza and Quattrini, 1964; Anderson, 1968). Although the fine structure of invertebrate sperm has been extensively investigated, the presence of glycogen has been reported in only a few—*Testacella* and *Helix* (Personne and André, 1964; André, 1965), *Lumbricus* (Anderson et al., 1967), *Paracentrotus* (Anderson, 1968). Based on morphological findings, glycogen-like granules have been demonstrated in sperm of the Platyhelminthes and Chilopoda (Silveira and Porter, 1964; von Bonsdorff and Telkka, 1965; Horstmann, 1968).

The recent development of satisfactory methods for the demonstration of polysaccharides at the electron microscope level (Hanker et al., 1964; Seligman et al., 1965; Thiéry, 1967) now makes it possible to localize polysaccharides in cells and tissues in which this substance formerly escaped detection. In the present study, the periodic acid-thiosemicarbazide-silver proteinate (PA-TSC-SP) technique of Thiéry has been applied to sperm of a wide variety of invertebrate and vertebrate species in order to determine how common glycogen is and where it is localized. Glycogen is demonstrated in the cavity of centrioles and basal bodies, in the matrix within the axoneme (and around it), and in the mitochondria and the surrounding cytoplasm. The cytochemical identification of glycogen has been verified in some instances by enzyme extraction procedures. The

methods of Takeuchi and Kuriaki (1955) and of Guha-Wegmann (1959) have been utilized to demonstrate in the sperm of certain species the presence of an active phosphorylase capable of catalyzing the synthesis of glycogen (Personne and Anderson, 1969 *a*). The observations are discussed in relation to the conditions of sperm storage and the environment in which fertilization takes place in the several species studied.

MATERIALS AND METHODS

Animal Material

The various specimens whose spermatozoa were investigated were collected in France or in the United States or purchased from dealers in those countries. They include the following:

ANNELIDA: *Lumbricus terrestris*; *Hirudo medicinalis*.

MOLLUSCA: (a) Marine Lamellebranchia: *Mytilus edulis*; *Ostrea edulis*; *Pecten maximus*; *Spisula solidissima*. (b) Marine Gastropoda: *Gibbula cineraria*; *Patella vulgata*; *Littorina littorea*; *Littorina obtusata*; *Purpura lapillus*; *Aplysia californica*. (c) Freshwater Gastropoda: *Lymnaea truncatula*; *Lymnaea auricularia*; *Pomacea cuprina*; *Viviparus malleatus*; *Viviparus japonicus*; *Viviparus viviparus*. (d) Terrestrial Gastropoda: *Arion aggericola*; *Helix aspersa*; *Helix pomatia*; *Otala lactea*.

ARTHROPODA: (a) Crustacea. Cirripedia: *Lepas anatifera*. Decapoda: *Cambarus clarkii*; *Astacus fluviatilis*; *Carcinus maenas*. (b) Insects: *Drosophila melanogaster*; *Locusta migratoria*; *Blatta orientalis*; *Periplaneta americana*.

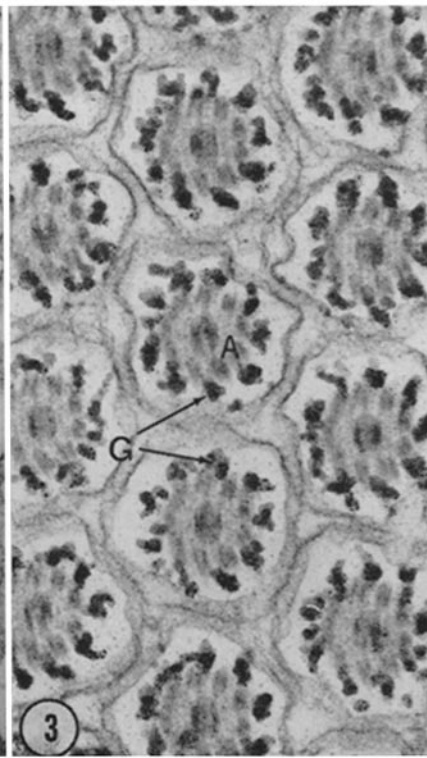
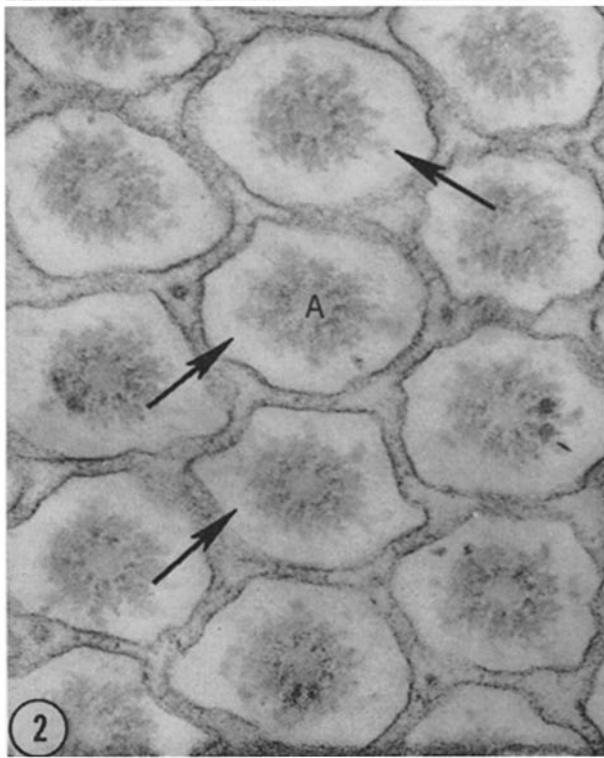
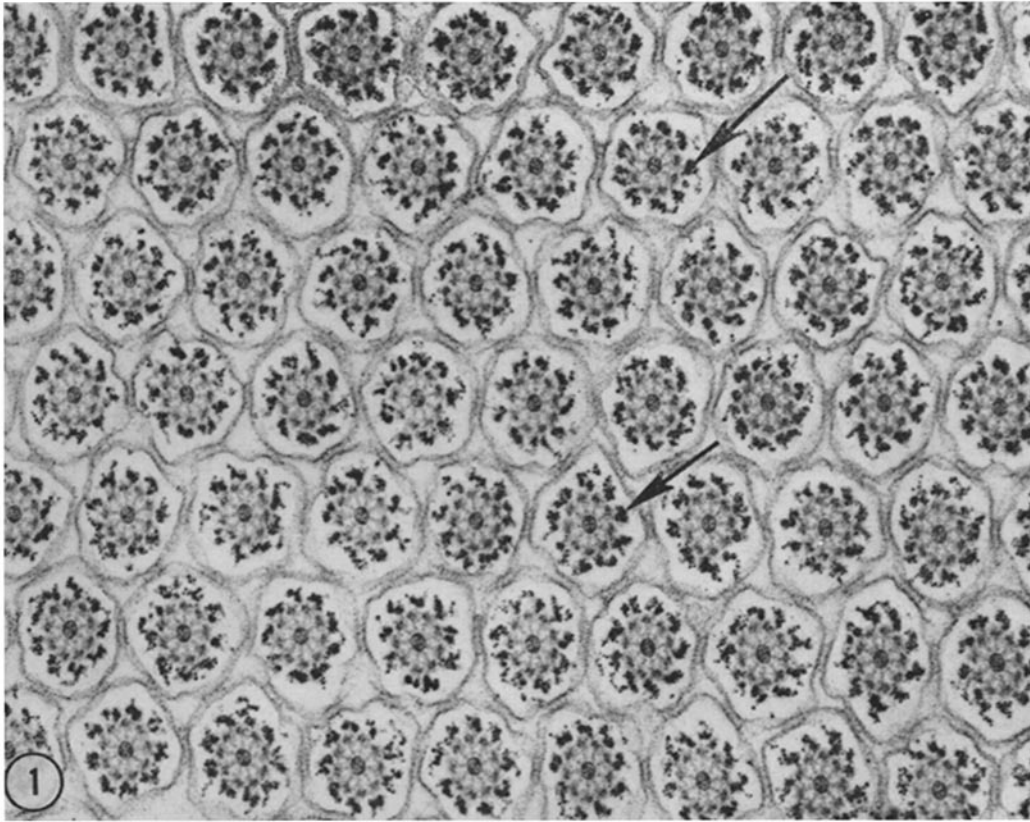
ECHINODERMATA: *Paracentrotus lividus*; *Arbacia lixula*.

All micrographs, except Fig. 4 *B*, were taken from sections that were treated with the periodic acid-thiosemicarbazide-silver proteinate procedure (PA-TSC-SP) for the demonstration of glycogen according to Thiéry (1967).

FIGURE 1 Transverse sections through flagella of sperm of *Hirudo*. After the PA-TSC-SP treatment, intensely stained glycogen granules (arrows) occupy the matrix between the outer doublets of the axoneme and the flagellar membrane. $\times 40,000$.

FIGURE 2 Following oxidation in 5% periodic acid solution for 10 min at 33°C, rinsing in distilled water for 30 min, and treatment of thin sections for 60 min with 0.5% α -amylase solution, the glycogen granules totally disappear from sperm tails. Following the PA-TSC-SP treatment and staining in 2% aqueous uranyl acetate solution for 5 min, the matrix surrounding the axonemes (*A*) remains clear (arrows). $\times 70,000$.

FIGURE 3 Glycogen granules (*G*) remain intact around the axonemes (*A*) of sperm tails in control sections that were incubated in heat-inactivated α -amylase solutions and treated in the same manner as those of Fig. 2. $\times 60,000$.



CHORDATA: (a) Ascidiacea: *Ciona intestinalis*; *Molgula manhattensis*. (b) Pisces: *Gasterosteus aculeatus*; *Xiphophorus helleri*; *Platypoecelia maculata*; *Lebistes reticulatus*; *Opsanus tau*. (c) Amphibia: *Triton cristatus*; *Rana pipiens*; *Bufo americana*. (d) Reptilia: *Holbrookia texana*; *Urosaurus ornatus*. (e) Aves: *Columba livia*. (f) Mammalia: *Rattus norvegicus*; *Mus musculus*. *Cavia porcellus*; *Meriones unguiculatus*; *Citellus tridecemlineatus*.

Preparation of Tissues for

Electron Microscopy

Small blocks of tissue (1–3 mm³), were fixed by immersion, or isolated sperm were suspended in fixative according to the following procedures. Samples of testes, seminal vesicles, and sperm receptacles of terrestrial and freshwater invertebrates were fixed for 30–60 min at room temperature in a 2% glutaraldehyde solution. The fixative contained 5.6 ml biological grade glutaraldehyde (36%), 50 ml 0.1 M sodium cacodylate buffer at pH 7.2, 44.4 ml of distilled water, and 1.5 g of sucrose. After fixation, the samples were rinsed in a solution containing 5.0 g of sucrose in 100 ml 0.1 M sodium cacodylate solution. Tissues from vertebrates and from some marine invertebrates were fixed for 30–45 min at 4° or at 27°C in 2% glutaraldehyde in 0.1 M sodium cacodylate made up in filtered seawater. The fixative contained 1.5 g of sucrose, and was adjusted to pH 7.0. In other instances, good preservation of tissues from vertebrates and invertebrates was obtained after fixation in the formaldehyde-glutaraldehyde plus trinitroresol mixture of Ito and Karnovsky (1968). Subsequent to aldehyde fixation, all specimens were postfixed in 2% osmium tetroxide solution. After postfixation, samples were dehydrated in acetone-water solutions and embedded in Epon or in an Epon-Araldite mixture (Voelz and Dworkin, 1962; Anderson and Ellis, 1965).

Cytochemical Procedures for the Demonstration of Glycogen

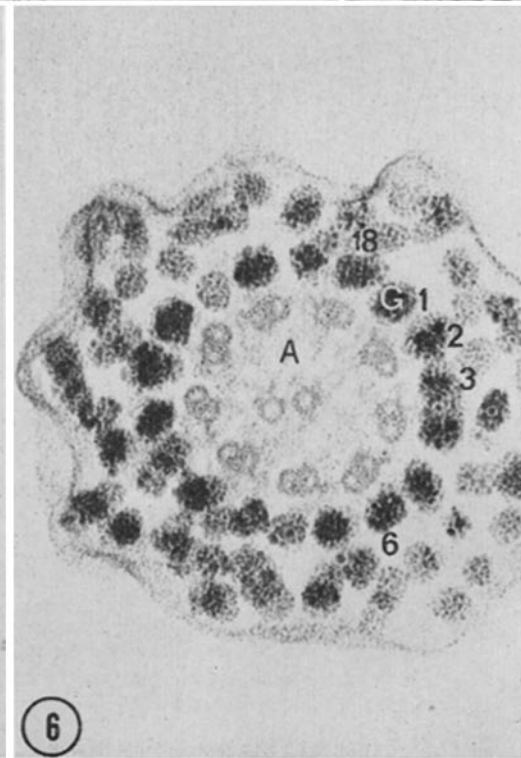
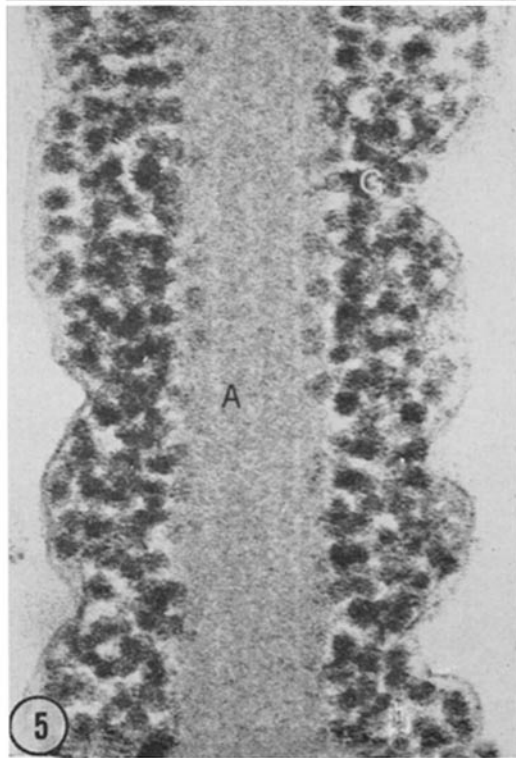
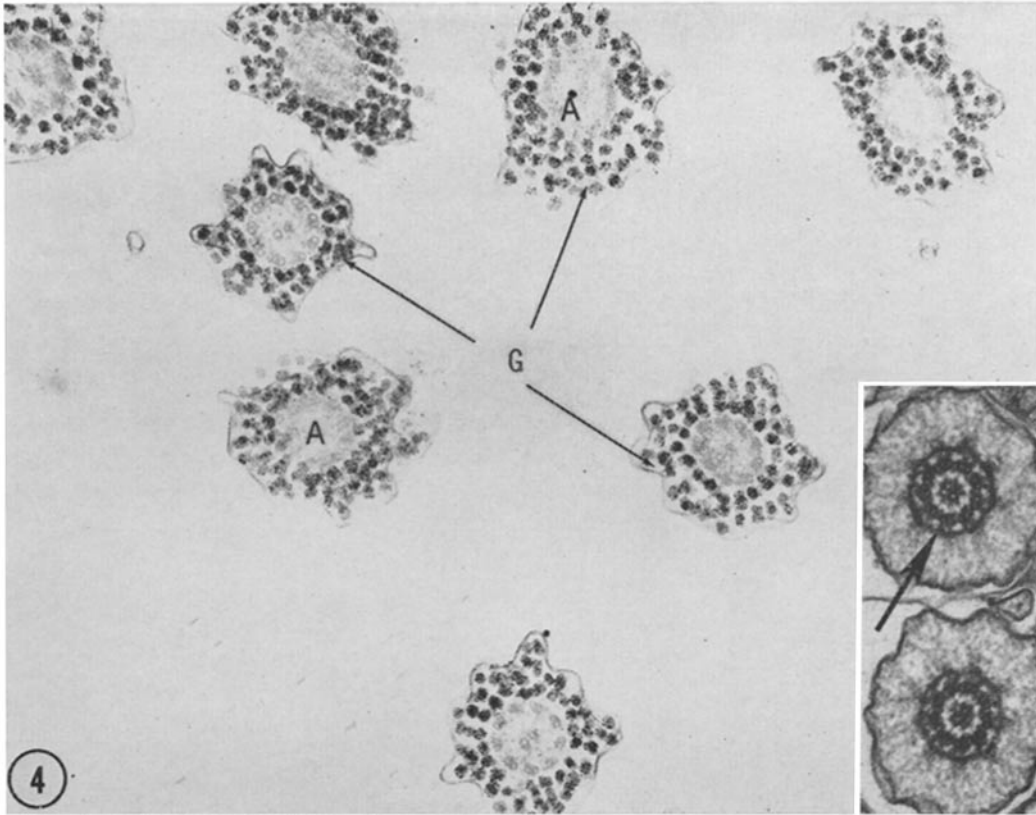
THE PERIODIC ACID-THIOSEMICARBAZIDE-SILVER PROTEINATE PROCEDURE (PA-TSC-SP): The details of this technique and the lengths of the respective reactions for the optimal demonstration of glycogen and of other polysaccharides in thin sections have been fully worked out by Thiéry (1967). In this study, except for some minor adjustments, Thiéry's procedure has been precisely followed. Thin sections of aldehyde-osmium tetroxide-fixed tissues were collected and transported to the respective incubation media within the lumen of polyethylene rings according to Marinozzi (1961). Sections were floated for 30–45 min on the surface of a solution of 1% periodic acid in distilled water. Thin sections were then rinsed thoroughly (approximately 30 min) in several changes of distilled water, and refloatated for 30–45 min on 1% thiosemicarbazide in 10% acetic acid. Subsequently, the sections were washed in three changes (approximately 10 min each) of 10% acetic acid solution, then for 5 min in 5 and 1% acetic acid solutions and finally in distilled water. The sections were then refloatated for 30 min on 1% silver proteinate in distilled water. Incubation in silver proteinate solution was performed in the dark. The sections were thoroughly rinsed in several changes of distilled water and collected on copper grids.

THE PERIODIC ACID-THIOSEMICARBAZIDE AND THE PERIODIC ACID-THIOCARBOHYDRIDE PLUS OSMIUM TETROXIDE PROCEDURES (PATO, PATCO) OF SELIGMAN ET AL., (1965) AND THE PAS-PHOSPHOTUNGSTIC ACID PROCEDURE OF THIÉRY (1967): These were also tried in the detection of glycogen in thin sections. Results with these techniques were identical with those obtained with the PA-TSC-SP procedure. However, since the PA-TSC-SP procedure yielded the most

FIGURE 4 Transverse sections through the sperm tails of *Littorina* are shown in this micrograph. The glycogen granules (G) are located around the axoneme (A). The inset (B) shows a specimen from sections that were stained for approximately 60 min in a saturated uranyl acetate solution in 40% ethanol. Glycogen granules are not stained, but a pepsin-digestible halo (arrow) surrounds the axoneme. $\times 55,000$.

FIGURE 5 A longitudinal section through a portion of the sperm tail of *Littorina* is shown here. Several layers of β -glycogen particles (G) are tightly packed between the axoneme (A) and the flagellar membrane. $\times 110,000$.

FIGURE 6 A transverse section through the flagellum of a spermatozoon of *Littorina* is shown here. 18 β -particles (as numbered) generally constitute the first row of glycogen particles (G) surrounding the axoneme (A). $\times 150,000$.



consistent and accurate results with a minimum of background precipitation, it was mainly used in the demonstration of glycogen in thin sections of spermatozoa.

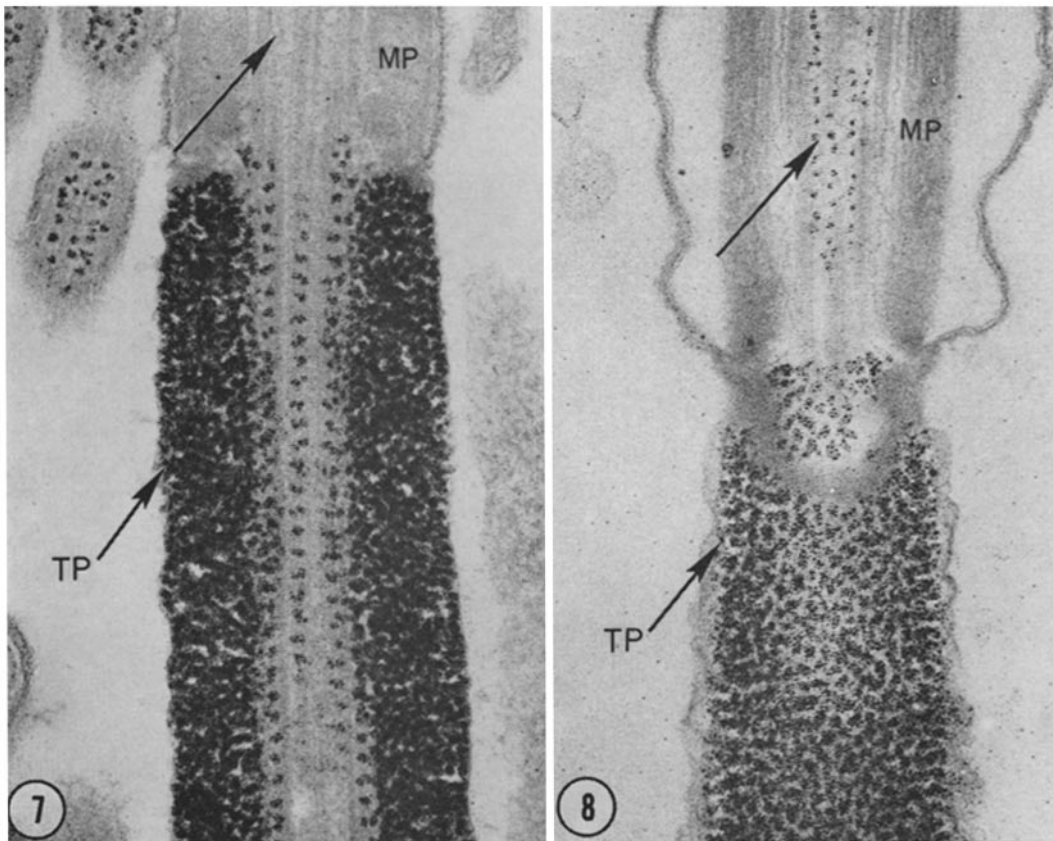
Enzymatic Extraction of Glycogen from Thin Sections

Cytochemical techniques for the enzymatic extraction of glycogen from Epon-embedded sections were performed according to Monneron and Bernhard (1966). Experimental and control sections were similarly carried in polyethylene rings according to Marinozzi (1961). After periodate oxidation and rinsing according to Monneron and Bernhard (1966), thin sections were incubated in 1, 0.5, 0.1, and 0.05% α - or β -amylase solutions in 0.1 M phosphate buffer at pH 7.0. The sections were in-

cupated at 37°C for 30 min, 1, 2, and 3 hr. Thin sections were also incubated in saliva for 3-4 hr at 37°C.

Control sections were incubated in heat-inactivated α - or β -amylase solutions and saliva, for the same times and under the same conditions. Enzyme- and saliva-treated, as well as control sections were then stained according to procedures described above for the detection of glycogen or were double stained in uranyl acetate and lead citrate solutions.

In some instances, tissues which were fixed for 30-60 min in 2% formalin in 0.1 M phosphate buffer at pH 7.0 and rinsed for 8-16 hr in buffer were incubated *en bloc* for 1-3 hr in 0.5-1% α -amylase solution or in saliva. Control samples were incubated in phosphate buffer only. After incubation, the tissues were rinsed in buffer, postfixed in 2% OsO₄ solu-



FIGURES 7 and 8 Longitudinal sections through the terminal portion of the mitochondrial sheath (*MP*) and the tail segment (*TP*) of sperm of *Pomacea* (Fig. 7) and of *Planorbis* (Fig. 8) are shown in these micrographs. Tightly packed β -particles comprise the thick mantle around the axoneme. In sperm of *Planorbis*, β -particles extend into the axoneme within the mitochondrial sheath (arrow, Fig. 8), but in sperm of *Pomacea* glycogen is not observed in the axoneme (arrow, Fig. 7) within the middle piece. $\times 70,000$; $\times 60,000$.

tion, and prepared for electron microscopy by routine procedures.

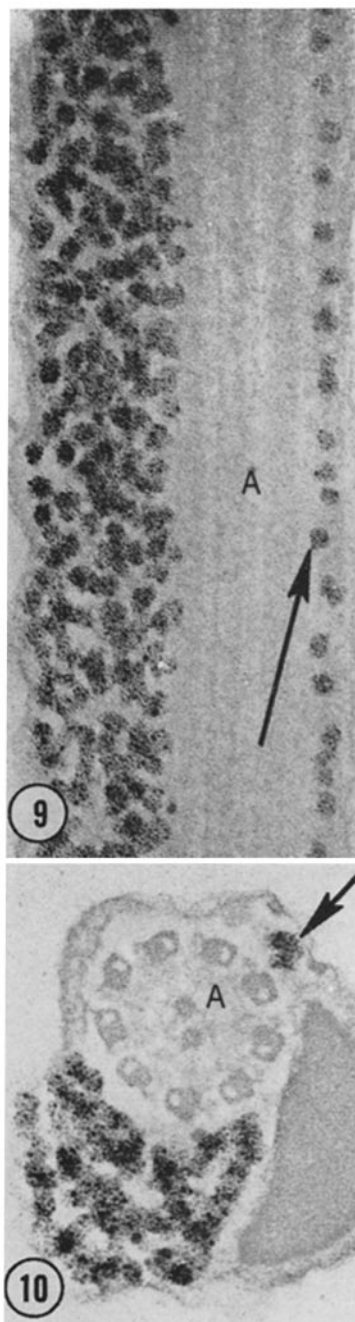
Chemical Isolation of Glycogen from Spermatozoa

Spermatozoa that were isolated from the hermaphroditic duct of *Lymnaea* and *Otala* were used in this study. After boiling the spermatozoa in 30% KOH solution, an ethanol precipitable fraction was obtained. This material was purified by repeated ethanol precipitation of the water-soluble extract. Drops of the extract suspended in 55% ethanol were spread on Formvar-coated grids. Excess fluid was removed from the surface of the grid and was replaced with a drop of 1% phosphotungstic acid (PTA) in 55% ethanol or in distilled water at pH 6.9. After 1-5 min, the PTA solution was removed and the grids were allowed to dry at room temperature.

Demonstration of Amylophosphorylase Activity Associated with Glycogen Synthesis

The Takeuchi and Kuriaki (1955) and the Guha and Wegmann (1959; 1961) procedures were used to demonstrate the synthesis of glycogen by an amylophosphorylase. Isolated spermatozoa were incubated in the following medium: 25 mg of glucose-1-phosphate, 5 mg of ATP, 10 mg of NaF, 4 mg of MgSO₄, 5 ml 0.2 M acetate buffer at pH 6.0, and 7.5 ml of distilled water. Sodium fluoride was added in order to inhibit phosphorylase phosphatase activity, thus blocking the conversion of active phosphorylase *a* to inactive phosphorylase *b*. ATP and Mg⁺⁺ ions activate phosphorylase kinase which catalyzes the conversion of inactive phosphorylase *b* to active phosphorylase *a*. Unfixed spermatozoa were also incubated in medium containing AMP (20 mg), but lacking ATP and MgSO₄. Control specimens were incubated in medium from which glucose-1-phosphate was omitted. Experimental and control specimens were incubated at 37°C from 30 min to 3 hr. After fixation in glutaraldehyde and osmium tetroxide solutions, samples were prepared for electron microscopy by the above-mentioned procedures. Pre-existing and newly formed glycogen were demonstrated in thin sections by the PA-TSC-SP procedure of Thiéry (1967). Some control and experimental sections were incubated for 1 hr in medium containing either 0.5% α - or β -amylase in phosphate buffer at pH 7.0.

A detailed study on the involvement of an amylophosphorylase in glycogen synthesis in sperm of pulmonate gastropods will be presented in a separate report (Personne and Anderson, 1969).



FIGURES 9 and 10 Longitudinal and transverse sections through the tail piece of sperm of *Lepas* are shown here. A single row of glycogen granules (arrows) exists on one side of the axoneme (A), while a stack of granules is located at the opposite side. $\times 120,000$.

OBSERVATIONS

Periaxonemal Distribution of Glycogen

Spermatozoa of the annelids *Lumbricus* and *Hirudo* conform to the plan of organization of other flagellate spermatozoa in that they are subdivided into acrosome, nucleus, middle piece, and tail segments (Anderson et al., 1967). The short cylindrical middle piece lies immediately behind the helical nucleus of *Hirudo* and the uniformly cylindrical nucleus of *Lumbricus*. The basal body of the axoneme is located at the base of the middle piece. Glycogen granules are consistently found in the sperm tails of *Hirudo* (Fig. 1) and of *Lumbricus* (Anderson et al., 1967). The irregularly shaped granules are approximately 200–300 Å in diameter and are identical to β -glycogen granules (nomenclature according to Drochmans, 1962). In thin sections prepared by conventional procedures for electron microscopy, the density of the periaxonemal granules is only moderately intensified with lead stains. These granules are much more opaque after treatment with the PA-TSC-SP procedure. The granules are distributed peripheral to the doublets and immediately beneath the flagellar membrane. In *Lumbricus*, the β -particles are distributed along the entire length of the flagellum, whereas in *Hirudo* they terminate at the junction of the axoneme with an homogeneous rodlike tip of the tail. This rodlike specialization is moderately contrasted after treatment with the PA-TSC-SP procedure.

For assessing further the specificity of the staining procedure in the detection of glycogen, thin sections were exposed to glycogen-extraction solutions. Following treatment in 0.5% α -amylase solution for 60 min at 37°C, the periaxonemal glycogen granules are totally extracted. When sections are treated first with α -amylase solution, followed by the PA-TSC-SP procedure, the region surrounding the outer doublets now appears clear (Fig. 2). When thin sections are treated with proteinases (according to Monneron and Bernhard, 1966; Anderson and André, 1968), the axonemal components are extracted while glycogen particles remain intact. In control sections that were incubated in heat-inactivated α -amylase solution or in water, the glycogen particles remained unaltered around the axoneme (Fig. 3).

Spermatozoa of the molluscs *Littorina* and *Purpura* do not conform morphologically in all

respects to the basic plan of flagellate spermatozoa. The axoneme originates at the base of the acrosome and runs the entire length of the cell. Anteriorly, it is surrounded by the nucleus for 40–60 μ , and by the mitochondrial derivative (Walker and MacGregor, 1968). Behind the middle piece, the flagellum continues for about 30–40 μ to form the tail. Axonemes of *Littorina* and *Purpura* have the 9 + 2 arrangement of microtubules. Walker and MacGregor (1968) have also described multiple coarse fibers around the axoneme, but in our opinion, what they interpreted as periaxonemal fibers are, in fact, deposits of glycogen. The particles are closely apposed to the doublets, and are present throughout the length of the tail (Figs. 4 and 6). Glycogen granules are clearly absent from that portion of the axoneme that is enveloped by the mitochondrial sheath or by the nucleus. The granules are not stained appreciably even after staining in uranyl acetate solution for extensive periods (30–60 min); however, moderately opaque, pepsin-extractable proteinaceous material is seen around the axoneme in such preparations (Fig. 4, inset). Upon close examination of sections treated by the PA-TSC-SP procedure the staining of the glycogen particles is punctate, suggesting the presence of subunits (Fig. 6). It is not certain, however, that this is other than a meaningless pattern of precipitation of the stain. The glycogen granules do not contact the doublets and are not present within the axoneme of these spermatozoa. The extensive deposits of glycogen do not appear to impede movement of the tail piece, for living spermatozoa of *Littorina* are actively motile throughout their entire length.

Glycogen granules are present within the tail piece of spermatozoa of all freshwater pulmonate gastropods studied so far, *Lymnaea*, *Helisoma*, and *Planorbis* (Fig. 8). Similarly, in typical flagellate spermatozoa of prosobranch gastropods, glycogen granules are also located around the axoneme of the tail piece (Fig. 7). In spermatozoa of these freshwater gastropods then, the glycogen granules form a thick mantle around the peripheral doublets. Glycogen particles are also observed within the axoneme of the tail piece of these spermatozoa.

Unlike other crustacean spermatozoa, those of cirriped Crustacea are flagellate (Brown, 1966) and motile. Significant amounts of glycogen are present within the spermatozoa of *Lepas*.

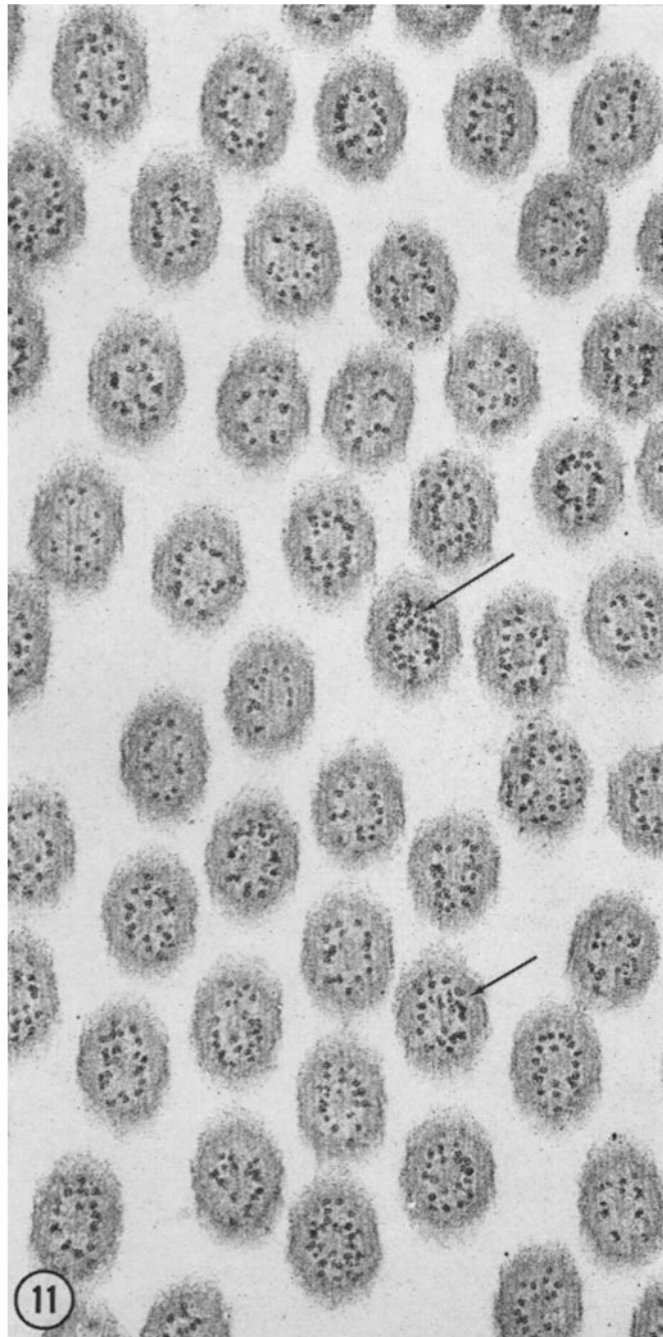


FIGURE 11 Transverse sections through flagella of atypical sperm of *Viviparus* are shown in this micrograph. Beta glycogen granules are located between the central pair and peripheral doublets of the axoneme (arrows). $\times 60,000$.

Glycogen particles are disposed in a single layer on one side of the axoneme, while on the opposite side they are densely packed in a prominent ridge (Figs. 9 and 10).

Intra-Axonemal Distribution of Glycogen

Atypical, vermiform spermatozoa of prosobranch gastropods possess multiple flagella. Glycogen granules are randomly distributed between the peripheral doublets and the central pair of microtubules in each flagellum (Fig. 11).

Material with the staining properties of glycogen is consistently present within the axoneme of spermatozoa in some pulmonate gastropods (Figs. 12-14). The granules conform to the usual description of β -particles and are intensely stained after the PA-TSC-SP treatment (Personne and Anderson, in preparation;¹ Anderson and Personne, 1969). They are not randomly distributed in the axoneme, for they consistently appear between the central tubules and the peripheral doublets (Figs. 13-14). In some species, the granules seem to be arrayed in parallel helices within the axoneme. They disappear after treatment with α -amylase solution.

Pericentriolar, Intracentriolar, and Perimitochondrial Distribution of Glycogen

Spermatozoa of the lamellibranch molluscs *Pecten*, *Mytilus*, *Ostrea*, *Spisula*, and of the gastropods *Gibbula*, *Patella*, and *Diodora* are divided into head, a short middle piece, and a simple flagellum. According to Franzen (1956), these spermatozoa are classified as "primitive." The middle piece is composed of relatively unmodified mitochondria surrounding the basal body and the proximal regions of the axoneme.

Spermatozoa of most Lamellibranchii and of the Gastropoda named above are similar in their general features to those of *Mytilus* and of *Crassostrea* which have been described, respectively, by Longo and Dornfeld (1967) and by Galtsoff and Philpott (1960); but they vary in shape of head and number of mitochondria. In sperm of *Mytilus*, an axial rod projects into a cylindrical space within the nucleus.

¹ P. Personne and W. A. Anderson. 1969. Existence de glycogène intraflagellaire dans le spermatozoïde des Gasteropodes Pulmones.

After double staining of thin sections with uranyl acetate and lead citrate, no unusual structures were observed. The mitochondria and centrioles were surrounded by material of low density or by finely granular or homogeneously dense material. After processing sections with the PA-TSC-SP procedure, α -amylase digestible glycogen particles are consistently observed within the middle piece. The glycogen granules are similar in appearance to those already described in sperm of other invertebrate species. The particles are arranged in compact clusters around the mitochondria and centrioles, and within the cavity of the centrioles (Figs. 15-18). In spermatozoa of *Pecten*, *Mytilus* and *Spisula*, glycogen particles are often in intimate contact with the outer membrane of the mitochondria (Figs. 17 and 18), in close apposition to the wall of the proximal and distal centrioles (Figs. 15 and 16), and within the central canal (of *Mytilus* sperm) and in peripheral indentations of the nucleus. Glycogen particles are not found in the flagellum of mature spermatozoa of these lamellibranch molluscs.

Glycogen is absent from the middle piece of spermatids and of immature spermatozoa obtained from the testis. Mature spermatozoa incubated in seawater for 6 hr contain varying amounts of glycogen. Glycogen disappears from sperm of some species under these conditions; in others, it is undiminished.

Intramitochondrial Localization of Glycogen

Spermatozoa of pulmonate gastropods possess an enormous middle piece that is largely composed of a highly modified mitochondrial sheath containing a paracrystalline component. Using classical techniques for the demonstration of polysaccharides, Personne and André (1964) and André (1965) demonstrated the presence of glycogen within a distinct compartment of the mitochondrial sheath. Other cytochemical tests indicate that the paracrystalline region is proteinaceous (Personne, 1965) and may contain the respiratory enzymes. In the present study, glycogen reserves are observed within the mitochondrial derivative of spermatozoa of all pulmonate gastropods studied so far (Figs. 12, 13, and 19). The number of glycogen-containing compartments varies with the species, but, in all cases, they run a helical course from the neck to the tail piece (Fig. 20). Although the glycogen stores are encased by the mitochondrial derivative, the com-

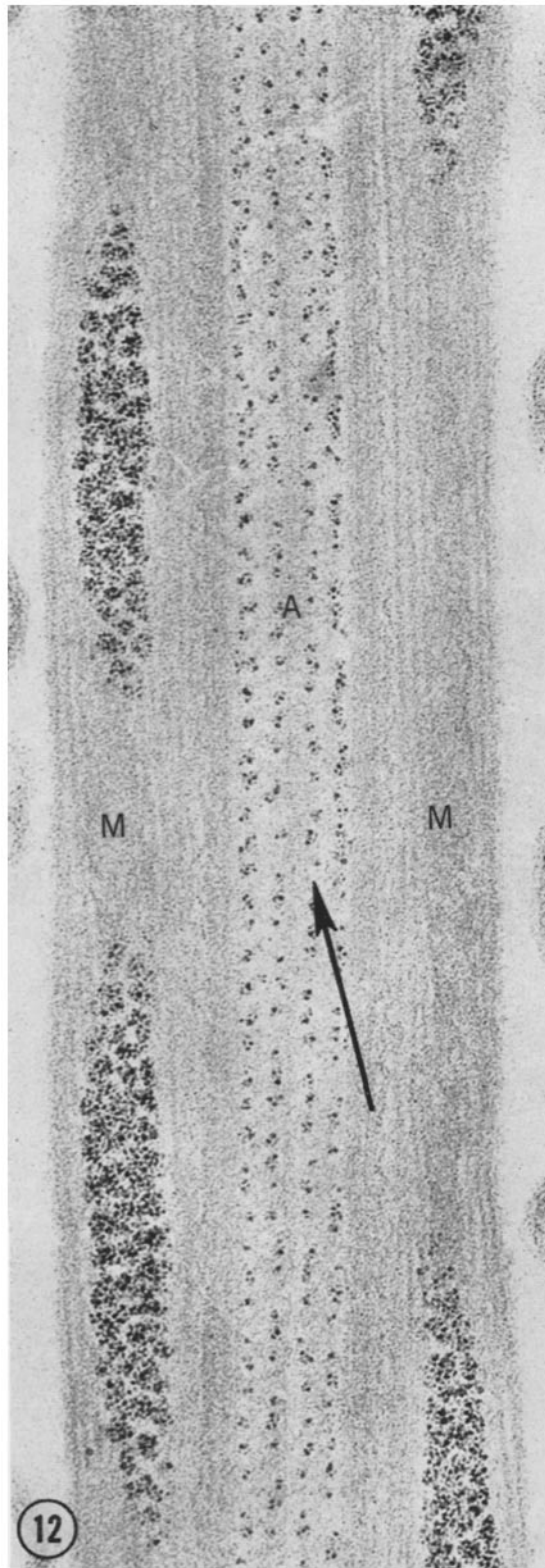
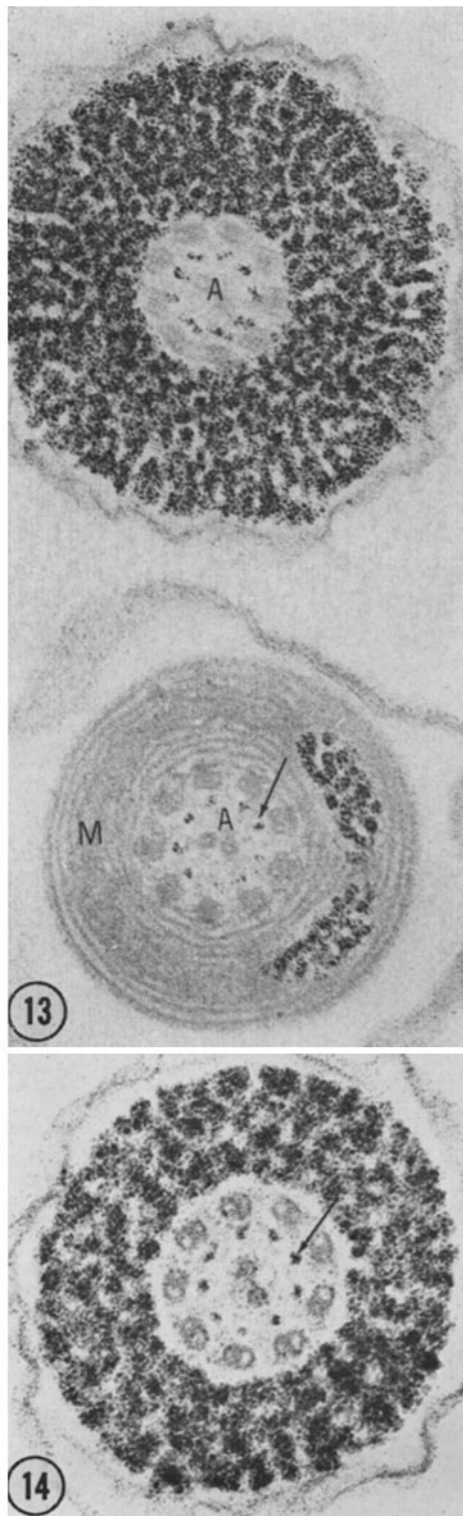


FIGURE 12 This micrograph illustrates a longitudinal section through the middle piece (*M*) of sperm of *Planorbis*. Opaque granules (arrow) with staining properties of glycogen are linearly arranged within the axoneme (*A*) of this spermatozoon. $\times 90,000$.



partment in which they reside is membrane-bounded and is thus set off from the other components of the mitochondrion.

Although the helical compartment is already identifiable within the mitochondrial derivative of spermatids and of immature spermatozoa, glycogen is usually absent at these early stages (Fig. 21). In *Lymnaea* and *Planorbis*, glycogen polymerization seems to commence in the tail piece and subsequently appears in the helical compartments of the mitochondrial derivative. It must be emphasized that in sperm of pulmonate gastropods glycogen accumulation commences after the completion of spermatozoan maturation. Therefore, intracellular glycogen reserves are most prominent in mature sperm in the hermaphroditic duct.

With the hot KOH procedure, ethanol-precipitable glycogen is extracted from spermatozoa of pulmonate gastropods. When samples of glycogen so precipitated are spread on Formvar-coated grids and treated with 1% PTA in 55% ethanol, β -glycogen granules are revealed.

Amylophosphorylase Activity Associated with Glycogen Synthesis

Phosphorylase activity has been demonstrated in spermatozoa of pulmonate gastropods at the light and electron microscope levels (Personne, 1966). Under normal *in vivo* conditions, the enzyme presumably functions in the catabolism of glycogen. Under certain defined conditions *in vitro*, phosphorylase can also catalyze the synthesis of glycogen (Cori and Cori, 1945; Mordoh et al., 1965; Wanson and Drochmans, 1968). Demonstration of phosphorylase activity associated with the *in vivo* synthesis of glycogen has been reported by Takeuchi and Kuriaki (1955) and by Guha and Wegmann (1959, 1961). Thus, when sperm are incubated in medium containing glucose-1-phosphate ATP, Mg^{++} ions, and NaF, the concentration of glycogen in sperm cells increases (Personne

FIGURE 13 Transverse sections through the middle (M) and tail segments of sperm of *Planorbis* are demonstrated. Intensely stained granules (arrow) are located in the matrix between the doublets of the axoneme (A) and the central pair of microtubules. $\times 90,000$.

FIGURE 14 A transverse section through the tail piece of a spermatozoon of *Lymnaea* is illustrated here. Glycogen granules are arranged as a thick mantle around the axoneme. Opaque granules (arrow) are shown at the level of the secondary fibers within the axoneme. $\times 120,000$.

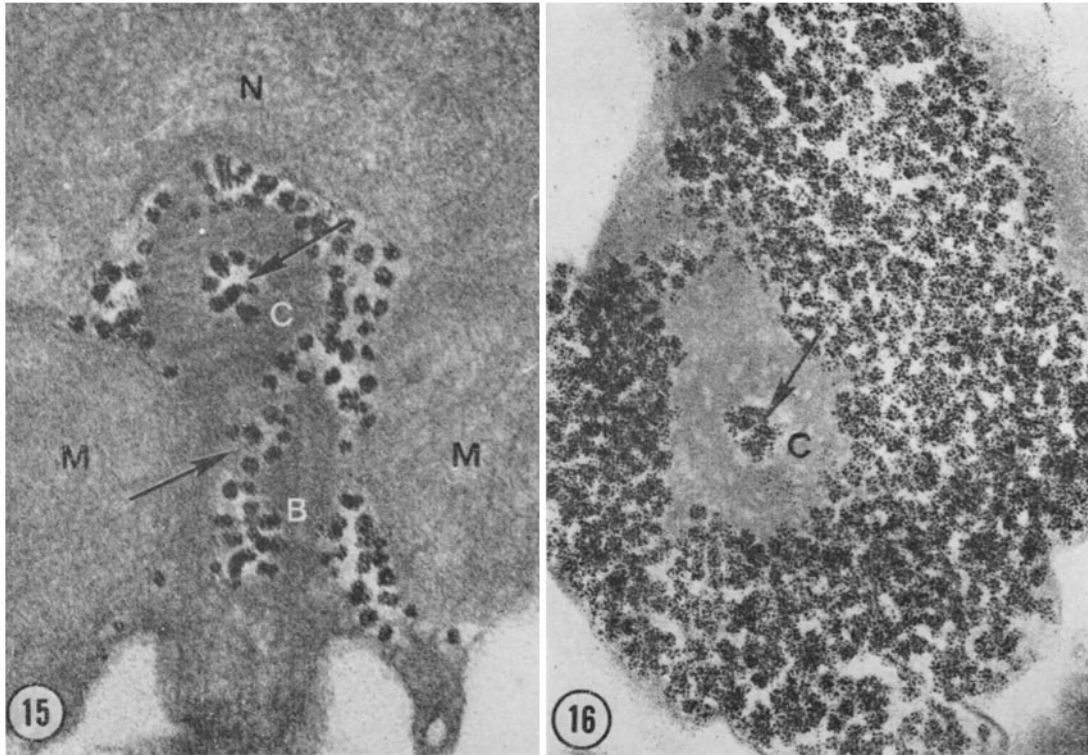


FIGURE 15 A longitudinal section through the basal region of a spermatozoon of *Mytilus* is demonstrated in this micrograph. Glycogen granules (arrows) occupy the concavity of the proximal centriole (C) and of the basal body (B). Glycogen granules are also present in the matrix between the nucleus (N) and mitochondria (M) and around the centrioles. $\times 130,000$.

FIGURE 16 This micrograph illustrates a transverse section through the centriole (C) of a spermatozoon *Pecten*. The glycogen granules are tightly packed in the lumen of the centriole (arrow) and are closely apposed to its outer wall. $\times 130,000$.

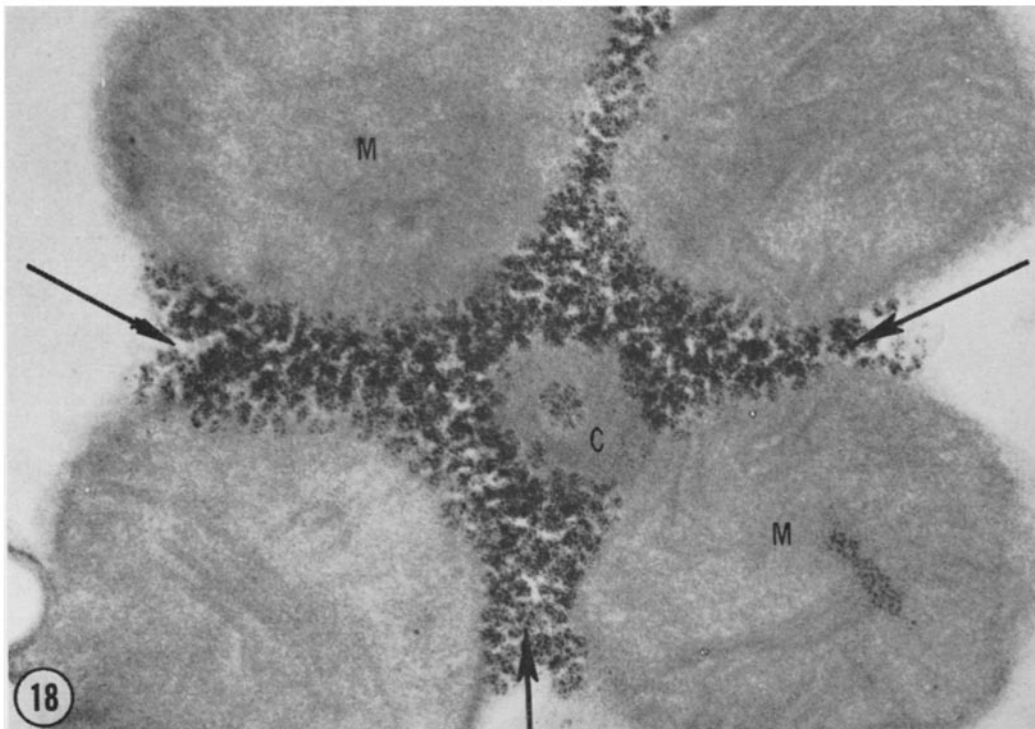
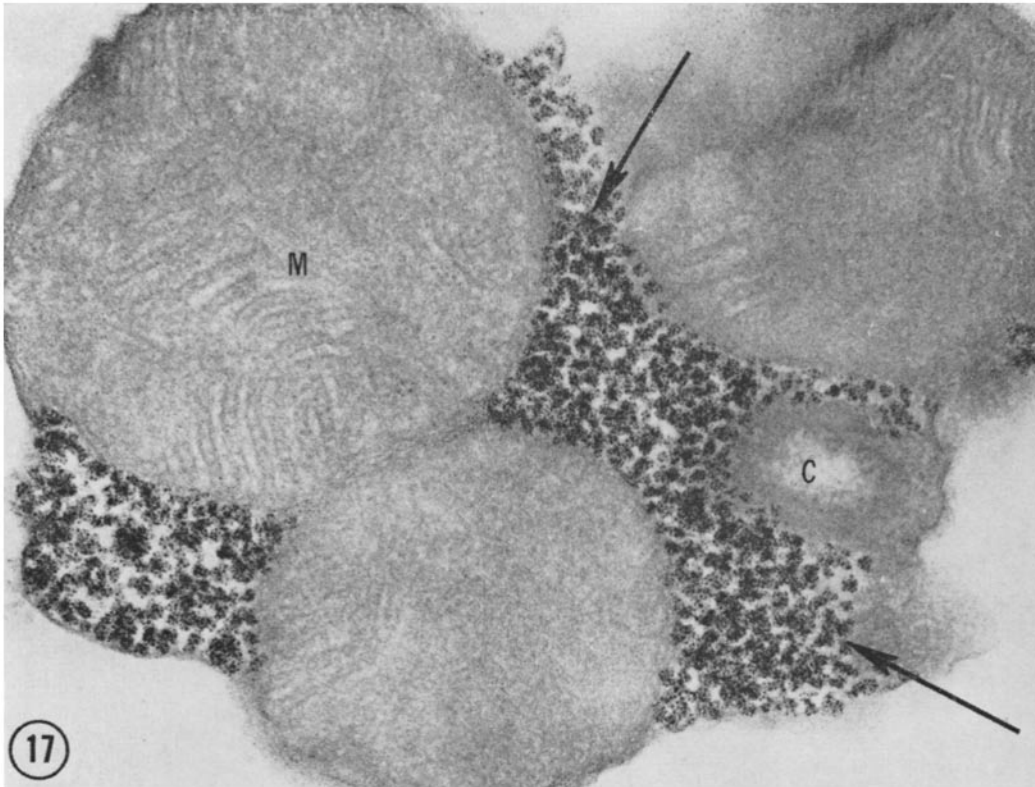
and Anderson, 1969). The newly formed product is finely granular (approximately 40 Å in diameter) and closely resembles the gamma particles described by Drochmans (1962). The fine-grained product of glycogen synthesis lies adjacent to pre-existing glycogen deposits. When AMP is substituted for ATP, the phosphorylase activity is weak. The newly formed particles believed to be glycogen are extracted after treatment with α -amylase and partially extracted by β -amylase. Control specimens incubated in substrate-free medium show no increase in glycogen concentration. A fuller report on the localization of amylophosphorylase activity in spermatozoa will be published separately (Personne and Anderson, 1969).

Glycogen in Atypical Spermatozoa of Prosobranch Gastropods

Prosobranch snails produce long threadlike,

uniflagellate sperm and enormous numbers of atypical, vermiform spermatozoa. Atypical spermatozoa lack distinct acrosomal and nuclear compartments and a well defined middle piece. In addition, these vermiform spermatozoa are multiflagellate and capable of vigorous movements throughout their entire length. Though metabolically active and motile, they are incapable of effecting fertilization. Detailed studies on the development and structural polymorphism of these spermatozoa have been made by Hanson et al. (1952), Kaye (1958), Gall (1961), and Yasuzumi (1960).

Atypical spermatozoa of *Pomacea* and *Viviparus* (*Paludina*) are quite similar in ultrastructure. They are composed of an anterior cylindrical portion and a posterior tuft of flagella. The anterior cylinder is composed of several rectangular or pyramidal structures that surround a central canal.



FIGURES 17 and 18 Intensely stained glycogen granules (arrows) are closely apposed to the peripheral leaflet of the mitochondrial envelope of these spermatozoa of *Pecten* Fig. 17) and *Mytilus* (Fig. 18). The granules are tightly packed between adjacent mitochondria (*M*) and around the centriole (*C*). $\times 80,000$.

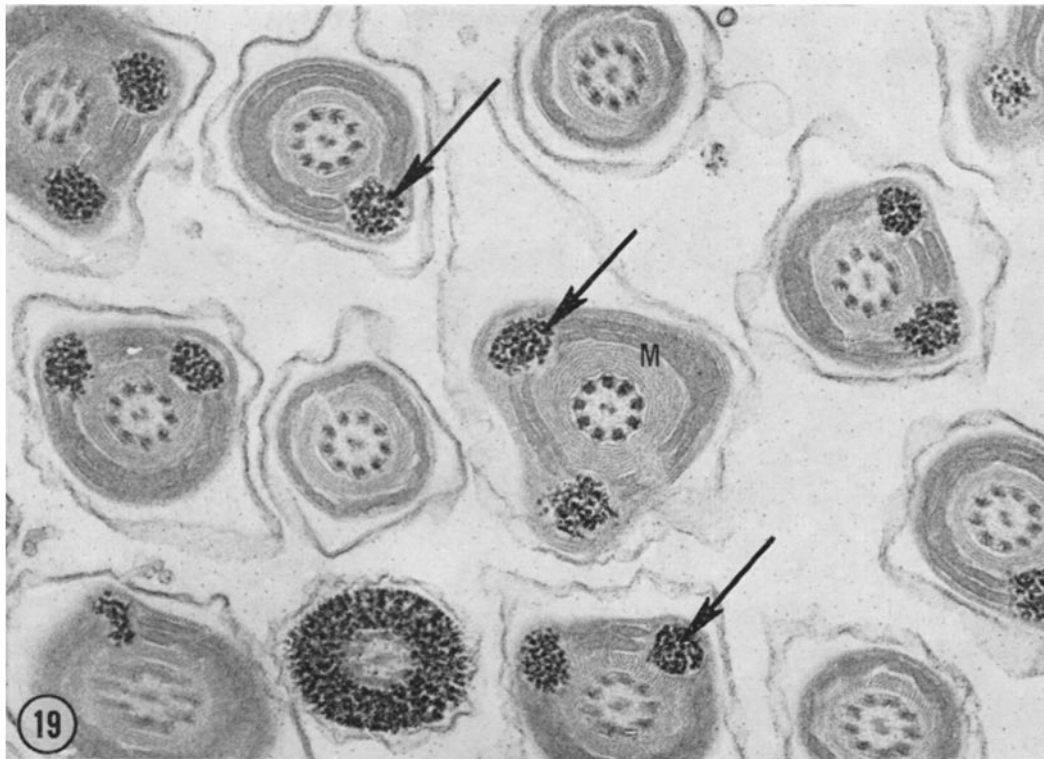


FIGURE 19 Transverse sections through the middle and tail piece of sperm of *Lymnaea* are illustrated here. In the middle piece, glycogen granules are ensheathed within special compartments (arrows) of the mitochondrial derivative (M). $\times 40,000$.

Mitochondria, dense rods, and several axonemes occupy this canal. Glycogen particles are distributed among the structures forming the anterior cylinder, within the axonemes, and in the central canal itself (Figs. 22-23). Glycogen granules appear within and around the axonemes of flagella at the base of these vermiform spermatozoa (Fig. 24).

Distribution of Glycogen in Spermatozoa of Chordates

Mature spermatozoa of the Ascidiacea, *Ciona* and *Molgula*, all contain rosettes of α -glycogen granules. These glycogen particles are located beneath the plasmalemma surrounding the nucleus and mitochondria. Glycogen is absent from the flagella of these spermatozoa.

Beta particles appear in the "packing" cytoplasm around the mitochondria in spermatozoa of the toad fish, *Opsanus tau*. Spermatozoa of the guppy, *Lebistes reticulatus* contain both α - and

β -glycogen granules. In this case too, the glycogen is located in the cytoplasm adjacent to the mitochondria.

Sperm of the amphibians *Rana* and *Bufo* retain some peripheral cytoplasm, and β -particles are randomly distributed in this residual cytoplasm.

The following table (Table I) presents a summation of the sperm of various taxa that do and do not contain glycogen. The classification (Types I, II, III) under Mollusca is not based on international rules, but is a convenience used by us to place sperm with similar mitochondrial derivatives into three distinct groups. Thus, Type I includes "primitive" spermatozoa with relatively unchanged mitochondria; Type II, sperm possessing an elongate, membranous mitochondrial derivative; and Type III, sperm possessing a paracrystalline mitochondrial derivative.

DISCUSSION

Previous electron microscope observations on

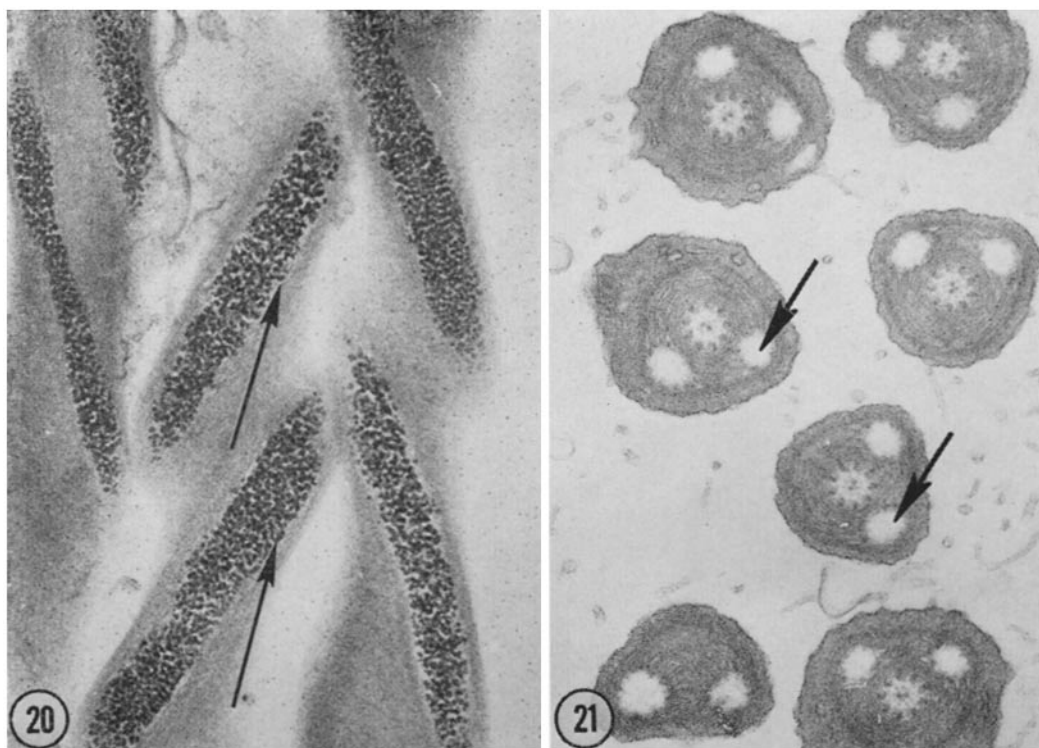


FIGURE 20 Tangential sections through the middle piece of mature spermatozoa isolated from the hermaphroditic duct of *Lymnaea*. The glycogen granules occupy compartments (arrows) that course low-pitched helices within the paracrystalline mitochondrial sheath. $\times 30,000$.

FIGURE 21 Transverse sections through the middle piece of immature sperm in the ovotestis of *Lymnaea*. After treatment with the PA-TSC-SP procedure, the compartments encased by the mitochondrial sheath appear clear (arrows) and lack glycogen deposits. $\times 30,000$.

spermatozoa of some of the species examined in this report have consistently failed to demonstrate the presence of glycogen. Failure to detect glycogen could have resulted from (1) the inability of routine techniques to demonstrate the very low concentrations of polysaccharide present, (2) a masked state of the polysaccharide, or (3) the extraction of glycogen following inadequate fixation. In addition, it must be emphasized that the factor of maturity is important in the accumulation of glycogen, since in specimens examined by us glycogen stores appeared only in spermatozoa that have completed their differentiation. Double fixation by aldehydes and osmium tetroxide may facilitate glycogen preservation by fixing associated proteins, but double fixation alone does not augment the contrast of the polysaccharides. It appears then, that negative results for glycogen with routine methods are not indicative of its absence.

Cytochemical tests that augment the contrast of glycogen at the electron microscope level are now well developed (Hanker et al., 1964; Seligman et al., 1965; Thiéry, 1967). It must be emphasized that the PA-TSC-SP procedure is a direct modification of the PA-TSC-OsO₄ procedure originally described by Hanker et al. (1964) and by Seligman et al. (1965). In this procedure, the aldehyde-thiosemicarbazones formed as a result of the periodate oxidation of 1, 2 glycol groups in polysaccharides and reaction with thiosemicarbazide are revealed by their affinity for silver proteinate. As Thiéry clearly indicated, when the thiosemicarbazide reaction is short (30–45 min), the PA-TSC-SP procedure is specific for components containing high concentrations of polysaccharide and for relatively pure polysaccharide complexes as glycogen. Based on its specificity and reproducibility, the periodic acid-thiosemicarbazide-silver proteinate pro-

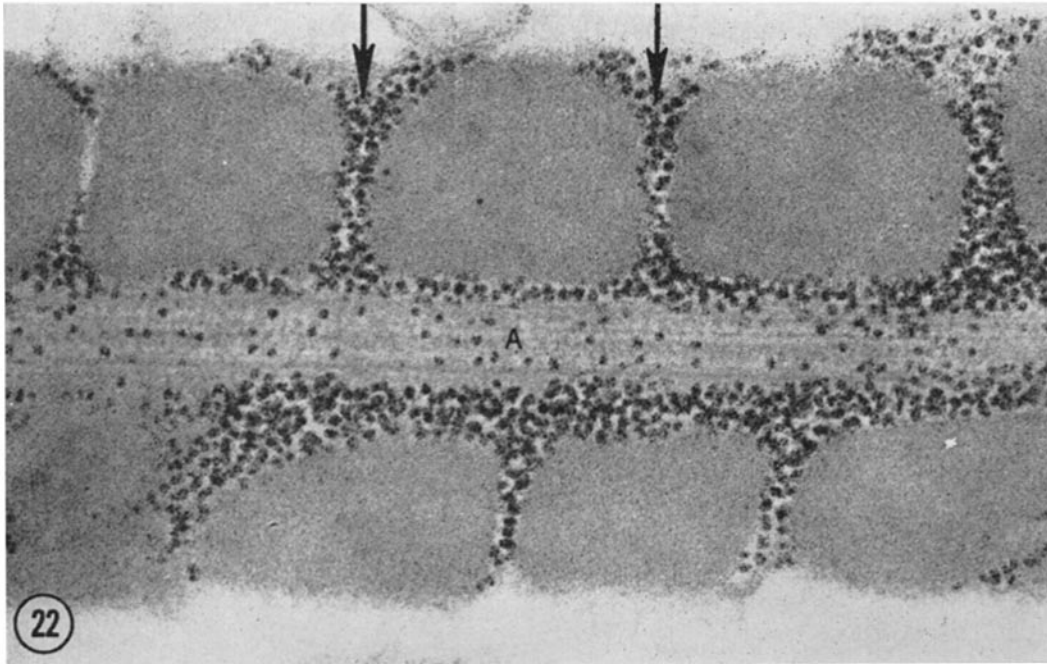


FIGURE 22 A longitudinal section through the anterior, cylindrical region of an atypical sperm of *Pomacea*. Glycogen granules are present within and around the axoneme (*A*), and in the matrix between the structures composing the walls of the cylinder (arrows). $\times 50,000$.

cedure of Thiéry (1967) proves most reliable for the detection of small amounts of glycogen.

Significance of Glycogen in Spermatozoa

Depending upon the species, the survival time of spermatozoa within gamete storage organs varies from weeks to months. Spermatozoa are consequently specialized to survive under conditions of high sperm density and of low oxygen tension. The relative absence of oxygen and of exogenous glycolyzable material suggests that spermatozoan survival in these forms depends neither upon aerobic oxidative processes nor upon the utilization of extracellular glycolyzable substances, but probably upon the anaerobic utilization of endogenous substrates. Glycogen located in the middle piece and flagellum of spermatozoa of some species may well be an important endogenous energy source.

Spermatozoa of some species are released into an aqueous environment that is devoid of metabolizable substances and varies extensively in its degree of oxygenation. These sperm reportedly depend entirely upon aerobic oxidative metabolic

processes (Rothschild, 1951; Rothschild and Cleland, 1951; Afzelius and Mohri, 1966); yet sperm of some species survive for several hours in the absence of oxygen (Barron, 1932). Moreover, biochemical analyses establish the existence of significant amounts of glycogen within spermatozoa of some of these species. Sperm of *Ciona* and *Phalusia* (Ascidacea), respectively, yield 2.8 and 1.6% glycogen on a dry weight basis (Restivo and Reverberi, 1957). Mature sperm of *Saxostrea* contain as much as 1% glycogen. These findings indicate that sperm of these species have stored glycogen. The presence of intracellular glycogen would seem to indicate a capacity for survival where no exogenous substrates are available. Under conditions in which aerobic oxidative processes are inhibited, sperm of the oyster metabolize exogenous materials (Humphrey, 1950). In the presence of fructose diphosphate, glycolytic processes account for $0.3 \mu\text{l}$ of lactic acid/ 10^8 sperm/hour. It seems possible, therefore, that, like most cells, sperm of some marine species can break down carbohydrates either aerobically or anaerobically.

Spermatozoa that effect internal fertilization

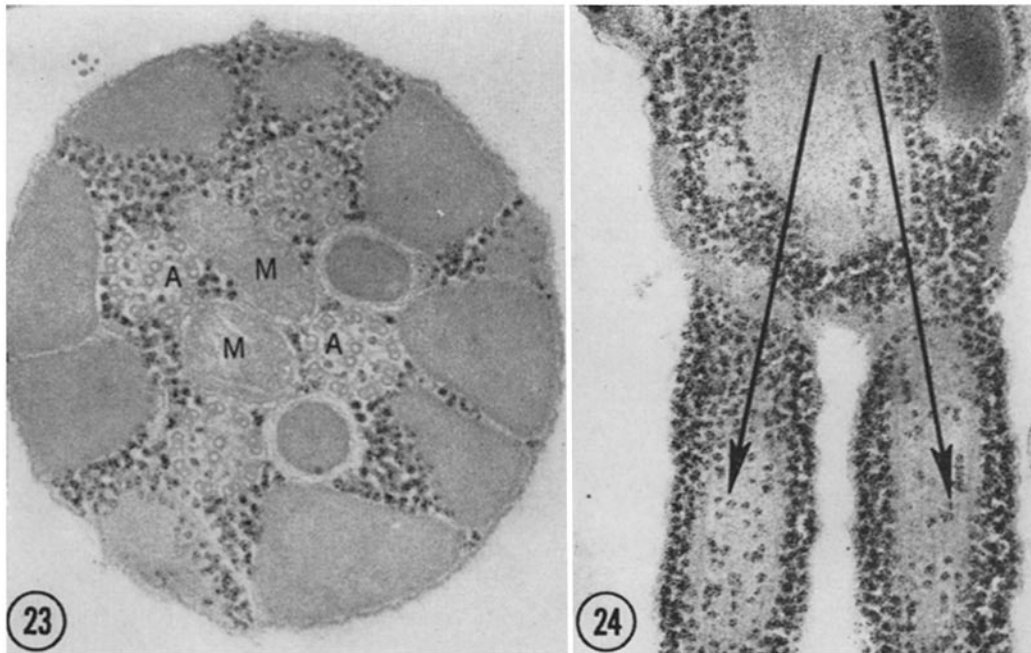


FIGURE 23 A transverse section through the anterior cylindrical region of an atypical spermatozoon of *Viviparus*. Glycogen granules are present between the components of the walls of the cylinder, and in the central canal surrounding the axonemes (*A*) and mitochondria (*M*). $\times 40,000$.

FIGURE 24 A longitudinal section through the basal region of an atypical sperm of *Pomacea*. β -particles occupy the central canal of the anterior cylinder and in the flagella that extend from it (arrows). $\times 40,000$.

are usually released in seminal plasma that is rich in hexoses and other metabolizable substances. Mammalian spermatozoa possess the full complement of enzymes for catalyzing glycolytic activities under aerobic or anaerobic conditions, as well as for oxidative processes under appropriate conditions (Mann, 1964). Since the prefertilization milieu is rich in metabolizable material, mammalian sperm do not rely on endogenous substrates for energy production except under unusual conditions. It is not surprising, therefore, that sperm of these species lack stored glycogen.

Spermatozoa of gastropod molluscs that effect internal fertilization possess relatively large stores of intracellular glycogen which is located within the mitochondrial derivative, as well as within and/or surrounding the axoneme. Thus, the glycogen stores are located in close proximity to the energy-producing and energy-utilizing components. The glycogen presumably serves as a source of energy for metabolism and motility. The existence of an active phosphorylase in the glycogen

compartment (Personne, 1966; Personne and Anderson, 1969) and of glucose-6-phosphatase activity in glycogen-containing mitochondria (Anderson et al., 1968; Anderson, 1968) strongly suggests the presence of pathways for glycogen metabolism in these cells.

The Distribution and Localization of Glycogen in Spermatozoa

The appearance and form of glycogen in spermatozoa vary with the species. However, it seems apparent that β -particles represent the most common form observed in both vertebrates and invertebrates. Among the species studied, α -particles are present only in spermatozoa of some pulmonate gastropods, in the Ascidiacea, *Ciona* and *Molgula*, and in the poeciliid fish, *Lebistes reticulatus*.

The arrangement and localization of glycogen in sperm is somewhat related to the degree of modification of their middle piece. In the so called "primitive" spermatozoa possessing typical mito-

TABLE I
Distribution of Glycogen in Spermatozoa

| Phylum | Species | Distribution of glycogen | Reference | |
|--|--|----------------------------|-----------------------------------|--|
| <i>Platyhelminthes</i> | | | | |
| Turbellaria | <i>Dugesia tigrina</i> | | | |
| | <i>Bdelloura candida</i> | Intraflagellar..... | Silveira and Porter 1964 | |
| | <i>Bdelloura propingua</i> | | | |
| Cestoda | <i>Diphyllobothrium latum</i> | Perinuclear..... | von Bonsdorff and Telkka, 1965 | |
| <i>Annelida</i> | | | | |
| Oligochaeta | <i>Lumbricus terrestris</i> | Periaxonemal..... | Anderson <i>et al.</i> 1967 | |
| Hirudinea | <i>Hirudo medicinalis</i> | Periaxonemal | | |
| <i>Mollusca</i> | | | | |
| Lamellibranchia | <i>Type I</i> ("Primitive" spermatozoa with relatively unchanged mitochondria) | | | |
| | <i>Pecten maximus</i> | Perimitochondrial, intra- | | |
| | <i>Mytilus edulis</i> | centriolar | | |
| | <i>Spisula solidissima</i> | | | |
| | <i>Ostrea edulis</i> | | | |
| Gastropoda | <i>Gibbula cineraria</i> | | | |
| | <i>Patella vulgata</i> | | | |
| | <i>Diadora cayensis</i> | | | |
| <i>Type II</i> (Sperm possessing elongate membranous mitochondrial derivative) | | | | |
| Gastropoda | <i>Littorina littorea</i> | Periaxonemal in the tail | | |
| | <i>Littorina obtusata</i> | piece; intra-axonemal in | | |
| | <i>Purpura lapillus</i> | tail piece | | |
| | <i>Nucella lapillus</i> | | Walker and MacGregor, 1968 | |
| | <i>Pomacea cuprina</i> | | | |
| | <i>Viviparus viviparus</i> | | | |
| | <i>Viviparus malleatus</i> | | | |
| | <i>Viviparus japonicus</i> | | | |
| | <i>Type III</i> (Sperm possessing a paracrystalline mitochondrial derivative) | | | |
| | Gastropoda | <i>Planorbis corneus</i> | | |
| <i>Lymnaea truncatula</i> | | Intramitochondrial; intra- | | |
| <i>Lymnaea auricularia</i> | | axonemal; periaxonemal | | |
| <i>Lymnaea stagnalis</i> | | in the tail piece | | |
| <i>Testacella</i> | | | Personne and André, 1964 | |
| <i>Vaginalus borellianus</i> | | | Lanza and Quattrini, 1964 | |
| <i>Arion aggericola</i> | | | | |
| <i>Helix aspersa</i> | | | | |
| <i>Helix pomatia</i> | | | André, 1965 | |
| <i>Otala lactea</i> | | | | |
| <i>Aplysia californica</i> | | | | |

TABLE I—Continued

| Phylum | Species | Distribution of glycogen | Reference |
|-----------------------------|--|--|-----------------|
| <i>Arthropoda Crustacea</i> | | | |
| Cirripedia | <i>Lepas anatifera</i> | Periaxonemal distribution | |
| Decapoda | <i>Carcinus moenas</i> <i>Cambarus clarkii</i> <i>Astacus fluviatilis</i> | Negative | |
| <i>Chilopoda</i> | <i>Geophilus linearis</i> | In canals in the middle piece. . . | Horstmann, 1968 |
| <i>Insecta</i> | | | |
| | <i>Drosophila melanogaster</i> <i>Locusta migratoria</i> <i>Blatta orientalis</i> <i>Periplaneta americana</i> | Negative | |
| <i>Echinodermata</i> | | | |
| Echinoidea | <i>Paracentrotus lividus</i> <i>Arbacia lixula</i> | Intramitochondrial Negative | Anderson, 1968 |
| <i>Chordata</i> | | | |
| Ascidiacea | <i>Ciona intestinalis</i> <i>Molgula manhattensis</i> | Perinuclear, perimito- chondrial | |
| Pisces | <i>Gasterosteus aculeatus</i> <i>Xiphophorus helleri</i> <i>Platylocelia maculata</i> <i>Lebistes reticulatus</i> <i>Opsanus tau</i> | Negative Perimitochondrial | |
| Amphibia | <i>Rana pipiens</i> <i>Bufo arenarium</i> <i>Triton cristatus</i> | Perimitochondrial Negative | |
| Reptilia | <i>Holbrookia texana</i> <i>Urosaurus ornatus</i> | Negative Negative | |
| Aves | <i>Columba livia</i> | Negative | |
| Mammalia | <i>Rattus norvegicus</i> <i>Mus musculus</i> <i>Meriones unguiculatus</i> <i>Citellus tridecemlineatus</i> <i>Cavia porcellus</i> | Negative | |

chondria at the base of the nucleus (Franzen, 1956), glycogen particles are tightly packed within all available space in the middle piece. Although glycogen is mainly located in the "packing" matrix between the mitochondria, it is even present in some instances within the centriole and basal body, and in indentations of the nucleus in sperm of some species. The presence of glycogen in centrioles and basal bodies may have no special metabolic signifi-

cance but may simply reflect storage in all available space. In the "primitive" spermatozoa, the glycogen is closely apposed to the external envelope of the mitochondria and to the base of the kinetic apparatus. The "primitive" sperm in question that store glycogen have some of the enzymes needed to break it down, such as glucose-6-phosphatase (Anderson, 1968).

Glycogen is usually absent from the middle

piece of more complex flagellate sperm. Instead, it is located adjacent to the axonemal components in the distal segments of the sperm tail in the region between the peripheral doublets and the flagellar membrane. This particular arrangement of glycogen is characteristic of spermatozoa of such widely separated species as terrestrial and freshwater annelids (*Lumbricus*, *Hirudo*), marine gastropods (*Purpura*), typical sperm of freshwater prosobranchs (*Viviparus*; *Pomacea*), and in sperm of the cirriped crustacean, *Lepas*. The close topographical relationship of glycogen to the axoneme suggests a functional relationship between the two.

Glycogen granules are specifically located in membrane-bounded compartments in typical mitochondria (Anderson, 1968) and in the complex paracrystalline mitochondrial derivative of sperm of pulmonate gastropods (Lanza and Quattrini, 1964; Personne and André, 1964; André, 1965). The presence of glycolytic enzymes (glyceraldehyde - 3 - phosphate dehydrogenase, LDH) in the immediate proximity of the glycogen granules was shown in a previous paper (Anderson and Personne, 1969). The presence of phosphorylase activity in the glycogen compartment also indicates the existence of glycogen synthetic and degradation enzymes within this compartment (Personne and Anderson, 1969). Hypotheses on the interrelations of glycogen and of other metabolic pathways have already been discussed (Anderson et al., 1968).

The intra-axonemal localization of glycogen in spermatozoa of pulmonate and prosobranch gastropods is highly unusual. In these sperm, granules with the staining properties of glycogen and which are extractable with α -amylase are located at the level of the secondary fibers between the central and peripheral doublets. The functional significance of this arrangement is unknown.

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Spermatozoa of some species (e.g. insects, birds, and mammals) do not contain stores of glycogen granules *per se*, even though, on the basis of their ability to flagellate actively, they are considered mature. In some specimens (e.g. insects, Baccetti et al., 1969 *a, b*; Cantacuzene, personal communication) the coarse fibers of the axoneme contain polysaccharide material that bears some similarity to glycogen. It may be unlikely, however, that polysaccharides in structural components of the axoneme are metabolized in energy production.

Glycogen storage, therefore, seems to be a relatively widespread phenomenon among spermatozoa of several invertebrate and vertebrate species (Anderson and Personne, 1969). Evidence that intracellular glycogen reserves may represent an important energy source will be presented in a subsequent report (Anderson and Personne, in preparation).

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