PROTEIN OF INSECT SPERM MITOCHONDRIAL CRYSTALS

Crystallomitin

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ABSTRACT

Mitochondrial derivatives of insect sperm usually contain a crystalline protein that shows a 45-nm main period, made up of 20-nm subperiods, determined by the coiling of filament bundles. Filaments are 2 nm thick and have a globular appearance. The crystals contain two main polypeptides, 52,000 and 55,000 daltons. These polypeptides are closely related, contain a high percentage of proline, and are insoluble in sodium dodecyl sulfate due to disulfide cross links. We suggest for this class of protein the name crystallomitin.

Most insect spermatozoa are characterized by a prominent crystal (or crystalloid) in the matrix of their modified mitochondria. In some species the crystal may be of truly enormous dimensions, approaching or even exceeding 1 cm, and it may make up most of the volume of the spermatozoon (2, 15). It plays a particular role, therefore, in tail movement, and other functions for it have been suggested as well (2).

It occurred to us that it should be possible to isolate these crystals and to characterize their contents by using standard chemical procedures. The obtained data could provide some information on the functional role and the evolution of insect sperm mitochondria. The present report shows the crystals to contain two main polypeptides, for which we propose the term "crystallomitin".

MATERIALS AND METHODS

Electron Microscopy

Adult backswimmers (*Notonecta glauca* L.) were caught in local ponds in the neighbourhood of Siena.

Males were dissected in Hoyle's medium (0.375 g KCl, 3.750 g NaCl, 0.110 g $CaCl_2$, 0.205 g $MgCl_2$, 0.170 g $NaHCO_3$, 0.415 g NaH_2PO_4, 500 cm^3 H_2O), and spermatozoa from vasa deferentia were prepared in the following ways: (a) Spermatozoa were fixed in 4%

paraformaldehyde and 5% glutaraldehyde at pH 7.2 in cacodylate buffer according to Karnovsky's method (8) for 1 h at 4°C. The material was then rinsed overnight in cacodylate buffer, postfixed in 1% osmium tetroxide for 1 h, dehydrated in a graded ethanol series, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate. (b) Spermatozoa were treated with 0.5 M EDTA for 5 h. After centrifugation, the material was rinsed in distilled water and fixed in 2.5% glutaraldehyde in Hoyle's medium, dehydrated, and embedded in Epon. (c) Spermatozoa were treated with 1% sodium dodecyl sulfate (SDS) in 0.75 M Tris HCl, pH 8.8, for 30 min, fixed as in (a), rinsed overnight in cacodylate buffer, postfixed, and embedded as in (a). (d) Spermatozoa were treated with EDTA as in (b), and subsequently with 0.1% SDS for 40 min. Fixation and embedding were carried out as in (c). For making negatively stained preparations, a drop of sperm dispersion was placed on a coated grid and washed with distilled water. A drop of 1.5% uranyl acetate or 1% phosphotungstic acid (PTA) at pH 6–7.2 was then added to the grid, the excess fluid was removed, and the preparation was allowed to air dry.

For freeze-etching the vasa deferentia were incubated for 1 h at 4°C in 25% glycerol in Hoyle's medium, transferred to small gold disks, and rapidly frozen (2–5 s) in Freon 22 cooled with liquid nitrogen. Freeze-etching was carried out on a Balzers apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.) as described by Moor et al. (12). The carbon-platinum replica was treated with

chromic acid, rinsed in distilled water, and picked up on copper grids.

All preparations were examined in a Philips 301 EM.

For electron probe X-ray microanalysis, vasa deferentia were fixed in 2.5% glutaraldehyde in Hoyle's medium. The material was dehydrated in ethanols, embedded in Epon, and 70- to 100-nm sections were cut on an LKB microtome (LKB Instruments, Inc., Rockville, Md.). The sections were mounted on Formvar films or on carbon-coated Formvar films (20 nm thick) on titanium, copper, or gold grids (according to the elements to be detected). Pellets of purified crystals obtained from SDS-treated spermatozoa (see above) were mounted on grids. X-ray microanalysis was carried out with the Philips 300 EM provided with a scanning attachment, an EDAX system, a double tilt, 50-200 s analysis time, and a 250 nm beam diameter.

Amino Acid Analysis

The SDS-resistant fraction of Notonecta sperm obtained as described above was washed with water, lyophilized, reduced with 0.1 N dithiothreitol in 7 M guanidine HCl and 0.5 M Tris HCl pH 8.5 for 3 h at 37°C, and alkylated by addition of iodoacetamide to 0.25 M; the protein was recovered from the reaction mixture by precipitation with 5 vol of absolute ethanol and dried in vacuo. Hydrolysis was performed with 6 N HCl for 24 h at 110°C according to Moore and Stein (13). The hydrolysates were brought to dryness under vacuum in a desiccator over P_2O_5 and NaOH. Amino acid analysis was performed, using the M 82 single column program on a Beckman Multichrom liquid chromatograph (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

Electrophoresis on SDS Polyacrylamide

Weighed amounts of SDS-resistant fraction from Notonecta sperm were dissolved in the "sample buffer" (0.0625 M Tris HCl buffer, pH 6.8, 2% SDS, 1% glycerol, 5% mercaptoethanol, and 0.001% bromphenol blue) described by Laemmli (9) at a concentration of 1 mg/ml by heating in a boiling water bath for 2 min. Electrophoresis was performed on 10% polyacrylamide separation gels, using the high pH discontinuous system described by Laemmli (9). Gels were stained with Coomassie blue in methanol and destained by the procedure of Meizel (11). For molecular weight determination, gels were calibrated with rabbit muscle myosin prepared by the method of Nauss et al. (14), rabbit muscle actin prepared according to Drabikowsky and Gergely (4), tubulin (present in whole sea urchin axonemes) purified according to Shelansky and Taylor (17), and with bovine serum albumin, γ-globulin, cytochrome C, which were obtained from Sigma Chemical Co., St. Louis, Mo.

Infrared Spectroscopy

Infrared spectra were recorded with a Perkin-Elmer 225 grating infrared spectrophotometer (Perkin-Elmer Corp., Electro-Optical Div., Norwalk, Conn.) from K Br pellets of lyophilized SDS-resistant fraction of Notonecta sperm.

RESULTS

Morphology

In a cross section of the Notonecta sperm tail, the mitochondrial derivative can be seen to contain three crystals (Fig. 1) which, in the negatively stained preparation show a characteristic longitudinal periodicity of 45 nm. Each period is divided into two subperiods of equal length, about 20 nm. The appearance is that of a textile with a fish-bone pattern (Fig. 3). The same pattern occurs in all three crystals. By freeze-etching, the crystals can be seen to be made up of longitudinal filaments (Fig. 5). Sonication does not effect the crystal structure (Fig. 4).

Extraction in 0.2 M SDS does not influence the disaggregation process of the crystals or the packing of the filament units, whereas other structures of the sperm tail, such as the axoneme or the membranes, are lost (Fig. 2). Hence, it has been possible to determine that the crystal accounts for 84% of the lyophilized weight of the sperm. This is in agreement with the volume calculated from electron micrographs, which is between 85 and 90%. This method of extraction provides pure preparations of mitochondrial crystals (Fig. 11).

Proteolysis with pronase and trypsin gradually solubilizes the crystal, leaving a residue with the same period as that of the starting material. On the other hand, the residue after papain action consists of crystals more or less dissociated into fibrils. In negatively stained preparations, the fibrils are 7 nm thick and appear helical, with a pitch of 20 nm, corresponding to the basic period of the crystal (Fig. 6). Some fibrils, if they have been mechanically fragmented, appear to give rise to a bundle of isolated filaments, 2 nm thick (Fig. 7). Treatment with EDTA results in mitochondrial derivatives in which the membrane is lost and in which the filamentous bundles are no longer in parallel register (Fig. 8). In negatively stained preparations, the individual filaments can be seen to be made up of globular subunits about 20 Å in size (Fig. 9).

Chemical Properties

The SDS-resistant fraction of the sperm tail (Fig. 11) is insoluble in 1 M KCl. This fraction is also insoluble in 7 M guanidin- HCl. However, if
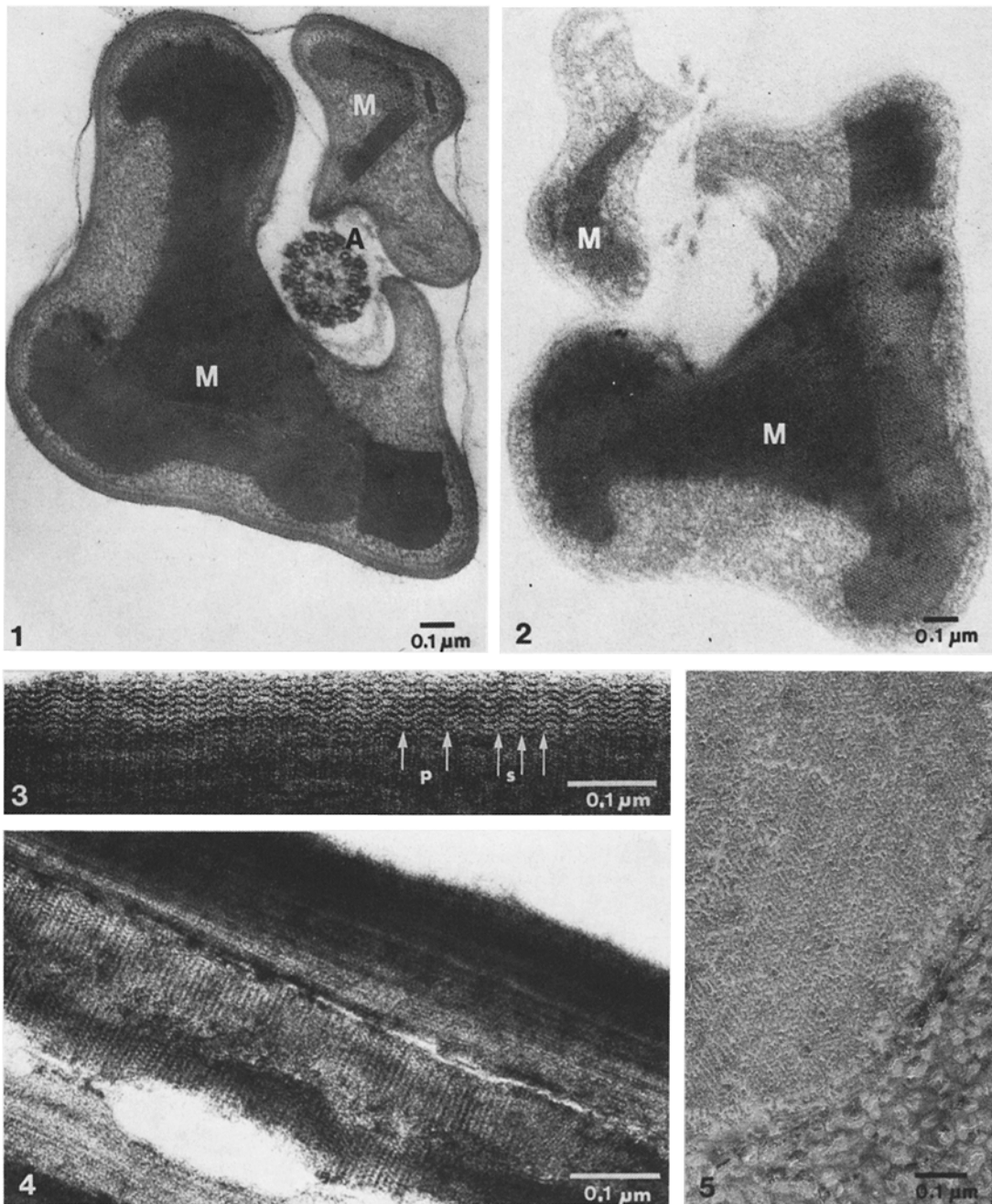
FIGURE 1   *Notonecta glauca* cross sectioned sperm tail. The two crystallized mitochondrial derivatives (*M*) are evident around the axoneme (*A*). × 50,000.

FIGURE 2   The same, after SDS extraction. Only mitochondrial derivatives (*M*) are evident; the axoneme has been removed. × 50,000.

FIGURE 3   Mitochondrial derivative after PTA negative staining. The 45-nm period (*p*) and the two subperiods (*s*) are evident. × 130,000.

FIGURE 4   Mitochondrial derivative after mechanical fragmentation and PTA negative staining. × 130,000.

FIGURE 5   Frozen-etched mitochondrial derivative. The crystalline texture is evident. × 75,000.
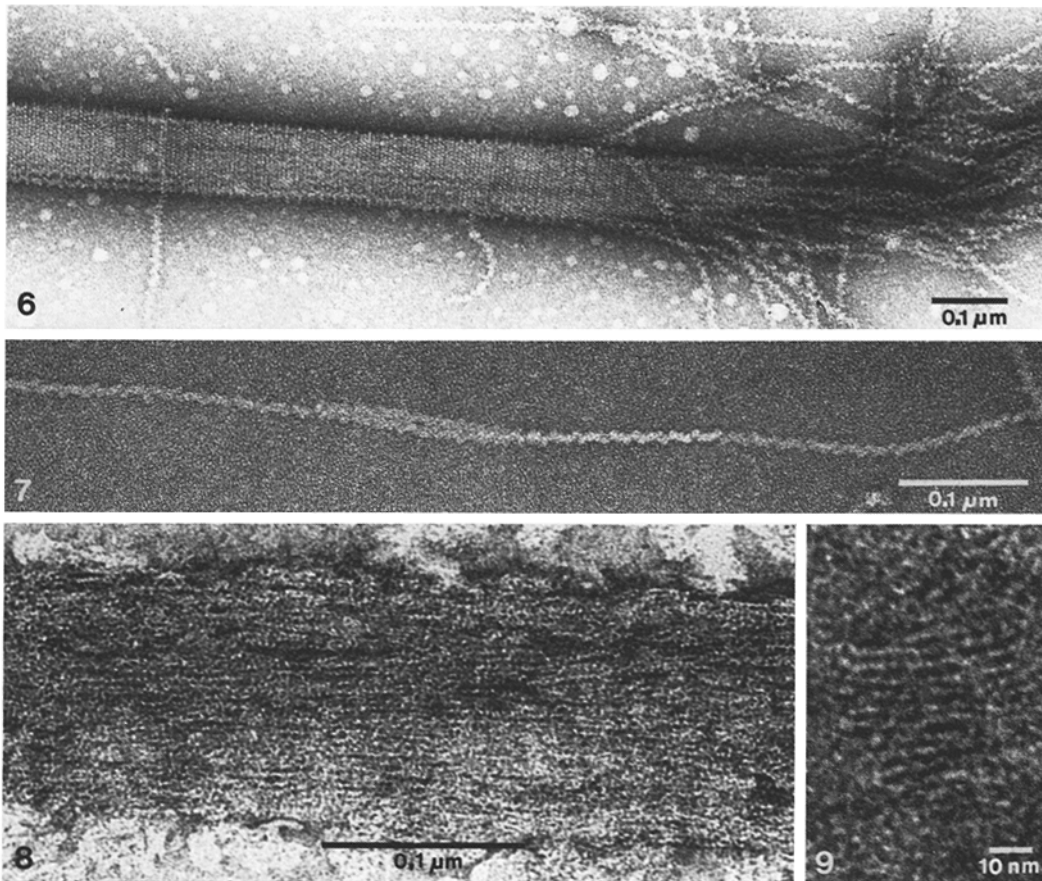
FIGURE 6 SDS-extracted, papain-digested mitochondrial derivative. Helicoidally coiled elements are evident, packed according to the period. PTA negative staining. × 100,000.

FIGURE 7 Same preparation. An individual coil appears to be made up of filaments. × 170,000.

FIGURE 8 EDTA-SDS-extracted mitochondrial derivative. The filaments appear to be made up of globular subunits. PTA negative staining. × 270,000.

FIGURE 9 The same, at higher magnification. × 560,000.

mercaptoethanol is added to the extraction medium (SDS or guanidine-HCl) to a final concentration of 0.1 M, a complete solubilization is rapidly achieved.

The occurrence of disulfide bonds in this structure is clearly demonstrated by chemical determinations which indicate that only about 30% of the total cysteine is present as —SH groups (0.08 μmol over 0.24 μmol/mg of lyophilized weight). 0.1-N HCl extracts of the crystal do not show absorbancy in ultraviolet light. By electrophoresis, the mercaptoethanol-solubilized, SDS-resistant fraction can be found to contain two

predominant components having molecular weights of 52,000 and 55,000 daltons (Fig. 12). Following the method of Sacharius et al. (16) and that of Fairbanks et al. (6), it has been demonstrated that these two polypeptides are not glycoproteins.

The amino acid composition of the whole crystal is given in Table I. The content of proline (7–8%) is notably high. Cysteine represents 3–4% of the total amino acid residues.

From the ultraviolet spectrum of the reduced alkylated protein dissolved in 6 M urea at pH 8.5, it can be deduced that tryptophan is very low in
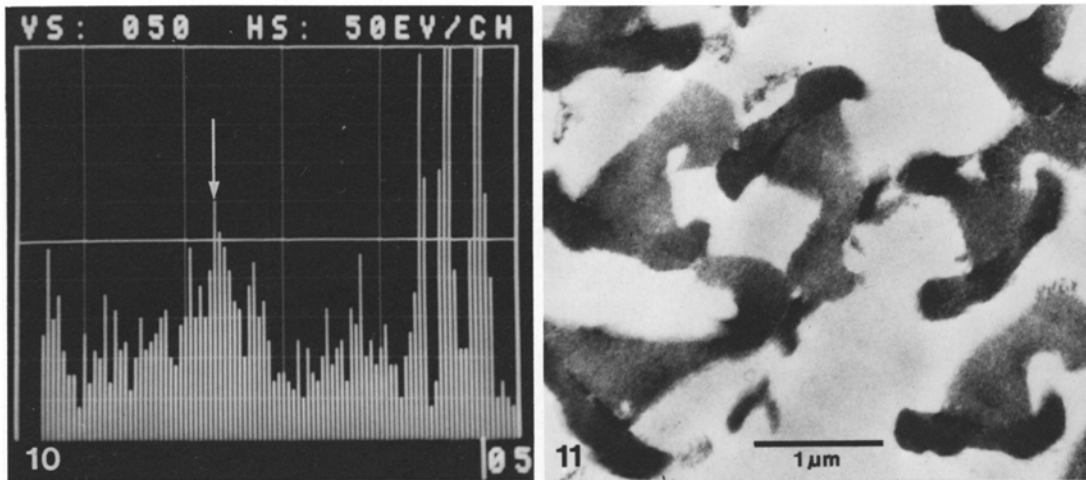
FIGURE 10 Electron probe microanalysis of purified *Notonecta* mitochondrial derivatives. The high content of sulfur (arrow) is evident.

FIGURE 11 Thin section of a pellet of EDTA-SDS-extracted, isolated *Notonecta* mitochondrial derivatives. The preparation is quite pure. × 17,000.

amount or absent. The absorption maximum occurs at 277 nm, with an absorbancy of about 0.8/mg per ml.

Electron probe microanalysis on thin sections and purified crystals has not demonstrated appreciable signals of Ca, Zn, or of any other metal. A clear signal of sulfur, however, has been recorded (Fig. 10).

The infrared spectrum of a KBr pellet of a *Notonecta* crystal is shown in Fig. 13. The amide I and amide II bands are clearly visible, with a maximum at 1,650 and 1,535 cm^{-1}, respectively. The peaks are complicated by the presence of shoulders, so that the occurrence of various conformations is to be suspected.

DISCUSSION

The most significant findings can be summarized as follows. (a) The mitochondrial crystals in insect spermatozoa show a 45-nm main period, made up of 20-nm subperiods, determined by the coiling of filament bundles. Filaments are 2 nm thick and have a globular appearance. (b) The crystals are comparatively simple and contain just two main polypeptides, of 52,000 and 55,000 daltons molecular weight. (c) The two polypeptides are closely related and contain a high percentage of proline. (d) The polypeptides are insoluble in sodium dodecyl sulfate and guanidine-hydrochloridine due to disulfide cross-links.

The amino acid composition of the two poly-

peptides, besides showing a relatively high proline content, does not have any striking peculiarity.

On the basis of the amino acid composition, the protein of the *Notonecta* crystal is to be distinguished from other insect protein, such as the resilin, arthropodin, and fibroin types. The occurrence of large amounts of leucine and glutamic acid might contribute to an α-helical secondary structure; however, the infrared spectrum indicates the occurrence of various conformations. The molecular weight of the two polypeptide chains present in *Notonecta* crystal resembles that of A and B chains of tubulin; tubulin fibers, however, have never been reported, to our knowledge, to resist the solubilizing action of SDS or to be disulfide cross-linked. The amino acid composition of the mitochondrial crystal is also different from that of tubulin in having a higher content of glycine, alanine, cysteine, and particularly proline. The recently described tubulin aggregates (10) show a clearly different organization.

Stabilization by -S-S cross-links occurs in several sperm structures in various zoological groups. Although it has a paraaxonemal localization, the *Notonecta* mitochondrial protein is clearly distinguished from the accessory fibers of mammals and cephalopods whose structure is definitely similar to that of keratins. Apparently, the described chains represent a class of polypeptides which has not heretofore been described. We propose the name "crystallomitin" for them. The name is de-

FIGURE 13 Amide I and amide II regions of an infrared spectrum obtained from a KBr pellet of *Notonecta* sperm mitochondrial derivatives. The frequencies of absorbancy maxima and shoulders are indicated by vertical lines.
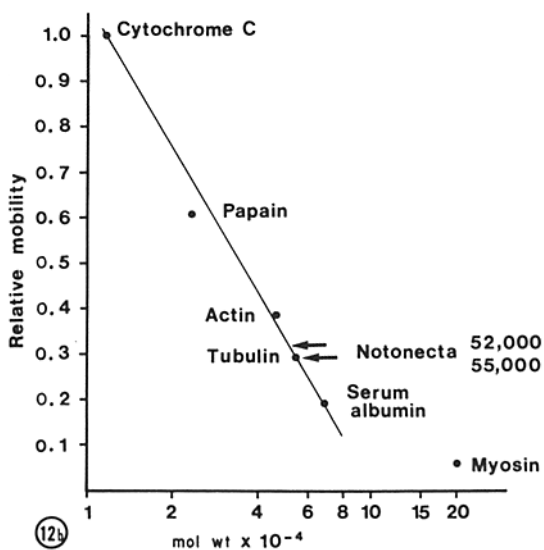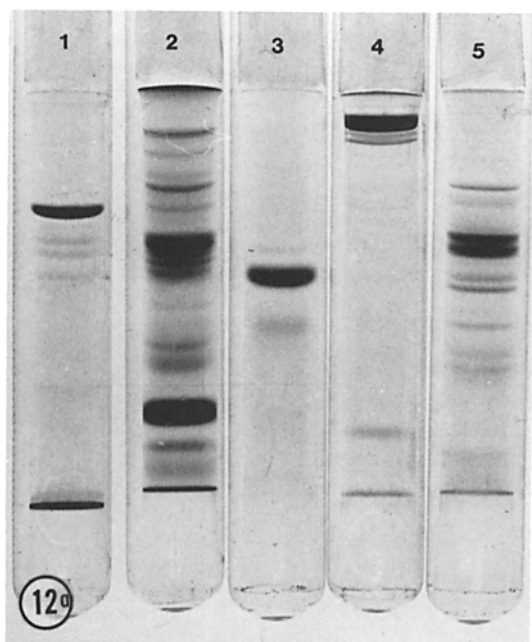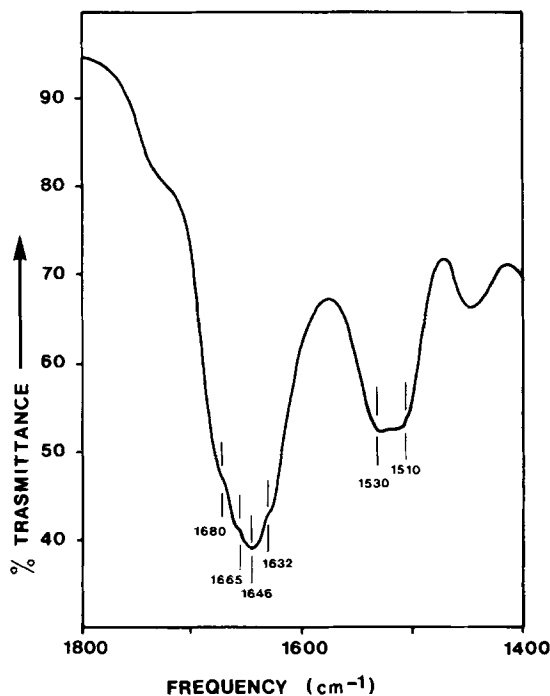

FIGURE 12 Electrophoresis on SDS-polyacrylamide gel and molecular weight determination of the polypeptides present in *Notonecta* sperm mitochondrial derivatives. Gel 1: bovine serum albumin (mol wt 68,000) and cytochrome C (mol wt 11,700); Gel 2: sea urchin sperm axonemes with tubulin (mol wt 54,500) and added papain (mol wt 23,000); Gel 3: rabbit muscle actin (mol wt 45,000); Gel 4: rabbit muscle myosin (mol wt 200,000 for the heavy chain); Gel 5: *Notonecta* sperm (SDS-resistant fraction).

TABLE I

Crystallomitin of Notonecta V. Amino Acid Composition

Residues/100 Residues	
Alanine	8.93
Arginine	4.68
Aspartic acid	9.45
Cysteine	3.84
Glutamic acid	9.21
Glycine	8.93
Histidine	1.85
Isoleucine	3.72
Leucine	8.40
Lysine	6.65
Methionine	2.34
Phenylalanine	3.54
Proline	8.04
Serine	5.96
Threonine	4.97
Tyrosine	2.90
Valine	6.60

rived from Greek κρύσταλλος - crystal and μίτος - thread.

The function of the crystallomitin proteins is a matter of guesses. Attempts to demonstrate in the crystal an enzymatic function have consistently given negative results (cytochrome oxidase, succinic dehydrogenase, and other respiratory enzymes). It appears that the mitochondrial crystal is metabolically inert. For a further discussion of its role, see reference 2. The presence of disulfide bonds suggests that the crystals may have an elastic role similar to that of the parergins in the mammalian and mollusk sperm. The present description is based on only one insect species, but we believe that crystallomitins may be widely distributed in those insect sperm endowed with crystallized mitochondrial derivatives. Previous data suggest that, in any case, the period is similar (1); preliminary investigations indicate the occurrence of similar characteristics and of similar polypeptide chains in the sperm mitochondrion of the Coleopteran *Divales*.

It may be asked whether the crystallomitins are typical for insect spermatozoa or whether they also occur in other cells and tissues. Crystals within the mitochondrial matrix have been found in a great many tissues such as the mammalian liver (18), mammalian kidney (19, 20), mammalian or crustacean skeletal muscle (7 and footnote 1) and *Hydraviridis* (3). There is a rather great variation in the morphology of the crystals from one cell type to another and from one investigator to another, yet most of these crystals are so similar in their basic organization to the crystals of the insect sperm mitochondria that it is likely that they contain a similar material. Because of the easy availability of crystalline mitochondria, the insect sperm provides a convenient system for studying intramitochondrial crystallization.

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