BRIEF NOTES

CYTOCHEMICAL AND BIOCHEMICAL PROPERTIES OF BASIC PROTEINS OF *URECHIS* ACROSOMES

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INTRODUCTION

It has been reported in earlier histochemical studies that acrosomes of some insect sperm contain an appreciable amount of basic proteins besides polysaccharides (9, 11). Recently, Bloch (4) and Chevaillier (6) detected such proteins in capsules, which may be homologous to acrosomes, of crustacean sperm. Results are presented in this report to show that acrosomes of *Urechis caupo*, a marine echiuroid worm, also contain basic proteins which are cytochemically different from basic proteins of sperm and spermatocyte nuclei. In addition, a method for isolation of *Urechis* acrosomes is described, and preliminary biochemical data on the amino acid composition are given in support of the cytochemical evidence.

MATERIALS AND METHODS

The maturation of *Urechis* sperm takes place in the coelom, and mature sperm are then stored in the storage sacs (10). Samples of mature sperm and coelomic fluid containing various types of male germ cells were smeared on slides, exposed to formalin or absolute methanol vapor to preserve sperm morphology, and postfixed in 10% neutral formalin or absolute methanol. Formalin-fixed samples were washed thoroughly in running water. These slides were then subjected to various cytochemical staining techniques used for detecting nucleic acids, proteins, and polysaccharides (see Table I for references to specific procedures).

Methods for isolation of newt (5) and ram (7) acrosomes have been described previously. In the present study the method described below was found to give the best results with respect to yielding whole acrosomes. It involves the use of an anionic detergent "7X" (Linbro Chemical Corporation) which releases the acrosome and dissolves the rest of the sperm. (*a*) Suspend mature sperm, collected from storage sacs, in 100 volumes of double distilled water to initiate acrosomal separation (Fig. 1). (*b*) Mix slowly with 7X until the final detergent concentration is 2–4% (v/v). (*c*) Force the viscous solution through a hypodermic needle (23G) to break sperm head and tail completely, and until the solution is no longer highly viscous. (*d*) Centrifuge at 2000 rpm for 1–2 min to precipitate cell debris. (*e*) Centrifuge supernatant at 8000 rpm for 30 min. (*f*) Wash (two to three times) precipitate in detergent solution (2–4%) and centrifuge at 8000 rpm for 20 min; this step is essential to wash out contaminating nucleoprotamines and also small cell particles. The precipitate contains ring-shaped acrosomes which show little cytoplasmic contamination when stained with acid fast green (Fig. 2). These acrosomes retain alkaline fast green and periodic acid–Schiff stainability. (*g*) Wash (three to four times) acrosomal precipitate in cold 95% ethanol or methanol to remove detergent and extractable lipids. In the present experiment steps *a–d* were done at room temperature rather than at 2–4°C to lower the viscosity of the solution; this does not affect the cytochemical staining characteristics of isolated acrosomes. (*h*) Remove any retained nucleoprotamine from acrosomal fraction by hydrolyzing in 5% trichloroacetic acid at 90°C (four to five times, 20 min each), wash out trichloroacetic acid in 70% ethanol, and dry acrosomal fraction under vacuum.

Since two separate structures, the nucleus and the acrosome, in the sperm head contain basic proteins, it was of interest to compare the amino acid compositions of these basic proteins. Amino acid contents of isolated acrosomes (dried powder) and of basic proteins of the mature sperm nuclei were analyzed with an automatic Beckman-Spinco amino acid analyzer.

Sperm powder was obtained by drying after removing tails and cytoplasm in distilled water and after removing lipids in cold methanol, methanol:ether (1:1), and ether. Nuclear basic proteins were extracted from dried sperm powder by 0.25 N sulfuric acid at 0–4°C for 30 min and precipitated in cold 95% ethanol (2 vol).

RESULTS AND DISCUSSION

The *Urechis* acrosome, before and after isolation, appears ring-shaped by any method used herein for visualization (acid fast green for total protein, alkaline fast green for basic proteins, and periodic acid–Schiff reaction for polysaccharides).

Table I shows various cytochemical staining characteristics of acrosomes and nuclei of mature sperm. *Urechis* acrosomes, like other acrosomes, are periodic acid–Schiff (PAS) positive for polysaccharides and they lack Feulgen-stainable DNA. They give a positive reaction (Fig. 3) for alkaline fast green (AFG)-stainable basic proteins without prior acid hydrolysis. These proteins are not bound to RNase-sensitive RNA, since RNase treatment does not increase this stainability. The AFG stainability of acrosomes is greatly enhanced after trichloroacetic acid, picric acid, or hydrochloric acid hydrolysis (Fig. 4); this increase does not seem to be due to the removal of polysaccharides since acrosomes stain with PAS following acid treatment. There is also no reason to believe that this increased stainability is due to removal of RNase-resistant RNA. According to Swift (personal communication), histone stainability *per se* is increased by mild hydrolysis which may result in the unmasking of stainable groups. In contrast to acrosomal basic proteins, the basic proteins of mature sperm nuclei stain intensely with AFG only after removal of DNA with picric acid, but not with hot trichloroacetic acid; this suggests the presence of protamine-like proteins in the nucleus; protamines are known to be leached out of nuclei during hot trichloroacetic acid hydrolysis (2). That the acrosomal and particularly the nuclear basic proteins have a high arginine content was evident from an intense Sakaguchi reaction and also from their ability to stain with AFG after deamination or acetylation of the epsilon-amino groups of lysine. The fluorodinitrobenzene reaction, however, shows that lysine is also present in these proteins.

TABLE I

Cytochemical Staining Characteristics of Acrosomes and Nuclei of Mature Sperm

Pretreatment	Staining	Intensity of stain: Acrosomes	Intensity of stain: Nuclei
None	Periodic acid–Schiff (8)	+	−
None	Feulgen	−	++
None	Alkaline fast green (1)	+	−
RNase, 2 hr at 37°C	Alkaline fast green (1)	+	
1 N HCl, 10 min at 60°C	Alkaline fast green (1)	++	−
5% trichloroacetic acid, 15 min at 90°C	Alkaline fast green (1)	++	±
Sat. picric acid, 6 hr at 60°C	Alkaline fast green (4)	++	++
Sat. picric acid, 6 hr at 60°C +acetylation or deamination	Alkaline fast green (4)	++	++
None	Sakaguchi (4)	+	++
None	Fluorodinitrobenzene (4)	+	+

Fig. 5 shows a sample of coelomic fluid containing clusters of primary spermatocytes, immature sperm, and mature sperm stained with AFG after removal of DNA and after acetylation. The AFG staining characteristics of developing and of well-developed acrosomes are similar, except that the latter are more intensely stained than the former. An increase in acidophilia of developing acrosomes in insects was previously reported by Moriber (9) with a different and less specific staining method. Acrosomal basic proteins differ from somatic histones of primary spermatocytes and immature sperm nuclei in not being affected by acetylation. They appear prior to the transition of histones to protamine-like protein which occurs late in spermiogenesis.

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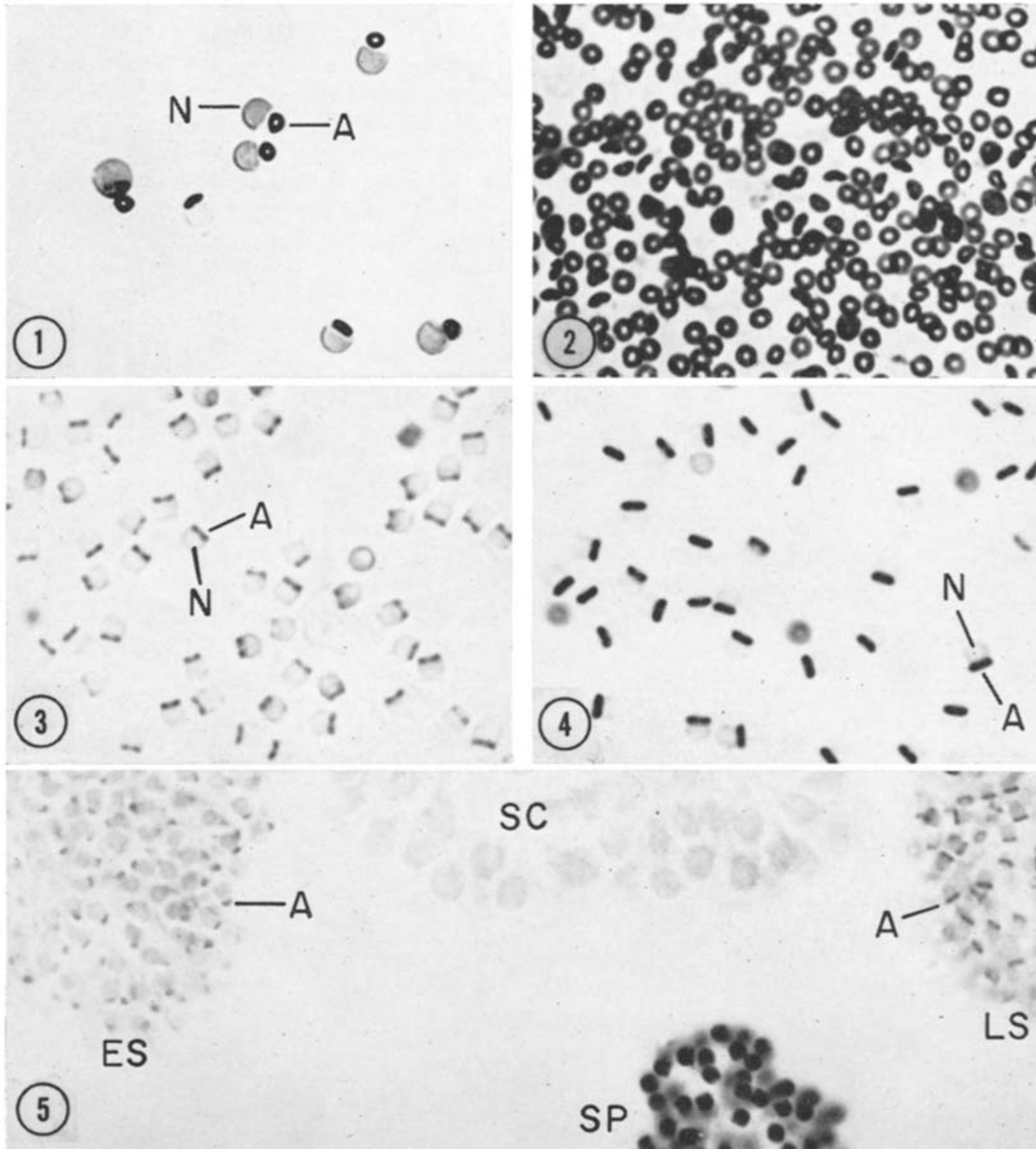

Unfixed sperm nuclei and acrosomes swell up in distilled water and particularly in detergent solution (Figs. 1 and 2). Cells in Figs. 3–5 were exposed to formalin vapor and postfixed in 10% neutral formalin.

FIGURE 1 Separation of acrosomes (*A*) from sperm nuclei (*N*) in distilled water. Unfixed cells stained with alkaline fast green. × 2900.

FIGURE 2 Isolated acrosomes (obtained at the end of step *f* of the isolation procedure) stained with acid fast green to show absence of appreciable proteinaceous contaminants. From top and side views acrosomes appear as ring-shaped and rod-shaped, respectively. × 1700.

FIGURES 3 and 4 Side views of alkaline fast green–stained acrosomes (*A*) which are attached to sperm nuclei (*N*). Fig. 3, photographed with a slightly lowered condenser to show nuclear outlines, shows weak staining of acrosomes without prior acid hydrolysis, and Fig. 4 shows an intense staining following 1 N HCl hydrolysis for 10 min at 60°C. × 2900.

FIGURE 5 Clusters of primary spermatocytes (*SC*), early spermatids (*ES*), late spermatids (*LS*), and mature sperm (*SP*) stained with alkaline fast green after removal of DNA in picric acid, followed by acetylation. Only acrosomes (*A*) of immature and mature sperm and nuclei of mature sperm are well stained. In the mature sperm intensely staining nuclei and acrosomes appear to be fused together. × 1900.

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TABLE II

Amino Acid Content in Per Cent of Total Moles Recovered

Amino acid	Acrosomal protein (A)	Nuclear protamine (P)	A/P
Alanine	4.1	11.4	0.4
Arginine	21.3	36.9	0.6
Aspartic acid	4.7	0.9	5.2
½ Cystine	0.4	—	
Glutamic acid	4.1	0.8	5.1
Glycine	15.2	2.7	5.6
Histidine	2.1	0.1	21.0
Isoleucine	2.1	0.3	7.0
Leucine	3.1	0.4	7.7
Lysine	23.2	19.2	1.2
Methionine	—	—	
Phenylalanine	1.4	0.2	7.0
Proline	2.2	0.9	2.4
Serine	7.4	22.4	0.3
Threonine	2.7	1.6	1.7
Tyrosine	1.1	0.2	5.5
Valine	4.8	2.0	2.4

Table II presents the amino acid composition of the isolated acrosome fraction and of basic proteins of mature sperm nuclei. These values should be considered tentative because of the absence of any check on the purity of the proteins. They do support the cytochemical observations that the acrosome contains at least a fraction of the cell's highly basic protein and that basic proteins of the mature sperm nuclei are of the protamine-like type (see reference 12). Compared to the nuclear basic proteins, the acrosomal proteins contain about one-half as much arginine and an equal amount of lysine. Other amino acids, except serine and alanine, are present in a higher proportion in acrosomal than in nuclear proteins. One acrosomal fraction, obtained at the end of step *f* of the isolation procedure and dried after washing in water, hot trichloroacetic acid, and 70% alcohol, contains by weight about 40% lipids (extractable in methanol and 1:1 methanol–chloroform). The tryptophan test of Badin et al. (3) indicates also the presence of 1–2% polysaccharides in this fraction.

The basic proteins of acrosomes, unlike histones and protamines, cannot be extracted by salt or dilute acid solution, perhaps owing to their strong association with a lipid or a carbohydrate fraction which is not removed during the isolation of acrosomes. The presence of acid glycoproteins in isolated acrosomes of ram sperm has been reported by Hartree and Srivastava (7).

Basic proteins have already been noted cytochemically in insect acrosomes (9, 11) and in capsules of crustacean sperm (4, 6). A number of lytic enzymes including ribonuclease, a basic protein, have also been found in isolated acrosomes of newt sperm (5). Further purification and characterization of basic proteins of *Urechis* acrosomes is necessary to elucidate their role in *Urechis* sperm function.

SUMMARY

The acrosome of *Urechis caupo* contains basic proteins which appear not to be bound to nucleic acids. Some cytochemical properties of these basic proteins differ from those of protamine-like proteins of mature sperm nuclei and from histones of spermatocytes and immature sperm nuclei. Preliminary biochemical analysis of an isolated acrosomal fraction and basic proteins of mature sperm nuclei support these cytochemical results.

This study was supported by a grant from the National Science Foundation (GB-4276).

We are grateful to Dr. J. S. Dixon for the amino acid analyses.

Received for publication 4 May 1967; revision accepted 26 June 1967.

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