Calcification in Sea Urchins—II.
The Seasonal Changes of Protein Concentration and Electrophoretic
Patterns of Both Proteins and Mucopolysaccharides
in the Perivisceral Fluid of a Sea Urchin

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In many ecological studies of sea urchins, their age determination has been made on the basis of statistical analysis of both test diameter and body weight. However, a physiological explanation for their test growth has not been clarified, though MOSS and MEEHAN¹⁾, and KOBAYASHI and TAKI²⁾ have initiated studies from this point of view.

It has generally been accepted that in both vertebrates and invertebrates, proteins, acid mucopolysaccharides and PAS-positive materials play important roles in the formation of organic matrix of hard tissues³⁻¹³⁾. In the case of urchins, these substances or their precursors are thought to be contained in the perivisceral fluid. Regarding this fluid of *Strongylocentrotus purpuratus*, HOLLAND *et al.*¹⁴⁾ have reported the occurrence of annual fluctuations of protein concentration and existence of three protein fractions by column chromatography. However, so far as the author is aware of, no attempt has been made to study on mucopolysaccharides of the perivisceral fluid of sea urchins. Previous workers^{2, 15, 16)} have reported that in *S. intermedius*, mature specimens more than 3 years old cease or almost cease their test growth in summer but grow actively in winter. In such cases, this species is an appropriate material for studying the mechanism of regulation of test growth.

The present paper deals with the seasonal changes of the protein concentration and electrophoretic patterns of both proteins and mucopolysaccharides in the perivisceral fluid of this species in relation to the test growth.

Materials and Methods

Mature specimens of *Strongylocentrotus intermedius* of about 4–6 cm test diameter and about 40–75 g body weight were used. Mature specimens of *S. nudus* were also used. These specimens were collected monthly throughout the year at littoral areas of Toi near Hakodate, Japan.

For the determination of protein concentration of each individual specimen, samples of the perivisceral fluid were prepared as follows. The fluid was gently withdrawn by

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inserting an injection needle on a syringe into the perivisceral coelom through the peristomial membrane. The fluid of about 1.5 ml thus collected from each specimen was filtered with a Toyo filter paper No. 50, and then centrifuged at 3000 rev/min for 10 minutes to remove cellular elements and tissue fragments. One ml of the supernatant was used for the determination of protein concentration by means of the micro-Kjeldahl method¹⁷⁾.

Samples of the perivisceral fluid for electrophoresis were obtained by the same method as mentioned above. However, the fluid collected from 2–3 specimens were pooled. The pooled fluid contained in a cellophane tube was concentrated to about 1/20–1/25 its original volume by dialysis against 20% gum arabic for about 48–60 hours at 4°C. The fluid sample thus concentrated was centrifuged at 3000 rev/min for 10 minutes, and the supernatant was used for electrophoresis.

A paper electrophoresis was performed by using a modified type of GRASSMANN-HANNING's apparatus. The constant current applied was 0.3 mA/cm width of paper strip (Toyo filter paper No. 51) for 5 hours at 4°C with HOLT's barbital buffer solution (pH 8.5 and ionic strength 0.045). The amount of the sample applied was 0.1–0.12 ml and that of human serum as a control was 0.001 ml. After the completion of the electrophoresis, paper strips were dried at about 100°C for 15 minutes, and stained with bromophenol blue for proteins, with either alcian blue (G. T. Gurr, Ltd., and Wako Pure Chem. Ind.) or toluidine blue (Kanto Chem. Co. Ltd.) for acid mucopolysaccharides^{18–21)}, and with periodic acid-Schiff reagents for PAS-positive materials²²⁾. Methylation and saponification were made under following conditions. Methylation: the strips were treated for 18 hours at 60°C in 0.8% HCl of methanol according to FISHER and LILLIE's method²¹⁾. Saponification: the methylated strips were treated in 1% KOH solution of 80% ethanol for 20 minutes, and dried in air according to LILLIE's method²¹⁾. Acetylation and saponification were made under the following conditions in accordance with MCMANUS and CASON's method²¹⁾. Acetylation: the strips were immersed for 2 minutes in absolute ethanol, and then in pyridine which was mixed with acetic anhydride at the ratio of 13 to 20, treated for 12 hours at 58–60°C, rinsed in absolute ethanol, and brought in water through 80–50–20% ethanol. Saponification: the acetylated strips were treated with 0.1N KOH solution for 45 minutes at room temperature and dried in air after washed in water. The digestion of acid mucopolysaccharides with testicular hyaluronidase (Worth. Biochem. Co.) was done by BÉLANGER's method²³⁾, and amylase (Wako Pure Chem. Ind.) digestion was made for PAS-positive materials in accordance with LISON²¹⁾. The scanning of paper strips was made with a Ozumor-82 densitometer (Asuka Manuf. Co. Ltd.) after clearing them in melted paraffin.

Results

Proteins As is seen in Table 1, protein concentration of the perivisceral fluid of *S. intermedius* clearly showed a seasonal change with the highest value of 0.40 mg/ml in December and the lowest of 0.14 mg/ml in August.

The electrophoretic patterns of proteins in the perivisceral fluid are presented in Fig. 1. A single protein fraction was found to occur in either species throughout the year. Migration rates of the protein fractions of both species were very similar to each other, the respective fraction moving to the location between α_2 - and β -globulins of human serum.

Any distinct seasonal change could not be found in paper electrophoretic patterns of

Table 1. Protein concentration in the perivisceral fluid of *S. intermedius* (mg/ml)

Month	Mar.	Jun.	Aug.	Oct.	Dec.
	0.24	0.08	0.09	0.35	0.58
	0.28	0.33	0.14	0.25	0.41
	0.35	0.40	0.25	0.19	0.53
	0.29	0.21	0.19	0.24	0.15
	0.47	0.23	0.02	0.33	0.38
	0.36	0.20	0.11	0.47	0.54
	0.27	0.18	0.12	0.21	0.48
	0.15	0.23	0.28	0.18	0.34
	0.45	0.10	0.10	0.14	0.25
	0.35	0.16	0.12	0.28	0.35
Mean $\pm$ SD	0.33 $\pm$ 0.09	0.21 $\pm$ 0.10	0.14 $\pm$ 0.07	0.26 $\pm$ 0.11	0.40 $\pm$ 0.13

Fig. 1. Paper electrophoretic patterns and their scanning diagram showing the protein fractions of the perivisceral fluid of *S. intermedius* and *S. nudus*, together with that of human serum as a control; these strips were stained with bromophenol blue. HS: human serum; BF (*S. int.*): perivisceral fluid of *S. intermedius*; BF (*S. nud.*): that of *S. nudus*. In the scanning diagram, the solid line indicates the perivisceral fluid of *S. intermedius*, the broken line that of *S. nudus*, the dotted line human serum. Arrow: origin of electrophoresis; alb: human serum albumin fraction.

protein fractions of the fluid. However, a decrease of the staining intensity for the protein fractions by bromophenol blue was appreciably noticed in the samples of summer specimens.

Mucopolysaccharides Electrophoretic patterns of the acid mucopolysaccharides are given in Figs. 2–4. In most fluid samples, paper electrophoresis revealed two fractions of acid mucopolysaccharides to occur. The faster fraction was deeply stained with alcian blue and metachromatic with toluidine blue, while the slower was stained lightly with alcian blue and orthochromatic with toluidine blue (Figs. 2 and 3). The faster fraction could not be found to reappear after methylation and saponification, and was hydrolysed by the hyaluronidase digestion though not completely in some cases. The slower fraction reappeared by the treatment of methylation and saponification, and was resistant to the digestion by hyaluronidase (Fig. 2).

Figs. 2–4. Paper electrophoretic patterns of acid mucopolysaccharides in the perivisceral fluid of *S. intermedius*. Arrow: origin. 2. “A type” of acid mucopolysaccharides. Two fractions were found; the slower fraction only reappeared after methylation (M) and saponification (S), and was resistant to hyaluronidase digestion (H-ase). ALBL: alcian blue staining; (A): “A type”. 3. The faster fraction was metachromatic and slower fraction was orthochromatic with toluidine blue. TLBL: toluidine blue staining. 4. “B type” of acid mucopolysaccharide. A single fraction of acid mucopolysaccharide was found to occur in a considerable number of fluid samples of summer specimens. This fraction moves a little faster than the slower fraction of “A type”. (B): “B type”.

It was noteworthy that a considerable number of the fluid samples examined during the period from June to September showed to contain only the slower fraction, whereas both faster and slower fractions occurred in all samples examined in the other months (Fig. 4). The fluid, though pooled from 2–3 specimens, containing two fractions of acid mucopolysaccharides was called temporarily “A type”, and that of a single fraction “B type”. Frequency of the occurrence of both types is presented in Fig. 5. The B type was found to occur only in summer with the following frequency: 9% in June (11 samples), 20% in July (20), 57% in August (21), and 25% in September (12).

Fig. 5. Monthly frequency of the occurrence of "A type" and "B type" of the electrophoretic patterns of the acid mucopolysaccharides in the perivisceral fluid of *S. intermedius*. Each sample was pooled from 2-3 specimens.

As is seen in Fig. 6, only one fraction of PAS-positive material was found, not showing any seasonal change of the electrophoretic pattern. PAS-positivity of this fraction was restored by saponification and acetylation, and was resistant to the amylase digestion (Fig. 6).

A schematic diagram showing the relative distribution of proteins, acid mucopolysaccharides and PAS-positive material is given in Fig. 7. Migration rates of both the protein fraction and the PAS-positive material in the paper electrophoresis were very similar to each other. With regard to acid mucopolysaccharides fractions, it was found that the slower fraction of A type moved with the protein fraction, though a single fraction of B type moved a little faster than the protein fraction.

Fig. 6. Paper electrophoretic patterns of PAS-positive material in the fluid of *S. intermedius*. This fraction was resistant to amylase digestion (A-ase), and restored by acetylation (A) and saponification (S). Arrow: origin.

Fig. 7. A schematic diagram of paper electrophoretic patterns showing the relative distribution of protein, acid mucopolysaccharides and PAS-positive material in the fluid of *S. intermedius*. BPB: bromophenol blue staining; PAS: PAS-reaction; ALBL (A): alcian blue staining, "A type"; ALBL (B): alcian blue staining, "B type"; TLBL: toluidine blue staining. Arrow: origin of electrophoresis.

Discussion

The mechanism of test growth in sea urchins has not hitherto been clarified. As

in the case of the extrapallial fluid in mollusca^{9,10,24)}, perivisceral fluid in urchins is considered to contain minerals in the state just prior to their deposition, precursors of the organic matrix of test, and other substances essential for test growth. At the same time, it is probable that the physico-chemical characteristics and composition of the fluid will affect the nature of organic matrix and regulate the test growth. However, no study has been made on test growth of urchins from this point of view. Nevertheless, LYONS²⁵⁾ has reported that proteins of the perivisceral fluid of *S. purpuratus* are immunologically identical with those of tissues. KOBAYASHI and TAKI²⁾ have indicated from their tetracycline labelling study that growth of the mature test of *S. intermedius* is found to occur in peripheral edges and the inner surface of each plate, which are provided with fibrous elements. Furthermore, these authors have suggested that these fibers play an important role in the test growth, and that there is a different mechanism of the test growth between summer and winter specimens. Their assumption seems to be substantiated by the finding of TRAVIS *et al.*²⁶⁾ that in *S. droebachiensis* and *Lytechinus variogatus* the calcite crystals of the plate are deposited in a collagenous matrix which appears to resemble that of mammalian hard tissue in structure and composition of amino acids.

It was noteworthy that, in a considerable number of the fluid samples of summer specimens, the paper electrophoresis showed to occur only a single fraction of the acid mucopolysaccharide, while two fractions were found in winter specimens without exception. From the histochemical tests of these acid mucopolysaccharides made on paper strips, the faster fraction was considered to be a chondroitin sulfate-like substance, and the slower fraction a complex of hyaluronate-like substance and some other acid mucopolysaccharide. A single fraction of the acid mucopolysaccharide found in summer specimens (B type; Fig. 4) moved a little faster than the slower fraction of winter specimens (A type; Fig. 2). Considering from the finding made by previous workers that frictional effects occur electrophoretically in the migration of mixture of hyaluronic acid and albumin²⁷⁻³¹⁾, such a different pattern of the acid mucopolysaccharides may be partly due to the decrease of protein concentration of the perivisceral fluid in summer specimens. Regarding both protein fraction and PAS-positive material, no distinct seasonal change was found electrophoretically. The PAS-positive material was found to move with the protein fraction. Possibly, these components may form a neutral heteropolysaccharide-protein complex.

It has generally been accepted that non-collagenous protein, acid mucopolysaccharides and PAS-positive materials elaborate an organic matrix of calcified tissue, in association with collagens^{6,7,23,32-34)}. From his *in vitro* experiment, GLIMCHER³⁵⁾ has proved the importance of collagen in calcification. Further, SOBEL and BURGER³⁾ have postulated that collagen-mucopolysaccharide complex takes an important part in remineralization of decalcified bone tissues. From the works of these investigators and others, the proteins,

acid mucopolysaccharides and PAS-positive material of the perivisceral fluid detected in the present experiment are considered also to serve formation of the organic matrix and to be closely involved in the test growth in collaboration with fibrous elements in the growing sites of plates. KOBAYASHI and TAKI²⁾ have wondered by what mechanism the cessation of mineralization of the test does occur in summer specimens, and assumed that the nature of the fibrous elements may change seasonally, along with the alteration of other matrix components. Possibly, the decrease of the protein concentration and appreciable decline of the amount of the chondroitin sulfate-like fraction in the perivisceral fluid of summer specimens may be related to the cessation of test growth.

Summary

1. Mature specimens of *Strongylocentrotus intermedius* are known to cease their test growth in summer. A study was made on seasonal changes of both the protein concentration and the electrophoretic patterns of proteins, acid mucopolysaccharides and PAS-positive materials in the perivisceral fluid, in relation to their test growth.

2. In coincidence with a cessation of their test growth, protein concentration of the perivisceral fluid in summer specimens was found to be much lower than that of winter specimens.

3. Paper electrophoresis of the perivisceral fluid revealed to present a single protein fraction. However, no distinct seasonal change of the electrophoretic pattern of the proteins was found. Similar results were obtained in *S. nudus* also.

4. In winter specimens, two fractions of the acid mucopolysaccharides were found, that is, a chondroitin sulfate-like substance and a complex of the hyaluronate-like substance and some acid mucopolysaccharide. However, in a considerable number of the fluid samples of summer specimens, the chondroitin sulfate-like substance disappeared in the paper electrophoresis.

5. A single PAS-positive fraction was found to move with the protein fraction.

6. Decrease of the protein concentration and decline of the amount of the chondroitin sulfate-like substance occurred in the perivisceral fluid of summer specimens may be closely related with the cessation of their test growth.

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