CALCIFICATION IN FISH AND SHELL-FISH—II. A PAPER
ELECTROPHORETIC STUDY ON THE ACID MUCOPOLYSACCHARIDES
AND PAS-POSITIVE MATERIALS OF THE EXTRAPALLIAL
FLUID IN SOME MOLLUSCAN SPECIES*

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Many studies have indicated that in the higher vertebrates acid mucopolysaccharides are necessary to initiate calcification of hard tissues, but their physiological roles have not yet been clearly explained¹⁾. With molluscs (cf. WILBUR²⁾, investigations made concerning acid mucopolysaccharides in relation to shell formation are very scanty, though from the comparative biochemical point of view several related substances have been identified³⁾⁻¹²⁾. Further, evidence has recently been accumulated on the association of collagen, cartilage, bone and other tissues with PAS-positive neutral heteropolysaccharides¹³⁾⁻¹⁸⁾.

In a previous paper, the author¹⁹⁾ reported that the extrapallial fluid of species with a calcite shell contains a single protein fraction, while that of species with an aragonite shell contains a complex system of three or more protein fractions. It is probable that in molluscs the main components or the precursors of the organic matrix of the shell are proteins, acid mucopolysaccharides and PAS-positive materials. However, no study has yet been made on the relative distribution of these three kinds of substances in relation to shell formation, especially taking into consideration the possible influence of these substances upon the crystal types of calcium carbonate. The present experiments were undertaken to detect the presence or absence of any correlation between the crystal types of calcium carbonate in shell and the distribution of both the acid mucopolysaccharides and the PAS-positive materials present in association with the proteins of the extrapallial fluid. Paper electrophoresis was used.

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Materials and Methods

The following fifteen species were used, most of which were the same as those in the previous experiments¹⁹⁾: (1) *Aequipecten irradians concentricus*, (2) *Chlamys nipponensis*, (3) *Crassostrea virginica*, (4) *C. gigas*, (5) *Ostrea edulis*, (6) *O. lurida*, (7) *Modiolus demissus*, (8) *Mercenaria mercenaria*, (9) *Busycon carica*, (10) *Spisula sachalinensis*, (11) *Elliptio complanatus*, (12) *Cristaria woodiana*, (13) *Corbicula leana*, (14) *Viviparus intertextus*, and (15) *Cipangopaludina malleata*. Of these, the first ten species are marine and the last five fresh-water forms. It is known that in the normal state, calcium carbonate crystals of the shell in species (1)–(6) are wholly composed of calcite, those of (7) of calcite in the prismatic layer and of aragonite in the nacreous layer, and those of (8)–(15) wholly of aragonite¹⁹⁾.

The method of collection of the extrapallial fluid was the same as described previously¹⁹⁾. The fluid was centrifuged and the supernatant was concentrated to about 1/5–1/10 its original volume by dialysis against 15–20% gum arabic for about 20 hours at 4°C. After dialysis, the concentrated fluid was centrifuged again and the supernatant was applied to electrophoretic strips. The conditions were as follows: E-C Apparatus Co. paper electrophoresis system; Holt's barbital buffer solution, pH 8.5 and ionic strength 0.045; constant current intensity applied 0.8 mA/cm width of the strip; duration of run 4.5 hours but 2.5 hours for the commercial acid mucopolysaccharides; cooling temperature for the apparatus 10°C; Whatman 3MM filter paper strip of 3.1×46.5 cm; amount of sample applied 0.02–0.05 ml and that of human serum 0.005–0.01 ml. The main differences between these conditions and those for the experiment in Japan were as follows: Toyo No. 51 filter paper strip of 3.1×26 cm; constant current intensity applied usually 0.8 mA/cm and duration of run 3 hours, often 0.6 mA/cm and 5 hours or 0.4 mA/cm and 7 hours; a modified type of GRASSMANN-HANNIG's electrophoresis apparatus. In each electrophoresis, four to six strips were run simultaneously, always including human serum as a control.

For staining acid mucopolysaccharides, the following six tests were made on paper strips. Toluidine blue (Nutritional Biochemical Co.): RIENITS²⁰⁾, LEITNER and KERBY²¹⁾, and HAMERMAN²²⁾; thionin (N. B. C.): LASH and WHITEHOUSE¹¹⁾; alcian blue 8 GS (G. T. Gurr, Ltd.): FEENEY and McEWEN²³⁾ and HEREMANS *et al.*²⁴⁾. The commercial toluidine blue was recrystallized more than three times from hot water and alcohol before use. PAS-staining for polysaccharides was made according to a modification of the McMANUS' method by MORI and KOBAYASHI²⁵⁾. HEREMANS' staining with alcian blue showed the clearest pattern, though protein inhibition of staining was found in various degrees. Among the three methods with toluidine blue, HAMERMAN's was best. However, metachromasia obtained by this method was often found to be less distinct than that of the RIENITS'. Metachromatic stain with thionin was not so distinct as in the case of toluidine blue. Consequently, in almost all the subsequent experiments, localization of every fraction of the acid mucopolysaccharides was

estimated by comparing the patterns of two halves of each strip (cut lengthwise) stained with both HEREMANS' alcian blue and RIENITS' toluidine blue, and further, by referring them to the patterns of both the PAS-reaction and the protein staining with methanolic bromphenol blue. When any distinct discrepancy was recognized between these stainings, electrophoresis was repeated using a fluid sample deproteinized by SOMOGYI's method²⁶⁾.

As a preliminary, paper electrophoresis of the commercial acid mucopolysaccharides was made under the conditions chosen, using 0.02–0.05 ml of 0.4% aqueous solution of hyaluronic acid (N. B. C.; Worthington Biochemical Sales), chondroitin sulfuric acid (N. B. C.) and heparin Na-salt (N. B. C.; Wako Pure Chemical Ind.), and also of mixtures of two and three of them.

Digestion of the acid mucopolysaccharides with testicular hyaluronidase (N. B. C.) was made according to BÉLANGER²⁷⁾. Amylase (N. B. C.) digestion of the polysaccharides was made according to LILLIE and GRECO²⁸⁾; digestion by saliva was made in accordance with a comment by LISON²⁹⁾. After completion of electrophoresis, each strip was cut in half lengthwise. One half was pretreated for methylation and the other for both methylation and saponification. Such strips were stained with either toluidine blue or alcian blue. Methylation: the strips were treated for 8 hours at 30°C in 0.8% concentrated HCl of methanol, with an exception in the case of *Cipangopaludina malleata*, when the period was 17–20 hours. Saponification: the methylated strips were fixed in 80% ethanol for 5 minutes, treated in 1% KOH solution of 80% ethanol for 20 minutes at room temperature, rinsed in 80% ethanol for 10 minutes, and dried in air. For PAS-reaction, histochemical techniques of acetylation and saponification were applied to the paper strips according to LISON²⁹⁾ and PEARSE³⁰⁾. Acetylation: the strips were immersed for a short time in absolute ethanol and then in pyridine (Wako Pure Chemical Ind.), treated with 40% acetic anhydride in pyridine at 58°C for 3 hours (with an exception in the case of *Cipangopaludina malleata*, when the period was 17–20 hours), rinsed in absolute ethanol, and brought to water through 80–50–20% ethanol. Saponification: the acetylated strips were treated with 0.1N KOH solution for 45 minutes at room temperature, and washed in water; treatments of both 10 and 25 minutes were also tested in accordance with a comment by CASSELMAN³¹⁾.

Results and Discussion

The electrophoretic patterns of the acid mucopolysaccharides and PAS-positive materials in the extrapallial fluid for each species are presented in Figs. 2–14 (PLATES I–IV). A schematic diagram showing the relative distribution of these substances and proteins in the fluid is given in Text-fig. 1. An example indicating migration rates of commercial hyaluronic acid and chondroitin sulfuric acid is presented in Fig. 1 for comparison. The values of the commercial ones relative to

that of human serum albumin (100%) under the conditions chosen were as follows: heparin Na-salt 209% (mean of 7 runs), chondroitin sulfuric acid 191% (23 runs), and hyaluronic acid 67% (23 runs); a mixture of the last two proved to be 175% and 86%, respectively (10 runs).

As is seen in Text-fig. 1, relative migration rates of the sulfated polysaccharides of the extrapallial fluid may be classified into two groups, that is, about 150% in *Aequipecten irradians concentricus* and 110–130% in other species. These two values somewhat resemble respectively those of the second and third minor components of the chondroitin sulfate-like substances of normal human urine which have been reported by HEREMANS *et al.*²⁴⁾ Further, several sulfated mucopolysaccharides such

Text-fig. 1. A schematic diagram of the relative distribution of proteins, acid mucopolysaccharides and PAS-positive materials in the extrapallial fluid of fifteen molluscan species. TLBL: toluidine blue staining; ALBL: alcian blue; PR: protein. alb: relative migration distance of human serum albumin (100%). Narrow bands of ALBL in 1, 2 and 3 mean that staining reaction is negative, but showed positive when the deproteinized fluid sample was applied to the paper strip. Arrow: origin.

as cellulose sulfate, polyglucose sulfate, limacoin sulfate, chondroitin sulfate, heparin, a heparin-like substance and a keratosulfate-like substance have been known to occur in the tissues and mucus of the molluscan species (*e.g.*, ref. 3-12). Identification of the sulfated substances in each species awaits further study.

When the PAS-positive materials are digested by amylase and saliva, the paper strips discolored. At least, their resistant state to the digestion was recognizable on paper. Further, as BJÖRNESJÖ³²⁰ has reported, the PAS-positive materials such as glycogen might be washed away from the paper during electrophoresis and staining processes. Acetylation and saponification suggested that the PAS-positive materials of the fluid, at least in all the species examined in Japan, contain amino groups, though these methods may not yet be practical³⁰. Furthermore, evidence has recently been accumulated on the association of some tissues such as skin, tendon, gingiva, vitreous humor, cartilage and bone with PAS-positive neutral heteropolysaccharides (*e.g.*, ref. 13-18). So, it is probable that the PAS-positive materials obtained are neutral heteropolysaccharide in nature. In agreement with the recent histochemical observations, the present experiment showed on paper also that commercial hyaluronic acid as well as chondroitin sulfuric acid and heparin Na-salt were entirely PAS-negative.

Calcite-shell group: In all four species of oysters, *Crassostrea virginica*, *C. gigas*, *Ostrea edulis* and *O. lurida*, a single protein fraction was found to move with both the PAS-positive material and the hyaluronate-like substance, showing a pattern of the simplest system of these three components among the species examined (Text-fig. 1; Figs. 3, 10). It was characteristic that alcian blue staining for this fraction was entirely negative but proved to be clearly positive when the deproteinized fluid sample was applied to the paper strip (Fig. 10a, c). In *Crassostrea virginica* and *C. gigas*, a sulfated fraction was rarely found to occur at the position about 1.1 times greater than human serum albumin, though no stainable protein was detected at the same site.

From the patterns presented in Fig. 9, the fast-running metachromatic fraction of *Chlamys nipponensis* is considered to be a sulfated mucopolysaccharide. A similar fraction of *Aequipecten irradians concentricus* also is presumed to be a related substance (Fig. 2b). This fraction of each species was PAS-negative and was found to move with the F-fraction of the protein as shown, for example, in Fig. 2c. Alcian blue staining for each of these fractions was entirely negative but showed positive by applying the deproteinized fluid sample to the paper strip (Fig. 9b, c). In both species, one more slowly migrating hyaluronate-like fraction was found (Fig. 9d). This fraction moved with PAS-positive material, though no stainable protein was detected at the same site (Figs. 2a, c; 9e).

Aragonite-shell and aragonite- and calcite-shell group: In each of the species, *Modiolus demissus*, *Elliptio complanatus*, *Viviparus intertextus*, *Corbicula leana* and

Cipangopaludina malleata, a fast-running sulfated fraction was found (Figs. 4, 7, 13, and 14 respectively). None of these fractions could be stained by protein dye and all were PAS-negative. In each of the species, *Mercenaria mercenaria*, *Busycon carica*, *Cristaria woodiana* and *Spisula sachalinensis*, rarely, a fast-migrating fraction was found to be very faintly metachromatic with toluidine blue.

In every species of this group, in addition to the occurrence of the sulfated polysaccharides described above, three or more slowly migrating hyaluronate-like fractions were found (Figs. 4-8, 11-14). The marked variety of the migration rates of these hyaluronate-like substances obtained may be due to various degrees of polymerization^{20), 33)}. Each fraction of these substances was found to move with both the protein and the PAS-positive material.

Previous workers have recognized that mucus is basically constituted of a mucoprotein-mucopolysaccharide complex^{34), 35)}. Possibly each separated fraction of the extrapallial fluid might form such a polyelectrolyte complex. When considered from such a standpoint, one can easily recognize that the fluid of the species with aragonite-shell contains a much more complex system of proteins, acid mucopolysaccharides and PAS-positive materials than that of the species with calcite-shell. Further, concerning acid mucopolysaccharides, the hyaluronate-like substances may play an important role in the formation of the organic matrix of the shell, if it is really true, as has been considered, that the template or matrix capable of shaping reticular and collagen fibrils is hyaluronic acid³⁶⁾. In addition, PAS-positive materials, possibly neutral heteropolysaccharides, might be also essential to the formation of the organic matrix of shell. The fact that both the hyaluronate-like substances and the PAS-positive materials were found in the extrapallial fluid of all the species examined seems to support this idea.

Summary

1. A study was made, by means of paper electrophoresis, of the relationship between the distribution of proteins, acid mucopolysaccharides and PAS-positive materials in extrapallial fluid, and the calcium carbonate crystals of shells. Fifteen molluscan species were used.

2. The extrapallial fluid of the species with aragonite-shell and aragonite- and calcite-shell contains a much more complex system of proteins, acid mucopolysaccharides and PAS-positive materials than that of the species with calcite-shell.

3. In all the species examined, hyaluronate-like substances and PAS-positive materials, possibly neutral heteropolysaccharides, were commonly found in the extrapallial fluid. This fact suggests that these two kinds of substances are components essential to the formation of the organic matrix of shell, in association with proteins. In some species, sulfated mucopolysaccharides were also detected.

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PLATE I

Fig. 1. Electrophoretic pattern of a mixture of commercial chondroitin sulfuric acid (fast fraction) and hyaluronic acid (slow fraction). ALBL: alcian blue staining.

Fig. 2. *Aequipecten irradians concentricus*. Fast-running fraction stained metachromatically with TLBL (b) is seen to move with F-fraction of the protein (c) and to be PAS-negative (a). Slow fraction of the hyaluronate-like substance is found to move with PAS-positive material (a, b); this fraction could not be stained by BPB (c). TLBL: toluidine blue; BPB: bromphenol blue.

Fig. 3. *Crassostrea virginica*. A single protein fraction is seen to move with both a hyaluronate-like substance and a PAS-positive material (a, b, c). (cf. Fig. 10). TH: thionin.

Fig. 4. *Modiolus demissus*. Fast-running sulfated fraction is clearly seen to be stained with both ALBL and TLBL (a, c); this fraction could not be stained by BPB, and was PAS-negative (b). ALBL staining and PAS-reaction for the slow fractions were indistinct, but TLBL stained these fractions dark blue (c).

PLATE I

1. Hyaluronic acid and chondroitin sulfuric acid

2. *Aequipecten irradians* 3. *Crassostrea virginica* 4. *Modiolus demissus*

PLATE II

Fig. 5. *Mercenaria mercenaria*. Patterns of both PAS-reaction and ALBL staining are very similar to each other (a, b). TLBL stained the slow fractions a deep dark blue (c), suggesting that ALBL staining for these fractions was inhibited by the co-existing proteins (b).

Fig. 6. *Busycon carica*. Stainings of this species were generally indistinct; PAS-reaction was found to be especially weak. However, one can recognize that both ALBL and TLBL stained three fractions, but their separation was not clear (a, b).

Fig. 7. *Elliptio complanatus*. Fast-running sulfated fraction is seen to be PAS-negative (a), metachromatic with TLBL (b) and as a clear band with ALBL (c); no stainable protein was detected at the same site. Slow fractions of the hyaluronate-like substances are rather clearly seen with ALBL staining (c).

Fig. 8. *Viviparus intertextus*. Fast-running sulfated fraction is seen to be metachromatic with TLBL (a), PAS-negative (b) and as a clear band with ALBL (c); no stainable protein was detected at the same site. Strong inhibition for TLBL staining of the hyaluronate-like fractions was found to make grayish patterns (a), though they appeared to be pinkish at the beginning of staining.

PLATE II

5. *Mercenaria mercenaria* 6. *Busycon carica* 7. *Elliptio complanatus* 8. *Viviparus intertextus*

PLATE III

Fig. 9. *Chlamys nipponensis*. TLBL stained two fractions of the fast-running sulfated and slow hyaluronate-like substances (a). ALBL staining was negative to the sulfated fraction which moved with the F-fraction of the protein (b); but, DPR revealed that this fraction is positive even with the same dye (c). Staining reaction of the hyaluronate-like fraction only reappeared by M&S (d). The sulfated fraction was PAS-negative (e). DPR: deproteinized fluid sample; M&S: saponification after methylation.

Fig. 10. *Ostrea edulis*. ALBL staining for the hyaluronate-like fraction was entirely negative (a), but showed clearly positive when DPR was applied to the paper strip (c). PAS-positive material is seen to move with both fractions of the protein and the hyaluronate-like substance (b). Fluids of both *Crassostrea gigas* and *Ostrea lurida* showed similar reactions. (cf. Fig. 3).

Fig. 11. *Spisula sachalinensis*. Protein inhibition for ALBL staining of the slow fractions is seen to be distinct when compared with that of TLBL (a, b). Staining reactions of all the fractions reappeared by M&S (d). PAS-positive materials are found to move with these hyaluronate-like substances and protein fractions (c).

PLATE III

9. *Chlamys nipponensis* 10. *Ostrea edulis* 11. *Spisula sachalinensis*

PLATE IV

Fig. 12. *Cristaria woodiana*. Staining reactions by M&S suggest that the fractions stained with ALBL are hyaluronate-like substances (a, b). No distinct difference of ALBL staining for these fractions was found between the fluid samples intact and deproteinized (b:d, a:c). TLBL staining for the slowest fraction showed grayish (e). Three fractions of PAS-positive materials are seen to move with the hyaluronate-like substances (e, f).

Fig. 13. *Corbicula leana*. Fast-running fraction is seen to be stained with both TLBL and ALBL (a, c) and to be PAS-negative (d); no stainable protein was detected at the same site; staining reaction of this fraction did not reappear by M&S (b). PAS-positive fractions are seen to move with the hyaluronate-like substances and proteins (d); protein inhibition for ALBL staining was found to occur in some of these fractions (c).

Fig. 14. *Cipangopaludina malleata*. Fast-running sulfated fraction is seen as a metachromatic band with TLBL (a) and is PAS-negative (d), though ALBL staining for it was not clearcut (c); no stainable protein was detected at the same site. Some of the hyaluronate-like fractions stained with TLBL showed grayish (a, b).

PLATE IV

12. *Cristaria woodiana* 13. *Corbicula leana* 14. *Cipangopaludina malleata*