Histochemical Studies on the Clam *Mactra solidissima.* (20510)

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For many years it has been known that certain species of molluscs are rich in acid mucopolysaccharide(1-6). The presence of a heparin-like factor in clams was shown by Thomas (7). Subsequently, Frommhagen *et al.*(8) isolated acid mucopolysaccharides from 2 species of clams and characterized them as sulfated polysaccharides similar to heparin and having a strong heparin-like activity in preventing the coagulation of blood.* In view of the supposed relationship between mast cells and heparin formation the following study was undertaken primarily to investigate the localization of mucopolysaccharide in the clam.

Materials and methods. Mactra solidissima was used for these experiments since this is the clam from which the anticoagulant substance (mactin-A) is routinely extracted in this laboratory.

Histochemical methods. Living clams were dissected and blocks of the mantle, syphons, palps, gills, foot, muscles, and body were fixed in 10% formaldehyde in saturated aqueous mercuric chloride for the preparation of routine paraffin sections which were stained with hematoxylin and eosin.

Blocks were fixed in Carnoy's fluid and 4% basic lead acetate and paraffin sections were prepared for the study of metachromasia. Sections of this material and of formol-sublimate fixed tissues were stained with 0.1% toluidine blue by the method of Bensley(9), dehydrated in alcohol and mounted in Permount. The distribution of the metachromatic substances was identical after all 3 fixatives, although diffuse granularity was present in all the lead acetate-fixed tissues and the Carnoy material cut poorly. For this reason, formol-sublimate fixed tissues were used for subsequent studies.

The solubility in alkali of the various metachromatic substances was tested by incubating deparaffinized sections for varying periods of time in N sodium hydroxide at room temperature. Attempts at extractions of unfixed material were vitiated by the resultant widespread diffusion of metachromatic material throughout the tissues.

The presence of ribonucleic acid and its effect on the staining reaction with toluidine blue was investigated by incubation of deparaffinized sections in 1% ribonuclease (Worthington Biochemicals) for one and two hours(10). The activity of the ribonuclease was confirmed by the ability of the solution to remove the cytoplasmic basophilia of plasma cells.

Sections were also stained by the periodic acid-Schiff method (PAS) of McManus(11) and by Mayer's mucicarmine and mucihematin methods(12). When unexpected PAS negative results were obtained with metachromatic substances, we considered the possibility that treatment of the sections in the process of staining may have removed the reactive substance. Accordingly, some of the sections were counterstained with toluidine blue. Deparaffinized sections were incubated for one hour in 0.1% malt diastase in phosphate buffer at pH 6.0(13) and stained by the PAS method. Control sections of clam were incubated in the buffer only and similarly stained. The efficacy of the enzyme was shown by the ability of the solution to remove glycogen from sections of chicken liver. Deparaffinized sections were incubated at room temperature for 24 hours in an aqueous solution containing 33 mg % of mammalian testicular hyaluronidase (Evans) and stained with toluidine blue. Paraffin sections were prepared of material fixed in cold acetone and stained as follows: 1) by Gomori's revised method for alkaline phosphatase using Fast Blue R.R. and incubating for a period of 2 hours(14), 2) by the

* In the paper by Frommhagen and co-workers (8), acknowledgement should have been made of the contributions of Thomas. The authors of paper(8) wish to express their regret at omitting not only the reference by Thomas(7) but also omitting mention of the fact that the idea of using the clam as a source of heparin-like anticoagulants was derived from a personal communication by Dr. Thomas.

Von Kossa method for phosphate-carbonate (15), 3) by Dahl's alizarin red S.(16), and 4) Schujeninoff's method(15) for calcium. Control sections of bone (for calcium) and of kidney (for phosphatase) were run simultaneously. Frozen sections of material fixed in formol sublimate were stained with Baker's sudan Black B(17). A saturated solution (100 mg/cc) of mactin-A was prepared in horse serum, smeared on slides covered with a thin film of gelatin, allowed to dry, and fixed over formaldehyde fumes. Control slides of serum alone were similarly prepared. The smears were then stained with mucicarmine and mucihematin. The effect of hyaluronidase on the anticoagulant was tested by comparing the colors of two aqueous solutions containing 0.4 cc of testicular hyaluronidase (33 mg %) and 0.5 cc of the anticoagulant (800 mg %), to each of which 0.1 cc of a 0.1% aqueous solution of toluidine blue was added before and after incubation for 30 minutes at room temperature.

Chemical methods. The periodic acid Schiff test was performed on an aqueous solution of the anticoagulant (mactin-A). The phosphatase activity of water extracts of the mantle tissue of *Mactra solidissima* was determined by the method of Bodansky(18). Inorganic phosphate was determined by the method of Fiske and SubbaRow(19). Clear water-white extracts of the mantle tissues at pH 5 were precipitated by elevation of the pH to 8.5 and the resultant solids analyzed for calcium and phosphate by microchemical methods.

Results. Mast cells are not found in the clam. Four different substances showing intense purple metachromasia are present in abundance: their histochemical properties and those of the anticoagulant (mactin-A) extracted from this species of clam are shown in Table I. Substance A is found in the mucous glands on the inner aspect of the mantle (Fig. 1), on the gills, palps, surface of the body, and in the genital tract. Substance B is observed only in the mucous glands on the outer (shell) side of the mantle (Fig. 2), and the third type (substance C) is found in the mucous glands of the gastrointestinal and urinary tracts. Substance D composes the reticulum fibers and large masses of ground substance in the mantle

TABLE I. Histochemical Properties of the Mucopolysaccharides of the Clam.

	Distribution	Toluidine blue	Ribonuclease + toluidine blue	Hyaluronidase + toluidine blue	Mucihematin	PAS	PAS + diastase	Solubility in N NaOH 2½ hr
Substance A (mantle & body)	Mucous glands of inner mantle, body surface, gills, palps, genital tract	Red-purple	Red-purple	Red-purple	Purple	—*	—	IS§
Substance B (mantle only)	Mucous glands of outer mantle	Blue-purple	"	"	"	—*	—	S
Substance C (body only)	Mucous glands of gastro-intest., urinary tracts	Red-purple	"	"	Pale purple blue	+	+	IS
Substance D (mantle & body)	Ground substance and reticulin fibers of all tissues	"	"	Pale blue	Pale purple blue	+	+	S
Anticoagulant mactin-A		"	NT‡	" †	Purple	+ †	NT	S

* Occasional trace of pink only. † Test tube reaction. ‡ NT = not tested. § IS = insoluble; S = soluble.
All substances gave positive mucicarmine tests.

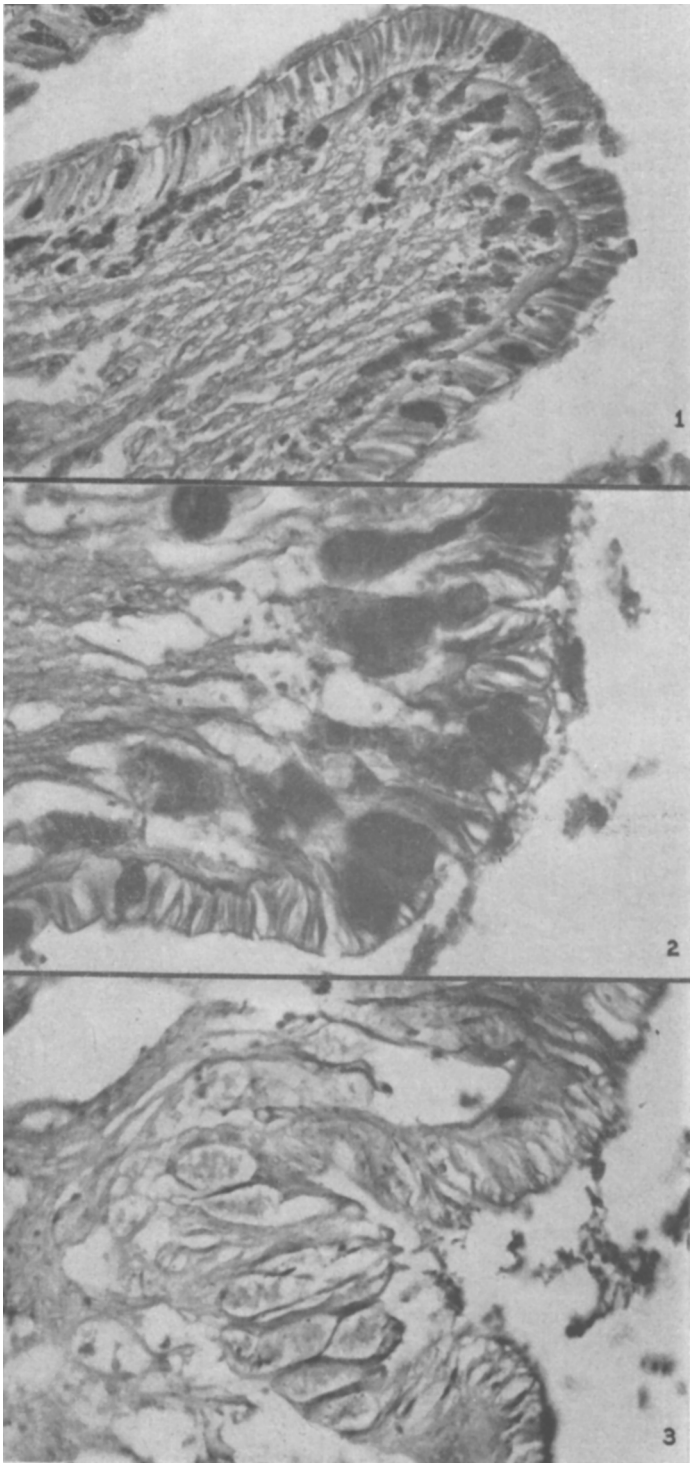

FIG. 1. Mucous glands on inner aspect of mantle. Toluidine blue × 235.
FIG. 2. Mucous glands on shell side of mantle. Toluidine blue × 350.
FIG. 3. Mucous glands on shell side of mantle after alkali extraction. Toluidine blue × 350.

and to a lesser extent in the other tissues of the clam. Extraction with alkali results in the disappearance of metachromatic substances B and D after 2½ hours (Fig. 3) and of all metachromatic substances after more prolonged treatment.

In the test tube the anticoagulant produces intense purple metachromasia which can be greatly reduced by hyaluronidase digestion. The serum controls for the slide tests stain faintly red with mucicarmine and blue with mucihematin; the experimental slides are, however, significantly different, indicating that the anticoagulant is responsible for a deep red color with mucicarmine and an intense purple with mucihematin.

It is not possible to demonstrate any calcium in the clam by Schujeninoff's method. The distribution of calcium by Dahl's alizarin method is identical with that of phosphate-carbonate by the Von Kossa method (Fig. 4 and 5). Considerable amounts of calcium phosphate-carbonate are present beneath the basement membrane of the epithelium on both sides of the peripheral rim of the mantle and, to a much lesser extent, in the form of fine granules in the epithelium and underlying connective tissue (Fig. 5). The largest aggregations are present at the tip of the papillary elevations of the underlying connective tissue (Fig. 5). No calcium is present in the region of the tentacle-like folds at the extreme periphery of the mantle (Fig. 6), which according to Vevelander and Benzer(20) is responsible for the elaboration of the organic matrix of the shell. Chemical analyses of extracts of the mantle indicate the following composition: Calcium 14.57%, phosphate 9.1%. Although carbonate was not determined, it is obvious that the phosphate, if it does exist as the inorganic calcium salt, must predominate.

Phosphatase is present in the same region of the mantle as calcium salts except that it is limited entirely to a small zone at the free border of the epithelial cell (Fig. 7). The presence of phosphatase was verified by the chemical method.

The persistence of metachromatic substances A and B in the tissues after the periodic acid-Schiff test was confirmed by counterstaining with toluidine blue. No sudanophilic lipid or glycogen can be demonstrated in the areas of metachromasia.

Discussion. Lison(21,22) has demonstrated that alcohol-resistant metachromasia can be due to the presence of sulfuric esters of large molecular size. In the absence of glycogen, ribonucleic acid is the only other substance likely to produce alcohol-resistant metachromasia in paraffin sections. The metachromasia of all 4 substances in the clam is not reduced by ribonuclease; it is therefore due to the presence of acid mucopolysaccharides. The 4 types of acid mucopolysaccharide differ from one another in regard to their solubility in alkali and in hyaluronidase, the availability of the 1-2 glycol groups for oxidation by the PAS method and in the color of their staining reaction with toluidine blue and mucihematin.

All of the mucopolysaccharides can be removed from the clam by intensive treatment with alkali, so that differential solubility is not of much assistance in determining the source of the anticoagulant (mactin-A). Since the latter is extractable from the body or from the mantle, substances B and C cannot be essential constituents. The anticoagulant is PAS positive and must, therefore, contain substance D (Table I). The presence of substance D in considerable amounts is further confirmed by the ability of hyaluronidase to remove the greater part of the metachromasia from solutions of the anticoagulant.

The very faint reaction of substances A and B with the PAS technic may indicate that they are highly sulfated (or phosphorylated) to such a degree indeed, that very few reactive hydroxyl groups remain(23). Confirmatory evidence for this explanation must await further chemical studies. The PAS positive and PAS negative mucopolysaccharides do not differ significantly in their degree of basophilia or metachromasia. The greater basophilia of the mucin on the shell side of the mantle (Table I) is abolished by treatment with ribonuclease, indicating that in this case at least, ribonucleic acid can even decrease, rather than produce, metachromasia.

Although the mucihematin method of staining is purely empirical, it is of interest to note that the most intense purple color is present in the mucin which is PAS negative. This

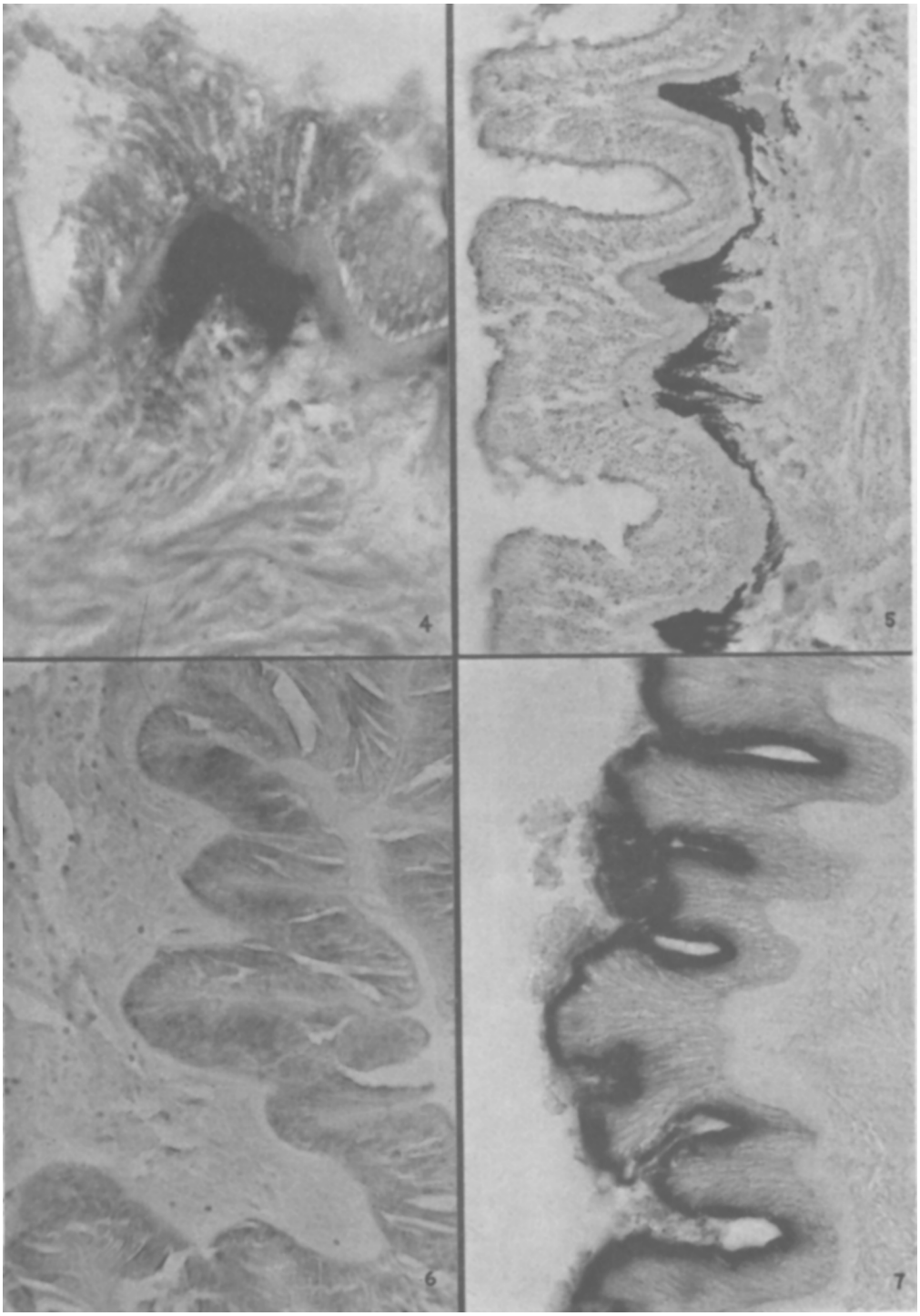

FIG. 4. Calcium deposits, mainly beneath basement membrane of inner aspect of mantle. Dahl's alizarin method × 350.

FIG. 5. Phosphate-carbonate deposits in inner aspect of mantle. Main deposits beneath basement membrane and fine granules in epithelium and subjacent connective tissue. Note

absence of calcium in mucous glands. Von Kossa and neutral red × 350.

FIG. 6. Same section as Fig. 5 showing absence of phosphate carbonate in tentacle-like folds at extreme periphery of mantle. Note absence of mucous glands. Von Kossa and neutral red × 350.

FIG. 7. Alkaline phosphatase in epithelium of inner aspect of mantle. × 350.

would suggest that the purple color with muci-hematin is indicative of highly sulfated muco-polysaccharides.

The histological demonstration of calcium phosphate-carbonate was confirmed by chemical procedures which suggested that the phosphate is predominant. Inasmuch as calcium and phosphate were shown to be localized in the same area, it seems probable that the deposits consist of some form of calcium phosphate. The presence of considerable amounts of calcium salts beneath the basement membrane suggests that this may be a storage area for calcium. Contrary to the reports of Bevelander and Benzer(20), it was not possible to demonstrate calcium salts in the mucous glands of the mantle (Fig. 5); it seems probable however, as they suggest, that the mucoid secretion of the glands plays a physical role in the transfer of calcium salts from the mantle to the shell. The calcium of the shell appears to consist mainly of calcium carbonate. whereas that of the mantle is mainly in the form of calcium phosphate(20,24,25). In view of the topographical relationship of phosphatase to the underlying calcium deposits the enzyme may take part in the "secretion" of calcium salts. The affinity of acid mucopolysaccharide for calcium ions and their importance in calcification has been the subject of recent research(26,27). The deeper deposits of inorganic calcium salts in the mantle are present in areas rich in acid mucopolysaccharide, but the overlying epithelium does not contain any of this material. If calcium salts are first formed in the deeper mucoid connective tissue and subsequently "secreted" by the epithelium, they may, as suggested by Thomas(28), be derived from a calcium mucopolysaccharide complex. The role of alkaline phosphatase in this process, is however obscure since mucopolysaccharide cannot be demonstrated in the cells which contain the enzyme, nor is there any evidence of enzymatic activity in the main deposits of calcium salts.

Summary. 1. Heparin-like substances in the clam do not arise from mast cells such as exist in higher vertebrates. 2. Four different acid mucopolysaccharides in the clam are described. The heparin-like anticoagulant (mactin-A) is probably a mixture of all 4 types but appears to consist mostly of the mucopolysaccharides of the ground substance of the connective tissues. 3. Ribonucleic acid is present in association with acid mucopolysaccharide in the mucous glands on the shell side of the mantle. 4. Calcium, phosphate and alkaline phosphatase are present in different sites in the epithelium and subjacent tissues of both sides of the mantle and are not topographically related to the mucous glands.

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Influence of Cortisone on Leptomeningeal Reaction Induced by Talc.* (20511)

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Ragan and co-workers(1) have demonstrated a marked retardation in the wound healing of rabbits treated with large doses of cortisone. This observation has been extended to the inflammatory and reparative processes of other mesodermal structures. Blunt(2) has studied delayed healing in experimental fractures of rabbits. Ducommun(3) and Scheinberg and Saltzstein(4) showed that ACTH and cortisone prevented the formation of intra-abdominal adhesions following the intraperitoneal instillation of talc, while Scheinberg, Saltzstein and Johnson(5) observed similar results in the prevention of pleural and pericardial adhesions.

Because cortisone diminished the inflammatory response of other serous surfaces, a study of its action on the meningeal reaction to the intrathecal administration of a sterile irritant was undertaken. Of several substances tried talc was the most effective in eliciting a meningeal reaction.

It was also felt that control of the meningeal reaction might be of value in the treatment of tuberculous meningitis as well as other granulomatous affections of the meninges. In such instances it seemed possible that the reduction of exudation and inflammatory response would make the offending organism more accessible to the action of the chemotherapeutic agent. Furthermore, the use of cortisone in the prevention of post-traumatic adhesions also suggested itself.

Materials and method. Eighteen white male rabbits weighing from 3.0 to 4.8 kg received a cisternal injection of 1 cc of a saline suspension of talc after a similar quantity of cerebrospinal fluid had been withdrawn. The talc suspension was sterilized by autoclaving. Ten animals were treated with cortisone, while 8 served as controls. In the cortisone-treated group 4 animals received a cisternal injection of 1 cc of a 1:9 suspension of talc, while 6

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