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STUDIES ON THE CEPHALOCHORDATES OF MADRAS COAST: VIII NATURE AND CHEMICAL COMPOSITION OF THE GILL BAR SKELETON AND THE NOTOCHORD IN THE LARVAL AND ADULT PHASES OF AMPHIOXUS *BRANCHIOSTOMA LANCEOLATUM**

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ABSTRACT

It is known that amphioxus in the course of its life history passes through a free swimming larval phase and a sedentary adult phase when it lies half buried in sand. The notochord is known to be soft and flexible in the larval stages and is hard and rigid in the adult condition. Similarly the gill bars show a change from a soft flexible condition to a hard and rigid structure as it passes from the larval to adult phase. Such changes are correlated with the requirements for their survival in the two respective phases. The transition from one condition to the other has been shown to be due to changes in the chemical composition of the respective structures involving a process of tanning in which the protein constituents of the gill bar and the notochord undergo first a condensation with a lipid to form a lipoprotein complex and later form cross linkages with quinones formed by the oxidation of phenolic substances present with the protein. The process strongly recalls the tanning of the arthropod cuticle resulting in mechanical rigidity and chemical resistance which are of functional significance to the animal in the larval and adult phases.

INTRODUCTION

IT is known that amphioxus, *Branchiostoma lanceolatum* passes through two different phases in its life history: a free swimming phase followed by a sedentary phase in which it remains half buried with a part of its cephalic end protruding above the sand level. Such a change in the mode of life may involve correlated modification in its structural organisation. But it is known from a study of morphology of amphioxus that the structural features may not change markedly although the requirements of a free swimming life may differ from those of a sedentary mode of existence (Barrington, 1965). The ability to penetrate and remain half buried in sand may involve the possession of a rigid notochord. While remaining half buried in sand the requirements for feeding have to be met. It is known that amphioxus is a filter feeder (Orton, 1913). The efficiency of filtration is closely related to the area available for filtration and the amount of water passing through the pharyngeal gills (Jorgensen, 1966). It is suggestive that the gill bars may play a role in keeping the pharyngeal area open for easy flow of water. How far this feature is ensured in the adult condition is not known.

Previous work on arthropods may suggest that chemical changes taking place in the cuticle bring about acquisition of new properties such as rigidity and mechanical resistance. It is felt desirable to investigate if such changes in chemical com-

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position occur in the gill bar and the notochord which may be correlated with the sedentary mode of life and the physiological requirements associated with such a mode of life.

There is very little information regarding the chemical nature of the gill bar and the notochord. Benham (1894), Lankester (1889) and Sannasi and Hermann (1970) considered that the gill bars are chitinous in nature. However, when the gill bars are treated with hot alkali they do not resist such treatment as that of the chitinous cuticle of arthropods (Azariah, 1969). In the light of such reports it is of interest to study the chemical nature of the gill bars and notochord which may have a functional significance in the larval and adult phases of the animal. Similarly the information available on the chemical nature of notochord is not extensive. Some information is available from the work of Flood (1968) who extracted a protein using 0.1 M/KCL in phosphate buffer at pH 7 and subsequent dialysis. The extracted protein was similar to paramyosin of molluscan muscle. The nature of protein and other chemical constitutents of the notochord and the changes in the chemical composition that they may undergo during growth were investigated.

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MATERIAL AND METHODS

Amphioxux, *Branchiostoma lanceolatum* used in the present study were dredged at a depth of 11m along the Madras Coast. 5% formalin and Bouin's (prepared in sea-water) fixed specimens were used for the preparation of sections by embedding them in gelatin as well as in polyester-wax after the method of Steedman (1957). The stains used were Mallory's triple stain, Masson's trichrome stain and Heidenhains haematoxylin. The details of the histochemical tests employed are mentioned in the text in relevant context.

STAINING REACTIONS AND HISTOCHEMICAL RESULTS

The chemical composition of the gill bars of the larva was investigated by application of appropriate staining and histochemical procedures. The results are recorded in Table I. With Mallory's triple stain and Heidenhains haematoxylin they stain blue and light brown respectively. From the results of histochemical tests it may be inferred from the positive biuret test in the entire gill bar that they may be composed of a simple protein. That the protein is lacking in phenyl groups is inferred from the negative reaction to Xanthoproteic, Millon's test. The gill bar was uniformly non responsive to the Sudan Black B test indicating the absence of lipids.

It is seen that the gill bars are formed primarily of proteins. Many exoskeletal structures of invertebrates are formed of proteins which in combination with lipids undergo hardening whereby they acquire mechanical rigidity and chemical resistance. In the exoskeletal structures of arthropods protein rich in tyrosine combines with a lipid forming a lipoprotein complex which is hardened by a process of phenolic tanning (Dennell, 1958). To find out if such a condition prevails in amphioxus sections of gill bar of adult amphioxus were used for a study of the chemical nature. The results are given in Table 2.

TABLE 1. *Results of staining reactions and histochemical tests obtained on the gill bar and notochord of larval Amphioxus*

Histochemical tests	Reference	Primary gill bar	Tongue bar	Noto-chordal plate	Noto-chordal sheath
Mallory's triple stain	Mallory 1938	Blue	Blue	Blue	Blue
Heidenhains haematoxylin	Lillie 1954	Light brown	Light brown	Light brown	Light brown
Masson's trichrome stain	Pantin 1948	Green	Green	Green	Green
Biuret test	Fearon 1946	+	+	+	+
Xanthoprotic test	Pearse 1961	—	—	—	—
Millon's test	Bensley and Gersh, 1933	—	—	—	—
Morner's test	Hawk *et al.*, 1954	—	—	—	—
Sudan Black B	Baker 1946	—	—	—	—

TABLE 2. *Result of staining reactions and histochemical tests obtained on the gill bar and notochord of adult Amphioxus*

Stains and histochemical tests	Reference	Primary gill bar	Tongue bar	Notochordal plate	Noto-chordal sheath
Mallory's triple stain	Mallory 1938	Red	Purple	Red	Blue
Heidenhains haematoxylin	Lillie 1954	Dark blue	Blue	Dark blue	Blue
Masson's trichrome stain	Pantin 1948	Red	Red	Red	Green
Chitosan test	Champbell 1929	—	—	—	—
Modified Chitosan test	Clark and Smith, 1936	—	—	—	—
Biuret test	Fearon 1946	+	+	+	+
Xanthoproteic test	Pearse 1961	+	+	+	—
Millon's test	Bensley and Gersh, 1933	+++	++	++	—

It is seen that in the adult the reactions to Mallory's stain undergo a change in that the sections of gill bars are fuchsinophil showing a differentiation in the intensity of colouration, the dorsal part of primary gill bar being red and the ventral part purple. The entire tongue bar takes up a purple colour. With Masson's trichrome

Stains and histochemical tests	Reference	Primary gill bar	Tongue bar	Noto-chordal plrce	Noto-chordal sheath
Morner's test	Hawk *et al.* 1954	+++	++	++	—
Sudan Black B	Baker, 1946	++	++	+++	+
Libermann Burchardt test	Lison, 1933	+	+	+	—
Argentaffin reaction	Lison, 1936	+	+	++	—
Ferric chloride test	Lison, 1936	++	+	++	—
Alkaline *nitro*-prusside	Pearse, 1961	++	+	++	—
Alkaline tetrazolium	Barnett and Saligmar, 1934	++	+	+++	—
Lead acetate	Lillie, 1954	+	+	+	—
Fast green von Gieson	Lillie, 1954	—	—	+	+++
Hcl-orange G methyl blue	Lillie, 1954	—	—	+	+++
Linder-Thomas phosphomolybdic acid haematoxylin	Linder, 1949	—	—	+	+++
In boiling water		—	—	—	Swells
Hot conc. H_2SO_4		Dissolves quickly	Dissolves quickly	Dissolves quickly	Dissolves quickly
Cold conc. H_2SO_4		Slowly dissolves	Slowly dissolves	Swells dissolves slowly	Swells and dissolves
Cold conc. Hcl.		Dissolves after a week	—	—	—
10% sodium hypo-chlorite		Dissolves in about 12 hrs.	Dissolves in about 12 hrs.	Dissolves in about 12 hrs.	Dissolves in about 12 hrs.

stain the entire primary gill bar and the tongue bar take up a red colour. The difference in the staining reactions of the gill bars in the larval and adult condition are reflected in their respective chemical composition.

In the adult condition both the primary and tongue bar yield a positive reaction to Millon's and Xanthoproteic tests. These reactions may suggest the presence of

a protein containing phenyl groups (Pearse, 1961; Bansley and Gersh, 1937). It is seen that the dorsal region of the primary gill bar reacts more intensely to the above tests than the ventral part of the primary gill and the tongue bar. It may be inferred that the distribution of the protein containing phenyl groups is not uniform throughout the length of the primary gill bar. The regions that contain this protein are also positive to Morner's reagent indicating the presence of tyrosine. The gill bar in the adult condition shows the presence of lipids as indicated by the positive reaction to Sudan Black B test. That the lipid may be of the nature of sterol is suggested by the positivity to Liberman-Burchardt test. The chemical composition of the adult gill bar appears to be more complex than in the larval condition.

In addition to proteins and lipids, positive reaction obtained with argentaffin test is indicative of the occurrence of reducing substances (Lison, 1936). Suggestive evidence that positivity to argentaffin test may be due to diphenols was indicated by the results of tests with ferric chloride. A green colour developed with a weak solution of ferric chloride may indicate the presence of diphenol (Lison, 1936; Pearse, 1961). The reaction with ferric chloride is more intense in dorsal part of the primary gill bar which assumed an intense greenish colour whereas the ventral part of the primary gill bar and the tongue bar took up a lighter green colour. On addition of sodium carbonate both primary and tongue bars turned red. The observations suggest the differential chemical composition of the primary gill bar and the tongue bar. It is of interest to mention in this context that the occurrence of polyphenols in the connective tissue of *B. lanceolatum* has been reported by Monne (1960).

The changes in the chemical composition noted above suggest that lipoprotein complex of the gill bar may combine with phenolic substances, indicative of a process similar to tanning. Support for such an assumption is provided by the results obtained by treating the gill bars of freshly collected amphioxus with 0.1% catechol at 40°C for a period of 20 minutes which brings about an amber colouration. It is known that such a result obtained with catechol treatment is due to the action of an oxidase (Krishnan, 1959; Symth, 1954). These results suggest that there may be a kind of hardening due to tanning which is of functional importance to gill bars in making them rigid so as to keep the pharyngeal cavity open when the animal is partially buried in sand. Such a feature may facilitate filtration of water (Jorgensen, 1966). The tanning process is possibly incomplete as inferred from the absence of sclerotization and the formation of a resultant amber coloured protein . The indications are that although the components for tanning are present tanning is incomplete. The results of treatment of the gill bars show that they have acquired some chemical resistance (Table 2).

In addition to the possible occurrence of a form of tanning in the gill bars there is indication of the presence of sulphur containing protein as evidenced by positive reaction to alkaline nitroprusside, tetrazolium and lead acetate tests. The intense reddish pink colouration of the dorsal part of primary gill bar with the application of nitroprusside test may indicate the probable aggregation of sulfhydryl groups. Further, the inner region of the primary gill bar is more intensively positive to the above test. Similar results were obtained with the alkaline tetrazolium test. It is suggestive from the observations that protein containing SH groups may be oxidized to give rise to –S–S– bonds which may contribute to the hardening of the gill bars. The occurrence of sulphur containing proteins in the endostyle and the presence of sulfhydryl groups in the skin of amphioxus have already been reported by Olsson (1961, 1963).

The involvement of sulphur in the hardening of the gill is suggested by the treatment of alkaline sodium sulphide which has the property of breaking the –S–S– bonds. It was noted after sodium sulphide treatment the gill bars both primary and tongue bars are rendered soft and the staining reactions of the gill bars are different in that stain blue and not red will Mallory's stain. Similarly, the occurrence of tanning may also be shown by its reversal on treatment with stannite solution as well as with 10% sodium hypochlorite. It is known that these detanning agents by their ability to destroy quinone binding bring about a reversal of staining reactions form red to blue (Dennell, 1958, Trim, 1941).

The relation between tanning and hardening by –S–S– bonding is not clear. It is suggestive that they may occur in different regions of the gill bar. It has been shown that in the exoskeletal structures of the arthropods a part of the cuticle may be hardened by –S–S– bonding and another part by tanning (Krishnan, 1953). Analogous condition is suggested in the gill bars, the effect being to strengthen them chemically and make them rigid mechanically.

A feature of chemical interest reported by earlier workers (Lankester, 1889; Benham, 1894; Sannasi and Harmann, 1970) in the gill bars of amphioxus is the occurrence of chitin. Lankester and Benham based their observations on the occurrence of increased rigidity of adult gill bars compared to that of the larval gill bars. Chitin has been shown to occur in invertebrates specially in arthropods annelids and molluscs (Richards, 1951; Dennell, 1958; Runham, 1961 a, b). It has not been reported in chordates (Rudall, 1955). The chitin test (Campbell, 1929) which involves the treatment of gill bars with concentrated potassium hydroxide solution at 180°C readily dissolved the gill bar within a period of 3 minutes. The tests for chitin was also negative. The modified chitosan test of Clark and Smith (1936) as well as treat ment with conc. alkali at 100°C (Sannasi and Hermann, 1970) with subsequent treatment with iodine/sulphuric acid gave only brown-amber colour and not violet. It is suggested that in the gill bar of adult amphioxus as well as in the larval stages chitin is absent. The rigidity of the gill bar attributed by earlier workers to chitin may be due to tanning or –S–S– bonded protein in it.

Studies on the chemical composition of the notochord in the larval stages of amphioxus and in the adult show marked differences in the two stages. The results of staining reactions and histochemical tests are given in Table 2. The results suggest that the notochord of larvae undergo changes in chemical composition during the transition from the larval to the adult phase. Some information is already available on the chemical nature of the notochordal sheath from the work of Eakin and Westfall (1962), Welsch (1968) who inferred the presence of a collagenous protein from electron microscopic studies on the notochord of amphioxus. Flood (1967, 1968) reported the paramyosin like protein in the notochord of amphioxus.

In the present study, from histochemical tests on the larval notochord it may be seen that it contains a simple protein reacting to bieuret test. There is no indication of the presence of lipids and phenolic groups in the larval notochord either in the plates or in the sheath. In the adult condition the notochordal plate and the notochordal sheath show staining reactions different from those in the larva (Table 2). With Mallory's triple stain the notochordal plate takes up a red colour while the sheath remains unaltered conforming to the condition noted in the larva. Similar results were obtained with Haematoxylin and Masson's trichrome stains. The notable changes undergone by the notochordal plates are in the protein composition.

The adult notochord reacts positively to Xanthoproteic and Millons tests as well as to Sudan Black B and Liebermann-Burchardt tests.

The presence of a protein containing aromatic groups and lipids together with argentaffin and ferric chloride positive substances, suggest a similarity to the condition reported in the adult gill bar where it has been suggested that they may be involved in a process of tanning. As in the latter tanning does not appear to be complete in that the notochordal plates still retain the reactivity to stains and do not assume an amber colouration and become insenstiive to chemical reagents. The notochord also shows the presence of organic sulphur. The possible occurrence of both-SH and -S–S– groups is indicated by the positivity to nitro-prussideand lead acetate tests. The positive reaction to the alkaline tetrazolium test recalls the reaction obtained with the adult gill bars. As in the latter the adult notochordal plates react to both stannite solution which detains and to alkaline sodium sulphide which tends to break up –S–S– bonds. The changes in the notochordal plates appear to recall strongly those reported in the gill bars during the growth stages. The changes in the chemical composition of both the gill bar and the notochordal plate confer rigidity to these structures in the adult condition and such changes involve a degree of both tanning and –S–S– bonding.

An interesting observation reported in previous studies is that the sheath is formed of a collagenous protein (Eakin and Westfall, 1962; welsch, 1968). The results obtained in the present study show that the notochordal sheath as well as the notochordal plates are presumably formed of a collagenous protein as inferred from the staining reactions of these structures. Fast green Von Gieson's test Hcl-Orange G methyl blue and Linder Thomas Phosphomolybdic acid haematoxylin (Lillie, 1954; Linder, 1949) are positive in the notochordal sheath and the notochordal plates of the larva. This condition persists in the sheath in the adult condition although the plates undergo modification. The above staining reactions may not by themselves indicate the collagenous nature of the protein in question, but they provide suggestive evidence for their presence since collagens have been reported to give positive reactions to these stains (Lillie, 1954).

DISCUSSION

In arthropods a close correlation between function and chemical composition of the exoskeleton has been shown by a number of workers (Wiggleswoth, 1948; Dennell, 1958; Beament, 1961). It has been shown in an intertidal crustacean like *Emerita asiatica* both tanning and calcification contribute to mechanical rigidity of the exoskeleton which is of advantage to animals in their burrowing mode of life and as protection against exposure to wave action (Chockalingam, 1967).

In contrast to the conditions seen in the adult, the exoskeleton of their larval forms is soft and flexible. In the transition from the larval condition to the adult phase the cuticle undergoes changes in chemical composition resulting in acquisition of mechanical rigidity and chemical resistance. This is brought about by a process of tanning of the protein constituents of the cuticle by quinones resulting in the formation of sclerotin. In a number of arthropods especially arachnids a similar change in the condition of the cuticle may be brought about by disulphide bonding comparable to a process of Keratinization in which a sulphur containing protein is involved.

An analogous condition has been suggested with reference to the skeletal parts of amphioxus. In the life cycle of amphioxus, there are two distinct phases, the larval phase in which it is free swimming and an adult condition when it is sedentary and burrowing. Although the transition from one phase to the other may not involve marked structural changes (Barrington, 1965), it is seen from the observations reported in the present study that chemical changes do occur in the skeletal parts, the gill bars and notochord which recall the changes taking place in the exoskeleton of arthropods when they pass from a larval to adult condition. The work of Olsson (1963) may suggest the presence of two different types of protein, one containing sulphur secreted by the endostylar cells of amphioxus. It is shown in the present study that a sulphur containing protein and a protein containing aromatic groups which are the essential components of the skeletal structures of amphioxus, undergo a process of hardening in varying degree in these structures in the adult thereby contributing to the rigidity of the structures. The significance of an hardened skeleton in the context of the mode of life of the adult amphioxus is that it provides the necessary rigidity for a sedentary and burrowing habit in a sandy substratum.

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