The Histochemistry of the Radula of *Patella vulgata*

By N. W. RUNHAM

*(From the Department of Zoology, University College of North Wales, Bangor, Caernarvonshire)*

**SUMMARY**

When first secreted the radula consists of chitin and protein. In the bases and cusps of the teeth the reactions for tyrosine, tryptophane, and chitin become negative, and are replaced by reactions for amino groups and argentaffin material. The bases of the teeth become impregnated with iron. Subsequently the reactions for amino groups and argentaffin material become negative in the cusps and they then become impregnated with iron and silica. Very few changes have been observed in the radular membrane.

It is suggested that the occurrence of quinone tanning might account for these histochemical changes observed in the radula, but phenols derived from tryptophane would appear to be more important in the tanning of the radula than those derived from tyrosine.

**INTRODUCTION**

In view of the great importance of the radula in the life of the gastropod and cephalopod molluscs it is surprising that the composition of this organ has been so neglected. A vast amount of work on its anatomy has been carried out owing to its importance in systematics. The growth and mode of secretion of the radula has been investigated by many authors, but the results have so far been very inconclusive. Thanks largely to the work of Spek (1921), Sollas (1907), Jones and others (1935), and Rudall (1955) there is little doubt that the radula contains chitin, protein tyrosine, and various minerals. In *Patella vulgata* large amounts of iron (14.3% dry wt. Fe₂O₃) and silica (8.7% dry wt. SiO₂) are found. Gabe and Prenant have carried out very extensive investigations of the molluscan radula but mainly on the cytology. Recently (1952) they have published the results of some histochemical studies and they conclude that mucopolysaccharides are present. It was decided therefore to investigate histochemically, first the radula of *P. vulgata* (the results of which form the substance of the present paper) and then to extend this study to various other molluscan species (the results to be published as a subsequent paper).

**MATERIAL AND METHODS**

Limpets (*P. vulgata* L.) were obtained from the Scottish Marine Biological Association, Millport. The radula of an adult limpet is between 5 and 8 cm long, and it is impossible to section most of this owing to its extreme hardness. Two pieces, each 1 cm in length, were cut therefore from the proximal end, and fixed. The majority of this investigation was carried out on material fixed in formaldehyde-calcium (Baker, 1944); where extraction of lipids was necessary weak Bouin's fixative was used (Baker, 1946), and for microincineration alcoholic buffered formalin (Glick, 1949). This material was then embedded in ester wax (Steedman, 1947) and sectioned at 8 μ. For the investigation of lipids
material fixed in formaldehyde-calcium was embedded in gelatin and sectioned on a freezing microtome (Baker, 1944).

Microincineration was carried out by the procedure given by Glick (1949). After incineration the slides were covered with a coverglass, ringed with paraffin wax, and examined by dark ground illumination. Some attempt was made to study the distribution of silica by the methods given by Sollas (1907).

The presence of chitin was investigated by microchemical techniques (Gatenby and others, 1953), and by paper chromatography (Smith, 1958).

The histochemical methods employed in this study are summarized in table 1, and the various controls and blocking reactions in table 2 (see appendix).

Attempts were made to investigate the stabilization of the protein in the radula by the methods suggested by Brown (1950a).

For the investigation of the effects of diaphanol on Mallory staining of the radula the material was fixed in formaldehyde-calcium, washed in water and 75% alcohol, and then placed in the diaphanol. The control and the pieces subjected to the action of diaphanol for 1, 2, 3, 4, 6, 8, or 11 days were washed in 75% alcohol and then embedded in ester wax.

RESULTS

The results of the histochemical reactions are summarized in fig. 1.

Minerals

By using Sollas’s procedure for investigating silica it was found that after the nitric acid treatment only cusps and small pieces of the bases of the teeth remained as a brown sludge. Treatment of the sludge with aqua regia dissolved the remains of the bases and also released iron from the cusps, as was shown by the reaction of the filtrate with potassium thiocyanate solution. The filtrate did not react with ammonium molybdate solution (Vogel, 1954), so that soluble silicates were absent. The residue consisted of colourless material which dissolved in hydrofluoric acid and was presumably the hydrated silica shown to be present by Sollas (1907). It was of interest, however, that the residue consisted of the posterior surfaces only of the older cusps. The methods employed did not allow any determination of the level along the radula at which silica appeared to be made.

Microincineration revealed a dense red ash in the cusps from row 45 onwards. Iron is the only mineral giving a red ash with this procedure (Glick, 1949). With the use of the Perl technique iron was shown to appear in the teeth bases from row 13 onwards and the teeth cusps from row 28. It never appeared in the radular membrane, however.

Carbohydrates

The microchemical reactions confirmed the presence of chitin in the radula, and chromatography has shown that the only sugar present in 0.1 N sulphuric acid hydrolysates of the radula is glucosamine. Chitin therefore
FIG. 1. A summary of the histochemistry of the radula of *P. vulgata*. The numbers refer to the tooth row, numbering from the proximal end of the radula. The thickness of the black in is proportional to the intensity of the reaction.

appears to be the only carbohydrate present in the limpet radula. The histochemical results for carbohydrates are in agreement with this conclusion. These results are analysed in detail elsewhere (Runham, 1960b) and only a summary is given here. The acetylamide-glycol groups of chitin are capable of giving a positive PAS reaction at least in this material. The alcin blue and Hale reactions are both due to intermolecular chelation between the acetyl group and the alcoholic hydroxyl group. Thus acetylation by blocking the hydroxyl group, and methylation which has been shown to hydrolyse the
acetyl group, prevent these reactions. Methylation releases amino groups, as shown by the bromophenol blue reaction. Deamination particularly after methylation causes hydrolysis of the chitin and probably conversion of the glucosamine to anhydro 2:5 D mannose, thus decreasing the intensity of the PAS reaction.

**Proteins**

The bromophenol blue reaction by means of a deamination control has been found to be specific for amino groups. The hydroxynaphthaldehyde reaction reveals only protein α acyl amino groups, and the amino groups revealed by this reaction largely parallel those obtained with the bromophenol blue technique. The ninhydrin / Schiff reaction was found to be very weak, so that only the presence of large numbers of amino groups was revealed.

The Morel–Sisley reaction paralleled the results obtained with the Millon reaction, but was far more intense.

**Lipids**

The reactions for lipids in the radula were all negative. It is possible that bound lipids are present although this is unlikely in view of the recent criticisms of Berenbaum's technique by Locke (1959). Locke has shown that chitosan will give the sudan black B / acetone reaction for bound lipids, and it is of interest that in this material the regions containing free chitin appeared to be stained by this technique.

**Other compounds**

With the diazonium reaction a fiery red coloration indicates the presence of polyphenolic material. In the case of the radula only a yellow to orange colour, rather more intense in the bases of the older teeth, was found: it was assumed that this reaction was negative. The argentaffin reaction was strongly positive in the older bases. A detailed investigation into the basis of the argentaffin reaction in this material is necessary before any definite conclusions can be drawn.

It was decided to investigate the possibility that the secretion of iron into the radula was responsible for some of the changes in the histochemical reactions. The results were inconclusive. The sections were treated for 24 h at room temperature with the solution devised by Warner and Weber (1953) for their studies on the iron binding of proteins. Subsequent to this treatment the Perl reaction revealed that the iron was distributed in a similar fashion to that found in the Hale reaction. The iron treatment blocked the alcian blue reaction, which indicated that the same chemical groups are responsible for the Hale and the alcian blue reaction in this material. The iron solution caused an increase in the bromophenol blue staining, an effect which warrants more detailed investigation. It is possible that iron secreted into the radula might cause the increase in the number of amino groups in the bases of the older
teeth, but as a similar sequence occurs in other moluscan radulae where iron is not found, this is considered to be unlikely (Runham, 1960a).

By use of the series of solutions suggested by Brown (1950a) for the investigation of the forces stabilizing proteins it was found that the radula was affected only by the sodium hypochlorite solution. This would suggest the presence of quinone-tanned material.

Histochemical reactions for tyrosinase were negative.

**DISCUSSION**

The radula when first secreted consists of chitin and protein, possibly in the form of a glycoprotein, as in the insect cuticle (Hackman, 1959). The acetamide and hydroxyl groups of the chitin are at least in part available for reaction, while the protein gives weak reactions for amino groups and tyrosine, and very strong reactions for tryptophane. Subsequently the radula becomes altered chemically in various ways. The end-result of these changes is a radula consisting of exceptionally hard radular teeth mounted on hard but brittle bases, and these are embedded in a tough, leathery, radular membrane.

In the invertebrates quinone tanning is a common method of hardening (Brown, 1950b). It was therefore of great interest to find out to what extent the histochemical results obtained here indicate that quinone tanning occurs in the radula.

Previously described examples of quinone tanning have the following properties in common: the enzyme responsible both for hydroxylation and for quinone formation is the phenolase complex (Mason, 1955); phenolic or polyphenolic material is present (perhaps in some cases aminophenols (Pryor, 1955); on exposure to air the untanned material often darkens; and the tanned material is soluble only in the sodium hypochlorite solution of Brown's series of solutions.

All attempts to demonstrate phenolase histochemically in the radula have given negative results. Further investigations must be carried out before this conclusion can be accepted. From the work of Whitehead and others (1960) and of Yasonobu (1959) it would appear that phenolases from different sources have differing substrate specificities. Therefore methods involving the use of catechol (Smyth, 1954) and haematoxylin (Lillie, 1956) as substrates may not demonstrate the enzyme in the molluscan radula. Further work has to be carried out with a wider range of substrates.

The argentaffin reaction was intensely positive in the bases of the older teeth, while the diazonium reaction was negative. Both of these reactions reveal aminophenols and polyphenols (Lison, 1936). In the absence of investigations on the specificity of the reactions, the meaning of these results is somewhat uncertain. Smyth and Clegg (1959) found that in cestodes one diazonium salt gave a strong reaction with the polyphenols of some species but not with others. Only fast red salt B has been used in this study. If a soluble phenol were involved it would presumably have dissolved out in the preparatory procedures. If aromatic amino-acids in the protein of the radula were oxidized by
an a phenolase to quinones they might react with the available amino and/or sulphhydryl groups of other protein chains as soon as they were formed, so that it may not be possible to detect them histochemically.

Exposure to air does not appear to cause a darkening of the limpet radula, but, as the only darkening in the radula of this species appears to be due to iron, this is to be expected. Of the series of reagents suggested by Brown for the identification of the forces stabilizing proteins, only sodium hypochlorite solution dissolved the radula. Boiling in saturated potassium hydroxide for 1 h does not affect the shape of the radula but dissolves out most of the protein, leaving the chitin. That only the sodium hypochlorite dissolved the radula implies that covalent bonds are responsible for its stabilization, but in this case they may well be the covalent bonds of chitin, not of a quinone-tanned protein. Experiments were carried out on the cuticle of *Locusta* to confirm these results. After exposure to the 10% sodium hypochlorite solution for 1 day the cuticle was bleached and after 2 days it had disintegrated into small pieces of which very few remained after 3 days.

On these criteria the evidence for the presence of quinone tanning in the radula is very slight and equivocal. There is, however, some indirect evidence that quinone tanning may occur here. Dennell and Malek (1955 a, b) divide the tanning of the insect cuticle into primary and secondary tanning thus:

**Primary tanning**

1. Sterol reactions at first positive become negative.
2. Protein tyrosine at first positive becomes negative.
3. Protein amino groups at first strongly positive become weaker.
4. An argentaffin reaction appears.
5. Mallory's triple stain stains the cuticle initially blue but this is later replaced by red staining. This change is reversed by treatment with diaphanol which destroys the aromatic amino-acids.

**Secondary tanning**

1. The red Mallory staining disappears, leaving the cuticle unstained.
2. The weak reactions for protein amino groups become negative.

Apart from the presence of sterols all these changes are found in the radula of the limpet. Diaphanol caused a gradual reversal of the changes in Mallory staining, the reversal being complete after 6 days' treatment. Kennaugh (1957) found that diaphanol first removed the aromatic acids from the insect cuticle and then more slowly the other ninhydrin-positive material. It would therefore appear that aromatic groups are responsible for the change in Mallory staining of the older radula. As the resemblances between the insect cuticle undergoing tanning and the radula are so great, it would seem likely that a similar mechanism is involved. In the radula, however, it would appear that quantitatively tryptophane is more important than tyrosine in hardening.

The sequence of changes that the author envisages as occurring in the radula of *P. vulgata* is as follows. The radula is first secreted as a chitin-protein
mixture or as a glycoprotein (Hackman, 1959). As far as has been observed in
this species very few subsequent changes occur in the radular membrane. The
bases of the teeth become impregnated with extra protein material rich in
amino groups and this becomes linked to the structural protein by its tyrosine
and tryptophane groups, presumably through phenolic derivatives. The cusps
appear to be similar to the bases of the teeth, but either tanning is more rapidly
completed, or the tanned material becomes linked to the impregnating silica.
It is not known which of these two alternatives is the more likely.

Previously described evidences of quinone tanning are believed to involve
tyrosine derivatives and are therefore related to melanin formation. However,
there is a group of pigments based on oxidation products of tryptophane,
namely the ommins (Forrest, 1959). A system of tanning based on trypto-
phane derivatives would appear to be possible and has previously been
suggested by Pryor (1955). The oxidation product of tryptophane found in the
ommins is 3-hydroxy kynurenine. If this oxidation occurred in the radula one
could explain the disappearance of reactions for tryptophane with the simulta-
aneous appearance of reactions for amino groups and the argentaffin reaction.
It is proposed to carry out a chemical investigation to determine if in fact this
mechanism of tanning occurs in the radula.

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APPENDIX

TABLE I. Summary of histochemical reactions

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Reference</th>
<th>Material demonstrated</th>
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<tr>
<td>Peri</td>
<td></td>
<td>ferric iron</td>
</tr>
<tr>
<td>periodic acid / Schiff (PAS)</td>
<td></td>
<td>glycol or glycolamine groups</td>
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<tr>
<td>alcian blue</td>
<td></td>
<td>acid groups of polysaccharides</td>
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<tr>
<td>Hale</td>
<td>Zugibe and others, 1959</td>
<td>acid groups of polysaccharides</td>
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<tr>
<td>mucinhyematin</td>
<td>Pearse, 1953</td>
<td>mucin</td>
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<tr>
<td>azure A metachromasia</td>
<td>Kramer and Windrum, 1955</td>
<td>acid groups of polysaccharides</td>
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<tr>
<td>sulphation, azure A</td>
<td>Kramer and Windrum, 1954</td>
<td>free hydroxyl groups</td>
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<tr>
<td>bromophenol blue</td>
<td>Mazia and others, 1953</td>
<td>amino groups</td>
</tr>
<tr>
<td>ninyhdrin / Schiff</td>
<td>Burstone, 1955</td>
<td>amino groups</td>
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<tr>
<td>hydroxynaphthaldehyde</td>
<td>Weiss and others, 1954</td>
<td>protein amino groups</td>
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<td>rosinole</td>
<td>Glenner, 1957</td>
<td>tryptophane</td>
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<td>Morel / Sisley</td>
<td>Lillie, 1957</td>
<td>tyrosine</td>
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<td>Millon</td>
<td>Pearse, 1953</td>
<td>tyrosine</td>
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<td>Sudan black B / 70% alc. (SBB)</td>
<td>Pearse, 1953</td>
<td>lipids</td>
</tr>
<tr>
<td>Nile blue</td>
<td>Pearse, 1953</td>
<td>acid lipids</td>
</tr>
<tr>
<td>burnt SBB</td>
<td>Pearse, 1953</td>
<td>bound lipids</td>
</tr>
<tr>
<td>SBB / 70% alc. 70° C</td>
<td>Berenbaum, 1958</td>
<td>bound lipids</td>
</tr>
<tr>
<td>SBB / acetone 70° C</td>
<td>Berenbaum, 1958</td>
<td>bound lipids</td>
</tr>
<tr>
<td>diazonium</td>
<td>Berenbaum, 1958</td>
<td>polyphenols</td>
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<td>argentaffin</td>
<td>Pearse, 1953</td>
<td>polyphenols, polyamines, &amp;c.</td>
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<td>aldehyde</td>
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<td>tyrosinase</td>
<td>Danielli, 1950</td>
<td>tyrosinase</td>
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<td></td>
<td>Smyth, 1954</td>
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<td></td>
<td>Lillie, 1956</td>
<td>tyrosinase</td>
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### Table 2. Summary of control procedures

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<th>Control reaction</th>
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<tr>
<td>diastase</td>
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<td>glycogen</td>
<td>1% BDH diastase in aq. dest. 37° C ½ h Benger’s testicular hyaluronidase obtained from N. W. Carter</td>
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<td>hyaluronidase</td>
<td>Pearse, 1953</td>
<td>hyaluronic acid; chondroitin sulphuric acids A and C</td>
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<td>pectinase</td>
<td>Grainger and Shillitoe, 1951</td>
<td>galactogen and pectin</td>
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<td>acetylation</td>
<td>Lillie, 1954</td>
<td>amino and hydroxyl groups</td>
<td></td>
</tr>
<tr>
<td>benzoylation</td>
<td>Lillie, 1954</td>
<td>amino and hydroxyl groups</td>
<td></td>
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<tr>
<td>deacetylation</td>
<td>Pearse, 1953</td>
<td>acetyl groups</td>
<td></td>
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<tr>
<td>deamination</td>
<td>Lillie, 1954</td>
<td>amino groups</td>
<td></td>
</tr>
<tr>
<td>methylation</td>
<td>Fullmer and Lillie, 1957</td>
<td>acid groups</td>
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<td>demethylation</td>
<td>Spicer and Lillie, 1959</td>
<td>methyl esters</td>
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<td>aldehyde blockade</td>
<td>Baker, 1946</td>
<td>aldehyde groups</td>
<td>material fixed in weak Bouin</td>
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<td>pyridine extraction</td>
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<td>lipids</td>
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<td>iron solution</td>
<td>Warner and Weber, 1953</td>
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