A Technique for the Histochemical Demonstration of Polyphenol Oxidase and its application to Egg-shell Formation in Helminths and Byssus Formation in *Mytilus*

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With one plate (fig. 2)

Summary

1. The distribution of polyphenol oxidase in quinone-tanning systems may be demonstrated in frozen-dried sections by incubation in 0·2 per cent. aqueous catechol at 40° C. for 15–60 minutes. A red colour develops at the enzyme site.

2. The evidence for the view that the egg-shell in trematodes, in certain cestode groups, and in turbellarians, is a quinone-tanned protein secreted by the so-called 'vitelline' glands, is summarized. The 'vitelline' cells, in addition to giving positive reactions for proteins and phenols, give a strongly positive reaction with the catechol polyphenol oxidase test.

3. The catechol technique may also be applied to whole helminths fixed in 70 per cent. alcohol, and serves as a useful whole mount stain for the shell-producing regions of the female genitalia.

4. In *Mytilus* the catechol technique reveals the presence of polyphenol oxidase in an 'upper' or enzyme gland in the foot.

5. It is suggested that in *Mytilus* the byssus is formed from a phenolic protein secreted from the phenol gland, which on contact with polyphenol oxidase can undergo 'auto-quinone tanning'.

Introduction and Previous Work

WITHIN recent years, particular interest has been shown in the structure and formation of quinone-tanned proteins known to occur in various invertebrate groups (Brown, 1950). The basis of quinone-tanning appears to be the interaction of a quinone and a protein, the former being formed enzymatically from an orthodiphenol in the presence of oxygen (Pryor, 1940). Only in the case of the ootheca of *Blatta* has the diphenol compound been identified; in this case, Pryor, Russell, and Todd (1946) isolated protocatechuic acid. There is evidence to suggest that, in at least one form, *Mytilus*, the diphenol may be an amino-acid or associated with a protein (Brown, 1952).

Evidence for the existence of a quinone-tanning system is based on:

(*a*) Macroscopic observation of the actual tanning process on exposure of the material to air.
(*b*) Positive histochemical tests for proteins.
(*c*) Positive histochemical tests for polyphenols.
(*d*) Disappearance of tanning on treatment with sodium hypochlorite.

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Brown (1950) neatly summarizes the criteria as follows: 'If it is found therefore that a structural protein dissolves only in sodium hypochlorite solution, and is secreted by tissues containing a polyphenol, it may be concluded that there is circumstantial evidence for aromatic tanning.'

Recent work in helminth physiology (Smyth, 1951*a*) has drawn particular attention to the structure of the so-called 'vitelline' glands which have long been suspected to play a major part in the formation of egg-shell material by a quinone-tanning process. Theoretical consideration of the mechanism of this process led to the development of a technique for the cytochemical localization of the oxidase concerned in such a system. This technique and its application to egg-shell formation in helminths and byssus formation in *Mytilus* form the basis of this paper.

Material

The bulk of the experiments on helminth material were carried out on *Fasciola hepatica*, but in the later stages confirmatory experiments were extended to other helminths, as follows: Trematoda, *Haematoloechus sp.* (lungs of frog); *Dolichosacchus rastulus* (gut of frog); *Diclidophora merlangi* (gills of whiting); pseudophyllidean cestode, *Schistocephalus solidus* (matured artificially *in vitro*), by using the cellulose tube technique (Smyth, 1954); tetraphyllidean cestode, *Proteocephalus filicollis* (gut of stickleback). When the technique was finally established, it was further tested on *Mytilus edulis*.

Preparation of Frozen-dried Material

Material was fixed in liquid oxygen (liquid nitrogen or liquid air not being available) for 1 minute, dehydrated in an Edward's tissue dryer for 3 days at −40° C., and embedded in wax in a vacuum by the usual procedure. Sections were cut at 7·5 μ and mounted, without floating on water, on slides smeared lightly with albumen and warmed slightly before mounting. Slides were left to dry, at least overnight, at 40° C. in a drying oven.

Frozen-dried sections were treated either (*a*) by removing the wax, as usual, with xylene, and passing down the alcohols—sections thus treated being in fact alcohol-fixed; or (*b*) by staining sections without first removing the wax. Occasionally sections stained through wax gave slightly sharper cytological pictures or were more reactive to cytochemical tests than alcohol-fixed sections, but, in general, little difference was apparent.

In addition to the freezing-drying method, the usual routine fixatives and routine wax or gelatine embedding procedures were used. When special methods were used they are referred to in the text.

Evidence for Quinone-tanning in Helminths

The question of obtaining concrete evidence for the specificity of a cytochemical test claiming to demonstrate the site of a particular enzyme is a peculiarly difficult one. The proof depends mainly on circumstantial evidence from other cytochemical tests indicating the presence of one of the normal

substrates of the enzyme, as well as theoretical considerations as to the likelihood of the enzyme having the distribution found. It is, therefore, important to establish that the helminth tissue is positive for the criteria for quinone-tanning systems established by Brown (1952) and mentioned earlier.

The eggs of the majority of trematodes and pseudophyllidean cestodes are colourless when laid, but turn brown or 'tan' on exposure to air; such eggs may be bleached by treatment with sodium hypochlorite. This immediately indicates protein tanning of some kind. The pseudophyllidean cestode, *Schistocephalus solidus*, when matured *in vitro* under anaerobic conditions, produces normal eggs which tan on exposure to air. When cultured under *aerobic* conditions (i.e. with a stream of air bubbling through the culture medium) normal eggs are not produced, the so-called 'vitellaria' become brown in colour and strings of brown material emerge from the uterine pore (Smyth, 1950). This result is interpreted as being due to the pre-tanning of the egg-shell material while still within the vitelline glands.

The histochemistry of the vitelline glands of a number of platyhelminths reveals that these glands are rich in proteins and polyphenols, according to the work of Vialli (1933, 4), Stephenson (1947), and Nurse (1950). Stephenson's results with *Fasciola* have been fully confirmed in this laboratory, by using mainly frozen-dried material, and applying the most critical histochemical tests available. A number of additional observations have also been made on other forms. Although the entire range of tests available has not been applied in every case, and although many of the tests alone are not specific, the accumulated evidence as summarized in Table 1 (see end of paper) leaves little doubt, on the whole, that the vitelline gland cells are rich in proteins and polyphenols. With frozen-dried material, the histochemical tests for both phenols and proteins which do not require severe techniques (e.g. Millon's) are particularly sharp. An exception is the so-called ferric chloride test for polyphenols, much quoted by previous authors in quinone-tanning studies. In this test the presence of an *o*-diphenol is supposed to be demonstrated by the appearance of a green colour with dilute ferric chloride (Lison, 1936), turning red on treatment with 2 per cent. potassium carbonate or purple with dilute ammonia. Microscopically, green is a most unsatisfactory colour to observe, and I have never been able to obtain a colour which could be called green with the certainty essential for histochemical tests. The red colour, after alkaline treatment, is, however, readily obtained and for this reason the ferric chloride test has been taken to be positive. Of the remaining phenol tests, the argentaffin, chromaffin, ammonium molybdate, and sodium iodate tests readily give strongly positive reactions. Although no single one of these is specific, taken as a group, together with a positive ferric chloride reaction (after alkali treatment), they provide strong evidence for the presence of *o*-diphenols. Of the protein tests, the Millon works particularly brilliantly with frozen-dried material, but is non-specific as the red colour produced is also given by diphenols. The xanthoproteic test is always positive but, again, is not entirely specific for proteins. The ninhydrin test was first attempted

on poorly hardened material and was only weakly positive. On the same material, the Sakuguchi and Biuret tests were negative. On theoretical grounds, this result was not unexpected as a secretory protein of this kind is likely to be in a highly labile and probably soluble state and would easily be lost in unhardened material. All these tests proved strongly positive in material that had been fixed in 5 per cent. formaldehyde-saline, embedded in gelatine, hardened for 8 weeks in formalin vapour, and cut as frozen sections. Baker's elegant modification (1947) of the Sakuguchi arginine test gave a particularly strong reaction with gelatine sections. The Biuret test, likewise, gave a strongly positive result, the granules appearing rose-pink, a colour indicative of lower proteins.

Only in the trematodes have tests for both phenols and proteins been carried out. In the turbellarians and cestodes, only tests for phenols have been carried out and positive results reported—except for a few results for *Polycelis*. The circumstantial evidence for quinone-tanning in these groups is, however, considerable.

The Catechol Technique for Detection of Polyphenol Oxidase in Tissues

Theoretical basis

The essential reactions in a quinone-tanning system may be summarized as follows:

$$\text{protein} + \text{diphenol} \xrightarrow{\text{polyphenol oxidase}} \text{protein} + \text{quinone}$$

$$= \text{tanned protein (sclerotin).}$$

In *Fasciola* rapid auto-tanning of sections does not occur (but see p. 144); so it can be assumed that although the polyphenol, enzyme, and protein constituents occur in the same globule (fig. 1), some blocking mechanism probably prevents the interaction of the phenol and enzyme.

Incubation of tissue containing such a system, with catechol as a substrate, should result in the formation of *o*-quinone at the sites of the enzyme; this quinone should then rapidly combine with and tan the adjacent protein to give rise to a reddish-brown colour. A prerequisite of such a technique would be that the enzyme should be preserved and the protein left still in a 'tannable' condition, i.e. with the imino and amino groups more or less intact. The reddish-brown colour should be very stable and only removable with sodium hypochlorite. The whole reaction should be inhibited by dilute cyanide.

Experimental results

In practice, the method has proved to be remarkably successful. The technique finally adopted for freshly prepared frozen-dried sections of *Fasciola* was as follows:

(1) Remove wax and bring down through alcohols to water.

(2) Incubate in 0·2 per cent. catechol (freshly prepared) at 40° C. for 15–30 minutes.
(3) Dehydrate, as usual, in 70 per cent., 90 per cent., and absolute.
(4) Treat with xylene and mount in balsam.

Within 2 minutes of incubation, with this technique, the globules in the vitelline cells became yellow, and after 10–15 minutes pinkish-red. The vitelline cells so coloured stood out clearly against the remainder of the tissue

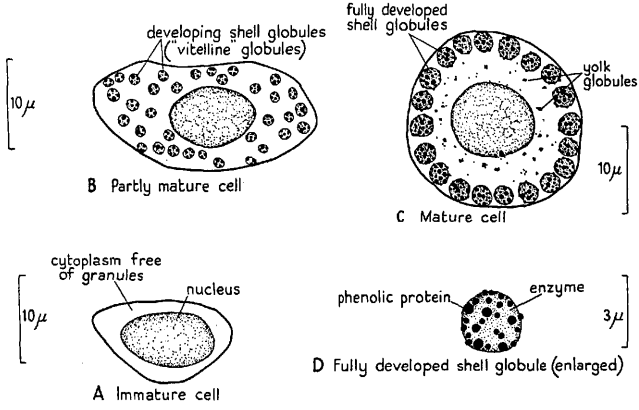

FIG. 1. Development of the 'vitelline' cells of *Fasciola* as shown in frozen-dried material stained in malachite green and counterstained in Gower's carmine. The large black dots within the shell globules are bright green; nuclei and yolk globules are red.

(fig. 2, B). In sections incubated for long periods (about 12 hours), the vitelline cells became darkish-brown and the remainder of the tissues greyish-brown, the whole almost having the appearance of a haematoxylin-stained section (fig. 2, F). Since, after several hours' exposure to air, catechol solutions become partly oxidized to quinone and this quinone solution will then slightly tan any tissues containing protein, irrespectively of the presence of the enzyme, this result is to be expected. It is essential, therefore, to use freshly prepared catechol solution for this test; a solution prepared in the morning may be used throughout the same day, provided it be stored in a reasonably cool place. The development of the red colour in *Fasciola* sections was inhibited by the addition of M/1000 KCN to the catechol solution. The red colour was also destroyed by 0·1 per cent. NaOCl—both these results being expected on theoretical grounds. Under low-power observation the red colour of the vitelline globules appeared sharp, but at oil immersion levels it seldom appeared so, a failing shared with the great majority of cytochemical tests involving proteins. The colour occurred only in the larger and mature, or

nearly mature, cells laden with globules of shell material, and not in the small and immature cells. This result is similar to that obtained with the malachite or methyl green techniques; but the red colour, although often having a mottled appearance, was not concentrated within definite granules within the globules as is the case with the former technique (fig. 1).

As is shown with *Mytilus*, catechol may form brown condensation-compounds with phenols, and the dark brown developed in the glands after about 12 hours in catechol may additionally be due to this reaction. This colour is, however, almost unaffected by NaOCl.

Effect of storage of material

As work with frozen-dried material progressed, two additional facts emerged: (*a*) that in sections cut and mounted on slides and stored at room temperature for several weeks, the 'vitelline' globules became slightly tanned, the deepest colour reached being yellow or pinkish-yellow. This result suggests that slow auto-tanning, which may or may not be enzymatic, can slowly take place; (*b*) that at the same time a gradual diminution of the effectiveness of the catechol technique took place after prolonged storage—a result interpreted as being due to loss of enzyme activity or denaturing of proteins, or both.

The Catechol Technique as a Whole-mount Stain for Helminths

Apart from its use as a histochemical method for demonstration of polyphenol oxidase in sections, it has been found that the technique is remarkably successful as a whole-mount stain for helminths containing quinone-tanning systems.

The simplest and most satisfactory fixative, so far developed for this purpose, is 70 per cent. alcohol, and the procedure adopted for flukes such as *Fasciola* and *Dolichosaccus*, and the cestode *Schistocephalus*, has been as follows:

(1) Fix in 70 per cent. alcohol—24 hours.
(2) Wash in water—30 minutes.

Fig. 2 (plate). A, *Fasciola* fixed 70 per cent. alcohol and incubated in 0·2 per cent. catechol for 90 minutes. Note unusual branching of left 'vitelline' duct.

B, *Fasciola*, frozen-dried section, incubated in catechol solution for 15 minutes. The 'vitellaria' stand out (red) against the almost colourless cytoplasm.

C, *Fasciola* treated as A.

D, *Schistocephalus*, treated as A.

E, *Mytilus* foot; horizontal section; frozen-dried section stained in malachite green. The phenol gland stains green and may be clearly seen to be secreting the byssus through posterior ducts.

F, *Fasciola* treated as for B, but incubated for 24 hours. Some tanning of cytoplasm has now also taken place.

G, *Mytilus* foot; hand section, fixed in 70 per cent. alcohol, incubated in catechol solution for 1 hour.

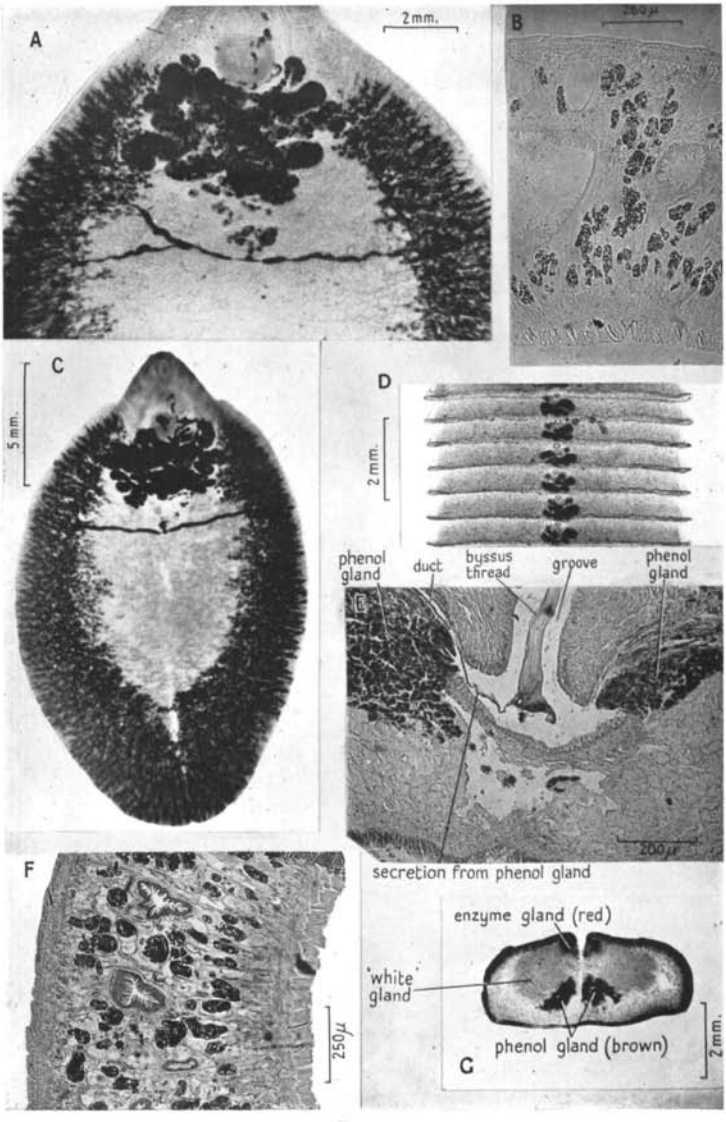
A
2mm.
B
200μ
C
5mm.
D
2mm.
phenol gland
duct
byssus thread
groove
phenol gland
200μ
secretion from phenol gland
F
250μ
enzyme gland (red)
white gland
phenol gland (brown)
C
2mm.

FIG. 2

(3) Incubate in 0·2 per cent. catechol (Analar, freshly prepared same day)—30–90 minutes.
(4) Wash in water—30 minutes.
(5) Upgrade—70 per cent., 90 per cent., absolute. Pass through xylene and mount in balsam.

Examples of specimens treated by this method are shown in fig. 2, A, C, D. In *Fasciola*, the 'vitelline' glands, 'vitelline' reservoir, and even the finest 'vitelline' ducts show up with remarkable clarity. Unexpected branching of the transverse 'vitelline' ducts are revealed in some specimens (fig. 2, A). Small flukes, such as *Dolichosaccus*, only require 30 minutes' incubation in the catechol solution, but *Fasciola* requires about 90 minutes'. The resultant colour is red to brown, depending on the incubation time. Since the colour is due to the quinone-tanned protein, it is virtually indestructible in any of the reagents commonly employed in staining techniques; it is, of course, easily bleached by NaOCl.

After specimens have been 'tanned' in the above manner by catechol treatment, they may be additionally stained (after stage 4) by any of the routine nuclear stains used for whole mounts—borax carmine, paracarmine, &c.—in order to stain the remainder of the genitalia not containing enzyme. It is recommended that, as a routine, trematodes should be treated with catechol in this way before normal staining.

The method may also be used for whole mounts of cestodes (fig. 2, D), but it must be emphasized that it is applicable only to groups forming a quinone-tanned shell similar to that of the trematode egg. The histochemistry of the cestodes is not sufficiently well known, as yet, to define these groups. It has been found that *Schistocephalus solidus* (Pseudophyllidea) and *Proteocephalus filicollis* (Tetraphyllidea) tan readily by this method, although there is some evidence to suggest that in *Proteocephalus*, at least, the enzyme is more difficult to preserve than in *Fasciola*. The catechol technique greatly simplifies the working out of the histological details of egg-shell formation. Thus, a helminth may be 'tanned' by the catechol technique, dehydrated and treated with xylene, and the actual region where the eggs first appear will become apparent. This region may be cut out, embedded, and sectioned. The shell-material, already reddish in colour, may clearly be seen in sections and the cytological details of the egg-shell formation worked out.

Confirmatory Experiments on *Mytilus*

Although the results obtained with *Fasciola* and other helminths were apparently conclusive, it was considered advisable to test the technique further in an organism which was not a helminth and in which a similar quinone-tanning system was known to occur. *Mytilus edulis* has proved to be most satisfactory for this purpose, for its byssus is formed from glandular tissue in the foot by a quinone-tanning process.

A very full account of byssus formation in *Mytilus* has recently been given by Brown (1952). As results obtained here differ in some points from hers, a summary of her findings is given below. For previous work on byssus-formation, reference may be made to her paper.

According to Brown, the byssus is formed in the posterior groove of the foot from the secretions of two glands: the 'white gland' which supplies protein, and the 'purple' gland which supplies the polyphenol constituent responsible for the tanning; the latter was found to be insoluble in alcohol, and there is evidence that it may be a phenolic amino-acid or protein.

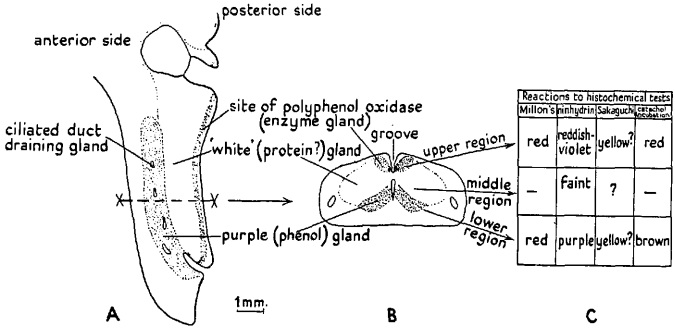

FIG. 3. Histochemical reactions of *Mytilus* foot. A is a reproduction of a figure by Brown (1952), as re-interpreted here. B is a transverse section at the point *X*–*X* in A. C shows histochemical reactions.

Brown believed that the protein material, after being secreted into the groove, is coated on the outside by a layer of phenol which forces itself through the groove epithelium 'to form a border to the mass of white gland material lying in the groove'. Unfortunately, the excellent photomicrographs in her paper are not provided with legends, so that it is difficult to obtain a completely clear picture as to her interpretation of the byssus formation process.

Histochemistry of the foot, and application of catechol technique

Histochemical studies of the foot have revealed the presence of *three* regions as seen in transverse section. These are shown in fig. 3. Fig. 3, A, is a re-drawing of Brown's figure with a re-interpretation. The histochemistry of these three regions is as follows:

Upper region. After catechol incubation of frozen-dried sections or alcohol-fixed hand-sections of the foot, a red region appears on each side of the posterior groove (fig. 2, G). The development of this red colour is inhibited by M/1000 cyanide. The colour is also destroyed by sodium hypochlorite, and it is concluded that it represents the site of polyphenol oxidase activity.

According to Brown (1952) this region is merely part of the phenol gland secreting into the groove. This view is difficult to understand, as there is no visible connexion between it and the phenol gland proper. We agree with her that this region gives a positive argentaffin reaction, but it also gives a particularly strong and unmistakable reaction with Millon's reagent and also with ninhydrin. But since polyphenol oxidase is well known to be a copper protein, this result is to be expected.

Middle region. This is the 'white' or 'protein' gland of Brown. According to her, this gland 'gave all the protein colour reactions but did not give any of the polyphenol reactions'. Unfortunately, she did not give details as to the range of tests covered by this general statement. All the common protein tests have been carried out on this region, on both frozen-dried material and gelatine-embedded material. The Millon test was completely negative: since this test works exceptionally well with frozen-dried material, it may definitely be concluded that tyrosine is lacking. Of the other protein tests, the Biuret, ninhydrin, and Sakaguchi tests invariably gave negative results or results which were questionable. The strongly positive protein reactions given in the same section by the upper and lower glands were never obtained. With the results on *Fasciola* in mind, the tests were repeated on gelatine-embedded material hardened in formalin for 2 weeks. Again, the results were consistently too indefinite to be considered positive.

Lower region. This is the 'purple' or 'phenol' gland of Brown. The results of histochemical tests in this region agreed with Brown's except that, again, a green colour was not obtained satisfactorily with ferric chloride, although after subsequent alkaline treatment the red colour was produced. In addition, this region gave strongly positive protein tests—the ninhydrin and Millon tests giving particularly brilliant reactions. These results thus confirm Brown's view that the polyphenol is a phenolic protein or phenolic amino-acid. After catechol incubation, this region became brown (in contrast to the enzyme region, which coloured red), owing to the condensation of the catechol with the phenol. This brown colour was remarkably stable and not easily affected by sodium hypochlorite.

According to Brown's account, quoted earlier, the material from the phenol gland forces its way through the epithelium of the groove. That this interpretation is incorrect is evident from fig. 2, G, which is a horizontal section of the foot, showing clearly that the phenol gland *opens by posterior ducts* into the groove in the region of the sucker-like depression. It is also evident from this section that a large amount of material is being secreted by this gland, which suggests that it is playing a major role in the formation of the byssus. Further along the groove the material appears thinned. The interpretation of these results is discussed later.

Effect of Phenylmethane Dyes on *Mytilus*

It has been shown that the globules in the 'vitelline' glands of *Fasciola* have a very marked affinity for methyl green, malachite green, and related

dyes in neutral solutions, provided that an approved sample of dye is used (Smyth, 1951*a*, 1951*b*, 1953). It was suspected that this phenomenon was in some way related to properties inherent in quinone-tanning systems, the most likely explanation being that the dye was held either by the protein or the phenol constituent, which, since they occur in the same globule, could not be distinguished in *Fasciola*. Application of the method to sections of *Mytilus* have revealed a similar result. The lower (phenol) gland shows a *very marked* affinity for these dyes (fig. 2, E), the middle ('white') gland has no affinity at all, and the upper (enzyme) gland has a slight, but constant, affinity. This result has been of considerable use in locating the phenol gland in difficult sections.

Discussion

The catechol technique. From the evidence quoted earlier, i.e. effect of KCN, NaOCl, and results of phenol and protein tests, there seems little doubt that the red colour produced after catechol incubation is indicative of oxidase activity of some kind. Since the catechol is oxidized to quinone, it is justifiable to term the enzyme a polyphenol oxidase, provided the term is used in a general sense, i.e. merely as a name for an enzyme that will oxidize catechol. Such a term would not exclude an enzyme like tyrosinase which is also capable of oxidizing diphenols under certain conditions (Sizer, 1953). The success of the technique in such widely differing organisms as *Fasciola* and *Mytilus* is particularly satisfying and has provided striking experimental confirmation of the soundness of the theoretical background built up by previous workers.

Since the colour produced by the catechol technique is due to the tanning of protein molecules, it is clear that it can never *precisely* indicate the enzyme site, but only the nearest protein molecule to such a site, although, on theoretical grounds, it is likely that the differences between enzyme and protein distribution would be on a molecular rather than a microscopic level. In any case, owing possibly to slight diffusion, the colour under oil immersion is never sufficiently intense to allow of more than general localization within the cell, and the red colour seen under low power is reduced to a rather diffuse apricot colour under oil immersion. For high-power work, then, the technique has its limitations.

Egg-shell formation in helminths. The catechol technique, when applied to whole helminths, suggests that a more rational approach to helminth staining may be possible. It also provides a biochemical approach to the problem of inter-relationships between helminths, since forms which produce eggs by quinone-tanning may be very readily detected. There appears to be a wide field here for further investigation, as it is likely that many variations of the basic principle of quinone-tanning will occur. For example, it is likely that there are marked differences between such forms as the anaerobic cestode *Schistocephalus* and the trematode *Haematoloechus* which lives aerobically in the lungs of the frog. Both tan readily in catechol and clearly produce eggs

by quinone-tanning processes. Since in *Schistocephalus* matured *in vitro* under aerobic conditions pre-tanning occurs in the vitelline cells, it may be assumed that normally the phenolic and amino groups and the enzyme are in close contact, but cannot react during life owing to lack of oxygen. In *Haematoloechus*, on the other hand, presumably some inhibitory mechanism must be developed to prevent premature tanning, since oxygen from the lung environment would normally be readily available in life.

From the histochemical results described in this paper, together with some unpublished observations, some idea of the processes occurring in the so-called 'vitelline' cell is now beginning to emerge. This is illustrated in fig. 1. In the mature cell the shell globules are large and occur almost exclusively at the periphery. Since these globules react to both protein and polyphenol tests and redden after catechol incubation, it is clear that the protein, phenol, and oxidase constituents occur in the same globule. None of the histochemical tests for either phenols or proteins has revealed any internal structure to these globules. Yet in frozen-dried sections of *Fasciola* stained in malachite green, it is found that instead of the globules showing a uniform affinity for green, the dye is held in large granules clearly visible within the globules. This observation, which was first made on *Fasciola*, has been repeated in several other trematodes with identical results. There is thus also morphological evidence for the complex structure of these globules. If, as the evidence from *Mytilus* suggests, malachite green is held by protein or phenolic material, these groups would appear to occur within definite areas of the globules. It may be that in helminths, as is suggested later for *Mytilus*, the phenol and protein constituents are the same substance, and the shell substance would thus be basically phenolic protein. On this view, the green granules would be phenolic protein and the unstained part of the globules, the oxidase.

Byssus formation in Mytilus. The observations made here suggest that the account of byssus formation put forward by Brown may not be correct. According to her interpretation, the 'upper' region of the foot (fig. 3, B) is part of the phenol gland. It has been shown here that this region reddens on catechol incubation and is, therefore, the site of polyphenol oxidase. It seems reasonable, therefore, to term this the 'enzyme' gland. Her conclusion that it was part of the phenol gland is understandable, for near to the point where its ducts open posteriorly into the groove, the phenol gland fills the bulk of the material on each side of the groove, and the 'enzyme' gland, which is large anteriorly, practically disappears (fig. 4).

Histochemical tests on the lower or phenol gland fully confirm Brown's view that it is a phenolic protein. If the upper gland is secreting an enzyme and the lower gland a phenolic protein, what then is the function of the middle gland—the so-called 'white' gland? In contradiction to Brown, this region has been found to give only questionable protein reactions, even in material subjected to prolonged hardening, in contrast to the strong protein reactions of the other two regions. If this region were pouring a protein secretion into the lower part of the groove, strong protein reactions might be expected. Yet

the cells of this gland appear to some extent to be orientated towards the lower part of the transverse groove, which may indicate that they open into it, although even in the best preparations an actual secretion in this region has

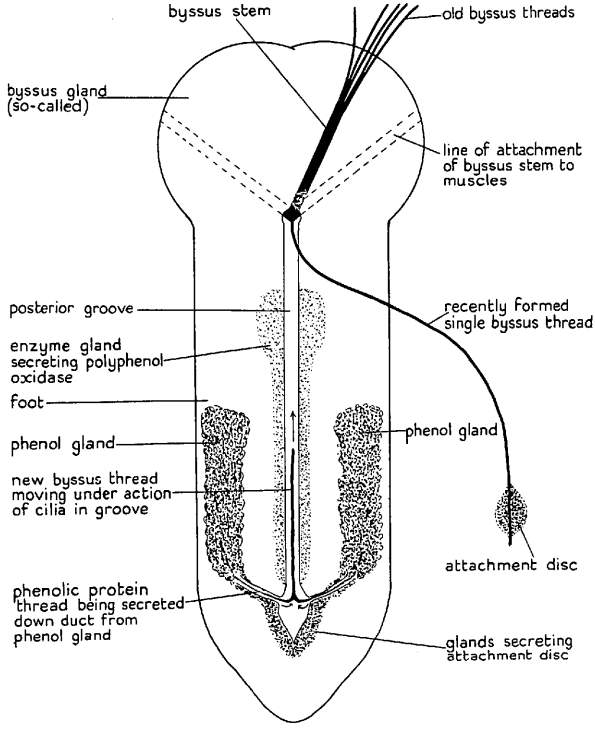

FIG. 4. Diagram to explain the method of byssus formation in *Mytilus*. The phenol glands open into the distal end of the groove in the foot, into which they pour a thread-like secretion of a phenolic protein. This secretion is drawn forwards along the foot groove by cilary action and is covered by a secretion of polyphenol oxidase from the enzyme glands on each side of the groove. The attachment disk is secreted by cells lining a sucker-line depression near the lower end of the groove. When a byssus thread fills the entire length of the groove, it is released except at the end attached to the byssus stem.

never been observed. There would appear to be two possible interpretations of these results. First, the enzyme gland produces polyphenol oxidase, the phenol gland produces phenol, and the 'white' gland produces protein. Apart from overlooking the enzyme gland, this interpretation is close to that of Brown.

TABLE 1

Results of histochemical tests on the 'vitelline' cells of platyhelminths. + = positive. A blank indicates tests not carried out. Negative results have not been reported

			Tests for phenols						*Tests for proteins*				
Group	*Organism*	*Worker*	*argentaffin*	*chromaffin*	*ferric chloride*	*ammonium molybdate*	*sodium iodate*	*diazo*	*xanthoproteic*	*Sakaguchi*	*ninhydrin*	*Millon*	*Biuret*
TREMATODA (Digenea)	*Fasciola hepatica*	Vialli (1933)	+	+	+	+	+	+					
	,, ,,	Stephenson (1946)	+	+	+	+	+		+	+	+	+	
	,, ,,	Smyth (this paper)	+	+	+	+	+		+	+	+	+	+
	Haematoloechus sp.	Smyth (this paper)	+	+	+	+	+		+		+	+	
TREMATODA (Monogenea)	*Diclidophora merlangi*	Rennison (1953)	+	+	+	+	+		+	+	+	+	+
TURBELLARIA	*Dendrocoelum lacteum*	Vialli (1933)	+	+	+	+	+	+					
	,, ,,	Nurse (1950)	+		+								
	Polycelis nigra	Vialli (1933)	+	+	+	+	+	+					
	,, ,,	Smyth (this paper)		+	+	+	+		+			+	
	Gunda segmentata	Vialli (1934)	+	+				+					
	Leptoplana tremallaris	Vialli (1934)	+	+				+					
CESTODA	*Triaenophorus nodulosus*	Vialli (1933)	+	+	+	+	+	+					
	Diphyllobothrium erinacei	Vialli (1933)	+	+	+	+	+	+					

If it were true, the 'protein' produced by the white gland would be endowed with peculiar properties, for it is tyrosine-free, extremely labile, and soluble. This interpretation can be rejected for the reason that a protein sufficiently soluble in water to disappear readily in histochemical tests is just as likely to go into solution in sea water. Since the byssus remains insoluble, even before it has become tanned and hardened, this view becomes untenable.

An alternative hypothesis is put forward—that the enzyme gland is producing a polyphenol oxidase and the phenol gland a phenolic protein, the 'white' gland is merely considered to be a developmental stage of the enzyme gland. On this view, the bulk of the byssus is secreted from the phenol glands through ducts opening into the distal region of the foot groove. Here the byssus material is wafted forwards and drawn out into fine threads which are moulded by the groove and covered by a secretion of polyphenol oxidase *en route* to the so-called byssus gland. When a byssus thread is formed along the whole length of the foot and an attachment disk secreted, it is released; the attachment disk becomes anchored to a rock and the proximal end of the byssus thread firmly bound to the byssus stem embedded in the byssus gland (fig. 4). This view essentially suggests that it is a phenolic protein—*without any further protein being added*—that under enzyme action becomes tanned by the combination of the amino-portion and the quinone nucleus of adjacent molecules. This process might well be termed 'auto-quinone tanning'.

This view that the byssus is made up, not of separate phenol and protein constituents, but of one phenolic protein is not unreasonable on theoretical grounds. Brown (1950) has already shown that the product of the phenol gland is insoluble in water—exactly the kind of material that would be suitable for secretion into sea water.

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