The occurrence and significance of phenolic hardening in the newly formed cuticle of Crustacea Decapoda

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Chemical tests have shown the resemblance of the amber-coloured epicuticle and pigmented zone of the fully formed cuticle of Crustacea Decapoda to the exocuticle of insects. The newly formed cuticle possesses a polyphenol oxidase like that found in the epicuticle of insects, and this, together with the previously recorded presence of a tyrosinase system in the blood, supports the view that a tanning action takes place at the time of molting. It is therefore possible to establish clearly the homology of the crustacean cuticle with that of insects, and it is suggested that the changes taking place in the new cuticle have the effect of limiting its permeability.

INTRODUCTION

In recent years attempts have been made to homologize the integuments of insects and Crustacea, but in spite of rapidly extending knowledge they have been only partially successful. For instance, although Yonge (1932) suggested that the two distinct layers, chitinous and non-chitinous, of the soft lining of the foregut of the lobster are probably to be found in all Arthropoda, Wigglesworth (1933) in the following year drew attention to the very different properties exhibited by the outer layer of the cuticle of the bug Rhodnius and that of the foregut of the lobster. Later, however, Pryor (1940) showed that notwithstanding the differences which had impressed Wigglesworth, the integuments of insects and Crustacea have much in common. In both the outer non-chitinous layer contains aromatic substances. Pryor clearly believes that the crustacean cuticle is hardened, if only to a slight extent, by the mechanism of phenolic tanning which he had shown to take place in insects. Previous to this, Drach (1939) had already expressed the view that the ‘pigmented layer’ of the hard integument of crabs is impregnated with the same amber-coloured substance which constitutes the outermost layer, and that this substance is identical with the ‘cuticulin’ of the insect cuticle (see Wigglesworth 1933). Although basing his views on little more than the resemblance in appearance of the outer zones of the two cuticles, Drach felt sufficiently convinced to propose the adoption in the Crustacea of the terminology applied to the insect cuticle by Campbell (1929) and now generally accepted by entomologists.
Throughout the period under review, therefore, while it became increasingly difficult to reject the view that the crustacean and insect cuticles are homologous, its complete acceptance was hindered by the lack of adequate information concerning their chemistry. Comparisons could be attempted only between those cuticles which were best understood, and in the light of present knowledge it is clear that comparison of the soft cuticle of the crustacean foregut (Yonge 1932) with the hard cuticle of the insect (Wigglesworth 1933; Pryor 1940) could not alone be expected to lead to a satisfactory conclusion.

But when the soft larval cuticle of an insect was studied it was at once possible to show the striking correspondence between the unhardened and unspecialized cuticles of insects and Crustacea (Dennell 1946). In view of this correspondence it became necessary to examine critically the hard integument of the Crustacea in order to discover whether, as Drach (1939) and Pryor (1940) had suspected, it gives indication of being hardened in the same manner as in insects. This has been attempted in the present work, and it will be shown that although calcification is the prime cause of hardening of the integument in Crustacea, particularly Decapoda, it is not the sole cause, for tanning, as in insects, takes place if only to a slight extent. The cuticle, although remaining soft and flexible for some time after each moult, clearly undergoes initial hardening by phenolic tanning, a polyphenol oxidase being located in the outermost layer. Only later do further extensive growth and calcification obscure the fundamental similarity of the decapod cuticle to that of the insect.

Material and methods

The work described here was begun on the crayfish, Potamobius (Astacus) pallipes, and later extended to the lobster, Homarus vulgaris, and to the crabs, Cancer pagurus and Carcinus maenas. Observations have also been made on Leander squilla, several species of Gammarus, Talitrus saltator, Ligua oceanica and Apus cancriformis. The methods and chemical tests employed do not call for detailed account here, being those already described (Dennell 1946, 1947). As in the examination of the insect cuticle, hand sections have been extensively used, and sections have been successsfully cut even of hard regions such as the merus of the first walking leg of medium-sized crayfish and other Decapoda. The soft cuticle immediately before or after a moult was conveniently rolled before hand sectioning. As an indicator of the presence and location of a polyphenol oxidase the ‘Nadi’ reagent has again been successfully used, and the results obtained have been verified by using catechol as a substitute for the natural substrate.

The structure of the fully formed cuticle

Although the calcified integument of the decapod Crustacea has been previously described, notably by Williamson (1860), Vitzou (1882), Herrick (1896), Pearson (1908), and Drach (1939), it will be convenient to give here a further description in
which features supporting the views put forward in this paper may be emphasized. The following description is based on a study of the exoskeleton of the merus of the first walking leg of Astacus but may be taken as representative of the hard exoskeleton of Decapoda generally.

![Diagram of cuticle structure]

**Figure 1.** To illustrate the structure of the fully formed Decapod cuticle. The pore canals are illustrated only on the right of the figure and the vacuolation of the pigmented zone only on the left. c.z. calcified zone; end. endocuticle; epd. epidermis; ep. 1, outer epicuticle; ep. 2, inner epicuticle; g. granules; n.c.z. non-calcified zone; p.z. pigmented zone; t.g.d. duct of tegumental gland; v. region of pigmented zone apparently vacuolated.

The structure of the cuticle is shown in figure 1. In hand sections of the untreated cuticle the outermost layer (ep. 2) is about 5 μ thick and of a clear amber colour. It shows no horizontal lamellae like those seen in the underlying layers, but it does often show faint vertical striae. Its outer surface, as seen in vertical section, appears to consist of a very thin but well-defined membrane (ep. 1). The clear amber layer contains no chitin, and obviously corresponds with the faint yellow outer layer of the soft lining of the foregut of Homarus which Yonge (1932), following the nomenclature of previous authors, refers to as the 'cuticle'. Drach (1939) and Pryor (1940) have pointed out the similarity between this layer and the epicuticle of insects, and Drach has proposed that, as in insects, the term 'epicuticle' be
adopted for this layer. Since the author has already shown (Dennell 1946) the similarity between this layer in the foregut of Homarus and its counterpart in the soft cuticle of Sarcophaga larvae, and since further resemblances will be described in this paper, this procedure will be adopted here. Drach's proposal is given further weight by his remark that the term 'cuticle' has no histological justification as applied to this layer.

The outer bounding membrane (figure 1, ep. 1) of the epicuticle stains strongly with Sudan Black B. With Mallory's triple stain, instead of staining red like the bulk of the epicuticle, it assumes a distinct blue coloration, and therefore clearly corresponds with the outer epicuticle of the larva of Sarcophaga (Dennell 1946). Drach (1939) has described in the Brachyura a very thin external layer of the epicuticle of about 0·25 μ in thickness, and Hass (1916) and previous authors have distinguished a thin bounding layer in the cuticle of Potamonius and other Crustacea.

The layers subjacent to the epicuticle all show a horizontally laminated structure and contain chitin. The outer pigmented zone (figure 1, p.z.) is about 30 μ thick in the sections described, and is light brown in colour, deepest at the outer surface where amber-coloured granules are often seen, and becoming paler towards the inner surface. In addition to its horizontal laminae it often shows in section a vacuolated appearance which led Williamson (1860) to term it the 'areolated layer'. Yonge (1932) describes its appearance as being caused by the presence of rounded inclusions, but fragments of cuticle from various parts of the body of Astacus show in surface view a complex system of horizontally disposed branching canals with blind and rounded ends. This is particularly well seen in the telson. Sections show that the canal system lies in the pigmented layer, and the vacuolated appearance presumably is due to the sectioning of the canals. Like the calcified zone beneath it, the pigmented zone is heavily calcified.

Beneath the pigmented zone occurs the extensive calcified zone (figure 1, c.z.) which forms the bulk of the cuticle and is therefore termed by Drach (1939) the 'couche principale'. In hand sections of the limb of Astacus studied it is about 150 μ thick. It contains, not only in Astacus but in other Decapoda, a diffuse blue pigment distributed throughout the outer third of its thickness.

The innermost zone of the cuticle (figure 1, u.c.z.) is uncalcified. It shows wide undulating laminae and is of variable but never considerable thickness.

Like the inner epicuticle, the horizontally laminated zones of the cuticle exhibit faint vertical striae, but it is not clear merely from an examination of untreated hand sections whether the striae are due to the presence of pore canals like those of the insect cuticle, or are due to the formation of cuticular prisms during the formation of the cuticle as described by Vitzou (1882). The pore canals and prisms of the Decapod cuticle will be described in a later section of this paper.

Also penetrating the cuticle vertically are the prominent ducts of the tegumental glands which Yonge (1932) believes are responsible for the secretion of the epicuticle.

This appearance of the cuticle in a moderately hard region such as the merus of the first walking leg is somewhat modified in softer or harder regions of the body.
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In very soft regions, such as the intersegmental membranes of the abdomen, the cuticle as a whole is thinner, and the outer epicuticle is visible in sections as a thin amber line, whereas the inner epicuticle and outer laminated zone—the so-called pigmented zone—are hardly coloured. This condition is similar to that described by Yonge (1932) for the foregut of Homarus. In very hard regions, such as the upper surface of the dactylus of the chela, the cuticle is very thick, and not only are both layers of the epicuticle deeply coloured amber, but the amber coloration extends throughout the pigmented zone so that the epicuticle is not readily distinguished. Every gradation from a soft and delicate membrane to a hard and massive exoskeleton is presented by different parts of the body, and with increasing hardness are exhibited greater thickness, more extensive calcification, and greater prominence of the amber coloration of the epicuticle and pigmented zone.

To summarize, the cuticle may be regarded as showing two primary and contrasting layers, the outer epicuticular layer shown by Yonge (1932) to be non-chitinous, and the inner and much more extensive and laminated chitinous layer. The two primary layers show differentiation into secondary layers. The epicuticle is actually a double layer, while the chitinous layer of the cuticle shows pigmented, calcified and non-calced zones. Expressed in this way a resemblance of the cuticle to that of the larva and puparium of Sarcophaga (Dennell 1946, 1947) is evident. From a study of the development of the cuticle in crabs, Drach (1939) holds that the three laminated and chitinous zones are to be regarded merely as structural modifications of the primary inner layer, the endocuticle, and regards the subdivision of the cuticle into pre- and post-ecdysial regions as of greater importance than the recognition of structural layers. Drach's view accords with that adopted by Wigglesworth (1933) for the insect cuticle, in which the exocuticle is regarded as a modification of the outer zone of endocuticle, and is in accordance also with the views presented here.

Decalcification and staining of paraffin sections in Mallory's triple stain gives suggestive information on the similarity of the Decapod cuticle to that of the insect. The outer epicuticle (figure 1, ep. 1), as already mentioned, stains blue, but in the harder parts the inner epicuticle (figure 1, ep. 2) remains amber coloured or merely stains a faint pink. The outer region of the pigmented zone (figure 1, p.z.), immediately beneath the epicuticle, however, stains a deep red, with a purple or blue-staining region beneath it. The remainder of the pigmented zone and the calcified and uncalcified zones stain light blue. These staining reactions strongly recall those found in the early puparium of Sarcophaga (Dennell 1947), in which the developing amber-coloured exocuticle stains only with difficulty, but red and blue staining regions mark its junction with the light blue-staining endocuticle. On the other hand, soft regions of the cuticle of Astacus, such as the intersegmental membranes, stain like the cuticle of the foregut of Homarus (Yonge 1932) and the larval cuticle of Sarcophaga (Dennell 1946), showing simply a prominent red epicuticle and blue endocuticle. These staining reactions give some support, by itself admittedly only slender, for the opinion that some tanning of the superficial
region of the cuticle, like that occurring in the insect, may take place in the hard parts of the body.

Examination of the short and blunt spines of the cheliped of Astacus and of the tips of the walking legs of Cancer and Carcinus, strongly confirms this opinion. The spines of the cheliped of Astacus are not entirely softened during decalcification, and after it present a clear amber appearance. Treatment with Diaphanol (chlorine dioxide in glacial acetic acid), however, completely softens and bleaches the spines.

The tip of the dactylus of a walking leg of Cancer or Carcinus presents a highly suggestive appearance. It is dark brown in colour and in appearance strongly resembles the insect cuticle. In large specimens of Cancer this resemblance is particularly marked, and the dark region, being thicker than the remainder of the cuticle of the dactylus, has the appearance of a massive conical sheath enveloping the tip of the joint. Treatment with glacial acetic acid alone softens the dactylus but not its tip, and since little evolution of gas is seen from the tip during this treatment it is clearly not heavily calcified. But prolonged treatment with Diaphanol entirely bleaches and softens the dactylus tip, just as it does the hard and dark insect cuticle. Its effect is very strongly suggestive evidence of hardening by a tanning reaction in the crustacean cuticle.

With a view to throwing further light on this possibility chemical tests have been carried out on hand sections of the fresh hard cuticle of the merus of the first walking leg of Astacus. The results obtained have been verified on other Decapoda and are expressed in table 1.

The result of the chitosan and Schulze tests for chitin, the application of ninhydrin, and staining with Sudan Black B, confirm Yonge’s statement (1932) that chitin is lacking from the epicuticle, which is composed largely of protein but contains lipoid substances. The pigmented zone appears to contain less chitin than the calcified and uncalcified zones of the cuticle. The application of alkaline pyrogallol as a test for the presence of calcium (Lison 1936) shows that the inner epicuticle and the pigmented and calcified zones of the cuticle are hardened by calcification. The strong positive reaction given to Millon’s reagent by the inner epicuticle and pigmented zone is entirely consistent with the view that these layers may contain aromatic substances. Fehling’s solution is strongly reduced by the inner epicuticle, just as it is by catechol, and its reduction here permits the supposition that it is effected by a phenol derivative. No free dihydroxyphenol, as indicated by the negative result of the ferric chloride test, is present in the fully formed cuticle, and this is in agreement with the condition in the fully hardened insect cuticle (Dennell 1947). The positive argentaffin reaction given by the epicuticle and to a less extent by the pigmented zone of the endocuticle is consistent with the presence in these layers of an aromatic substance.

As is to be expected from the results obtained by treating the tips of the walking legs of crabs with Diaphanol, this reagent bleaches those zones, the epicuticle and pigmented zone, which are suspected to be hardened by phenolic tanning. It may be mentioned that after its use these regions of the hard cuticle assume the staining
reactions to Mallory characteristic of the soft cuticle (Yonge 1932; Dennell 1946). The inner epicuticle stains red and the pigmented zone blue. A similar result is obtained when the puparia of Sarcophaga and the hard cuticles of other insects are treated with Diaphanol (Dennell 1946).

Additional evidence that the amber region of the cuticle owes its coloration to the oxidation product of a phenol is furnished by the use of the ‘Nadi’ reagent. Even in the presence of cyanide a blue coloration is rapidly given, indicating that the reaction is not due in the fully formed cuticle to the presence of an oxidase. From what is now known of the hardening of the insect cuticle it is not to be expected that, even if the crustacean cuticle is partially hardened in a similar manner, a polyphenol oxidase is still to be found in the fully formed cuticle. But the hard cuticle of the insect, tanned by an orthoquinone, gives a positive reaction with the ‘Nadi’ reagent in the presence of cyanide, and Szent-Gyorgi (1925) has pointed out that orthoquinones give positive tests with diphenylenediamine. The positive test obtained in the crustacean cuticle is therefore in full agreement with the presence of an orthoquinone.

| Table 1 |
|---|---|---|---|
| test | epicuticle | endocuticle |
| | outer | inner | pigmented zone | calcified zone | uncalcified zone |
| chitosan (Campbell) | — | — | + | ++ | ++ |
| chitin (Schulze) | — | — | — | ++ | ++ |
| ninhydrin | ? | +++ | — | + | + |
| Sudan Black B | ++++ | +++ | ++ | — | — |
| alkaline pyrogallol | — | ++ | +++ | +++ | — |
| Millon | ? | +++ | ++ | — | + |
| Fehling | + | +++ | — | — | — |
| argentaffin | + | +++ | — | — | — |
| ferric chloride | — | — | — | — | — |
| Diaphanol | — | +* | +* | — | — |
| Nadi + KCN | +++ | +++ | — | — | — |

* Indicates bleaching of amber coloration.

The foregoing observations furnish strong presumptive evidence that hardening by quinone tanning may take place in the outer zone of the crustacean cuticle, just as it does in the cuticle of insects. In order to obtain further evidence of this possibility observations have been made on the soft developing cuticle in order to discover whether, as in insects (Dennell 1947), a polyphenol oxidase located in the epicuticle is concerned in the process.

**The Polyphenol Oxidase of the Epicuticle and the Initial Hardening of the Cuticle**

The thin and flexible cuticle of the newly moulted decapod crustacean is not only uncalcified but consists merely of the epicuticle and the pigmented zone of the endocuticle. Calcification and the addition of the further zones of the endocuticle
take place later (Pearson 1908; Drach 1939). The layers of the thin and delicate new cuticle both show, however, a slight amber coloration which is most marked in the epicuticle, and it is of interest to note that even in the soft but fully formed cuticle lining the foregut of *Homarus* the epicuticle has a faint yellow colour (Yonge 1932). These colorations indicate that tanning of the cuticle takes place at an early stage independent of calcification.

Hand sections of untreated new cuticles of *Cancer pagurus, Carcinus maenas* and *Homarus vulgaris* have been studied with the aid of the ‘Nadi’ reagent (α-naphthol and dimethyl-p-phenylenediamine). This reagent was found valuable in studies on the insect cuticle (Dennell 1946). Regions of high oxidative activity become rapidly and intensely coloured blue owing to oxidation of the reagent which results in the formation of indophenol blue. The results obtained are entirely consistent with the occurrence of oxidase activity in the cuticle, and are capable of concise description.

Shortly after moulting, the new slightly amber-coloured inner epicuticle gives an immediate positive reaction with ‘Nadi’. It is stronger than that given by the older calcified cuticle, but, unlike that in the older cuticle, it is almost completely inhibited in the presence of cyanide, when the epicuticle becomes only very faintly blue. These results indicate that an oxidase inhibited by cyanide is located in the epicuticle, but the fact that inhibition of oxidation of the reagent is not complete points to the fact that the orthoquinone responsible for tanning the cuticle is already present to some extent.

More conclusive evidence of the occurrence of an oxidase in the epicuticle is given by specimens which have just begun to moult, and even more satisfactorily by those which are preparing to moult. In the latter the new cuticle, like that of the former, consists of epicuticle and underlying pigmented zone. Again, the epicuticle gives a strong positive response with the ‘Nadi’ reagent, but now complete inhibition of the oxidation of the reagent is obtained with cyanide. An orthoquinone can only be present, if at all, in very small amount at this stage.

The results of experiment with the ‘Nadi’ reagent may be summarized by stating that, while the epicuticle always gives a positive reaction, the coloration produced becomes less intense and inhibition by cyanide progressively less with increasing age of the cuticle, indicating oxidation of the reagent by an oxidase alone in the very young cuticle, and in older cuticles non-enzymatic oxidation by the orthoquinone resulting from earlier oxidase activity.

That the oxidase of the epicuticle is truly a polyphenol oxidase is shown by experiments with catechol as a substitute for the natural substrate. Immersion of hand sections of newly formed cuticles in dilute catechol (pyrocatechol) solution rapidly produces a reddish brown coloration in the epicuticle. The oxidation of catechol does not proceed as rapidly as that of the ‘Nadi’ reagent, and is completely inhibited by cyanide. Inhibition is also effected by heating the cuticle for some minutes in water at 65° C.

The above experiments, taken in conjunction with the results of chemical tests on the fully formed cuticle, leave little doubt that the initial hardening of the
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decapod cuticle is effected by a mechanism corresponding with that found in insects, in which a polyphenol produced in the blood by tyrosinase activity is oxidized by a polyphenol oxidase in the epicuticle, the resulting orthoquinone hardening the protein constituents of the cuticle by a tanning action (Pryor 1940; Dennell 1946). It is accordingly to be expected, if the above opinion is justified, that the blood of decapod Crustacea should show tyrosinase activity and exhibit a physiological constitution similar to that found in insects. The work of Pinhey (1930) shows that this expectation is well founded.

THE PHYSIOLOGY OF THE BLOOD

Pinhey (1930) found that the blood of the crabs *Maia squinado* and *Cancer pagurus* contains a tyrosinase system responsible for its blackening when shed. The enzyme is contained in leucocytes, and, under the experimental conditions described, only becomes active on their cytolysis. The use of narcotics, such as various alcohols, thymol, phenyl urethane and urethane as inhibitors of tyrosinase activity, gives variable results depending on their concentration. Thus thymol in small concentrations diminishes the oxidation of tyrosine, but increases it 'to an astonishing extent' in higher concentrations. Phenylurethane and urethane give similar but less regular results. Pinhey suggests that in dilute solutions the narcotic becomes adsorbed on the surface of the colloidal enzyme particles, separating them from the substrate, but that in higher concentrations the narcotic forms an emulsion and the enzyme and substrate become adsorbed on the surface of the narcotic globules. The enzyme and substrate are therefore brought into intimate contact and increased oxidation is effected.

These results show a strong resemblance to those obtained in experiments with *Sarcophaga* larvae (Dennell 1947), although in the latter narcotics were found to diminish the reducing power of the blood and so permit of the oxidation of tyrosine through the activity of tyrosinase. It is of interest here to note that, as mentioned later, crustacean blood possesses reducing powers. Toluene blue is reduced to its colourless leucobase. Although Pinhey makes no suggestion as to the possible function of tyrosinase in the blood, her work shows clearly that an enzyme mechanism like that present in insects occurs also in decapod Crustacea. Confirmation of the presence in the blood of *C. pagurus* of an oxidase capable of accelerating the oxidation of monohydric phenols is given by Bhagvat & Richter (1938), who suggested, however, that the physiological importance of phenol oxidases in the blood of arthropods may be the activity as respiratory carriers or the bactericidal action of the orthoquinones to which they give rise.

The variability in the tyrosinase system of crabs recorded by Pinhey (1930) is fully consistent with the view that its function is the production of a phenol ultimately responsible for hardening the new cuticle. Enormous differences were found in the rate of discoloration of blood taken from different animals, and from the same animal at different times, but in general it was found that blood collected
in the spring discolours less than that collected during autumn and winter. It is
striking that Pearson (1908) states that the frequency of moulting in _C. pagurus_ is
greater in the latter part of the year than at other times, and Pinhey herself
remarks: ‘It is not improbable that there exist shorter cycles related to the
variation in the number of the leucocytes, upon whose cytolysis, as will be shown
later, the tyrosinase activity of the blood depends.’ It seems probable that the
amount of tyrosinase in the blood will prove to undergo cyclical changes in harmony
with the moulting cycle, and it may appropriately be commented here that in the
larva of _Sarcophaga_ the cells responsible for the elaboration of tyrosinase are not
a constant feature of the blood. They appear only in late larval life and diminish
in number before pupation (Dennell 1947).

Further work on the physiology of crustacean blood is clearly necessary. Informa-
tion is particularly required on the nature of the inhibition of tyrosinase activity
which prevents the darkening of unshed blood. In this connexion preliminary tests
have shown that the blood of _Carcinus_ and the amphipod _Gammarus pulex_ is
capable, like the blood of _Sarcophaga_ larvae (Dennell 1947) of reducing tolylene
blue. Probably, therefore, tyrosinase activity is held in check by the reducing
power of the blood, as in the insect, and not, as Pinhey (1930) suggests, merely by
its leucocytes remaining intact.

However this may be, the presence of tyrosinase in crustacean blood, together
with the early occurrence in the epicuticle of a polyphenol oxidase and later of
aromatic substances, leaves little doubt that initially the cuticle is hardened by
phenolic tanning as in insects.

**The pore canals and cuticular prisms**

The vertical striae commonly seen in sections of the decapod cuticle have a two-
fold origin. Not only is the cuticle penetrated by vertical canals like the well-known
pore canals of the insect cuticle, but its outer layers at least are usually composed
of vertical prisms of cuticular material with well-defined boundaries. The literature
relating to the canals and prisms is reviewed by Drach (1939) in his comprehensive
paper, and from his own work Drach believes that the pore canals, at any rate when
first formed, are occupied by cytoplasmic filaments arising from the epidermal cells.
Further, he supports Vitzou (1882) in his contention that the prisms represent the
outer portions of the elongated epidermal cells which have been transformed into
cuticular substance. In some Decapoda, however, the prisms formed in this way
from adjacent cells may undergo fusion to a greater or less extent (see Yonge 1932),
but in spite of this it seems probable that in most Decapoda the outer portions of
the prisms remain distinct for some time at least. Drach has pointed out that the
amber-coloured substance which is found in the epicuticle, and which as shown in
this paper is almost certainly an orthoquinone—the coloured oxidation product
of a polyphenol—also occupies the interprismatic spaces of the pigmented zone.
The pore canals penetrate the prisms and never occur in the interprismatic spaces.
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Observations made during the present work entirely confirm Drach’s statements. The justification for their brief presentation here will be seen in the discussion in connexion with the origin of the epicuticle.

The pore canals of the fully formed cuticle of *Astacus*, as of other Decapoda, are visible in paraffin sections only as faint vertical striae which pass from the epidermis and extend through the endocuticle. They appear to pass into the inner epicuticle, for this layer also shows faint closely spaced vertical striae. Hass (1916) has stated that the pore canals of *Potamobius* pass into the ‘aussenlage’ (which, although stated to be laminated, appears to be the inner epicuticle). As far as can be seen from Mallory-stained sections the canals are occupied by strands of cuticular material which stain blue like the remainder of the endocuticle. The solid nature of the canal contents is suggested in sections where the laminae of the endocuticle have parted during preparation and the gaps so formed are bridged by fine strands.

An entirely different picture of the pore canals is obtained when pieces of the fresh hard cuticle of *Astacus* are treated overnight with 70% alcohol to which a few drops of glacial acetic acid have been added. The diffuse blue pigment of the calcified zone becomes converted into the red pigment astacin, and in hand sections is seen to have accumulated in the canals, revealing them clearly. Whether the pigment is absorbed by the canal contents, or occurs in the space between them and the canal wall, has not been discovered. At first sight the canals appear to have an undulating form, but as Drach (1939) observes, each canal constitutes an extended helix. The pore canals of the brachyuran cuticle are revealed by similar treatment.

Examination of young developing cuticles gives evidence that the solid strands of the canals are preceded by cytoplasmic filaments. Sections of the soft cuticle of a large *Homarus* 5 days after molting, in which the calcified zone was still only of very small extent, were stained in Mallory’s triple stain. The inner ends of the filamentous canal contents stained brilliantly red in the inner half of the calcified zone, while the remainder of the contents stained blue like the endocuticle. In some areas, however, the canals appeared to be empty distally. The canals are clearly seen in these sections to pass into the inner epicuticle. The basal red-staining contents occupy but do not entirely fill the clearly defined canals, so that an annular space is left between them as in the larva of *Sarcophaga* (Dennell 1946). It appears that, as in *Sarcophaga*, the canals are first occupied by cytoplasmic filaments which as the cuticle increases in thickness are progressively withdrawn and cuticular material left behind.

In order to discover whether the contents of the canals of the fully formed decapod cuticle truly consist of cuticular material, the cuticle of *Astacus* has been examined after testing for the presence of chitin. The results, however, were inconclusive. After neither the Campbell chitosan test nor the Schulze test (see Lison 1936) were the canal contents more deeply coloured than the surrounding endocuticle, as was the case when the larval cuticle of *Sarcophaga* was tested (Dennell 1946). If the canals are occupied by cuticular material it has a chitin content no greater than that of the remainder of the cuticle. Huxley (1880)
observes that the canals of the dried cuticle of *Astacus* are filled with threads of air, and deduces that they are ordinarily filled with fluid. But the presence of air after drying is not inconsistent with the occupation of the canals by cuticular threads, for, as has already been stated, the threads do not entirely fill the canals, and in any case they will certainly contract on drying.

The cuticular prisms are not clearly distinct in the fully formed cuticle of *Astacus*. They have not been distinguished in paraffin sections, but in surface view of the decalcified cuticle are visible over small regions. They appear in end view as a number of polygonal areas with more or less clearly defined boundaries. It is noteworthy that the scattered regions in which the prisms have been seen also possess granular amber deposits, presumably of phenolic origin. Since Drach (1939) observes that the interprismatic spaces are often filled with amber-coloured material it seems likely that the prisms in these regions, as compared with those elsewhere, are clearly seen because their fusion has been prevented by the intervening walls of tanned protein. Huxley (1880) appears to have observed cuticular prisms in *Astacus*, for he states that the pores of the cuticle 'are seen to be disposed in distinct areas circumscribed by clear polygonal borders. These perforated areas appear to correspond with individual cells of the ectoderm....'

In agreement with the observations of Vitzou (1882) and Yonge (1932) no prisms have been observed in the cuticle of *Homarus*, suggesting, as Yonge remarks, that these structures may readily undergo fusion.

In the Brachyura studied, however, the prisms are particularly clearly seen. Mallory-stained sections of the new cuticle of *Cancer* and *Carcinus* immediately after moulting show comparatively widely spaced blue-staining vertical lines in the pigmented layer. These represent the boundaries of the prisms. Their continuation inwards into the only slightly developed calcified zone has not been observed. In a surface view of the intact and untreated cuticle the prisms are represented by well-defined lines enclosing polygonal areas (figure 2b).

A point of interest was found in the appearance of the prisms in a specimen of *Cancer pagurus* which was just beginning to moult. In surface view the bounding membranes of the prisms were seen to be extensively corrugated (figure 2a), thus providing for expansion of the prisms during extension of the new cuticle immediately following the moult. The condition of the cuticle some time after the moult is seen in figure 2b.

It must be emphasized that during the present work no indication of the extension of the prisms outwards into the inner epicuticle have been encountered. Drach (1939) illustrates in *Maia squinado* the amber-coloured material of the outer region of the pigmented zone as extending inwards some distance into the interprismatic spaces, and thereby clearly defining the prisms. The outermost amber-coloured zone of the cuticle, including the epicuticle, does not therefore show a segmented appearance.

As already mentioned, the implications of these observations will be discussed in considering the origin of the epicuticle.
Phenolic hardening in the cuticle of Crustacea Decapoda

Observations on other Crustacea

In order to discover whether tanning of the cuticle takes place in Crustacea other than the larger Decapoda the following species have been less fully examined: *Leander squilla* (Decapoda Natantia), *Gammarus pulex*, *G. locusta* and *G. duebeni*, *Taliurus saltator* (Amphipoda), *Ligia oceanica* (Isopoda), and *Apsi cancriformis* (Branchiopoda). Owing to the difficulty of working with thin yet often brittle cuticles they have not been as extensively studied as those already described, but the results obtained are in entire agreement with the views already expressed and merit brief description.

*Figure 2.* a. The new cuticle of *Cancer pagurus* in surface view at the time of moulting. The pore canals are represented by dots. b. The slightly older cuticle of *C. pagurus* after moulting.

The cuticle of *Leander squilla* is about 40 μ thick and has a transparent glassy appearance which suggests that it is not heavily calcified. Treatment with acid, however, results in the vigorous evolution of gas from the cuticle, showing the falsity of this impression. Hand sections of the fresh cuticle show a dark yellow-brown inner epicuticle, and the endocuticle beneath is amber coloured in its outer region, constituting a pigmented zone. This coloration is removed by treatment with Diaphanol. With the ‘Nadi’ reagent the inner epicuticle gives a strong positive reaction, and the amber zone of the endocuticle a much feeble one, even in the presence of cyanide. The occurrence of an orthoquinone in the cuticle is therefore indicated. The surface of the cuticle is greasy, and the ready staining of the inner epicuticle with Sudan Black B points to its impregnation with lipidoid substances.

The cuticle of the species of *Gammarus* and of *Taliurus* are very similar to that of *Leander*, except that they are darker in colour and less transparent. In appearance they resemble the hard insect cuticle. They are calcified, but not heavily, and possess a deep amber-coloured inner epicuticle and pigmented zone of the endocuticle. The epicuticle gives a positive response with the ‘Nadi’ reagent in the
presence of cyanide, and the pigmented zone a less vigorous response. The coloration of the cuticle is entirely removed by Diaphanol. A further indication of phenolic tanning is furnished by the observations that, as in the larva of *Sarcophaga* (Dennell 1946), the exposed blood of *Gammarus pulex* darkens readily on exposure to the air, gives a positive response with the ‘Nadi’ reagent, and readily oxidizes catechol. These reactions are inhibited by cyanide, and the existence in the blood of a tyrosinase system capable of providing the polyphenol responsible for hardening the cuticle is clearly indicated. Further, the blood is capable of reducing the oxidation-reduction indicator tolylene blue, suggesting that as in *Sarcophaga* tyrosinase activity may be held in check by the reducing power of the blood until the time of hardening of the new cuticle after a moult.

In *Ligia* the cuticle is calcified but lacks the brown coloration seen in *Gammarus*. The epicuticle has, however, a definite yellow coloration. The setae and tips of the limbs are a clear amber, and, like the epicuticle, give a positive test with ‘Nadi’ in the presence of cyanide.

The cuticle of *Aplys* is interesting. It is thin and delicate, and the shape of the body is preserved only by the pressure of the body fluid within. Those parts of the cuticle, however, which are subjected to abrasion, such as the biting regions of the mouthparts and gnathobasic setae, possess a clear amber coloration and recall the insect cuticle.

The foregoing results and observations indicate that hardening of the cuticle in the manner characteristic of insects is of wide if not universal occurrence in the Crustacea.

**DISCUSSION**

If the observations described in this paper are well founded, the cuticles of Crustacea and insects present striking points of similarity. Both consist primarily of two layers, the epicuticle,* not containing chitin, and the endocuticle, a laminated chitin-protein complex. The epicuticle in both possesses a very thin but distinct surface layer of different composition, and so may be regarded as double. The outer thin epicuticle, always rich in lipid substances, may be termed the ‘lipoid epicuticle’, and the inner, basically composed of protein, the ‘protein epicuticle’ (Dennell 1946). The inner epicuticle typically carries a polyphenol oxidase before hardening begins, and together with the outer zone of the endocuticle becomes tanned owing to the formation in it of an orthoquinone as the result of the oxidation of a polyphenol derived from the blood. In the Crustacea, therefore, as well as in insects, the term ‘exocuticle’ may justifiably be applied to the outer tanned region of the endocuticle.

There is, then, a very close resemblance between both the soft and the hard cuticles of Crustacea and insects. The soft integument lining the foregut of *Homarus* (Yonge 1932) must be compared not with the hard cuticle of the insect, but with

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* Throughout this discussion, as in the foregoing description, the adoption for the Crustacea of the terminology applied to the insect cuticle will be adhered to.
Phenolic hardening in the cuticle of Crustacea Decapoda

the soft cuticle such as that of the larva of Sarcophaga (Dennell 1946). The hardening of the soft cuticles is carried out in a similar manner, although the similarity is soon masked by extensive calcification in the Crustacea. The importance of calcification in the crustacean cuticle in conferring rigidity on the exoskeleton is probably, however, to be regarded as a peculiarity related to the aquatic mode of life and the ready availability of calcium salts. It is noteworthy that some insect larvae accumulate lime in the cuticle (see Wigglesworth 1939).

Other important resemblances are that in Crustacea, as in insects, part of the endocuticle is laid down before mouling and the remainder after. The isoelectric points of the epicuticle and endocuticle are the same in Crustacea (Yonge 1932) as in insects (Dennell 1946), thereby accounting for the similarity in staining with Mallory's triple stain. And finally in both helical pore canals, occupied at first by cytoplasmic filaments extending from the epidermal cells, pass through the cuticle (Drach 1939; Dennell 1946).

In spite of these close resemblances, however, the origins of the epicuticle in Crustacea and in insects have previously been stated to be remarkably different. In 1932 Yonge stated that specialized tegumental glands situated beneath the epidermis are responsible for the secretion of the crustacean epicuticle, whereas in 1933 Wigglesworth had expressed the view that in insects this layer is secreted by the epidermal cells themselves, the dermal glands being responsible only for the production of the mouling fluid. In the crustacean Homarus dissolution of the old endocuticle is carried out by invading nuclei. The tegumental gland of Crustacea and the dermal glands of insects do not, according to these views, appear to be homologous.

More recently, however, Drach (1939) has advanced views on the origin of the epicuticle which are radically opposed to those of Yonge, while, as will be discussed later, Wigglesworth (1947) has recently shown that the dermal glands do actually secrete one constituent of the epicuticle—the cement layer—in Rhodnius. It will be appreciated that the mode of formation of the epicuticle in insects and Crustacea still presents difficulties.

The opposing claims of Yonge and Drach on the formation of the crustacean epicuticle call for critical examination. Working on Homarus, Yonge holds that the tegumental glands produce a fluid of low-surface tension which passes through the ducts of the glands to the surface of the cuticle, over which it spreads as a uniform thin film and later solidifies as a constituent layer of the cuticle—the epicuticle. The three major observations on which Yonge bases his claim are that the glands show cyclical activity in relation to mouling, that the contents of their ducts has the same properties as the epicuticle, and that in the early development of the cuticle a layer of endocuticle no more than 2 μ thick without an overlying epicuticle has been observed. It seems unlikely, therefore, that the epidermal cells, separated from the future epicuticle by the intervening endocuticle, can be responsible for its production. In additional support of this Yonge has actually observed the appearance of secretion at the openings of the tegumental gland ducts, and has
noted the progressive increase in thickness of the epicuticle while endocuticle secretion is proceeding. Finally, Yonge points out the shortcomings of previous suggestions as to the functions of the tegumental glands.

Drach (1939), on the other hand, maintains that it is surprising that glands which are so irregularly distributed should produce a layer of uniform thickness, indeed, that the secretion should even form a continuous layer. The cyclical activity of the glands, he says, is capable of various interpretations, for many cyclic phenomena are observable in Crustacea in relation to moulting. Most important, however, he categorically affirms, in direct opposition to Yonge, that in the developing cuticle of the crab *Maia squinado* a red staining epicuticle is formed *before* the endocuticle appears, and accurately follows all the undulations of the epidermis. Since it is unlikely that within the Crustacea Decapoda two distinct modes of epicuticle formation are to be found, the present position is clearly unsatisfactory.

In favour of Drach's contention is the presence of pore canals which appear to penetrate the epicuticle. Hass (1916) refers to their presence in the 'aussenzelage' of *Potamobius*, although he admits that the thinness of this layer makes it difficult to study. In the present work they have been clearly seen in the epicuticle of the growing cuticle of *Homerus*. The presence of pore canals in the epicuticle, produced by filamentous extensions of the cytoplasm of the epidermal cells, is in entire agreement with the production of the epicuticle by the epidermis, but is difficult to reconcile with its formation by the tegumental glands.

The fact that the epicuticle does not appear to share in the segmentation of the cuticle into vertical prisms is inconclusive. It is, however, in no way inconsistent with the production of the epicuticle by the conversion of the outermost portions of the epidermal cells into cuticular substance. The prisms of the pigmented zone are marked by the interprismatic septa which appear to consist of material similar to the inner epicuticle, that is, tanned protein probably impregnated with polymerized lipoproteins, and it is not to be expected therefore that the septa should be distinguishable in the epicuticle. On the other hand, however, the occurrence of interprismatic septa in the pigmented zone might, in support of Yonge's views, be interpreted as due to the penetration of tegumental gland secretion between the newly formed prisms of the exterior part of the endocuticle. It is to be noted that the prisms are not apparent in the inner part of the endocuticle remote from the epicuticle.

Perhaps supporting Drach is the presence of a thin surface layer of different constitution from the remainder of the epicuticle. A similar double epicuticle is found in the larva of *Sarcophaga* (Dennell 1946) which has no tegumental glands. In this connexion it should be noted that while Drach describes a very thin surface layer of the epicuticle he believes that many authors have attributed too great a thickness to the epicuticle as a whole. Since the epicuticle becomes difficult to distinguish from the developing pigmented zone (his 'external zone' of the 'pre-exuvial layer') he thinks it likely that one or more laminae of the endocuticle have been included with the epicuticle in their measurements. It may be added that the
use of Diaphanol followed by Mallory staining, however, removes any confusion between the epicuticle and pigmented zone.

If the difficulty presented by the occurrence of pore canals in the epicuticle can be surmounted, it is perhaps possible to reconcile the observations of Yonge and Drach in the following manner. If the epicuticle is truly produced, as Yonge describes, by the tegumental glands, then variation in the time of onset of the tegumental gland activity and endocuticle secretion by the epidermis may well result in the appearance described by Drach. There seems no obstacle, other than that presented by the pore canals, to the view that in *Maia* tegumental gland secretion may precede endocuticle formation and result in an epicuticle in contact with the epidermis, whereas in *Homarus* it may be somewhat preceded by the activity of the epidermis so that before the epicuticle is formed a thin layer of endocuticle has been secreted.

In the insects also a somewhat similar divergence of opinion arises. As a result of recent work on *Rhodnius*, Wigglesworth (1947) shows clearly that the epicuticle is more complex than previously thought. It comprises four distinct layers. A cuticulin layer composed of polymerized lipoproteins tanned by quinones is overlain by a layer rich in polyphenols. In turn the polyphenol layer is surmounted by a wax layer which has a protective covering of cement of unknown composition. The dermal glands secrete the protective cement and to this extent participate in the formation of the epicuticle.

The epicuticle of *Rhodnius* therefore differs from that of the cockroach (Richards & Anderson 1942) and the larva of *Sarcophaga* (Dennell 1946) in which only two constituent layers were recognized. It is possible that further investigation may reveal the existence of wax and cement layers in *Sarcophaga*, but even discounting these the underlying cuticulin and polyphenol layers are not readily homologized with the two layers of the epicuticle of *Sarcophaga*. Whereas in *Rhodnius* the cuticulin layer appears to be penetrated by the pore canals, these structures do not enter the epicuticle of *Sarcophaga*, and have not been observed to exude from their tips a polyphenol-containing substance as described by Wigglesworth (1947) for *Rhodnius*. No dermal glands have been observed in *Sarcophaga*, and the entire cuticle appears to be the product of epidermal activity. Wigglesworth (1947) has pointed out the difficulty of satisfactorily defining the epicuticle: in this paper and in the work on *Sarcophaga* the epicuticle has been regarded as the chitin-free outermost region of the cuticle. The very difficulty of definition points to the need for further investigation, as in the Crustacea, on the origin of the epicuticle.

It is difficult to avoid the view that in Crustacea the activity of the tegumental glands is closely connected with the structure of the cuticle. Yonge (1938) has shown that the outermost membrane of the eggs of *Homarus* is produced by tegumental glands and has properties identical with those of the epicuticle. The tegumental glands occur in profusion in areas where the epicuticle is thick and the need for resistance to abrasion is great, such as the mouth parts and labrum, and Yonge (1932) has pointed out the importance of the epicuticle, even in such a soft
region as the lining of the foregut, in protecting the underlying endocuticle against abrasion. The heavily tanned tips of the walking legs of Homarus and Cancer, subject to great abrasion, have been observed in the present work to be penetrated by conspicuous tegumental gland ducts. Since a tanned and hardened epicuticle is even better adapted to withstand abrasion than the soft epicuticle of the foregut, the suspicion arises that the tegumental glands may perhaps be concerned in the hardening process. During the present work the source of the polyphenol oxidase of the crustacean epicuticle has not been demonstrated, but if it should prove that it is elaborated by the tegumental glands and not by the epidermis as in Sarcophaga larvae (Dennell 1947), this might be regarded as support for the views expressed by Yonge.

The significance of the crustacean epicuticle in modifying the permeability of the cuticle, as clearly demonstrated by Yonge (1936), is still further established by the recognition in the exposed parts of the body of a tanned epicuticle of the insect type. The effectiveness of the lipoids of the insect cuticle in controlling permeability has been clearly shown by Wigglesworth (1942, 1945), Alexander, Kitchener & Briscoe (1944a, b) and Beament (1945). Further, Pryor (1940) has pointed out the importance of phenolic tanning of the insect cuticle in rendering it more strongly lipophilic. The tanning of the crustacean cuticle, in addition to providing resistance against abrasion, may be regarded in this light. Initial hardening by tanning, by facilitating impregnation with lipoid substances, may be expected to afford effective control of permeability while leaving the cuticle sufficiently soft and elastic to allow of its extension before the onset of calcification.

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References

Quantitative studies on the wetting of leaves by water

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Quantitative studies on the wetting by water of the exterior surfaces of leaves of Sinapis arvensis, Triticum vulgare, and other plants, have been made, using advancing contact angle as a measure of the extent to which wetting takes place.

The magnitude of the contact angle of water on a leaf surface has been found to vary regularly with the position of the leaf on the plant and to show also a characteristic diurnal fluctuation through a range which may be as much as 30°. The diurnal variations in contact angle are shown to be correlated with changes in leaf water content.

On detached leaves contact angle varies markedly as wilting proceeds, this change being reversible on recovery of turgor.

Evidence is presented to show that these variations in the behaviour of water on leaves are caused by changes in the degree of corrugation of the leaf surface produced by changes in the water content of the tissues. The events in the diurnal cycle are explained on this basis.

The significance of the observed phenomena in connexion with the retention of water by leaves, the exchange of water and dissolved substances between leaf and water, and stomatal behaviour, is discussed.

Under natural conditions the external surface of a normally aerial leaf is frequently more or less covered with liquid water. Since under these circumstances gaseous diffusion through the stomata must be impeded and since exchange of dissolved substances and water may occur between the leaf tissues and the water drops, the physiological effects of wetting can be important in the life of the plant. It is therefore of interest to know something of the factors determining the extent to which a leaf is wetted by water. In the course of investigations primarily concerned with the behaviour of toxic solutions sprayed on foliage, it became evident that certain