Chemistry of Insect Cuticle

3. HARDENING AND DARKENING OF THE CUTICLE

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Since the work of Pryor (1940*a, b*) a number of papers have been published on the nature of the process of hardening and darkening, which takes place in the cuticle of most insects after moulting or pupation. Previously it was thought that the formation of melanin products was solely responsible for darkening, the melanin resulting from the oxidation of tyrosine. The formation of hard cuticles was attributed to impregnation of the chitin framework with insoluble materials. These insoluble materials were believed by some authors to be carbohydrates (Schulze, 1922; Kühnelt, 1928), and later Wigglesworth (1933) gave the name 'cuticulin' to the amber-coloured substance occurring in the exocuticle and epicuticle. Cuticulin was considered to have a fatty or waxy component. More recently, Wigglesworth (1947) has shown that the epicuticle does contain lipid material chemically incorporated in its substance, probably as a lipoprotein.

Campbell (1929) clearly demonstrated that there is no relation between the hardness and the chitin content (implied by the term 'heavily chitinized') of the cuticle of the cockroach (*Periplaneta americana* L.). Much later Pryor (1940*a*) showed that the ootheca of the cockroach is formed from the secretion of two glands, one of which secretes a water-soluble protein and the other an *o*-dihydric phenol, later shown to be 3:4-dihydroxybenzoic acid (Pryor, Russell & Todd, 1946). Pryor considered the phenol to be oxidized enzymically to a quinone. This reacted with, or 'tanned', the protein, thus rendering it hard, dark and insoluble in water. A decrease in the activity of the amino groups of the 'tanned' protein was shown by use of the ninhydrin reaction and, by X-ray diffraction and other methods, Pryor showed the presence of a very well-developed system of primary-valence cross-linkages. There is no doubt that these cross-linkages are responsible for the hardened protein being insoluble in water and for its resistance to enzymes and chemical reagents. Pryor (1940*b*) suggested that normal hardening of the insect cuticle is due to a similar process, the phenol being produced by deamination of blood tyrosine or dihydroxyphenylalanine. He proposed the name 'sclerotin' for the tanned protein. Pryor's conclusions are supported by the work of Lafon (1943*a*). Fraenkel & Rudall (1940, 1947) have shown that, coincidentally with darkening, the blowfly larval cuticle (which forms the puparium)

undergoes a pronounced structural change. The arrangement of the chitin crystallites changes from random orientation in planes parallel to the surface in the larval cuticle to an arrangement where they lie approximately parallel to the transverse axis. The contraction of the larval cuticle prior to pupation is explained by this change of orientation. At the same time the water content of the cuticle decreases, which, incidentally, is to be expected if the protein becomes tanned.

Before pupation, there is an increase of tyrosine in the haemolymph of all insects which have been examined (Fraenkel & Rudall, 1947; Hackman & Lazarus, unpublished). The phenolic substances occurring in the cuticle are, in all probability, derived from this tyrosine which has been specially produced. A number of such phenols has been isolated from various insect cuticles (Hackman, Pryor & Todd, 1948; Pryor, Russell & Todd, 1947). The phenols which have been isolated are 3:4-dihydroxybenzoic acid, 3:4-dihydroxyphenylacetic acid, 3:4-dihydroxyphenyllactic acid and β -(3:4-dihydroxyphenyl)alanine.

Several workers (Hackman, 1953*b*; Fraenkel & Rudall, 1947; Trim, 1941*b*; Evans, 1934) have pointed out that, after hardening of the cuticle has taken place, the protein of the epicuticle and exocuticle has become insoluble in water. Trim (1941*b*) suggested that the protein in the hardened cuticle is bound to chitin. However, it now seems established that it is combined with quinones, although there does appear to be a weak bonding between chitin and the protein chains in soft cuticles. Fraenkel & Rudall (1947) showed that the gain in weight of the cuticles of blowflies at pupation is accounted for by the loss of free tyrosine from the blood, but, since the weight increase adds no nitrogen to the cuticle, the tyrosine must be deaminated.

Although the cuticle may contain protein and *o*-dihydric phenols, it is considered that no darkening and hardening takes place until the latter are oxidized enzymically to the corresponding quinones. Before pupation the inner epicuticle of the *Sarcophaga* larva contains a polyphenol oxidase, while the future exocuticle (the outer endocuticle) contains dihydric phenols (Dennell, 1946, 1947, 1949). The inner epicuticle is the only region of high oxidation potential in the cuticle, hence the phenol passes unchanged through the endocuticle. At

pupation, darkening commences where enzyme and substrate meet (oxidation of the phenol followed by tanning of the protein) and gradually spreads inwards through the outer endocuticle. The quinone tans the protein with the formation of a coloured exocuticle and epicuticle. The deposition of phenol in the cuticle follows closely on the appearance of a related phenol in the blood. Although enzyme and substrate are both present in the blood before pupation, reaction is inhibited by the presence of a dehydrogenase system, which acts by lowering the relevant oxidation-reduction potential below the point at which the polyphenol oxidase can operate. The onset of pupation is governed by the liberation of a hormone (Fraenkel, 1935; Becker & Plagge, 1939) which coincides with the abrupt and complete termination of dehydrogenase activity. Many workers have observed that catechol, benzoquinone or quinol bring about a darkening of the cuticle similar to natural darkening and Fraenkel & Rudall (1947) have brought forth evidence which suggests that such compounds stabilize the protein structure in a manner similar to that occurring in natural hardening.

This paper describes an investigation of the reaction taking place during the hardening and darkening of an insect cuticle (i.e. the tanning of the cuticular proteins). The mechanism of the reaction is considered and the relation between sclerotin (tanned protein) and melanin is discussed.*

EXPERIMENTAL

Materials and Methods

The absorption spectra were determined in a Beckman photoelectric quartz spectrophotometer (model DU) using matched quartz cells with a light path of 1 cm.

The enzyme, polyphenol oxidase (prepared from *Psalliotia campestris*), was supplied through the courtesy of Prof. D. Keilin of the Molteno Institute, Cambridge. 3:4-Dihydroxyphenylacetic acid was prepared from hydroferulic acid by demethylation with boiling hydrobromic acid and *N*-acetyl-L-tyrosine by the method of du Vigneaud & Meyer (1932). The phosphate buffers used had the following composition: pH 5.6, 0.003 M- Na_2HPO_4 , 0.063 M- KH_2PO_4 ; pH 6.98, 0.04 M- Na_2HPO_4 , 0.027 M- KH_2PO_4 . For pH 8.4, 0.1 N-NaOH was added to 50 ml. 0.1 M- KH_2PO_4 to adjust the pH to 8.4 and the mixture was diluted to 100 ml. with water (cf. Mason, 1948a).

Tanning of cuticular protein

Water-soluble insect cuticular protein (*Diaphonia dorsalis* Don. (Scarabaeidae) and *Cyclochila australasiae* Don. (Cicadidae)) (Hackman, 1953a) (30 mg.), polyphenol oxidase (30 mg.) and catechol (3 mg.) were aerated in phos-

* Footnote added in proof. Recently, Malek reported that in the cockroach cuticle the substance tanned is a lipoprotein complex, which is, of course, part of the cuticular proteins; Malek, S. R. A. (1952), *Nature, Lond.*, **170**, 850.

Fig. 1. Light absorption of *Diaphonia dorsalis* cuticular protein after 5 min. aeration with catechol and polyphenol oxidase in aqueous buffer at: A, pH 8.4, —; B, pH 6.98, ----; C, pH 5.6, - · - ·. For details see text.

Fig. 2. Light absorption of insect cuticular proteins after aeration for 24 hr. in aqueous buffer (pH 6.98) with polyphenol oxidase. A, —, *Diaphonia dorsalis*; B, ----, *Cyclochila australasiae*; C, - · - ·, *C. australasiae* with catechol added. For details see text.

phate buffer (200 ml.). The experiments were carried out at pH 5.6, 6.98 and 8.4 with aeration for either 5 min. or 24 hr. After centrifugation the absorption spectra of the solutions were determined.

A set of parallel experiments was run in which no catechol was added to the solution containing the protein and enzyme and the absorption spectra were determined after aeration for 24 hr. The absorption curves are given in Figs. 1 and 2. As controls, the absorption spectra of the proteins and the enzymes were determined in phosphate buffer at pH 6.98, both before and after aeration. In both of these controls aeration was shown to have no effect on the absorption

Action of polyphenol oxidase on simple phenolic compounds

N-Acetyltyrosine (50 mg.) and polyphenol oxidase (25 mg.) in 200 ml. phosphate buffer (pH 6.98) were aerated for 24 hr. The solution became reddish brown and the absorption spectrum of the solution was determined after centrifugation. The same experiment was repeated using equal weights of catechol, *p*-cresol, 3:4-dihydroxyphenylacetic acid and, as a control, *L*-tyrosine. The absorption curves are given in Fig. 3.

Absorption spectra of hardened protein

The water-soluble protein isolated by Hackman (1953*a*) from elytra of *Aphodius howitti* Hope was very dark in colour, the colour being considered to be due to a small amount of tanned protein. The absorption spectrum of this compound in a phosphate buffer (pH 6.98) is given in Fig. 4. For comparison the absorption spectrum of the abdominal wall of *Drosophila melanogaster*, redrawn from the curve given by Durand, Hollaender & Houlihan (1941), is given together with the spectral curves of 'synthetic' 3:4-dihydroxyphenylalanine melanins at pH 6.8 and 8.4, redrawn

Fig. 3. Light absorption after aeration for 24 hr. with polyphenol oxidase in aqueous buffer (pH 6.98). A, —, *N*-acetyltyrosine; B, ---, 3:4-dihydroxyphenylacetic acid; C, . . . , catechol; D, - · - ·, *p*-cresol; E, — — —, *L*-tyrosine. For details see text.

curves. Neither mixture showed any absorption above 300 $m\mu$. and both showed single absorption peaks. The protein solution had λ_{max} at 273 $m\mu$. and λ_{min} at 242 $m\mu$.; the enzyme solution had λ_{max} at 275 $m\mu$. and λ_{min} at 250 $m\mu$. No tyrosine could be detected in the acid hydrolysate of the enzyme by paper-partition chromatographic methods.

The absorption spectrum of the solution obtained by aerating a mixture of catechol and polyphenol oxidase was also determined. At all pH's and after the two different times of aeration the curves were almost the same as those obtained when the insect cuticular protein was present. For this reason the absorption curves are not shown in the figures.

Fig. 4. Light absorption in aqueous buffer: A, *Aphodius howitti* protein; B, *Drosophila melanogaster* abdominal wall; C, melanin, pH 8.4; D, melanin, pH 6.8. For details see text.

from the curves given by Mason (1948*a*). The absorption curve for the reaction between tyrosine and polyphenol oxidase given in Fig. 1 has almost the same slope as that given by Mason for melanin at pH 8.4. The exact shape of the absorption curve for melanin formed enzymically from tyrosine or β -(3:4-dihydroxyphenyl)alanine was found to depend upon the period of oxidation, the temperature and the pH of the solution (see also Nickerson, 1946; Mason, 1948*a*).

All the materials studied in Figs. 1, 2 and 4 showed absorption in the region 220–320 $m\mu$. However, as all the reaction mixtures contained catechol or other phenol, free or combined tyrosine, or other compounds showing absorption in this region, comparisons based on absorption spectra in this region are of doubtful value.

Qualitative tests

Several qualitative tests which are said to be given by melanin were tried on a number of insect cuticles. The insect materials used were the elytra of adult *Aphodius howitti* and *Tenebrio molitor* L., the puparia of *Musca domestica* L. and *Lucilia cuprina* Wied. and the cast skins of *Locusta migratoria* L. all of which were finely ground and freed of lipid material by extraction with light petroleum. Melanin and tanned gelatin were used as controls. To prepare the melanin, polyphenol oxidase (25 mg.) and β -(3:4-dihydroxyphenyl)-alanine (50 mg.) were aerated for 24 hr. in 50 ml. of phosphate buffer (pH 6.98). The tanned gelatin was prepared by immersing a very thin sheet of gelatin in 50 ml. of phosphate buffer (pH 6.98) containing catechol (50 mg.) and polyphenol oxidase (25 mg.) and aerating the mixture for 7 days. The tests were: (i) to boil with 4*N*-ethanolic KOH; melanin is said to give a deep-red solution (Verne, 1926); (ii) to heat with a conc. aqueous solution of KOH; melanin-containing material is bleached (Lison, 1936); (iii) to immerse the material in 0.1% (w/v) aqueous KMnO_4 for 24 hr., rinse in distilled water and immerse in 0.3% (w/v) aqueous oxalic acid; after 24 hr. melanin is completely decolorized (Lison, 1936); (iv) to reduce with sodium dithionite in 2*N*-NaOH; melanin gives a brownish leuco compound which can be reoxidized to melanin (Figg, 1939, 1940).

All the insect materials, the tanned gelatin and melanin reacted positively in each of these tests.

DISCUSSION

In the two previous papers (Hackman, 1953*a*, *b*) the proteins of insect cuticle have been examined. The water-soluble, alkali-soluble and alkali-insoluble proteins are all very similar, especially in their amino-acid content. It would appear that any of these proteins can be considered to be typical of those taking part in the tanning reaction which occurs during the hardening of insect cuticle.

Tanning of proteins

The absorption curves given in Fig. 2 show clearly that the presence of an added *o*-dihydric phenol is not essential for the tanning of the protein. In these experiments catechol was used as the *o*-dihydric phenol because it has been shown (Hackman, 1949; Hackman & Todd, 1953) that, when 3:4-dihydroxybenzoic acid is oxidized to the corresponding *o*-quinone and reacts with a compound containing an amino group, it undergoes decarboxylation. For example, with aniline 3:4-dianilino-1:2-benzoquinone is formed, the same compound as if catechol had been used as the phenol.

Spectroscopic evidence indicates that, in the presence of the enzyme polyphenol oxidase, the cuticular protein becomes tanned in a manner very similar to that when catechol is added. However, the colour is less intense than when an added *o*-dihydric phenol is present; this is probably due to the absence of polymerized quinones. That this development of colour in the absence of an added

phenol is not due to the enzyme or protein alone is shown by comparison of the results obtained after aerating the protein and enzyme solutions. In both cases aeration was shown to have no effect on the absorption spectra and none of the spectra showed absorption above 300 $\text{m}\mu$. No tyrosine could be detected in the enzyme after acid hydrolysis, which may be the reason why the enzyme cannot tan itself. However, in the presence of catechol the protein of the enzyme becomes tanned and the absorption spectrum of the solution is very similar to that obtained when cuticular protein is present. In this case, undoubtedly, the catechol is converted to *o*-benzoquinone, which reacts with the protein of the enzyme.

In the system (cuticular protein + enzyme) the enzyme has probably oxidized the phenolic groups of the tyrosine in the protein to an *o*-quinone. This quinone then acts as the tanning agent. The high tyrosine content (approx. 11%) of some cuticular proteins (Hackman, 1953*a*; Trim, 1941*b*) would enable the proteins to take part more effectively in this type of hardening process. This interpretation would lend support to the suggestions of several workers. First, Brown (1949, 1950) and Blower (1950) suggest that in some invertebrates the quinone responsible for the tanning of the proteins is probably an aromatic amino-acid or protein and probably one in which the phenolic groups of tyrosine have become oxidized to the quinone without removal of tyrosine from the protein chains. Brown suggests that the tanning mechanism may have evolved through such a step as this. Secondly, the work of Sizer and colleagues (Sizer, 1946, 1947; Sizer, Brindley & Wagley, 1949; Hass, Sizer & Loofbourow, 1951) and of Kertesz (1950) suggests that tyrosinase (polyphenol oxidase) attacks a number of proteins, presumably through their tyrosine residues, with at least partial loss of the identity of the protein substrates.

It has now been shown (Hackman, 1953*a*) that the terminal carboxyl groups in the proteins obtained from *Diaphonia dorsalis* larval cuticles are those of tyrosine, glycine, lysine, serine and glutamic acid. Tyrosine is of interest because of its phenolic properties. Reference has already been made to the role played by tyrosine, even when an integral part of the protein molecule, in the tanning of proteins, and it has been shown (Hackman, 1949; Hackman & Todd, 1953) that compounds such as *N*-(3:4-dihydroxyphenyl)glycine are unstable and readily give indoxyl-like structures. The reaction was formulated as proceeding through 3:5:6-trihydroxyindole. It is of interest to know if a similar type of reaction could occur with a terminal tyrosine residue (free carboxyl).

N-Acetyltyrosine was used as a type compound and it was aerated with the enzyme polyphenol

oxidase in a phosphate buffer. Comparison of the absorption spectrum of the resulting solution with those obtained from catechol, *p*-cresol and 3:4-dihydroxyphenylacetic acid by a similar procedure (see Fig. 3) suggests that essentially the same reaction has occurred with all these compounds. However, all the absorption spectra are obviously different from that given by the solution obtained by the enzymic oxidation of tyrosine (which would form melanin) and from the spectra given by Hackman (1949) and Hackman & Todd (1953) for the compounds formed by the oxidation of *N*-(3:4-dihydroxyphenyl)glycine and the subsequent polymerization. These results indicate that a *C*-terminal tyrosine residue behaves very similarly to catechol and of itself does not give rise to indoxyl-like structures on enzymic oxidation.

The relation of sclerotin to melanin

Reference to the curves in Figs. 2 and 4 shows that the hardened protein from *Aphodius howitti* elytra and the protein tanned by *o*-benzoquinone are quite different from melanin derived from tyrosine or β -(3:4-dihydroxyphenyl)alanine. The major differences between the absorption spectra for melanin and the hardened protein (sclerotin) are that in the curve for the hardened protein the maximum is at a shorter wavelength, and there is much less absorption in the visible region of the spectrum than with melanin. That the absorption spectrum of the abdominal wall of *Drosophila melanogaster* follows closely that of the hardened protein serves as confirmatory evidence.

In an attempt to find some method to distinguish between sclerotin and melanin many qualitative tests were investigated. Unfortunately many of the 'tests' for melanin are in fact tests for sclerotin because they were worked out using insect material, e.g. decolorization with diphenol (Campbell, 1929) or concentrated aqueous solutions of potassium hydroxide, which is the accepted method for the estimation of chitin in insect material. Again, it is to be expected that almost all insect material will reduce ammoniacal silver nitrate because of the *o*-dihydric phenols present in the cuticle. It has now been shown that sclerotin, like melanin, gives a deep-red solution when boiled with ethanolic potassium hydroxide, is decolorized by aqueous potassium permanganate and is reduced by sodium dithionite. Sclerotin thus gives all the tests for a typical melanin, as prepared from tyrosine or β -(3:4-dihydroxyphenyl)alanine. However, from spectroscopic evidence, sclerotin and melanin are different. These qualitative tests do not detect the presence of melanin as such, but merely show the presence of reducible material. This work clearly shows the futility of applying these tests as a means

of showing the presence of melanin as distinct from sclerotin.

Sclerotin has properties resembling melanin, namely dark colour and great resistance to diverse solvents and chemical reagents. The question then arises what melanin is. The term melanin has become largely meaningless due to the tendency of biologists to place in this class any dark pigment which is highly stable and insoluble in most solvents. Although the formation of melanin from tyrosine has been extensively studied, the structure of the pigment is unknown; however 5:6-indolequinone is known to be an intermediate. Furthermore, there is still controversy about the relation between natural and synthetic melanins (Mason, 1948*b*; Ginsburg, 1944). It is probable that there are many pigments of this class and that they may be derived by different methods from more than one source. The occurrence of differently coloured pigments obviously closely related supports this view (Clemo & Duxbury, 1950; Smyth, Porter & Bohren, 1951; Nickerson, 1946; Figg, 1939, 1940). Melanin is often bound to proteins to give melanoproteins. In fact, it is difficult to imagine that there could exist in a biological system indolequinones and proteins without some reaction taking place between them. Sclerotin, on the other hand, indicates a class of pigments formed by a particular type of chemical reaction (see later). It has protein incorporated in its structure and as Hackman (1949) and Hackman & Todd (1953) have shown may, in some instances, possess an indole-like structure. Thus even though the *o*-quinones in most cases contain no nitrogen, the reaction with the protein chains can be such as to form an indole derivative. From the method of formation of sclerotin and the range of phenols (or quinones) and proteins which may participate, it is certain that sclerotin cannot represent a single compound, but must represent a group of structurally related pigments, which are at the moment indistinguishable.

The tanning mechanism

The work of Pryor (1940*a, b*), Dennell (1946, 1947), Hackman (1949) and Hackman & Todd (1953) clearly indicates that the tanning (i.e. hardening and darkening) of insect cuticles is due to the interaction of the cuticular proteins and quinones. The quinones are produced from *o*-dihydric phenols by the action of the enzyme polyphenol oxidase, which is plentiful in the cuticles of arthropods (Bhagvat & Richter, 1938; Dennell, 1947). The presence of oxygen is also necessary for the hardening of the cuticle (Henke, 1924).

Failure to detect free amino groups in the hardened protein obtained from *Aphodius howitti* elytra (Hackman, 1953*b*) and the decrease in the

activity of amino groups during the hardening of the cockroach ootheca (Pryor, 1940*a*) indicates that reaction has taken place at these amino groups. As *o*-quinones are involved, cross-linked structures are formed in which the nitrogen of the amino groups of the protein chains has become attached directly to the aromatic nuclei of the quinones. The failure to detect lysine in the hydrolysate of the hardened protein would be due to reaction of the ϵ -amino group with a quinone. That such a reaction is possible has been shown by Hackman (1949) and Hackman & Todd (1953) and the reaction is analogous to the reactions of *o*-benzoquinone and substituted *o*-benzoquinones with aniline to give 4:5-dianilino-1:2-benzoquinone, 4:5-dianilino-1:2-benzoquinone anil, etc. (Hackman & Todd, 1953; Hackman, 1949; Fischer & Hepp, 1888; Jackson & Porter, 1903). In this manner very stable covalent links would be formed. The ϵ -amino group of lysine is of great importance in this reaction as it provides a means of cross-linking the protein chains at points other than the ends. The presence of polymerized quinones probably contributes to the colour of very dark cuticles.

The origin of the *o*-dihydric phenols is not known with certainty, but there is strong evidence (Fraenkel & Rudall, 1947; Dennell, 1949; Pryor *et al.* 1947) to suggest that they are derived from tyrosine or β -(3:4-dihydroxyphenyl)alanine by oxidative deamination. Such oxidative deaminations can be brought about enzymically or by a variety of organic carbonyl compounds (Herbst, 1944; Trautner & Roberts, 1950). *o*-Quinones are capable of bringing about the reaction. Trautner & Roberts have investigated the oxidative deamination of certain amino-acids by the system catechol-polyphenolase and they formulated the reaction as proceeding through *N*-(3:4-diketocyclohexa-1:5-dienyl)glycine, the quinone formed from *N*-(3:4-dihydroxyphenyl)glycine. Although there is no direct evidence to show that oxidative deamination does occur in insects, the fact that compounds capable of bringing about the reaction are known to be present renders the mechanism highly probable. Herbst considers the reaction to lead to the formation of a pyruvic acid, and for β -(3:4-dihydroxyphenyl)alanine it would be β -(3:4-dihydroxyphenyl)pyruvic acid. The lactic acid derivative isolated by Pryor *et al.* (1947) would be formed by reduction of this pyruvic acid, while further oxidation would lead to 3:4-dihydroxyphenylacetic acid. This may account for the failure to isolate β -(3:4-dihydroxyphenyl)propionic acid from insect cuticles.

Available evidence (Hackman & Todd, 1953; Hackman, 1949; Jackson & Kendal, 1949; Cushing, 1946; James, Roberts, Beevers & de Kock, 1948; Beevers & James, 1948) clearly indicates that

reaction first takes place at the 4 and/or 5 position in the *o*-benzoquinone nucleus. Initially, structures such as I and II are formed, R—NH₂ representing a free amino group of a protein chain. Then reaction could take place at the quinonoid oxygen atoms to give finally III. Possible variants in this type of reaction are discussed by Hackman (1949).

Covalent bonds, due to their extreme stability, are by far the most important type of link between protein chains or between parts of a protein chain responsible for the hardening of insect cuticles. Such bonds are formed when a quinone reacts with a protein. However, there are two other types of linkage capable of holding together protein chains which undoubtedly play some part in insect cuticle. These are coulombic forces and van der Waals forces. Specific reference will be made to the first (salt links) in regard to glutamic acid, but such linkages are possible between any adjacent —NH₃⁺ and —COO[−] groups in the protein side chains. The hydrogen bond is a special case of this type of linkage. Electrovalent linkages are greatly influenced by the ionic content of the surrounding medium. Van der Waals forces are those which hold together non-polar molecules and would presumably exist between, say, terminal methyl groups in protein side chains.

In regard to glutamic acid, as it is probably a constituent amino-acid (as distinct from a terminal amino-acid) of cuticular proteins, possibly there is branching from the γ -carboxyl groups. A number of proteins have been shown to contain γ -glutamyl bonds (Haurowitz & Bursa, 1949). Since there is little or no sulphur in the insect cuticle or in the cuticular proteins (Hackman, 1953*a, b*; Trim, 1941*a, b*; Lafon, 1941) cross-linking by the formation of disulphide bonds, as in keratin, cannot occur to any appreciable extent. The absence of sulphhydryl groups is perhaps significant because they inhibit the action of polyphenol oxidases (Flesch & Rothman, 1948) and hence would interfere with the hardening mechanism. The almost total absence of sulphur in the cuticular proteins is in marked contrast to the proteins of the shell of some insect eggs and of the cuticle of the primitive arthropods of the genus *Limulus*, which contain several per cent of sulphur (Lafon, 1943*b*).

Cross-linking of the protein chains in the insect cuticle would decrease its ability to retain water, would cause brittleness, loss of elasticity and reduce solubility in water. Subsequent impregnation of the cuticle with lipid material would be facilitated

since most of the strongly hydrated groups of the protein chains would have been utilized in the formation of cross-linkages. The observed physical characteristics of insect cuticle are thus those which would be expected if the protein chains are held together by many stable cross-linkages.

SUMMARY

1. For an insect cuticular protein to become tanned in the presence of the enzyme polyphenol oxidase, the presence of an added *o*-dihydric phenol is not essential. It is concluded that the phenolic groups of the tyrosine residues in the protein become oxidized to an *o*-quinone. This quinone then acts as the tanning agent.

2. Possible roles played by glutamic acid and tyrosine in this tanning (hardening) process are considered in some detail.

3. The mechanism of the tanning reaction and the origin of the *o*-dihydric phenols is discussed.

4. The tanned protein (sclerotin) is considered on spectroscopic evidence to be quite different from melanin. Spectroscopy is the only method known which can be used to distinguish between these two types of pigment. Sclerotin indicates a group of pigments formed by reaction between a protein and a quinone, whereas melanin is an ill-defined term which has come to include besides the pigment formed by the enzymic oxidation of tyrosine almost any naturally occurring pigment which is dark in colour.

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