STUDIES ON THE EXPRESSION OF THE PHENOLASE COMPLEX IN INSECTS

by

WILLIAM SINCLAIR

B.Sc. (Edin.)

Department of Zoology, University of Edinburgh.

THESIS submitted for the degree of Doctor of Philosophy at
Edinburgh University. December, 1961.

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INTRODUCTION

There is an abundant literature on the related processes of melanin formation and the hardening of cuticle in insects. Knowledge of the nature of these processes has been derived largely from histo-chemical and histological investigations of cuticles in different stages of development, or from the employment of chemical methods designed to differentiate between the various components of cuticle.

It is generally accepted that the two processes involve reactions catalysed by one and the same enzyme, tyrosinase (Pryor, 1940a, 1940b; Mason, 1955a). It is likely that this enzyme has the ability to catalyse two important sequential steps, the conversion of tyrosine to an ortho-dihydroxyphenol, and this in turn to a corresponding ortho-quinone. When catalysing the second step, the enzyme is often referred to as a polyphenol oxidase. Since there is still room for controversy over whether one or two separate enzymes are involved, the term phenolase complex has been used (Mason, 1955a).

Whereas there is strong circumstantial evidence for tyrosinase being actively concerned with the deposition of melanin as well as with the hardening of cuticle, Mason (1955a) pointed out that the enzyme had not been isolated from insects, and since that time little advance has been made in this sphere. Notwithstanding, it seems certain /

certain that the enzyme is involved in the hardening and melanising processes.

In most insects, the degree of hardening of the cuticle and the amount of melanin deposited in a fixed pattern are fairly constant for a given species. An exception to this is in the case of the locust. Whereas the degree of hardening of the cuticle appears to be constant, there are obvious differences in the amount of melanin deposition, as is illustrated by the gregarious and solitary phases of the same species. In the former phase, the deposition is considerable and this appears to be coupled with a higher rate of metabolism. In the latter, the deposition is light or absent, and the metabolic rate is lower (Kennedy, 1956). However, the cuticles of both the gregarious and solitary phases of locust are apparently equally hard, although evidence on this point is rather scarce.

In view of the above considerations, the occurrence of a non-melanised mutant, or "albino", strain of the Desert Locust Schistocerca gregaria Forskål suggested that it might be possible to induce the formation of melanin patterns in these albinos by experimentally introducing phenolic substrates of the phenolase complex, provided that an active tyrosinase or polyphenol oxidase was present. Since albinos were capable of performing normal movements there seemed every reason for supposing that the cuticle was hardened and consequently that tyrosinase was present.

When hardening and melanin deposition occur, it has been contended that the two processes take place simultaneously (Wigglesworth, 1948). This raised the question of how hardening could take place in an albino locust, while at the same time melanin formation was suppressed. Here, then, was an excellent medium for attempting to study these processes by experiments devised to test whether the albino condition was due to the absence of the phenolase complex or the appropriate substrate. If the phenolase complex was present, then was the albinism due to the absence of a substrate necessary for melanin formation but not for cuticle-hardening? If so, the reason for albinism in this insect would be different from that in human albinos, in which the enzyme is apparently absent.

The chief aim of the work presented in this thesis has therefore been to design a technique for experimentally inducing excess hardening of the cuticle, or melanin deposition, or both, by the introduction of substrates of the phenolase complex. In conjunction with these procedures, it would also be possible to test the effect of enzyme inhibitors as a means of detecting the nature of the enzyme involved in the natural processes.

That a technique for inducing the formation of melanin patterns in albino locusts by experimentally introduced substrates was devised opens the way to the use of labelled substrates to follow the possible routes, and deposition, of the substrates in the living insect. By utilising albino locusts as a medium for study in this way, it seemed likely /

likely that the problem of which substrates the insect is capable of employing in the hardening of cuticle and the deposition of melanin could be approached from a new angle, when experiments were accompanied by detailed histochemical and histological studies of natural and treated cuticles.

Enzyme inhibition studies have also been made on Phormia to try to throw some light on the question of whether the hardening of cuticle and the deposition of melanin are the results of two separate processes catalysed by the same enzyme complex.

It is interesting to recall that in the last few years biochemists have devoted a great deal of attention to the fact that quinones are widespread in cells. It has now transpired that a quinone referred to as coenzyme Q appears to be implicated in oxidative phosphorylation. It is also likely that tyrosinase is involved. In view of the accumulating information on the polyphenol-polyphenoloxidase system, any new approach to studying the expressions of this system in insects is therefore always likely to have wider implications.

MATERIALS AND METHODS

In this study, the Desert Locust, Schistocerca gregaria Forskål and the blowfly, Phormia terrae-novae were used. The albino strain material was generously provided by the Anti-Locust Research Centre in London. This strain breeds true, and melanin did not appear to be present in any stage of the albinos' life-cycle. Neither could they be forced to deposit this pigment in response to extreme crowding (Uvarov, 1957; Hunter-Jones, 1957). In this respect it differs radically from the lightly melanised form, solitaria, of the normal strain, which can be forced by crowding to increase the extent of its pigmentation until the insect is almost entirely black. This deeply pigmented state is typical of the wild type in the gregaria phase.

Cultures of the wild strain were maintained in this laboratory, using standard breeding techniques (Hunter-Jones, 1956). However, for some completely unknown reason, it has been impossible to maintain the albino strain here, despite every attempt to provide suitable conditions. The only explanations that can be given are that there was either lack of some critical humidity condition required by the albinos, or insufficient numbers of albinos present in the cages to promote successful breeding. When relatively large numbers of albinos from the Anti-Locust Research Centre were obtained and kept in crowded conditions it did not, however, prevent a high mortality rate in all stages reared through from egg to adult. Gregarine parasites /

parasites were found in equal quantities in both wild and albino strains. It is unlikely that this common locust infection could have been responsible for the losses of albinos unless they were more susceptible to the adverse effects of the parasites.

This difficulty in rearing the albinos might, however, have been associated with the lowered vitality of albino animals in general. That this condition was the case in the albino insects was reflected in the extreme ease with which even minute injury could kill the albino S. gregaria. This susceptibility unfortunately also presented considerable difficulties when experiments were performed on the albinos.

The blowfly, P. terrae-novae was maintained in large numbers. The eggs were collected on raw meat as often as they were needed. In this way, uniformity of larval age within any batch was obtained. The eggs were then transferred to glass jars containing dried milk and yeast, as a paste made with water.

The immersion technique devised for experimentally introducing agents into the living insect entailed the removal of the wax layer of the cuticle from the tip of the abdomen. This de-waxed portion of the abdomen was then immersed in a solution of the agent to be introduced into the haemocoel by passage through the cuticle. Further details of this technique are given in the section dealing with the experiments carried out on melanin-induction in the albinos.

Tyrosinase inhibitor tests were largely carried out on the blowfly /

blowfly larvae. The inhibitor was injected in solution in saline, from an "Agla" micro-syringe, using a No. 30 needle. Although there was usually some slight loss of haemolymph from the puncture, the wound healed rapidly. To off-set the loss of inhibitor, the needle was left inserted in the insect for a few seconds to allow the substance injected to disperse in the blood. After being injected, the larvae were kept at 30'C. until development to the adult stage.

The micro-syringe was calibrated by weighing known deliveries of fluid.

THE PHENOLASE COMPLEX

Since various names have been used to describe the enzyme system responsible for melanisation and the hardening of the cuticle in insects, some difficulty is experienced in defining precisely the nature and functions of this system. The term "phenolase complex" has been used by Mason (1955a) in the sense of its embracing "that pair of enzymatic activities occurring together, associated with copper-protein, and responsible both for the ortho-hydroxylation of mono-phenols, and dehydrogenation of ortho-diphenols". However, this definition has the disadvantage that it regards the conversion of ortho-dihydroxyphenols to quinones as a dehydrogenation, since Dawson and Tarpley (1951) have pointed out that this process is better regarded as an oxidation on the grounds that it cannot be performed anaerobically.

The phenolase complex catalyses the oxidation of mono-phenols, for example, tyrosine and para-cresol, as well as ortho-dihydroxyphenols such as catechol, 3, 4-dihydroxyphenylalanine (dopa), and proto-catechuic acid. The ability of the complex to catalyse these two distinct reactions has led to attempts to separate two enzyme components, one catalysing the oxidation of mono-phenols, the other the oxidation of ortho-dihydroxyphenols. Up to the present, the evidence is strongly in favour of both these reactions being catalysed by one and the same copper-protein enzyme (Dawson and Tarpley, 1951). Further support of this conclusion is provided

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by the results of recent work by Kertesz and Zito (1957). In using electrophoretic analyses, they showed that the purified active fraction from mushrooms was homogeneous, and that solutions of the fraction gave a single, sharp, symmetrical peak in the sedimentation pattern obtained in the ultracentrifuge.

The adjectives cresolase and catecholase have been used to designate the types of reaction with which the phenolase complex is concerned. It shows cresolase activity when the substrate is a mono-phenol, and catecholase activity when oxidising an ortho-dihydroxyphenol (Mason, 1955a). Similarly, the terms polyphenol oxidase and dopa oxidase have been applied to an enzyme catalysing the oxidation of ortho-dihydroxyphenols to quinones, or to higher products, when no cresolase activity is apparent. But in such cases it is likely that a monophenolase activity may have been overlooked, since this activity is much less rapid than the catecholase one (Dawson and Tarpley, 1951).

The enzyme, when it occurs in vertebrates and insects, is usually referred to as tyrosinase (Brown, Ward and Griffin, 1959; Pryor, 1940a; Richards, 1958). Dawson and Tarpley (1951) regard polyphenol oxidase and tyrosinase as the same enzyme. In the literature on the phenolase complex in insects, however, the terms polyphenol oxidase, tyrosinase, and phenolase complex all appear to be used without resort to defining what is understood by the term used. Wigglesworth (1957) uses the names polyphenol oxidase and /

and tyrosinase in the sense that they are synonymous. Dennell (1958d) used the term polyphenol oxidase to indicate the phenolase activity in the epicuticle of insects, and tyrosinase for that in the blood. This distinction is probably drawn from his observations showing that the mono-phenol tyrosine in the blood of blowflies is oxidised to dopa, while the oxidation of this ortho-dihydroxyphenol seems to be confined to cuticle. Mason (1955a) prefers to regard the enzyme in the insect blood as identical to that present in the epicuticle. However, these different usages may have been applied simply to emphasise the points these authors wished to bring out.

It seems, therefore, that the term phenolase complex is synonymous with tyrosinase, or polyphenol oxidase. However, the enzyme in different organisms varies in its specificity. That extracted from fungi, for example, has a very low specificity, while that in vertebrates is highly specific for tyrosine (Mason, 1955a). The cresolase to catecholase ratio may vary widely, and it can be affected radically by extraction methods (Dawson and Tarpley, 1951). It has also been suggested that the enzyme in the epicuticle of *Drosophila* may have a different substrate specificity from that in the blood (Ohnishi, 1954).

The term phenolase complex has therefore been used in this thesis in the sense that it is an enzyme system which has the ability to catalyse two distinct steps in the process of hardening of insect cuticle and of melanogenesis. The term embraces both the so-called cresolase and catecholase activities.

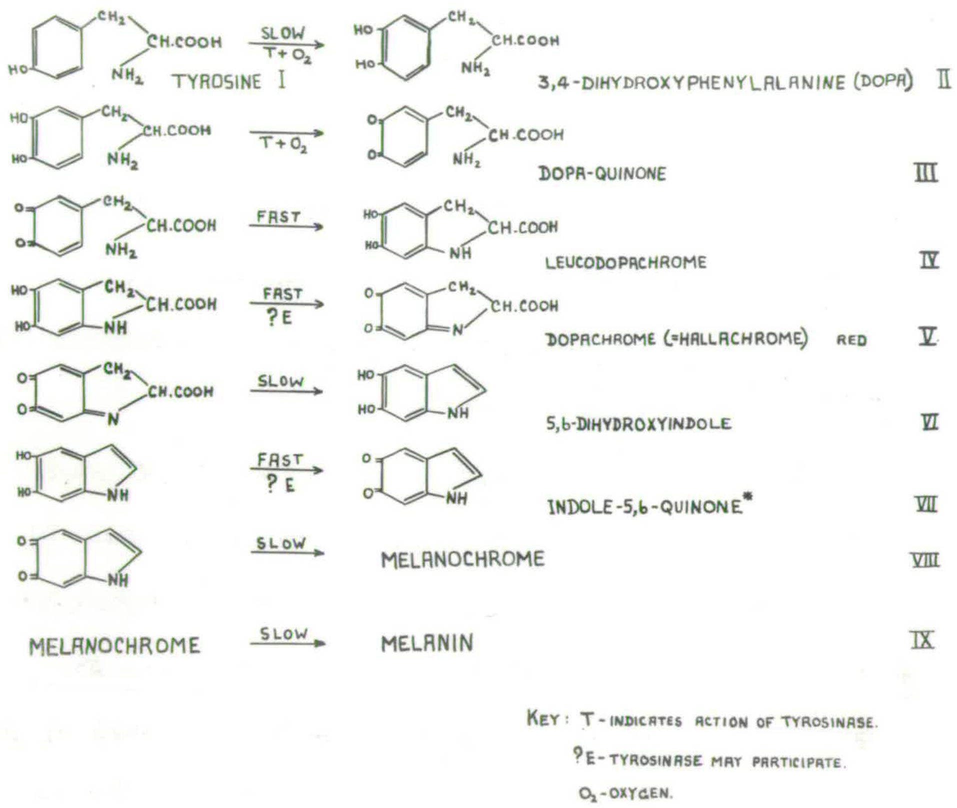

Figure 1. Formation of melanin from tyrosine (after Mason, 1959; Fitzpatrick and Kukita, 1959).

* According to Mason (1959), indole-5,6-quinone is purple; Fitzpatrick and Kukita (1959) say it is yellow.

ON THE CHEMISTRY OF THE RELATED PROCESSES OF MELANIN FORMATION AND THE HARDENING OF INSECT CUTICLE

The important function of the phenolase complex in insects, then, lies in its ability to catalyse the conversion of the amino-acid tyrosine to di-hydroxyphenylalanine (dopa), and of this product, dopa, to dopa-quinone. It is believed that the substances which produce melanin pigments, as well as those which are involved in the hardening of insect cuticle, are largely derived from dopa-quinone (Mason, 1955a, 1959; Fitzpatrick and Kukita, 1959). The nature of the later intermediate stages in melanogenesis and in cuticle hardening or sclerotisation is still open to controversy (Mason, 1955a, 1959; Yasunobu, 1959).

Fukuda (1956) has demonstrated the conversion of phenylalanine to tyrosine in the silkworm, Bombyx mori. The first step in the conversion of tyrosine to a corresponding di-hydroxyphenol involves the insertion of an hydroxyl group into the ortho position. This yields dopa. Tyrosinase catalyses this reaction as well as the conversion of dopa to its quinone (Fig. 1, I - III). Since the enzyme catalyses both these oxidation reactions aerobically, and moreover since these reactions cannot be brought about anaerobically by utilising reducing agents such as methylene blue, the enzyme is best regarded as an oxidase in this case and not a dehydrogenase (Dawson and Tarpley, 1951). The conversion of dopa to dopa-quinone is sometimes described as a dehydrogenation (Mason, 1955a), but it is best to avoid this.

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From this stage, there is a divergence in the reaction chains of the processes leading to melanisation and sclerotisation. To take melanisation first, dopa-quinone is believed to undergo a rapid spontaneous ring closure, to give initially leucodopachrome, or, as it was often called in the older literature, Hallachrome (Fig. 1, IV and V). For a long time this red substance was thought to be identical to a similarly coloured pigment in the annelid Halla, from which the name of the pigment was derived. This synonymity, however, is invalid (Brunet, 1959). The pigment in Halla is stable, while the dopachrome formed in the melanogenetic sequence spontaneously goes on to be converted into melanin (Sumner and Somers, 1953). It is possible that the conversion into melanin might take place without depending on further assistance from tyrosinase. It is likely, however, that the enzyme may be involved in the conversion of 5, 6-dihydroxyindole to indole-5,6-quinone. From dopachrome, further reactions involve the loss of the carboxyl group and the formation of indole-5,6-quinone, which is thought to produce melanin ultimately by a process involving polymerisation (Fig. 1, VI - IX). The suggested ways in which the still obscure later reactions occur have been reviewed in detail (Mason, 1955a).

The sequence of reactions at present accepted as fitting most satisfactorily the evidence available, is shown in Fig 1. This reaction chain (Mason, 1955a, 1959) applies chiefly to chordate /

chordate melanins. It is accepted throughout an extensive literature on the subject that, apart from variations in substrate specificity of tyrosinase in invertebrates and plants from that in vertebrates, the tyrosinase in all organisms is operating in the same general way (Mason, 1955a). In fact, a great deal of the work on the chemistry of tyrosinase has been done on the extracts obtained from fungi, mainly the wild and cultivated mushroom, and Neurospora (Dawson and Tarpley, 1951; Sumner and Somers, 1953; Horowitz, 1956; Gest and Horowitz, 1958).

In the process of hardening of insect cuticle, a different series of reactions is thought to take place. It was Pryor (1940a, 1940b) who first proposed a plausible explanation for the way in which cuticle became hardened. His model experiments were based on the hardening process associated with cockroach oothecae. The protein of which the ootheca is mainly composed is secreted by the left colleterial gland of the female. Mason (1955a) misquotes Pryor on this point. When initially produced by the insect, this protein is in the form of a viscous solution. However, Pryor concluded that the right colleterial gland gave rise to an ortho-dihydroxyphenol which, in the presence of an active phenolase, produced quinone substances which tanned the proteins to form a hardened ootheca. The finer details of how the ortho-quinone precursor was released from its initially inactive /

inactive form occurring as a glucoside in not the right gland, but the left one, were later presented by Brunet and Kent (1955). Cohen (1950) mentioned that hydroquinone, a dihydroxyphenol regarded by Dennell (1958c) as important in protein tanning in the blowfly, occurs as the glucoside arbutin in the bearberry (Ericaceae : *Arctostaphylos* sp.), and certain other plants. Considering the theories involving tyrosinase and phenols in oxidative enzyme systems in plants (Baker and Nelson, 1943), it is tempting to speculate whether arbutin is involved in such cycles.

Brunet and Kent (1955) showed that Pryor had been mistaken in interpreting the occurrence of a very weak chromaffin reaction in the right gland of the cockroach as indicating the presence of a dihydroxyphenol. The cause of the weak reaction is not known. Brunet and Kent elegantly showed that although the dihydroxyphenol came from the left gland, it was not in fact free in the secretion. Only when the secretion of the right gland was mixed with that of the left was the dihydroxyphenol formed. It was also shown that the phenol existed as a glucoside, alongside the structural protein of the ootheca, and a polyphenol oxidase, in the secretion of the left colleterial gland. The secretion from the other gland contained a β-glucosidase which, in the formation of the ootheca, split the glucoside of protocatechuic acid. The liberated dihydroxyphenol was subsequently oxidised by the polyphenol oxidase, and /

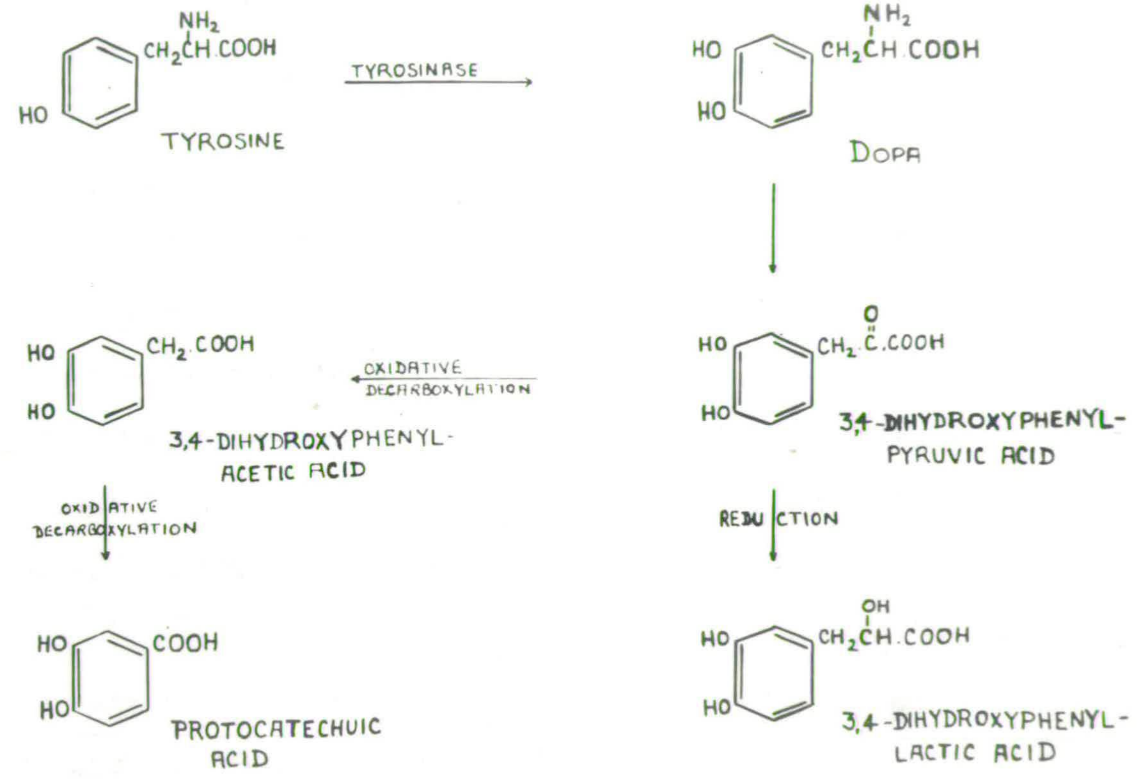

FIGURE 2. Probable method of formation of the various 3,4-dihydroxyphenyl acids in the cuticle (Based on Gilmour, 1961).

and the resulting quinone tanned the protein of the ootheca.

In an attempt to characterize in detail the ortho-quinone immediately responsible for the hardening of the protein of cockroach ootheca and insect cuticle in general, Pryor (1940a, 1940b) was able to eliminate both tyrosine and dopa as being the immediate precursors of the quinone, although not eliminating them as members of possible related reactions. He further proved that the unknown precursor of the hardening quinone had two hydroxy groups in the ortho position, and had a carboxyl group in a side chain. Of the alternatives possible from the evidence then available to him, he suggested that the tanning phenol was 3,4-dihydroxyphenylacetic acid, whose quinone would be formed by tyrosinase oxidation and would then tan the protein of the ootheca or cuticle.

Several times before and since Pryor's experiments, various similar phenols have been demonstrated from insect cuticle. These include all the substances to be expected in a logical chain of reactions resulting in the deamination of dopa, and eventual production of 3,4-dihydroxybenzoic acid, usually called protocatechuic acid (Fig. 2). All of these intermediates can produce ortho-quinones, but it was not clear whether all of these quinones could react with cuticular protein. Richards (1958) rejects the view that all of the possible quinones in insect cuticle can tan proteins, and on the basis of the work of Hackman (1953a, 1953b, 1953c) suggests that Pryor was almost certainly in error in assuming /

assuming that the quinone of 3,4-dihydroxyphenylacetic acid could link proteins in cuticle or ootheca. Richards (1958) agrees with the view that only the quinone of protocatechuic acid can link proteins. This argument is based on the theory that the quinone structure of this molecule makes the carboxyl group sufficiently reactive to allow the molecule to possess two reactive sites. Such an activation would not be possible with longer side chains such as that of Pryor's 3,4-dihydroxyphenylacetic acid. However, Hackman (1958) states categorically that not only protocatechuic acid, but the various 4-substituted dihydroxyphenols such as 3,4-dihydroxyphenylacetic acid, should be capable of linking two or three protein chains in the presence of a polyphenol oxidase.

This information now makes it possible to understand how tyrosinase may be involved in cuticle hardening, when consideration of the mechanisms involved is resumed from the point where tyrosinase action on tyrosine had led to the formation of dopa. While melanogenesis involved internal structural changes in the dopa molecule, it appears that for cuticle hardening to occur, dopa must be deaminated, to release, eventually, protocatechuic acid, or possibly some other similar acid in the group shown in Fig. 2. So far as I have been able to discover, this mechanism has not been elucidated. However, protocatechuic acid and various other 3,4-dihydroxyphenols have been isolated from hard cuticles many times (Mason, 1955a; Dennell, 1958d), and there seems no doubt that they are formed. There remains the question of whether or not they are derived from dopa.

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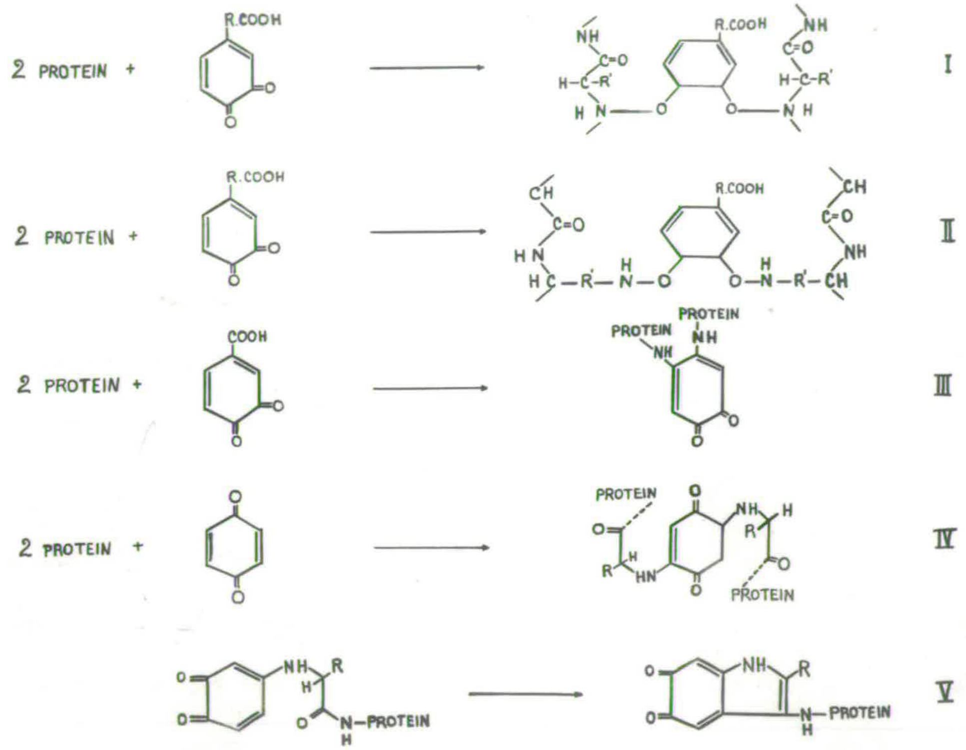

Figure 3. Concepts of the linkage of quinones to proteins.

I and II - linkage of imino and amino groups respectively (Pryor, 1940a).

III - linkage of amino terminal groups (Hackman, 1953).

IV - linkage by a para-quinone (Dennell, 1958d).

V - linkage of ortho-benzoquinone to one protein, followed by indole formation to form a coloured compound (Hackman and Todd, 1953).

The protocatechuic acid found in the cuticle is believed to be oxidised to the corresponding ortho-quinone by the tyrosinase in the epicuticle. Thence, the ortho-quinone is believed to diffuse inwards through the cuticle to react with proteins, cross-linking them to give a resistant, relatively insoluble mass. Spectroscopic evidence for this process has been obtained (Fox, 1953; Hackman, 1953c).

There is little agreement on the mechanism of this linking. Pryor (1940a) used comparison of the action of tanning in the ootheca with results in leather tanning by vegetable complex dihydroxyphenols to suggest that the quinone which tans the cockroach oothecal protein does so by linking to the imino groups of the protein backbone (Fig. 3, I) and to the amino groups of basic side-chains (Fig. 3, II). He confirmed the involvement of at least one of these reactions by blocking the amino and imino groups of untanned oothecal protein by formol, treatment which does not block carboxyl groups. No tanning on addition of extracts of right colleterial glands, or of ortho-benzoquinone, which he had shown to give closely similar results to the natural agent, took place. Pieces of left gland added as controls tanned normally.

In the light of Brunet and Kent's later work (1955), it is clear that the fact that formol-treated left gland did not react to the right gland secretion might have been due to the formol rendering the polyphenol oxidase of the treated left gland inactive, or /

or to leaching of the glucoside of protocatechuic acid. However, it still cannot be refuted that lack of response by the treated left gland to the artificial tanning agent, ortho-benzoquinone, would seem to substantiate that the blocking of the imino and amino groups prevented tanning.

Hackman (1953c) agreed that the linking of protein chains in cuticular tanning is by way of amino groups, and showed the disappearance of amino groups in the tanning process. Mason (1955a) emphasised the finding of Hackman (1953a, 1953b) that the available free amines in the water soluble protein, arthropodin, of the insect cuticle are N-terminal amino groups, which are plentiful in the arthropodin before tanning. However, Hackman (1953c) proposed the method of involvement of the tanning quinone shown in Fig. 3, III, in preference to Pryor's suggested methods (Fig. 3, I and II).

An alternative hypothesis involving the participation not of orthoquinones, but of para-quinones, was put forward by Dennell (1958d) to explain the results of his work on the chemistry of blowfly cuticle. This suggestion required that ortho-benzoquinone, formed by the tyrosinase-catalysed oxidation of catechol, in turn oxidised the para-dihydroxyphenol, hydroquinone to its quinone. Mason (1955a), in considering the possible reactions of quinones, showed that this reaction is possible in biological material. Dennell (1958d) then postulated that the para-quinone derived /

derived from the hydroquinone links amino groups of proteins as shown in Fig. 3, IV. He substantiated the hypothesis by showing that larval blowfly cuticles artificially tanned in hydroquinone, on hydrolysis, yielded an aminophenol indistinguishable from that recoverable from naturally tanned cuticles.

It is therefore conceivable that two types of sclerotisation are present in insects, one method using an ortho-dihydroxyphenol, the other a para-dihydroxyphenol. Thus far, however, the involvement of para-dihydroxyphenols in tanning does not seem to have been shown in any insects apart from the Diptera, although Brunet and Kent (1955) noted the presence of unknown phenols in small amounts in cockroach oothecal fluid. The occurrence of para-quinones in insects, however, seems to be fairly common, but hithertofore their function has been ascribed to the production of noxious materials used probably as defence mechanisms (Pavan, 1958; Roth and Stay, 1958).

It must not be overlooked that the chitin, as well as the protein, in the cuticle seems to be involved in sclerotisation. Details of how this occurs are not thus far known; it is even doubtful whether the form of bonding between chitin and protein is chemical or physical (Richards, 1958).

There are some variations on the theme discussed so far. Hackman and Todd (1953), cited by Mason (1955a), have suggested, for example, that after ortho-benzoquinone has been linked by one bond /

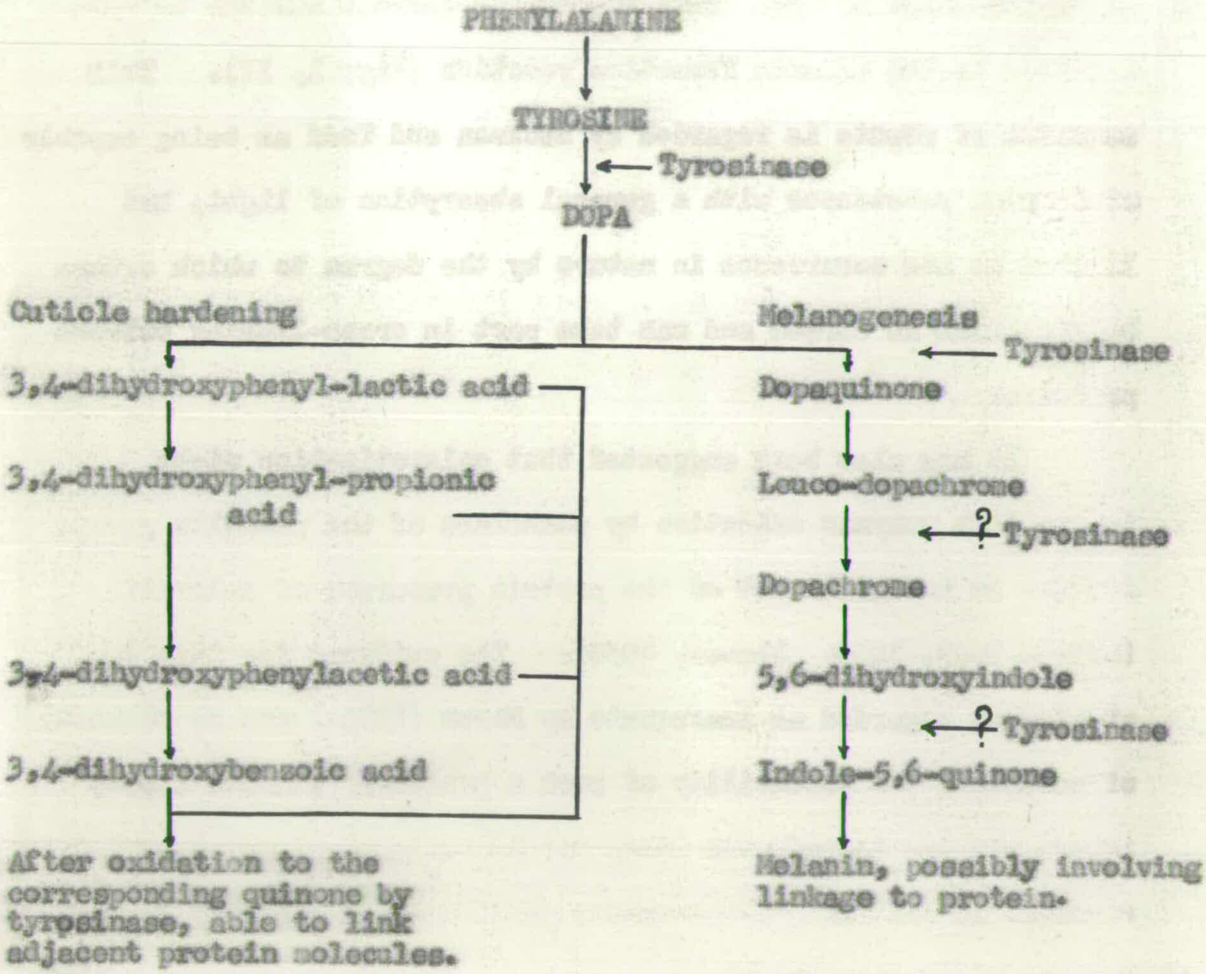

Figure 4. Diagram to illustrate the early reactions common to both melanin production and sclerotisation, the point of divergence between the two processes, and the probable subsequent reactions.

bond to a free amino group of a protein, it may be possible for it, instead of joining to another amino group, to undergo indole-formation (Fig. 3, V). This process is closely similar to that involved in the melanin formation reaction (Fig. 1, IV). This sequence of events is regarded by Hackman and Todd as being capable of forming substances with a general absorption of light, but limited in its occurrence in nature by the degree to which ortho-benzoquinone is formed and can take part in cross-linking between proteins.

It has also been suggested that sclerotization might involve the enzymic oxidation by phenolase of the phenolic groups forming an integral part of the protein precursor of sclerotin (Brown, 1949, 1950; Blower, 1950). The evidence for this is at present regarded as inadequate by Mason (1955a) who is cautious of accepting the possibility of such a process; Dennell (1956) is sympathetic towards the idea, but indicates that it cannot be regarded as proved. The concepts discussed in this section are summarised in Fig. 4.

DEFINING THE COMPONENTS OF INSECT CUTICLE RELATIVE TO THOSE INVESTIGATED IN NORMAL AND ALBINO LOCUSTS

In the literature on insect cuticle, many terms have been used to describe its various component layers. Hard cuticles have been described as "heavily chitinised", as if the polysaccharide chitin was responsible for the hardness and chemical stability of the cuticle. Dennell and Malek (1953), as well as Richards (1951), however, have done much to stabilise the usage of terms. A simple scheme based on their views has been outlined by Lower (1956). It is essentially Lower's view of cuticle terminology that has been adopted in this thesis. It is as follows.

The insect is regarded as being enclosed in an "integument". This is composed of a single layer of cells, which secretes distally a "cuticle". The layer of cells is bounded proximally by a "limiting" or "basement" membrane. The cellular layer is called either "epidermis" or "hypodermis". Although the epidermis signifies the origin and functions of this layer, Lower prefers the term hypodermis because it indicates the position of the cellular layer relative to the cuticle.

In the cuticle itself, two major sub-divisions are recognised. The outer of these two regions is known as the "epicuticle", and the inner as "procuticle", a term suggested for this purpose by Richards (1951). So far, however, the latter term has not found general acceptance.

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The epicuticle is very thin, of the order of less than three microns; it does not apparently contain chitin. However, Krishnan (1956) reported that chitin is present in the epicuticle of scorpions, and it may yet prove to be present in the epicuticle of some insects.

In comparison with the epicuticle, the procuticle is relatively very thick. It is accepted now as being capable of differentiating into three sub-layers in the fully developed cuticle. That one which is chemically unchanged after its initial deposition by the epidermis is the "endocuticle", a name derived originally from the fact that this region almost always lies innermost of the three sub-layers. When the cuticular components of a sub-layer are hardened, or "sclerotised", or "tanned", by quinonoid materials, and are impregnated by materials such as sterols, which seem to participate in the hardening process (Dennell and Malek, 1955a, 1955b), the changed layer is known as the "exocuticle". The term again arose originally from the occurrence of such a layer in the outer region of the procuticle. The exocuticle is commonly amber, brown or black in colour. It is also refractory to staining (Richards, 1958).

Intermediate between these two conditions is a procuticle which has been impregnated but not sclerotised. This stains with acid dyes, such as acid fuchsin (Lower, 1956). When such a layer is present, it is called the "mesocuticle".

All /

All three sub-layers of the procuticle are found in the cockroach, Periplaneta (Dennell and Malek, 1954). In Sarcophaga, on the other hand, the mesocuticle is seen only for a very brief period during the conversion of the final instar larva into the puparium, and is not extensively developed (Dennell, 1947).

When the sub-divisions of the epicuticle are considered, there is seen to be little agreement among authors. Much of this is due to the lack as yet of a sufficiently wide range of studies, and the doubt that prevails over the chemical composition of the substances which form the various sub-layers. Wigglesworth (1947, 1949) recognised four layers in the epicuticle of the blood-sucking Hemipteran, Rhodnius, as well as in that of the mealworm, Tenebrio. The layer next to the exocuticle was termed the "cuticulin layer", and was thought to be lipoprotein; the next layer, rich in polyphenols, was called the "polyphenol layer". Outside this was a "wax layer", which was in turn protected by a "cement layer". While the cuticulin and polyphenol layers were believed to be concerned closely with the hardening of the newly secreted cuticle to form meso- or exo-cuticle, a water-proofing function has been ascribed to the wax layer. The cement layer is probably protective, as the method employed for demonstrating its presence tacitly implies.

This method involves immersion of the cuticle in ammoniacal silver nitrate solution, when the polyphenol layer is regarded as being /

being able to reduce the solution to give metallic silver. If the polyphenols are covered by wax, then no reaction will be obtained unless the wax is removed by cold chloroform. However, if the wax in turn is protected by a cement layer, cold chloroform will not remove the wax. The cement can be removed only after boiling the cuticle in chloroform for an hour. Consequently, if a positive reaction is obtained to the silver solution after both cold and hot chloroform treatment, then it is assumed that no cement layer is present. If, on the other hand, only boiling chloroform is capable of exposing the underlying polyphenols, this is taken as implying the occurrence of a cement. The nature of this cement has not been clear, but the latest work on it by Beament (1955) suggests that it is composed of a shellac-like material.

Dennell and Malek (1953, 1955) do not agree with Wigglesworth's suggestions regarding the structure of the epicuticle, and do not admit the evidence for a distinct polyphenol layer as being adequate. In its place, they see a "paraffin layer".

Lower (1956) takes the view that it is not yet practicable to erect a rigid terminology for the epicuticle. However, he does suggest that distinctive layers found to be present in any particular species of insect should be named according to their chemical composition.

In the light of these considerations, the cuticles of both the /

Figure 5. Epicuticular pattern of wild locust to show how areas containing multiple-tipped plaques may be surrounded by untipped plaques. The sample has been removed on nitric acid, and the treatment is sufficiently gentle to allow setae to remain attached.

Figure 6. Epicuticular pattern of wild locust, after removal of epicuticle on nitric acid. The left half of the photograph shows a large area of melanin.

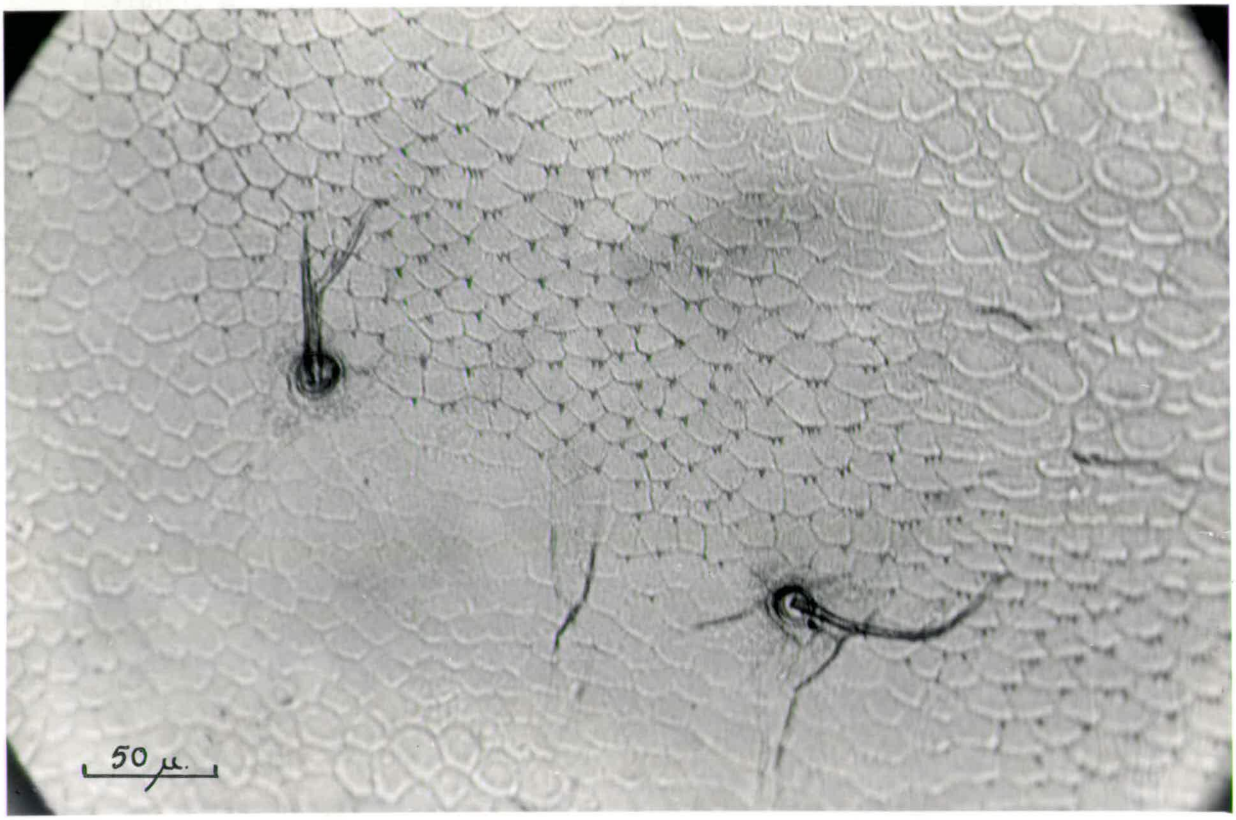


Figure 7. Epicuticular pattern of albino locust. Sample removed on nitric acid. Several types of plaque are visible, and no difference is apparent between this strain and the wild one.

the wild and albino strains of Schistocerca gregaria were examined. They showed that the albino cuticle was completely colourless. The yellow or brown pigments visible externally in the living insect were in fact present in the epidermis, the cuticle being transparent. In the wild strain, the only difference in the colouration of the cuticle lay in the presence of melanin patches in all instars, although the melanisation was much reduced in the adult. Macroscopically, these patches appeared black, and were not altered by removal of the epidermis. They were retained in exuviae, and therefore were not components of the endocuticle, which is dissolved by the moulting fluid during ecdysis.

The extreme outer surface of the cuticles of both strains displayed similar patterns at a micro-level (Figs. 5 to 7). On whole mounts of cuticle these patterns were seen most easily over the melanin patches of the wild strain, but, under very carefully adjusted illumination such that excess refraction was deliberately introduced, they could be seen to cover virtually the entire cuticle. A useful method of demonstrating the pattern in isolation from the procuticle consisted of heating the sample of integument in concentrated nitric acid very gently, until the procuticle only just dissolved. The acid was then cooled, and poured quickly into a large volume of cold water. The epicuticle remaining after this treatment was very hydrophobic, and floated on the water. There /

There it could be washed to remove all traces of the acid, and subsequently mounted by floating on to a slide. After drying, the epicuticle then showed its pattern distribution very clearly. During the process of removal the thin epicuticle expands its area by about twice, and therefore no estimations of size of the units of the micropattern were ever made on isolated samples, but always on the intact total cuticle. Comparison of the isolated with the _in situ_ epicuticles showed that the variation in micropattern about to be described was not an artifact due to the removal process. The isolated epicuticles provided better material for photographing the micropatterns.

The units of which the patterns were composed were called "plaques", such a term being descriptive without introducing any pre-conceived ideas as to their origin or structure. The only disadvantage of the term lay in that it was also used by Wigglesworth (1954) to describe much larger areas of the cuticle, in _Rhodnius_, but no parallelism between the two uses is intended here. The plaques of the micropattern on the locust epicuticle could be divided into two types: in one, each plaque was polygonal, with fairly sharply angled margins, and frequently with one or more pointed projections at the posterior; the other series had plaques which were approximately semi-circular in outline, and lacked the points found in the polygonal types (Figs. 5 and 7). Often, as on the cuticle from the posterior femur, areas composed entirely of /

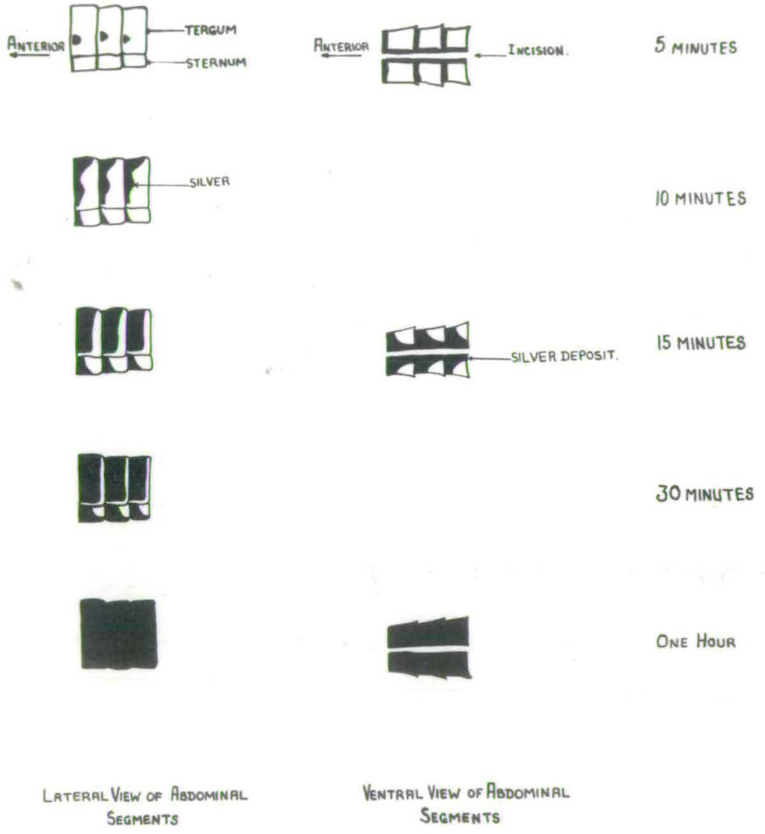


Figure 8. Diagram of the development of silver deposition in the integument of wild or albino nymphs of *Schistocerca gregaria*.

of polygonal plaques were seen to occur regularly along the length of the femur, the remainder of the surface being covered mainly by the semi-circular type. There was no correlation visible between the areas showing any particular type of plaque and the underlying structures, but there was a tendency, albeit not highly developed, for the melanin-containing spots of the wild abdominal sclerites to be overlaid by epicuticle containing a very high percentage of multiple-spined polygonal plaques.

To try to detect polyphenols in the cuticles, they were subjected to a series of tests involving the use of ammoniacal silver nitrate (Lison, 1936). When pieces of integument with epidermis attached were treated with the silver nitrate solution, silver was deposited in the epidermis. The silver first became visible on the cut surfaces, and at the front of each abdominal tergite (Fig. 8). At the same time silver was also laid down at the anterior border of the sternites. The silver deposited extended from these sites. On the tergites, the deposition extended along the anterior margin, and then spread backwards. On the venter, it spread backwards from the anterior margin, and laterally from the cut surfaces at the position of the mid-ventral incision used to open the insects.

After fifteen minutes, all of the tergal epidermis was silver-stained except for a posterior margin of about one millimetre. About two-thirds of the ventral area was stained in each segment.

In /

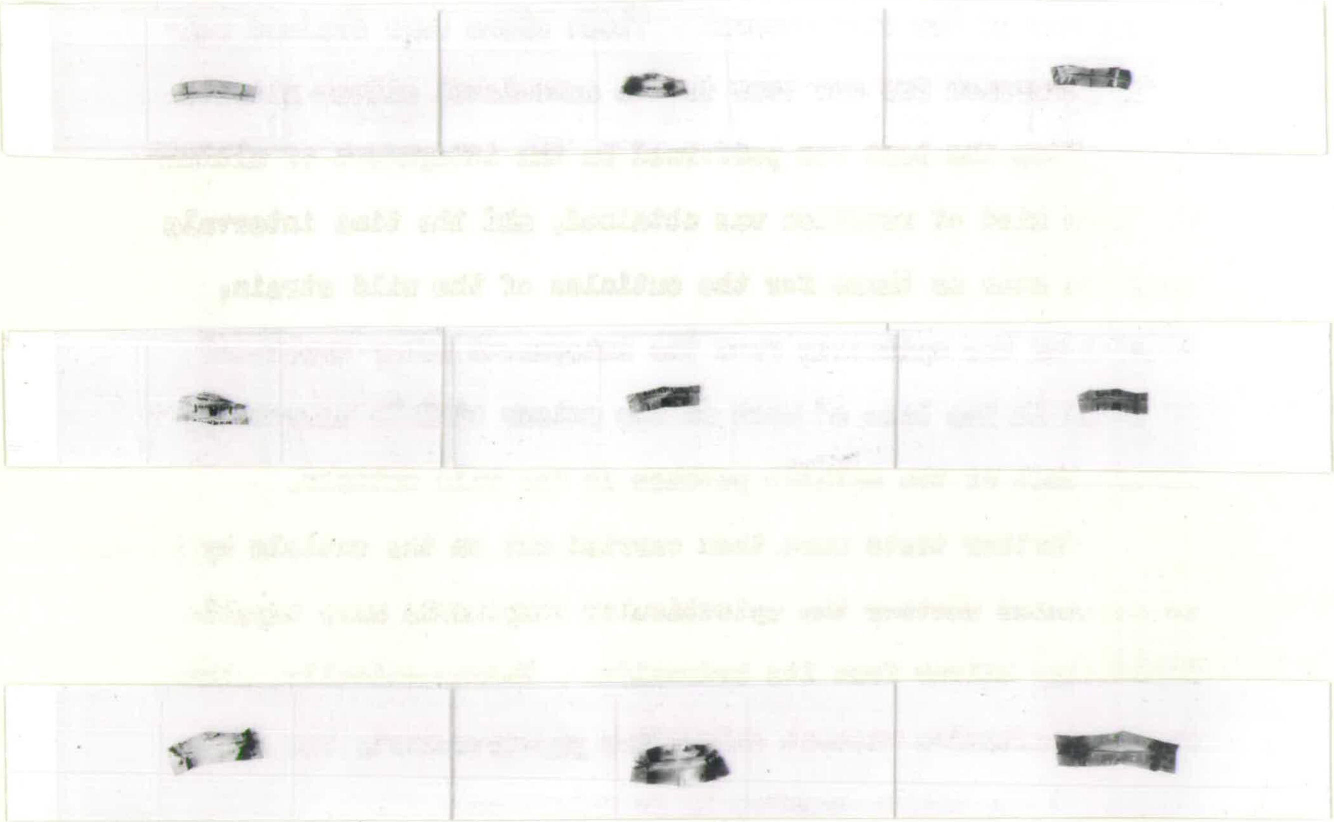


Figure 9. Synopsis of various treatments of wild and albino cuticles with ammoniacal silver nitrate. Top row - albino nymph; middle row - wild nymph; bottom row - wild adult. Left column - no pre-treatment; middle column - pre-treatment with cold chloroform for one hour; right column - pre-treatment with boiling chloroform for one hour.

In forty minutes, almost all of the ventral epidermis contained deposited silver, but the narrow posterior margin of the tergites remained unstained, as did the epidermis under the white lateral tergal bar of the wild strain. These areas were stained only after immersion for one hour in the ammoniacal silver nitrate.

When the test was performed on the integument of albinos, the same kind of reaction was obtained, and the time intervals were the same as those for the cuticles of the wild strain. Removal of the epidermis from the integument after treatment resulted in the loss of most of the colour visible macroscopically, except that of the melanin patches in the wild cuticle.

Further tests were then carried out on the cuticle by itself to determine whether the epicuticular components were capable of depositing silver from its hydroxide. Macroscopically, albino and wild cuticles without chloroform pre-treatment, but after immersion for thirty minutes in freshly prepared ammoniacal silver nitrate solution, showed a faint colouration. This colour was distinctly intensified after pre-treatment by cold chloroform for one hour, before transfer to the silver solution. The use of boiling chloroform resulted in a much stronger general colouration of the cuticles. The macroscopic effects of the various treatments are summarised in Fig. 9. There was a slight indication macroscopically of a lesser degree of colouration in albino material. The reasons for this were sought microscopically.

Examination showed that cuticles which had not been treated with chloroform after removal of the epidermis had no deposit of silver on their outer surface, except where there were abrasions. Here the polyphenols were exposed and as a consequence the silver solution was reduced. The procuticle deposited silver in a layer some twelve microns below the surface of the epicuticle, corresponding to the junction of the endocuticle and outer procuticle. This was in agreement with the results obtained in sections stained by Lower's technique, where the inner ends of the pore canals at the endocuticle/outer procuticle junction stained prominently, as shown in Fig. 27. The appearance of the plaques on the surface of the cuticle was not altered by the silver treatment on the whole cuticles. Some of the longer setae showed some areas at their bases, or near their tips, where silver had been deposited strongly. Whether the deposition near the tips of some of the stouter, but shorter, of these setae represented an effect due to their tips being highly permeable, as suggested by Richards (1958), is an interesting conjecture.

The cuticles of both the normal and albino strains reacted similarly to the silver test, except that the abrasion marks in the albino stained less strongly, and lacked the continuity along their length as in the cuticle of the wild strain.

When the cuticles were treated with cold chloroform for periods from one to several hours, no deposition of silver in the /

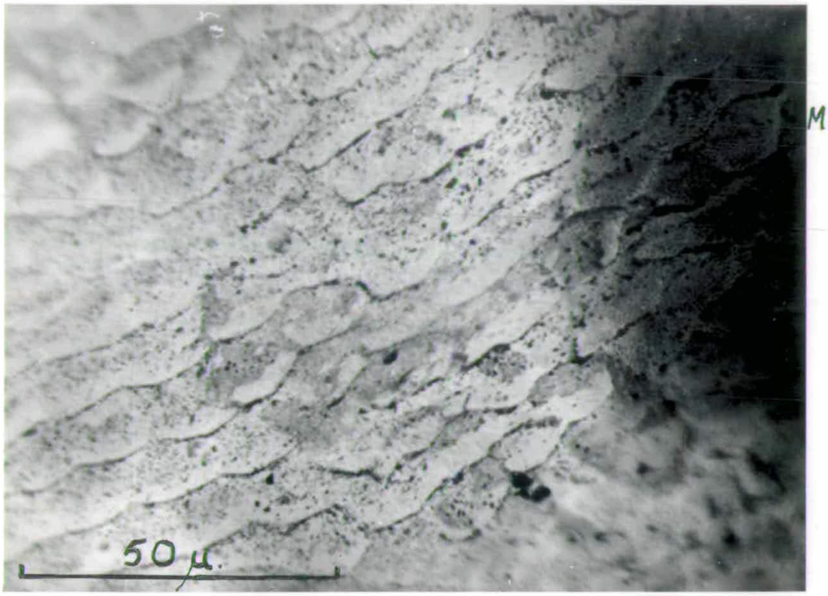
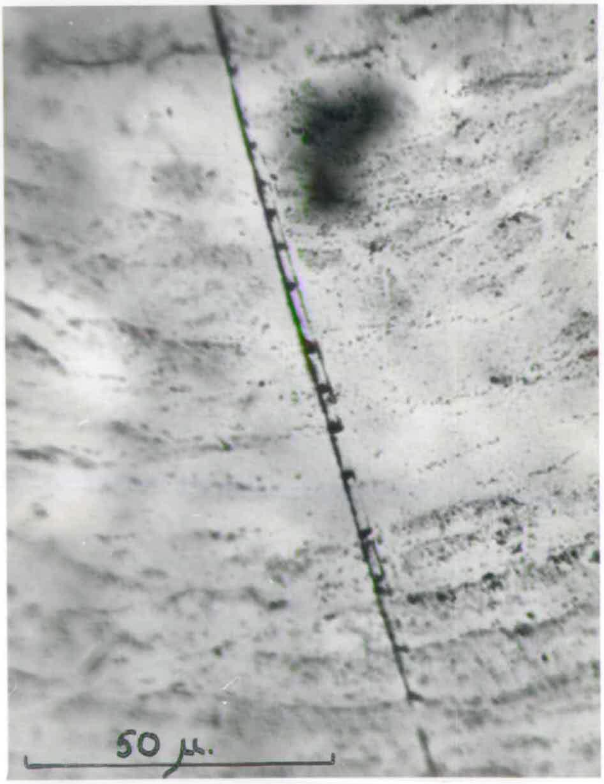


Figure 10. Surface of cuticle of wild locust, after treatment with boiling chloroform for one hour, followed by ammoniacal silver nitrate for thirty minutes. The reaction is weak, but decisive. Part of a melanin patch (m.p.) is included.

Figure 11. Subject and treatment as in Fig. 10. An abrasion mark, where exposed polyphenols have caused the deposition of silver is shown. The regularly occurring spots along the scratch represent the openings of the ducts of dermal glands.



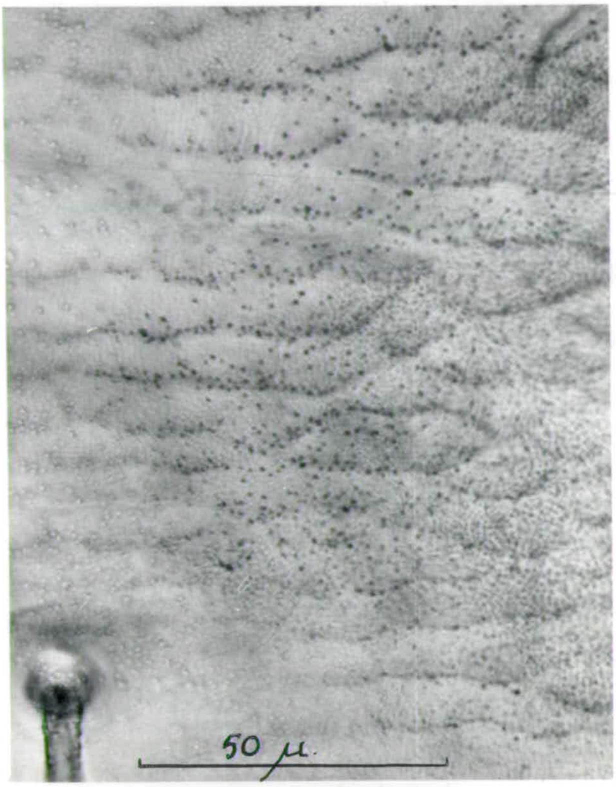
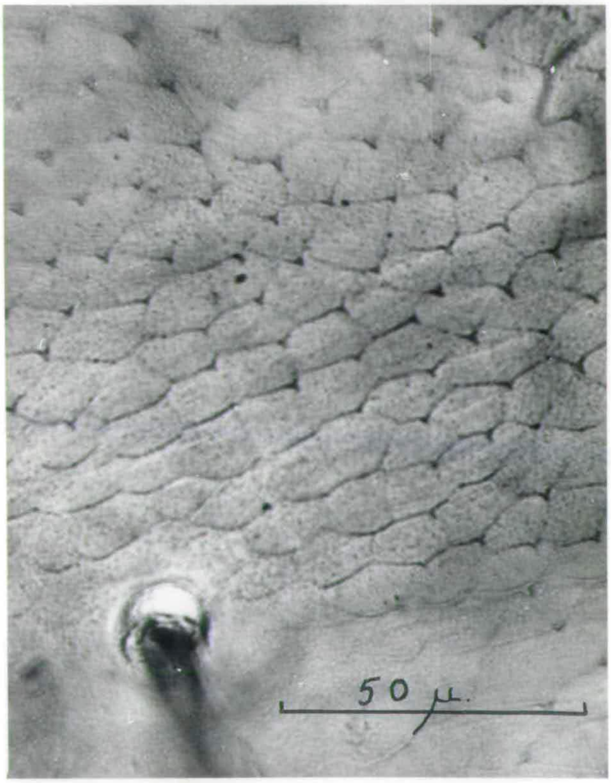


Figure 12. Surface of cuticle of albino locust, treated with boiling chloroform for one hour, followed by immersion in ammoniacal silver nitrate for thirty minutes. Note relative paucity of reaction.

Figure 13. Subject and treatment as in Fig. 12. This area of the cuticle shows virtually no reaction in the epicuticle.



the albino took place on the surface of the epicuticle, but in the wild strain there was a slight argentaffin reaction on the epicuticle. Similarly, after treatment for the same times with boiling chloroform under reflux, a weak response over the general cuticle surface was obtained, in the wild strain (Figs. 10 and 11). A much lesser response at epicuticular level was obtained in the albino (Figs. 12 and 13). This suggested the presence of small quantities of polyphenols. These reactions in the locusts were very much less intense, however, than was found in pieces of cockroach cuticle treated similarly.

The deposition in the endocuticle/outer procuticle junction was not removed, or visibly altered, by the hot chloroform treatment. Also, silver was detectable very slightly below the epicuticular surface silver, and estimations on the vertical focussing scale of the microscope showed the staining to lie some two microns below the surface silver. The restriction of the response in the "sub-epicuticular" layer to small spots, of the order of one micron diameter, and in such a position, suggested that the silver was being deposited in the extreme outer ends of the pore canals, and possibly in the extreme inner layer of the epicuticle. Since the pore canals could be demonstrated to stain with ammoniacal silver nitrate applied according to Lison's technique (p.48.) to sections of the cuticle, and since the epicuticle in these sections took up the stain slightly, it seems that /

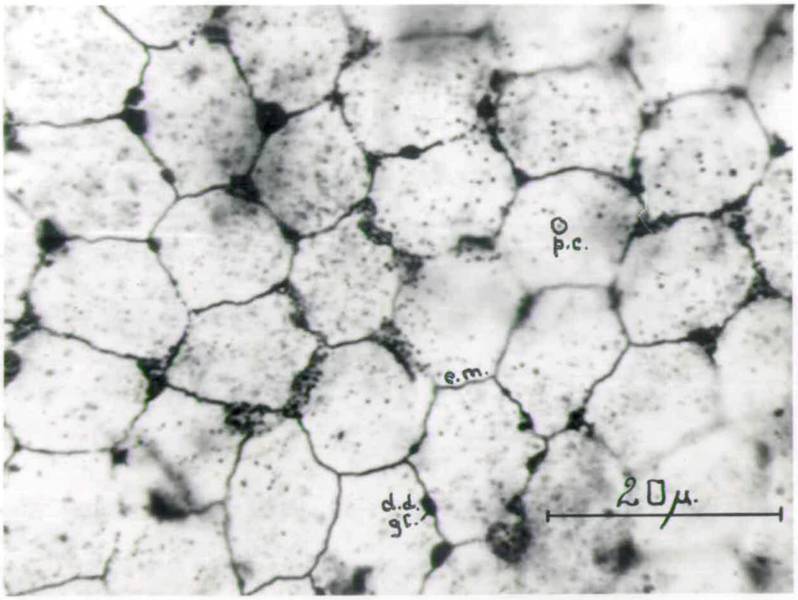


Figure 14. The inner surface of the albino locust cuticle after treatment with ammoniacal silver nitrate, followed by stripping of the epidermis.

d.d.gl. - apertures of ducts of dermal glands; p.c. - stained pore canals; c.m. - position in life of interfaces between cells.

that this hypothesis is not untenable. On the inner surface of the cuticle, silver was deposited in a pattern representing the position of the interfaces between the epidermal cells, in the ducts of the dermal glands, and in small spots which may represent pore canals (Fig. 14).

Varying the time of exposure to the silver solution showed that essentially similar results were obtained irrespective of whether the duration was ten or thirty minutes. The activity of the solution used in the tests was checked before and after use by the addition of a minute crystal of catechol. Copious deposition of silver occurred in the solution, confirming its activity.

The results, therefore, draw one into concluding that reducing substances are present in the cuticles of both the wild and albino locust. The pore canals in the outer procuticle in both types of locust also reduced the silver solution. When the cuticle had not been pre-treated with chloroform, only the inner ends of the pore canals stained, while the epicuticle stained very weakly. Treatment with cold chloroform slightly increased the power of the epicuticle to reduce the solution. Hot chloroform, on the other hand, removed any protective cement layer present, so that the outer ends of the pore canals and the epicuticle itself, in exposing more reducing substances, caused the deposition of considerably more silver.

The /

The albino seemed to possess less reducing material in both its epicuticle and its pore canals. This observation confirms the findings from applying Lison's argentaffin test to sections of cuticle, as described on page 48.

HISTOLOGICAL AND HISTOCHEMICAL STUDIES ON THE CUTICLES OF NORMAL AND ALBINO LOCUSTS

Since the mature cuticle of the insect may be regarded as an end-product of a process catalysed by tyrosinase, considerable importance may be attached to discovering whether the lack of melanin in the albino locusts reflected any lessening in the hardening of the cuticle. Accordingly, the reactions of the cuticles of the wild and albino locust strains to various tests were investigated. It so happened that this revealed in the cuticles examined the presence of structures hitherto seldom encountered in insect cuticle, and not, to my knowledge, reported previously in Orthopteran cuticle. These particular structures were common to both the wild and albino insects. However, significant differences were also found between the staining reactions of the cuticles of the two strains of locusts.

In the tests, samples of cuticle were taken from the abdomen. In some cases, thoracic tergites provided the cuticle. The abdominal cuticle was chosen because it is relatively uncomplicated by apodemes. It has a more uniform general structure, an important consideration when comparisons are to be made between two strains of insect. Abdominal cuticle can also be removed as a complete ring, so sections of this include both the sclerites and the arthrodial membranes. One can therefore test simultaneously cuticle in varying states and degrees of hardening under identical conditions.

When the cuticle was removed, it was washed and carefully cleaned to get rid of any attached tracheae and fat body. It was then placed in Duboscq-Brasil fixative solution kept at 60'C. for one hour. The fixative was then allowed to cool to room temperature, but the material was left in it for a further twenty-four hours. The fixed material was then washed several times in 70% ethyl alcohol over a period of 48 hours until most of the yellow residue due to the picric acid in the fixative had been washed out. Next, the material was brought up through the alcohols, usually three hours in each. These were followed by xylol for three hours, or overnight, when the material was passed through three changes of 56'C. melting point paraffin wax. When cooled and trimmed, the block was sectioned on either a Cambridge Rocking Microtome, or a Spencer Rotary Microtome, usually at eight microns section thickness. This thickness was chosen after some experiment as being that which gave good clarity of detail in the sections without leading to excessive shattering of the cuticle due to the knife's difficulty in cutting the hard material. In this work, it was never found possible consistently to get perfect sections, due to shattering of the cuticle, and parts of it then gouging the remainder of the embedded material. Allied to this was the problem of delamination of the cuticle by separation of its component layers. However, these problems were not particularly significant from the point of view of this histochemical study of

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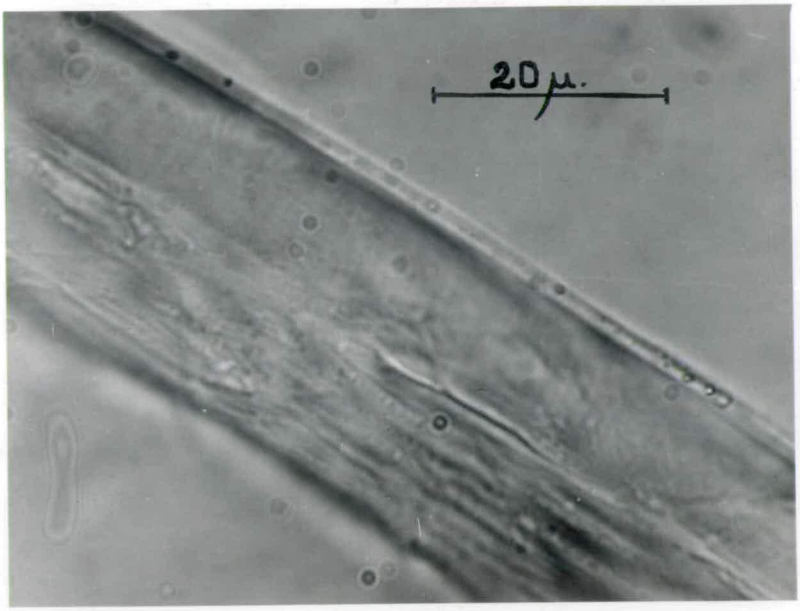

Figure 15. Unstained adult locust cuticle to show the extent of the epicuticle.

the cuticle as their effects were relatively localised in any given section.

Subsequent de-waxing and hydration of the sections followed normal routine methods.

Characteristics of unstained sections of the cuticles

In the wild strain, the general appearance of unstained sections of its cuticle was as to be expected in a hard-bodied insect. The usual division into epicuticle, an outer, non-laminated portion of procuticle, and an inner, laminated region of procuticle was seen clearly, even in the absence of staining. The epicuticle, in this locust, however, is very often difficult to distinguish conclusively in the mature cuticle. It is of two to three microns thickness, and is shown in Fig. 15. Only too often it separated from the underlying procuticle, when it became visible as a highly refractile bar lying close to the surface of the main mass of the section. This feature indicated the presence of the epicuticle in these sections.

In the wild strain, the unstained outer procuticle, which was found to be composed of mesocuticle and exocuticle, was homogeneous. It averaged about fifteen microns in thickness. However, a prominent lamellar structure was present in the endocuticle, which was twenty to twenty-five microns thick.

In these sections, there was a regular fragmentation of the exocuticle and mesocuticle, but not of the endocuticle, in most /

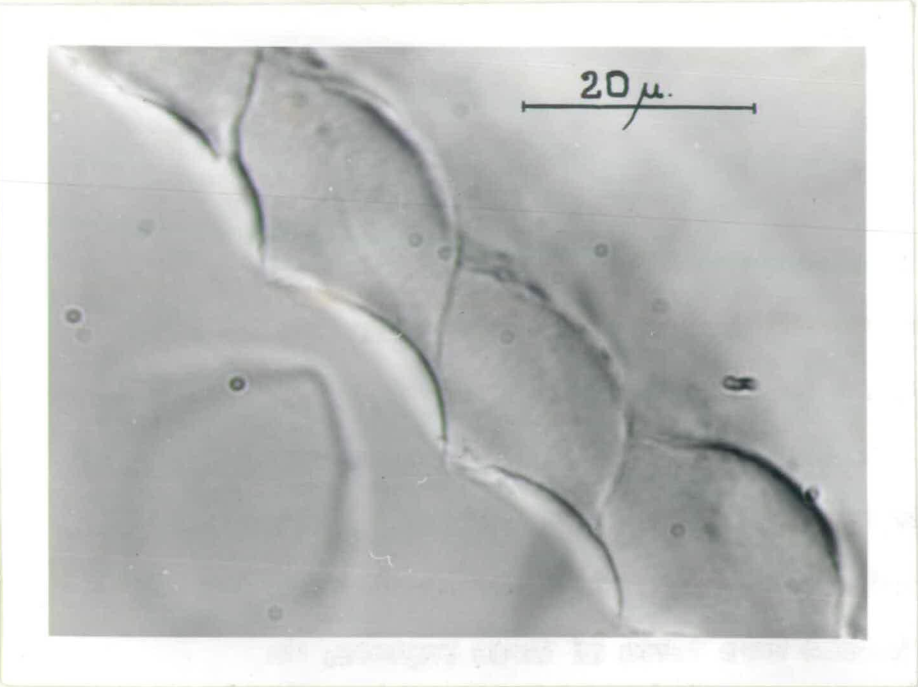

Figure 16. Unstained wild cuticle, where the outer layers have become free and fallen over. The formation of blocks is seen, their shape not being consistent with effects due to simple fracturing.

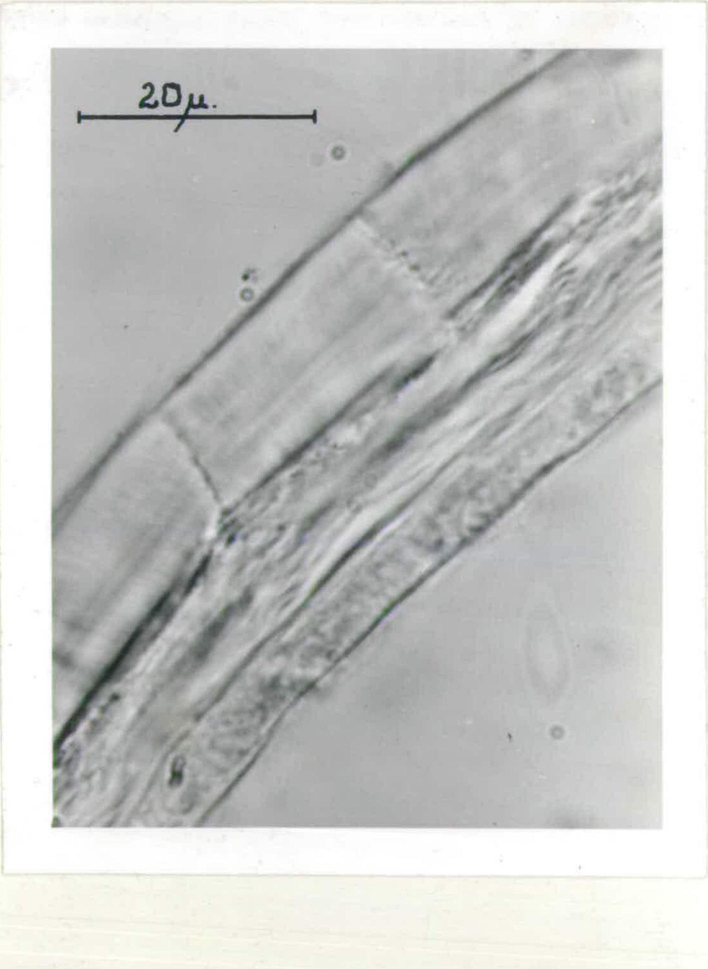

Figure 17. Unstained wild adult cuticle showing incipient fracture lines in the outer procuticle.

most cases (Figs. 16 and 17). This fragmentation was first thought to be some artifact due to sectioning this brittle cuticle. However, a more thorough examination revealed that while the actual breakage of the exocuticle and mesocuticle was due to shearing from the cutting process in these layers, there was a structural arrangement which produced lines of weakness in certain areas of the cuticle. These sites were predominantly in the mid-dorsal and mid-ventral lines, or just above the level of the white tergal bar of the tergites. Most of the photographs used to illustrate this thesis were taken of these regions, to illustrate simultaneously the staining reactions of the cuticle layers and of these areas of weakness in the cuticle. These lines of mechanical weakness have been named "fracture lines" in the text from now on. It will be seen later in this section that they could be distinguished even in intact cuticle by their staining reactions.

In this text, the description of the "outer, non-laminated procuticle" embraces the exocuticle and mesocuticle, the "inner, laminated procuticle" the endocuticle. The term "arthrodial membrane" is used in the anatomists' sense as a junctional area between the main plates of the body, either between segments, or between the sternite and tergite of any one segment. The term "pleural region" has been used to describe the more flexible cuticle between the sternite and tergite of the same segment.

Sections of the pleural region of the wild strain locust were mainly composed of endocuticle, displaying typical lamellar structure. However, the edge of the pleural cuticle was translucent and not lamellated. The surface of this pleural region was intricately folded. It was therefore difficult to measure the thickness of the outer translucent layer of the pleural cuticle accurately, but it seemed to be within the range of three to five microns. This layer did not show any tendency to fragmentation.

Sections of unstained cuticle derived from adult albinos possessed the same basic structure as those of the wild strain. The relative thicknesses of the outer, non-laminated and the inner, laminated layers, however, were the same. But there was a less marked tendency for the albino cuticles to fragment during sectioning. The structure of the pleural cuticle resembled that of the wild strain.

Reactions of the cuticles to various stains

Acid Fuchsin: One of the stains which has been employed by various workers to determine the nature of the components of cuticle is acid fuchsin, one of the dyes present in Mallory's Triple stain. It is usually accepted, for example by Dennell and Malek (1956), that this dye reacts with protein impregnated with an ortho-quinone. This situation corresponds to that found in Lower's (1956) mesocuticle. In the present work, the acid fuchsin was made up as a ½% solution. The cuticle to be stained was exposed to the dye for two minutes.

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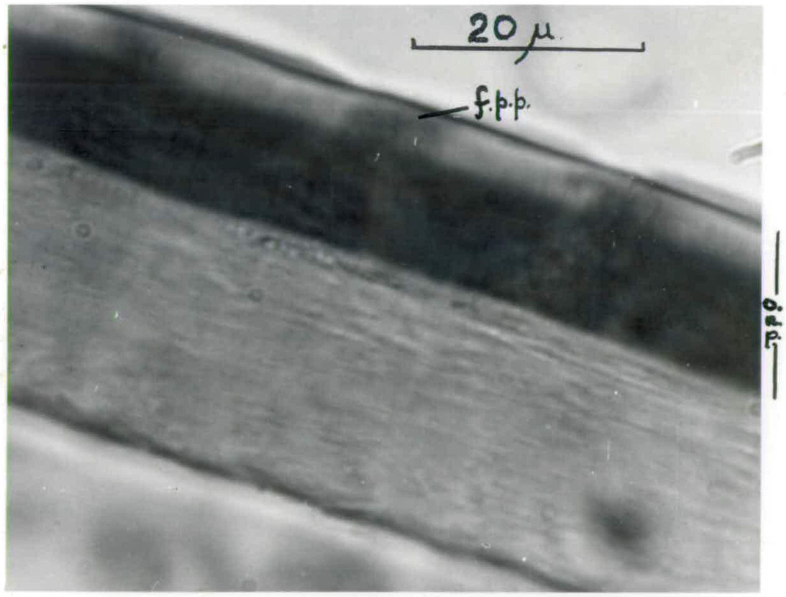


Figure 18. Wild adult cuticle stained with Acid Fuchsin. o.n.p. - outer nonlaminated procuticle; f.p.p. fuchsin positive regions of the exocuticle.

The greater proportion of the pleural cuticle of the albino was completely refractory. However, an extremely thin outer layer, about one to two microns thick, was strongly fuchsinophil. Most of the outer procuticle of the sclerite regions was deeply stained, except for some areas bordering the epicuticle. In contrast to the results in the wild strain, the outer layer of the epicuticle was easily demonstrated to be fuchsinophil. The endocuticle was at most very weakly stained. The epidermis remained refractory.

The cuticle of the wild strain reacted similarly to the dye, except that the outer region of the procuticle of the sclerites could be further divided into an inner strongly fuchsin-positive part and an outer fuchsinophobe one. The latter had fuchsinophil triangles running vertically through it, dividing the unstained layer into many regular blocks (Fig. 18). These triangles and blocks were present on tergite and sclerite alike. The term "triangle" in this connotation refers to those regions in the exocuticular layer which retained staining properties resembling those of the mesocuticle, as well as, to some extent, those of the endocuticle.

The epicuticle remained refractory throughout most of its thickness, but an extremely thin outer layer, approaching the limits of resolution of the oil immersion lens, was seen to be fuchsin positive. The epidermis did not accept the stain. In those areas of this cuticle which showed fragmentation, it was conspicuous that the fuchsinophil triangles in the exocuticle were always involved in the line of fracture.

These results began to suggest that an exocuticle and a mesocuticle were present in the wild strain S. gregaria, in the homogeneous outer procuticle. Further, it was suspected that exocuticle development in the albino lagged behind that of the wild strain in extent.

Aniline Blue / Orange G: Aniline Blue distinguishes between untanned and tanned cuticle since the stain reacts only with basophil protein (Lower, 1957b). This stain may also be used in conjunction with Orange G. When examined after two minutes' staining, found to be the optimum when cuticle from the wild strain was treated with these dyes, the outer procuticle took up the Orange G to give a strong yellow colour. The Aniline Blue was taken up by the fracture-sensitive triangles previously described. The endocuticle stained blue. The epidermis and other soft tissues were strongly blue. In the pleuron, the fuchsin-positive thin outer layer stained yellow. The endocuticle of this region also took up the Aniline Blue strongly,

In sections of albino cuticle, the outer procuticle stained yellow, as it did in that of the wild strain. The endocuticle, however, showed a weak affinity for Aniline Blue. In sections of the pleuron, the endocuticle, unlike that of the sclerite, showed a strong affinity for Aniline Blue. However, the outer layer, which stained strongly yellow in the wild strain, did not do so in the albino, but was indistinguishable from the blue of the pleural endocuticle. Only the pleural spines stained very slightly yellow.

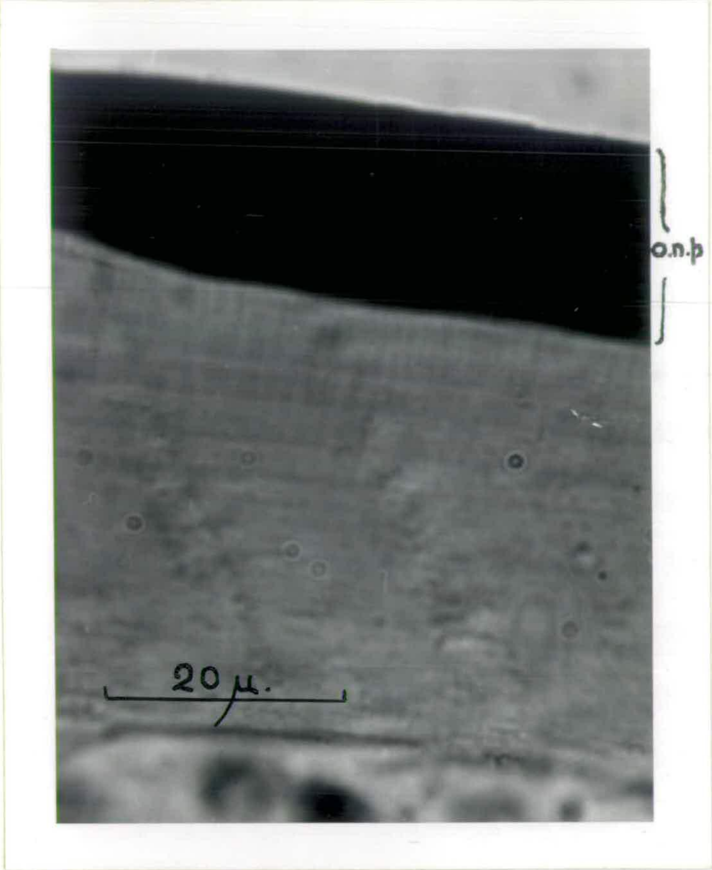
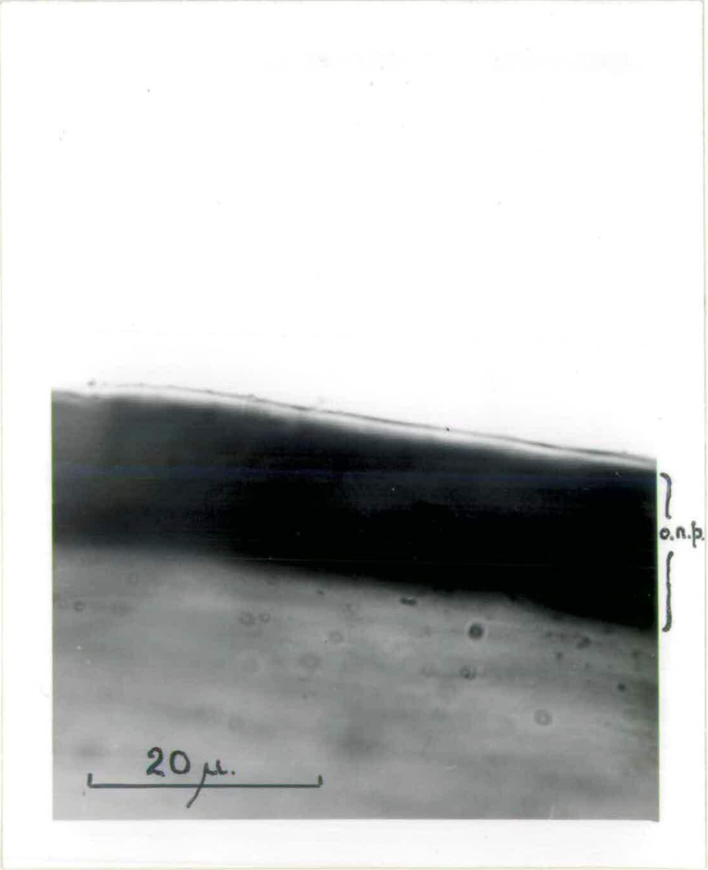


Figure 19. Wild adult cuticl
stained in Heidenhain's Iron
Haematoxylin.

o.n.p. - outer nonlaminated procuticle.

Figure 20. Albino adult cuticle, stained in Heidenhain's Iron Haematoxylin.

o.n.p. - outer non-laminated procuticle.



Heidenhain's Iron Haematoxylin: Dennell and Malek (1954a), using cuticle fixed in alcoholic Bouin, showed that Heidenhain's Iron Haematoxylin stained both the hardened but uncoloured region of the exocuticle, and the epicuticle.

The stain used here was prepared as recommended by Gatenby and Beams (1950). Since progressive, unlike regressive, staining is very unpredictable in its results with Heidenhain's Iron Haematoxylin, the latter method was used, section thickness was kept below ten microns, and xylol was used for clearing (Gatenby and Beams, 1950). After mordanting in 3% iron alum for thirty minutes, the sections of cuticle were stained in the haematoxylin for another thirty minutes. Differentiation was carried out in 1½% iron alum.

In the wild cuticle, the outer, non-lamellar cuticle stained strongly (Fig. 19). The endocuticle showed no reaction. In comparison, the albino cuticle was less easily stained. In that the method involved differentiation, this result has to be accepted with reservation. The outer procuticle showed a strong affinity for the stain, as did the outer region of the epicuticle (Fig. 20). Only the extreme outer surface, and spines, of the pleural cuticle from both wild and albino strains stained.

Eosine: This very common cytoplasmic counterstain seems to have had no part to play in previous investigations of cuticular staining reactions. For that reason, and because it is normally used to stain the untanned proteins of cells, it was tested on several samples /

samples of both strains of locust cuticle. Alcoholic solutions were used, and sections were stained for thirty seconds.

Surprisingly, a very distinct difference between the reactions of the two cuticles was found. In wild adult material, the non-lamellar outer layer of the cuticle had affinities for the stain at different levels; the inner half stained very strongly, but the outer basically did not. Further, the latter region, although predominantly unstained, showed stained triangles running from the outer surface to the junction of the outer and inner layers of the outer procuticle, and these triangles could normally be seen to have their bases on the junctional interface between the outer and inner procuticles. Significantly, however, where exocuticular fracturing occurred, the breaks were always precisely down the middle of the eosine-positive triangles in the outer cuticle. The boundary between the inner stained and the outer unstained layers of the outer-non-laminated cuticle was sharply defined, whereas that between eosine-negative and eosine-positive regions of the outer layer was less sharp. The epicuticle showed inner stained and outer unstained layers.

The reactions were the same in dorsal or ventral sclerite. The endocuticle of both sclerite and pleural membrane was stained about half as deeply as the inner layer of the non-laminated procuticle. The outer region of the pleural membrane stained relatively strongly, suggesting its similarity in eosine affinity

to /

There seems to be no record of previous work using eosine on cuticle. This may be due to the stain's solubility giving different results from the same material at different times. However, when used on a strictly controlled comparative basis, it would seem to have some use in demonstrating variations between cuticles. While the precise interpretation of the chemical basis underlying the action of the stain in the cuticle is not known, the results from observations on wild cuticle suggest that eosine has a greater affinity for impregnated cuticles than for untanned, non-impregnated protein. On this deduction, the data obtained from eosine staining of albino material can be provisionally interpreted to suggest that there is less exocuticle development, and correspondingly more development of mesocuticle, in the albino.

Lower's iodine silver stain: Lower (1957a) published a technique for rendering permanent the results of iodine staining of insect cuticles. In this paper, he stated that the binding of iodine into the cuticle was most strongly developed where phenolic groups were present. But he emphasised that plant and vertebrate material known also to be rich in such groups failed to respond; and that the precise nature of the reaction with insect cuticle was not known. On the basis of these observations and his several years of experience of the new technique, he suggested that it was not the phenolic groups themselves which bound the iodine, but that a particular combination of these phenols with other cuticular components did so.

The results of his own work he summarised by stating that only a "limited number of materials respond. Only exceptionally are the endocuticle and the cuticulin layer of the epicuticle stained. Depending on their composition, the contents of the pore canals may or may not stain. The paraffin layer of the epicuticle appears always to be strongly iodophil". The reactions given by this stain were described by Lower. Iodophil regions were stained deep purple to black. The remainder of the cuticle was a pale pink, or colourless. Various soft tissues, especially muscle, may also be stained.

On considering these observations, it seemed that differences in the phenolic composition of albino and wild cuticles of locusts might be demonstrable by this technique, although the meaning of such differences would not be clear until the chemistry of the staining method was elucidated. Lower's comment, "... the technique has enabled me to define regions in the cuticle which other techniques tried have either failed to do or did so less effectively" encouraged the hope that use of the new method might prove rewarding. Unfortunately, he did not specify to which regions he was referring in the remark quoted, nor were any references given in either this paper (Lower, 1957a) or in another where he discusses the results obtained from the technique (Lower, 1957b).

The method presented for rendering permanent the iodophilia of sections of insect cuticle involved the replacement by metallic silver /

to the inner layer of the outer procuticle. The epidermis was stained a pink typical of eosine.

Comparison of these results with those from albino material showed that there was a wide difference in eosine affinity between the two strains of cuticle. In the albinos, the sclerite endocuticle stained the same pink as in the wild, but the outer cuticle differed significantly. The outer procuticle showed no differentiation into two sub-layers, and was more intensely coloured than the endocuticle. The entire outer procuticle, in fact, stained like the mesocuticle of the wild strain. In a few small areas a slight difference in intensity was found, but this was exceptional. It was difficult to distinguish the epicuticle in these sections, but it was believed that this was due to its inner layer staining like the outer procuticle. The outer epicuticle was refractory. Confirmation of this was obtained in that pieces of epicuticle which had separated from the main mass exhibited a pink colour, convertible to purple in blue light. The extremely refractile nature of the fragments, however, made it impossible to see whether they were totally pink, but focussing through their depth showed that the strong pink colour was restricted to the interference lines on one side only. This would suggest differentiation into stained and unstained layers.

The pleural endocuticle was weakly stained, but the outer margin accepted stain more strongly, as in the wild locust.

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silver of iodine bound from solutions of the latter element. When the halogen is replaced by a noble metal, most uncertainty about the fading of the image can be eliminated.

The various reactions of Lower's technique required, first, the conversion by silver nitrate solution of the iodine in the cuticle into insoluble silver iodide. This halide was then reduced by a fine grain photographic developer, I.D. 11, made up according to the standard formula distributed by Messrs. Ilford, Ltd. The metallic silver so obtained was intensified by gold chloride toning, while, subsequently, any halides still present were removed by sodium thiosulphate solution. After this, any "unwanted coloration due to the reducing agent" (Lower, 1957a) was removed by bleaching in slightly acidified potassium metabisulphite solution. This bleach is commonly used in photographic practice to keep silver images free of extraneous precipitates, and it is presumed that this function is that intended by Lower.

Those solutions in the iodine-silver process which required particular care in their preparation were made up as follows. The iodine stain was prepared by grinding together one gram of elemental iodine with two grams of potassium iodide. Small portions of distilled water were gradually stirred into this until all the solids had dissolved. The volume was then made up to 100 ml. with distilled water. The I.D. 11 developer was prepared according to the published formula. The usual precautions with regard to preventing oxidation and precipitation of metol were taken.

The /

The acidified potassium metabisulphite solution required to be prepared immediately before use. One gram of potassium metabisulphite was dissolved in 50 ml. of distilled water, 1 ml. of concentrated hydrochloric acid was added, and the total volume made up to 100 ml. with distilled water.

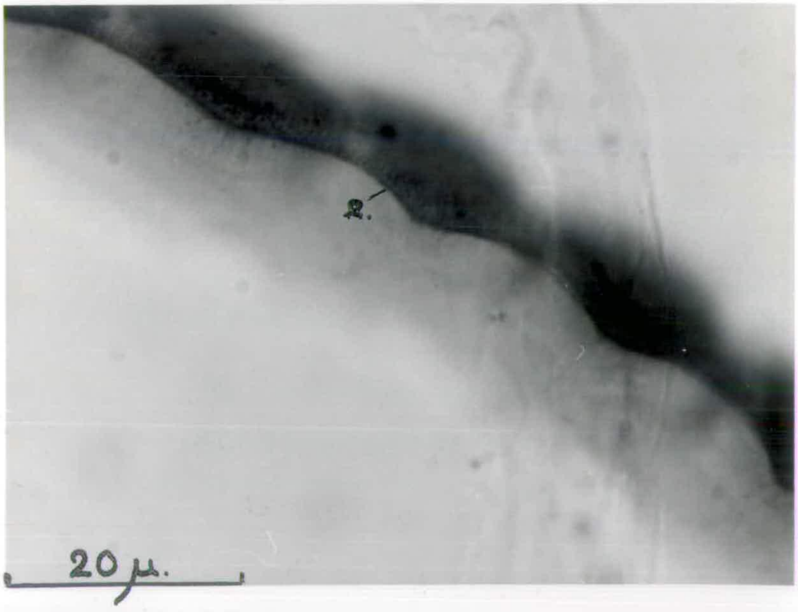
Working with this combination of solutions required scrupulous cleanliness, and very careful washing between the various solutions to avoid any spurious precipitates.

The procedure used on the deparaffined and hydrated sections of cuticle consisted of first staining them in the iodine solution for ten minutes. After rinsing thoroughly in distilled water to remove any free iodine which might react with the subsequent solution to form a non-specific precipitate, the sections were passed into a 1% solution of silver nitrate in distilled water for ten minutes, and then washed in distilled water. The silver iodide present in the cuticle was reduced to elemental silver by developing for ten minutes at 18°C. in I. D. 11 developer. After washing the sections again in distilled water, they were intensified in a 0.1% solution of gold chloride for fifteen minutes. Further rinses in distilled water were followed by fixation, in the photographic sense, in a 5% solution of sodium thiosulphate in distilled water, for two minutes. Lower pointed out that this time must not be exceeded, as the thiosulphate tends to detach the sections from the slides. The sections /

Figure 21.

Wild adult cuticle stained by Lower's method. In Fig. 21, the stain can be seen to be attaching to the outer region of the non-laminated procuticle (o.n.p.). Pore canals (p.c.) are visible. Block formation is pronounced. Fig. 22 demonstrates that the epicuticle (e.) is stained, and also shows that the block formation seen in Fig. 21 is not due to fracturing, but to specific variation in the staining of the cuticle. The photographs differ only in the plane focussed.

Figure 22.



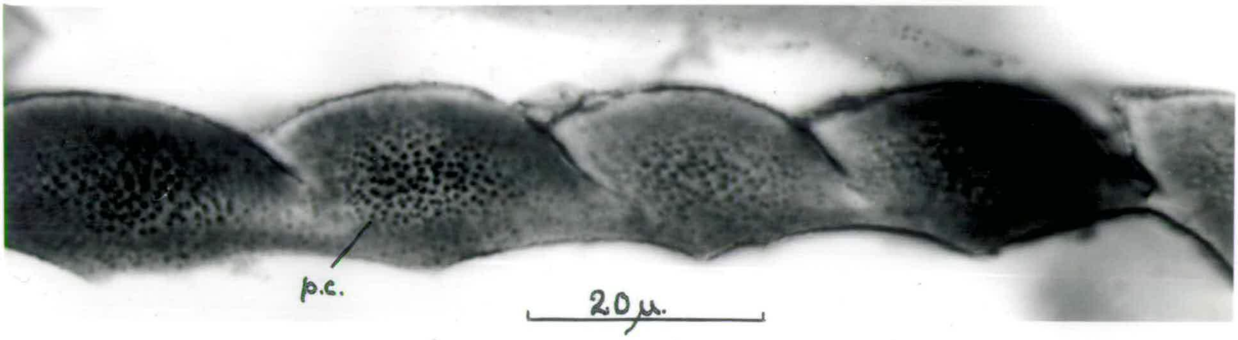


Figure 23. Wild adult cuticle stained by Lower's method. In this case, the exocuticle and epicuticle have separated from the remainder of the cuticle in sectioning, and have fallen over. Consequently, the general reaction in the cuticle and the very strong reaction in the pore canals can be differentiated more conclusively than in intact sections. The "block formation" in this area of cuticle is also demonstrated.

p.c. - pore canals.

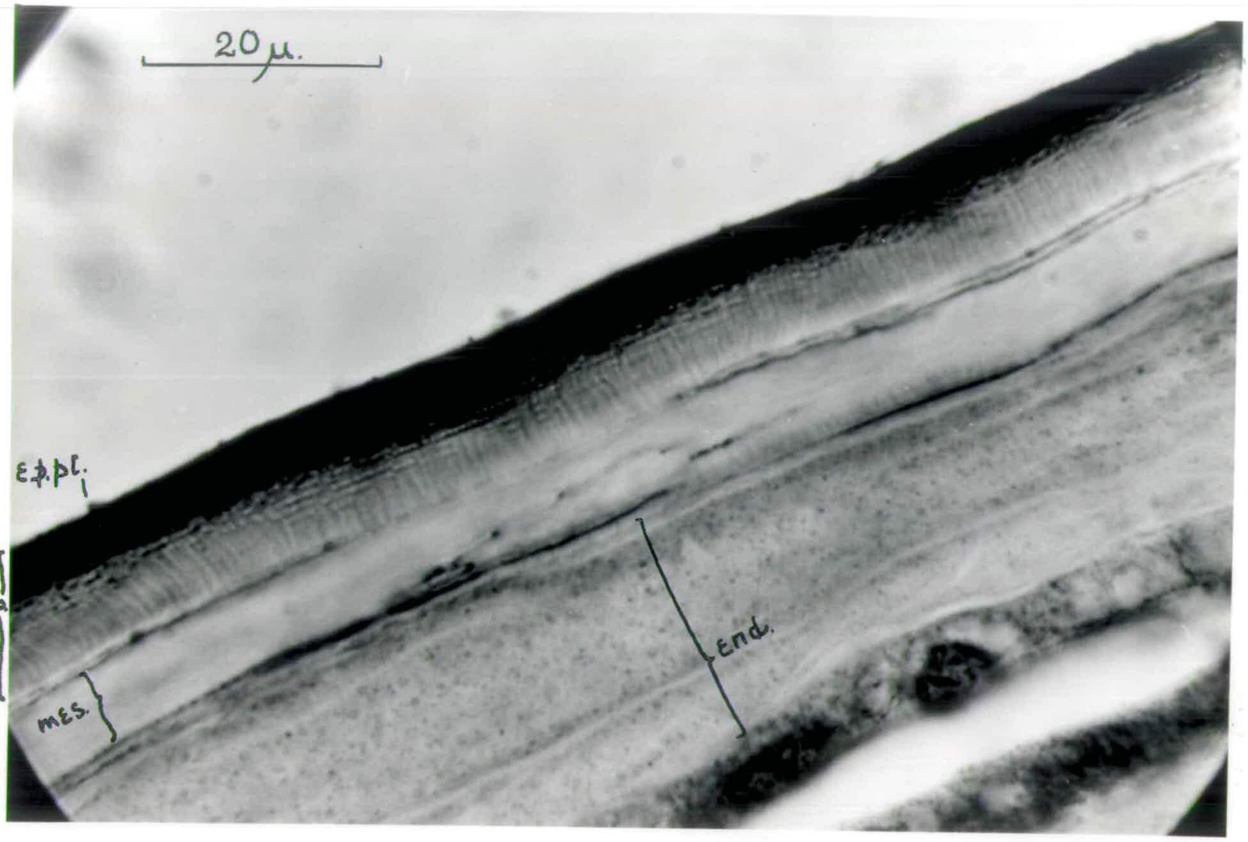


Figure 24. Wild adult cuticle stained by Lower's method. In this sample, the epicuticular plaques can be seen, and differentiation between exocuticle and mesocuticle is apparent.

ep.pl. - epicuticular plaques; exo. - exocuticle; mes. - mesocuticle; end. - endocuticle.

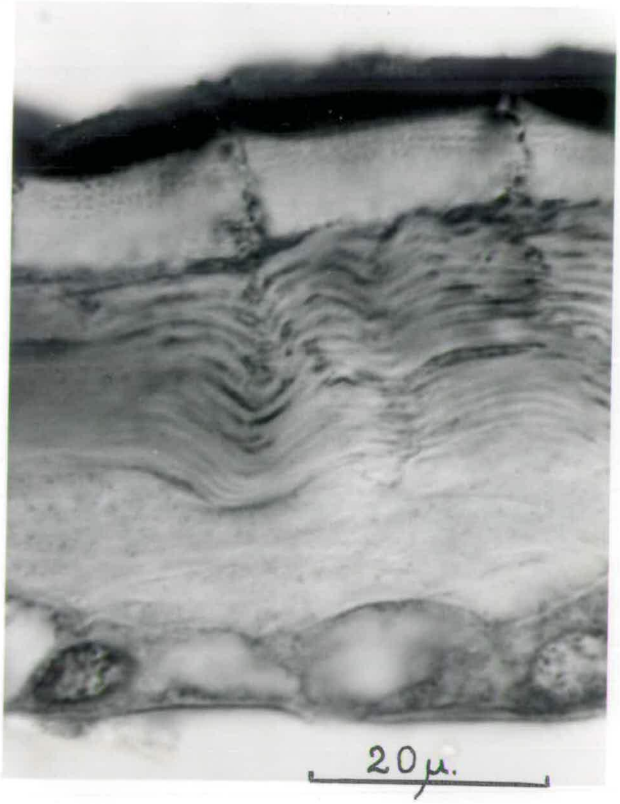


Figure 25. Wild adult cuticle stained by Lower's method. A sample from the lateral area of the tergite to demonstrate the staining reaction of the fracturing regions, and some modification of the corresponding regions of the endocuticle.

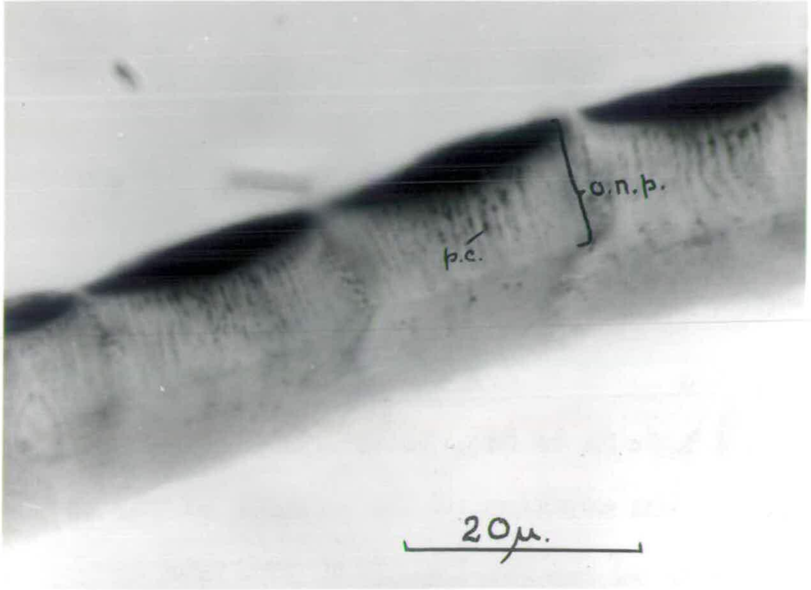
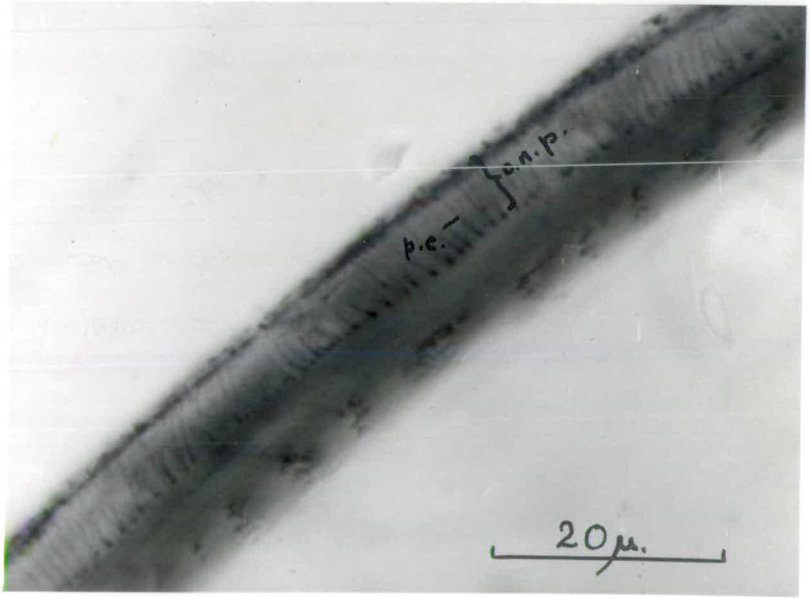


Figure 26.

Figure 26. Wild adult cuticle stained by Lower's method. o.n.p. - outer, non-laminated procuticle; p.c. - pore canals.

Figure 27. Albino adult cuticle stained by Lower's method. The reaction is much more limited than in the wild strain. Abbreviations as in Fig. 26.

Figure 27.



sections were next washed in running tap water for ten minutes, passed through distilled water, and cleared for two minutes in the acidified potassium metabisulphite solution. They were finally washed, dehydrated, and mounted in balsam.

In wild strain cuticle (Figs. 21 to 26), Lower's method yielded results which varied according to the position of the cuticle on the body. In general, the outer layer of the non-laminated procuticle stained a deep purplish black, while the inner layer of this region either did not, or showed a yellowish- or brownish-purple colour. In this latter layer the pore canals were prominent due to their being darkly stained (Figs. 21, 26). The endocuticle stained a light purple. The inner epicuticle was refractory, but the outer was strongly stained.

Superimposed on this general pattern was the variation due to the source of the cuticle. Just dorsal to the pleural border of the tergite, the dense staining traversed about half of the thickness of the non-laminated outer procuticle. At the mid-dorsal region, however, the stain distribution had altered so that the outer one third of the non-laminated procuticle was stained. Exact measurement and description of this staining was complicated by the fact that it was not possible to focus the darkly-stained regions of the outer cuticle, just as it is not possible to focus the so-called "light line" in the columnar epithelial cells of some leguminous seeds (Morris, 1928). A similar effect in cuticle has been noted by Lower (1957b).

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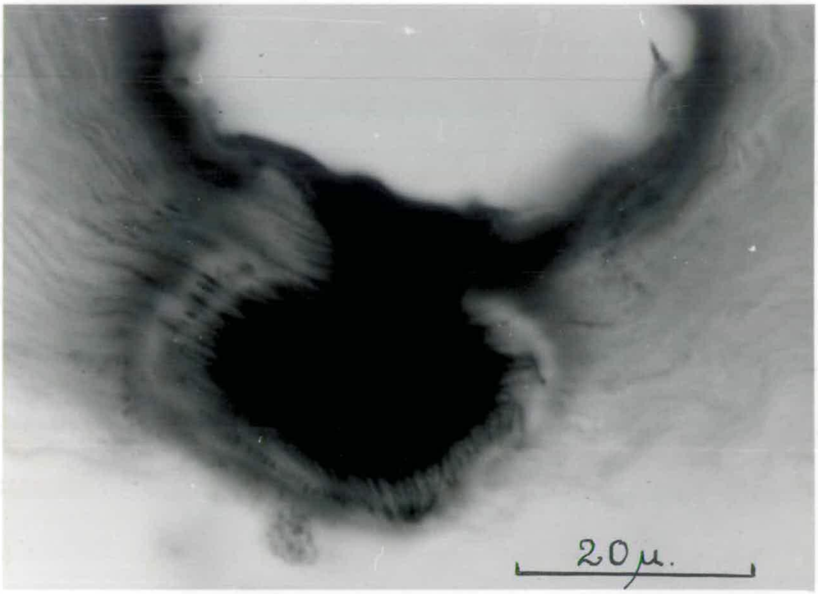

Figure 28. Albino adult cuticle, stained by Lower's method. The apodeme reacts very strongly : compare with the results of Lison's method on apodemes in Fig. 33.

In the pleuron, most of the cuticle stained weak purple. The extreme surface and spines were highly refractile and apparently unstained.

When this condition was compared with that in the albino, various differences emerged. The amount of dark staining in the outer procuticle was relatively less. While the outer one-third of the non-laminated procuticle had been stained in the wild cuticle, only the outer one-fifth was stained in the albino (Fig. 27). The pleural cuticle was virtually unstained, except that a region about two to three microns thick at the outer surface stained strongly. The spines on the pleural cuticle, like those of the normal insect, remained unstained. In apodemes, it was also possible to see the range of staining reactions normally seen from one side of the cuticle to the other (Fig. 28). The inner mass of an apodeme was black, suggesting it was an exocuticular type of material. Surrounding this was a translucent-yellow brown region suggestive of affinities with the inner non-laminated procuticle. This in turn was bounded by the weakly purple staining endocuticle.

Lison's argentaffin test: To compare the occurrence of reducing agents, including melanin and its propigments, in sections of the cuticle of wild and albino adults, a histochemical test of known efficacy was required. Such a test was described by Lison (1936) in his classic work, and the technique has been widely used to demonstrate /

demonstrate the occurrence of reducing substances in insect cuticle. The test depends on the deposition of silver by reducing agents, usually of polyphenol nature, from ammoniacal silver nitrate (Fontana's solution) according to the formula:

$$2\ Ag(NH_3)_2OH + R = 2\ Ag + H_2O + 4\ NH_3 + RO,$$

where R represents the reducing agent.

Immediately Fontana's solution is used, criticisms may arise unless every care is taken throughout the experiment. Perhaps the most important source of error to be eliminated is that due to non-specific impregnation by the solution. To this end, the experiment must run for not more than forty hours. The solution also must not be used on unsectioned blocks of tissue, as there is then considerable risk of non-specific deposition of silver at sites which are not in the least degree argentaffin (Lison, 1936).

Safeguards are needed in the handling of the stain. Apart from the need for careful preparation (_Vide infra_.) of this easily spoiled solution, the material being stained must never come near or above the surface of the solution, when massive deposition of silver usually occurs; and staining bottles must be kept tightly shut as far as possible, and in darkness.

When all these conditions were satisfied, Lison considered it safe to accept the results obtained. He quoted the results of earlier workers and their opinions to substantiate this statement.

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When a technique is so sensitive, it might seem that the nature of the fixatives used on the tissue before sectioning would be of importance. Nevertheless, it appears that there is little risk therein. The original work quoted by Lison (1936) used Bouin fixative. The Duboscq-Brasil derivative of the Bouin formula was therefore considered satisfactory for the work on adult material described in this thesis, while, when the potential colouring effect due to the presence of picric acid was found to be undesirable, 70% ethanol was used on albino nymphal cuticles.

The method finally adopted in this part of the work can now be described. Duboscq-Brasil- or ethanol-fixed sections were deparaffined, hydrated, and washed carefully in distilled water. They were then placed in the ammoniacal silver nitrate solution, in a tightly stoppered staining bottle. This bottle was wrapped in black paper, and was stored for 36 hours in a dark cupboard. The slides were then transferred to distilled water, and given several rinses. The stain was toned by immersion of the sections in a 0.1% solution of gold chloride for five minutes. They were then fixed in a 5% solution of sodium thiosulphate for two minutes. Since counterstaining was not required, the slides were next thoroughly washed to remove the thiosulphate and thus to prevent any subsequent complexing with silver, dehydrated, and mounted in Canada Balsam.

To prepare Fontana's solution for this stain, concentrated ammonia /

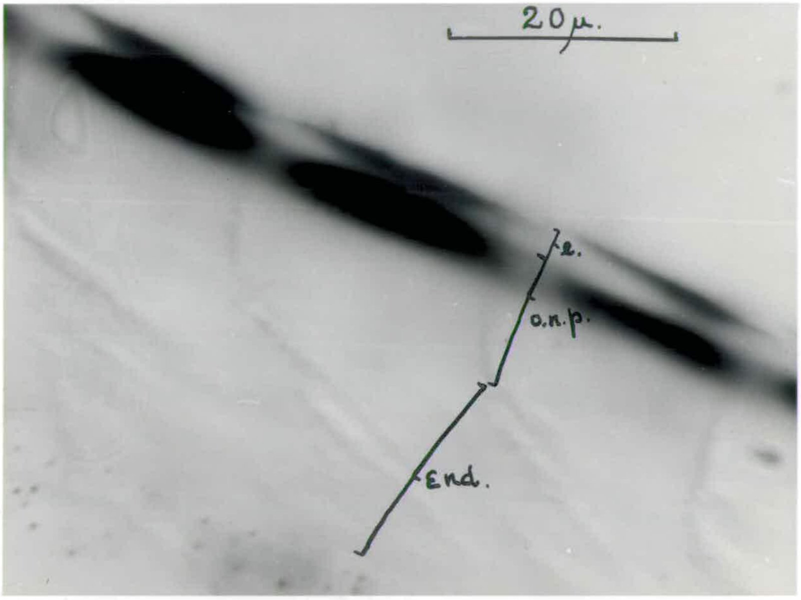

Figure 29. Wild adult cuticle, stained by Lison's method. The epicuticle and outer third of the non-laminated procuticle react intensely. The stain is not evenly distributed in this area of the cuticle, but shows the "block formation" found with other stains.

o.n.p. - outer, non-laminated procuticle; e - epicuticle; end. - endocuticle.

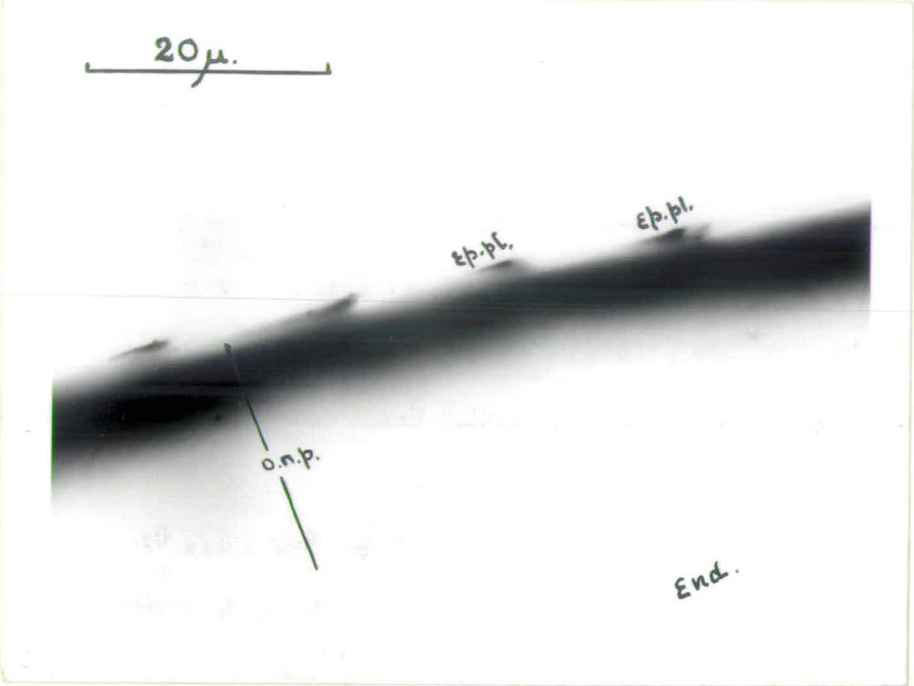

Figure 30. Wild adult cuticle, stained by Lison's method. The reaction is strong, and virtually restricted to the outer third of the non-laminated procuticle. The epicuticular plaques stain deeply, and their relationship to the remainder of the cuticle is clearly shown here.

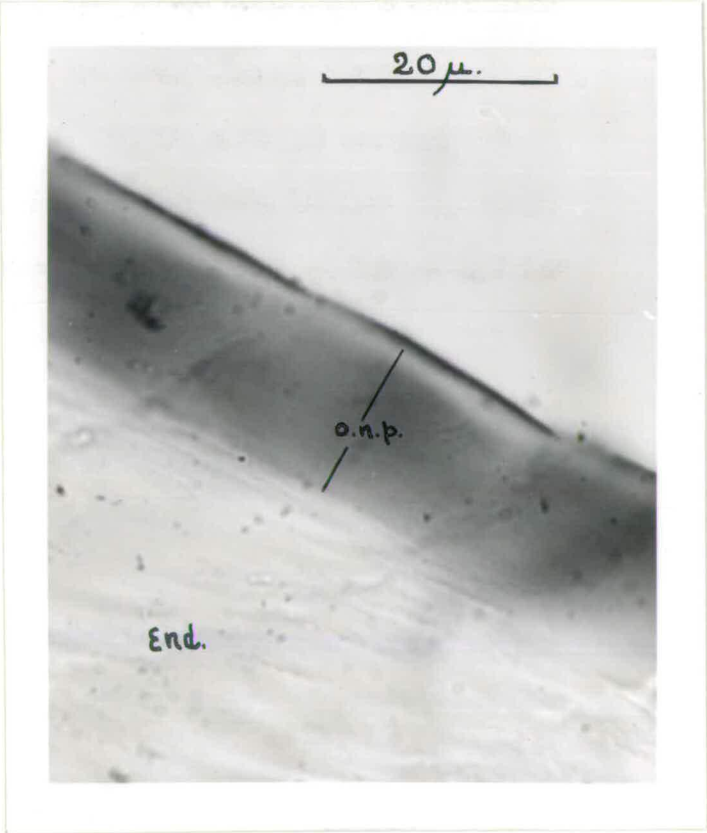

Fig. 31. Albino adult cuticle, stained by Lison's method. The diffuse nature of the reaction in the non-laminated procuticle differs sharply from the localised reaction in the wild cuticle, shown above.

o.n.p. - outer, non-laminated procuticle; end. - endocuticle;

ep.pl. - epicuticular plaques.

ammonia solution was added carefully to a 5% solution of silver nitrate until the heavy precipitate was just re-dissolved. To this was then added, drop by drop, 5% silver nitrate solution until a persistent cloudiness appeared. At this point the solution was balanced, the absence of excess ammonia being of paramount importance for the success of the stain.

Results somewhat similar to those described for Lower's technique were obtained with Lison's method. In the wild cuticle, the exocuticle in general gave many foci of dark brown stain, separated by relatively unstained areas (Figs. 29 and 30). The extent to which this colour extended downwards into the cuticle again varied considerably. In the stained areas, pore canals could with some difficulty be distinguished as even denser staining strands. The stain in them sometimes ceased abruptly in the areas beneath the argentaffin layer of the cuticle, and sometimes persisted to a level considerably further down through the cuticle. The epicuticle stained a reddish brown, but much less intensely than the stained foci in the outer procuticle. The epicuticle was not discontinuous, although over the densely stained areas of the procuticle the epicuticular stain appeared thicker than between them. The plaques were strongly stained, and their relationship to the underlying cuticle was easily seen (Fig. 30). Endocuticle and hypodermis were not stained. The pleuron showed affinity for the stain only in the tips of the larger spines.

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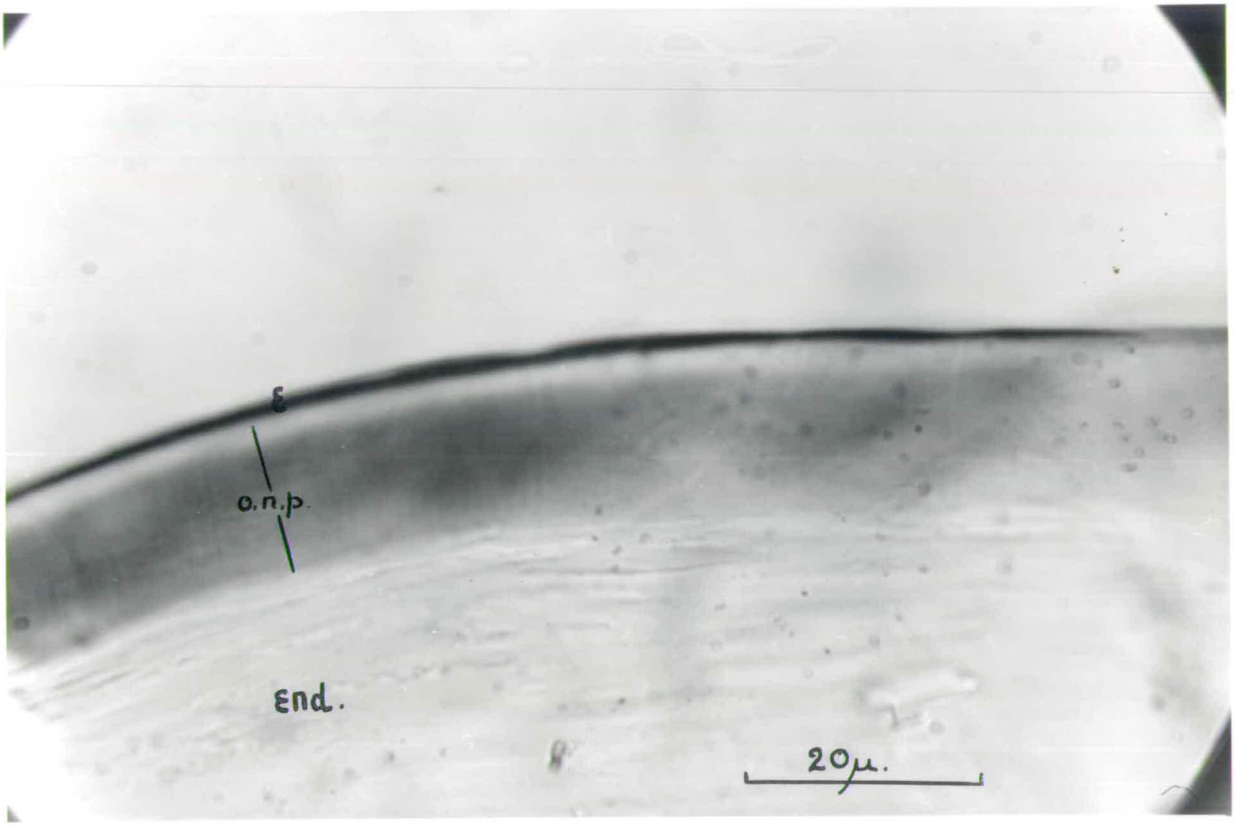

Figure 32. Albino adult cuticle, stained by Lison's method. The epicuticle stains strongly, but the outer, non-laminated procuticle is only weakly stained.

e. - epicuticle; o.n.p. - outer, non-laminated procuticle; end. - endocuticle.

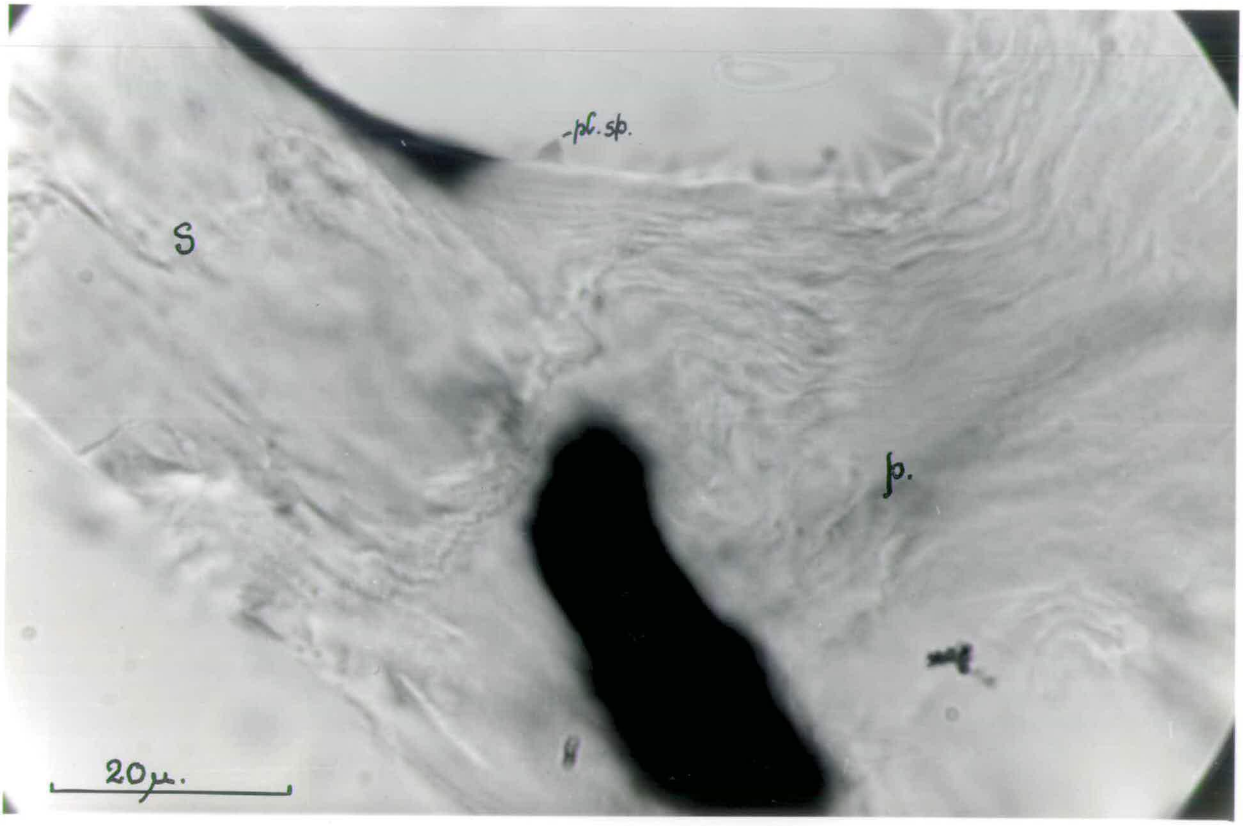

Figure 33. Adult albino cuticle, stained by Lison's method. To show the intense reaction in an apodeme, while the remainder of the pleural and sclerite cuticle is refractory.

p - pleural cuticle; s - sclerite cuticle; p.sp. pleural spine.

In albino cuticle (Figs. 30, 31, 32) there was little staining. The strongest reaction was in the epicuticle. The regions of the outer procuticle below the maxima in the epicuticle varied from a weak purple stain to no reaction. The endocuticle was not stained, nor was the pleuron. Apodemes were rendered almost black (Fig. 33). In the sclerite cuticle the acetabula of the setae and the surrounding rings of superficial sense organs were rendered strong purple. The presence of these localised areas of intense staining confirmed the validity of the weak reactions from the remainder of the cuticle.

INDUCTION OF MELANIN PATTERNS IN ALBINO LOCUSTS BY EXPERIMENTALLY INTRODUCED PHENOL SUBSTRATES

The immersion technique

In an attempt to determine which of the substances accepted as being substrates for tyrosinase *in vitro* (Sumner and Somers, 1953) could be used by the insect in melanisation, various substrates were introduced into the unmelanised strain of the Desert Locust. Since solutions cannot successfully be injected into this strain of locust in more than a few percent of cases at best, a technique had to be devised to overcome this problem. The method was as follows. The wax on the abdominal cuticle was removed from the surface using cotton wool moistened with chloroform. The de-waxed abdomen and posterior legs of the locust were then passed through a ring of "Plasticene" which closely fitted the body. The insect was cross-pinned down to the plasticene, and its de-waxed abdomen and posterior legs were dipped below the surface of the solution to be tested. In this position, the test insect was able to move its front legs and head normally.

This method had several advantages. It did not involve wounding of the insect, as in the case of injections. Thus the violent fluctuations in body metabolism associated with even minor wounding (Wigglesworth, 1939; Sussman, 1949; Williams, 1952; Schneiderman and Williams, 1953) were avoided, and the phenolase complex /

complex was not activated into depositing melanin at punctures. Treating cuticles after removal from the insect, as was done in experiments on blowfly larval cuticle (Fraenkel and Rudall, 1947), has various disadvantages. The cuticle is remote from all control by the insect. It is difficult to know how this may be affecting the results of experiments. When the cuticle has been removed, post-mortem changes may be present, and cannot be ignored. By subjecting the cuticle, while it was still on the living insect, to various experimental treatments, the results would therefore seem to be much more reliable.

In exploratory experiments with the immersion technique, using M/20 solutions of substrates in distilled water, test albinos survived for only three to five days. However, survival was prolonged by increasing and varying the concentrations of sodium chloride in test solutions. When sufficient sodium chloride was used to bring the total concentration of external solutes up to 0.13M, the insects survived for at least one instar, except when exceptionally toxic compounds such as o-benzoquinone were used. Not only was survival prolonged, but it was also possible to obtain the exuviae of the test animals for microscopic examination.

In the immersion experiments, the effects of catechol, 3,4-dihydroxyphenylalanine (dopa), tyrosine, and protocatechuic (3,4-dihydroxybenzoic) acid were tested. Other substances used were hydroquinone, a di-hydroxyphenol of the same empirical formula as /

as catechol; o-benzoquinone, a reagent capable of tanning proteins; and tryptophan, an aromatic amino acid possibly linked with melanin synthesis (Brunet, 1959). Where these substances were not soluble to the extent of producing M/20 solutions, saturated solutions were used.

All experiments were carried out at room temperature of 18'C. to 20'C., and, when necessary, the insects were left in a constant temperature room (18'C.) overnight and throughout week-ends.

Control insects were also exposed to the substances tested on the de-waxed insects. Some, however, were de-waxed with chloroform, and suspended in air. This control is an important one in view of the fact that chloroform is listed by Bodine and Allen (1941) as one of the substances capable of activating pro-tyrosinase. Some insects were de-waxed and put in 0.13M. saline solution, without added substrates, thus keeping the total osmotic pressure of the control solution the same as that of solutions containing salts as well as test substances. Controls which had not had their abdomens de waxed were also immersed in the solutions. In none of these controls was a change in pigmentation detected.

The saline solution in which certain control insects had been immersed for several days was tested for the presence of ortho-di-hydroxyphenols which might have been released from the insect. Addition of dilute ferric chloride solution to a solution containing ortho-di-hydroxyphenols, followed by the addition of sodium bicarbonate, gives /

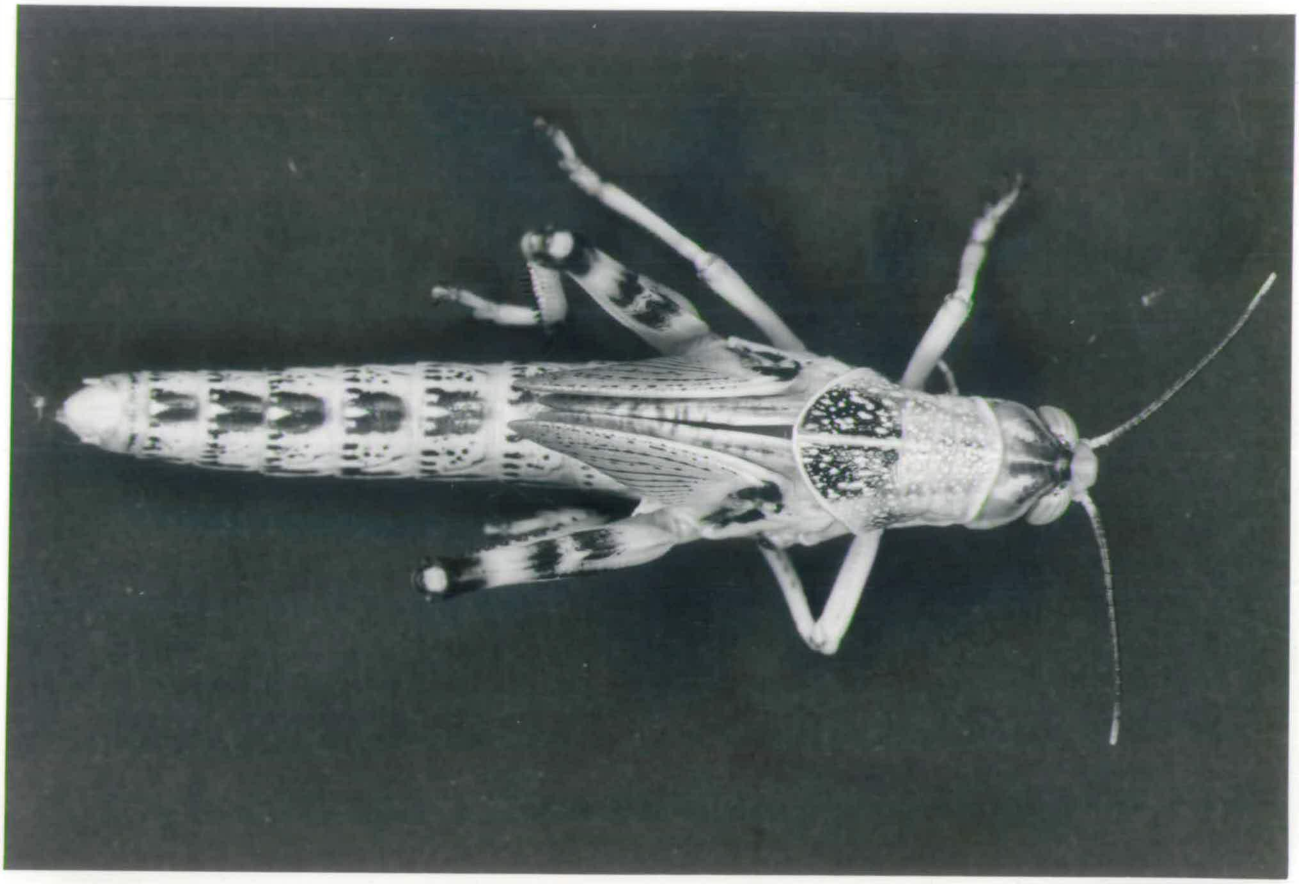

Figure 34. Wild final instar nymph of Schistocerca gregaria, showing the degree of melanin deposition typical of those used in the present work.

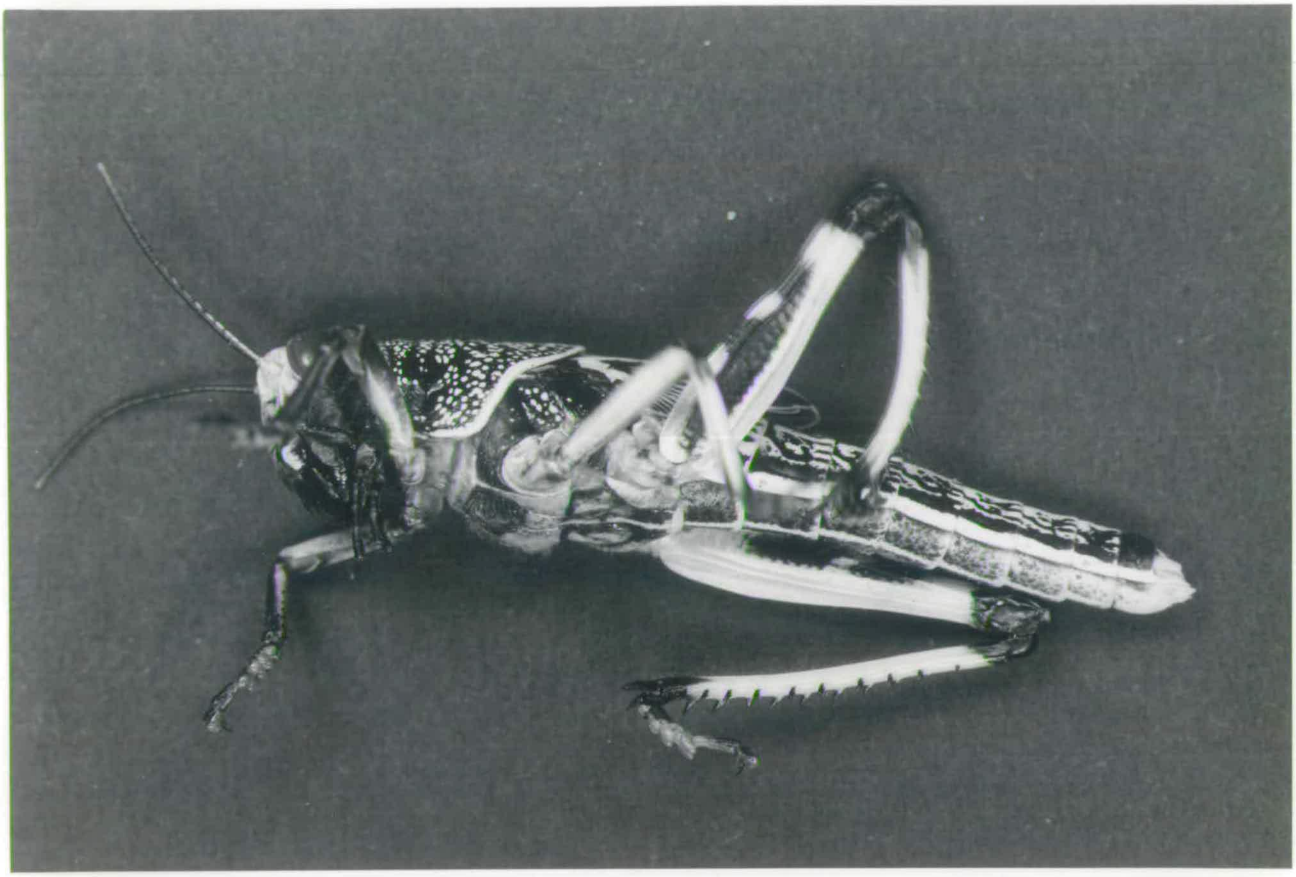

Figure 35. Wild nymph to demonstrate the ventral abdominal pattern, and also a more extensive degree of melanin deposition than in the nymphs shown in Figs. 34 and 36.

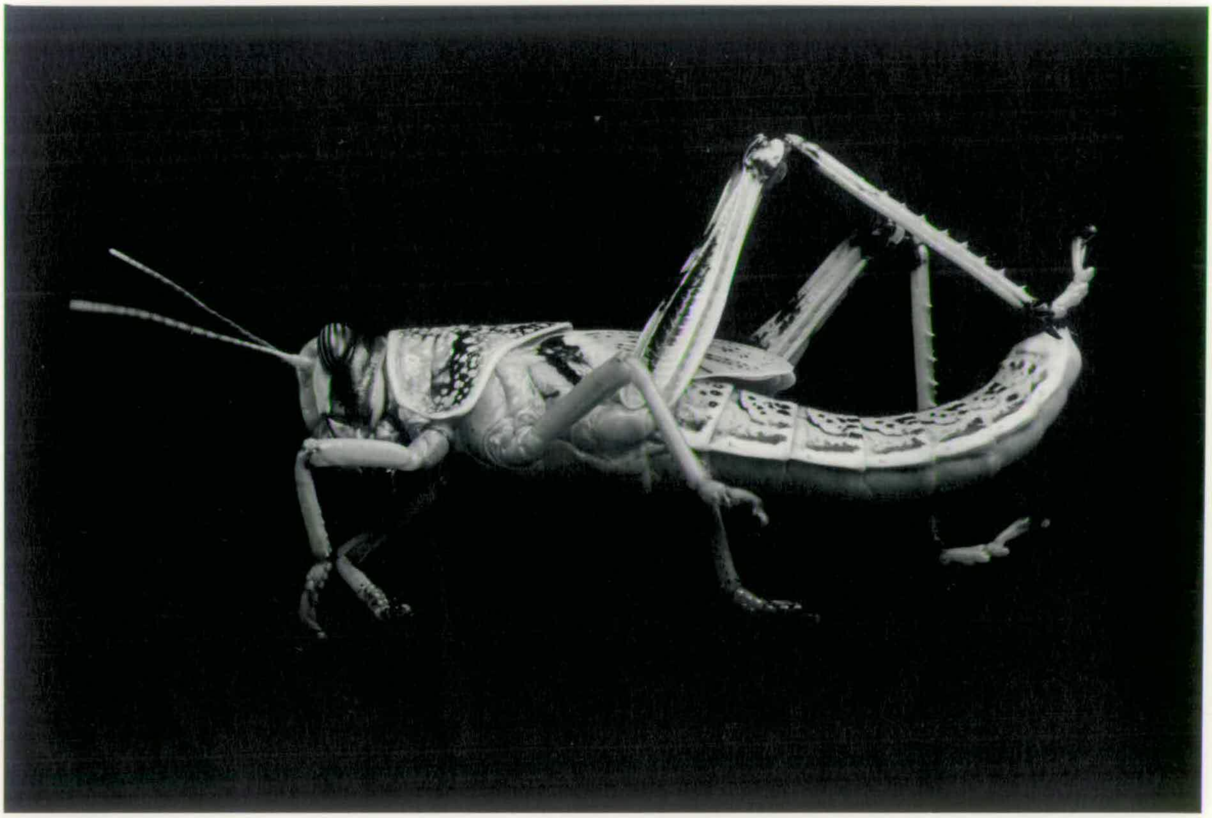

Figure 36. Wild nymph showing a more restricted melanin pattern. The pattern induced in albinos by catechol closely resembles this.

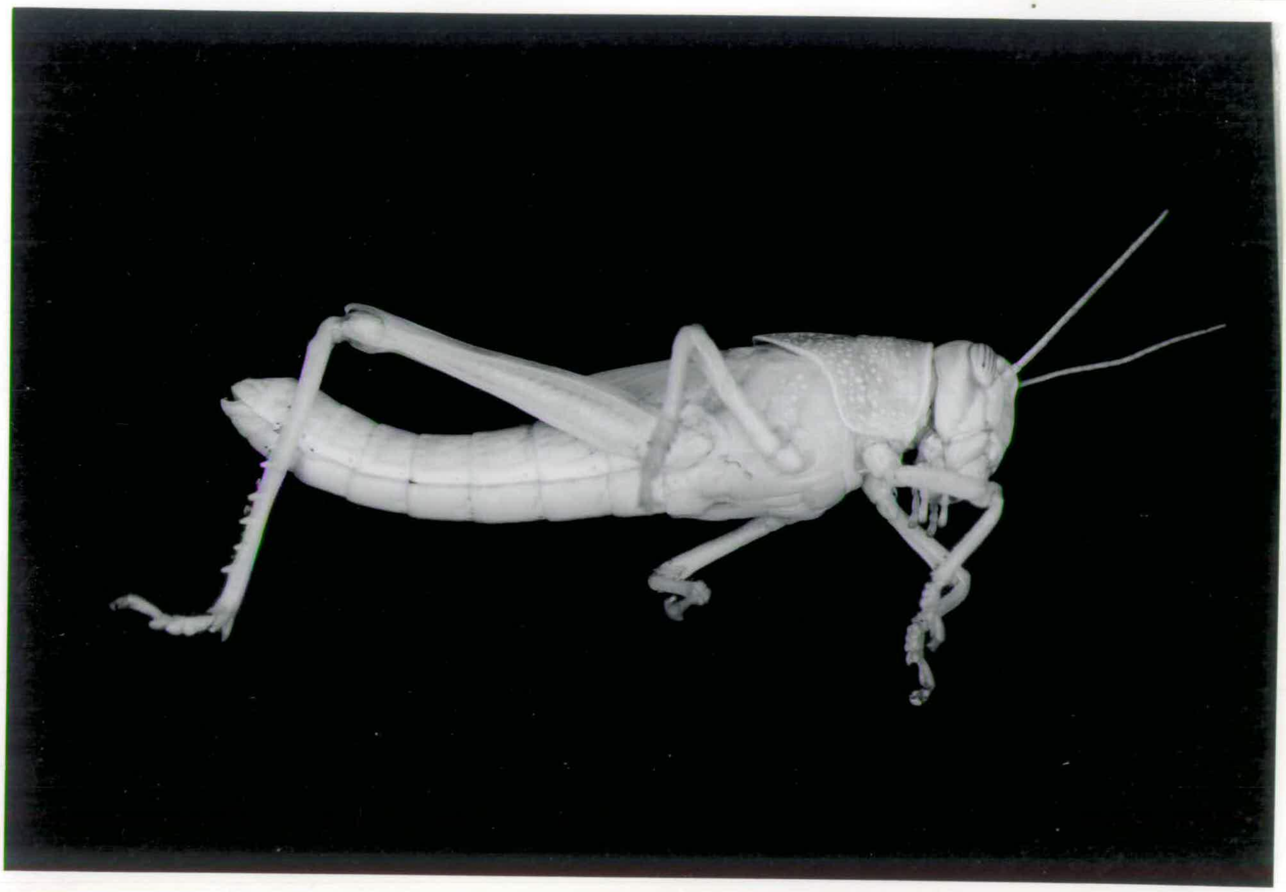

Figure 37. The untreated albino mutant of *Schistocerca gregaria*.

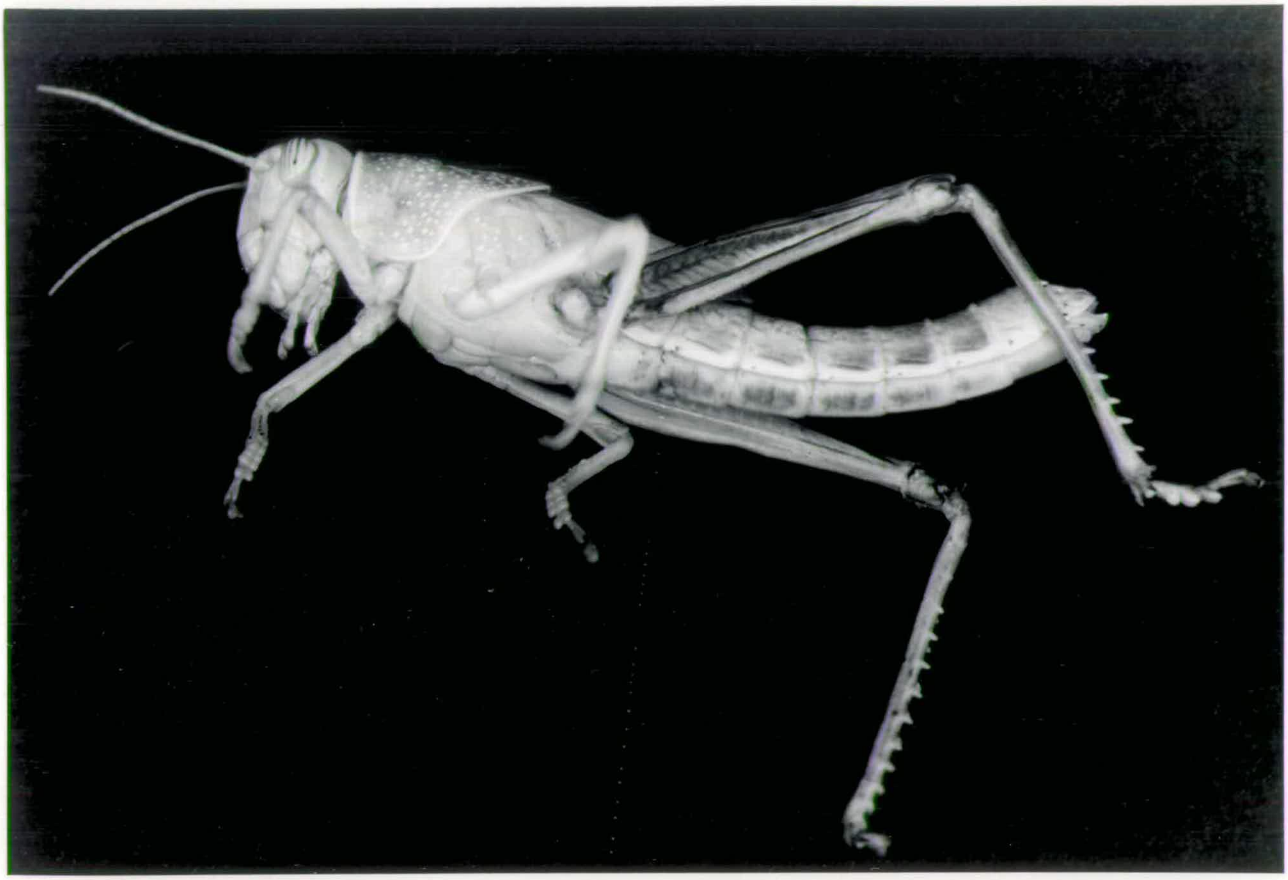

Figure 38. Albino nymph after treatment with catechol for about thirty-six hours, to show the early development of the lateral tergal bars, and of the pattern on the sternites. Liminal spots and the general pigmentation of the tergites not yet fully developed.

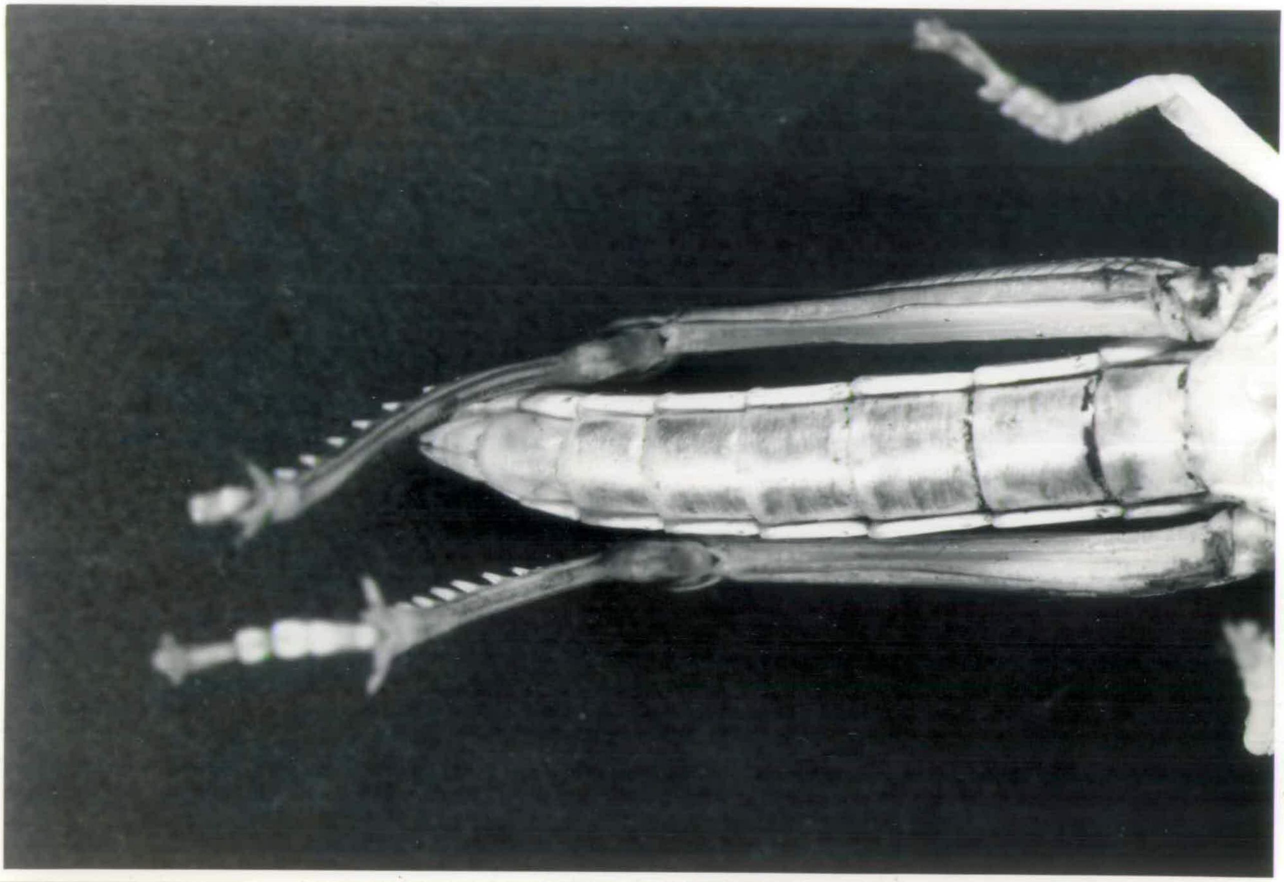

Figure 39. Albino nymph after treatment with catechol, to show the diffuse pigmentation induced on the abdominal sternites.

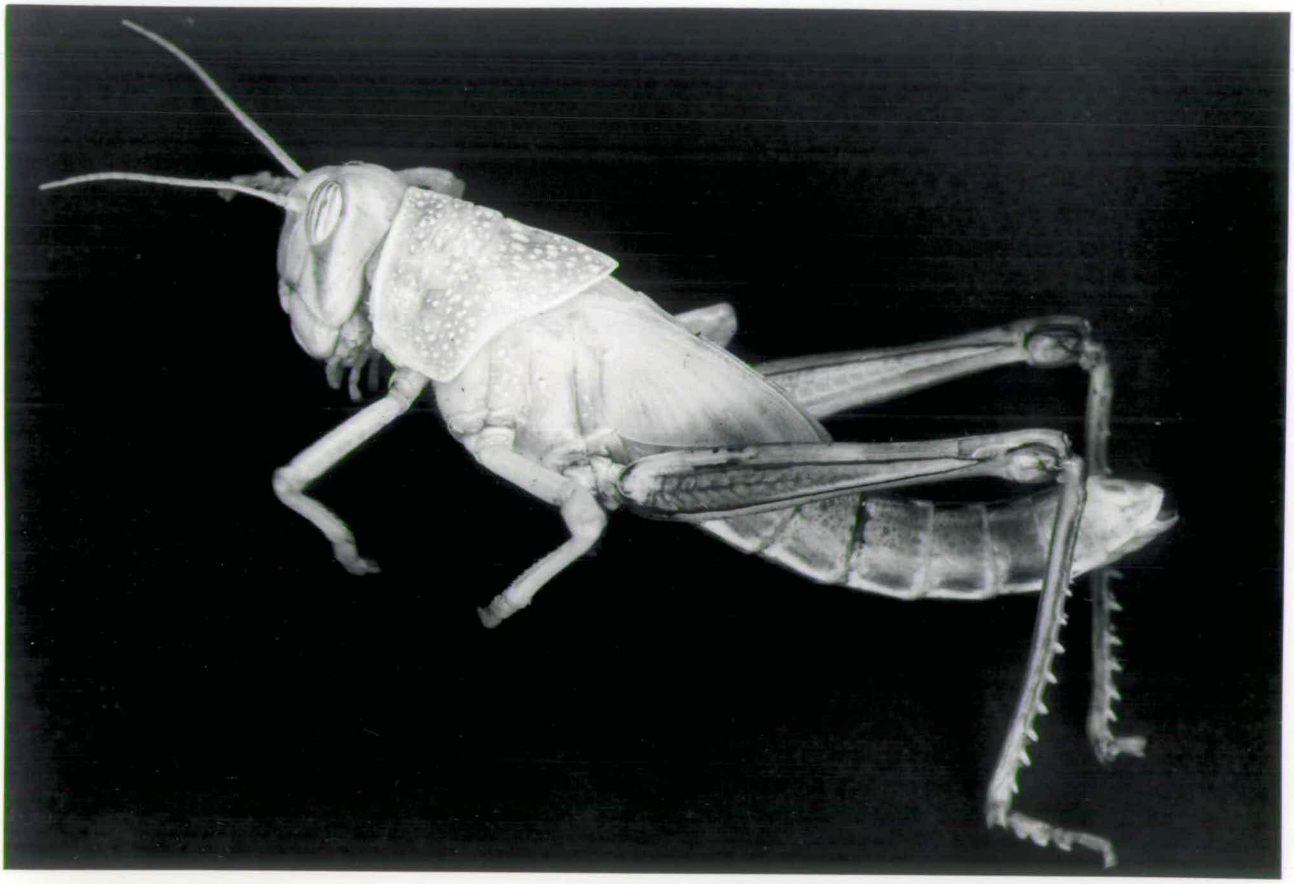

Figure 40. Albino nymph after treatment with catechol, showing clearly the lateral bars of induced pigment on the abdominal terga, the liminal spots, and the scattered spots of the remainder of the pattern.

gives first a green colour, which is changed to a red or purple on addition of the bicarbonate (Cohen, 1950). The test gave negative results.

Experimental effects of catechol

Albinos immersed in catechol solution developed a strong pigmentation pattern (Figs. 38 to 43). The initial deposit was discernible some distance above the lower margin of the tergites of the abdomen as brown patches. This was followed by the development of smaller areas of colour, the liminal spots of Nickerson (1956), along the posterior border of the same tergites. Pigmentation also became apparent at the same time on a longitudinal area parallel to, and centrally coincident with, the mid-dorsal line of the abdomen. Small streaks and spots of colour also occurred on the central region of each side of the tergite. The area in the albino corresponding to the white lateral longitudinal tergal bar in the wild insect (Figs. 35 and 36) did not produce a deposit. On the venter, about the same time as the more general brown of the terga was appearing, strong bars of rather diffuse brown developed. These bars ran longitudinally on each side of the sternites, but did not fuse in the longitudinal mid line (Fig. 39). No sub-pattern within the two blocks on any one sternite was detected.

The overall pattern was produced in 24 to 48 hours, thereafter remaining apparently constant in distribution, but gradually gaining intensity. However, a deposit as dense and black as that in the /

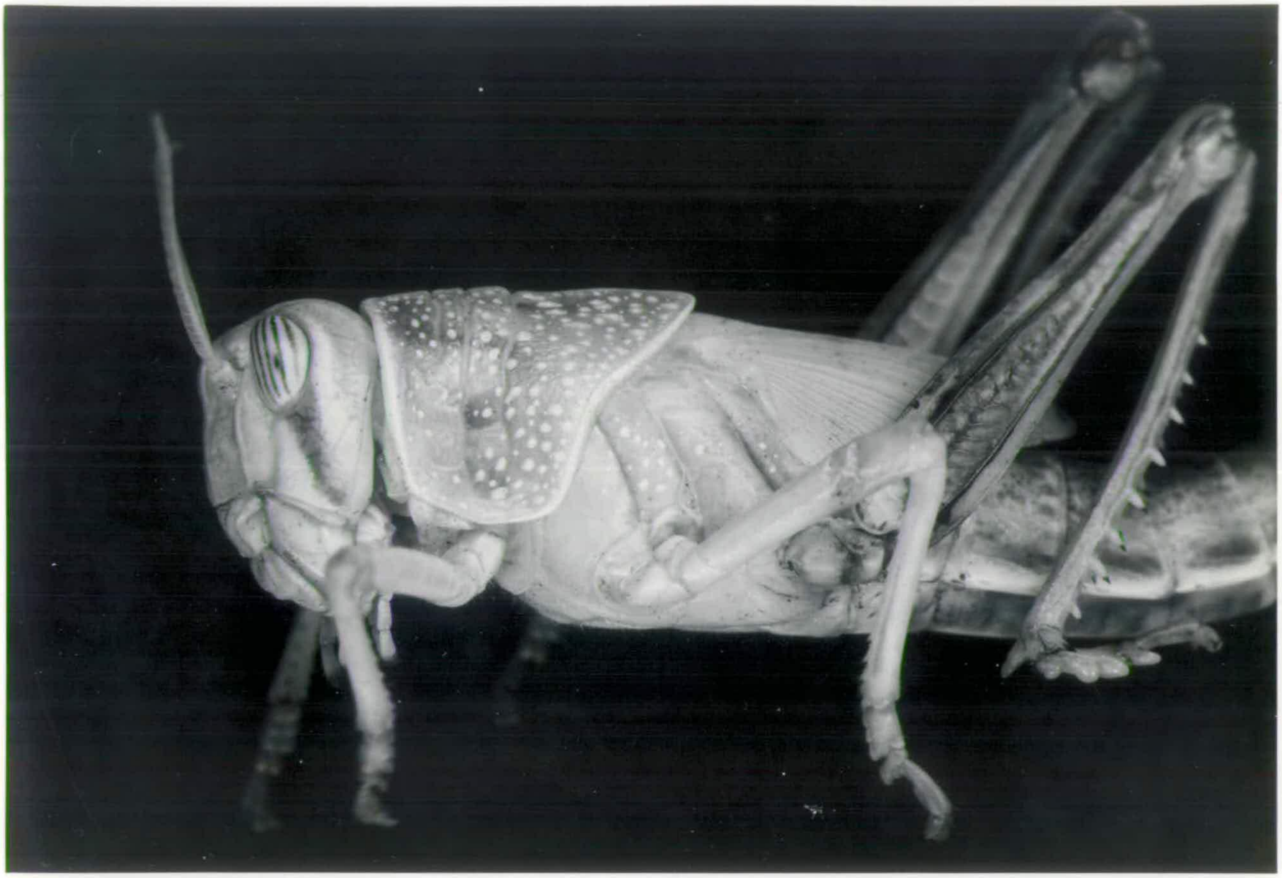

Figure 41. Albino nymph after treatment in catechol, showing the beginning of an induced pigmentation on the prothorax.

Figure 42. Facial melanin induced in albinos treated with catechol.

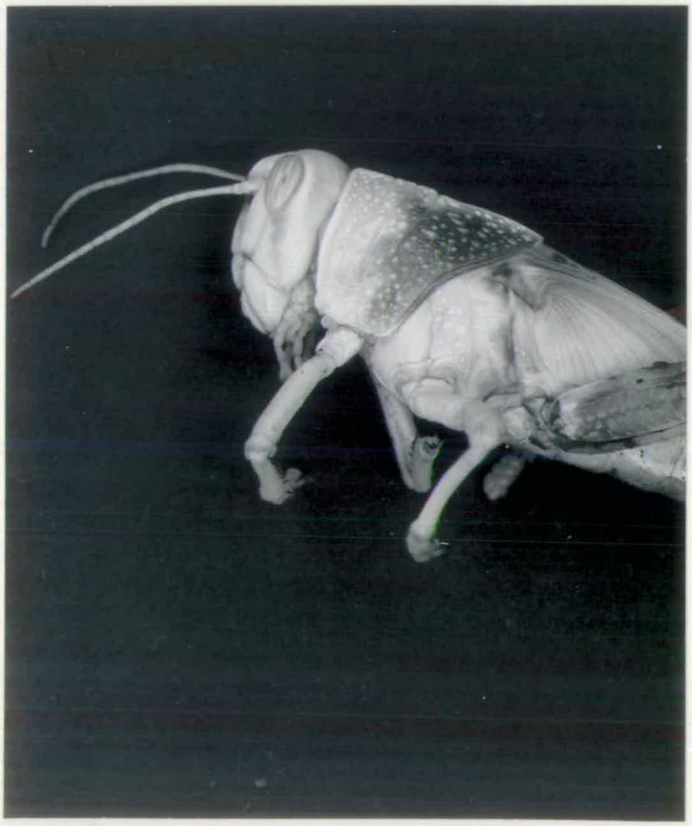

Figure 43. Prothoracic melanin induced in albinos treated with catechol.

the wild strain was not induced in the test albino. The deepest colour was a dark brown.

This overall abdominal pattern developed in all the insects tested. However, when test insects were allowed to remain in catechol for a longer period, up to fourteen days, depending on their position in the moulting cycle, not all of them deposited melanin in the non-immersed anterior regions. The percentage that did was about 10% to 15%. When the pattern developed in the thorax it invariably involved a darkening of the posterior half of the prothoracic shield, varying in different individuals from a light to a very deep brown (Figs. 41, 43). Small sclerites in the middle of the face became a very dense brown (Fig. 42). Some involvement of small sclerites of the thorax was also frequent. The final pattern was very closely similar in distribution to that illustrated by Goodwin (1952) as occurring in wild strain Schistocerca gregaria raised at 40'C. At this temperature, the wild strain develops its minimum melanin pigmentation, and it seems that the albinos have been forced to change from zero melanisation to that minimally occurring in the wild strain.

Just which biochemical phenomenon is responsible for the prevention of the expression of melanisation in the untreated albino, and whether it is an extra-developed version of the naturally occurring pigment control mechanism remains to be examined by the biochemist.

The location of the induced pigmentation in the catechol immersed albinos was seen to be exo- or epi-cuticular, as on moulting, the /

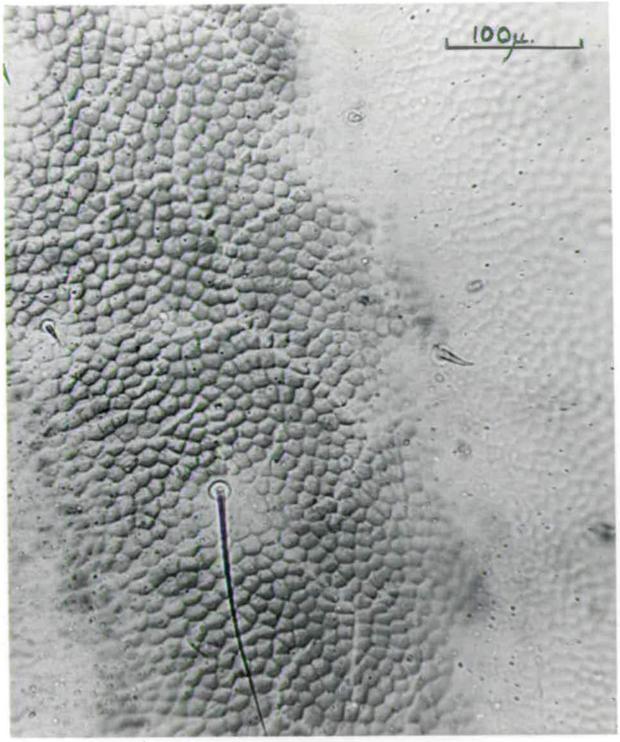

Figure 44. Melanin deposits and surface pattern of albino nymph treated with catechol.

the exuvia retained the induced colour. When the intact integument was removed from the insect and whole-mounted for microscopic examination, it was in part scraped clear of all epidermal constituents, and in the remaining areas was allowed to retain these cells. Removal of the epidermis resulted in the loss of all the insectorubin present, but did not destroy the induced pigmentation, thus further confirming the latter to be in the cuticle. This applied to all the cuticles showing induced pigmentation, irrespective of the solution in which they had been immersed (Cp. data on effect of tyrosine, p. 63).

Close examination of the relative positions of the pigmented areas in the intact cuticle again showed that the colour was located very close to the surface of the cuticle, that is to say, the outer exocuticle and epicuticle. Examination of sections of the cuticle, described fully on p. 69, confirmed this. However, it was more significant that the pigment was not laid down in an even deposit, but as a pattern which, at micro-level, resembled a scaling. This is shown in Fig. 44. The resemblance of this pattern to that in the cuticle of the wild strain S. gregaria is clear. Patterns of this nature have been attributed to the activity of the underlying cells (Kramer and Wigglesworth, 1950). On this view, it is therefore likely that the cells of the epidermis of the albino locust were controlling the deposition of pigment in the overlying cuticle. Whether this was due to direct control from the epidermis, or to an earlier /

Figure 45. Albino nymph after treatment with catechol and phenylthiourea. The induced pigmentation has not been prevented by the presence of the inhibitor of the phenolase complex.

earlier control by the epidermis of the deposition of superficial enzyme, cannot be resolved by the experiments described here. On the other hand, it is obvious that the pigment pattern is unlikely to be due to autoxidation of the catechol and random deposition of colour, since one would then expect a diffuse brown colour over the entire cuticular surface.

There was no constant morphological difference detectable between the areas of sclerite cuticle which bear induced pigment, and those which remained colourless. This does not rule out the possibility that such differences may exist. It does, however, suggest that any controlling influence between the regions is on a physiological rather than a structural level.

In order to prove that the deposition of colour in the albino after catechol treatment was indeed phenolase-catalysed, two inhibitors of the enzyme system were tested. Use of phenyl thiourea at concentrations in the immersion solution of 10^{-3}M. did not change the rate of deposition of melanin or alter the induced pigmentation pattern.

At 2.5 x 10^{-2}M., however, phenylthiourea noticeably slowed, but did not stop, the induction of melanin (Fig. 45). This conclusion is based on the observation that albinos in catechol with 2.5 x 10^{-2}M. phenylthiourea took three days to develop the same intensity of melanin deposition as developed in control de-waxed albinos dipped in catechol lacking the inhibitor in two days. Phenylthiourea in the absence of catechol did not induce pigmentation.

Sodium /

Sodium diethyldithiocarbamate gave comparable results to phenylthiourea.

Use of 10^{-2}M. or 10^{-3}M. solutions of ethyl carbamate (urethane), which is not specifically listed as a tyrosinase inhibitor, did not inhibit the induced pigmentation. Neither did its presence in test solutions containing catechol prevent them turning brown.

The catechol solutions used in the immersion experiments with albino locusts eventually turned brown, which colour was conspicuously more intense than that developed by autoxidation in catechol solutions in control tubes without insects. This suggested that the presence of the insect had increased the rate of oxidation of the catechol. By itself, this might be regarded as of little significance, but the importance of this observation was brought out when it was noted that in the presence of either phenylthiourea or sodium diethyldithiocarbamate in the solutions, there was a reduction in the intensity of the colouration developed. The presence of 10^{-3}M. inhibitor in solutions effected only a slight but constant diminution. When the concentration of the inhibitors was raised to 2.5×10^{-2}M. for phenylthiourea, or 10^{-2}M. for sodium diethyldithiocarbamate, the intensity of the brown colour of the solutions did not exceed that of the control without insects. In these controls, autoxidation was slight.

The survival of the insects in these solutions containing the inhibitor was related to the concentration of the inhibitor. In the case of phenylthiourea, the higher concentrations, 2.5×10^{-2}M., were /

were lethal to seventy percent of the insects after six days, at which time the controls were all alive. The inhibitor had this effect without the presence of catechol. The lower concentration, 10^{-3}M., gave forty percent mortality in six days. In the case of the sodium diethyldithiocarbamate, this correlation seemed to be less marked.

The immersion solutions at the end of eight days were tested for the presence of ortho-dihydroxyphenols with ferric chloride and sodium bicarbonate. This test confirmed the presence at this time of excess unchanged catechol in the solutions. The presence or absence of sodium chloride in the immersion solutions made no difference to the results of these tests.

When these darkenings in the cuticle of the catechol-treated albino locusts took place, it remained to prove that they were melanic, and not merely fortuitous depositions of insectorubin or other pigments. Since there is no satisfactory positive test for melanin, all proof obtained depended on the failure of the pigment to respond positively to tests for other pertinent substances.

The most obvious alternative to the pigment's being melanin by experimental induction was that it was insectorubin, an orange to brown pigment normally present in _Schistocerca_, and many other Orthoptera (Goodwin, 1952). On this assumption, it would have been deposited as a response to the immersion of the insect in catechol. This seemed a case of _reducto ad absurdum_, since insectorubin is a

pyrrole /

pyrrole derivative bearing little resemblance chemically to melanin. Nevertheless, it is found closely associated with the areas of Schistocerca integument depositing melanin, but always in the epidermis (Nickerson, 1956). Insectorubin was easily removed by treatment with 5% v/v. hydrochloric acid in absolute ethanol, or 5% v/v. acid methanol. In these reagents, melanin was unchanged, while the insectorubin-protein complex turned pink, and was removed (Goodwin, 1952). Related to this test is the effect of numerically low pH, an effect which is not found on melanin. When treated with 20 parts of hydrochloric acid in 100 parts of water, the former pigment was changed to a bright orange-red.

In the tests with acid ethanol, acid methanol, or low pH, the induced pigmentation in the treated albinos remained unchanged. The insectorubin naturally occurring in albinos became red, and leached out. Consequently, it seemed proved that insectorubin was not responsible for the changed aspect of the cuticle in the albinos.

Certain pigments found in locusts are carotenoids. These are frequently yellow or pink substances derived almost directly from pigments in the food plants. They are at most only slightly altered chemically by the insect, but may be complexed with a protein, thus yielding variations in colour (Goodwin, 1952). These pigments therefore seemed unlikely to be responsible for the dull brown colour of the treated albinos, but could be objectively ruled out by their solution in alcohols and acetone (Goodwin, 1952). The induced

browning /

browning of the albino was unaltered in these reagents.

Of the remaining pigments found in locusts, insectoverdin is absent in the fifth instar *gregaria* hoppers of *Schistocerca*, but present in the *solitaria*. The complex is restricted to the blood, and plays no part in the general pigmentation (Goodwin, 1952).

Therefore, it seemed proved that the induced colour in the albino cuticle was melanin, known to be a highly insoluble, stable, usually dark-coloured pigment. The fact that the induced pattern mirrored so closely that of the wild strain reared at high temperatures substantiates this conclusion.

Experimental effects of tyrosine, dopa, ortho-benzoquinone, protocatechuic acid, and tryptophan.

When tyrosine was used as the substrate in the induction experiments, the results obtained were similar to those when using catechol, except that the pattern developed at a much slower rate. In addition, the deposit of melanin was less intense.

Dopa solutions did not induce pigment deposition. This is surprising considering that dopa is known to give rise to melanin (Mason, 1955a). In solution, dopa tends to autoxidise quickly, and precipitates are deposited in the crevices of the immersed insects' bodies. Whether tyrosinase in the insects' cuticle is also coupled with this extraneous deposition is an open question.

In considering the apparent lack of effect of dopa on the albino cuticle, it must not be overlooked that in some of the cases which /

which have been examined catechol is oxidised one thousand times more rapidly than is dopa (Yasunobu, 1959). An induced pigmentation developing at a comparable rate in the albinos tested would not be detected by the present technique.

Immersion of the test albinos in ortho-benzoquinone resulted in the cuticle becoming very hard. This compound was also very quickly lethal. Since ortho-benzoquinone is highly toxic, and reacts readily with proteins to form a hardened conjugate, these results were to be expected.

Protocatechuic acid, as in the case of dopa, did not, when used as a substrate in the immersion experiments, induce the deposition of melanin in the test albinos. This compound was thought to be implicated with the process of hardening of cuticle. The results of examining histologically the cuticle of albinos treated with protocatechuic acid are presented later in this thesis.

Tryptophan is utilised by insects to synthesise kynurenine types of pigment (Forrest, 1959). Apparently, a connection seems to exist between the synthesis of kynurenine pigment and that of melanin. Forrest (1959) pointed out that tyrosinase may be involved in the conversion of hydroxykynurenine to brown ommatins. He adds that no specific enzyme for the oxidation of 3-hydroxykynurenine has yet been isolated, but that tyrosinase, in the presence of small amounts of dopa will bring about the oxidation of 3-hydroxykynurenine to xanthommatin. These results were obtained in in vitro experiments.

Forrest /

Forrest mentioned that it remains to be seen whether this reaction is significant *in vivo*.

Brunet, in conversation with me about his work, pointed out that tryptophan was involved in the production of yellow pigments, the ommochromes, in human "red" hair. He postulated that an aminophenol derived from tryptophan might reduce dopa quinone formed by tyrosinase action back to dopa, the aminophenol itself being oxidised to aminophenoxazone. This last compound could then polymerise to form ommochromes. He substantiated this view by the results of his work, which showed that if tyrosine was provided for incubation with the follicles of red hairs, a black pigment was formed. However when the aminophenol derived from tryptophan was provided along with tyrosine a red pigment was produced until the former substrate was used up, to be followed by the deposition of a black pigment produced from tyrosine. It is interesting to recall that Dennell (1958b) demonstrated that tryptophan could be non-specifically hydroxylated by blowfly larval cuticle to form pigment precursors of the kynurenine type.

It was possible then that tryptophan might be involved with the production of the brown pigments in the cuticle of albinos immersed in catechol. The fact that it was not possible to inhibit completely the production of this pigment by the use of tyrosinase inhibitors also suggested that a process not catalysed by tyrosinase might be producing pigments derived from tryptophan.

When /

When test albinos were treated with tryptophan, pigment was not deposited in the cuticle. The inference to be drawn from this is that tryptophan, under the conditions of the experiment was incapable of serving as a substrate for melanin formation. Neither was it capable of producing pigments of the ommochrome type in the test albinos. It is possible that kynurenine and its relatives were produced, but this was not considered of sufficient relevance to the present work to warrant investigations by histochemical or chromatographic methods.

On the specificity of the experimentally induced melanin deposits in albinos.

That some of the substrates tested were utilised by the albinos to lay down melanin deposits strongly suggests that tyrosinase is present despite the natural lack of melanin in these insects. However, laccase, a copper-protein enzyme similar to tyrosinase, is often present along with tyrosinase. This is the case in plants (Dawson and Tarpley, 1951). The two enzymes were distinguished by colorimetric methods since laccase, unlike tyrosinase, can catalyse the oxidation of hydroquinone, a dihydroxyphenol which differs from catechol in being the para instead of the ortho isomer. Yasunobu (1959) has confirmed this difference.

The work of Dennell (1958b) has shown that hydroquinone can be produced in the cuticle of blowfly larvae. This para-dihydroxyphenol was formed non-enzymatically from para-tyrosine. Although it could not /

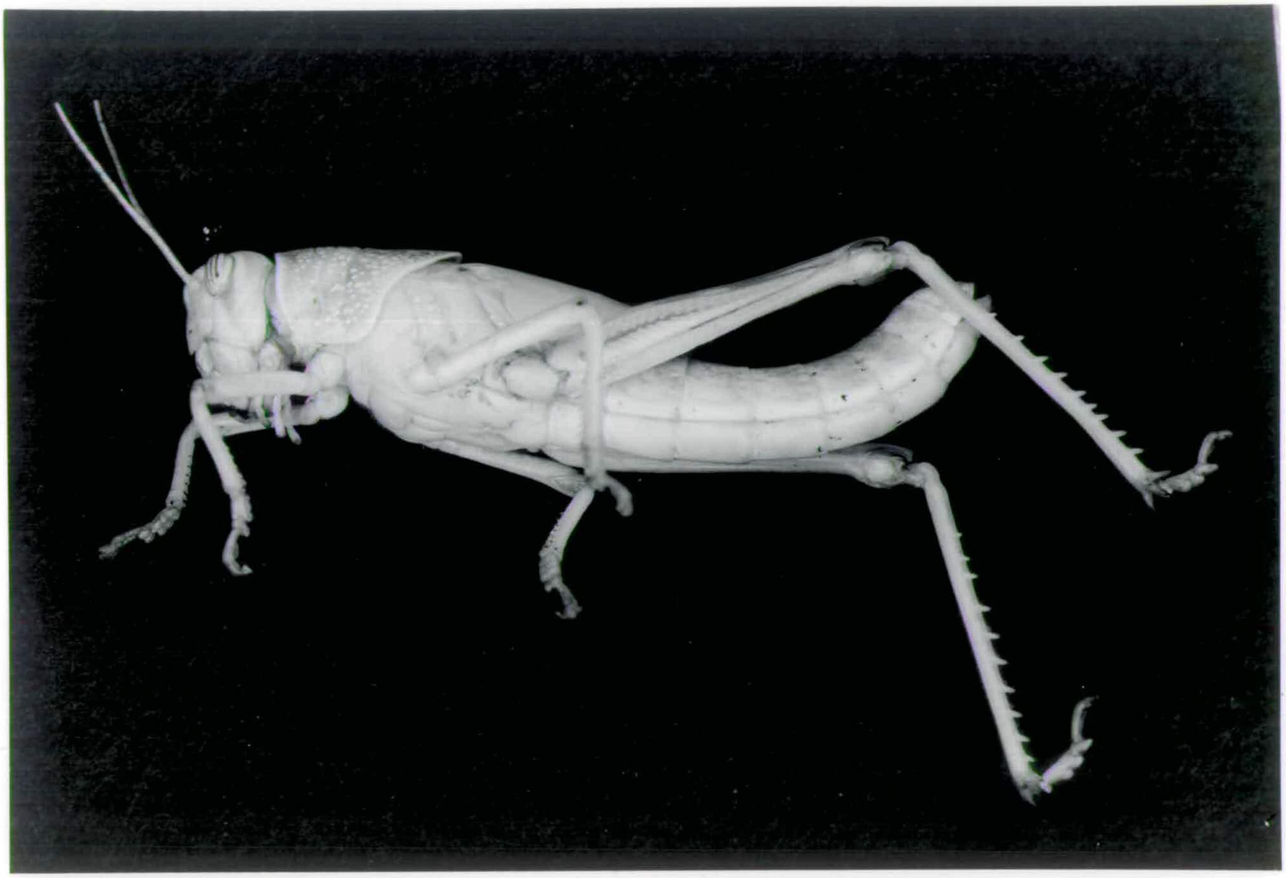

Figure 46. Albino nymph after treatment with hydroquinone for seven days. No pigmentation has been induced.

not be oxidised directly by tyrosinase (Mason, 1955a), it was oxidised, in the blowfly larvae, to the para-quinone in the presence of ortho-benzoquinone, derived from the tyrosinase-catalysed oxidation of catechol (Mason, 1955a; Dennell, 1958d).

If immersion of the albinos in hydroquinone resulted in the deposition of pigment in the cuticle,it would suggest the possibility of laccase being involved. Alternatively, if deposition of a coloured material did not occur, it would suggest, when a pigment was deposited when catechol and tyrosine were provided, that tyrosinase-catalysed the processes. But it had to be kept in mind that the provision of excess hydroquinone could result in the formation of para-benzoquinone by oxidation due to the presence of any orthoquinones free in the cuticle. This was unlikely, however, in the intermoult cuticle, since, if any orthoquinones were produced, they would probably have been used up in the hardening of the cuticle.

When hydroquinone was provided as a substrate in tests on the albinos, pigment deposits were not laid down in the cuticle (Fig. 46). This suggests that the deposits derived from tyrosine and catechol in identical tests were due to reactions catalysed by tyrosinase, and that laccase was not involved. In support of this is the observation that laccase cannot catalyse the insertion of a hydroxyl group into a position ortho to that in a monohydric phenol (Dawson and Tarpley, 1951). The utilisation of tyrosine by the albinos to form a melanin pigment could not therefore have been due to laccase.

The test insects were removed from the hydroquinone solution after immersion for seven days. The test solution in the tubes was then examined to determine whether any ortho-dihydroxyphenols were present, since they might have been produced by interaction between the insects and the hydroquinone. When dilute ferric chloride was added to the test solution, a murky green colour was produced. This indicated the formation, in the presence of the ferric chloride, of quinhydrone (Cohen, 1950). A red or purple colour was not produced when sodium bicarbonate was added. This indicated the absence of ortho-dihydroxyphenols.

EXAMINATION OF CUTICLES OF ALBINO NYMPHS EXPERIMENTALLY INDUCED TO LAY DOWN MELANIN PATTERNS

After the albino nymphs had been forced to deposit melanin in their cuticle, it remained to check the precise location of the pigment, and to discover whether the inductions had led to significant changes in the histochemistry of the treated cuticles. Also, the effect of protocatechuic acid on the albino integument was investigated.

The cuticles used were fixed in 70% ethyl alcohol to avoid the addition of any spurious colours, as is possible when Bouin fixative is used. Otherwise, standard techniques as described in the section on the histology of the adult cuticle were used, with a similar series of stains.

Characteristics of unstained sections of nymphal cuticles

In control nymphs, no pigmentation was seen in sections of the cuticle. Structurally, the outer procuticle was homogeneous, while the inner was prominently lamellar. Both showed pore canals, more so the inner procuticle. Regular fracturing of the cuticle could be obtained here, and was similar to that in the adult, again confirming that more than mere chance was involved in this process.

Cuticle derived from nymphs which had been treated with protocatechuic acid for thirty-six hours showed no difference from that of the control.

In catechol-treated insects, the outer procuticle showed deep /

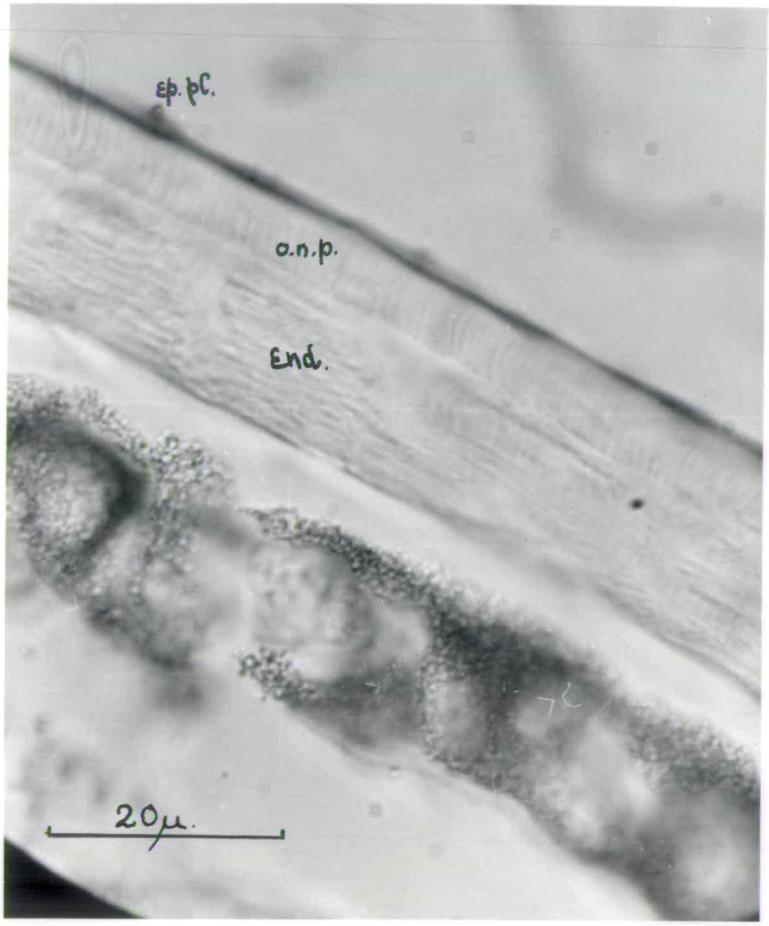

Figure 47. Nymphal cuticle from insect treated with catechol. o.n.p. - outer non-laminated procuticle; end. - endocuticle; ep.pl. - epicuticular plaque.

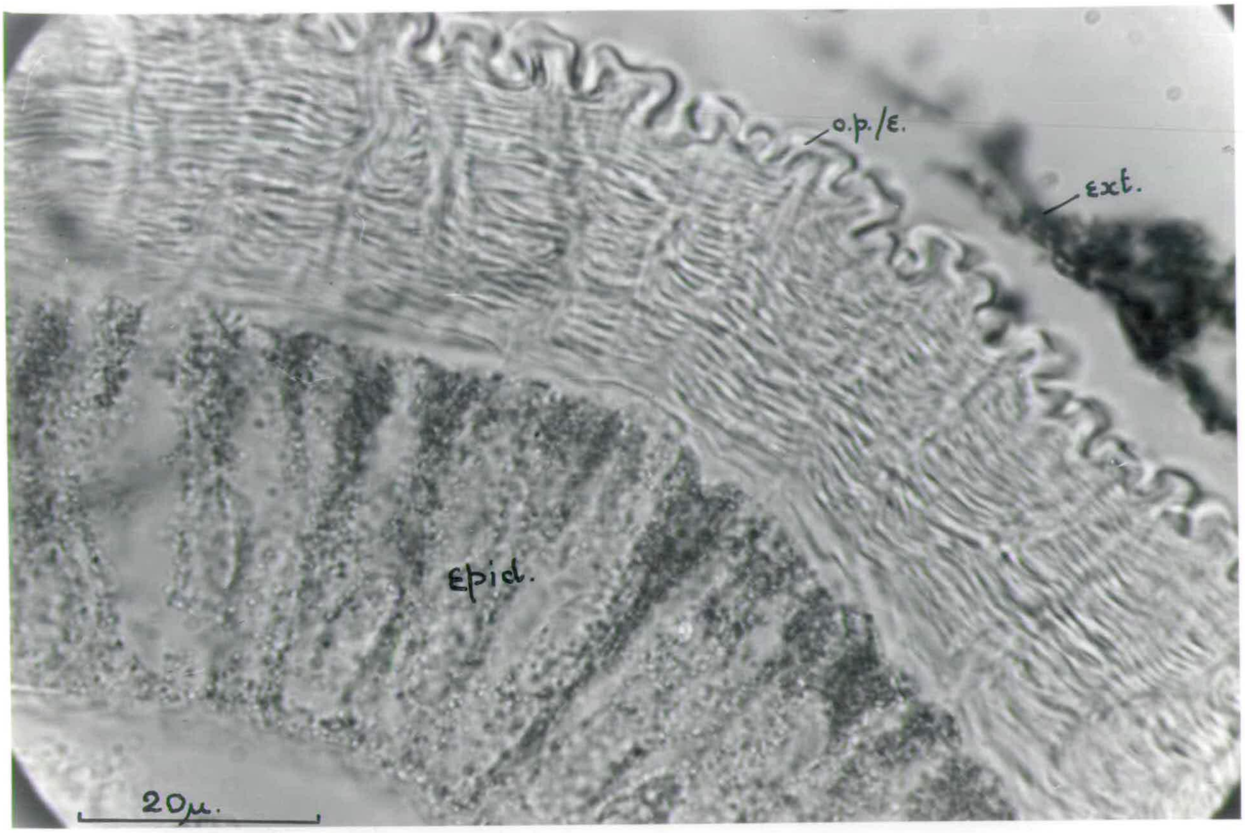

Figure 48. Nymphal pleural cuticle from insect treated with catechol, showing absence of reaction in this region. Extraneous precipitates are seen, as mentioned in the text.

epid. – epidermis; o.p./e. – outer procuticle and epicuticle; ext. – extraneous precipitate.

deep colouration over considerable areas of the tergum, but very little on the sternal cuticle (Fig. 47). This colouration was a strong homogeneous brown, occupying the outer border of the procuticle and the epicuticle. It was fairly sharply demarcated from the underlying portions of the cuticle. Perhaps the most cogent single piece of evidence to confirm that this colour represented that of the pattern seen macroscopically on the intact insect after treatment was the absence of colour for some 600 microns dorsal to the pleural junction on either side. This region would be occupied in life by the prominent white band seen in both treated albinos and wild individuals (Figs. 35, 36, 38, 40, 44), and the measurements made microscopically compared closely with those of the width of the band on the living insect. Dorsal to the white band, the induced pigment commenced rather sharply in the section, and was strongly developed. Just lateral to the heart, pigment was also plentiful, but elsewhere the density was lower. No changes were seen in the arthrodial cuticle (Fig. 48).

Some precipitation of coloured material occurred in these experiments involving catechol, but in sections the precipitates were seen quite clearly to be surface contamination, occurring especially in the groove of the pleural junction. It was impossible to confuse them with cuticular colouring.

Reactions to various stains

Acid Fuchsin: Acid fuchsin on control albinos gave intense staining /

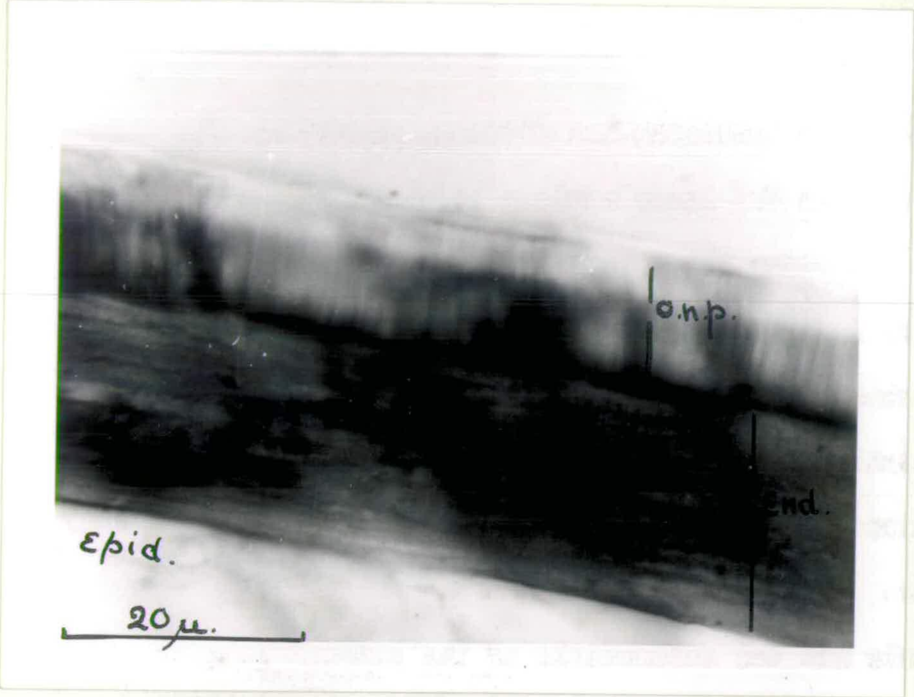

Figure 49. Control albin cuticle stained with Acid Fuchsin. The endocuticle is strongly stained.

o.n.p. - outer non-laminated procuticle; end. - endocuticle; epid. - epidermis.

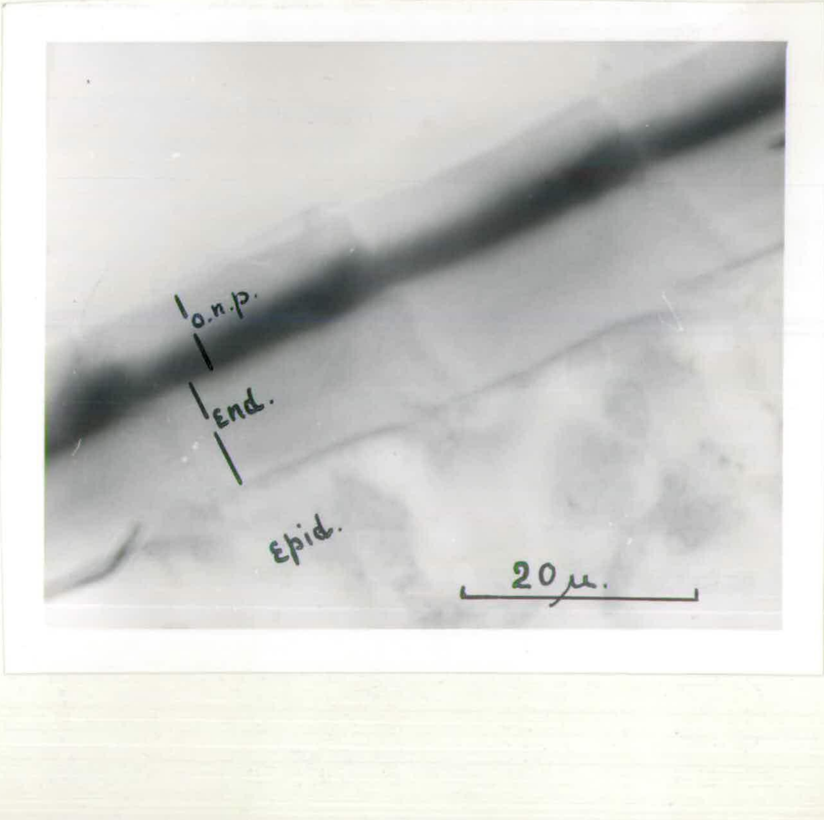

Figure 50. Albino nymph treated with protocatechuic acid, cuticle stained with Acid Fuchsin. The endocuticle has lost its affinity for the dye.

staining in the whole of the endocuticle and diffuse, poorly defined, but strongly stained blocks in the inner regions of the outer procuticle (Fig. 49). The stain also attached to areas around the regularly spaced fracture lines obtainable in the exocuticle. The stain in the outer procuticle was present predominantly in the pore canals. The inner epicuticle stained, and its morphologically thickest, fuchsin-positive areas overlay those most intense areas in the outer procuticle.

The pleural cuticle and the endocuticle of the extreme anterior and posterior borders of the sclerites were refractory. In these places, the thin outer layer overlying the endocuticle was as strongly fuchsinophil as the inner procuticle of the sclerite.

The protocatechuic acid treated cuticles differed sharply from the controls in reaction to acid fuchsin. The stain was present in only traces in the general endocuticle, and was totally absent from the outer portion of the outer procuticle. The inner half of the outer procuticle, however, reacted very strongly indeed (Fig. 50).

The epicuticle showed no affinity for the fuchsin, unlike that in the control. If the slides were grossly overstained, still no fuchsin was taken up in the epicuticle, but soft tissues and endocuticle were forced to take up the stain. The inner half of the outer procuticle in such cases was so intensely coloured as to appear purple.

In the pleuron, the endocuticle, as in the controls, was totally /

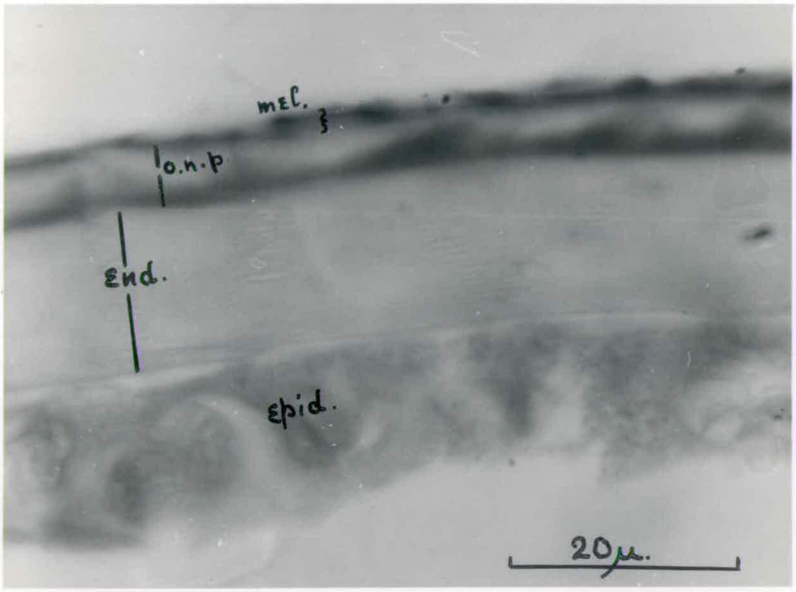

Figure 51. Albino nymph treated with catechol, cuticle stained with Acid Fuchsin.

o.n.p. - outer non-laminated procuticle; end. - endocuticle; epid. - epidermis; mel. - induced melanin.

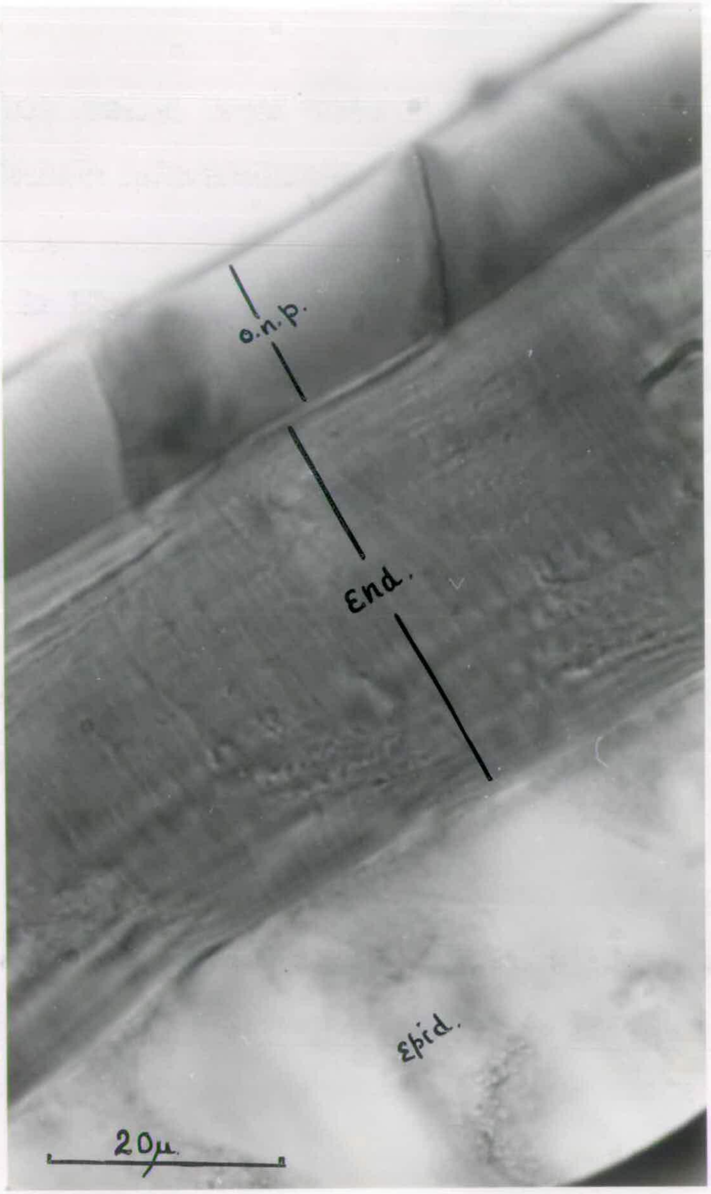

Figure 52. Albino control nymphal cuticle stained with Aniline Blue / Orange G. The Orange G is taken up by almost all the cuticle. o.n.p. - outer non-laminated procuticle; end. - endocuticle; epid. - epidermis.

totally refractory, while the thin outer layer stained strongly.

Fuchsin distribution in the catechol-treated albinos was even more sharply circumscribed. The stain in this material, in fact, became virtually restricted to the inner half of the outer procuticle. Triangles at the usual intervals were seen, and any fracture lines present bisected these (Fig. 51).

The pleural endocuticle was unstained, and sharply delimited from the fuchsin-positive outer layer. The anterior or posterior margins of the terga showed a less clear-cut localisation of the stain, which was fairly uniformly distributed throughout the outer procuticle, and faint or absent in the endocuticle.

Aniline Blue/Orange G: Control albino material stained yellow almost in its entirety (Fig. 52). This reaction was in line with the unexpected staining of most of the cuticle with acid fuchsin. The inner half of the outer procuticle of the sclerites stained more strongly with Orange G. than did the outer half. The inner procuticle stained a uniform yellow.

In the pleuron the endocuticle stained blue. The pleural surface was yellow only in a thin layer, and in its spines. Apodemes had a central unstained core, suggestive of true exocuticle, surrounded by a yellow-stained area. The epicuticle was not easy to distinguish, but appeared to be positive to the Orange G. Soft tissues stained blue.

This /

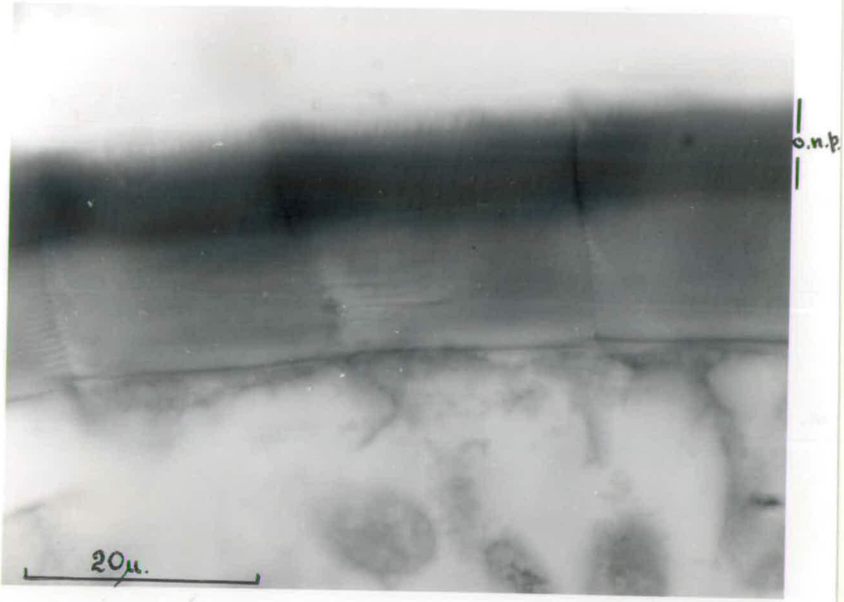

Figure 53. Albino nymph treated with protocatechuic acid, cuticle stained with Aniline Blue / Orange G. The Orange G has become more strongly restricted to the outer non-laminated procuticle (o.n.p.).

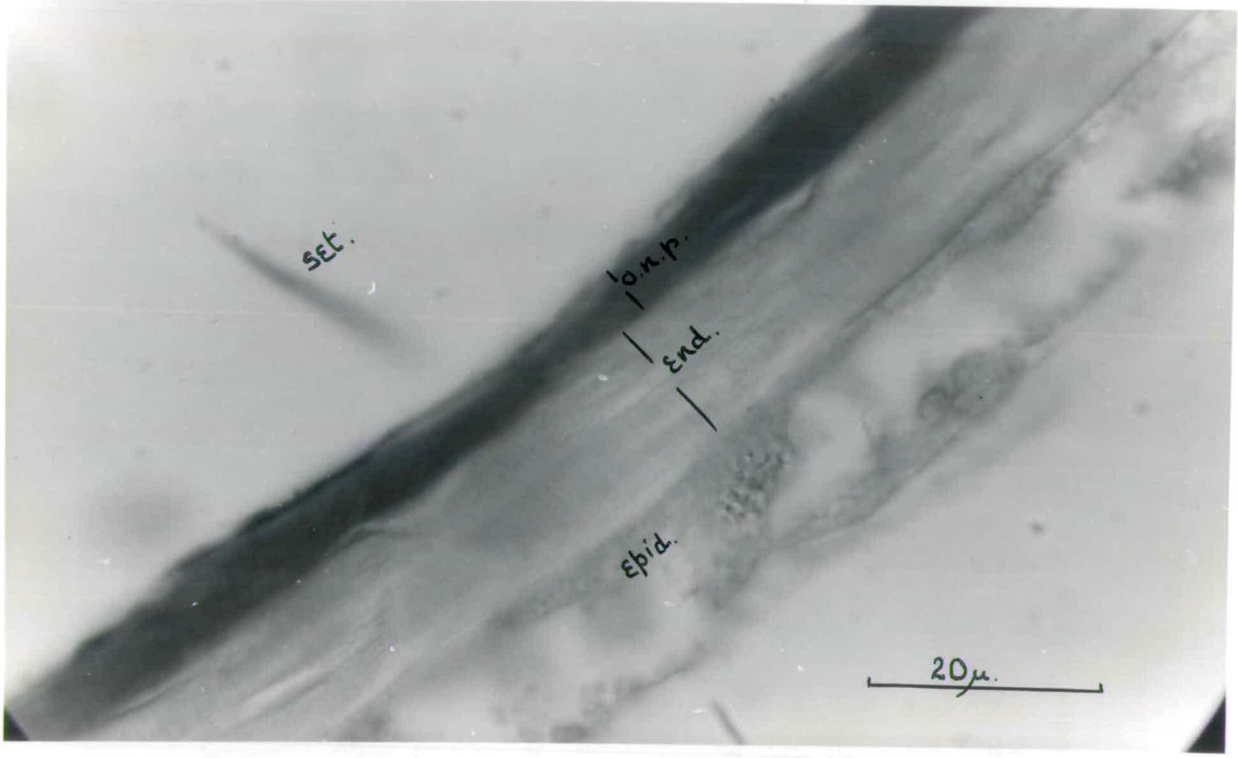

Figure 54. Albino nymph treated with catechol, cuticle stained with Aniline Blue / Orange G. The endocuticle has ceased to accept Orange G, and accepts the Aniline Blue.

o.n.p. - outer non-laminated procuticle; end. - endocuticle; epid. - epidermis; set. - portion of a seta.

This unusual combination of reactions was changed after treatment of the albinos with protocatechuic acid. The response of the outer procuticle became almost uniform, but remained more intense than that of the endocuticle, which was now less strongly stained with Orange G, and showed signs of blue admixture (Fig. 53).

Comparison with control material therefore showed that the inner procuticle had been fairly radically altered, although this test demonstrated no great change in the outer procuticle.

The catechol-treated cuticles showed an even greater change towards homogeneity within its various layers (Fig. 54). Most of the outer procuticle stained yellow, but between the induced pigment and the outer surface of the yellow-staining material, there was a narrow unstained zone. The endocuticle stained bright blue, with no detectable yellow, unlike the previously described materials.

Neither of the treatments given to these cuticles in life altered the reactions of the hypodermis, or other soft tissues to Aniline Blue/Orange G. However, the results obtained from the above two histochemical tests showed very important changes had been induced in the cuticles.

<u>Lower's iodine-silver stain</u>: This test also showed a tendency in the cuticle layers towards greater homogeneity as a result of the experimental treatments. The control stained very intensely throughout the outer procuticle. This was broken in continuity by triangular regions of weaker staining, so that the general appearance /

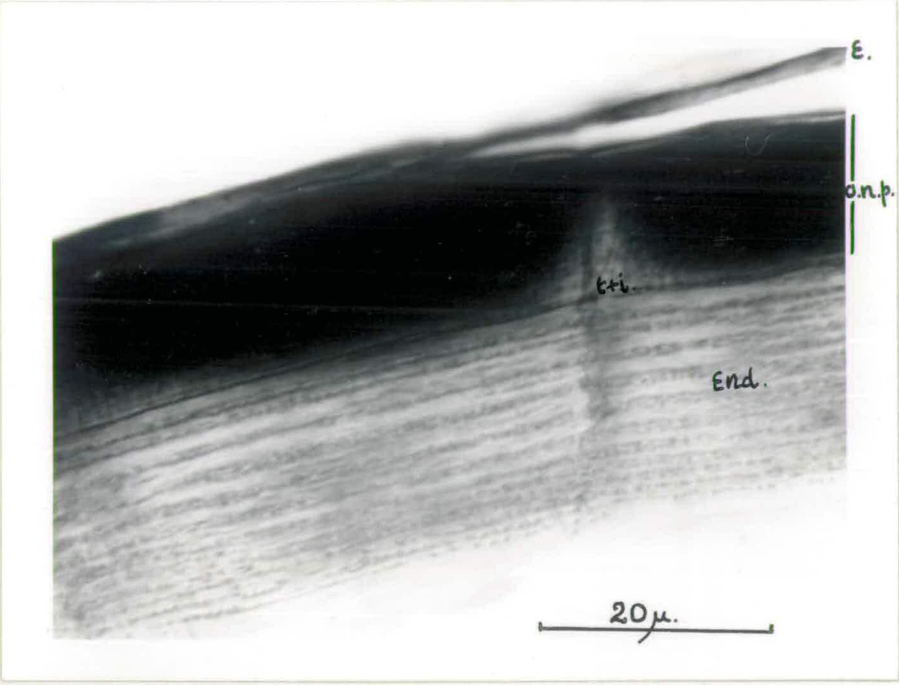

Figure 55. Control nymphal cuticle stained by Lower's method. The unstained triangle shows well in this photograph.

o.n.p. - outer non-laminated procuticle; end. - endocuticle; tri. - unstained triangle; e. - epicuticle.

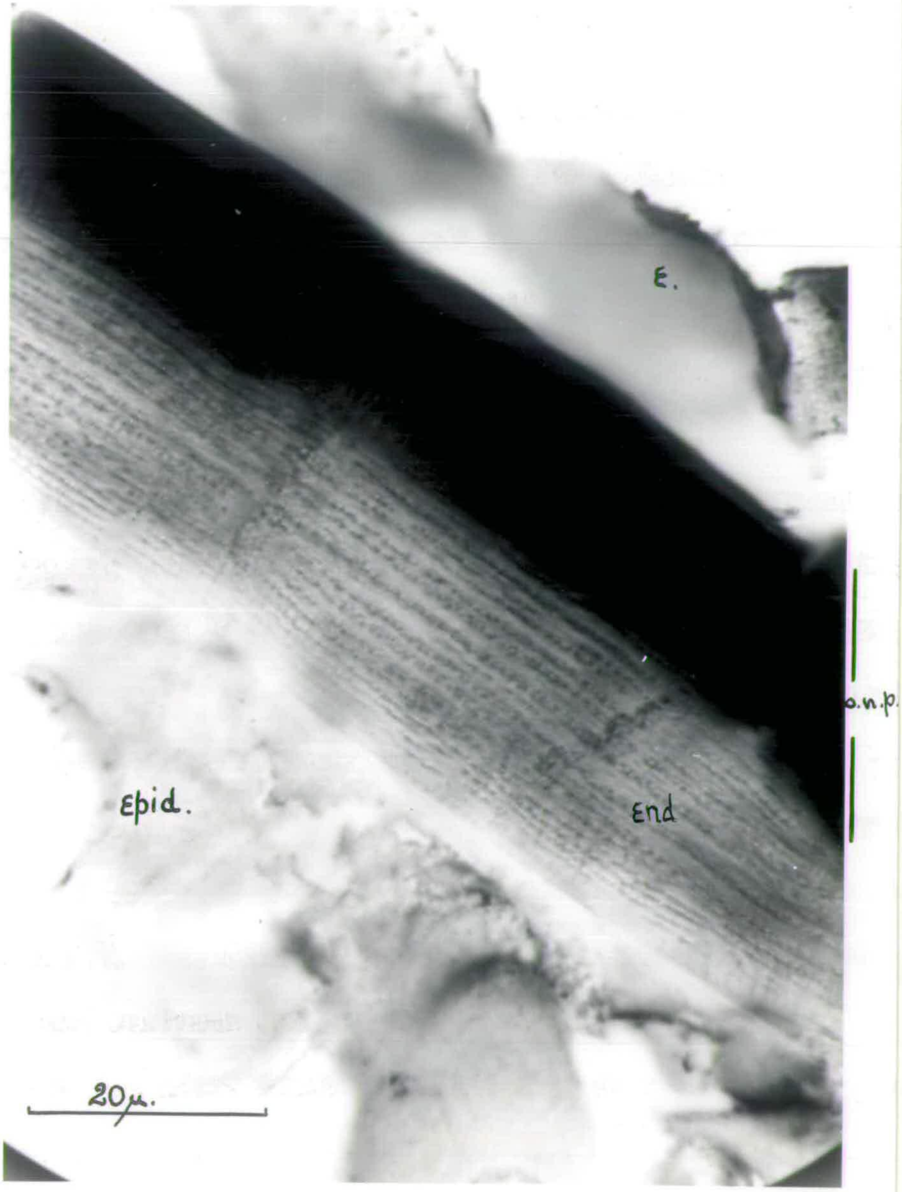

Figure 56. Albino nymphal cuticle stained by Lower's method. The epicuticle has stripped off, and the typical shape of its blocks can be seen, as well as its strong reaction to Lower's stain.

e. - epicuticle; o.n.p. - outer non-laminated procuticle; end. - endocuticle; epid. - epidermis.

appearance suggested a series of elongate trapezia separated by relatively small triangles (Fig. 55). Examination showed most of the staining to be resident in the pore canals. These canals could sometimes be seen to be linked together by material staining identically to the pore canals, running parallel to the surface of the cuticle.

The triangular regions between the trapezia corresponded to the position of lines of fracture, where these were present, and otherwise occurred at regular intervals of the same order as previously described.

The endocuticle stained weakly. The epicuticle was stained strongly (Fig. 56).

The source of mechanical weakness in the fracture lines could be seen to advantage in this series of slides. At any one point of focus, the appearance of the incipient fracture line suggested a section of a coiled duct, and there could be little doubt that it was to such a structure, with surrounding modification of cuticular structure and chemistry, that the production of regularly occurring fractures in certain areas was due. The large ducts carrying the cellular processes up into cuticular setae had straight walls, and were always easily visible. The only other duct of sufficient size to warrant identification with that in the incipient fracture planes is that of the dermal gland (Malek, 1957). It is clear that the passage of these ducts through the /

the cuticle leads to more change in structure in their neighbourhood than seems to have been appreciated.

In the pleural cuticle of control albinos, the endocuticle was totally refractory, and the surface region showed only slight traces of stain.

Perhaps the most striking difference between the inner procuticles of pleuron and sclerite lay in their 'mobility'. This descriptive term was applied to an optical phenomenon present in the endocuticle of the sclerite, though not in the pleuron, when Lower's staining method was used. In the endocuticle about ten to fifteen "smooth" layers alternated with an equal number of "rough" layers, parallel to the surface of the cuticle. On focussing throughout the thickness of the section, the smooth layers showed no appearance of movement, but the rough layers did. The layers were so arranged that when a "rough" one appeared to move one way on being focussed, the next simultaneously moved the other way. This alternation of direction of apparent movement was maintained across the thickness of the endocuticle.

The pore canals ran straight through these layers of material. Consequently, the optical behaviour of the cuticle would seem to be due to some more deep-seated property of the lamellae. Such stratification is probably universally found in this situation, but within the lamellae in this case it seemed that there was some orientation of the molecules along definite directions. A similar condition is found, at a higher grade of organisation, in the layers of nematode cuticles (Bird, 1956).

The /

The apparently fibrous nature of the lamellae in the albino locust could be seen in the separate rough layers as a serious of tiny spots which moved laterally on focussing. That they did so at equal speed in opposite directions suggested that they were arranged at equal angles to the path of light in the section. The smooth layers, in comparison, would be those whose fibres lay parallel to the plane of section.

It is important to note that the 'mobility' in the albino cuticle stained by Lower's method was not found when otherwise identical material was examined unstained, stained in Aniline Blue, Eosine, or Haemalum; and only very slightly when the slides were stained in Acid Fuchsin. In the last, the effect was restricted to the innermost few lamellae, and to very localised regions of the integument. Therefore, it would seem to be an effect dependent very closely on the staining method used.

The protocatechuic acid treated albino material showed slight differences from the controls. In the endocuticle, there was no staining. Furthermore, the 'mobility' in this region of the controls had been eliminated. In the outer procuticle, the large black stained blocks, separated by relatively unstained triangles remained fairly obvious. But while the pore canal reaction was not altered from the control state, the background colour was reduced.

Most of the pleuron was unstained, except a thin outer layer, the outer epicuticle. The external openings of the pore canals also reacted.

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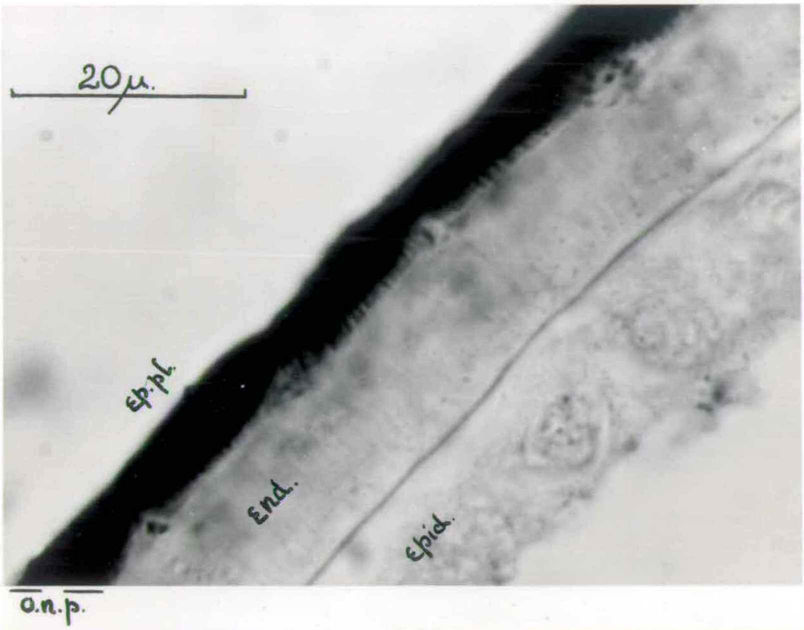

Figure 57. Albino nymph treated with catechol, cuticle stained by Lower's method. The typical unstained triangles are seen. The presence of stained epicuticular plaques confirms the staining of the epicuticle by this technique.

o.n.p. - outer non-laminated procuticle; ep. pl. - epicuticular plaque; end. - endocuticle; epid. - epidermis.

Figure 58. Control albino nymph, cuticle stained by Lison's method.

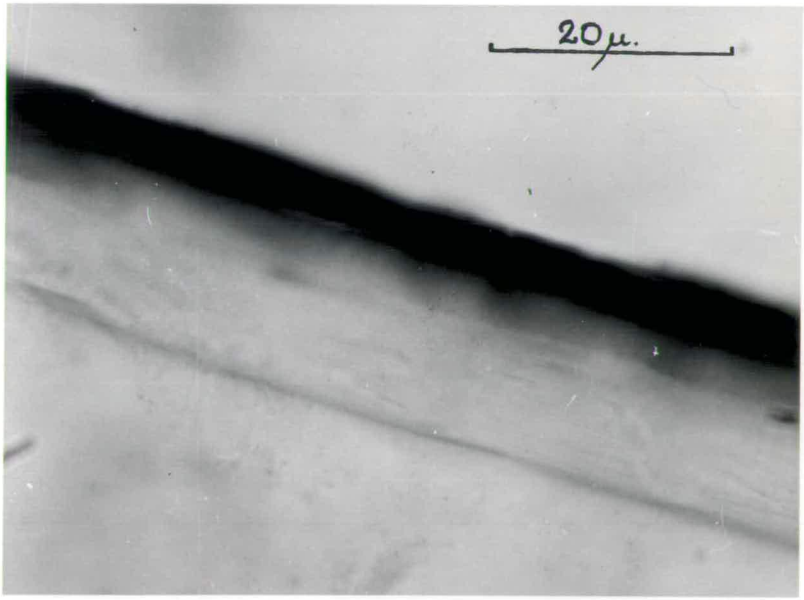

Figure 59. Albino nymph treated with protocatechuic acid, cuticle stained by Lison's method.

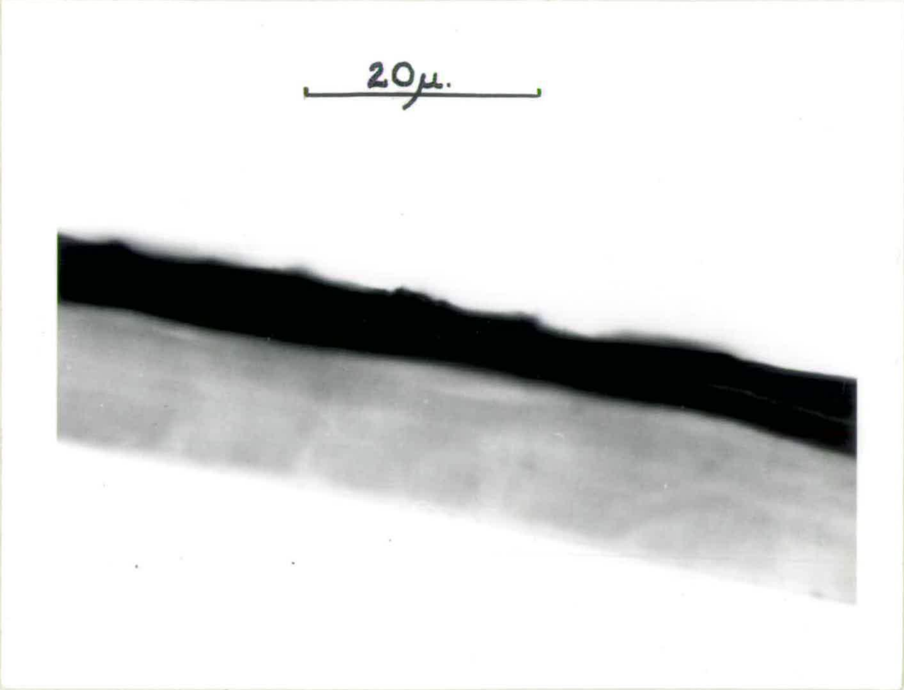

Figure 60. Albino nymph treated with catechol, cuticle stained by Lison's method.

The pattern of staining in the cuticle of albinos immersed in catechol was similar. In the endocuticle, there was no 'mobility', and the pore canals stained faintly (Fig. 57).

In all three types of material, it was possible to show in thin sections and surface preparations that the dark blocks in the outer cuticle were due to a large extent to bunches of deeply stained pore canals. The areas of cuticle between the main blocks seemed not to lack pore canals, but those present stained much more weakly.

Lison's argentaffin test: In controls, the results were somewhat similar in appearance to those obtained by Lower's method. Outer procuticle and outer epicuticle were a strong brown-purple (Fig. 58). The endocuticle was not stained in either sclerite or pleuron. The pleural surface stained weakly, as it did also in the albinos treated with protocatechuic acid. In the latter, the endocuticle was again refractory, but the outer procuticle differed by being strongly stained in the outer half while being only very faintly stained in the inner half (Fig. 59). The epicuticle was unchanged. After catechol treatment, the whole outer procuticle, and the outer region of the pleuron stained very intensely (Fig. 60). The outer procuticle of the sclerite seemed always to be divided by a very thin non-staining layer, the outer of the two stained regions thus formed being the more intensely coloured.

Demonstration of triangular areas of different stain affinity was not as convincing with Lison's method as with Lower's. In local regions the effect could be detected, but this was exceptional.

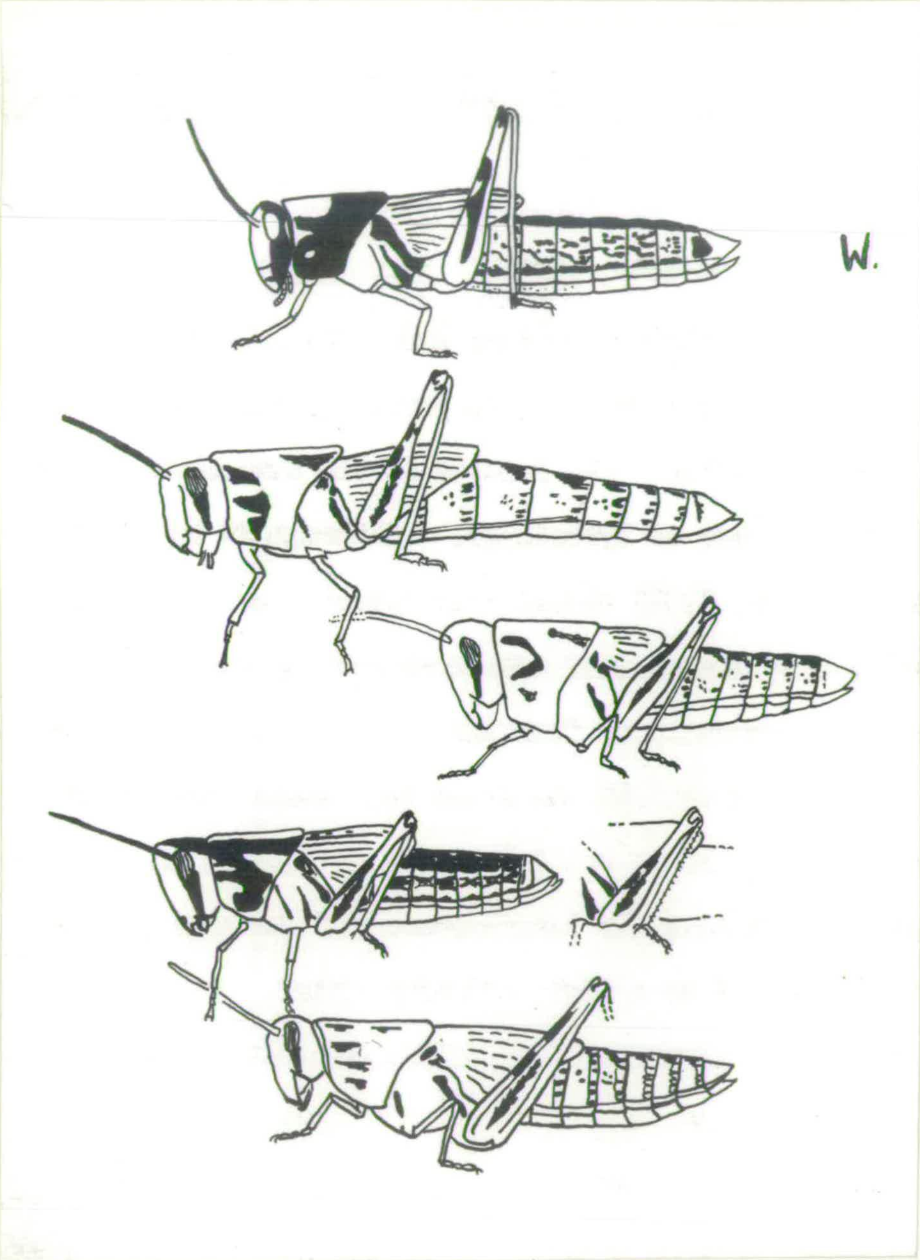

Figure 61. The melanin patterns induced in albino nymphs after treatment of the eggs at 20°C. for three weeks. A final instar nymph (W) of the wild strain is included for comparison.

INDUCING THE FORMATION OF MELANIN PATTERNS IN POST-EMBRYONIC STAGES OF ALBINO LOCUSTS BY PREVIOUSLY EXPOSING THE EGGS TO LOW TEMPERATURES

Low temperatures encourage, in general, the deposition of melanin in insects (Kennedy, 1956). A pre-requisite for albinos to produce a melanin pigment pattern under the influence of exposure to low temperatures was the presence of an essential phenol-phenolase system. The results of the immersion experiments indicated the presence of tyrosinase. Although it has limitations, Baker's test (Baker, 1956) showed that tyrosine was present.

Exploratory experiments were conducted by exposing eggs of various ages to temperatures from 10'C. to 4'C. for periods ranging from one to seven days. It was found that these conditions of low temperature did not induce the deposition of pigment in the insects which emerged. Excessively long exposure of the eggs to such temperatures resulted in a high mortality rate.

Exposure for three weeks at 20'C., despite subsequent exposure to 30'C., also resulted in a high mortality rate, but it was significant that the survivors produced a melanin pattern to a varying degree. A typical series to illustrate the results is shown in Fig. 61. A wild strain locust of the same stage as the test albinos may be compared. However, wild strain individuals vary in their patterns. The one serving as illustration shows an average pattern, under the conditions of breeding used in this laboratory.

It /

It will be seen that the pattern induced in the albino by prolonged exposure to a temperature of 20'C. resembles that of the wild type. The pigment appeared, in less extensively melanised albinos, as isolated areas in sites which corresponded to those where the most intense pigmentation in the wild strain occurred. As more extensively developed patterns were induced, the areas of pigment spread. For example, on the thorax the pigment areas coalesced so as to resemble more closely the wild pattern. The coalescence of pigment areas on the abdominal cuticle was obscured, to some extent, in the living albino by the presence of the insectorubin-protein complex. When the latter pigment was removed, the induced pigment remained. That the induced pigment was melanin was confirmed by the tests for melanin already described.

ESTIMATIONS OF THE RELATIVE CONCENTRATIONS OF TYROSINASE IN EXTRACTS FROM ALBINO AND WILD STRAIN LOCUST NYMPHS

Attention was next focussed on the possibility of determining whether the tyrosinase in the tissues derived from wild strain locust nymphs was more active than that from albino nymphs. It was reasoned that if the activity of the enzyme in the albino was suppressed by inhibitory influences, the enzyme might display an activity near to normal in a brei kept at a fixed pH, and containing an adequate amount of suitable substrate. In addition, excess substrate might, of itself, activate the enzyme (Dubois and Erway, 1946).

A known weight of locust tissue, including cuticle, was homogenised in Sorensen's buffer at pH 6.6 to 6.7. The pH was measured using a pH meter as well as a comparator. The homogenate was filtered and diluted with buffer. Equal weights of wet tissue, albino or wild, were maintained in each sample.

The substrate used was catechol, at a final concentration of 1.25×10^{-2} M., the substrate being dissolved in buffer at pH. 6.6. to 6.7. Five millilitres of this solution were put in matched colorimeter tubes, and a five millilitre sample of the brei was then added to each tube. The optical density of the solutions was measured immediately on an E.E.L. colorimeter fitted with a green filter. Buffered catechol solution acted as a control to indicate the effect of autoxidation of the substrate, and distilled water was used as a standardising solution for calibration of the stability of the colorimeter.

As /

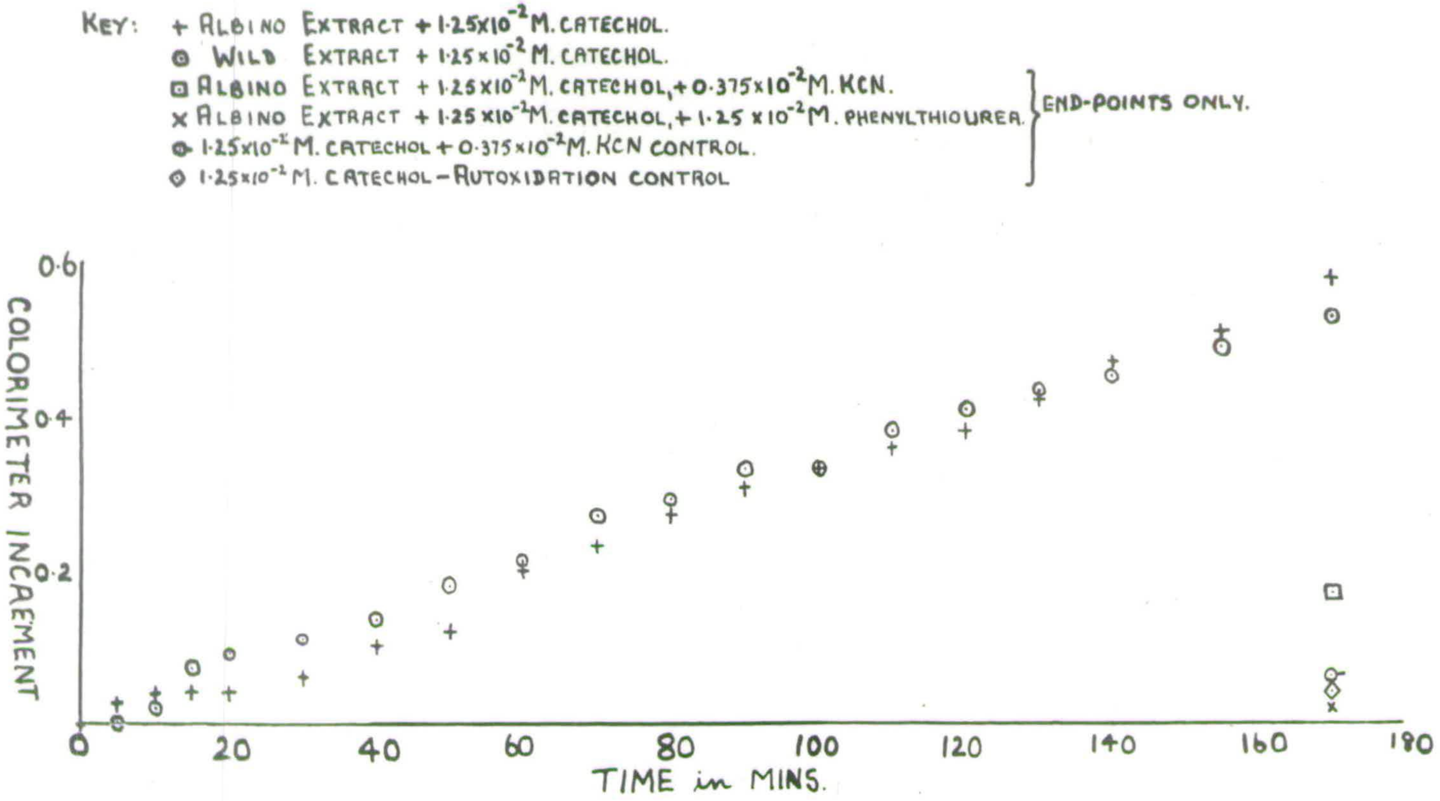

Figure 62. Relative activities of breis of albino and wild strain locust tissues towards catechol; the effect of cyanide and phenylthiourea on these activities.

As the oxidation of catechol took place the increase in light absorption was measured. Control solutions containing only albino extract and buffer gradually altered in their light absorption. The use of these extracts therefore meant that a correction had to be applied to allow for this variation. The light absorption values in the extracts of wild nymphs did not alter, in the absence of substrate. The results of the experiments are summarised in Fig. 62. In this graph, all the curves are corrected to compensate for the alteration in the absorption values of albino extracts.

The plots of the increments in light absorption of the breis against time show that the activities of the tissues of the two strains towards catechol are of the same order. Light absorption due to autoxidation of the catechol in solution is very small, and this made little difference to the values estimated for the extracts acting on catechol.

Since cyanide will inhibit both cytochrome oxidase and the phenolase complex (Sumner and Somers, 1953), the use of potassium cyanide in these tests would indicate only whether an oxidative enzyme was operating. To rule out any possibility that the cytochrome system might be involved, phenylthiourea was also used. This substance inhibits tyrosinase, but not cytochrome oxidase or succinic dehydrogenase (Dubois and Erway, 1946). Potassium cyanide was used at a final concentration of 0.375×10^{-2}M., and phenylthiourea at a final concentration of 1.25×10^{-2}M. The results are shown in Fig. 62. It /

It will be seen that phenylthiourea completely halted the oxidation of the catechol by the tissue homogenates. Cyanide at the concentration used depressed the oxidation.

One is therefore drawn to the conclusion that the tissue homogenates derived from both the wild and albino strains of locust nymphs will oxidise catechol. It seems also that the oxidation is an enzymatic one, and the enzyme involved is tyrosinase. Moreover, the enzymatic activity is similar in breis derived from wild or albino locusts.

COMPARISON OF THE RELATIVE AMOUNTS OF ASCORBIC ACID IN ALBINO AND WILD STRAIN LOCUST NYMPHS

Reducing conditions in the epidermis of insects have been suggested as the mechanism whereby the expression of the phenolase complex has been suppressed (Dennell, 1947). This effect is due not to inhibition of the enzyme, but to the reduction of the orthoquinones formed by its activity, in the presence of ascorbic acid (Dennell, 1947). Baldwin (1953) stated that if ascorbic acid is present in a test system where tyrosinase is acting on tyrosine, no melanin formation will occur until all the ascorbic acid has been oxidised. Consequently, it was of interest to compare the relative amounts of ascorbic acid occurring in the wild and mutant strains of locust.

Since a comparative result was desired, a simple technique due to Levine (1936) was used. Levine tested the ability of various substances to reduce sodium selenite to brick-red elemental selenium in neutral, acid, and alkaline media. He found that, of the many substances he tested, acidified sodium selenite solution was reduced in the cold by only ascorbic acid. The neutral and alkaline solutions could be reduced by many other agents in the cold.

In the present work two applications of Levine's technique were used. In one series of experiments attempts were made to measure /

measure colorimetrically the amount of selenium produced when locust blood or tissue extracts were added to acidified selenite solution. However, this was not possible, for two reasons. When the acid solution was added to the blood, or tissue extracts, the presence of the acid caused precipitation of protein in such quantities as to block the possibility of the detection of colour change due to any selenium produced. Additionally, since the selenium was being released in its elemental form, it tended to precipitate at very low concentration, colloidal suspension not being maintained.

As an alternative to the colorimetric method, which had proved able to detect 0.008% w/w of ascorbic acid in solution in synthetic mixtures (personal finding), the even simpler technique of Levine (1936) was used, whereby he merely added the selenite solutions to the test tissues to detect, non-quantitatively, the presence of various groups of reducing agents. Since the indication specifically of ascorbic acid had to be done in an acid medium, the tests on the locust material using this method involved the addition of pieces of tissue to the acidified sodium selenite solution.

Considerable care had to be taken, however, to exclude spurious colours similar to those due to the presence of elemental selenium. The acid nature of the solutions caused the insectorubin present in the nymphal epidermis to assume its bright red colour (Goodwin, 1952). This tended to mask any reaction due to selenium.

Control material exposed to twenty parts of hydrochloric acid added to 100 parts of water gave a good indication of the extent to which the colour due to the effect of the acid in the selenite reagent on the insectorubin was involved. Along with this, if the test solutions were left with the tissues for several days, the insectorubin pigments were gradually removed, when all doubt as to the implication of spurious responses from this source was eliminated.

The acidified selenite reagent as specified by Levine (1936) contained two per cent sodium selenite, to each 100 ml. of which were added 20 ml. of concentrated hydrochloric acid. Albino tissue and wild nymphal tissue were added to samples of the reagent. Controls were exposed to acid in the absence of selenite.

The wild nymphs showed some slight precipitation of selenium. In the epidermis it was not easy to detect, but where compressed tissues, such as wing rudiments, were examined, the colour became more apparent. Selenium was also precipitated on the glass of the containing vessel, as happened in the colorimetric experiments when syhthetic mixtures were used to estimate the efficiency of the technique as an indicator of ascorbic acid. The wild control material in acid, but without selenite, showed no similar effect.

In the albino there was conspicuously more selenium deposition. In this case, the wing rudiments became strongly brick-red. Again, it was not easy to pinpoint the deposition in thin layers of tissue, such as /

as the epidermis, unless several thicknesses were crinkled together. The glass of the containing vessel carried a substantial deposit of selenium. This response was totally lacking in the acid-only controls, and control samples of selenite solution without added tissue showed almost no spontaneous deposit of selenium in the same time.

It would seem, therefore, that there is significantly more ascorbic acid in the albino strain nymphs than there is in the wild ones. On the basis of these results, it is hoped to do further work to measure quantitatively the extent of the difference in ascorbic acid content between the two insects, using the method of Schaffert and Kingsley (1954).

EXPRESSION OF THE PHENOLASE COMPLEX IN THE LARVAL AND PUPARIAL CUTICLES OF PHORMIA

When the final instar larva of the black blowfly *Phormia terrae-novae* (Diptera:Calliphoridae) pupates there are two phases of development visible. The first starts with the contraction of the larva to the puparial shape, a process which takes only a matter of minutes. Then, as immobility becomes complete, eight transverse melanin bands appear on the puparium. The second phase, occurring after formation of the bands has become effectively complete, consists of the hardening, and the deposition of the general background colour, of the cuticle.

The observation that regionally restricted melanisation occurs here before sclerotisation becomes apparent would, of itself, suggest that the two processes are distinct. However if it were possible to inhibit one of the processes differentially, good evidence would be provided that they can act independently of each other *in vivo*. But the very nature of the reactions makes this difficult because both sclerotisation and melanisation depend on the same enzyme, tyrosinase (Mason, 1955a; Wigglesworth, 1957), and, in the early stages, the precursors of both processes are identical.

It was on the blowfly *Sarcophaga*, an insect closely related to *Phormia*, that Fraenkel and Rudall (1947) demonstrated the parent substance for sclerotisation to be tyrosine. They showed that there was /

was a fall in tyrosine content of the blood at pupation which was paralleled by the increase in weight of the puparium, when this latter increase was corrected for the loss to be expected in the deamination and degradation of the amino-acid to the simple aromatic acid, ortho-dihydroxybenzoic acid. This acid was thought to link adjacent protein chains after it had been oxidised to the corresponding quinone by tyrosinase.

The evidence for this sequence of events is mostly indirect, and is based on the fact that this acid was, and is, commonly regarded as the one derivative of tyrosine and dopa which possessed the necessary chemical property of having two reactive sites whereby it could become joined to two separate protein groups. And free protocatechuic acid could be isolated from the hardened cuticle in considerable quantities. This latter fact has now led Dennell (1958a; 1958b; 1958c; 1958d) to propose that protocatechuic acid is not responsible for tanning. He argued that if it were used in this way it would not be so plentiful as a free substance in the cuticle.

Consequently, Dennell suggested that some other system is operating in the blowfly cuticle, and proposed that non-specific hydroxylation of para-tyrosine, phenylalanine, or perhaps even tryptophan, is producing, not protocatechuic acid, but hydroquinone and catechol. When cuticle hardening takes place, he visualised the tyrosinase in the epicuticle of the blowfly oxidising the catechol

to /

to ortho-benzoquinone. This ortho-quinone then oxidises the hydroquinone, the latter not a tyrosinase substrate, to para-benzoquinone, which in turn reacts with, and tans, the proteins of the cuticle. The catechol, as a result of the oxidation of hydroquinone, is regenerated. The free protocatechuic acid present in the cuticle was seen as being derived from deamination and degradation of dopa. In other words, Dennell (1958d) saw the cuticle as being tanned, not by ortho-dihydroxyphenols but by para-dihydroxyphenols.

To explain melanin formation, Dennell postulated that dopa from the blood is oxidised by the epicuticular tyrosinase without undergoing the non-specific hydroxylation process, and thus gives a precipitate of melanin according to the normally accepted chain (Fig. 1). This process would not require deamination of the dopa, as ring closure in the molecule is an important step in the process of melanin formation.

Logically, it could be that both non-specific hydroxylation and the normally accepted deamination and degradation processes are both present in normal cuticle hardening in the blowfly and possibly in other insects. However, this interpretation does not allow of blocking the processes of hardening or melanisation selectively, as the same enzyme is still participating in both reactions to produce oxidation of dihydroxyphenols to quinones. Although its involvement in the oxidation of hydroquinone is indirect, it would appear to be, nevertheless /

nevertheless, essential. Therefore, if the enzyme were blocked, reduction in the hardness of the cuticle compared with that in controls should result, and be accompanied by a comparable suppression of melanin formation. It is not easy to see, on the other hand, how suppression of the tyrosinase activity could lead to inhibition of melanin formation while not having any apparent effect on cuticle hardening. Yet Dennell (1958d) reported just this effect in Phormia at pupation when the tyrosinase was blocked by phenylthiourea. Since this result was so unexpected to the present writer, it was decided to repeat this experiment to try to discover whether any similar effect existed on the background melanin formed in the puparia as they aged. It was also hoped to extend the use of inhibitors as reported by Dennell (1958d) to include others such as sodium diethyldithiocarbamate.

Phormia was particularly suited for this experiment in view of its depositing its melanin bands very quickly and before any apparent sclerotisation takes place. Background melanin is visible only after more extended periods of time. Any effects on either banding or sclerotisation would therefore be easy to detect. If the larvae contracted prior to pupation, and were unable either to deposit melanin or to sclerotise their cuticle, it was not clear whether this would be detectable. Intermediate stages, where some degree of reduction of the process of hardening was accompanied by recognisable variation from the normal shape of

the /

the puparium, coupled with lack, or reduction, of melanin, were the best prospect for detection of the effects of the inhibitors under these circumstances.

Variations in the melanin bands of the puparia in nature

Before preparing a prolonged series of experiments to try to elucidate the expression of tyrosinase in *Phormia* larvae and puparia, information on the normally occurring variation in the banding and background colour of these stages was necessary. Random samples of puparia were taken from the breeding cages. It was expected that the puparia would all be strongly banded, and that the background colour would be very dark. However, this was not the case. Not only did the intensity of the bands vary between individuals, but melanin was often totally absent, leaving a translucent, light brown puparium. The presence of such variation added considerably to the difficulties of experimenting with these puparia, because it was impossible to obtain uniformly pigmented controls.

In order to investigate more closely the relationships between the various aspects of darkening and hardening in these puparia, they were divided into those which had been vacated by the adults, and those which had not. The empty ones were further divided somewhat arbitrarily into two groups: those which were lighter in general appearance, and those which were darker. The dividing /

dividing line between the groups was subjective, and was chosen to be a fairly rich brown colour. In this way, examinations within the two series might yield differences in the proportions of banded and unbanded individuals, if the assumption were made that the puparia which were darker overall would also have stronger banding. If, on the other hand, this correlation did not exist, it would be suggestive that melanin expression in the puparia was undergoing a variation in control with time. In other words, if some puparia formed strong bands, and the tyrosinase in their cuticle was then for some reason rendered less active, for example, by products of its own activity, the background melanin would be relatively weak. Conversely, lightly melanised bands might exist due to a low initial tyrosinase activity at the early stages of pupation, followed by a later increase to boost the background colour. Several other combinations of colouring could exist. It should be possible, therefore, from examining the puparia to tell whether band and background melanisation varied independently.

Of the sixty-five puparia in a particular sample, fourteen were set aside temporarily, because they were not empty. Of the fifty-one empty puparia, twenty-eight were classified as dark, and twenty-three as light, but it may be emphasised that this differentiation was merely subjective, and was confined to the actual sample. The light and dark puparia were then examined for /

for the presence or absence of bands, or variations in banding intensity. The results are shown in the following table:

PUPARIA	LIGHT		DARK		DEAD	
	No.	%	No.	%	No.	%
Banded fully	12	52	16	57	9	64.3
Bands reduced, but evenly distributed	5	21.8	0	0	1	7.2
Bands very reduced, or irregular	2	8.7	2	7.2	0	0
Bands absent	4	17.4	5	17.8	4[a]	28.6
Puparia totally black			5	17.8	0	0
TOTALS	23	99.9	28	99.8	14	100.1

Footnote: [a] - contains only light puparium in the sample of dead puparia

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This table shows that any combination of intensity of background colour in the puparium can occur alongside any degree of banding. It also indicates that there is little difference in the type of banding variation between the dark background, and light background, puparia. The figure indicating the absence of reduced bands in dark puparia is not regarded as having much meaning, as the darker background masks differences between the levels of banding, so that only a major reduction becomes sufficiently obvious to be accepted as valid. Similarly, the very dark pupae could not be read at all.

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The column headed "Dead" lists those puparia in the original sample which were still closed. All were opened and found to be non-viable. Two contained dead adults, the remainder only dessicated larval material. It is interesting to note in passing that it was almost always found in experiments with these pupae that those which had died early in the pupal stage were completely dried out, although kept under conditions identical to their living, fully hydrated counterparts.

Although the figures from this particular examination of a random sample of unoperated insects do not include any dead puparia showing very reduced or irregular bands, these were commonly seen in other samples, and in uninjected controls in different experiments.

The existence of variability of this nature and extent in *Phormia* immediately raised problems in estimating the effect of injected solutions in subsequent experiments. But its presence also yielded information on the nature of the changes in the cuticle of the puparium, which could be obtained in no other way. That background melanin can be produced more or less independently of band melanin and *vice versa* is clearly obvious. The factor which does most to suggest the causal relationships between the two processes is time. As band melanin is always produced considerably before background melanin, there is ample opportunity for any mechanism /

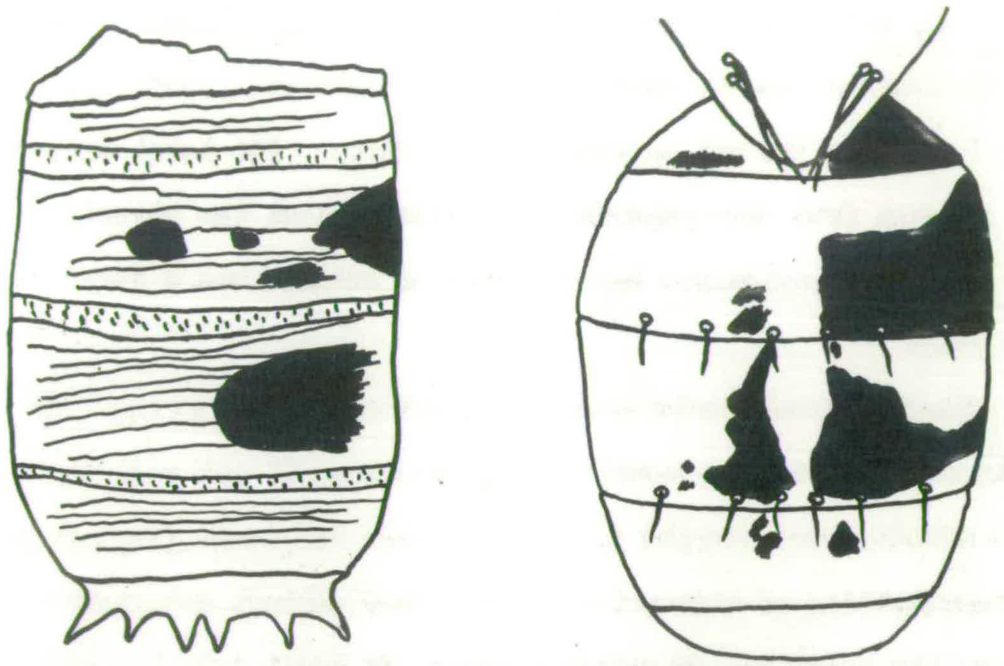

Figure 63. Teratological effects on the pigmentation in *Phormia* puparium and adult. The imbalance is similar in both instars.

mechanism supporting or suppressing tyrosinase to decay, or be reinforced, before the general background darkening process takes place. That viable puparia with no bands or general blackening could exist, and show no obvious lack of hardening, suggests that even when maximum natural suppression of melanin was occurring, cuticle hardening was not appreciably affected. The total absence of all melanin from some puparia also revealed that the structural colour of the non-melanised but sclerotised cuticle was a very light brown.

Teratological variants occur occasionally in <u>Phormia</u>. Of those found involving asymmetry of pigmentation, it was seen that similar effects were carried in both puparium and adult (Fig. 63). That abnormalities of this nature could occur without detectable effect on the hardening of either puparial or adult cuticle, and without introducing structural abnormalities, would seem to assist interpretation of melanogenesis and sclerotisation as independent processes.

Another naturally occurring phenomenon in <u>Phormia</u> involves the correlation between the size of the puparium and the degree of banding developed. On examining the puparia used in various experiments on the effect of inhibitors, it became apparent that the larger the puparium the more likely it was to be banded, regardless of the presence or absence of injected solutions. To confirm /

confirm this impression objectively, the puparia from batches of 500 larvae used in particular experiments were measured after eclosion of the imagos.

The lengths of the puparia were estimated under a binocular microscope. The length used was that between the anterior border of the puparium after the cap had split to release the adult, and the basal ring of the posterior processes.

Analysis of the results showed that in uninjected controls the fully banded puparia were very significantly longer than the unbanded ($P << 0.01$). Similarly, larvae injected with 0.002 ml., or 0.004 ml., of saturated solutions of phenylthiourea in 0.75% saline showed differences in size between the banded and unbanded groups significant at $P << 0.01$. In the controls injected with equivalent volumes of saline solution the differences in size between the groups of banded and unbanded larvae was of the same highly significant order.

Comparison between groups injected with comparable volumes of saline or phenylthiourea showed no significant differences between the samples, nor did these samples differ from the controls significantly in size. This correlation of banding with size of the larvae does not seem to have been noted before. Its presence adds another interesting facet to the study of the expression of the phenolase complex in insects. Whether the size of the larva and /

and the degree of banding it displays at pupation are causally related, or whether both are dependent on some common controlling mechanism in larval growth and development, cannot be suggested at present, but it would appear unlikely that mere size could affect the degree of banding directly to the extent it was found in Phormia.

Effects of low temperature on melanin patterns in Phormia

Many insects show an increase in melanin deposition if maintained at lower temperatures (Kennedy, 1956; Wigglesworth, 1939). Consequently, if Phormia larvae at the stage just prior to pupation were kept at temperatures much lower than those in which they were normally bred and maintained in the laboratory, it was hoped to show an increase in the intensity of the melanogenesis. If such a reaction to cold could be obtained, it would help to substantiate that the background colour in the puparia of these flies was indeed melanin. It has not been possible to find conclusive proof in the literature that the black bands and background darkening on Phormia are due to melanin, and reliance has again been placed solely on the evidence obtained personally by the techniques described for proving the induced pigmentation in albino locusts to be melanic. Additional corroboration by reaction of Phormia to cold would therefore be helpful.

Early attempts to achieve this result by continuous exposure of late larvae to temperatures of 5°C. failed, due to mortality /

mortality of the larvae, and their inability to form a significant number of puparia at this degree of cold. As in the experiments with cold-treatment of albino locust eggs, a compromise was struck by exposing the larvae to 5°C. for approximately twelve hours per day, and to 15°C. for the remainder of the daily period. Under these conditions puparia were formed very readily. Whereas pupae at 30°C. took about five to six days to produce adults, those treated at 5°C. / 15°C. took seventeen to nineteen days. The yield of imagos from the puparia was of the order of 80%.

The results of this experiment indicated that a marked increase in the darkening of the puparia took place in response to low temperatures. This was seen in particular in the background colour, and, subject to the qualification that increased background tended to mask band variation, also in the bands.

The point of major significance in this experiment is that the cold brought up the general body colour so prominently. This very strongly corroborates that the pigmentation was indeed due to melanin. It also emphasises that the enzyme/substrate system responsible for general melanin production is widely distributed over the cuticle, and is not restricted to the regions which lay down melanin bands in the white pupa. Since it is this same enzyme which is responsible for the production of tanning quinones, it would, on this latter count, be expected to be present over the whole /

whole area, and this as a consequence only emphasises the need to understand how melanin comes down, in the control insects, first of all in well-defined bands before it does so over the whole cuticle.

Chemical interference in the puparia

The method used in attempting to inhibit the tyrosinase in the larvae was simple injection of various concentrations of the inhibitors, phenylthiourea (Dubois and Erway, 1946) and sodium diethyldithiocarbamate (Mason, 1955a). Dennell (private communication) stated that he injected 0.05 ml. of a 0.01 M. solution of phenylthiourea into larvae within a day or two of pupation. Under these conditions, he was able to demonstrate that band formation was blocked in the puparium, while the sclerotisation involved in puparium-formation proceeded.

In the present work it was not found possible to use such large volumes. This amount of inhibitor gave 100% mortality of the injected larvae. The most obvious reason for this was that the relatively vast amount of solution injected distended the larvae grossly, and they never recovered. Lesser volumes were tried, down to 0.002 ml., and the concentrations were increased in other experiments with different volumes to saturated solutions of phenylthiourea, which are 0.025M. at room temperature. It soon became obvious that it was preferable, for maximum survival of the larvae, to use the saturated solution in smaller volumes. Controls were /

were run with samples of uninjected larvae, and larvae injected with a volume of 0.75% saline equal to that of the inhibitor used. Since all inhibitors used were dissolved in the same 0.75% saline, this second type of control would give a good indication of the amount of mortality or abnormality introduced by dilution of the larval blood, or distension of the body, while leaving the osmotic balance fairly constant.

This latter possibility of distension effects was considered, as Wigglesworth (1934, 1954) has shown that such a mechanical effect is predominant in the control of ecdysis in *Rhodnius prolixus* (Hemiptera). It would not be expected to be important here as the moult at pupation is determined considerably earlier in larval life by the distension of the crop, but the possibility that a second distension, or compression, of any organ might affect pupation could not, nevertheless, be ignored.

Any effects of injections on the banding were read at pupation, and after eclosion of the imagos. Tests were made to confirm that these latter readings were valid by immersing the cast puparia in benzene, chloroform, or water, to eliminate any colour due to trapped air or to other possible structural effects. Puparia were not read when the adult inside was approaching full development, as the presence of the adult masked the true puparial bands.

Another technique tested to detect any effect on banding

was /

was to measure the time taken from the initial contraction of the larva until the melanin bands on the puparium were just detectable. After this, the time to complete development of the bands was read, and the results of both measurements compared between controls and the various groups of treated larvae. In this way, any slowing, though not totally inhibiting, effect of the inhibitors should be detectable. However, this technique suffered from its cost in time, as, to be accurate, the time measurements had to start precisely from the period of initial contraction. Unless the larvae were examined every five minutes this requirement could not be met.

It was soon found that the scatter of the results prevented this technique being of much use here. Control insects commenced to deposit bands thirty to forty-five minutes after initial contraction, at room temperature, and the various groups injected with saline as control, or phenylthiourea as inhibitor, showed times of the same order. The time for full development of the bands for the majority of puparia in all these groups was from 80 to 120 minutes, with the control, uninjected insects tending to group at 100 to 120 minutes. Of these groups of larvae, however, all showed insects producing no bands, and some produced bands only after much longer periods, although this latter occurrence was relatively rare.

Within /

Within the limits of this technique, therefore, it is clear that there were no changes in the timing of band formation in the larvae containing inhibitor from those found in the controls.

Effects of phenylthiourea: It has already been noted that it was not possible to use 0.05 ml. of 0.01 M. phenylthiourea in this work. The larvae used with this volume of inhibitor all died quickly. Consequently 0.025 ml. at 0.02 M. per larva was tried, thus keeping the absolute amount of phenylthiourea injected constant. Again, a very high mortality was encountered. This discrepancy between the results of Dennell's work and of those presented here suggested that the method of culture of the larvae in my case was not satisfactory, and every care was taken to eliminate crowding, to supply fresh medium copiously, to try variations in the composition of the medium, and to pick out for experimentation only those larvae which were fully fed and migrating.

After this there was still no worth-while survival following injections of 0.025 ml. of 0.02 M. solutions of phenylthiourea, and none at all after 0.05 ml. of 0.01 M. solutions. Therefore, experiments were performed using saturated solutions of phenylthiourea, 0.025 M. Varying amounts were injected, from 0.002 ml. to 0.025 ml., using injections of corresponding volumes of 0.75% saline as controls. In this case, it was soon clear that the inhibitor, at the volumes which permitted survival of the insects, was having no detectable effect /

effect on the pigmentation of the bands in the puparia. Wound spots at the site of injection were always melanised. No viable misshapen puparia were obtained, as was the case when sodium diethyldithiocarbamate was used.

Dennell (1958d) mentioned that Colvard Jones had informed him that pupation in Sarcophaga bullata was not prevented by injection of phenylthiourea. At the time the work was done, the only explanation for this was that the amount of inhibitor used was not sufficient (Dennell, 1958d). Prevention of pupation might have been expected on the grounds that tyrosinase activity was believed to be required for the production of tanning quinones to convert the larval cuticle of the final instar into the puparium. If the enzyme was inhibited, then pupation would be expected to be upset, or prevented. No mention was made of any change in the background colouration of the resulting puparia, which, unlike those of Phormia, do not have bands of melanin.

In view of the failure in the work done here, on Phormia, to block banding or sclerotisation by the use of phenylthiourea, it was therefore of interest to spend a short time confirming this finding of Colvard Jones, and endeavouring to find out whether the final colour of the puparia in Sarcophaga was altered.

Accordingly, 0.01 ml. of a saturated solution of phenylthiourea in 0.75% saline was injected into the larvae of Sarcophaga carnaria.

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This particular volume was chosen on the basis of results previously obtained from work on *Phormia* as being that most likely to exercise an effect on the tyrosinase in the larvae without causing total mortality, and the choice was found to be justified. Some considerable mortality in the experimental animals was encountered, being evidenced by their puparia not yielding adults, although almost all the larvae pupated. However, a loss of insects as puparia was also present to a lesser degree in the controls. Further, the control adults emerged over a period of three days, although the larvae used in the experiments had been chosen carefully for cessation of feeding. The injected insects yielded imagos in the same period as the controls. This confirmed that the injected larvae of *Sarcophaga carnaria* under the conditions used could pupate, and it was found that the background colour, under the same conditions, was unaltered.

The conclusion would seem to be that phenylthiourea injection into two further dipterous species, *Sarcophaga carnaria* and *Phormia terrae-novae*, does not prevent pupation. When coupled with the results of Dennell and Colvard Jones, the apparent conclusion would be that Calliphorid larvae show a mechanism of tanning in their cuticle, when they are undergoing puparium formation, which is not tyrosinase catalysed. However, such a conclusion would be contrary to much other evidence (Fraenkel and Rudall, 1947; Dennell, 1947). A much more reasonable interpretation of the results /

results is presented in the discussion to this thesis.

Since it had proved impossible to duplicate Dennell's results, even when using half the injection volume with twice or two and a half times the concentration of inhibitor, it was necessary to look more closely into the details of phenylthiourea concentration in the operated insects, and to compare this figure with that found to be satisfactory in *in vitro* work. To carry this out, no attempt was made to measure directly the final concentration of phenylthiourea in the larvae or pupae. An estimate of sufficient accuracy for the purpose in hand was made on the following basis.

When the larva contracts to form the pupa, there is no change in the volume of the body tissue. On the other hand, the puparium formed is of such a shape that its volume can be estimated from linear measurements. It is virtually a cylinder whose radius and length can be measured under a low power microscope. The volume of this cylinder must represent also that of the larva immediately prior to pupation.

Since the object was to confirm that a sufficiently high concentration of phenylthiourea for tyrosinase inhibition was present in the larva after injections such as those described above, all estimates of body volume were made on larvae from the higher end of the size range, and no correction was made for the volume error introduced by the fact that the puparium had

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tapered ends. In this way, a figure utilising the maximum dilution would be obtained for the final concentration of any injected solutions. Similarly, the larvae were assumed to consist entirely of water, an assumption which again gave no reason to under-estimate the dilution of the inhibitor by the body fluids.

The measurements of the defined puparium used here were:-

Length overall, excluding posterior processes, 8 mm.:
Maximum diameter ... ... 3.5 mm.

Therefore the volume of the defined puparium was 0.077 ml.

If 0.025 ml. of 0.025 M. phenylthiourea were injected,

Total volume of larva and injected solution = 0.102 ml.

Therefore the final concentration of phenylthiourea in the larva

$$= \frac{0.025 \times 0.025}{0.012} \text{ M.}$$

$$= \underline{\underline{6.1 \times 10^{-3} \text{ M.}}}$$

This figure is the maximum used in the experiments.

Similarly, if 0.002 ml. of 0.025 M. phenylthiourea were injected, the final concentration of phenylthiourea in the larva is ... ... $= \underline{\underline{6.3 \times 10^{-4} \text{ M}}}$.

This figure is the minimum used in the experiments.

Noting again that these figures for estimated final concentrations of injected inhibitor under various conditions give minimum values, it can been seen that they fall within what might be called the "typical range" of concentrations for many enzyme inhibitors.

On /

On considering in detail the findings of other workers using specifically tyrosinase and phenylthiourea, the work of Dubois and Erway (1946) shows that the final concentrations estimated here were not too low to inhibit tyrosinase. These authors found that 50% inhibition of tyrosinase _in vitro_ could be obtained by using a final concentration of 2.5×10^{-7} M. phenylthiourea. Consequently, any doubt as to whether a sufficiently high concentration of phenylthiourea had been used in the present experiments would seem to be dispelled.

It is of interest, in this consideration, to note that Dubois and Erway quoted a figure of 10 mg. per kilo of body weight as being "an uniformly fatal dose" for the rats used in their experiments. When the minimum dose giving 100% mortality of the _Phormia_ larvae was calculated, a minimum figure of approximately 1250 mg. per kilo body weight was indicated. Whether this radical difference in lethal level between mammal and insect can be interpreted as mirroring differences in delicacy of homoeostasis is a moot point. Jenkins (1960a, 1960b), working on the effect of mammalian goitrogens on planarians, showed that exposure of these animals to concentrations of phenylthiourea equivalent to 76 mg. per kilo, for ten days, did not have a significant lethal effect. This figure is already almost one order of dimensions higher than that ensuring total mortality in rats. Such an indication helps to corroborate the estimations of the lethal level in _Phormia_.

The /

The only conclusion from the results of the experiments reported on the injection of phenylthiourea into *Phormia* and the comparison of the estimated final concentrations of the inhibitor in the insects, seems to be that the tyrosinase in the insects used here was not being inhibited, due perhaps to the inhibitor's not being able to reach the epicuticular enzyme, or that alternatively the inhibition was reversible. These possibilities are argued in detail more appropriately in the discussion to this thesis.

Effects of sodium diethyldithiocarbamate: Since the use of phenylthiourea in the work presented here had failed to bring about inhibition of the banding in *Phormia* puparia without affecting sclerotisation, it was decided to try to do so by the use of another inhibitor. This was sodium diethyldithiocarbamate, listed by Mason (1955a) and Lerner and Fitzpatrick (1950) as an inhibitor of the phenolase complex. If it were injected into late *Phormia* larvae, and the pattern of results obtained was similar to that when phenylthiourea was used, then some confirmation of the inability to block melanin formation in these insects in preference to the sclerotisation process would be obtained.

When experiments were done with sodium diethyldithiocarbamate, using constant injection volumes of 0.002 ml., and varying the concentration injected from 10^{-3} M. to 10^{-1}M., no significant reduction /

reduction of the bands on the treated puparia was found. These volumes and concentrations were such that they gave minimum figures of 2.5×10^{-6} M. to 2.5×10^{-4} M. as estimates for the final concentrations of inhibitors in the insect. Using a constant concentration of 10^{-2} M., and varying the volume injected from 0.002 ml. to 0.01 ml., estimated final concentrations were from 2.5×10^{-4} M. to 1.25×10^{-3} M.

In these latter experiments, a distinct trend was seen towards the formation of misshapen puparia. When 0.002 ml. of a 10^{-2} M. solution of the inhibitor was used, almost all the puparia were normally shaped, and no consistent difference could be seen between their banding and that of controls. When 0.004 ml. of the same inhibitor was used fewer puparia were formed, and these varied from the typical puparium to types showing less contraction and streamlining than controls. If 0.01 ml. of inhibitor was injected, the resulting puparia were rarely typically shaped, most being elongate, and showing more clearly the spinose areas of the larvae from which they had been derived. These misshapen puparia were brown, and could not be reliably differentiated from control puparia in colour. Since working with this amount of inhibitor introduced heavy mortality, only 25% of the larvae injected produced puparia. But, of these, all were unbanded. All, nevertheless, like those of the groups with lower volumes of injected sodium diethyldithiocarbamate, showed a typical black spot /

spot at the site of injection. The larval types of puparium were capable of producing living adults. These adults appeared normally developed and coloured, but were very short-lived in comparison with control adults produced in the same experiments.

The results of this experiment indicate several conclusions of interest. The mortality of the larvae, and the reduced viability of the adults from the injected puparia, indicate an anti-metabolic effect of the sodium diethyldithiocarbamate. This effect is apparently being carried over into the adult, but there is no evidence to suggest whether the injected inhibitor persisted into the adult. Further, these results indicate that, contrary to what might be forecast from considerations of the involvement of tyrosinase in both sclerotisation and melanisation, it is possible to prevent melanisation without blocking cuticle hardening completely. However, this effect is obtainable only when the amount of inhibitor injected is very close to that giving total mortality. The discrepancy between Dennell's (1958d) obtaining melanin inhibition without preventing pupation in Phormia, when using phenylthiourea as inhibitor, and the inability in the present work to duplicate his results, is the more puzzling in view of the fact that suppression of melanogenesis when using sodium diethyldithiocarbamate at high concentrations was produced. It would appear that the larvae used here were not unable to demonstrate such an alteration in melanin expression, but could not be induced to do so under the influence of phenylthiourea.

Effects of catechol: Since catechol had been found to be capable of exercising such a strong effect on the melanin producing system in locusts, it was of interest to confirm that Phormia larvae could metabolise this substance. On injection of 0.01 ml. or 0.02 ml. of 3×10^{-2} M. catechol, the larvae were all killed in less than twenty-four hours. The larvae all turned dark brown, and then rapidly became an intense black.

Tests for ortho-diphenols in the dead larvae by treatment with ferric chloride followed by sodium bicarbonate gave negative results in all cases. This therefore suggests very strongly that ortho-dihydroxyphenols were absent from the dead larvae, as control tests using volumes of catechol identical to those injected into the larvae gave the strong green and purple colours indicative of ortho-dihydroxyphenols (Cohen, 1950).

This experiment does not prove that the injected substance was oxidised, but merely that it was metabolised. Since catechol is capable of acting as a substrate for tyrosinase, an enzyme plentiful in blowfly larvae just before pupation (Dennell, 1947; Fraenkel and Rudall, 1947), it seems logical that oxidation of the injected catechol can explain its removal, and also that this oxidation aids the production of the dense blackening of the larvae on death. Dennell (private communication) has subsequently informed me that injected catechol is oxidised by Calliphora larvae, and that the insects all died quickly. The results from experiments on the two genera therefore agree closely.

DISCUSSION

In studying a problem which has invited the proposition of several theories, it is preferable to base investigations, at least initially, on those concepts which have had substantial backing. This is particularly necessary in the present work because there is still a great deal of controversy over the proposed theories on both melanin formation and the hardening of cuticle in insects. However, the arrangement suggested for the various steps in the formation of melanin is generally accepted (Mason, 1955a). The participation of quinones, derived from tyrosine, in the process of tanning also seems certain.

Views on various details of these processes have not been so readily substantiated. For example, there is some uncertainty as to whether the phenolase complex responsible for cuticle hardening resides in the epicuticle (Dennell, 1947; Malek, 1952). Mason (1955a) and Hackman (1958) maintain that while the evidence brought forward for the theory that the tyrosinase in the epidermis of the young dipterous larva is later transferred to the inner epicuticle is reliable enough, the methods used can be faulted because they are not specific. It is admitted, however, that the view that tyrosinase is present in the epicuticle is consistent with sclerotisation being initiated at the outer surface of the procuticle, before it extends inwards. It might be interesting to learn, nevertheless, whether the positive reaction to the Nadi reagent /

reagent used to locate tyrosinase might not also be given by quinones that were formed in the epidermis before being passed to the outer surface of the procuticle.

The question of whether ortho- or para-quinones are responsible for tanning or hardening remains to be answered unequivocally. The work of Pryor (1940a, 1940b), and of Brunet and Kent (1955) strongly suggests that ortho-quinones are responsible. Dennell (1958a, 1968b, 1958c, 1958d) puts forward in detail a case for the participation of para-quinones. Karlson (in a discussion in a paper by Hackman, 1958) contradicts Dennell, saying that work on the incorporation of tyrosine-2-C^{14} into the puparia of *Calliphora* indicates that dopa is incorporated to a high extent, without loss of the side chain. However, it is not clear whether Karlson may not be measuring the incorporation of dopa into melanin in the cuticles. Nevertheless, if dopa is indeed being incorporated in such a relatively unchanged state into the puparium, Dennell's argument that protocatechuic acid would not be present in such large amounts in puparia if it were the tanning agent receives additional support.

It would be very revealing to combine the experiments of Dennell and Karlson, and investigate whether the radioactivity in generally labelled para-tyrosine injected into *Calliphora* larvae could be recovered from hydroquinone and catechol in the cuticle, or from the aminophenol derived from hydrolysis of puparia.

The /

The results of the present work show that in the wild strain of Schistocerca gregaria the enzyme has led to the formation of a fully tanned but colourless exocuticle in the outer region of the outer procuticle. The inner layer of this region is retained in the mesocuticular condition, as demonstrated by its affinity for acid fuchsin (Lower, 1956). The staining of the inner procuticle by Aniline Blue, and of the whole outer procuticle by Heidenhain's Iron Haematoxylin agrees with the interpretation of a three layered cuticle in the wild strain locust.

In the albino, the fuchsinophilia indicated that the exocuticle was less developed. Since the regions which were fully sclerotised were adjacent to the epicuticle, it is likely that the supply of the tanning phenols is derived from the epicuticular region. A reduction in the formation of exocuticle in the albino was also shown by the results from the use of eosine. However, the action of this stain on cuticle is not known, so the results are really acceptable only on a comparative basis. This stain was used only to demonstrate differences between the cuticles of the wild and albino strains of the same species of locust.

The precise nature of the reaction of the cuticle to Lower's staining technique is not known (Lower, 1957a), therefore it is impossible to verify which phenolic material is taking up the stain. Lower pointed out that the results seemed to vary according to the way /

way in which the phenolic material was bound into a structure. However, the significant depression of the reaction in the albino compared with that in the exocuticle of the wild strain may be explained in two possible ways. Either there is a reduction in the absolute amount of phenolic material, or the material may be combined in a manner which renders it incapable of reacting with the stain used. Since the apodemes, representing exceptionally hardened parts of the cuticle, were strongly iodophil, it is likely that the stain reacted most readily with structures which showed a high degree of tanning. On this interpretation, it is therefore possible that while the outer half or third of the outer procuticle derived from the wild strain resembled exocuticle, only the outer one-fifth or less of the outer procuticle of the adult albino resembled exocuticle. That this exocuticular region of the albino cuticle also took up acid fuchsin suggests that it was not fully tanned, as was the case of the exocuticle of the wild strain.

If we accept Lison's argentaffin method as a means of demonstrating the presence of reducing substances of the polyphenol type, a comparison of the results of using the method on the cuticles of the two locust strains indicates that the cuticle of the albino contains considerably less polyphenols than that of the wild strain.

When the results of the different staining tests described

are /

are collated, there is a very strong indication that the albino is not producing an adequate supply of polyphenols in its epicuticular region. As a consequence, the exocuticle in the albino can be formed only on a scale below that found in the cuticle of the wild strain. Such an explanation fits in with the results obtained. It does not explain, however, why the requisite quinones are not being produced. Whether the epicuticular tyrosinase is absent or deficient in supply was not deducible, but total absence of the enzyme would seem to be ruled out by the presence of a certain degree of hardening. It may be that a restricted supply of substrate from the blood and epidermis is responsible for the limited amount of tanning which takes place. Partial inhibition of the action of the blood tyrosinase on tyrosine to provide dopa for the epicuticular enzyme might also explain the results obtained. It is interesting that the albino had a higher content of ascorbic acid than the wild strain. This substance has been implicated as a reductant of the quinones formed by tyrosinase. Its presence in the albino suggests that tyrosinase expression in this strain may owe at least some of its modification of expression to the ascorbic acid.

Since the methods adopted in the past were unable to provide answers to such questions, it seemed possible that a method designed to introduce excess substrates of various kinds into the albino so that it could utilise them <u>in vivo</u> might give results which /

which would elucidate the nature of the processes responsible for melanin deposition and the hardening of the cuticle. Injection methods are undesirable because of the effects on body metabolism (Williams, 1952; Schneiderman and Williams, 1953). In any case, the albino locusts succumbed to the after effects of being punctured with a needle. All attempts to link the blood streams of the wild and albino nymphs by parabiosis, a technique which has been so helpful to insect physiologists, were unfortunately unsuccessful. The only alternative seemed to lie in passing the test substrates directly through the cuticle. There is now a growing body of evidence to show that substances can cross the intact cuticle, to enter the haemocoel. For example, Rajindar (1947) showed that large quantities of water could be transported through the cuticle of _Periplaneta_. Organic substances such as acetic acid and ether were also readily transported. However, ether, being a fat solvent, could at the same time have been radically altering the structure of the cuticle. Glycerine failed to penetrate the cuticle. Eisner (1961) does not state explicitly that acetic and caprylic acid penetrate the cuticle of cockroaches, but his descriptions clearly imply that he considers this to be the case.

In the immersion experiments, it was shown that orthobenzoquinone entered the insect rapidly, and was lethal. The concept that the various compounds tested were penetrating the cuticle and entering the haemocoel was supported by the fact that the /

the anterior region of the albino, which was not in contact with the test solutions of catechol, produced a pigment pattern. The prolonged survival of the test albinos, moreover, indicated that such pigment deposits were in no way the result of a pathological response.

In turning to a consideration of the effects of the different compounds experimentally introduced into the albinos on the cuticle of these insects, the most puzzling result was the inability of the insect to utilise 3,4-dihydroxyphenylalanine (dopa). One would have expected this compound to have been converted readily into a melanin which would have been deposited in the cuticle and there to have produced a pattern. That this did not prove to be the case invites explanation. This, however, is not easy to provide. One may recall that Yasunobu (1959) showed that mushroom tyrosinase catalysed dopa oxidation one thousand times less rapidly than it did the oxidation of catechol. It would be interesting to learn whether the tyrosinase in the albino has the same relative activities towards these substrates. Preliminary results obtained from estimating the activity of breis towards dopa seem to be in line with this possibility. However, tests with breis are not the most reliable means of obtaining information having a bearing on such questions.

The inability of the albino to utilise protocatechuic acid to lay down a deposit of melanin in its cuticle is in agreement with /

with the accepted conclusion that, in insects, this compound can tan cuticles without, at the same time, being implicated with the process of melanin formation. In the test albinos, the degree of hardening of the cuticle was increased by the added protocatechuic acid. The results obtained in the immersion experiments also confirm the view that tanned cuticles need not necessarily be coloured (Dennell, 1958d; Hackman, 1958; Mason, 1955b).

Whether tryptophan can be utilised only at an exceedingly slow rate by the albino for conversion to a melanin product, as suggested in the case of dopa, it is difficult to say. On the evidence produced, it is certain that either this, or the complete failure of the albino to use the amino-acid, was reflected in the absence of a pigment deposit when tryptophan was introduced.

The results obtained with catechol as the test substrate reveal two important changes in the cuticle of the test albinos. Tanning is increased, as shown particularly by the results of using acid fuchsin stain. And secondly, a pattern of pigment is laid down, that conforms closely to that naturally present in the wild strain locust. In addition, the development of the pattern shows a remarkable resemblance to the way the pattern develops in the wild strain locust.

Another feature of considerable importance also arises from these results with catechol. Whereas the increased tanning effect on /

on the cuticle was uniformly distributed, melanin deposition was restricted to regions where the colour was relatively intense. The extra tanning of the cuticle would not seem to result in any colouring of the cuticle in those regions where melanin was not deposited.

It seems likely that in the albino there is a lack of requisite quinones for changing the mesocuticular condition to an exocuticular one. Mason (1955b) showed that proteins joined by a single bond to a quinone nucleus, the catecholic-proteins, cannot act as substrates for phenolases. They can be oxidised to the quinonoid-protein state only if there is a high ortho-quinone to protein ratio, the ortho-quinone carrying out the oxidation while itself being reduced in the process. This quinonoid-protein can then re-act with another terminal amino group of another protein to form a di-substituted derivative.

That the albino cuticle is predominantly of a mesocuticular nature may be due to the presence in it of proteins, which, although combined with an ortho-quinone, are not in fact fully tanned. According to Mason (1955b), this could be explained by the supply of free quinones, formed in the epicuticular region containing the tyrosinase, being insufficient.

In view of the above considerations, it seems then that one of the functions of the quinones in the wild strain is to convert catecholic-proteins to quinonoid-proteins. It is likely that /

that the quinones formed in the epicuticular region in the presence of tyrosinase carry the enzyme's oxidising energy to the impregnated proteins in the outer procuticle. There, the quinones oxidise the proteins to the quinonoid-protein state, while they are themselves reduced to dihydroxyphenols. On this view, the occurrence of large amounts of free protocatechuic acid in the tanned puparia of blowflies (Dennell, 1958d) might represent the spent energy-carrier coupling the oxidative power of the epicuticular tyrosinase to the non-tyrosinase-oxidisable catecholic-proteins of the outer layers of the cuticle.

On the basis of the results of the present work on both the albino and wild strains of Locusts one is therefore drawn into concluding that the albino wild cuticle, although basically colourless, is tanned. Malek (1957) came to the same conclusion. In addition, the results would seem to favour the view that in the same cuticle the two processes of melanisation and sclerotisation might be capable of proceeding independently, despite the same enzyme being involved in the initial reactions of both processes.

The inhibitory studies on _Phormia_, designed to throw further light on the question of to what extent melanisation could be distinguished from the process of hardening of the cuticle, gave results which were unexpected. _Phormia_ deposits melanin in the puparium prior to this structure becoming truly hardened. Here then presumably were the two processes taking place sequentially. Dennell (1958d) reported that he had obtained results showing that

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the deposition of the melanin bands in Phormia could be halted by phenylthiourea without interfering with the subsequent hardening of the puparium.

For several reasons pertinent to the present work, and particularly to the work on the experimental induction of melanin patterns in albino locusts, as already discussed, inhibition studies were carried out on the phenolase system operating in Phormia. However, it was disconcerting to find that with this insect the results obtained by Dennell (1958d) could not be repeated, as had been hoped. However, if sodium diethyldithiocarbamate was used as the tyrosinase blocking agent instead of phenylthiourea, at concentrations approaching a lethal level, it was possible to obtain corresponding results.

It is significant that, in the puparia of untreated Phormia, there was a wide range of variation in the intensity of the banding. At one extreme, bands were formed, along with an intense deposition of black background melanin. At the other, no bands at all were produced, and in addition no melanin was deposited in the background. Further, even when banding occurred, the background melanin deposit tended to be of varied intensity. In other words, variation in the intensity of banding seemed to be independent of the degree of intensity of the background melanin colouration. This suggests, therefore, that in Phormia the amount of substrates available may not /

not be fixed. Or, if it is, it is likely that the ability to utilise it varies from individual to individual. However, as the results of the present work show, it was possibleto intensify the deposition of background melanin by exposing the late larvae and pupae to low temperatures. Unfortunately, there is no information that points to how low temperatures might be responsible for causing a greater development of melanin.

It is interesting to recall that Hackman and Todd (1952) have suggested that an ortho-quinone linked by one bond to a protein might be able to undergo ring closure and oxidation, as mentioned earlier, to give a dark-coloured compound. They also pointed out that the extent of this occurrence must be affected by whether there were adjacent amino-groups available for linkage to the quinone. If there were adequate groups, then the quinone would be expected to join to these. If, however, there were few such groups available, it would not be possible for this linkage to occur, and, under these circumstances, ring closure might take place, to give a product with general absorption of light.

It is significant that in <u>Phormia</u> the background darkening of the cuticle occurs fairly late in puparial development, when most of the sclerotisation has been completed. In such a case, the number of adjacent sites available for quinone linkage would be low, and consequently mechanisms of the type proposed by Hackman and Todd (1953) would be favoured. The process would still require the presence /

presence of active tyrosinase, not to oxidise the catecholic-proteins, which Mason (1955b) has shown not to be substrates of tyrosinase, but to oxidise free ortho-quinones at the epicuticle. These ortho quinones would then be able, if present in relatively high concentration, to oxidise the catecholic protein to the quinonoid protein (Mason, 1955b). The fact that puparia showing a strong development of the background colour could also be totally unbanded suggests that if the above mechanism were operating to produce general melanisation effects in Phormia, the active enzyme necessary was not able to produce banding at the same time. Consequently, the puzzle as to why these bands were not produced remains as unbroken as before.

The non-banded puparia with no background melanin might, on this interpretation, be regarded as a result of the overall depression of tyrosinase activity. This hypothesis implies that a lesser degree of sclerotisation may also be present. Such an effect was not detectable, but it is extremely difficult to find a method of measuring the degree of sclerotisation. Attempts to do so using the Young's Modulus, M, of the cuticle failed, due to the reduction in extensibility of the sclerotised cuticle of Phormia being so great that the methods used, whereby extension of loaded samples of cuticle was measured microscopically, could not read the extensions produced in the tanned cuticle on loading. It was, of course, comparatively straight-forward to measure the modulus of larval cuticle along the breadth /

breadth of any segment, when figures ranging from 1.69×10^7 dynes per sq. cm. to 3.3×10^7 dynes per sq. cm. were obtained (Personal finding). These numbers immediately confirm the elastomeric nature of the untanned cuticle, such a range of M being highly characteristic of elastomers (Stacy, Williams, Worden and McMorris, 1955). Under these conditions, Hooke's Law is obeyed only during very limited degrees of extension. Internal yield values are low, and this can be awkward in attempts to obtain accurate values of M, particular care to work within the elastic limit of the cuticle being essential.

In the puparial cuticle, a very different state of affairs exists, when all elastomeric properties are hidden by the relatively rigid nature of the tanned proteins. When sclerotisation occurs, it is no longer possible, with the techniques used, to measure the Young's Modulus of the cuticle, and thus to establish whether the Modulus might be used as an index of the degree of sclerotisation. There is, therefore, no objective proof that unbanded puparia with no background melanin are, or are not, fully sclerotised, although subjective observations suggest that they are not altered in hardening from the normally pigmented ones.

To return to the work on phenylthiourea injection into *Phormia*, it would seem to be proved, from the discussion of the estimated final concentrations of the inhibitor in the insects, that the concentration of phenylthiourea in the larvae and pupae was /

was not too low to inhibit tyrosinase. It now remains to explain this inability to stop the melanisation.

It is known that in the blood of the blowflies Calliphora and Sarcophaga at pupation there is a high concentration of tyrosine and tyrosinase (Dennell, 1947; Fraenkel and Rudall, 1947). It is therefore logical to assume that there is a similar occurrence of these substances in Phormia, a closely allied genus. In other words, the phenolase in the blood is in the presence of a large amount of substrate, but is, immediately prior to pupation of the larva, unable to act on it. At pupation, the tyrosine is oxidised to dopa. The release of the activity of the tyrosinase is now claimed to be under humoral control (Karlson, 1958). The dopa then moves into the cuticle. There, according to earlier theories based on Pryor's (1940b) concepts of tanning, it would be deaminated and degraded to produce protocatechuic acid. However, the mechanism whereby this is done is not known, but Hackman (1958) points out that an excess of ortho-quinones could bring about the reaction. In Dennell's schema, on the other hand, (Dennell, 1958d), dopa in the cuticle of the blowfly larva can undergo non-specific hydroxylation to form hydroquinone and protocatechuic acid; but it can also be used by tyrosinase in the epicuticle to produce melanin.

If it is accepted that dopa is produced in the blood of Phormia larvae just prior to pupation, then injected phenylthiourea may block tyrosinase in the blood without having any appreciable effect /

effect on the supply of substrates to the cuticle, if the dopa formation has been completed. Therefore, the next stage at which melanogenesis and sclerotisation in the larva can be blocked is that where the epicuticular tyrosinase converts dopa to dopa-quinone, or catechol, derived from non-specific hydroxylation of ortho-tyrosine or dopa, to ortho-benzoquinone. If the phenylthiourea injected into the blood is capable of permeating the cuticle, and blocking the enzyme in the epicuticle, then hardening and melanin production should be prevented, regardless of whether inhibition of the blood tyrosinase had been successful, and regardless of whether tanning involves protocatechuic acid or hydroquinone.

Such an inhibition was not found in the present work. Similarly, Colvard Jones (personal communication to Dennell, 1958d), was unable to block puparium formation in Sarcophaga falculata by injecting phenylthiourea, and Dennell (1958d) was unable to block puparium formation in Calliphora. This series of findings is not easy to understand. In the present work, injections were made at various times before pupation, such that adequate inhibition of the formation of dopa in the blood was expected to have been achieved. Nevertheless, the end result was unchanged.

The fact that dopa can be oxidised by cytochrome oxidase (Zimmerman 1959) must be kept in mind in this connection. Evidence regarding this reaction appears to be scanty. Although cytochrome oxidase /

oxidase in the epidermis might conceivably oxidise dopa, it is not easy to see how it could effect the production of ortho-benzoquinone in the epicuticle.

It therefore appears that two possible explanations exist for the inability to block melanisation in the Phormia larvae. The simpler is that the injected phenylthiourea was applied too late to prevent the formation of dopa in the blood, and was not able to reach the epicuticular phenolase to block quinone and melanin formation. This interpretation is contra-indicated, however, by experiments in which feeding larvae of Phormia were injected with the inhibitor. At that stage, it is known that dopa has not yet been formed from tyrosine (Dennell, 1947), but, of those larvae pupating, there was no inhibition of banding apparent. However, both controls and injected animals tend to produce more unbanded forms when they are forced to cease feeding earlier than they would do if left in the medium.

The second explanation requires that the inhibition of tyrosinase be not total, or can be reversed. Evidence concerning this possibility comes from various sources. Dubois and Erway (1946) found that in in vitro experiments, the inhibition of tyrosinase from potato by phenylthiourea was not reversed in two hours. But these workers also quote the results of Bernheim and Bernheim (1944), where a reversal of inhibition was found, and the effect was also discovered to be dependent on the substrate concentration.

Allied to this question, is the work of Chmurzynska and Wojtczak (1959) and Wojtczak and Chmurzynska (1960). These authors were studying /

studying the inhibition *in vitro* of the tyrosinase of the wax moth, *Galleria mellonella*, and found that while various substances including thiourea inhibited the enzyme at first, the inhibition quickly was reversed, and that the activity of the enzyme could regain, and surpass, the activity of uninhibited control samples. This reversal in the case of thiourea took two to three hours. After using sodium diethyldithiocarbamate as inhibitor, these workers found that tyrosinase from wax moths regained its activity in the same time range. This ability of the enzyme to regain its activity was not found when very high concentrations of the inhibitors were used, that in the case of thiourea being 5×10^{-3} M.

If such a reversal of inhibition was occurring in the *Phormia* larvae and puparia after injection with phenylthiourea or sodium diethyldithiocarbamate, the lack of effect in the former case, and the limitrophe effect in the latter, can be explained very easily. It was for this reason that attempts were made to measure the effects of the inhibitors on the timing of band formation in the pupation sequence of these insects. The results, however, showed that even this technique was not capable of showing any apparent effect on the banding after injection of the larvae with phenylthiourea.

It is clear, therefore, that the phenolase complex in insects is a system whose expressions are not easy to elucidate, but in view of the overall results of the present work, one is drawn /

drawn into concluding tentatively that melanisation and the process of hardening of the cuticle seems capable of differential control by the insect. Although in the early stages of work on insect cuticle, this was regarded as being improbable, evidence is accumulating in support of such a conclusion. The next question to be posed is how the differential control of the two processes is brought about. There is a great deal to be learned about the phenolase complex before the answer is forthcoming.

SUMMARY.

1. Current concepts of the involvement of the phenolase complex and its substrates and products in the production of melanogenesis and sclerotisation in insects are discussed.

2. A non melanised mutant of the Desert Locust, Schistocerca gregaria is described, and designated as "albino", although this does not imply that the phenolase complex is absent from the mutant.

3. The gross appearance of the cuticle of the albino locust is compared with that of the wild strain, when the major point of difference is shown to be the total lack of melanin patches in the albino. Comparative tests between the two strains show that the albino does not contain as much reducing material as the wild insect.

4. Histological tests, to demonstrate the degree of sclerotisation present, indicate that the albino cuticle is less heavily sclerotised than is the case in the wild strain. A corresponding reduction is found in the amount of reducing substances present in sections of the cuticles. Information derived from the use of /

of a relatively recently developed "iodophil" technique demonstrating as yet unspecified combinations of phenols with proteins agrees with the other tests in suggesting reduction of the amount of phenols in the albino cuticle. The summation of the various histological tests indicates that in the mutant strain there is a strong reduction of true tanning, combined with total absence of melanin. But the albino cuticle is impregnated and therefore corresponds predominantly to the mesocuticular condition.

5. The albino is used as a unique experimental animal in which to study the effects of various substrates of the phenolase complex. This is done by means of a very simple technique developed to introduce substances in solution into the insect by means of immersing the dewaxed abdomens of living nymphs in solutions of the chemicals to be used.

6. When catechol or tyrosine are used as test substrates, the phenolase in the albino cuticle causes the deposition of a well-defined melanin pattern. The induced pattern is stronger with catechol than with tyrosine. It is suggested that this is due to the widely-observed higher activity of the phenolase complex towards diphenols than towards monophenols. Proof of the melanic nature of the induced pigmentation is presented. That the inductions are catalysed by the phenolase complex is proved by the ability to inhibit them partially by addition of phenylthiourea or sodium diethyldithiocarbamate to the immersion solutions. When the final /

final concentrations of either of these inhibitors is 10^{-3} M., no effect is seen on the melanogenetic process, but when the final concentrations are 2.5×10^{-2} M., a distinct, though not total, inhibition of melanin production is obtainable.

7. It is shown that 3,4-dihydroxyphenylalanine does not have any melanin-inducing effect on the albino locust, contrary to predictions based on its being an utilisable substrate of the phenolase complex. It is suggested that the lack of induction is due to the substrate's being oxidised at a relatively much slower rate, such that the present technique would not be sufficiently sensitive to detect the effect. Evidence from other workers is quoted to support this hypothesis.
   3,4-dihydroxybenzoic acid does not induce any colouration in the albino cuticle, either in discrete patterns, or generally.

8. Histological examination of albino cuticles treated with catechol and protocatechuic acid shows that radical changes in the chemical nature of the cuticle have occurred. The melanin induced by the catechol is visible in the epicuticle and outer exocuticular regions. The results of several staining techniques indicate that the originally mesocuticular condition of the outer portion of the outer procuticle, corresponding in anatomical position to the exocuticle of the wild strain locust, is converted to the exocuticular condition. That portion of the albino procuticle corresponding in anatomical /

anatomical position to the mesocuticle of the wild strain is not affected by the conditions which converted the outer regions. These effects on the degree of sclerotisation of the cuticle are apparently identical after treatment with either catechol or 3,4-dihydroxybenzoic acid.

9. It is pointed out that the lack of general colour induction in the cuticle after treatment with catechol or 3,4-dihydroxybenzoic acid is good evidence to support the growing body of opinion that sclerotisation need not obligatorily involve concomitant colouration of the tanned cuticle.

10. The ability to induce melanisation in albino locusts by exposure of the eggs to low temperatures is reported, and used as additional evidence for the presence of both phenolase complex and its normal substrate(s) in the albino, although their interaction is apparently very much reduced.

11. Comparison of the relative tyrosinase activities of breis of wild and albino locusts indicate that there is no significant difference between the two strains, under these conditions. The presence of the enzyme in the albino is therefore further indicated.

12. The process of pupation in <u>Phormia-terrae novae</u> is briefly described. Melanin is produced in this insect at two different times; it is laid down as a series of eight transverse bands immediately after larval contraction, and before sclerotisation is apparent. It is also /

also laid down over the whole cuticle later in pupal life, after most of the sclerotisation has been completed. The types of variation occurring naturally in this sequence of events gives evidence that the amount of melanin deposited on either occasion is independent of the amount laid down on the other. Both would appear to be independent of the degree of sclerotisation, but it is pointed out that there is not yet any reliable objective method of measuring the degree of sclerotisation. Some doubt as to the amount of sclerotisation present in any puparium must therefore remain. It is suggested that the dark colour developed later in pupal life may be formed by oxidation of catecholic-proteins to form coloured compounds.

13. It is shown to be possible to increase the intensity of the general melanin by exposure of the late larvae and pupae to low temperatures. This agrees with the common observation that lower temperatures tend to increase melanin production in insects.

14. The injection of solutions of phenylthiourea in various concentrations and in various volumes into <u>Phormia</u> late larvae did not permit of blocking the action of the phenolase complex towards melanin band formation in preference to sclerotisation. The reasons for this are discussed, when the result is seen to be not unexpected.

15. The injection of solutions of sodium diethyldithiocarbamate into similar larvae permitted of blocking melanin band formation in preference to sclerotisation, but the effect was marginal.

16./

16. An unexpected and somewhat puzzling finding is presented, where the size, measured as the length, of Phormia puparia is found to be closely correlated with the presence or absence of bands. This effect is present whether the larvae be unoperated, or injected with various volumes of phenylthiourea or saline.

17. Catechol injected into Phormia late larvae is rapidly metabolised, and kills the larvae. They become a very intense black after death.

18. Injection of phenylthiourea into Sarcophaga larvae in doses which are approaching the lethal limit does not prevent sclerotisation.

19. The results of the various experiments are discussed. A suggestion is presented to explain the inability to block melanin formation and sclerotisation in Phormia larvae and puparia, and sclerotisation in Sarcophaga larvae. This explanation is based on the biochemical findings of other workers on the phenolase complex in insects, where the enzyme was found to overcome in a relatively short time, its inhibition by various inhibitors. Since the experiments using these inhibitors in the present work, and similar experiments reported from the literature, were run over considerable periods of time, it is suggested that the enzyme under these conditions was overcoming the effect of the inhibitors.

20. Throughout this thesis, the evidence available has tended to point towards the two aspects of the expression of the phenolase complex /

complex in insects, namely, melanogenesis and sclerotisation, as being independent processes. The belief is expressed that work on the problem of differentiating between the two processes is accumulating sufficient evidence to make this conclusion more widely accepted than it is at present.

ACKNOWLEDGMENTS.

It is my pleasure to offer my thanks to the many people, both within and without this department, who have helped me in this work. Professor Michael Swann provided facilities in his laboratory. My supervisor, Dr. B. M. Jones, now Professor of Zoology at the University College of Wales, Aberystwyth, introduced me to the problems concerning tyrosinase expression in insects. Dr. Philip Hunter-Jones of the Anti-Locust Research Centre in London very generously supplied albino locusts on numerous occasions.

In other spheres, I wish to thank Mr. D. L. Doxey, B.V.M.S., M.R.C.V.S., for doing so much to assist with the checking of the text, and for discussing pathological melanogenesis in animals. Dr. L. J. Hale assisted me with statistical problems, and made this subject much less forbidding. Mr. D. Cremer patiently coped with photographic problems, and produced the prints of locusts and blowflies. Mr. A. Gall made up pieces of specialised apparatus. Mr. T. Macrae cultured the Phormia larvae, and obtained and cultured the Sarcophaga material for me.

Miss H.R. Wilson, who did the typing, is thanked for the very generous way in which she gave up much of her own time to meet submission dates for the thesis.

My indebtedness to my wife, who provided so much enthusiastic encouragement when things did not go according to plan, is gratefully acknowledged.

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