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Chemistry of Insect Cuticle

1. THE WATER-SOLUBLE PROTEINS

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During the last decade there has been a great increase in knowledge of the insect cuticle, which is now recognized to possess an exceedingly complex structure (see Wigglesworth, 1948, 1949). The cuticle consists of two layers, an inner endocuticle and an outer epicuticle. The endocuticle is composed of chitin and protein; the protein in its outer part is sometimes hardened (tanned) to form a dark exocuticle. The epicuticle is composite, consisting of an inner tanned lipoprotein layer, followed by a polyphenol layer which is overlaid by a wax layer. In some insects, but not all, there is also a cement layer which covers and protects the wax layer.

The most important properties of the cuticle are its flexibility and elasticity (which allow of movement but of only limited extension), its hardness (giving a rigid support for the attachment of muscles as well as a protective covering) and its impermeability, especially to water. This last property has enabled insects and related arthropods, in spite of their small size and relatively enormous surface area, to colonize far drier environments than most other small invertebrates. The epicuticle is mainly responsible for the impermeability to water. It is the cuticle which has first to be penetrated by contact insecticides before their toxic effects can be exerted.

The non-chitinous material in the cuticle is mainly protein (in the insects which have been studied, the protein content varied from 25 to 37% (Odiar, 1823; Lafon, 1943a)); the inner parts of the cuticle readily give various protein colour tests, such as the biuret, Millon's and xanthoproteic reactions (Kühnelt, 1928; Campbell, 1929; Wigglesworth, 1933; Pryor, 1940a, b).

The water-soluble proteins from several insect cuticles have been isolated and examined by Trim (1941a, b) and by Fraenkel & Rudall (1940, 1947). However, these workers made no mention of the fact that the protein, as extracted from the cuticles, was not homogeneous. It is shown below that the extracted protein can be separated electrophoretically into a number of component proteins; hence it is desirable to reconsider the conclusions of Trim and of Fraenkel & Rudall. The extractable proteins fail to coagulate in hot water and are soluble in hot, but not cold, aqueous trichloroacetic acid. They are precipitated from aqueous solutions by ethanol in concentrations above 45%, by one-third-saturated ammonium sulphate and by saturated sodium chloride. After precipitation by either trichloroacetic acid or ethanol the proteins readily redissolve in water; even after heating in boiling water they remain in solution. Heating does not alter their reaction with quinones. The cuticular proteins thus show physical properties unlike those of any other known proteins.

Trim (1941a, b) characterized the cuticular proteins from two insects (*Sarcophaga falcitata* and *Sphinx ligustri*) by determining nitrogen distribution, tyrosine, tryptophan, glycine and β -hydroxy- α -amino-acids. He concluded that the protein was quite distinct from collagen in its chemical composition and that it bore some resemblance to the sericin of silk.

Fraenkel & Rudall (1940, 1947), using X-ray analysis methods, have shown that, unlike most vertebrate skeletal proteins, insect cuticular proteins from soft (*Sarcophaga*) cuticles, give β -type diffraction. By X-ray and related studies they

have shown that there is present in the cuticle a polysaccharide-protein complex consisting of alternating monolayers of chitin and protein. Electron microscope studies by Richards & Anderson (1942) support such a laminated structure. Fraenkel & Rudall consider this cuticular 'protein' to occur in all arthropods and they have called it 'arthropodin'.

EXPERIMENTAL

Preparation of cuticles

Soft cuticles. Scarab larvae of three species (*Diaphonia dorsalis* Don., *Dasygnathus* sp. and *Mastochilus* sp.) were collected in the field. To obtain the cuticles, the larvae were decapitated, opened longitudinally and the body contents removed in a washing medium of 65% (v/v) aqueous ethanol. The cuticles were transferred to fresh aqueous ethanol and the remaining tissues, muscles and hypodermal cells were removed by scraping with a blunt scalpel, the last stage of removal being carried out under a binocular microscope. After a final rinsing in aqueous ethanol the cuticles were dried in a vacuum desiccator over P_2O_5 and KOH. By use of 65% aqueous ethanol as the washing medium, loss of cuticular protein was avoided. At the same time, muscle protein and cell contents were denatured, which facilitated their removal. In the cleaning process it is possible that some of the endocuticle (inner layers) was sacrificed in order to ensure that all muscle protein and other body tissues were removed.

Hard cuticles. The cast skins of larvae of *Tenebrio molitor* L. (Coleoptera), final instar nymphs of *Chortocetes terminifera* Walk. (Orthoptera) and the final nymphal instar of *Cyclochila australasiae* Don. were used as representing the hardened parts of soft cuticles, while the elytra of adult *Aphodius howitti* Hope (Coleoptera) represented a hard cuticle.

Extraction of the proteins

Preliminary work confirmed the observations of Trim (1941*b*) that the protein fractions extracted by water and by a buffer solution at pH 9.2 (5:5:4:1 parts by vol. of borax solution (19 g./l.), water, ethanol and ether, respectively) are indistinguishable by N content or general physical and chemical properties. However, more colour was extracted

from dark cuticles by buffer than by water. It was further shown that these two fractions yield on hydrolysis the same amino-acids. By use of the buffer solution the protein was obtained more readily.

The dried cuticles were ground with sand in a mortar and extracted with light petroleum (b.p. 40–60°) to remove lipid material. The residue was extracted twice for 36 hr. at 50° with the buffer solution mentioned above, after which it was shown that no further material precipitable by cold aqueous trichloroacetic acid was extracted. The borax extract was filtered, acidified to pH 3–4 with HCl and the protein precipitated by one-third saturation with $(NH_4)_2SO_4$. The protein was collected by centrifugation, resuspended in distilled water and dialysed in a cellophan sac against distilled water for 2 days. To obtain the water-soluble fraction the dialysed solution, together with the undissolved material, was evaporated *in vacuo* and dried in a vacuum desiccator over P_2O_5 and KOH. Any remaining lipid material was removed by extracting successively with ethanol and ether. The residue was finally dried to constant weight. The soft cuticles gave almost colourless brittle solids, the cast skins pale-brown solids and the elytra of *A. howitti* a dark-brown solid. The yield of protein from each kind of insect cuticle is given in Table 1.

Overall composition of the proteins

All seven proteins gave negative tests for S, P and carbohydrate (Pirie, 1936). The protein from *A. howitti* elytra gave 1.68% ash but the other proteins were apparently ash free. The total N and amide N contents are given in Table 1. Amide-N values were determined by estimating the ammonia produced when the protein was heated with 2N-HCl for 3 hr. at 100°. Where no N values are given for the proteins, insufficient material was available for estimations. Solubility behaviour in salt solutions showed that the proteins contained several components (Northrop & Kunitz, 1930).

Detection of amino-acids present in the proteins

One- and two-dimensional filter-paper partition chromatography (capillary ascent method of Williams & Kirby (1948)) was used for the detection of the amino-acids present in the hydrolysates of the proteins. The following systems were used as solvents: phenol 74%, water 26%

Table 1. *The water-soluble proteins of insect cuticles*

Insect material	Protein isolated (% of dry insect material)	% of isolated protein	
		N (Kjeldahl)	Amide N
<i>Diaphonia dorsalis</i> Don. (Coleoptera) larval cuticles	24.0	14.0	1.01
<i>Dasygnathus</i> sp. (Coleoptera) larval cuticles	20.1	15.5	1.05
<i>Mastochilus</i> sp. (Coleoptera) larval cuticles	22.7	—	—
<i>Tenebrio molitor</i> L. (Coleoptera) larval cast skins	8.9	—	—
<i>Chortocetes terminifera</i> Walk. (Orthoptera) final nymphal instar cast skins	7.7	—	—
<i>Cyclochila australasiae</i> Don. (Hemiptera) final nymphal instar cast skins	3.7	14.0	—
<i>Aphodius howitti</i> Hope (Coleoptera) adult elytra	2.1	13.7	—

(w/w); acetone 60%, water 40% (v/v); pyridine 65%, water 35% (v/v); *n*-butanol 77%, acetic acid 6%, water 17% (v/v). For buffered filter-paper chromatography (McFarren, 1951) the mixture used was phenol (74%) and a buffer of pH 10.0 (26%, w/w) (0.053M- H_3BO_3 and KCl, 0.047M-NaOH in final concentrations). The acetone and pyridine solvents have been described by Bentley & Whitehead (1950) and the butanol solvent by Fromageot, Jutisz, Meyer & Penasse (1950). Whatman no. 1 filter paper was used and the chromatography chambers were kept at $25^\circ \pm 1^\circ$.

The protein (50 mg.) was hydrolysed by refluxing in 6N-HCl (5 ml.) for 24 hr. The solution was evaporated to dryness under reduced pressure, the residue dried *in vacuo* over solid KOH to remove all trace of free HCl, then dissolved in water (3 ml.) and filtered. To prevent mould growth a drop of toluene was added. In later experiments the residue was dissolved in 10% (v/v) aqueous isopropanol. 5 μ l. of solution was used for each chromatogram. After development with the solvent for the required time (15–20 hr.) the papers were freed from solvent by repeated washing in ether and then air-dried. In the earlier experiments the amino-acids were detected by spraying the paper with a solution of ninhydrin (0.25%) in water-saturated *n*-butanol and subsequently heating them for 5 min. at 95° . Later it was found that better results were obtained by spraying the paper with ninhydrin in dry *n*-butanol, heating it and then hanging it over a vigorously boiling water bath for 5 min. By this method tailing of the spots was avoided (Hackman & Lazarus, unpublished observations). When spraying buffered filter paper, 5% (v/v) of acetic acid was added to the ninhydrin solution.

In all seven proteins the same amino-acids were detected, viz. aspartic acid, glutamic acid, serine, glycine, threonine, alanine, tyrosine, valine, phenylalanine, leucine and/or isoleucine, proline, hydroxyproline and lysine. Proof for the presence of these amino-acids was obtained by preparing a mixture of these thirteen amino-acids and showing that such a mixture behaved in the same manner as the hydrolysates from the insect proteins. Then, after mixing the made-up mixture with the protein hydrolysates, it was shown that spots to the same number and in the same places on the filter paper were obtained and that no additional spots appeared. A sample of hawk moth (*Sphinx ligustri*) larval protein supplied by Dr Trim was also hydrolysed, and only the above amino-acids were detected. Tryptophan, which is destroyed during acid hydrolysis, was shown after alkaline hydrolysis to be present in all proteins by the method of Lugg (1937, 1938).

The free amino groups of the proteins

To detect those amino-acids in the proteins which have free amino groups the method described by Sanger (1945) with the modifications devised by Blackburn (1949) and Blackburn & Lowther (1951) was used. The silica gel and the *N*-2:4-dinitrophenyl derivatives of the amino-acids (DNP-amino-acids) detected by filter-paper partition chromatography (see above) were prepared by the published methods. The DNP-amino-acids were required for use as markers in the chromatographic procedure and for identification of DNP-amino-acids obtained by hydrolysis of the reaction product between the proteins and 1-fluoro-2:4-dinitrobenzene.

The DNP-proteins were prepared by the method given by Sanger (1945) for DNP-insulin. The fluorodinitrobenzene was prepared from the corresponding chloro compound by the method described by Cook & Saunders (1947). Amide N determinations showed that 100 mg. DNP-protein from *Diaphonia dorsalis* (0.624% amide N) corresponded to 61.5 mg. protein. The DNP-protein (100 mg.) was hydrolysed by refluxing for 8 hr. in 6N-HCl (10 ml.). Ethereal extraction of the cooled hydrolysate gave an ether layer which contained the DNP derivatives of the acidic and neutral amino-acids and an aqueous residue which contained the free amino-acids and the DNP derivatives of the basic amino-acids.

The ethereal extract was evaporated to dryness, the residue dissolved in ether saturated with water and the DNP-amino-acids separated on buffered columns of silica gel (Blackburn, 1949). Buffer solutions used were 2M- NaH_2PO_4 and phosphate buffers of pH 6.98 (0.040M- Na_2HPO_4 , 0.027M- KH_2PO_4) and pH 6.24 (0.013M- Na_2HPO_4 , 0.053M- KH_2PO_4). 0.66 ml. buffer solution was used for each gramme of silica gel. As solvents, ether, chloroform and 5% (v/v) *n*-propanol-cyclohexane (each saturated with water) were used. Alternatively, the paper-chromatographic method described by Blackburn & Lowther (1951) was used. By these methods the DNP derivatives of glutamic acid, glycine, serine and alanine were shown to be present in the proteins obtained from *Diaphonia dorsalis*, the *Dasygnathus* sp., *Cyclochila australasiae* and *Aphodius howitti*. The quantity of proteins available from the other three insects was insufficient to permit of further study. The identification of these DNP-amino-acids was based on the position of the bands or spots and was confirmed by mixed chromatograms. Further hydrolysis of the DNP-amino-acids brought about no change, thus showing the absence of DNP-peptides.

The aqueous solution (obtained after extraction of the DNP-protein hydrolysate with ether) was evaporated to dryness under reduced pressure and the residue dissolved in ethyl methyl ketone-ether (2:1 by vol.). This solution was passed through a column of silica gel containing 50% by weight of water. *N* ϵ -DNP-lysine was detected in all four DNP-proteins and its identity confirmed by mixed chromatograms. As before, further hydrolysis showed the absence of DNP-peptides.

Terminal carboxyl groups

The terminal carboxyl groups in the proteins obtained from the *Diaphonia dorsalis* larval cuticles were characterized by the method of Fromageot *et al.* (1950). The *N*-ethylmorpholine was prepared from morpholine by ethylation with ethyl iodide. In this method the carboxyl groups of the proteins were reduced to $-CH_2OH$ groups by lithium aluminium hydride. The following amino alcohols were detected by the technique of paper-partition chromatography: tyrosinol, ethanolamine, lysinol, serinol and 2-aminopentan-1:5-diol. This indicates that the carboxyl groups of tyrosine, glycine, lysine, serine and glutamic acid occur as terminal carboxyl groups in the protein chains.

Electrophoretic examination

Electrophoresis was carried out in a Tiselius apparatus equipped with a Philpot-Svensson scanning system (Holden, Ada & Pye, 1952). Analytical runs were done in

a 2 ml. fused Pyrex cell and separation runs were carried out in a 10 ml. two-piece cell. Only the protein obtained from the *D. dorsalis* cuticles was examined.

In a preliminary analysis the protein (40 mg.) in phosphate buffer (3 ml., pH 7.0, ionic strength = 0.2, 0.091 M- KH_2PO_4 , 0.0545 M-KOH) was dialysed to equilibrium against 1 l. of the same buffer. The diagrams shown in Fig. 1 were obtained. Three major components, all migrating towards the anode, were found. The fastest component, estimated from the diagram to account for 46% of the total protein present, was separated more completely from the slower components by electrophoresis in a phosphate buffer (pH 5.8, ionic strength = 0.2, 0.167 M- KH_2PO_4 + 0.0167 M-KOH). Protein (150 mg.) in buffer (11 ml.) was dialysed against 2 l. of the same buffer. After 400 min. at 3.1 V./cm., a better separation was achieved and a sample of the fastest component, estimated to contain less than 10% of the next main component, was taken off. In this manner 0.75 mg. of the fastest component was obtained from two runs.

Fig. 1. Ascending (A) and descending (B) patterns of *D. dorsalis* cuticular protein after submission to electrophoresis in phosphate buffer (pH 7.0) for 176 min. at 3.15 V./cm. (migration towards anode). For details of buffer see text.

Acid hydrolysis followed by partition chromatography on filter paper showed that this fast component contained very little, if any, serine, threonine, phenylalanine, proline or hydroxyproline. Otherwise its component amino-acids were the same as those of the entire protein fraction. By the use of the methods of Moore & Stein (1948), Awapara (1949) and Landua & Awapara (1949) the protein was shown to contain 8.9% aspartic acid, 11.5% tyrosine and 3.7% valine.

DISCUSSION

A chemical examination has been made of the water-soluble proteins isolated from the cuticles of seven insects representing three orders, viz. Coleoptera, Orthoptera and Hemiptera. The percentage yields of water-soluble proteins are in agreement with the theory that it is the protein of the epicuticle and exocuticle which is rendered water-insoluble when the cuticle is hardened. On this theory the protein of the endocuticle only should be extracted from the

A. howitti material; however, from the colour and solubility behaviour of the proteins from the hard cuticles a small amount of some other material was also extracted. Since protein could be extracted from the cast skins of *Tenebrio molitor*, *Chortoicetes terminifera* and *Cyclochila australasiae* it appears that either the exocuticle contains untanned protein or that the moulting fluid does not digest the entire endocuticle.

The water-soluble cuticular proteins of insects contain no carbohydrates or sulphur. In fact, the intact cuticles are free or almost free from sulphur (Pryor, 1940a; Trim, 1941a, b). This is in marked contrast with the proteins of the egg shells of some insects (Verson, 1884; Tichomiroff, 1885; Farkas, 1903) and of the cuticle of *Limulus* which contain several per cent of sulphur (Lafon, 1943b).

The proteins obtained from the seven cuticles are very similar in physical and chemical properties and they all appear to be built up of the same fourteen amino-acids, viz. aspartic acid, glutamic acid, serine, glycine, threonine, alanine, tyrosine, phenylalanine, valine, leucine and/or isoleucine, proline, hydroxyproline, lysine and tryptophan. The presence of proline and hydroxyproline is of interest as proline and hydroxyproline are considered to modify considerably the structure of protein chains (Pauling & Corey, 1951; Coleman & Howitt, 1945; Mizushima *et al.* 1952). The results obtained for these seven cuticles are qualitatively similar to those obtained by Johnson, Pepper, Botkin Banning, Hastings & Clark (1952) for the amino-acid content of the cast skins of the mormon cricket, *Anabrus simplex* Hald. However, no valid comparison can be made as these workers used the entire cast skins from which most of the water-soluble protein would have been removed by the insect before moulting.

Electrophoretic examination of the protein obtained from *Diaphonia dorsalis* cuticles has shown it to consist of a number of components. A small amount of the fastest anodic-migrating component was isolated. This fraction was not tested for electrophoretic homogeneity and thus does not necessarily represent a single molecular species; it is considered to be slightly contaminated with the next-fastest fraction. Paper-partition chromatography showed the almost complete absence of the β -hydroxy- α -amino-acids (serine and threonine), phenylalanine, proline and hydroxyproline. The traces found are probably explained by the slight contamination. Otherwise its component amino-acids are the same as those of the entire protein preparation.

Further light is thrown on the chemical composition of the proteins by an examination of the free amino and carboxyl groups in the intact proteins. Amino-acids which are α -substituted with 2:4-dinitrophenyl groups must be assumed to have had

free α -amino groups and, therefore, if they are mono-aminocarboxylic acids, to have been at the ends of protein chains. The free α -amino groups of the proteins isolated from the insect cuticles are located on glutamic acid, glycine, serine and alanine residues. This indicates that either the proteins contain a number of chain ends in each molecule or that there are a number of component proteins. Solubility methods and, in the case of *D. dorsalis*, electrophoretic examination have in fact shown the proteins to be composed of a number of components. It has been assumed that the N^{ϵ} -DNP-lysine is derived from lysine residues located in the protein chain. The amount of DNP radicals in the proteins from *D. dorsalis* is high, which probably indicates a high proportion of free amino groups. However, it must be borne in mind that the method of calculating original protein from DNP-protein by amide-N content assumes that no deamidation occurs during the substitution. The terminal carboxyl groups in the proteins are those of tyrosine, glycine, lysine, serine and glutamic acid. This again suggests that there are several component proteins, which is in agreement with the results obtained above. Of the reactive groups those of lysine, glutamic acid and tyrosine are of most interest as they are capable of undergoing further interesting reactions which involve cross-linkages. The significance of these results is discussed in a later paper (Hackman, 1953).

The study of the chemistry of a protein has only begun when its composition in terms of amino-acids and reactive groups is known. Many of the properties of a protein must depend, not only on its amino-acid composition, but also on the arrangement of the residues within the molecule. The fact that the proteins have been shown to have free α -amino groups and free α -carboxyl groups does not necessarily indicate a structure which consists of parallel chains held together by cross-links. Possibly there also exist in the protein molecule cyclic chains or chains branching off the ω -carboxyl groups of glutamic acid. Available evidence suggests that a number of proteins and polypeptides have a cyclic structure (Synge, 1949; Bailey, 1951; Akabori, Ohno & Narita, 1952) while Haurowitz & Bursa (1949) have shown the presence of a small number of γ -glutamyl bonds in five proteins and a much higher number of such bonds in a sixth, viz. the capsular substance of *Bacillus anthracis*.

A problem which has recently come into prominence when considering the amino-acid composition of proteins is that of variable composition. Several amino-acid analyses for insulin have been reported (Rees, 1946; Macpherson, 1946; Tristram, 1946; Brand, 1946; Miller & du Vigneaud, 1937; Sahyun, 1941). Several different methods for estimating the amino-acids were used; for most amino-acids there is reasonable agreement, and for some, quite good

agreement. For threonine, amide nitrogen and sulphur, however, the variations exceed those which would be expected. These differences suggest that all preparations of insulin may not in fact be identical. Perhaps one or more 'non-essential' amino-acid has been replaced by another. Again, it has long been realized that the composition of different wools may vary considerably in respect to their cystine content, and this variability may necessitate corresponding changes in other amino-acids. There are other indications in the literature of a similar effect with other proteins; thus Csonka (1948) has suggested that the composition of crystalline egg albumin can be significantly affected by the hen's diet. It thus appears that it is possible to replace certain amino-acids in a protein with others without affecting its role in a biological process. The actual structure of a protein therefore may vary, depending upon the source from which it has been obtained. As this study is concerned primarily with the role the cuticular proteins may play in the hardening process undergone by insect cuticles, greater importance has been placed upon the determination of those groups which are chemically reactive, viz. the free amino groups and free carboxyl groups.

The seven water-soluble insect cuticular proteins examined are remarkably similar in their physical and chemical properties, including component amino-acids, free amino groups and free carboxyl groups. There is reasonable agreement between the results reported in this paper and the less detailed data reported by Trim (1941*a, b*) and Fraenkel & Rudall (1940, 1947). General physical and chemical properties are the same, the total nitrogen and amide nitrogen values reported are very similar while the very high tyrosine content (11.5%) of the fast anodic-migrating fraction of *Diaphonia dorsalis* protein closely approximates that of *Sphinx ligustri* entire protein (11.6%).

SUMMARY

1. The water-soluble proteins have been isolated from the cuticles of seven species of insects representing three orders.
2. The protein preparations have been shown to be heterogeneous.
3. By paper-partition chromatography the amino-acids present in acid hydrolysates of the proteins were identified. The amino-acids with free α -amino groups were recognized in four of the preparations and the amino-acids with free carboxyl groups in one.
4. All the water-soluble proteins are remarkably similar in their physical and chemical properties, including component amino-acids and free amino groups. Although the protein fractions as first

isolated contained the same component amino-acids, one fraction, obtained by electrophoretic separation, was shown to differ considerably, the serine, threonine, phenylalanine, proline and hydroxyproline being absent.

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Chemistry of Insect Cuticle

2. THE WATER-INSOLUBLE PROTEINS OF THE CUTICLE OF *APHODIUS HOWITTI* HOPE (COLEOPTERA)

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In the previous paper (Hackman, 1953a) the structure of the insect cuticle was outlined and information was presented on the water-soluble proteins obtained from the cuticles of a variety of insects. It was pointed out that the exocuticle and epicuticle contain hardened ('tanned') protein which is water-insoluble. That there are water-insoluble proteins in the insect cuticle is clearly

indicated by the work of Kühnelt (1928), Pryor (1940) and Wigglesworth (1933, 1947). However, the only paper describing any attempt to examine the nature of these proteins is that of Trim (1941).

Trim isolated a fraction from *Sphinx ligustri* larval cuticle which was soluble in warm 5% (w/v) aqueous sodium hydroxide solution but insoluble in water. He estimated the total nitrogen, sulphur and