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.

The author is indebted to Messrs G. L. Ada and J. Pye of the Walter and Eliza Hall Institute of Medical Research, Melbourne, for carrying out the electrophoretic separation and to Mr P. B. Carne of this Division for identifying the scarab larvae.

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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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(Received 30 June 1952)

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

carbohydrate contents of this fraction but provided no further data on its composition. He also showed the presence of a protein fraction in *Sarcophaga falcata* larval cuticles which was soluble in dilute alkali but insoluble in water. Both of these cuticles may be classed as soft cuticles, with relatively little water-insoluble protein, as distinct from hard cuticles where a far higher proportion of the protein in the outer layers has been rendered insoluble (by tanning). As a characteristic source, therefore, of a well-tanned insoluble protein from a hard cuticle, the dark-brown elytra of the scarab beetle *Aphodius howitti* Hope were selected for study. An examination has been made of the water-insoluble proteins present in the elytra of these beetles.

EXPERIMENTAL

Separation of protein fractions

The elytra were dried, ground to a powder and extracted with light petroleum to remove lipid material and then with a borate buffer solution of pH 9.2 to remove water-soluble proteins (cf. Hackman, 1953*a*). A second protein fraction was obtained by extracting the residue (56 g.) from these purification operations with *N*-NaOH at 50° for 5 hr. The insoluble material was then separated by centrifugation and washed with water by centrifugation and decantation until the washings were no longer alkaline. The filtrate and washings were brought to pH 3-4 with H₂SO₄, the solution half-saturated with (NH₄)₂SO₄ and the precipitate which separated collected by centrifugation. Concentration of the supernatant liquid *in vacuo* at 30°, after neutralization with BaCO₃ and separation of the precipitated BaSO₄ and excess BaCO₃, did not yield any further material precipitable by trichloroacetic acid. The protein was resuspended in distilled water, dialysed against distilled water for 2 days, evaporated to dryness *in vacuo*, extracted with ethanol and ether and dried to constant weight in the same way as the water-soluble protein (Hackman, 1953*a*). Yield: 5.26 g. (9.4% calculated on the weight of cuticle after removal of the

water-soluble protein) of a very dark material. Much coloured material was lost during dialysis.

The residual cuticular material, which contained chitin and hardened protein (insoluble in water or dilute alkali) was dried and the amount of chitin present estimated. The method suggested by Fraenkel & Rudall (1947) was used, the chitin being that fraction which was insoluble in 5% (w/v) aqueous KOH at 100°.

Overall composition

Alkali-soluble protein. N (Kjeldahl), 11.04%; S, approx. 0.3%; ash, 9.26%; total carbohydrate, 3.0% (as glucose) determined by the orcinol-H₂SO₄ method (Pirie, 1936). The absorption was measured at 430 mμ.

Residual cuticular material. N, 9.11%; S, nil; ash, 1.81%; chitin, 55.1%.

Detection of amino-acids

The amino-acids present in the acid hydrolysates obtained from each of these two fractions were identified using one- and two-dimensional partition chromatography on filter paper. For methods and solvents used see Hackman (1953*a*). Tryptophan was shown to be present after alkaline hydrolysis by the method of Lugg (1937, 1938).

The amino-acids detected in the hydrolysates from both the fraction soluble in dilute alkali and the insoluble fraction are presented in Table 1. For comparison, the results obtained from the water-soluble protein are included together with those amino-acids which have free amino groups. No other amino-acids than those listed could be detected, but hydrolysis of the residual cuticular material gave two additional spots which were shown to be produced by the hydrolysis of chitin.

Free amino groups

Although the two protein fractions were insoluble in water and dilute aqueous NaHCO₃, the method used to identify the free amino groups in the water-soluble proteins (Hackman, 1953*a*) was used with slight modification. The reaction with 1-fluoro-2:4-dinitrobenzene was repeated

Table 1. *Amino-acids and free amino groups present in protein fractions isolated from the elytra of Aphodius howitti Hope*

(+, Present; ±, present in traces only; -, apparently absent.)

Amino-acid	Protein fraction			Free amino groups	
	Insol. in water, sol. alkali	Insol. in water and alkali	Sol. in water	Insol. in water, sol. alkali	Sol. in water
Aspartic acid	+	+	+	-	-
Glutamic acid	+	+	+	+	+
Serine	+	-	+	+	+
Glycine	+	+	+	+	+
Threonine	±	+	+	-	-
Alanine	+	+	+	+	+
Tyrosine	+	+	+	-	-
Valine	+	+	+	-	-
Phenylalanine	+	+	+	-	-
Leucine and/or isoleucine	+	+	+	-	-
Proline	-	-	+	-	-
Hydroxyproline	-	+	+	-	-
Tryptophan	+	+	+	-	-
Lysine	+	-	+	+	+

several times. Even after repeated reaction with fluorodinitrobenzene the residual cuticular material failed to yield any *N*-2:4-dinitrophenylamino-acids (DNP-amino-acids) on hydrolysis except for the occasional appearance of traces of DNP-glycine. On the other hand, the alkali-soluble protein fraction readily gave a DNP derivative which on hydrolysis yielded the DNP derivatives of glycine, serine, alanine, glutamic acid and lysine.

Tyrosine estimations

The method employed was that described by Lugg (1938), viz. alkaline hydrolysis and estimation of the tyrosine by the $\text{HgSO}_4\text{-HNO}_3$ technique. Trim (1941) has shown that the presence of chitin has a negligible effect on the estimation of tyrosine by this method. The residual cuticular material was found to contain 1.96% tyrosine (duplicates 1.96, 1.96%) which is equivalent to 4.4% in the hardened protein calculated to be in this fraction.

As a check on the above method the tyrosine content was estimated by the quantitative paper-partition chromatographic method of Awapara (1949) and Moore & Stein (1948). A sample of the hydrolysate was subjected to paper chromatography, ascending technique, on Whatman no. 1 filter paper with *n*-butanol-acetic acid-water as the developing solvent. Found: 1.3% tyrosine in the residual cuticular material (mean of six estimations, varying from 1.29 to 1.31%). This corresponds to 2.9% tyrosine in the hardened protein in the fraction.

DISCUSSION

The water-insoluble proteins of the elytra of adult *A. howitti* have been separated into two fractions, viz. that soluble in warm *N*-sodium hydroxide solution and the residue which is insoluble under these conditions.

Alkali-soluble protein fraction

An examination of the protein material soluble in dilute alkali has shown it to be very similar to the water-soluble proteins both as to constituent amino-acids and to those amino-acids with free amino groups. However, proline and hydroxyproline were not detected in the alkali-soluble proteins and the amount of threonine present was very small. The ash, nitrogen, sulphur and carbohydrate contents are similar to those of the alkali-soluble proteins isolated by Trim (1941) from *Sphinx ligustri* larval cuticles (see Table 2). If cuticular protein is considered to contain 15% total nitrogen then the alkali-soluble protein fraction must contain 18.9%

of other non-nitrogenous material. It is probable that this additional material is phenolic and/or quinonoid in view of the dark colour, which is soluble in alkali, and of the tanning reaction which is known to take place in insect cuticles (see Hackman, 1953*b*). The greater insolubility of part of the protein of *Aphodius howitti* cuticles could be a property of the fraction *per se* or could be due to the linking of the protein to some other cuticular component. This latter explanation is favoured; the protein chains are thought to be linked together by reaction of a small number of their free amino groups with *o*-quinones (Hackman, 1953*b*).

It is of great interest that this protein fraction only should show the presence of sulphur and carbohydrates other than chitin. The form in which the sulphur occurs is unknown and, in view of Trim's work, it is highly improbable that the sulphur is present in inorganic form.

Residual cuticular material

The properties of the residual cuticular material, which is insoluble in dilute alkali, are of interest because this material contains the hardened protein. This material contained 55.1% chitin, the remainder being fully hardened protein ('sclerotin'). As the sources of error in chitin estimations cannot be readily evaluated, this percentage can only be taken as approximate. Correcting for ash, this hardened protein would contain 11.9% nitrogen; again assuming a cuticular protein to contain 15% nitrogen, this hardened protein would contain 20.4% of additional nitrogen-free material which is equivalent to 9.0% in the entire residual cuticular fraction. Fraenkel & Rudall (1947) recorded the nitrogen content of the pigment fraction obtained from *Sarcophaga puparia* as varying from 8.6 to 11% depending upon the method of extraction. The figure of 11.9% nitrogen calculated for the hardened protein from *Aphodius howitti* compares favourably with their result.

That the added material is probably phenolic in nature is supported by the analyses for tyrosine. Lugg's method gives 4.4% tyrosine in the hardened protein whilst a quantitative partition-chromatographic method gave 2.9% tyrosine. The latter method estimated tyrosine alone whereas in Lugg's method phenolic material other than tyrosine would also be estimated. This may account for the higher

Table 2. Composition of alkali-soluble proteins from insect cuticle

Insect	Ash (%)	Total nitrogen* (%)	Total sulphur* (%)	Total carbohydrate* (as glucose) (%)
<i>Aphodius howitti</i>	9.26	12.17	Approx. 0.3	3.3
<i>Sphinx ligustri</i> †	11	12.4	0.57	3.2

* Calculated on ash-free basis.

† Trim (1941).

result and can be taken as evidence to support the presence of other phenolic material in the hardened (or tanned) protein. Such phenolic material would be introduced during the tanning reaction (Hackman, 1953*b*; Fraenkel & Rudall, 1947).

The amino-acid residues of the residual cuticular material are very similar to those of the water- and alkali-soluble protein fractions. However, serine and threonine are absent, as also is lysine. The absence of lysine is highly significant as its ϵ -amino group would have reacted with the quinones during the tanning reaction and on hydrolysis no lysine could be formed. Likewise the absence of free amino groups is important as this indicates that the free amino-groups have also reacted with the quinones. However, failure of the fluorodinitrobenzene to react with any free amino groups might possibly have been due to steric factors. This and the absence of sulphur are discussed in the following paper (Hackman, 1953*b*). Acid hydrolysis of the residual cuticular material led to the formation of a black solution, whereas alkaline hydrolysis gave a pale-brown solution. This may be due to some of the phenolic decomposition products being stabilized as sodium salts and hence prevented from undergoing further reactions.

An attempt was made to remove the chitin from admixture with the hardened protein. Orthodox chemical methods are unsatisfactory because all reagents capable of rendering chitin soluble bring about hydrolysis of the proteins. Enzymes appear

more promising and a chitinase was prepared from almonds (Tauber, 1932; Zechmeister & Toth, 1938, 1939). However, this particular chitinase could not be used as it could not be freed of proteases.

SUMMARY

1. An examination has been made of the protein fractions isolated from the elytra of adult *Aphodius howitti* which are (i) insoluble in water but soluble in warm dilute alkali, and (ii) insoluble in both water and warm dilute alkali. These two fractions have been analysed for ash, total nitrogen, total sulphur, total carbohydrate and chitin, and the constituent amino-acids and free amino groups have been identified.

2. The amino-acids identified in the acid hydrolysates of the fraction soluble in alkali are very similar to those of the water-soluble proteins (Hackman, 1953*a*), the major difference being the absence of proline and hydroxyproline. In both the water-soluble and alkali-soluble proteins the same amino-acids have free amino groups.

3. The fraction insoluble in both water and alkali contained chitin in addition to tanned protein. The tanning reaction introduces much phenolic material. The amino-acids detected on acid hydrolysis are similar to those of the water- and alkali-soluble proteins but serine, threonine and lysine are absent. Free amino groups could not be detected in this material.

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