123. STUDIES IN THE CHEMISTRY OF THE INSECT CUTICLE

I. SOME GENERAL OBSERVATIONS ON CERTAIN ARTHROPOD CUTICLES WITH SPECIAL REFERENCE TO THE CHARACTERIZATION OF THE PROTEINS

By A. R. TRIM

From the Biochemical Department and the Biological Field Station, Imperial College of Science and Technology, Slough

(*Received 1 September 1941*)

Previous workers have shown that, besides the nitrogenous polysaccharide chitin, the cuticle of insects contains high proportions of other substances, including protein. In his classical survey of the insect cuticle Odier [1823] recognized five general categories of substances in the elytra of cockchafers and other insects; an inert substance, which he proposed to call chitin, pigments, fatty material, albuminous substance and mineral components. Of these the chitin proper formed the skeletal structure and was, in the cases investigated, impregnated with large quantities of brown-pigmented protein removable by heating with potash. This was overlain by a thin layer of fat-soluble material often containing brightly coloured pigments.

Among the large number of chitin-containing organs of both plants and animals two main characters are apparent. (1) As far as they have been studied all specimens of chitin have been shown to be almost identical, both chemically and crystallographically. (2) The chitin always occurs associated with protein. The chitin itself has been studied very closely and is known to be composed of chains of condensed *N*-acetyl glucosamine units and is identical with cellulose except in that the secondary —OH at C_2 is substituted by an acetamide group. Krawkow [1892] showed that chitin is present in all groups of Arthropods and in certain Annelids and the Cephalopod Molluscs. Further, he showed that wherever chitin structures occur protein is also present. Iwanoff [1923] obtained chitin from fungi and showed that protein was associated with it, probably through an organic phosphate linkage. Gonell [1926] suggested from a review of the X-ray diffraction properties of a large number of chitin preparations from plant and animal sources that their micro-crystalline structures are, with rare exceptions, identical. Campbell [1929] made a quantitative investigation of the chitin content of the insect integument, using a method based on the van Wisselingh reaction; in the cockroach he found large variations (e.g. exocuticle 23% chitin, endocuticle 57% chitin).

In most of the above-mentioned studies attention was focussed on the chitin proper. Wigglesworth [1933] obtained qualitative evidence for the presence of protein in the cuticle of *Rhodnius* (Hemiptera) at several different stages of the life history. Pryor [1940] obtained similar data for other insects. Fraenkel & Rudall [1940] obtained some general quantitative data on the composition of the cuticle of the blowflies *Sarcophaga falculata* and *Calliphora erythrocephala* and using X-ray analysis threw light on the distribution and function of protein

in the general structure. They showed that 60% of the dry weight of the larval cuticle of *Sarcophaga* and 47% of the puparium is chitin. In both cases the non-chitin material has the typically high N content of protein.

Apart from its academic interest the insect cuticle has been studied in the present work with a view to contributing to the elucidation of certain physiological problems. These are concerned with its permeability as a membrane and in particular with the penetration of a large group of insecticidal agents which are known to act through the cuticle.

It has now been possible to characterize the proteins of the larval cuticle of *Sarcophaga*; the first is water- and borate-soluble, the second is extracted from the residue by 5% NaOH and contains carbohydrate. Similar fractions have been prepared from the hawk moth (*Sphinx ligustri*) and a water-soluble protein from the cuticle of the lobster (*Homarus*). Certain specific differences in the compositions of the proteins from the three sources have been noted. It appears that the hard melanized protein of the puparium of *Sarcophaga* is similar to the soluble protein of the larval cuticle, and leads to agreement with the view that the correlated hardening and darkening of the puparium is due to the tanning of the protein-chitin complex by diphenolic and quinonoid substances derived from tyrosine.

Experimental

Preparation of proteins for analysis

The skins were prepared by the method of Fraenkel & Rudall [1940] except where otherwise stated. The majority of them were very kindly provided by Dr Fraenkel. As described by Fraenkel & Rudall, part of the protein of the larval cuticle of *Sarcophaga* is extremely soluble in water and the bulk of it may be obtained by grinding the cuticle with sand in cold water for a short time. The whole of the protein is not removed by this treatment, however, and further yields can be obtained by soaking in water at 0° for several days, and following this by extraction with boiling water. The three fractions thus obtained are identical so far as total N and general physical and chemical properties are concerned. The protein is obtained more readily and without risk of degradation by extracting the skins at 50° with a buffer solution at *p*H 9·2, consisting of borax solution (19 g./l.), water, alcohol and ether in the proportions of 5 : 5 : 4 : 1. After 36 hr. no further material precipitable by trichloroacetic acid is extracted unless the skins remain in contact with the solvent for many weeks at room temperature. The borate extract is then filtered through paper pulp, the filtrate acidified with HCl to *p*H 3–4 and precipitated by 1/3 saturation with $(NH_4)_2SO_4$ or full saturation with NaCl. The precipitate is then centrifuged, resuspended in distilled water and dialysed against distilled water for 2 days. During such treatment the precipitate tends to go into solution; the mixture was therefore reduced *in vacuo*, transferred to a flat dish and kept in a vacuum desiccator over NaOH. The brittle translucent and almost colourless solid thus obtained was ground up with alcohol, extracted twice with boiling alcohol and then with boiling ether to remove fat. A similar product was prepared by a like treatment of the hawk moth cuticle. In the case of the lobster the integument was first finely ground in a mechanical mortar with the borate buffer mixture so as to give a soft mud.

The second protein was prepared from *Sarcophaga* cuticles by extracting the residues from the first with 5% NaOH at 50° for 5 hr. The extract was then filtered and the protein precipitated by adding several volumes of acetone. The fine precipitate was next suspended in distilled water and dialysed against

distilled water for 2 days. The procedure then followed that described above for the borate-soluble protein. Insufficient material from *Sarcophaga* was obtained for characterization by chemical analysis, but this was possible with the product derived from *Sphinx ligustri*.

Methods of analysis

The following techniques were used: Total N, micro-Kjeldahl; total S, micro-Carius (Weiler); total P, Fiske & Subbarow [1925]; total carbohydrate, Pirie's [1936] modification of Tillmans' & Philippi's method [1929]; cystine and methionine, Lugg's [1938, 2] modification of Baernstein's [1936] volatile iodide method; amino-sugar, Nilson [1936], Elson & Morgan [1933].

The N distribution in the proteins was determined by a modification of Thimann's [1926] method for the determination of the Hausmann number. Humin was neglected and accurate ammonia values were determined on separate hydrolysates of about 50 mg. protein. These were brought to *p*H 6–7 with NaOH, using bromothymol blue as indicator, transferred to a micro-Kjeldahl distillation apparatus and the ammonia distilled from an excess of borax solution into standard acid in the usual way. The ammonia-free hydrolysates for base determination were reduced to 10 ml., acidified and precipitated with phosphotungstic acid according to Thimann's procedure. The phosphotungstic acid was removed from both the base and monoamino-acid fractions by extraction with an amyl alcohol-ethyl alcohol-ether mixture according to Van Slyke [1915]. The removal of the phosphotungstic acid facilitated incineration for N determinations and comparison of the N distribution of edestin determined by this method with and without the removal of the phosphotungstic acid showed that a negligible quantity of N was lost during the extraction. The amino-N and hence the non-amino-N of the monoamino fraction was determined by the usual Van Slyke constant volume gasometric method. True amide-N values were determined by heating about 50 mg. protein with 5 ml. 2*N* HCl for 3 hr. on a boiling water bath and estimating the ammonia produced as in the previous determination.

Glycine was determined by Patton's [1935] colorimetric method, using 300–500 mg. protein instead of the 3 g. employed by that author, the amounts of reagents employed being reduced accordingly. Good values were obtained for duplicate analyses of gelatin (23·8 and 24·6%).

Tyrosine and tryptophan in the proteins of the whole cuticle were determined by Lugg's [1937] method, with minor modifications. The chitin residues after alkaline hydrolysis in the usual way were thoroughly washed with hot water and finally with dilute H_2SO_4 to remove the persistently adhering alkaline hydrolysate. The large bulk of washings and hydrolysate was then reduced to a small volume *in vacuo* and finally made up to 25 ml. The employment of Lugg's [1938, 1] alkaline stannite reducing reagent gave no marked difference in the tyrosine value obtained. The possible effect of chitin on the estimation of the two amino-acids was investigated by adding chitin to casein in the proportions in which it occurs in the cuticles under investigation. The effect was negligible as shown by Table 1.

Table 1. *The effect of chitin on the estimation of tyrosine and tryptophan*

Material	Chitin mg.	Protein mg.	Tyrosine %	Tryptophan %
Crab chitin	202	0·0	0·0	0·0
Insect chitin	181	0·0	0·0	0·0
Casein (N = 15·65%)	0·0	196	5·74	1·52
Casein + insect chitin	145	201	5·74	1·49
Casein + crab chitin	195	188	5·72	1·51

To determine sulphate the hydrolysates were adjusted and precipitated according to the standard methods of quantitative inorganic analysis. They were checked by precipitation with benzidine hydrochloride [Cole, 1933].

The proteins of the whole cuticle

Analytical data for the various intact Arthropod cuticles, the residues obtained from them by extraction for 30 hr. with borate buffer at 50°, and the residues obtained from these by further extraction with 5% NaOH for 5 hr. at 50°, are given in Tables 2–4. In all cases the borate buffer extracted a protein

Table 2. *Analysis of the intact cuticles of certain Arthropods*

Material	Total N %	Total S %	Total P %	Total carbohydrate as glucose %	Ash %
Sarcophaga falculata (blowfly) larval cuticle	9·0	0·56	0·0	0·4	0·56
Calliphora erythrocephala (blowfly) larval cuticle	10·7	0·40	0·0	0·5	—
Phormia terra novae (blowfly) larval cuticle	11·3	0·32	0·0	0·5	—
Sarcophaga puparium	11·3	0·59	Trace	—	1·9
Sphinx ligustri (hawk moth) larval cuticle	10·9	0·30	Trace	0·8	1·5
Dixippus (stick insect) adult abdominal cuticle	11·8	0·21	0·0	0·9	—
Locust adult abdominal cuticle	11·8	0·34	0·0	0·6	—
Prawn cuticle (decalcified)	9·4	—	+ve	+ve	—

Table 3. *The composition of cuticle residues after extraction with borate buffer mixture*

Material	Total N %	Total S %	Total carbohydrate as glucose %	Ash %
Sarcophaga larval cuticle	7·8	0·25	0·9	0·6
Sphinx larval cuticle	7·9	0·38	1·0	1·8
Dixippus abdomen	10·5	0·20	0·9	—
Locust abdomen	11·8	0·33	0·6	—
Lobster residue	6·9	0·44	0·7	—

Table 4. *The composition of cuticle residues after extraction with 5% NaOH*

Material	Total N %	Total S %	Total carbohydrate as glucose %	Ash %
Sarcophaga larval cuticle	6·5	0·0	0·9	2·5
Sphinx larval cuticle	6·5	0·24	0·9	2·1

having general properties similar to the product obtained from *Sarcophaga* larval cuticle, though the amount obtained from *Dixippus* and locust was small. The reagent, however, has left a large proportion of the carbohydrate and of the S-containing component intact. The data given in Table 5 are almost identical with those obtained from the residues of both *Sarcophaga* larval cuticle and puparia after extraction with 5% NaOH for 2 days at 100°, or after prolonged

diaphanol treatment [Fraenkel & Rudall, 1940]. All the residues still contain about 0·8% of material giving the orcin reaction for carbohydrate, and this is not removed by more prolonged treatment with any of the reagents concerned.

The borate- and water-soluble proteins

The proteins of Sarcophaga. The observations of Fraenkel & Rudall [1940] on the general precipitation properties of this protein were confirmed and extended; in addition it was found that these properties were shared by all the borate- and water-soluble proteins prepared from other insects. The protein is extremely soluble in water, and is not coagulated by heat. It is precipitated from solution completely by 10% trichloroacetic acid and also at *p*H 3–4, but not at higher values, by one-third saturation with $(NH_4)_2SO_4$ and saturation with NaCl. Many volumes of alcohol and of acetone are required to precipitate it from either acid or alkaline solution. Wet trichloroacetic acid precipitates will gel in alcohol or acetone, but water gels similar to those of gelatin have not been observed.

Table 5. *Nitrogen distribution in the water-soluble proteins of the cuticle*

Nitrogen values given are in percentages of total protein-N.

Material	Total N % protein	Amide-N†	Ammonia-N‡	Base-N	Mono-amino-N	Non-amino-N
Edestin (Osborne & Harris)	—	10·1	—	31·6	57·7	—
Edestin (Thimann [1926])	18·4	—	11·1	28·8	58·7	—
Edestin (modified Thimann method)	18·4	—	10·3	29·8	58·3	—
Sarcophaga protein	15·0	5·3	6·2	23·0	72·4	4·3
	—	—	6·2	26·4	70·0	3·5
Sphinx protein	15·1	7·4	8·5	18·0	75·0	—
	—	7·4	8·5	21·0	68·2	—
Lobster protein	15·4	8·5	9·5	18·1	70·4	—
Sarcophaga larval cuticle*	—	—	—	20·5	75·2	2·0
	—	—	—	19·3	77·8	3·0
Sarcophaga puparium*	—	—	—	22·7	67·0	3·5
Sericin A (Ito & Komori [1939])	17·37	—	12·14	16·91	70·10	0·61
Sericin B (Ito & Komori [1939])	16·88	—	12·22	18·20	69·19	1·16

* 5% HCl hydrolysis for 48 hr. to separate chitin, chitin removed and values calculated on percentage of total N of non-chitin fraction.
† Ammonia given after hydrolysis with 2*N* HCl for 3 hr.
‡ Ammonia given after hydrolysis with 20% HCl for 24 hr.

Table 6. *Some amino-acids and other components in the water-soluble cuticle proteins and in sericin*

Protein	Tyrosine %	Trypto-phan %	Glycine %	Hexos-amine as glucos-amine %	Carbo-hydrate as glucose %	Total S %	Total P %	Ash %
Sarcophaga protein	5·2	1·4	0·67	0·0	0·0	0·0	0·0	0·4
Sphinx protein prep. 1	11·7	0·5	1·32	0·0	0·0	0·87	0·0	0·2
Sphinx protein prep. 2	11·6	0·5	—	0·0	0·0	0·34	0·0	0·2
Lobster protein fraction 1	6·0	+ve	—	0·0	+ve	+ve	0·0	—
Sericin A	6·0*	1·0*	1·2†	0·45§	0·37§	1·2*		
Sericin B			1·5‡	1·08§	1·25§			

* Alders [1927].
† Abderhalden & Worms [1909].
‡ Strauch [1909].
§ Ito & Komori [1939].

Chemically the protein exhibits weak biuret and xanthoproteic reactions similar to those given by gelatin. Tests for carbohydrate, P, S, and for amino-sugars by the methods of Elson & Morgan [1933] and Nilson [1936] were negative. The product obtained by ice-cold water extraction of the living skin gives the same reactions; it is unlikely therefore to be the degradation product of a coagulable protein, though certain of its physical properties and the absence of S do, it is true, suggest a similarity to gelatin. The analytical data given in Tables 5 and 6 however do not bear out such a relationship; the glycine content is low, as also must be the proline content (non-amino-N, Table 5). The data are, indeed, more in keeping with the suggestion that the Arthropod cuticular proteins are akin to sericin (silk gelatin), but again the analogy must not be pressed too far, for they do not contain the very high proportion of serine that is characteristic of the latter protein. The data for threonine-N, and for β-hydroxy-α-amino-N given in Table 7 were obtained by the indirect method of Martin & Synge [1941]. The difference between the two values for any one protein probably

Table 7. *Minimum values for the distribution of nitrogen between α-amino-β-hydroxy-N and amide-N in some proteins*

Values given are in percentages of total protein-N.

Protein	Total N %	Amide-N	Total α-amino-β-OH-N	Threonine-N
Casein [Martin & Synge, 1941]	13·4	10·4	6·3	2·1
Casein	15·65	10·0	6·2	2·2
Sericin 1	17·1	10·6	21·4	4·4
Sericin 2	17·0	10·4	24·4*	1·9
Sarcophaga larval protein	15·0	6·9	6·8	1·8
Sphinx larval protein	15·1	7·8	6·5	2·5

* Allowing the reaction and aeration to continue for another hour this value was increased to 28·7% although continued aeration for the same period in the case of the amide determination and the hydroxyamino-N of other proteins did not give this large increase.

represents serine-N, and the high content recorded for each of the two samples of sericin confirms the recent findings of Nicolet & Saidel [1941]. It should be noted that the products referred to as sericin 1 and 2 in Table 7 were not prepared in the same way as the A and B of Ito & Komori [1937]. 1, was more soluble in water than 2, whereas B was precipitated by acid at *p*H 4·1 and A by adding alcohol to the mother liquor.

The protein of the hawk moth, Sphinx ligustri

A protein similar in physical and certain chemical properties to that of *Sarcophaga* was obtained from the larval cuticle of this insect. It exhibited certain marked and also certain less pronounced differences however; the tyrosine content, for instance, was extremely high. Two products made from different broods of larvae gave identical analytical data with the exception of total S, and the variations here may be due to the method of preparation. In the first the protein was precipitated from the borate extract by one-third saturation with $(NH_4)_2SO_4$ at *p*H 3–4: in the second the reagents employed were S-free and the precipitation was carried out at *p*H 3–4 with saturated NaCl. The high S content of preparation 1 therefore may have been due to retention of sulphate ions though one would have expected these to have been removed by the subsequent dialysis. Whether or not the S-containing component occurs in easily dissociable form is not known, nor is there as yet any indication of the

type of compound concerned. Experiments have shown that it contains organic S, and that this is not in the form of ethereal sulphate, cysteine, cystine, sulphydryl or methionine. The sulphur reactions for cystine, and Fink's [1927] test for sulphydryl groups, for instance, were negative for both the isolated protein and the intact skin; likewise a distribution of S by Lugg's [1938, 2] modification of Baernstein's [1936] volatile iodide method gave a negative result in both cases. The absence of inorganic S was shown in the following way. Duplicate dried samples of preparation 2 were dissolved by warming with a little distilled water and made up to about 50 ml. in *N* HCl: 2 ml. of a solution containing 36 mg. $BaCl_2, 2H_2O$/ml. were added and the whole kept. After 36 hr. the precipitate was centrifuged and the protein solution decanted. The precipitate was washed with hot water, transferred to a crucible and ignited. There was no appreciable ash, indicating the absence of free inorganic sulphate from the protein preparation. Duplicate hydrolyses each with about 500 mg. of protein were made by boiling it with 15 ml. 5*N* HCl for 12 hr. One hydrolysate was precipitated with $BaCl_2$ under standard conditions; no appreciable residue remained after ignition. The second hydrolysate was freed from excess HCl and treated with benzidine hydrochloride, but no precipitate was obtained. These experiments suggest that ethereal sulphate is not present in the protein. Negative results were also obtained from the intact cuticle of *Sarcophaga*.

The protein of lobster cuticle is similar to that of the two insects, the tyrosine content being comparable with that of *Sarcophaga*. Glycine was not determined.

The protein fraction soluble in warm alkali

Protein from the hawk moth. The original protein preparation effervesced when treated with acid; it was therefore redissolved in water and dialysed against distilled water. The dried preparation contained 11% ash, which was completely soluble in water and strongly alkaline. It was probably Na_2CO_3 derived from the alkali used for extraction. On the ash-free basis the preparation analyses as follows: total N, 12·4%; total S, 0·57%; carbohydrate (as glucose), 3·2%. The carbohydrate content of this fraction is higher than that of any other, showing that in the cuticle the carbohydrate is present either directly combined with the protein or in the form of polysaccharide. Tests for amino-sugars [Nilson, 1936] were negative. The sulphur values indicate that the S-containing component is more closely associated with the carbohydrate-rich fraction than with any other.

Protein from Sarcophaga *puparium*

It has been shown by Fraenkel & Rudall [1940] that a certain amount of the puparium protein may be extracted with cold water. This was confirmed by the fall in the total N from 11·0 to 9·3% after grinding with distilled water in a mechanical mortar for about 1 hr. As described below, all attempts to obtain the remaining protein in an intact state were unsuccessful, suggesting that the protein and chitin are not chemically separable entities, but are firmly bound by linkages at least as strong as those between the amino-acids of the protein.

Ammoniacal copper solutions, ethylene glycol, dioxan, anhydrous formamide, anhydrous formamide containing a little formic acid, saturated urea and strong cyanide solutions were found to have little or no solvent action upon intact powdered puparia. Acid solutions, even conc. HCl, had only a slow solvent action upon the residual water-extracted puparia compared with their action upon the soft larval cuticle of the same animal. Their action is not noticeably increased by the presence of reducing agents such as SO_2. Alkalis have much

more effect, but even when dilute (0·5*N* NaOH) have a strong disrupting action upon the protein. Here, again, reducing agents such as $SnCl_2$ do not promote solution.

Clark & Smith [1936] showed that pure chitin is readily dispersed at 95° in aqueous LiCNS previously saturated at 60°. Von Weinarm [1912] showed that proteins like keratins and silk fibroin were not dispersed by this reagent until very high concentrations of LiCNS and a temperature of 170° had been reached. Experiments with the puparia showed them to be similar to the latter substances in their dispersal properties. The dispersed product was partially reprecipitable with acetone after the removal of LiCNS by dialysis. However, the precipitated product and that remaining in solution both had a total N of about 11%, the same as the untreated material; consequently it appears that no effective separation of the protein and chitin had been obtained.

No further attempts to separate the protein from the chitin were made, but work was continued on partial hydrolysates of the whole skin. 5% HCl and 5% NaOH remove the protein by hydrolysis on heating to 100° for 48 hr. whereas the chitin is unaffected and can be filtered off so as to permit the hydrolysis of the protein being taken to completion in the usual way. Chitin residues obtained by this treatment contained 6·3–6·5% N (purified chitin 6·9% N) and are comparable with products obtained by much milder treatment. As shown in Table 5 a fully hydrolysed 5% HCl extract of *Sarcophaga* puparia has a N distribution comparable with that of a similar extract from the larval cuticle and of the protein isolated from it by borate.

Chemical changes on pupation

There was a decrease in the amount of tyrosine from 3·5 to 2·0% in the cuticles of a brood of *Sarcophaga* on pupation. Previous experiments had shown that chitin has no effect on the estimation of the tyrosine in casein; consequently it is unlikely that it had any upon the determinations with the cuticle protein. A comparison of prepupae and pupae from the same brood shows that there is a corresponding decrease in the total tyrosine in the whole organism, from 1·70 to 0·87%.

There is little doubt that much of the lost tyrosine, part of which must have come from the protein in the cuticle, has been oxidized to form pigmented products. That the oxidation has proceeded as far as the melanin stage is shown by the fact that the tyrosine value for the puparia is not increased if the extraction is made with alkaline stannite [Lugg, 1938, 2]. The stannite reduces the whole of the dark brown pigments, giving a clear, golden-yellow hydrolysate and an almost colourless chitin residue. On exposure to the air the hydrolysate rapidly resumes a dark brown colour indistinguishable from that of the normal alkali-hydrolysed puparia, showing that the pigment (melanin) is an oxidation product of a precursor held reduced by the action of the stannite. This oxidation is retarded but not completely inhibited if the reduced solution is acidified. Ether will extract from the reduced and acidified solution a small proportion of this easily oxidized material, which is in turn extracted from the ether by an alkaline solution, and in the latter state gives Arnow's [1937] characteristic reaction for dihydric phenols. The bulk of the reduced pigment remains in the original aqueous acid phase, from which it may be sharply and completely separated by extraction with amyl alcohol. Butyl alcohol was also found to have similar powers of extraction although amyl alcohol gave a better separation. The fresh amyl alcohol extracts are red but gradually turn brown on contact with the air. No reduced pigment can be extracted directly from the alkaline stannite hydro-

lysate and if the amyl alcohol extract from the acidified solution is removed and treated with alkaline solutions the colour changes to a light olive-brown and the pigment passes into the aqueous phase once more. The brown pigment extracted by acid hydrolysis of the puparia is also insoluble in amyl alcohol. Mazza & Stolfi [1931] extracted from the skin of the marine worm *Halla parthenopea* a red pigment, hallachrome, which was reversibly oxidized and reduced (see also Friedheim [1933]) and had similar amyl alcohol solubility properties to the above reduced pigments. They showed that it was the 5:6-quinone of dihydro-indole-2-carboxylic acid, previously shown by Raper [1926] to be the red intermediate in the oxidation of tyrosine to melanin under the action of tyrosinase. Schmalfuss and co-workers [1927; 1933; 1935; 1937] have isolated 'dopa' and 3:4-dihydroxyphenylacetic acid from the hard, dark cuticles of certain insects. The above evidence suggests that a small proportion of the tyrosine derivatives in the puparium may be in the form of such dihydric phenols but that the majority of them are probably indole derivatives similar to hallachrome. Further, it is likely that they are formed by polyphenol oxidase in the cuticle, for Bhagvat & Richter [1938] have shown that the enzyme is present in high activity in the unmelanized skins of certain insects.

A few further experiments were made on the amino-acid content of the puparium protein. 3·4 g. of puparia were boiled under reflux with 20 ml. of 5*N* HCl for 24 hr. and cooled; the residual chitin was filtered off and washed with water. The filtrate and washings were reduced to a small volume, made up to 20 ml. with 20% HCl and boiled under reflux for a further 24 hr. Excess HCl was then removed by evaporation in the usual way and the dicarboxylic acids precipitated according to Foreman [1914]. The residual lime carried down the melanin pigments and the humin, and clean extracts were obtained, from both the dicarboxylic acid precipitate and the filtrate, on removing the Ca^{++} as oxalate. From the former 52 mg. of glutamic acid hydrochloride (N = 7·62%) and 36 mg. copper aspartate ($C_4H_5O_4NCu$, $4{\cdot}5H_2O$ requires H_2O, 29·2%; the anhydrous salt N, 7·2%: found 29·2% and 7·1% respectively). Repeated esterification of the clarified Foreman filtrate and saturation with gaseous HCl at low temperature failed to give any glycine ester hydrochloride. From the experience of Fischer [1902] and later workers it may be concluded that the puparium protein does not contain more than a few per cent of glycine.

Discussion

It will be seen that the amino-acid analyses of various Arthropod cuticles, though somewhat limited in scope on account of shortage of material, have shown that the constituent proteins differ from the collagens such as gelatin and are in certain respects akin to sericin. There is, for instance, general agreement in the values for amide-N, basic N, non-amino-N, tyrosine and tryptophan, and in the absence of cystine and methionine. On the other hand there is a sharp differentiation in the high serine content of sericin.

More interesting perhaps is the analogy that can be drawn between the insect cuticle and the sericin-fibroin complex of raw silk. The micro-crystalline skeletons of the two complexes are crystallographically comparable in some respects. The fibroin skeleton of silk differs from the majority of proteins in the predominance of the simple amino-acids glycine and alanine (glycine 43·8%, alanine 26·4% [Bergmann & Niemann, 1937]) and as Meyer & Mark [1930] have indicated, it is also structurally anomalous among protein fibres in that its X-ray diffraction photographs may be interpreted directly on the assumption that the chain is

composed of alternating glycine and alanine residues. In this sense fibroin is the only protein directly comparable with cellulose and chitin.

Silk is secreted by glands derived from the epidermal layer, as are the cells of the hypodermis which secretes the cuticle. Foa [1912] has shown that unsecreted silk in the seripterium of the silk-worm larva is a homogeneous substance soluble in boiling water, 5–20% NaCl and 5–10% Na_2CO_3, non-dialysable and non-heat-coagulable. Silk therefore must be formed on spinning by crystallization of fibroin within a sericin matrix. Ramsden [1938] has suggested a mechanism for this process. It is possible that chitin crystallizes in a similar way from a homogeneous fluid polysaccharide-protein complex secreted by the hypodermis, in other words, that the cuticle is preceded by a stage comparable with the known mucopolysaccharide-protein salts.

According to Meyer [1938] the mucopolysaccharide of synovial fluid may be spun to produce a material comparable with chitin in some of its physical properties. It is composed of long, doubly refracting fibres of considerable tensile strength. Alsberg & Hedblom [1909], moreover, have shown that chitin may be redispersed after prolonged treatment with dilute HCl in the cold to give a water-soluble form comparable with a soluble mucopolysaccharide. A further similarity between the vertebrate tissues in which mucoproteins occur and the chitin-protein complexes of invertebrates is the fact that they are both selectively calcified.

Summary

Protein, carbohydrate and a sulphur-containing component have been shown to be present in the cuticles of a variety of insects.

The protein fractions are usually large and when extracted show similar general properties.

The protein of the larval cuticle of *Sarcophaga falculata* was characterized by the determination of its nitrogen distribution, tyrosine, tryptophan, glycine and β-hydroxy-α-amino-acid values. Sulphur and conjugated non-protein components were absent. The data showed that the protein was quite distinct from collagen in its chemical composition and that it bears a close resemblance to sericin, although the characteristically high serine content of the latter was not recorded for the cuticle protein.

Similar proteins from the hawk moth larva *Sphinx ligustri* and the lobster were characterized in the same way. Certain marked differences from the blow-fly protein were recorded in each case. The tyrosine value of 11·7% of the weight in the case of the hawk moth protein is remarkably high.

The protein of the hard, melanized puparium of *Sarcophaga* appeared to differ from the larval protein mainly in a loss of solubility and the replacement of a proportion of its tyrosine by melanoid oxidation products. These latter were reduced, extracted and shown to have some of the properties of hallachrome.

The possible formation of chitin from a homogeneous fluid polysaccharide-protein complex secreted by the hypodermis is briefly discussed.

I am greatly indebted to Prof. J. W. Munro for continued interest and encouragement in this work, to Prof. A. C. Chibnall for his kind interest and advice, the hospitality of his laboratory and the gift of certain amino-acids and protein preparations. I wish to thank Dr G. Fraenkel for a large proportion of the insect material used in this work, Drs K. M. Rudall, K. Bailey and R. L. M. Synge and Messrs E. F. Williams and M. W. Rees for their help. Finally I am indebted to the Agricultural Research Council for a grant to carry out this work.

REFERENCES

Abderhalden & Worms (1909). *Hoppe-Seyl. Z.* **62**, 142.
Alders (1927). *Biochem. Z.* **183**, 446.
Alsberg & Hedblom (1909). *J. biol. Chem.* **6**, 483.
Arnow (1937). *J. biol. Chem.* **118**, 531.
Baernstein (1936). *J. biol. Chem.* **115**, 25.
Bergmann & Niemann (1937). *J. biol. Chem.* **122**, 577.
Bhagvat & Richter (1938). *Biochem. J.* **32**, 1397.
Campbell (1929). *Ann. ent. Soc. Amer.* **22**, 401.
Clark & Smith (1936). *J. phys. Chem.* **40**, 863.
Cole (1933). Practical Physiological Chemistry, p. 360. Cambridge: Heffer.
Elson & Morgan (1933). *Biochem. J.* **27**, 1824.
Fink (1927). *Science*, **65**, 143.
Fischer (1902). *Hoppe-Seyl. Z.* **35**, 229.
Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
Foa (1912). *Z. Chem. Industr. Kolloide*, **10**, 7.
Foreman (1914). *Biochem. J.* **8**, 463.
Fraenkel & Rudall (1940). *Proc. roy. Soc.* B, **129**, 1.
Friedheim (1933). *Biochem. Z.* **259**, 257.
Gonell (1926). *Hoppe-Seyl. Z.* **152**, 18.
Ito & Komori (1937). *J. Agric. Soc. Japan*, **13**, 115.
—— —— (1939). *J. Agric. Soc. Japan*, **15**, 50.
Iwanoff (1923). *Biochem. Z.* **137**, 320.
Krawkow (1892). *Z. Biol.* **29**, 177.
Lugg (1937). *Biochem. J.* **31**, 1422.
—— (1938, 1). *Biochem. J.* **32**, 775.
—— (1938, 2). *Biochem. J.* **32**, 2114.
—— (1938, 3). *Biochem. J.* **32**, 2123.
Martin & Synge (1941). *Biochem. J.* **35**, 294.
Mazza & Stolfi (1931). *Arch. Sci. biol. Italy*, **16**, 183.
Meyer (1938). *Cold Spr. Harb. Symp.* **6**, 91.
—— & Mark (1930). Der Aufbau der Hochpolymeren organ. Naturstoffe. Leipzig.
Nicolet & Saidel (1941). *J. biol. Chem.* **139**, 477.
Nilson (1936). *Biochem. Z.* **285**, 386.
Odier (1823). *Mem. Soc. Hist. nat. (Paris)*, **1**, 29.
Patton (1935). *J. biol. Chem.* **108**, 267.
Pirie (1936). *Brit. J. exp. Path.* **17**, 259.
Pryor (1940). *Proc. roy. Soc.* B, **128**, 393.
Ramsden (1938). *Nature, Lond.*, **142**, 1120.
Raper (1926). *Biochem. J.* **19**, 735.
Schmalfuss & Muller (1927). *Biochem. Z.* **183**, 362.
—— (1935). *Hoppe-Seyl. Z.* **231**, 161.
—— (1937). *Biochem. Z.* **294**, 112.
—— & Winkelmann (1933). *Biochem. Z.* **257**, 188.
Strauch (1909). *Hoppe-Seyl. Z.* **62**, 142.
Thimann (1926). *Biochem. J.* **20**, 1190.
Tillmans & Philippi (1929). *Biochem. Z.* **215**, 36.
Van Slyke (1915). *J. biol. Chem.* **22**, 281.
von Weinarm (1912). *Industr. Engng Chem.* **19**, 109.
Wigglesworth (1933). *Quart. J. micr. Sci.* **76**, 269.