STUDIES ON CHITIN

IV. THE OCCURRENCE OF COMPLEXES IN WHICH CHITIN AND PROTEIN ARE COVALENTLY LINKED

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[*Manuscript received June* 29, 1960]

Summary

Samples of chitin have been prepared from the cuticles of insects and crustacea, from cuttlefish shell, and from the skeletal pen of squid. In every case protein was bound by covalent bonds to the chitin so forming stable complexes (glycoproteins). Glycoproteins from different sources contained differing amounts of chitin and protein, the ratio of chitin to protein varying from 1 : 1 to 20 : 1. The protein would appear to be linked to the chitin through aspartyl or histidyl residues or both and each glycoprotein is polydisperse. The chitin preparations included glycoproteins which contained α- and β-type chitins and also a third and different type of chitin. The amino acid composition of the protein components of the glycoproteins has been determined. It is probable that chitin does not occur uncombined with protein.

I. INTRODUCTION

Chitin occurs in the cuticles of all arthropods and in certain other invertebrates, e.g. squid and cuttlefish. It is always associated with protein and, for example, in crustacea the cuticle may also be heavily calcified. For reviews on the distribution of chitin in nature see Richards (1951, 1958) and Rudall (1955). Chitin, which was originally defined as the cuticular material insoluble in hot aqueous alkali, is a polymer composed of 2-acetamido-2-deoxy-α-D-glycopyranose (i.e. *N*-acetyl-D-glucosamine) residues linked in the 1,4-β-glucosidic manner of cellulose. Its molecular weight is not known with any degree of accuracy and estimates range from a chain composed of from several hundred to one thousand residues.

Chitin is usually prepared from cuticles by repeatedly extracting them with hot dilute alkali (e.g. 1N aqueous sodium hydroxide at 100°C). If necessary the cuticles are decalcified prior to treatment with alkali. Such drastic treatment may well bring about structural changes in the chitin, in particular removal of pendent groups (e.g. proteins, peptides, or acetyl groups) and fragmentation. The presence of pendent groups may be important biologically and so it is of interest to know if such groups are present and if so the manner in which they are linked to the chitin chain.

The chitin which occurs in arthropods and also in some other invertebrates is designated α-chitin to distinguish it from the β-chitin which occurs in some parts of certain invertebrates, e.g. the skeletal pen of the squid and the chaetae of annelids. There is no record of β-chitin occurring in arthropods. β-chitin, which is distinguished from α-chitin by its X-ray diffraction pattern, was first described by

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Lotmar and Picken (1950). Work on β-chitin has been continued by Rudall (1955) who concluded that the β-chitin structure appears to be associated with collagen-type cuticles, whereas the α-chitin structure replaces collagen-type cuticles. β-chitin is converted to α-chitin by the action of cold 6N hydrochloric acid (Rudall, personal communication).

For more than one hundred years there has been speculation in the literature concerning the interaction between components of the insect cuticle. The old idea that the insect cuticle is a chitinous matrix impregnated with other materials has gradually given way to a consideration of the existence in the cuticle of a glycoprotein—a complex formed from chitin and protein, the two major components of the cuticle. Hackman (1955) and Hackman and Goldberg (1958) have shown that there is a weak bonding between chitin and some of the proteins in the insect cuticle, while in the larval cuticle of *Agrianome spinicollis* McL. much of the protein is bound to chitin in the form of a glycoprotein. Foster and Hackman (1957) have shown that in the cuticle of another arthropod, the crab *Cancer pagurus* L., part of the protein is bound chemically to chitin and the glycoprotein so formed is polydisperse.

In this paper a study has been made of the chitin-containing glycoproteins which occur in the cuticle of insects and crustacea, in the skeleton of cuttlefish, and in the skeletal pen of squid. Glycoproteins containing α- and β-type chitins have been investigated.

II. Experimental and Results

(a) Lucilia cuprina (*Wied.*)

Chitin was prepared from washed empty puparia of *L. cuprina* by extracting the powdered material with 1N aqueous sodium hydroxide at 100°C until no further colour was extracted. The residue was collected by centrifugation, washed once with water, and dialysed against running tap water for several days until the pH of the supernatant was no longer alkaline. The chitin was then dialysed against distilled water for 24 hr, changing the distilled water several times during this period, collected by centrifugation, washed three times with ethanol, three times with ether, and finally dried *in vacuo* over phosphorus pentoxide. Yield 36·6 per cent. (Found: N, 7·0%; ash, nil. Calc. for $(C_8H_{13}O_5N)_x$: N, 6·9%).

A sample of this chitin (20 mg) was hydrolysed by heating for 17 hr with 5·7N aqueous hydrochloric acid (20 ml) in a sealed evacuated tube at 105°C. The hydrolysate was evaporated to dryness *in vacuo* at 30°C, dried *in vacuo* over solid potassium hydroxide, and the residue dissolved in 10 per cent. *iso*propanol (1 ml). The solution was subjected to ionophoresis on paper (Whatman No. 3 filter paper, acetate buffer pH 5 and ionic strength 0·19, potential 20 V/cm for the effective length of 50 cm for 60 min (Foster 1952, 1957)). Three spots were detected with ninhydrin, one intense spot (glucosamine) and two weak spots (one acidic and one basic amino acid). The amino acids, by comparison with the behaviour under similar conditions of known amino acids were identified as aspartic acid and histidine. Glucosamine was identified by the position it occupied on the paper and by its reaction with the Elson and Morgan reagent.

To confirm the presence of aspartic acid and histidine the ionophoresis on paper was repeated using an acetate buffer of pH 3·6, a phosphate buffer of pH 7·5, and carbonate buffers of pH 9·7 and 10·7. All the buffers had an ionic strength of 0·2. In these experiments aspartic acid, glutamic acid, lysine, arginine, histidine and glucosamine were included as controls. At pH 3·6 glutamic acid, at pH 7·5 histidine, at pH 9·7 lysine, and at pH 10·7 arginine remained at the origin. At all these pH values the spot corresponding to the acidic amino acid behaved in a manner identical with that of authentic aspartic acid and the basic amino acid in a manner identical with that of authentic histidine. At none of the pH values did any additional spots appear.

Further confirmation of the identity of the amino acids was obtained by filter paper partition chromatography. The hydrolysate was subjected to ionophoresis on paper, the position of the two amino acids determined by the use of guide strips, and the areas of paper containing the amino acids cut out. These pieces of paper were either woven into a strip of Whatman No. 1 filter paper (Boggs 1952) or the amino acids eluted and spotted on to a strip of Whatman No. 1 filter paper. The paper strips were subjected to chromatography using the solvent systems and spray reagents described by Hackman and Lazarus (1956) together with the additional solvent system methanol–water–pyridine (Smith 1958). Appropriate amino acids were used as controls on all paper strips and the two amino acids were identified as aspartic acid and histidine.

(*b*) Agrianome spinicollis

(i) Larval cuticles were prepared as described by Hackman and Goldberg (1958) and subjected to digestion with papain. Experiments showed that papain removed more protein from the cuticles than did either pepsin or trypsin. Finely powdered cuticle (100 mg) was digested at 70°C with papain (10 mg) in phosphate buffer (25 ml, Sörensen 1909) containing 0·01M sodium sulphide. At 2-hourly intervals a further 10 mg papain and 30 mg sodium sulphide were added and the total time of digestion was 6 hr. Soluble nitrogen was determined after 2, 4, and 6 hr. After 2 hr no more protein was digested and the nitrogen content of the solution corresponded to a residue containing 95 per cent. chitin and 5 per cent. protein. The residue was washed thoroughly with water, alcohol, and ether and dried *in vacuo* over phosphorus pentoxide. The absence of sulphur-containing amino acids in this residue established the absence of papain (*A. spinicollis* larval cuticles after acid hydrolysis do not give any cystine, cysteine, or methionine). The cuticular residue was hydrolysed with hydrochloric acid as described in Section II(*a*), the amino acids present in the hydrolysate identified (filter paper partition chromatography), and their concentrations estimated by visual comparison of the intensities of the coloured spots with those given by standard solutions of the amino acids. The results expressed as mg amino acid obtained from 1 g protein (associated with the chitin) are given in Table 1.

(ii) Chitin was prepared from the larval cuticles of *A. spinicollis* by the method described above for *L. cuprina* puparia. When subjected to hydrolysis with hydrochloric acid the degradation products were identified as glucosamine, aspartic acid,

and histidine by ionophoresis and chromatography on paper as described in Section II(*a*). The aspartic acid and histidine were present only in small amounts. Because of the unknown amount of decomposition which occurred during hydrolysis it was not possible to determine accurately the concentration of the aspartic acid and histidine present in the sample of chitin. However, an estimate of the amount of each present in the hydrolysate was obtained by visual comparison of the intensities

TABLE 1

AMINO ACID COMPOSITION OF PROTEIN COMPONENTS OF GLYCOPROTEINS

Amino acid composition expressed as mg amino acid obtained from 1 g protein

Amino Acid	Source of Glycoprotein			
	Larval Cuticle of *Agrianome spinicollis* (papain)	Cuticle of *Scylla serrata* (EDTA)	Cuttlefish Shell (EDTA)	*Loligo* Skeletal Pen (lithium thiocyanate)
Alanine	84	90	95	121
Arginine	82	190	47	24
Aspartic acid	125	109	142	55
Cystine and/or cysteine	Absent	Absent	43	20
Glutamic acid	139	97	52	42
Glycine	Trace	Trace	80	102
Histidine	37	51	55	43
Hydroxyproline	Absent	Absent	Absent	Absent
Leucine and/or isoleucine	103	72	47	89
Lysine	Trace	Trace	39	20
Methionine	Absent	Absent	Absent	Absent
Phenylalanine	78	109	35	28
Proline	55	38	41	58
Serine	Trace	Trace	38	29
Threonine	113	78	26	33
Tyrosine	171	120	129	62
Valine	138	129	63	56

of the coloured spots with those given by standard solutions of the amino acids. There were two histidine and one aspartic acid residue for each 400 glucosamine residues.

(*c*) Scylla serrata (*Forskål*)

(i) Chitin was prepared from the finely powdered, hair-free carapace of the crab *S. serrata* by extraction with aqueous ethylenediaminetetra-acetic acid (EDTA) at pH 9 and pH 3 as outlined by Foster and Hackman (1957).

Uncooked shell from recently caught crabs was cleaned of all flesh, air dried, and ground to a powder in a vibrating ball mill. Powdered shell (50 g) was added to a solution of EDTA (235 g) in water (1·5 l.) which had been adjusted to pH 9. The solution was shaken gently overnight, the insoluble material collected, and washed three times with very dilute ammonia (pH 9) and three times with water.

The residue was shaken gently overnight with a solution of EDTA (2·5 g) in water (500 ml) which had been adjusted to pH 3. The insoluble material was washed three times with water, three times with dilute ammonia (pH 9), and three times with water again. Pigment was removed by extracting the residue repeatedly with ethanol, and free lipoid by extraction with ether. Yield 7·1 g (14·2 per cent.). (Found: N, 8·3% (on ash-free basis) and ash (550°C) 0·43%). A sample of this chitin was hydrolysed with hydrochloric acid and the amino acids in the hydrolysate identified and their concentrations estimated as described in Sections II(*a*) and II(*b*). The results are given in Table 1.

(ii) A second sample of this chitin was heated with 1N aqueous sodium hydroxide at 100°C for 60 hr changing the alkali five times during this period. The residue (Found: N, 6·9%) was isolated and hydrolysed as described in Section II(*a*). The 10 per cent. *iso*propanol extract was subjected to ionophoresis and chromatography on paper. Glucosamine and small amounts of aspartic acid and histidine were identified, no other compounds were detected.

(*d*) *Cuttlefish*

(i) Cuttlefish shell, collected from beaches on the south coast of New South Wales, was ground to a fine powder in a vibrating ball mill. The finely powdered shell was decalcified with aqueous EDTA at pH 9 and pH 3 as described in Section II(*c*) for powdered crab shell. The yield of chitin was 4·4 per cent. (Found: N, 11·6% (on ash-free basis) and ash (550°C) 5·2%). A sample of this chitin was hydrolysed with hydrochloric acid and the amino acids in the hydrolysate identified and their concentrations estimated as described in Sections II(*a*) and II(*b*). The results are given in Table 1.

(ii) A second sample of cuttlefish chitin was heated with 1N aqueous sodium hydroxide at 100°C for 60 hr, changing the alkali three times during this period. The residue was isolated (Found: N, 7·1%), hydrolysed with hydrochloric acid, and the 10 per cent. *iso*propanol extract subjected to ionophoresis and chromatography on paper. Only glucosamine, aspartic acid, and histidine were detected and the concentrations of aspartic acid and histidine were noticeably less than those obtained from *L. cuprina* or *S. serrata* chitins prepared in a similar manner.

(*e*) *Squid Skeletal Pen* (Loligo *sp.*)

(i) The skeletal pen contained 12·7 per cent. nitrogen and left no residue on heating at 550°C. A sample of the pen was hydrolysed with hydrochloric acid and the amino acids in the hydrolysate identified. The amino acid composition of the protein present in the intact pen was qualitatively similar to that of the protein in the glycoprotein prepared by the method described in Section II(*e*)(ii). Quantitatively the only marked difference was in the tyrosine content. The protein of the intact pen contained about twice as much tyrosine as the protein of the glycoprotein.

(ii) Powdered skeletal pen (250 mg) and aqueous lithium thiocyanate (50 ml, saturated at room temperature) were heated at 130°C for 2 hr with intermittent

shaking. A clear colloidal syrupy solution formed which was poured into 50 per cent. aqueous acetone (150 ml) and allowed to stand overnight. The precipitate was collected by centrifugation, washed with water until free from lithium thiocyanate, then with ethanol and ether and dried *in vacuo* over phosphorus pentoxide. Yield 102 mg. (Found: N, 8·1%; ash, nil). A sample of this chitin was hydrolysed with hydrochloric acid and the amino acids in the hydrolysate identified and their concentrations estimated. The results are given in Table 1.

(iii) The skeletal pen (500 mg) was heated with 1N aqueous sodium hydroxide (25 ml) at 100°C for 5 days, changing the alkali each day. The residue was isolated as described in Section II(*a*). (Found: N, 6·8%; ash nil). Acidic hydrolysis (HCl) of the residue gave only glucosamine, aspartic acid, and histidine.

(*f*) *Other Chitin Preparations*

Chitins prepared by the alkaline digestion of the decalcified shell of the marine crayfish *Jasus verreauxi* H. M. Edw., of the elytra of adult *Aphodius howitti* Hope, and of the decalcified shell of *Spirula spirula* L. were subjected to acidic hydrolysis and the hydrolysates examined by ionophoresis on paper. Only glucosamine, aspartic acid, and histidine were detected.

(*g*) *Extraction with Aqueous Phenol*

An attempt was made to extract protein from chitins prepared as described in Sections II(*b*)(i), II(*c*)(i), II(*d*)(i), and II(*e*)(ii).

Chitin (500 mg) was extracted with 90 per cent. aqueous phenol (20 ml) for 24 hr at room temperature in an atmosphere of nitrogen. The chitin was collected by centrifugation and re-extracted twice more with 20-ml portions of aqueous phenol, the duration of each extraction being 24 hr. The residue was thoroughly washed with methanol and ether and dried *in vacuo* over phosphorus pentoxide. Recovery was almost theoretical and the amino acid content of the residue was not materially altered from that of the chitin before extraction with phenol.

(*h*) *Solution in Aqueous Lithium Thiocyanate*

The four samples of chitin (Sections II(*b*)(i), II(*c*)(i), II(*d*)(i), and II(*e*)(ii)) were dispersed in aqueous lithium thiocyanate, saturated at room temperature as described in Section II(*e*)(ii). A syrupy colloidal solution formed within 1 hr. Any insoluble material was removed by centrifugation and the solution fractionally precipitated with acetone (see Foster and Hackman 1957) to give a series of chitin fractions. Each fraction was collected by centrifugation, washed with water until free from lithium thiocyanate, then with acetone, and dried. The fractions were hydrolysed with hydrochloric acid and the amino acids in the hydrolysates identified. In no case was a protein-free chitin fraction obtained and the amino acid composition of each fraction appeared to be similar to that of the chitin preparation from which it was prepared.

III. Discussion

Samples of chitin which have been prepared by alkaline digestion of insect cuticles (puparia of *Lucilia cuprina*, larvae of *Agrianome spinicollis*, and elytra of *Aphodius howitti*), of decalcified crab and crayfish cuticles, of decalcified cuttlefish and *Spirula* shell, and of the skeletal pen of the squid have been subjected to acidic (HCl) hydrolysis and the hydrolysates examined by ionophoresis and chromatography on paper. In every case very small amounts of aspartic acid and histidine were identified in addition to glucosamine and no other amino acids were present. Chitin samples, such as those mentioned, give a positive colour reaction with ninhydrin thus indicating the presence of a free amino group and possibly of a free amino group alpha to a carboxyl group.

Chitin has also been prepared from the larval cuticles of *A. spinicollis*, from the shell of the crab *S. serrata*, from the shell of cuttlefish, and from the skeletal pen of the squid (*Loligo* sp.) without resorting to digestion with hot aqueous alkali. Use was made of ethylenediaminetetra-acetic acid to decalcify the crab and cuttlefish materials and enzyme to remove protein from the *A. spinicollis* material. The skeletal pen of the squid was reprecipitated from aqueous lithium thiocyanate. The association of the chitin and protein in these four preparations is best considered when taken together with the results already published for the cuticle of the crab *Cancer pagurus* (Foster and Hackman 1957) and the larval cuticles of *A. spinicollis* (Hackman and Goldberg 1958). The protein contents of all these chitin preparations are given in Table 2.

The protein content of each of the chitin preparations given in Table 2 has been calculated from the nitrogen content of the chitin preparation. Chitin contains 6·9 per cent. nitrogen and the protein component has been considered to contain 16 per cent. nitrogen. Taking these calculated percentages of protein the results given in Table 1 have been expressed as mg amino acid obtained from 1 g protein. However, when these chitin preparations are hydrolysed an unknown amount of decomposition occurs because of the presence together of carbohydrate and amino acids. That this decomposition occurs is shown by the formation of a dark-coloured solution or sometimes of a black precipitate. Consequently the results given for the amino acid composition of each protein do not add up to 100 per cent. Moreover, since it is not known which amino acids have contributed to the formation of the products of decomposition it is not known which of the values given are low or by how much. Tryptophan, of course, has not been estimated. Nevertheless, certain conclusions can be drawn from the results and these are given below.

In all six chitin preparations it appears that chitin and protein are bound together by covalent bonds. The evidence for this conclusion is: (1) The methods of preparation exclude the presence of the more labile types of linkages such as would be broken by extraction with water, salt solution, aqueous urea, aqueous phenol, and other organic solvents. (2) The protein content of all the chitin preparations can be substantially reduced but the amino acid content has never been completely eliminated by repeated extraction with hot (100°C) aqueous alkali. (3) All the chitin preparations can be dispersed into aqueous lithium thiocyanate and the solutions on progressive dilution with acetone give a series of fractions

which contain both chitin and protein. In no case was a protein-free chitin fraction obtained. The presence of protein and chitin in the fractions was established by hydrolysing (HCl) each fraction and detecting, by ionophoresis and chromatography on paper, amino acids and glucosamine in the hydrolysates. Trim (1941) has reported that the puparia of the blowfly *Sarcophaga falculata* can be dispersed in aqueous lithium thiocyanate at 170°C and reprecipitated without effecting a separation of the chitin and protein components.

The observations made on the two types of chitin preparations (with or without the use of hot aqueous alkali) suggest that the protein of the cuticle is bound by covalent bonds to chitin so forming a glycoprotein or mucopolysaccharide. The difficulty experienced in removing the last traces of amino acids from the chitin by prolonged treatment with hot dilute alkali clearly shows how strongly the protein is bound to the chitin. The fractionations from lithium thiocyanate solutions indicate the polydisperse nature of the glycoproteins. The amount of protein covalently

TABLE 2
PROTEIN CONTENT OF GLYCOPROTEINS CONTAINING CHITIN

Source of Glycoprotein	Percentage Protein (calc. from nitrogen content)
Agrianome spinicollis larval cuticle*	50
Agrianome spinicollis larval cuticle (papain)	5
Scylla serrata cuticle (EDTA)	15·6
Cancer pagurus cuticle (EDTA)†	7·5
Cuttlefish shell (EDTA)	51·2
Loligo sp. skeletal pen (lithium thiocyanate)	13·2

* Hackman and Goldberg (1958). † Foster and Hackman (1957).

bound to the chitin varies considerably. In the larval cuticles of *A. spinicollis* and in the cuttlefish shell about an equal weight of protein and chitin are bound together while in the cuticle of the crab *C. pagurus* the protein content of the glycoprotein is as low as 7·5 per cent. The protein content of the glycoproteins in the cuticle of *S. serrata* and the pen of *Loligo* are intermediate in value.

Nomenclature in the field of carbohydrate–protein complexes is in a state of flux and some recent classifications have been given by Kent and Whitehead (1955), Pigman (1957), and Bettelheim-Jevons (1958). According to the schemes outlined by Kent and Whitehead and by Bettelheim-Jevons the complexes formed from chitin and protein would be classified as either mucopolysaccharides or mucoproteins depending not only on the amounts of chitin and protein present but also on the "completeness of the protein structure". According to the scheme given by Pigman these covalently bound, stable combinations would be classified as glycoproteins. The results given in Table 2 show that the ratio of chitin to protein can vary greatly and so the less specific term, glycoprotein, as used by Pigman would appear to be preferable for these chitin–protein complexes. Moreover, the term glycoprotein has the added advantage of indicating a stable, covalently bound complex.

In this study chitins have been prepared from a number of different sources. The arthropods (insects and crustaceae) contained α-type chitin and the squid skeletal pen contained β-type chitin. X-ray diffraction studies of the cuttlefish shell chitin showed that it was poorly orientated, that it differed in detail from β-chitin, and that it corresponded in general features to a third and as yet unnamed type of chitin (Rudall, personal communication). All chitins prepared by extraction of the cuticles (or decalcified cuticles) with hot aqueous alkali gave aspartic acid and histidine on acidic hydrolysis. However, differences do exist in the amino acid composition of the glycoproteins. The protein components of both the cuttlefish and squid glycoproteins have a higher glycine content than those of the crab or insect glycoproteins (Table 1). Of the amino acids estimated in the protein of the squid preparation glycine accounts for 13 per cent. by weight or 20 per cent. of the total number of amino acid residues present. For the cuttlefish protein component the figures are 8·6 per cent. and 14·1 per cent. respectively. Piez and Gross (1959) have shown that in invertebrate and vertebrate collagens one-third of the total number of amino acid residues are glycine. The squid and cuttlefish protein components do not contain this amount of glycine and in view of the absence of hydroxyproline these proteins cannot be described as being of the collagen type. Cysteine or cystine or both are present in the protein components of the cuttlefish and squid glycoproteins but are absent from the chitin-containing glycoproteins of arthropods. Although there are similarities in the amino acid composition of the glycoproteins from cuttlefish shell and squid skeletal pen there are two marked differences, namely the higher aspartic acid and tyrosine content of the former. The protein components of both arthropod glycoproteins have a high aspartic acid and tyrosine content.

Although glycoproteins, in which the carbohydrate fraction is non-acidic, have been described from many sources very little is known of the nature of the bond or bonds linking the protein to the carbohydrate. Johansen, Marshall, and Neuberger (1958) obtained a peptide–carbohydrate complex from egg albumin which contained mannose, glucosamine, leucine, and aspartic acid. As a result of degradative experiments they concluded that an aspartic acid residue is linked directly to the carbohydrate through one of its carboxyl groups. Glycoproteins of similar composition have been obtained from the same source by Cunningham, Nuenke, and Nuenke (1957) and Jevons (1958). Rosevear and Smith (1958) isolated a glycoprotein from human γ-globulin in which they concluded that the peptide was linked with the carbohydrate residue through the β-carboxyl group of aspartic acid. It is probable that these links are of the amide or ester type.

The results described above for the chitin-containing glycoproteins indicate that the protein is linked to chitin through aspartyl or histidyl residues or both. In view of the stability of the link to hot alkali and its instability to hot acid this link could be in the form of an *N*-acylglucosamine. The *N*-acetyl link in chitin shows this type of behaviour although the acetyl group of *N*-acetyl-D-glucosamine is lost when heated with acid or alkali. Attempts to identify free amino groups, by the dinitrofluorobenzene method, in the chitins prepared by extraction of the cuticles with hot alkali were not successful. Because an unknown amount of decomposition occurs during the acid hydrolysis of these glycoproteins it is not possible to determine accurately the ratio of amino acids to glucosamine. However, an estimate was

obtained of the amino acids in the chitin prepared by alkaline extraction of the larval cuticles of *A. spinicollis*, it being one amino acid residue per 200–300 glucosamine residues. Although the length of the chitin chain is not known it is generally considered to consist of several hundred *N*-acetyl-D-glucosamine residues and so it is possible that only one or two protein chains are linked to each chitin chain.

The work described above makes it probable that chitin does not occur uncombined with protein. This raises the question of terminology. Chitin, by common usage, refers to a polymer composed only of *N*-acetyl-D-glucosamine residues. It is suggested that for convenience the glycoproteins, the chitin–protein complexes, be referred to as "native chitin" and a distinction is therefore made between "native chitin" and "chitin". Since the chitin-containing glycoprotein is polydisperse one or more of the glycoproteins may be more readily attacked by the enzyme chitinase. Jeuniaux (1959) has described a membrane in the cuticle of crustacea which is resistant to the action of chitinase and it contains 73 per cent. chitin and 12–15 per cent. protein. Removal of the protein by alkaline digestion permits the chitin to be degraded by the enzyme. The peritrophic membrane of insects may represent another chitin-containing glycoprotein which is not attacked by the enzyme chitinase.

IV. Acknowledgments

The author thanks Dr. M. V. Tracey, Wheat Research Unit, C.S.I.R.O., for supplying the *Loligo* skeletal pen and *Spirula* shell and Dr. K. M. Rudall, University of Leeds, England, for determining the type of chitin in the *Loligo* and cuttlefish preparations.

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