Biochem. J. (1971) **125**, 703–716
Printed in Great Britain

The Composition of Peptidochitodextrins from Sarcophagid Puparial Cases

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(*Received* 26 *April* 1971)

1. *N*-Bromosuccinimide cleaved proteins and pigments from fly puparia, increasing the chitin:protein ratio from 0.5 to 1.5. The product afforded subfractions (ratio 5:1) of molecular weights of 1200 and 1600 devoid of aromatic residues and *N*-terminal β-alanine, direct aryl links between polysaccharide chains being discounted. 2. The chitin–protein complex decreased in molecular weight when treated with Pronase, which suggested polypeptide bridges within the native chitin micelle. The limit dextrins generated by chitinase were mixtures of unsubstituted dextrins and peptidylated oligosaccharides, with the former predominating. 3. Peptidochitodextrins of similar molecular weight but markedly different solubility were prepared, which were indistinguishable with respect to amino acid, glucosamine, acetyl, X-ray or infrared characteristics. It is suggested that physical interactions contribute to the stability of the integument in addition to the covalent bonds that form during sclerotization.

The occurrence of amino acids in hydrolysates of native chitin suggests that covalent polysaccharide–protein bonds contribute to the stability of the arthropod exoskeleton (Hackman, 1960; Attwood & Zola, 1967; Rudall, 1970). Adding to the evidence is the limited hydrolysis observed with chitinases or proteases, which suggests masking of hydrolase-sensitive sites (Hackman & Goldberg, 1965; Jeuniaux, 1965; Lipke & Geoghegan, 1971). For these investigations, the chitin–protein aggregates were prepared by digestion in concentrated sodium hydroxide and dispersion of the aggregates in hot lithium thiocyanate or 12M-hydrochloric acid. The rigour of this procedure is against the general acceptance of a chitin–protein bond, since a framework of insoluble polysaccharide could protect small amounts of unglycosylated peptides from proteolysis and contribute low concentrations of amino acids to acid hydrolysates (Hunt, 1970). Confirmation of a conjugate requires characterization of soluble glycopeptides of low molecular weight (Spiro, 1966). To this end, soluble fractions were prepared by Andersen (1970) and Lipke (1971) by the use of dilute hydrochloric acid and subtilisin. The preparations were unsuited to linkage or sequence analysis owing to the aryl substituents laid down during sclerotization (Brunet, 1965). This process, together with melanization (Kuo & Alexander, 1967), renders the bulk of the protein and polysaccharide chains resistant to those hydrolases most useful as reagents for linkage analysis (Lipke, 1971; Lipke & Geoghegan, 1971).

In addition to primary linkages, physical forces contribute to the stability of the integument. In the course of sclerotization, the cuticle increases in density, extrudes at least 40% of the water and retains a second portion of the water in the chitin–protein micelle (Fraenkel & Rudall, 1940, 1947; Rudall, 1963). X-ray and i.r. spectra of high-molecular-weight material established that the newly compacted macromolecules assumed new orientations dependent not only on covalent links bridging macromolecules but also on realignments favouring an increase in hydrogen and hydrophobic bonds (Rudall, 1965). In contrast to chitin prepared by digestion in alkali, the loci in native cuticle which are associated by secondary interactions have not been defined, since simple models such as synthetic chitohexaose do not show the line spectra or infrared bands characteristic of the native polysaccharide (Pearson, Marchessault & Liang, 1960; Falk, Smith, McLachlan & McInnes, 1966).

Iwasaki & Witkop (1964) and Wilchek, Spander, Milne & Witkop (1968) reported that tyrosyl, phenylalanyl, histidyl, indolyl and phenoxy residues were cleaved by *N*-bromosuccinimide without significant alteration of other components of a peptide chain. The one exception, cysteine,

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constitutes a minor component in cuticle protein (Hackman, 1964; Lipke & Geoghegan, 1971). By fission of aromatic residues, including the closely associated melanic pigments, this reagent has afforded a route to soluble chitin-like fragments that are susceptible to enzymic attack. The fragments retain a portion of the cuticle protein and aggregate in a manner similar to that presumed to occur *in situ*. The molecular-weight range of the oligosaccharides is 1000–2000 and the aggregation is pH-dependent and reversible, providing a material suitable for physical studies of the complex.

MATERIALS AND METHODS

Preparation of tissue. Sarcophaga bullata was maintained on horse meat and the empty pupal cases were harvested from the top of the cultures. After elimination of particulate impurities with a stream of air, the cases were washed with methanol and ethyl ether, dried in air, and crushed to a coarse powder with a roller. Washing was continued on a fritted funnel with 5 litres of hot neutral detergent and water for each 200 g batch. Soluble protein, oligosaccharides, urates and other contaminants were extracted with 5 litres of 1% NaCl containing 0.05 M-EDTA, pH 8, at 50°C, followed by 4 litres of 0.1 M-NaOH at 2°C for 6 h with agitation by a stream of N_2. The residue was washed with 3% acetic acid and water until the washings were neutral, followed by alcohol, ether and air-drying. After extraction with 19 M-formic acid at 25°C, the residue was again washed, dried and ground in a ball mill at 2°C to 50–100 μm. The powdery residue was extracted for 10 h at 55°C under N_2, with 4 M-HCl in aq. 30% (v/v) ethanol (solids:solvent ratio 1:7). After centrifugation the precipitate was washed copiously with water, dried and reground. Yield, 56%, based on coarse-ground puparial cases. This material is referred to as 'cuticle residue'. Subsequent fractionation is outlined in Scheme 1.

Oxidation by N-bromosuccinimide. Oxidations were performed in a bath maintained at 80°C in foil-shaded flasks equipped with glass stoppers, with stirring provided by a glass-enclosed magnet. Cuticle residue (35 g) was suspended in 400 ml of 10 M-acetic acid and an equal volume of 8% *N*-bromosuccinimide in the same solvent was added gradually. The flask was stoppered tightly and held at 80°C for 30 min. The reaction was quenched with 2 litres of cold 5 M-formic acid, starch–KI paper being used to verify complete decomposition of the *N*-bromosuccinimide. The insoluble residue was harvested by centrifugation at 5000 *g* and washed three times each with 1 litre quantities of 5 M-formic acid, 0.05 M-imidazole, pH 7.0, water, alcohol and ether. The yellow, insoluble powder, fraction E65P, was ground and dried *in vacuo* at 50°C over P_2O_5. Yield, 52%, based on cuticle residue. The material released from the cuticle by *N*-bromosuccinimide was recovered from the combined reaction medium and washings by concentration on a rotary evaporator followed by chromatography on Sephadex G-15 with 0.05 M-LiCl as eluent. The fractions of low-molecular weight co-chromatographing with [^{14}C]leucine were discarded.

Preparation of peptidochitodextrins. Fraction E65P (20 g) was extracted with 1 litre of 12 M-HCl at 0°C for 8 h and the insoluble material, fraction NS, was removed by centrifugation. Yield, 2.1 g. Cold water (1 vol.) was added, followed by powdered Na_2CO_3 to pH 7.0. Neutralization was performed in an ice bath. The precipitate that formed between pH 2 and 7 was removed by centrifugation, washed with water and alcohol and dried: fraction P7; yield, 8.1 g. The material remaining in solution at pH 7 was dialysed against distilled water and freeze-dried: fraction S8; yield, 316 mg.

Both P7 and S8 were reprecipitated separately with 4 vol. of ethanol after rapid solution in 4 M-HCl at 0°C. A fourth reprecipitation was effected at 0°C by the addition of M-KOH to the fractions taken up in HCl. Yield for both fractions, 90–92%. Chitin and chitosan were prepared from both the crustacean carapace and cuticle residue from sarcophagid puparia by digestion with KOH as described previously (Lipke, Grainger & Siakotos, 1965).

Enzyme digestion. Chitinase from *Streptomyces griseus* was purchased from Gallard-Schlessinger (New York, N.Y., U.S.A.) and purified as previously described (Lipke & Geoghegan, 1971). The preparation consists of a mixture of chitinase and chitobiase. Digestion was performed at 40°C in 0.02 M-potassium phosphate buffer, pH 7.0, at a substrate:enzyme ratio of 7:1. For measurements of the relative rates of chitinolysis reactions were terminated by heating to 100°C for 5 min and insoluble materials removed by centrifugation. For exhaustive digestion, substrate–buffer suspensions and chitinase solutions were sterilized by boiling and membrane filtration respectively. Incubations extended to 48 h, under toluene, at which time further additions of enzyme failed to yield an increase in the amount of monosaccharide. Deproteinization was accomplished by boiling followed by the addition of trichloroacetic acid to 5% final concentration. The digest was extracted with ethyl ether, and traces of insoluble peptides at the interface were discarded. Proteolysis with trypsin, subtilisin, Pronase and thermolysin was performed at a substrate:enzyme ratio of 25:1 in the presence of Ca^{2+} (Lipke & Geoghegan, 1971).

Acid hydrolysis. Acid concentrations for maximum release of amino acids and glucosamine at 105°C were determined separately for cuticle residue and fractions E65P, P7 and S8. Cuticle residue required 6 M-HCl for amino acids and 8 M-HCl for glucosamine, for a period of 20 h. Fractions treated with *N*-bromosuccinimide were hydrolysed in sealed tubes under N_2 in 3 M-HCl for 4 h for glucosamine and in 5 M-HCl for 12 h for amino acids. Insoluble fractions were agitated with a 'flea'. Recoveries of aromatic amino acids were verified with duplicate determinations in the presence of 1% thioglycollic acid (Matsubara & Sasaki, 1969). No tryptophan was detected. For identification of sugars by paper chromatography, hydrolysis was performed in 3 M-HCl at 90°C for 3 h. Acid was removed with Dowex 1 (HCO_3^- form). Spots were revealed with ninhydrin, $AgNO_3$ and aniline–trichloroacetic acid.

Analytical methods. Reducing sugar, glucosamine, *N*-acetylglucosamine, neutral sugar, amino N, total N and pentose were determined as previously reported (Lipke *et al.* 1965; Lipke, 1971). Non-acylated glucosamine residues were assessed by conversion into anhydro-

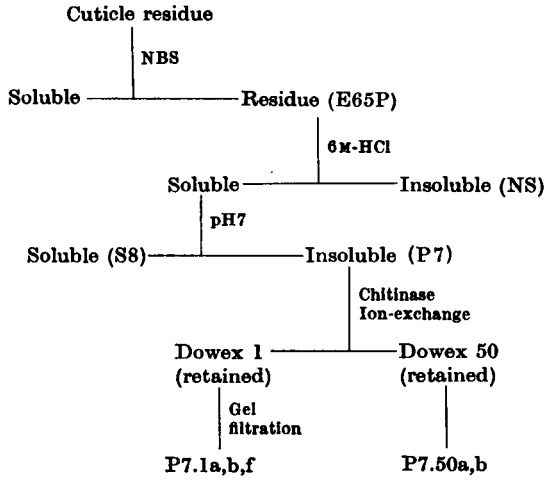
mannose (Lee & Montgomery, 1961) with corrections for chromogen formed without the deamination step. Anhydromannose recovery from chitosan was 82%. Insoluble preparations were reground to 30μm before deamination, and removed from the reaction medium by centrifugation before colour development. Glucosamine was detected by chromatography according to Gardell (1963) and with a Phoenix amino acid analyser, which was also used for amino acid determination according to Spackman, Stein & Moore (1958). Losses of hydroxy amino acids were corrected as described by Perham (1967). Ultraviolet spectra were obtained in a recording Gilford model 2400 instrument. Infrared spectra were examined by using KBr pellets in a Perkin–Elmer model 225 spectrophotometer. Elemental analysis and *N*-acetyl determinations by distillation and titration (Gibbons, 1959) were performed by M–H–W Laboratories, Garden City, Mich., U.S.A., and the University of Massachusetts Microanalytical Laboratories, Amherst, Mass., U.S.A.

X-ray diffraction. Photographs of the powdered specimens were taken with Ni-filtered Cu K_2 X-rays (λ=1.5418 Å). A Supper cylindrical film-holder with specimen-to-film distance of 2.87 cm was employed.

Chemicals. A.R.-grade reagents were used wherever possible. Pyridine, butan-1-ol, acetic acid, formic acid, acetic anhydride, acetylacetone and ethyl ether were redistilled. *N*-Bromosuccinimide (K & K Chemicals, Plainview, N.Y., U.S.A.) was recrystallized from water and stored at −20°C for periods no longer than 60 days. Ehrlich's reagent, *p*-dimethylaminobenzaldehyde, was recrystallized from ethanol after purchase from Matheson, Coleman & Bell, Cincinatti, Ohio, U.S.A. Reference carbohydrates were purchased from sources previously described, and recrystallized (Lipke *et al.* 1965). Thermolysin and Pronase B were products of Calbiochem, Los Angeles, Cal., U.S.A. Trypsin (3×recrystallized) was purchased from Worthington Chemical Co., Freehold, N.J., U.S.A., and subtilisin BPN from Nagase Ltd., Osaka, Japan.

Paper and column chromatography. Paper chromatograms were run by the descending method with three passes of solvent and Whatman no. 1 or 4 paper. The solvent for amino acids was butan-1-ol–pyridine–acetic acid-water (30:20:6:24, by vol.) and for carbohydrates was butan-1-ol–pyridine–water (6:3:2, by vol.) and butan-1-ol–ethanol–water (4:1:1, by vol.). Gel filtration was performed according to the manufacturers' instructions with Sephadex cross-linked dextrans (Sephadex, Uppsala, Sweden) and Bio-Gel P-2 (BioRad Laboratories, Richmond, Calif., U.S.A.). Columns were calibrated with respect to molecular weight by using ^{3}HOH, raffinose, Dextran 10 and Dextran Blue 2000 (Cifonelli, 1968; Raftery *et al.* 1969).

Ultracentrifugation. Molecular weights were estimated by the high-speed-sedimentation-equilibrium technique with a Spinco model E analytical ultracentrifuge equipped with Raleigh interference optics (Yphantis, 1964; Roark & Yphantis, 1969). The concentration was 0.7 mg/ml in 4M- and 6M-HCl for S8 and P7 respectively. Rotor speed was 60000 rev./min at 0°C. The interference patterns were read on a microcomparator and the data analysed by a program devised for an IBM 360/67 computer. The partial specific volume was taken as 0.56 cm^3/g (Tanford & Davidson, 1964). The molecular-weight averages obtained from this program were further analysed by using a two-species plot to estimate the molecular weight of a single chain (Roark & Yphantis, 1969).



Scheme 1. Flow chart for cuticle fractionation NBS, *N*-bromosuccinimide.

RESULTS

Fragmentation of the polysaccharide–protein complex. Like most polyphenol-tanned cuticle from arthropods, puparia from *S. bullata* failed to release significant quantities of hexosamine-containing fragments on incubation with proteases and glycosidases of assorted specificities (Lipke & Geoghegan, 1971). Partial disruption of the cuticle matrix with 0.2M-sodium hydroxide, 3M-hydrochloric acid, 87% sulphuric acid or 8M-guanidine did not stimulate enzymolysis because of the stability of the benzenoid tanning links. Partial deacetylation with 3M- and 5M-hydrochloric acid failed to render the complex susceptible to deaminative cleavage with nitrous acid (Stacey, 1958). In tanned cuticles prepared without exposure to alkali, controlled removal of *N*-acetyl groups could not be effected, and the reactivity of the few amino groups that were liberated by brief exposure to acid was low. Cuticle residue was treated with 5M-hydrochloric acid at 2°C for 100 days under nitrogen. About 80% of the sample was converted into soluble fragments of molecular weight 10^2–10^4. All of the glycopeptides of molecular weight $>10^3$ remained resistant to proteolytic and chitinolytic enzymes, were heavily pigmented and showed amino acid: hexosamine ratios similar to the parent material.

Effect of N-bromosuccinimide. In contrast to the indiscriminate action of mineral acid, cleavage of the aromatic constituents of the complex with *N*-bromosuccinimide separated the cuticle into a

Table 1. *Composition of fractions after treatment with* N-*bromosuccinimide*

Cuticle residue (100 mg) was suspended in 10 ml of medium with 1.2 mequiv. of *N*-bromosuccinimide (NBS). The interval was 180 min and 30 min for reactions at 25°C and 80°C respectively. The reaction was quenched with 5 mequiv. of formic acid, centrifuged, and the pellet washed and dried. Reduction with $NaBH_4$ (200 mg) in 0.1 M-pyridinium acetate buffer, pH 7.4, proceeded for 120 min. Fractions were hydrolysed for amino acid and hexosamine analysis as described in the Materials and Methods section. Molar ratio refers to glucosamine: α-amino nitrogen.

				Fractions			
				Soluble		Residue	
Preparation	Reaction medium	NBS	Temp. (°C)	Dry wt. (mg)	Molar ratio	Dry wt. (mg)	Molar ratio
Cuticle residue	4 M-HCl	–	60	10.4	0.2:1	87.4	0.6:1
	10 M-CH_3CO_2H	–	80	8.1	0.1:1	90.6	0.5:1
	10 M-CH_3CO_2H	+	25	2.5	0.08:1	97.2	0.8:1
	10 M-CH_3CO_2H	+	80	28.8	0.05:1	70.2*	1.6:1
	0.1 M-Pyridinium acetate	+	25	6.1	0.1:1	93.1	0.7:1
		+	80	12.5	0.08:1	86.7	1.5:1
Borohydride-reduced	10 M-CH_3CO_2H	+	80	25.6	0.09:1	74.7	1.5:1
Chitin		–	85	9.1	—	90.8	50:1
(NaOH-digested)		+	85	9.9	—	92.3	50:1

* Fraction E65P.

soluble fraction, which contained 5% peptide-bound glucosamine, and an acetic acid-insoluble residue, which consisted primarily of chitin (Table 1). In comparison with results for proteins, elevated temperatures and 10 M-acetic acid were required to effect maximum solubilization (Wilchek *et al.* 1968). When *N*-bromosuccinimide was omitted, more peptide remained in the insoluble fraction and no visible loss of colour was observed. Since sclerotized protein may include bound quinones (Hackman & Todd, 1953), cuticle residue was treated with borohydride before exposure to *N*-bromosuccinimide to convert these into phenols. No change in the distribution of peptide and carbohydrate, and no increase in solubilization, was observed. For comparison, alkali-purified chitin from crab carapace was treated with *N*-bromosuccinimide. In the presence of *N*-bromosuccinimide, the release of soluble material was similar to that effected by the solvent alone, suggesting that the reagent acts principally on the protein and pigments moieties rather than on the polysaccharide.

Treatment with *N*-bromosuccinimide produced a fall in the titre of indolyl and aryl components as shown by the spectrophotometric method of Iwasaki & Witkop (1964). Fig. 1 shows that in the 260–300 nm portion of the spectrum, the extinction of a 1% (w/v) solution of fraction E65P in 5 M-hydrochloric acid was one-thirtieth that of an equivalent concentration of cuticle residue not treated with *N*-bromosuccinimide. For this measurement the cuticle residue was solubilized by partial hydrolysis with 5 M-hydrochloric acid at 2°C for 100 days in an atmosphere of nitrogen; the other samples were readily soluble in hydrochloric acid. The loss in extinction was proportional to the time of exposure to *N*-bromosuccinimide and was more pronounced for the polysaccharide-enriched fraction E65P than for the material rendered soluble by *N*-bromosuccinimide. Two subfractions, P7 and S8, prepared from E65P by precipitation from acid solution (Scheme 1), were essentially devoid of aromatic absorption. Examination of these fractions in the i.r. spectrum showed a complete loss of the absorption band at 1515 cm^{-1}, where the residual band of tyrosyl residues is detectable (Bendit, 1967).

Composition and solubility after treatment with N-*bromosuccinimide.* Cleavage with *N*-bromosuccinimide released a substantial portion of the cuticle polypeptide and small amounts of glucosamine (Table 2). The insoluble and residual fractions differed not only in the protein:polysaccharide ratio but also in the relative amounts of specific amino acid residues. The results for cuticle residue are in excellent agreement with those reported for a similar fraction from *Calliphora erythrocephala* (Karlson, Sekeris & Marmaras, 1969). The solubilized material was enriched with respect to serine, glutamic acid, glycine, leucine and β-alanine. The sum of the tyrosine, phenylalanine and histidine contents was 4.39 residues/10 mg for the untreated material, and 2.49 and 1.06 for the *N*-bromosuccinimide-derived soluble and insoluble fractions respectively, showing that *N*-bromosuccinimide had attacked these amino acids. None of the

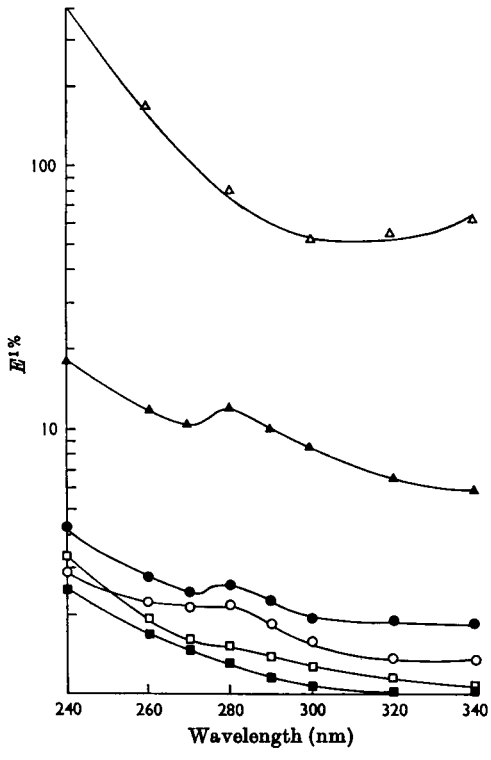


Fig. 1. Ultraviolet spectra of fractions treated with *N*-bromosuccinimide (NBS) in 5M-HCl. Details of the preparative procedure are given in the text. △, Partial hydrolysate of cuticle residue; ▲, NBS-solubilized glycopeptides; ●, residue after treatment with NBS at 65°C; ○, residue after treatment with NBS at 85°C (fraction E65P); □, acid-soluble fraction S8; ■, acid-soluble fraction P7.

Table 2. *Composition of fractions derived from N-bromosuccinimide*

Fractions were prepared and analysed as described in the Materials and Methods section. Values for cuticle residue are from Lipke & Geoghegan (1971). NBS, *N*-bromosuccinimide.

	Composition (residues/10 mg)		
Amino acid	Cuticle residue	NBS-released	NBS residue*
Asp	4.27	7.61	1.97
Thr	2.48	3.36	0.94
Ser	3.63	6.72	0.73
Glu	5.17	10.55	2.59
Pro	5.02	5.22	1.31
Gly	4.26	12.67	2.36
α-Ala	3.57	6.71	1.64
Val	3.52	7.81	2.01
Met	0.44	0.13	—
Ile	1.84	1.91	0.93
Leu	1.64	8.06	1.03
Tyr	1.58	0.49	0.03
Phe	1.27	0.60	0.47
β-Ala	5.47	2.24	0.06
Lys	1.49	3.40	4.88
His	1.54	1.39	0.56
Arg	0.97	3.11	1.47
GlcN	24.1	0.88	35.2
Unidentified amines	2.81	3.11	0.21

* Fraction E65P.

unidentified substances reacting with ninhydrin was detected at concentrations greater than 0.6 residue/10 mg. Glucosaminic acid was absent from the hydrolysates, oxidation of glucosamine at C-1 being discounted. Examination of unhydrolysed preparations for hexose, ketose and pentose with both the phenol–sulphuric acid and orcinol reagents failed to reveal any neutral monosaccharides. Since these criteria would also indicate the presence of 2-oxo artifacts, the evidence is fairly conclusive that little or no destruction of amino sugar occurred via action of *N*-bromosuccinimide. Paper chromatography of hydrolysates showed glucosamine and *N*-acetylglucosamine to be the only substances present reacting with alkaline silver nitrate.

Chitin prepared from arthropod cuticle by the use of concentrated alkali can be dispersed in 12M-hydrochloric acid and lithium thiocyanate (Tracey, 1957). These preparations are heterodisperse aggregates that are readily sedimented at low values of *g* (Attwood & Zola, 1967). Chitosan, on the other hand, dissolves readily in 0.5M-acid as a result of chain fission and the protonation and hydration of the amino groups. Table 3 shows that chitin and chitosan prepared from *Sarcophaga* puparia with alkali exhibited a solubility and a glucosamine:*N*-acetylglucosamine ratio similar to that from crab carapace. Exposure of crab chitin to *N*-bromosuccinimide at 80°C did not alter the solubility or the peptide and glucosamine titre significantly. When protein was split from the cuticle residue with *N*-bromosuccinimide at 60°C or 80°C, the residue could be dissolved in 6M-hydrochloric acid at 2°C. Acetic acid, lithium chloride, guanidine and ammonium sulphate at 20°C did not dissolve the *N*-bromosuccinimide-residue, and 4M-hydrochloric acid at 50°C was the best solvent although degradation of the polysaccharide may have occurred at this temperature. Deacetylation of the chitin was not the cause of the increased solubility, since the material treated with *N*-bromosuccinimide contained little or no non-acetylated glucosamine.

Amino acid and glucosamine composition of acid-soluble peptidochitodextrins. The puparial residue

Table 3. *Effect of* N*-bromosuccinimide on solubility and composition of cuticle*

Solubility was determined by extraction of 500 mg for 120 min in 10 ml of solvent with mechanical agitation. After centrifugation at 10000*g*, the dry weight of the supernatant was determined. Molar proportions refer to the sample before extraction. GlcNAc was estimated as the difference between total GlcN in hydrolysates and anhydromannose in the intact material. Amino acid α-amino N was determined with the amino acid analyser. Values for *N*-bromosuccinimide (NBS)-treated samples refer to the insoluble residue remaining after removal of the NBS-solubilized fraction (see the Materials and Methods section).

Materials	NBS	Molar proportions (GlcN:GlcNAc:α-amino N)	Solvent	Solubility (mg/ml)
Chitin crab	–	1:17:0.08	10 M-CH_3CO_2H (20°C)	0.11
	–		6 M-HCl (20°C)	0.31
	+(80°C)	1:20:0.02	10 M-CH_3CO_2H (20°C)	0.20
	+(80°C)		6 M-HCl (20°C)	0.38
	+(80°C)		Water (80°C)	0.06
puparial	–	1:14:0.09	10 M-CH_3CO_2H (20°C)	0.26
			6 M-HCl (20°C)	0.53
Chitosan crab	–	3.9:1:0.04	10 M-CH_3CO_2H (20°C)	21.4
			6 M-HCl (20°C)	28.4
puparial	–	4.7:1:0.13	10 M-CH_3CO_2H (20°C)	26.7
			6 M-HCl (30°C)	32.2
Cuticle residue	–	0.02:1:1.7	10 M-CH_3CO_2H (20°C)	0.04
			6 M-HCl (2°C)	2.52
			14 M-LiCl (20°C)	3.47
			4 M-$(NH_4)_2SO_4$ (20°C)	0.32
			(80°C)	2.02
			8 M-Guanidine (20°C)	0.87
	+(20°C)	0.02:1:1.6	10 M-CH_3CO_2H (20°C)	0.73
			6 M-HCl (2°C)	3.08
	+(60°C)	0.02:1:0.8	6 M-HCl (2°C)	10.4
	+(80°C)*	0.03:1:0.6	10 M-CH_3CO_2H (20°C)	0.78
			6 M-HCl (2°C)	26.4
			4 M-HCl (50°C)	36.7
			14 M-LiCl (20°C)	1.2
			4 M-$(NH_4)_2SO_4$ (20°C)	0.48
			(80°C)	3.49
			8 M-Guanidine (20°C)	0.88

* Fraction E65P.

after treatment with *N*-bromosuccinimide was extracted with 6 M-hydrochloric acid at 2°C to yield acid-insoluble fraction NS, and a soluble portion. Neutralization of the hydrochloric acid-soluble portion precipitated fraction P7, which was reprecipitated with sodium hydroxide and ethanol. The fraction remaining soluble at neutrality was dialysed and reprecipitated with ethanol before spectrophotometric and amino acid analysis. The aromatic amino acid and pigment contents were further decreased from the values in the parent *N*-bromosuccinimide-residue (Fig. 1 and Table 4). Conversely, the chitodextrin content was higher in P7 and S8 than in either the acid-insoluble fraction (NS) or the parent material (E65P). Fractions P7 and S8 were similar in amino acid composition except for lysine and proline, both minor constituents. Free peptides, amino acids or carbohydrates could not be detected in unhydrolysed preparations, indicating that the components present in hydrolysates were firmly bound to the oligosaccharide. Unacetylated glucosamine residues appeared slightly more abundant in fraction S8, with anhydromannose as the criterion, but this result was not confirmed with the method based on alkaline deacetylation. Fraction S8 was much more soluble in water and 4 M-sodium carbonate. No increase in the amount of dissolved P7 was observed at 80°C in water, 0.1 M-sodium chloride or potassium phosphate, pH 7. Found, for P7: C, 45.1; H, 6.1; N, 6.48; $C_{48}H_{80}O_3N_6$ requires C, 46.6; H, 6.72; N, 6.75%. $[M]_D$ in 6 M-hydrochloric acid was more negative than −40°, in agreement with a β-linkage. No colour was produced with the Morgan–Elson or Elson–Morgan reactions, indicating that the reducing moiety was linked through position 4 (Barker, Foster, Stacy & Weber, 1958).

Infrared spectroscopy. The extent of deacetylation and hydrogen bonding within the complex can be estimated from i.r. spectra (Pearson *et al.* 1960:

Table 4. *Solubility and composition of fractions NS, P7 and S8*

Preparations of fractions were described in the Materials and Methods section. Solubility was determined by extraction at 20°C for fractions NS and S8 and at 2°C for P7 as described for Table 3. *N*-Acetyl groups were determined by alkaline hydrolysis and unacetylated glucosamine as anhydromannose.

	Residues/10 mg		
Property	Fraction NS	Fraction P7	Fraction S8
Amino acid			
Asp	5.55	1.17	1.33
Thr	3.11	0.55	0.71
Ser	3.82	0.87	0.77
Glu	7.52	1.43	1.88
Pro	5.23	0.96	0.32
Gly	8.53	1.39	1.15
α-Ala	6.52	1.08	1.04
Val	3.74	1.02	1.24
Ile	0.76	0.16	0.11
Leu	1.85	0.46	0.43
Tyr	0.12	Trace	Trace
Phe	0.12	Trace	Trace
Lys	2.28	0.06	0.23
His	0.98	Trace	0.07
Arg	1.02	0.04	0.09
GlcN	1.28	0.46	1.76
GlcNAc	21.1	43.3	40.3
N-Acetyl		35.7	34.8
GlcN:GlcNAc:α-amino N	0.06:1:2.4	0.01:1:0.21	0:04:1:0.23
Solubility (mg/ml)			
Water	0.12	0.35	9.2
4M-HCl	0.07	36.2	44.8
4M-Na_2CO_3	0.02	0.16	12.2

Rudall, 1963). The characteristics of E65P, P7 and S8 appear essentially identical and also resemble those of alkali chitin from the crab cuticle (Fig. 2). No difference at frequencies characteristic of N–H stretching (3100 cm^{-1}) or bound water (1650 cm^{-1}) were evident. Except for a weakening at 1560 cm^{-1} the spectrum did not differ from that described for chitin prepared from the cuticle of the immature stage of *Sarcophaga* or from *N*-acetylchitohexose (Barker *et al.* 1958; Pearson *et al.* 1960).

X-ray diffraction. The X-ray diffraction powder lines of P7 and S8 confirm the similarity of these fractions derived from *N*-bromosuccinimide to crab chitin prepared by alkaline digestion (Table 5). The strong powder lines observed at 9.4, 4.6 and the medium line at 3.34 Å are common to the three samples, and weaker reflexions at 2.3 and 2.5 Å coincide. These characters are in excellent agreement with those reported by Falk *et al.* (1966) for the crustacean polysaccharide. Two values appear unique to fraction S8 at 7.41 and 3.07 Å, where weak lines indicate a structural difference that is supported by the occurrence of fewer reflexions in this soluble fraction.

Enzymolysis of cuticle-treated with N-bromosuccinimide. Hot alkaline digestion of cuticle removes pigment and polypeptide conjugates that mask the β-1,4-*N*-acetylglucosamine residues from chitinase (Fig. 3). Thus fraction E65P and chitin prepared by reflux in 2M-sodium hydroxide was hydrolysed four times faster than cuticle residue. The most rapid rates were observed with P7 and S8. Fraction NS, which retained high amounts of peptides, was resistant to chitinase although derived from E65P. The striking stimulation resulting from acid purification of the *N*-bromosuccinimide-treated cuticle was not due to differences in solubility, since P7, NS, alkali chitin and E65P were all insoluble and were all ground to about the same particle size. Preincubation with subtilisin did not facilitate chitinase action. At pH 5 or 7, only 2% of the *N*-acetylglucosamine was hydrolysed by crystalline egg-white lysozyme in 48h. Incubation of S8 with Pronase increased the elution volume on Sephadex of that portion of the complex degradable by chitinase (Fig. 4). The distribution of the chitinase-susceptible fragments found with either the Morgan–Elson or the ferricyanide procedures was essentially the same. No chitinase or deacetylase activity could be detected

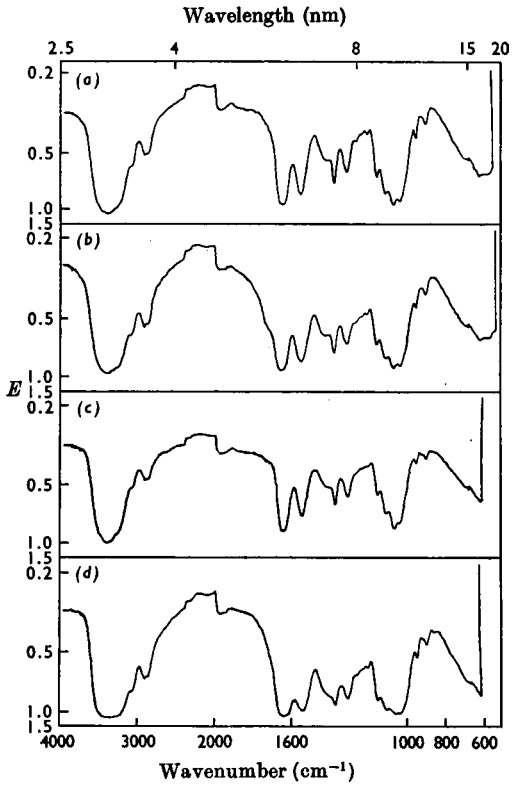


Fig. 2. Infrared spectra of *N*-bromosuccinimide-treated fractions: (*a*) alkali chitin (crab); (*b*) P7; (*c*) E65P; (*d*) S8.

Table 5. *X-ray reflexions of alkali chitin (crab) and fractions P7 and S8*

Abbreviations: vs, very strong; s, strong; m, medium; w, weak; vw, very weak.

	Diffraction lines (Å)	
Chitin	Fraction P7	Fraction S8
9.21 s	9.40 s	9.31 s
		7.41 w
6.66 m	6.97 w	
4.62 vs	4.61 vs	4.59 vs
3.93 w	3.75 w	
3.34 m	3.34 m	3.43 m
	2.80 w	3.07 vw
2.60 vw	2.55 w	2.53 w
2.35 vw	2.30 w	2.22 w
	2.09 vw	
1.83 vw	1.86 w	

in the Pronase preparation. Although the bulk of the peptidochitodextrin decreased in size, a portion was unaffected by proteolysis, suggesting that the

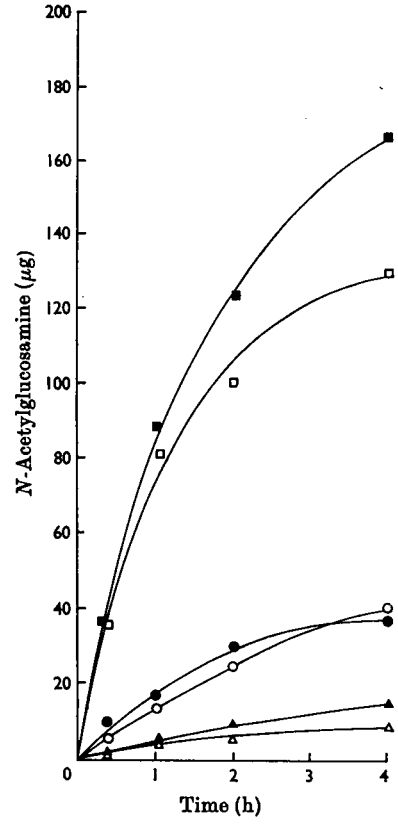


Fig. 3. Release of *N*-acetylglucosamine from cuticle fractions by chitinase–chitobiase preparations. Fractions (400 μg) were incubated at 40°C with 80 μg of purified chitinase–chitobiase preparations from *S. griseus*. For details see the Materials and Methods section. ■, Acid-soluble fraction P7; □, acid-soluble fraction S8; ○, *N*-bromosuccinimide residue (fraction E65P); ●, alkali-digested crab chitin; ▲, acid-insoluble fraction NS; △, cuticle residue.

preparation was a mixture of unsubstituted and peptide-substituted oligosaccharides. In contrast, the solubility properties of fraction E65P were unaffected by proteolysis. Incubation with Pronase, thermolysin, trypsin or subtilisin, either singly or in series, failed to solubilize this fraction. Since measurements of molecular weight require a soluble polymer, the degree of polymerization of the chitin chains in E65P remains to be established.

Molecular weight of P7 and S8. Differences in solubility at pH 7 between P7 and S8 were at first believed to be the result of major differences in molecular weight. The degree of polymerization of the two complexes was estimated from the total hexosamine:hemiacetal ratio (Tsai, 1970) and

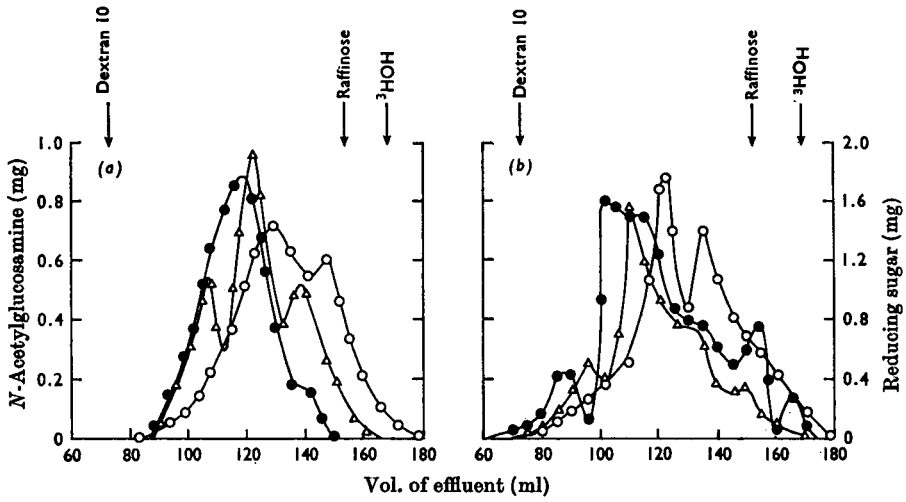


Fig. 4. Effect of Pronase on mobility of chitinase-sensitive polysaccharides on Sephadex G-50. Fraction S8 (5mg) was incubated with Pronase, deproteinized and chromatographed as described in the Materials and Methods section. Fractions were incubated with chitinase and assayed for (*a*) *N*-acetylglucosamine and (*b*) reducing sugar. Treatment with Pronase was for 0 (●), 8 (△) and 24 (○) h.

found to be 4–7 for both samples. A column of Sephadex G-50 (1.5cm × 100cm) was calibrated with mono-, tri- and oligo-saccharide standards at pH7 for runs with S8 and in 4M-hydrochloric acid at 2° for P7. Both P7 and S8 eluted in the 1000–2000 daltons region. By meniscus depletion ultracentrifugation in acid medium, 80% of fraction P7 was found to consist of material with a molecular weight of 1200±150. For fraction S8, a value of 1600±150 accounted for 80% of the sample, the remaining 20% of P7 and S8 consisting of higher-molecular-weight species that may have been aggregates of the predominant species. Of particular note was the inverse relation of size to solubility at pH2–8.

Removal of chitodextrins from peptidochitodextrins. The distribution of chitinase-susceptible and chitinase-resistant fractions in the Pronase digest (Fig. 4) suggested that the *N*-bromosuccinimide preparations were mixtures of oligosaccharides of varying degrees of amino acid substitution. Passage of 1g of a 48-h chitinase–chitobiase digest of fraction P7 through a 2cm × 6cm column of the sulphonic resin Dowex 50 (H^+ form) yielded a fraction that could be eluted with aq. M-ammonia solution. After removal of ammonia on the rotary evaporator, the mixed cationic glycopeptides were chromatographed on Bio-Gel P-2. Fig. 5(*b*) shows that the two prominent peaks reacting with ferricyanide were present, as well as a third consisting of a small amount of free glucosamine. The ratio of glucosamine to total glucosamine plus *N*-acetylglucosamine was 1:35. The aqueous effluent and washings from the cation-exchange column were passed through a column of the basic resin Dowex 1 (formate form) and eluted with 2M-formic acid, evaporated and chromatographed on Bio-Gel P-2 (Fig. 5*a*). The anionic peptidochitodextrins comprised 0.5% of P7, and the cationic conjugates about 2.3%. Chitinase action afforded fractions with lower glucosamine: α-amino N ratios than the fractions before enzyme treatment. In each fraction a different amino acid predominated, although all the fractions were rich in aspartic acid, serine, proline, alanine and glycine. The ratio of glucosamine to the most abundant amino acid was 5–8:1.

That the amino acids of fractions P7.1a and P7.50a were present as polypeptides of significant length was verified by following the change in molecular weight of the peptidochitodextrins resulting from proteolytic digestion. Each fraction was passed through a column of Sephadex G-10 and the elution profile delineated with the alkaline ferricyanide reagent for reducing sugar. When the largest discrete peak in the molecular-weight range 600–1000 was digested with Pronase and subtilisin, the elution volumes of both the cationic and anionic conjugates increased significantly (Fig. 6). Too little material remained to determine which residues were still fixed to carbohydrate. The fractions studied to date all appear heterogeneous both with respect to composition and location of the peptide moiety.

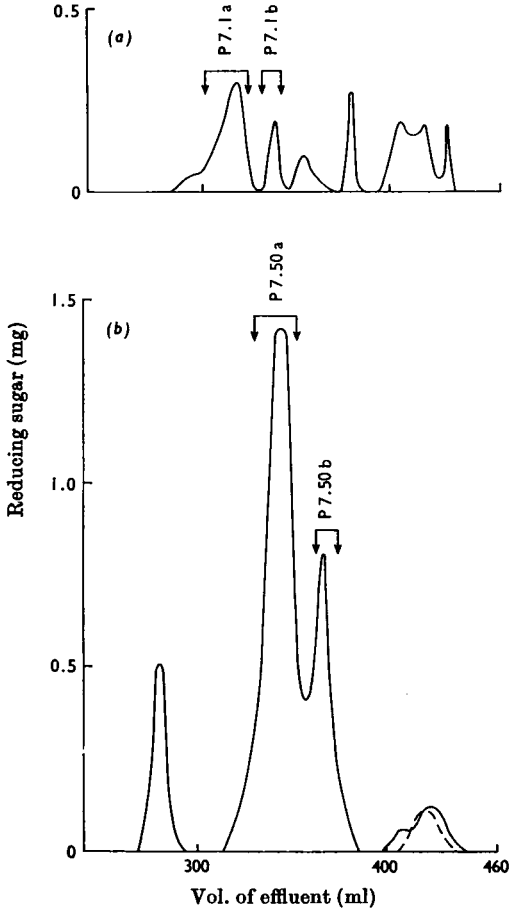


Fig. 5. Bio-Gel P-2 chromatography of glycopeptides retained by (*a*) Dowex 1 and (*b*) Dowex 50. Fraction P7 was digested with chitinase and passed through ion-exchange resins before gel fractionation. For details see the text. Fractions were assayed without acid hydrolysis for ——, reducing sugar, and – – –, glucosamine.

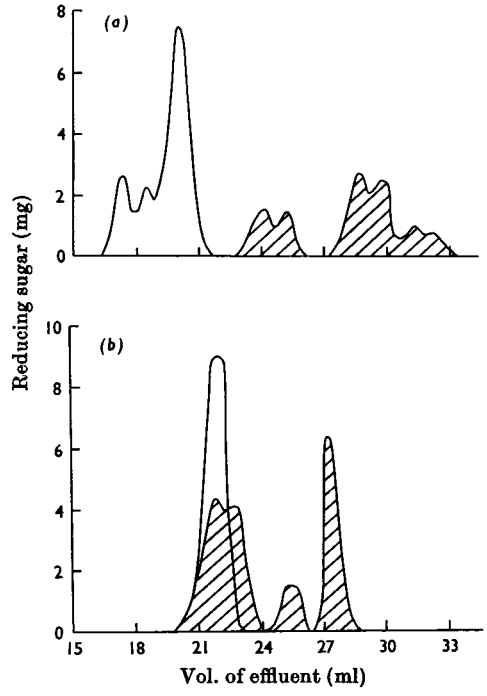


Fig. 6. Mobility of cationic and anionic glycopeptides on Sephadex G-10 after proteolytic digestion. Fractions P7.50a (*a*) and P7.1a (*b*) were chromatographed on columns (1 cm × 55 cm) and the material indicated under the non-hatched curves was digested with Pronase and subtilisin. The digests were rechromatographed on the same columns. The hatched areas represent amounts of reducing sugar after proteolysis.

Distribution of peptides in oligosaccharide chains. The hexosamine content of fraction P7 exceeded that of any one amino acid by a factor of 30–40, and of the summed amino acids by a factor of 4 (Table 4). Given an average molecular weight corresponding to hexaose, possible details for the two extreme structures are: (*a*) each group of six distinct hexaose chains consists of five unsubstituted chitodextrins and one hexaose linked to polypeptide; (*b*) one out of every four acetylglucosamine residues is amino acid-substituted, implying that most of the hexaose chains are linked to amino acids. The two alternatives could be distinguished with the chitinase–chitobiase reagent, which for (*a*) would produce an excess of unsubstituted *N*-acetylglucosamine, whereas in (*b*) chitinolytic enzymes would release a mixture consisting predominantly of lower oligosaccharides. These substituted di- and tri-saccharides would result from the inability of the chitinases to cleave amino acid-substituted *N*-acetylglucosamine residues by virtue of the interference of amino acid chains with the action of chitobiase. Exhaustive digestion of P7 with chitinase solubilized the entire sample and released 61% of the total *N*-acetylglucosamine as the monosaccharide, and an additional 33% as uncharged trisaccharide, as established by passage through a mixed-bed cation- and anion-exchange resin and by ultracentrifugation, which gave a value of 700 for the molecular weight of the oligosaccharide fractions. This observation indicated restriction of the peptide chains to a minority of the hexaose moieties as in (*a*) above, and also showed that the insolubility of P7 at neutral pH was dependent on a critical molecular size between 700 and 1200 molecular-weight units.

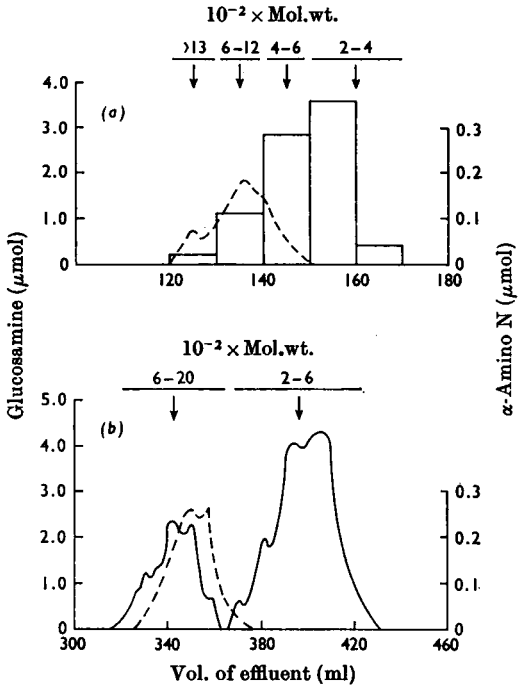


Fig. 7. Distribution of amino acid N and glucosamine on molecular sieves after digestion of P7 with chitinase. The digest was deproteinized as described in the text and chromatographed (*a*) on Sephadex G-50 (1.7 cm × 100 cm) or (*b*) on Bio-Gel P-2 (2.6 cm × 170 cm). The molecular-weight distribution of the oligosaccharides remaining after chitinase treatment was determined from the reducing value before acid hydrolysis and the yield of glucosamine after hydrolysis (Raftery *et al.* 1969). Peptide N was determined as α-amino N after acid hydrolysis and was corrected for colour contributed by glucosamine. Before acid hydrolysis the total α-amino N content of the digest was 0.038 μmol. ——, Glucosamine present as chitodextrins; – – –, amino N present as peptide.

After chitinase treatment it can be seen (Fig. 7) that amino acid N was found principally in those fractions of molecular weight 600–2000 rather than in those of the 200–600 molecular-weight range. Before hydrolysis of the gel fractions in 4M-hydrochloric acid little or no amino acid N could be detected in the fractions, in agreement with the value of 0.038 μmol of α-amino N for the sample before exposure to chitinase. Amino acid analysis of acid hydrolysates of gel fractions chosen in the 600–1000 molecular-weight range revealed aspartic acid, serine, alanine and lysine in addition to glucosamine, the amino sugar values exceeding that for the amino acid by 10:1. The lower-molecular-weight fractions contained only glucosamine and traces of amino acids. These data favour the restricted structure (*a*) above, which confines the amino acids to higher-molecular-weight components, since peptides of even moderate size, such as penta- or hexa-peptides, would substantially increase the molecular weight of the oligosaccharide to which they were appended.

DISCUSSION

The removal of cuticle pigment and proteins with halogenated oxidizing agents is an established procedure, but since this step followed rather than preceded alkaline digestion, details of the chitin-protein relationship were overlooked (Tracey, 1957). This study establishes that the association of chitin and protein postulated by Hackman (1960, 1964) can be retained after extensive chemical and enzymatic degradation of cuticle mucoprotein. Hackman (1960) observed only aspartic acid and histidine in hydrolysates of alkali chitin from insects and crustaceans. Table 6 shows that histidine was a minor component of the glycopeptides, in keeping with the susceptibility of this residue to *N*-bromosuccinimide. Since the fractions retained the peptide component, assignment of histidine to the linkage region is questionable (Attwood & Zola, 1967; Lipke, 1971). Preparations of increasing purity retained amino acids in conjugation with poly(*N*-acetylglucosamine) in the course of purification by precipitation from true solutions, molecular-sieve and ion-exchange chromatography and enzymic removal of polysaccharide and protein. In the course of purification, the ratio of carbohydrate to amino acid increased from 0.5 to 10:1 with substantial decrease in the abundance of leucine and isoleucine, valine, arginine, lysine, melanin, *N*-terminal β-alanine (Bodnaryk & Levenbook, 1969) and the components sensitive to *N*-bromosuccinimide: tyrosine, phenylalanine, histidine and polyphenols. The absence of u.v. absorption in the fractions treated with *N*-bromosuccinimide (Fig. 1) discounts chitin–protein bonding by a polyphenol-derived bridge (Iwasaki & Witkop, 1964) or by common linkage to melanin (Hackman & Goldberg, 1968; Fogal & Fraenkel, 1969). On the basis of the isolation of 2-amino-3′,4′-dihydroxyacetophenone from sclerotized cuticle, and the masking of the carbonyl function before extraction, Andersen (1970) suggested that this compound or a derivative was involved in cross-linking cuticle components via the β-carbon atom. The proposed structure and similar arylations involving protocatechuyl and tyrosyl (Hackman & Todd, 1953; Hackman, 1964) or *N*-acetyldopamine-derived tanning agents (Karlson, Sekeris & Sekeri, 1962) are all susceptible to *N*-bromosuccinimide via fission of *C*-peptide bonds to form soluble halogenated spiro-γ-iminolactones (Witkop, 1968).

Table 6. *Analysis of glycopeptides isolated by chromatography on Bio-Gel P-2*

Fractions were prepared as described in the text and are as designated in Fig. 5. Values represent molar ratios, a value of unity being assigned to the most abundant amino acid (**bold** type).

	Fraction				
	P7.1a	P7.1b	P7.1f	P7.50a	P7.50b
Amino acid					
Asp	0.654	0.702	0.122	**1.000**	0.973
Thr	0.147	0.313	0.021	0.018	0.497
Ser	**1.000**	0.445	0.794	0.459	0.546
Glu	0.097	**1.000**	0.022	0.523	0.464
Pro	0.630	0.615	**1.000**	0.539	0.989
Gly	0.565	0.419	0.298	0.991	**1.000**
α-Ala	0.481	0.362	0.744	0.801	0.434
Val	0.177	0.119	0.014	0.461	0.136
Ile	—	0.142	0.010	0.042	0.501
Leu	—	0.178	0.007	0.094	0.152
Lys	—	0.192	0.021	0.222	0.417
His	—	0.063	—	0.074	0.038
Arg	—	0.223	—	0.018	0.078
GlcN	7.24	5.44	3.60	4.66	8.12
GlcN:α-amino N	2	1.1	1.2	1.1	1.4

Although not entirely in accord with the accepted mechanism of *N*-bromosuccinimide oxidation of aromatic rings, the spectral and analytical data do not eliminate linkage by ring fragments derived from aromatic bridges which were rendered non-absorbent in the u.v. region and unreactive to ninhydrin. The bulk of the cuticle proteins and a minor portion of the polysaccharide were, in fact, converted into fractions soluble in 10M-acetic acid (Table 2). It is clear furthermore that quinones are not a major component of sclerotized cuticle, although a role as an intermediate may be indicated. Degradation with *N*-bromosuccinimide was not stimulated by pretreatment with borohydride, in agreement with Attwood & Zola (1967).

The high order of susceptibility of P7 and S8 to chitinase (Fig. 2) is in marked contrast with that recorded for crude puparial material, for alkali chitin or for crab integument, all of which are refractory to hydrolysis (Waterhouse, Hackman & McKellar, 1961). The increased chitinolysis may, in part, be due to depolymerization of native chitin during the course of *N*-bromosuccinimide cleavage and acid fractionation. In the absence of information on the molecular weight of fraction E65P before solution in hydrochloric acid, it would appear that increased susceptibility to chitinase was due to peptide removal rather than decrease in the size of the polysaccharide, since *N*-bromosuccinimide did not alter the solubility or hexosamine:amino acid ratio of crab chitin prepared by digestion in sodium hydroxide (Tables 1 and 2). The progressive broadening of the elution pattern on Sephadex G-50 after treatment of S8 with Pronase indicated that a portion of this fraction was glycopeptide. Since the ratio of carbohydrate to peptide in S8 was about 5, the increased retention of the oligosaccharide moiety by the gel could result from hydrolysis of peptide bridges between chitodextrins by Pronase. Fraction P7 was composed predominantly of unconjugated chitodextrins, which were not retarded by cation- or anion-exchange resins, plus a lesser number of oligosaccharides to which peptide was appended. Incubation of the isolated conjugates from P7 with subtilisin and Pronase again resulted in a decrease in molecular weight of a portion of a sample (Fig. 6).

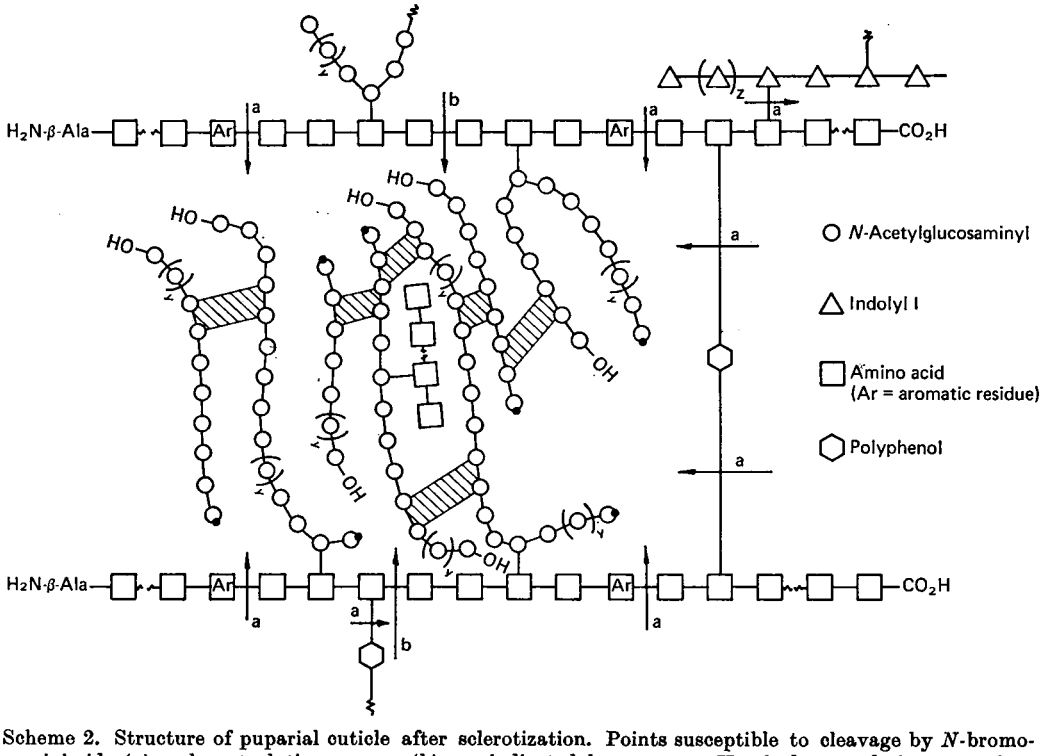
Exposure of many glycoproteins and mucopolysaccharide–protein complexes to 0.4M-sodium hydroxide at 2°C releases the polypeptide chains by β-elimination of glycosylated seryl or threonyl hydroxyl groups. Sarcophagid cuticle treated with alkali in the course of sample preparation retained protein (Table 1); in fact, Karlson *et al.* (1969) have solubilized only 5–10% of the puparial case protein with M-sodium hydroxide at 40°C. Furthermore, the dipteran samples were devoid of neutral sugar, including pentose, which together with alkali-stability, discounts chitin–protein linkage via a neutral sugar bridge to serine or threonine, although a masked or unreactive linkage of this type has not been ruled out. Non-covalent interchain forces sequester some of the polypeptide from proteolytic agents (Lipke & Geoghegan, 1971). This factor must be overcome before further study of the linkage can proceed.

The number of unacetylated glucosamine residues in the polyacetylglucosamine chains appeared so low as to be of doubtful authenticity. Alkaline hydrolysis, nitrous acid deamination, i.r. spectrophotometry and glucosamine release by chitinase failed to reveal glucosaminyl in amounts greater

than 1–3% (Table 1, Fig. 5). Fractions E65P, P7 and S8 were exposed to high concentrations of acetic acid or mineral acid in the course of preparation, which may have generated a small number of amino groups from fully acetylated polymer. Hunt (1970) failed to couple dinitrofluorobenzene with chitin and concluded that the number of unsubstituted amino groups in the chain was well below the number required for the verification of the partially unacylated structures proposed by Rudall (1963).

Fraction P7, but not S8, could be precipitated from 4M-hydrochloric acid by neutralization; the procedure could be repeated without significant loss of carbohydrate or peptide (Table 4). In alkali chitin, presumably of high molecular weight, the same effect is manifest. The chitonium–hydronium ion equilibrium, in part, determines the solubility of the polysaccharide in strong acid (Zimmerman, 1953), the other factor being the hydrogen bonds between solvent hydrochloric acid and polysaccharide (Lee & Kaplow, 1970). A two-dimensional representation of the structural features contributing to the insolubility, chemical stability and hydrolase resistance of sclerotized cuticle is presented in Scheme 2. The chitin chains are linked to protein via occasional non-aromatic bridges and to each other via non-covalent interactions to form polysaccharide micelles (Rudall, 1963). This arrangement does not require the participation of hexoses or pentoses. The physical forces leading to micelle formation are those conferring insolubility and an ordered structure to fraction P7. The peptides are arylated with the polyphenol-derived tanning agents joining the protein chains (Andersen, 1970), which terminate with β-alanine (Bodnaryk & Levenbook, 1969). In melanized structures, additional linkages to polyindole pigments are indicated (Hackman & Goldberg, 1968). For clarity, the model omits additional stabilizing influences such as non-covalent interactions between protein chains (Lipke & Geoghegan, 1971). The arrangement, however, provides for the increased susceptibility to protease and chitinase action after *N*-bromosuccinimide cleavage.

In the absence of information on the chemical structure of chitin–protein complexes, X-ray-diffraction and i.r. radiation has provided useful information on the molecular dimensions of the exoskeleton. However, as shown by Neville & Luke



Scheme 2. Structure of puparial cuticle after sclerotization. Points susceptible to cleavage by *N*-bromosuccinimide (a) and proteolytic enzymes (b) are indicated by arrows. Hatched areas designate regions bonded by non-covalent interactions. Subscripts represent arbitrary polymerization indices for chitin (y) and melanin (z).

(1969), major features of architecture have been overlooked by these approaches. Conversely, Falk *et al.* (1966) have shown that undue reliance has been placed on the use of these tools as indicators of specific structures. Thus the marked differences in molecular weight and solubility of alkali chitin, E65P, P7 and S8 are not revealed by the i.r. spectra (Fig. 2), which show only a slight weakening at 1560 cm^{-1} for S8 to distinguish this material from cogeners. X-ray patterns for S8 reveal the usual strong lines characteristic of alkali chitin at a *d*-spacing in the general vicinity of 9.5, 4.6 and 3.4 Å (Winkler, Douglas & Pramer, 1960). These spacings are common to the less-soluble preparations described in Table 5 as well. The spacings which are characteristic of S8 are the weak reflexions at 7.41 and 3.07 Å; these features would benefit from re-examination as drawn fibres. Fractions P7 and S8 are more promising subjects for a study of interchain forces in structural polysaccharides than either alkali chitin or acid mucopolysaccharide–protein complexes, since the forces which lead to aggregation are expressed in a low-molecular-weight oligosaccharide.

The authors thank Professor D. Laufer and Professor T. Margulis of this institution for guidance with the i.r. and X-ray studies respectively. Ultracentrifugal analysis was suggested and performed by Professor E. A. Davidson and Professor D. Roark of the Hershey Medical Center. Technical assistance was provided by Miss E. B. Wilkes and Mr E. Deitch. This investigation was supported in part by National Science Foundation Grant GB-7462.

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