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PEPTIDOCHITODEXTRINS OF SARCOPHAGID CUTICLE
II. STUDIES ON THE LINKAGE REGION

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"It ts suggested that the dehydration of the cuticle, the growing insolubility of its constituents and the loss in swelling power are due to the protein hydrophilic groups reacting with one another and/or with the chitin side chains. " G.S. Fraenkel, 1940.

ABSTRACT

1. Glycopeptides generated from sclerotized cuticle **of *Sarcophaga bullata*** with N-bromosuccinimide are uniform with respect to carbohydrate and **amino** acid composition 2. Heterogeneity is due to the degree of polymerization **of** a) the polysaccharide chain and b) the peptide subunit to which the chitodextrins are appended. 3. The peptide subunit is comprised principally of aspartic and glutamic acids, serine, glycine and alanine, mole ratios remain constant in fragments **of low** molecular weight released by proteolytic enzymes or chitinase. 4. The linkage between N-acetyl-glucosamine and the peptide subunit is retained following elimination **with** alkaline-borohydride or alkaline-sulflte, discounting serine or threonine as a constituent of the bridge. 5. Partial digestion with proteases or dilute acid does not **afford** subfractions enriched in any particular amino acid, suggesting an additional constituent in the **linkage** group. **6.** On the basis of the masking of amino termini by **tills** subsitueirt, **a** model is proposed relegating the chitin chains to a peptide distinct **from** the **arylatsd** proteins joined by polyphenol or polyindole bridges.

INTRODUCTION

Pigments and arylated proteins are cleaved **by** N-6romosuccinimide **(NBS)** without significant loss of non-aromatic amino acid rtsidnw (Witkop, 1968). The polypeptides released from the insoluble ehitin-pfoteih matrix **of** the puparial case **of *Sarcophaga bullata*** contain the bulk **of** the .....-alanine but few amino sugar residues (Lipke and Geoghegan, 1971a) and probably represent the peripheral rather than the core region of the sclerotized matrix. The insoluble residue withstanding treatment with NBS is greatly enriched with respect to .....***-1,*** 4-poly N-acetylglucosaminyl residues and exhibits the X-ray, infrared and substrate characteristics of chitin prepared by digestion in hot concentrated sodium hydroxide (Pearson ***et al,*** I960), the classical procedure. In addition, the NBS-generated polysaccharide is firmly bound to peptide, this relationship is apparently via covalent bonds, since the carbohydrate and amino acids remain associated after precipitation from true solution, digestion by proteases and carbohydrases and chromatography on ion exchange resins and molecular sieve gels.

Carbohydrate-protein links in insect cuticle have not been characterized, although several structures have been proposed (Hackman, 1960; Lipke *et al.*, 1965; Attwood and Zola, 1967; Sinohara, 1972). The retention of the chitin-protein bond following digestion in mild alkaline solution is against the sugar-O-seryl or threonyl couple as found in the proteoglycans of cartilage. Unsclerotized tissues from crustaceans (Kimura, 1972) and lepidopterous larvae (Spiro and Fukushi, 1969) contain hexosyl-hydroxylysines, while aspartamido-N, N′-diacetylchitobiose has been identified in sericin (Sinohara *et al.*,1971). In NBS-treated cuticle, the high ratio of chitodextrin to the candidate amino acids described above, as well as the chemical properties of the complex (Lipke and Geoghegan, 1971a) has indicated that the carbohydrate-protein link in the puparial cuticle had unique features worthy of further investigation of the linkage region.

MATERIALS AND METHODS

The origin and method of preparation of the puparial fractions have been previously described (Lipke and Geoghegan, 1971a). In brief, P7 and S8 were obtained by partial acid hydrolysis of the larger complex, E65P, in turn provided by treatment of cuticle residue with NBS. Limit peptidochitodextrins were prepared from P7 and S8 by exhaustive enzymolysis with chitinase, chitobiase and lysozyme (Lipke and Geoghegan, 1971a,b). These fractions are designated as P7ch and S8ch. Where indicated, the polysaccharase digests were separated into charged and uncharged species by chromatography on Dowex-50 and Dowex-1 by the procedures previously detailed. Sizing by chromatography on Sephadex and Bio-Gel was performed according to the manufacturers' instructions using tritiated water, sucrose, raffinose, and commercial dextrans of known molecular weight as reference compounds. Acid hydrolysis of glycopeptides and analysis of amino acids and hexosamines on the amino acid analyzer are as before (Lipke, 1971, Lipke and Geoghegan, 1971a,b; Lipke *et al.*, 1965). Reducing sugar was assayed with neocuproine (Dygert *et al.*, 1965) using N-acetylglucoasamine as a standard, and nitrous acid oxidations were according to Dische and Borrenfreund (Dische and Borrenfreund, 1950). Alkaline elimination of carbohydrate links to hydroxy amino acid residues was executed in the presence of borohydride (Mayo and Carlson, 1970) or sulfite (Simpson *et al.*, 1972). Aspartamido-N-acetylglucosamines were determined on the amino acid analyzer using citrate buffer, pH 2.8 (Tarentino *et al.*, 1970). The standard was a gift from Dr. Karl Schmid of Boston University.

RESULTS

Distribution and homogeneity of the peptide

Peptidoglycans from puparial cuticle were shown to be 80% monodisperse by sedimentation equilibrium, the remaining 20% were oligomers of the predominant species (Lipke and Geoghegan, 1971a). Digestion with chitinase yielded amino-acid substituted oligosaccharides of a wide range of molecular weights. The cause of the newly acquired heterogeneity could originate in the length of the oligosaccharide or the peptide chain or in the composition of the peptide. Since the mobility of

peptidogylcans on molecular sieves is established by the length of the carbohydrate chain (Constantopoulos *et al.*, 1969), a column of Bio-Gel P-2 (145 x 2 cm) was standardized with oligosaccharides and dextrans in 0.03 M ammonium acetate buffer prior to application of fraction P7ch. As shown in Table I, development of the column

Table 1: Molecular weight distribution and composition of fraction P7 following digestion with chitinase.

Fractions of 40 ml each were collected at a rate of 0.5 ml per minute and analyzed for reducing sugar and for amino acids and glucosamine after hydrolysis in 4 N HCl. Values represent mole ratios.

	Relative Molecular Weight			
Amino acids	Very high	High	Medium	Low
Asp	1.0	1.3	1.2	1.0
Ser + Thr	1.3	1.1	1.5	1.4
Glut	1.1	1.2	1.2	0.73
Gly	2.0	2.0	2.0	2.0
Ala	0.66	0.55	0.61	0.50
Val	0.41	0.34	0.22	0.33
Glucosamine	7.4	8.3	53	168
Chitin:peptide	1:1	1:1	8.1	29:1

separated the mixture into components of different molecular weight but similar amino acid composition. These data are in accord with a peptidogylcan preparation heterogeneous with respect to molecular weight of the carbohydrate rather than the polypeptide. Table I also shows that the medium and low molecular weight fractions contained substantial levels of di- and trisaccharides substituents as evidenced by a high chitin:peptide ratio. In this respect the volume of eluant required for elution, rather than the glucosamine titer of each fraction establishes the relative molecular weight of the glycopeptide. In Table 1, leucines, lysines, histidine, arginine, and sulfur-containing amino acids together comprise less than 10 per cent of the total amino N and are not listed. The aromatic residues are degraded by NBS during sample preparation. The observed regularity in amino acid composition would obtain from scission of a polypeptide backbone in the native integument into subunits of similar amino acid composition. Each subunit would include a constituent that would serve as a point of attachment of the chitin chains(s). For the purpose of further consideration, the subunits can be visualized as in Scheme 1 as either

(a) -NH-abXdfh-CO-
|
G - G - G

(b) -NH-abdfh-CO-
|
X - G - G - G

with amino acid and (acetyl)-glucosamine residues designated by lower case letters and G, respectively. The linkage between sugar and peptide is represented by -X-.

Oligosaccharide distribution

For a complex consisting of polysaccharide-substituted, repeating peptide subunits, in the extreme cases a) many subunits may be unsubstituted by poly N-acetylglucosaminyl chains or b) the carbohydrate can be equally distributed, with each subunit in the glycosylated condition. The two alternatives can be distinguished by digestion with proteolytic enzymes, which would generate peptides from unglycosylated regions of the complex, as in a). On the other hand, in b) proteolysis would not effect fully glycosylated subunits by virtue of the masking effect of the carbohydrate on the peptide chain. The intermediate situation, with glycosylated and unglycosylated subunits distributed along a single chain would yield both glycosylated and unglycosylated fragments in proportion to their occurrence in the parent material. Fraction P7ch was again digested with chitinase, chitobiase, and lysozyme (a chitodextrinase) and chromatographed on a column of Bio-Gel P-2. The higher molecular weight components were identified by analysis for reducing sugar and glucosamine, then chromatographed with a first pass on a column of Sephadex G-10 (145 x 2.5 cm). Two components were collected, I and II, of relatively high and intermediate molecular weight respectively. Each component was incubated separately with one twentieth weight of pepsin at pH 2, followed by subtilisin at pH 7. After deproteinization, the digests were subjected to a second pass on the same column of Sephadex G-10 for segregation into classes of decreasing molecular size base on the volume required for elution. The amino acid and hexosamine composition of the various classes is presented in Table 2. It can be seen that approximately 2.64 μmoles,

Table 2: Relative molecular size of peptides released from P 7ch by proteolytic digestion.

Glycopeptides were separated into high (I) and intermediate (II) molecular weight components on Sephadex G-10 columns. Each component was digested with pepsin and subtilsin and the digests rechromatographed on the same column for resolution into size classes. Values represent μmoles after hydrolysis for α-amino N and glucosamine. Reducing sugar determined without hydrolysis.

			Relative Molecular Weight			
	Component	Proteolysis	Very high	High	Medium	Low
α-Amino N	I	–	3.78	0.0	0.0	0.0
	I	+	2.64	0.53	0.18	nd
	II	–	0.0	0.84	0.0	0.0
	II	+	0.0	0.41	0.21	0.31
Glucosamine	I	–	3.68	0.0	0.0	0.0
	I	+	2.70	0.62	0.01	nd
Reducing sugar	I	–	1.64	0.0	0.0	0.0
	I	+	1.24	0.22	0.08	0.0
	II	–	0.0	0.36	0.0	0.0
	II	+	0.0	0.28	0.06	0.0

or 70% of the higher molecular weight glycopeptide (I) is unaffected with respect to elution volume, 30%, however, appears as less polymeric material. The smaller Component, II, is split into more mobile fragments to the extent of 0.41 μmoles, about 50%. In each case, a significant amount of I and II were digested to lower molecular weight material, in keeping with the presence of chitodextrins of diverse chain length in the parent material. The low recovery of glucosamine in the fragments derived from I can be explained by the inhibition of proteolysis by glycosylation with the more extensively polymerized dextrin. The data also suggest that the limit dextrin prior to the first pass on Sephadex that resolved components I and II, consisted of a mixture of long and short chains of peptide subunits, each size also heterogeneous with respect to chitodextrin substitution. The amino acid composition of the cleavage products closely resembled that of the parent material, discounting autodigestion of pepsin and subtilisin as a source of peptides.

The formation of lower molecular weight peptides from the peptidochitodextrin by proteolytic digestion usually provides a convenient route to the isolation of the linkage region. Ideally a chitodextrin with only the linking amino acid remaining bound to the polysaccharide is obtained. Removal of amino acid residues from the parent puparial material, furthermore, should afford fractions on Sephadex columns containing various amounts of glucosamine and greatly enriched with respect to the amino acid involved in the linkage, since carbohydrate-amino acid links, unlike peptide links are protease-resistant. This approach affords easy removal of the amino acids, lower peptides, and moni, di- and tri-saccharides, from the larger products of digestion, since the smaller material is relegated to the later column fractions. Fraction S8ch-D1, a negatively charged glycopeptide isolated from S8ch by chromatography on Dowex-1 (formate), as well as component II, (see Table 2) were subjected to exhaustive digestion by pancreatin and thermolysin and rechromatographed on Sephadex G-10. These proteases were chosen because of the broad range of specificity exhibited for peptide cleavage. Table 3 shows that these enzymes failed to produce a significant enrichment of any amino acid in any of the digest fractions. Since the chitin chains have previously been reduced to a minimum length by treatment with polysaccharases, a strong indication is provided that the bulk of the peptide links in the peptidochitodextrin are resistant to proteolysis by some means other than failure of the proteases to gain access to the amino acid portion of the complex. Both pancreatin and thermolysin preparations were without demonstrable chitinase activity, thus the production of lower molecular weight material of similar amino acid composition was probably due to the splitting of subunit chains into smaller species rather than the digestion of the chitin chains. The failure of both endo- and exopeptidases as well as those acting on both N- and C- terminal loci to affect cleavage further suggests that protection of the peptide from complete removal of those amino acids which do not bear a link to a sugar moiety is afforded by the structure of -X- which is presumed not to be an amino acid and which interferes with protease action, probably by steric factors. These considerations point to the importance of the degree of continuity of the subunits within the complex.

Table 3: Mole ratios of amino acids following digestion with pancreatin and thermolysin.

Molecular weight ranges estimated by rechromatography of chitinase limit peptidodextrins on Sephadex G-10. Values represent mole ratios.

Material	Component II			Fraction S8chD-1			
	Untreated[1]	Treated		Untreated[2]	Treated		
	Relative molecular weight						
	Medium	Medium	Low	Large	Large	Medium	Low
Asp	1.3	1.3	1.2	1.6	1.6	0.83	1.5
Thr	1.2	1.2	1.0	0.34	0.30	0.38	0.68
Ser	1.1	1.2	1.0	0.47	2.06	1.0	0.88
Glut	1.5	1.4	1.6	1.0	0.82	0.31	0.39
Gly	*2.0*	*2.0*	*2.0*	*2.0*	*2.0*	*2.0*	*2.0*
Ala	1.0	1.4	1.2	0.23	0.45	0.25	0.24
Val	1.0	0.89	0.64	0.47	0.67	0.79	0.77
Glucosamine	12.0	12.4	2.4	10.8	2.6	4.7	11.1

[1]No carbohydrate or peptide of low molecular weight was present in Component II before treatment.

[2]No carbohydrate or peptide of medium or low molecular weight was present in S8chD-1 before treatment.

Continuity of the peptide subunits

The subunits can exist as discrete entities of varying content of N-acetylglucosamine or the units may be members of a larger complex with the subunits joined. The junction point, in turn, can be to the next subunit or to a common nucleus as in the case of the spokes of a wheel. Indications favoring the linear array were available from the very low levels of free amino groups in unhydrolyzed components I and II as determined with ninhydrin. Additional evidence was obtained by subjecting a peptidochitodextrin of high molecular weight derived from P7ch to oxidation with nitrous acid. This reagent detects amino-terminal groups by subtractive means, deamination rendering the N-terminus unreactive to ninhydrin following hydrolysis and amino acid analysis. In addition, unacetylated glucosaminyl residues are converted to anhydromannose which can be detected with indole-HCl. Table 4 shows that the amino acid composition of the glycopeptides was unaffected by this procedure, a result best fitted to a linear, head to tail arrangement of subunits. The data also show that deactylated residues did not occur in the carbohydrate chain.

Table 4: Effect of nitrous acid on the amino acid and carbohydrate content of P7ch

The sample was treated with nitrous acid and a portion assayed for anhydromannose with indole-HCl. A second portion was chromatographed on Sephadex G-10 and the fractions containing reducing groups were pooled and hydrolyzed for amino acid and glucosamine analysis. Values are in nanomoles.

	Nitrous acid treatment	
	Before	After
Asp	17	15
Thr	12	11
Ser	27	22
Glut	21	18
Gly	43	45
Ala	16	15
Val	7	5
Ile	4	4
Leu	6	6
Glucosamine	5920	6480
Anhydromannose	0	6820

Properties of the linkage region

In alkaline solution, 0-glycosyl links to the hydroxy amino acids undergo beta elimination with release of the oligosaccharide moiety. In the presence of borohydride, alkali-stable reducing termini are converted to sugar alcohols. Seryl residues linked to carbohydrate appear in hydrolysates as additional alpha alanine, while threonyl linkages are detected as α-amino butyric acid. On the other hand, aspartamido-carbohydrate bridges withstand alkaline conditions, retaining the carbohydrate-peptide link. Complexes of relatively high molecular weight were isolated on Sephadex G-10 as previously described and treated with 1M KBH_4 in 0.1 N NaOH for 18 hours. Incubations were performed both at 0° and 37°, following which, the samples were acidified and borate ion removed by repeated evaporation from acidified methyl alcohol. The samples were then chromatographed on the same Sephadex columns as before and fractions in the various molecular weight classes hydrolyzed and analyzed. Table 5 shows no significant decline of seryl or threonyl residues compared to untreated material and no major increase in alanine levels. No α-amino butyric acid was detected in the borohydride-treated samples. Exposure to alklaline conditions at both 0° and 37° generated glycopeptides of intermediate molecular weight from larger material but without significant loss of any one amino acid residue. This finding suggests that some component or some linkage in the polypeptide backbone was base-labile. It is also evident that the substantial formation of glucosaminitol, usually indicative of reducing chain termini, was not accompanied by a corresponding decline in an amino acid residue. Attachment of carbohydrate to peptide via alkali-stable non-glycosidic bonds or the presence of a highly branched region at that portion of the

Table 5: Effect of alkaline borohydride on amino acid and carbohydrate composition of P7ch.

A high molecular weight fraction was harvested from P7ch on Sephadex G-10 and treated at the indicated temperatures. After sizing by rechromatography on Sephadex G-10 pooled fractions were assayed for reducing sugar and for amino acids and hexosamines after hydrolysis in 4 N HCl. Values are in nonomoles.

Relative molecular weight							
Temperature		0°			37°		
	Untreated	Treated					
	High[1]	High	Medium	Total	High	Medium	Total
Asp	56	28	13	41	24	16	40
Thr	38	15	22	37	12	22	34
Ser	102	36	58	94	37	62	99
Glut	108	45	58	103	43	44	86
Pro	64	24	36	60	24	54	78
Gly	184	80	84	164	70	62	132
Ala	49	24	31	55	18	26	44
Val	31	9	14	23	9	11	20
Ile	17	6	8	14	6	10	16
Leu	23	10	18	28	7	15	22
Glucosamine	590	560	0	560	130	150	280
Glucosaminitol	0	910	0	910	600	350	950

[1]No carbohydrate or peptide of intermediate molecular weight was present in the starting material.

chitin chain proximal to the peptide is thus indicated. The increase in total carbohydrate from 0.59 μmoles for the control to 1.47 and 1.23 μmoles for the treated samples probably is due to the greater susceptibility of the BH_4^- -reduced material to hydrolysis and the greater resistance of the hydrolysis products to reversion and deamination during subsequent handling.

Alkali-sensitive links between hydroxy amino acids and carbohydrate can also be detected in the presence of sulfite. Sulfitolysis in alkaline solution converts seryl links to cysteic acid and threonyl links to 2-amino-3-sulfonylbutyric acid (Simpson *et al.*, 1972). As shown in Table 6, sulfitolysis at 0° did not alter the amino acid ratio of

Table 6: Effect of alkaline sulfite on amino acid and carbohydrate composition of P7ch

The sample was treated with Na_2SO_3 at 0° for 72 hours in 0.1 N NaOH. A control was treated at pH 3 to serve as a check without sulfitolysis. The samples were hydrolyzed for amino acid and carbohydrate analysis in 4 N NCl. Values represent mole ratios.

	Control	Treated
Cysteic acid[1]	0.0	0.35
Asp	0.90	0.60
Thr	0.72	0.63
Ser	1.6	1.5
Glut	1.2	1.1
Gly	*2.0*	*2.0*
Ala	0.91	1.1
Glucosamine	25	20

[1]Includes 2-amino-3-sulfonylbutyric acid and other sulfonic acid derivatives that co-chromatography on the amino acid analyzer.

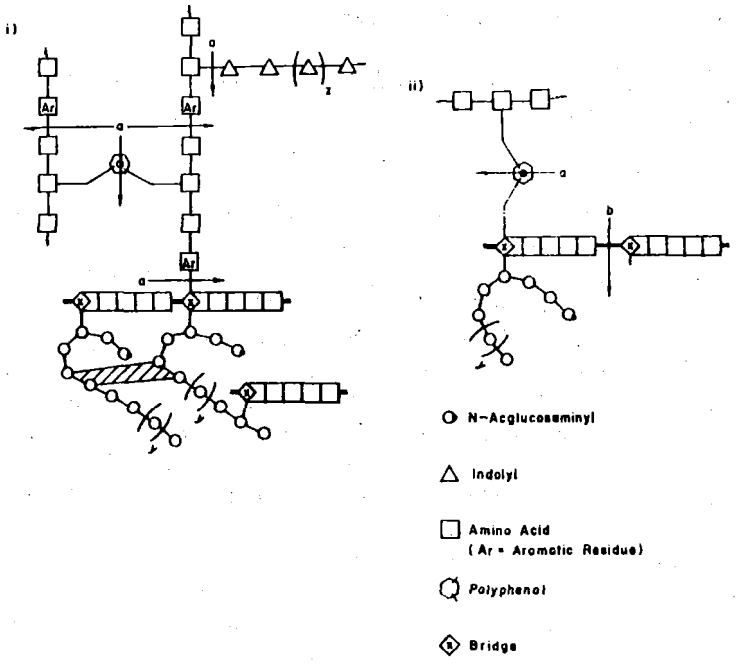
serine or threonine to glycine. These ratios were unchanged at 37° and 60° as well. Although 0.35 μmoles of a sulfonic acid derivative were formed and a comparable amount of aspartic acid was lost at 0°, further studies (not shown) at the higher temperatures did not form additional sulfonyl derivatives at the expense of aspartyl residues. At 37°, for example, 4.6 μmoles of a sulfonic acid derivative was formed with a decline in amino sugar recovery. No change in amino acid levels was observed. That partially oxidized derivatives of N-acetylglucosamine are subject to β-elimination in alkaline solution has been reported (Pravdic, 1971), presumably this route was the source of the uncharacterized acidic material. (Weber and Winzler, 1970).

Several preparations have been examined for the presence of β-aspartamido N-acetyl glucosamine bridges without definitive results. Small peaks with the mobility of the mono- and disaccharide derivatives of asparagine have been observed in partial hydrolysates of S8ch, P7ch and in cationic subfractions of these preparations isolated on columns of Dowex-50 (H+). Exhaustive digestion of these entities with Pronase, papain, subtilisin, pepsin or thermolysin followed by chromatography of the fragments on gels, charcoal, or ion exchange resins did not liberate significant levels of glycopeptides composed principally of aspartic acid, ammonia and N-acetyl-glucosamine. The most abundant fraction with the chromatographic properties of aspartamido N-acetyl-glycosamine was recovered from a papain digest. The peak was isolated with a stream splitting device and hydrolyzed. Amino acid analysis revealed this peak to contain four amino acids, including aspartic acid, but devoid of glucosamine. Similar results were obtained when partial hydrolysis was performed with 1 M HCl for 30 minutes instead of proteolytic enzymes.

DISCUSSION

The peptidochitodextrins prepared from the dark, sclerotized puparial case of *Sarcophaga bullata* by NBS oxidation, acid fragmentation and other purification steps retain many of the properties of the native integument. Substantial levels of chitin are present, the chemical and physical properties of which are identical to that of the polysaccharide prepared in alkali. Although of low molecular weight, the peptidylated oligosaccharides aggregate to form crystalline arrays in a manner resembling the alignment of chitin chains during formation of the puparium (Lipke and Geoghegan, 1971a). The purified fractions remain resistant to proteolytic digestion, although the effect is less pronounced than in untreated cuticle (Lipke and Geoghegan, 1971b). Inhibition of proteolysis could be ascribed to D-amino acid residues, but the occurrence of high levels of D-amino acid oxidase coupled with the absence of D-serine and D-alanine in the body fluids of dipterans (Corrigan, 1968) do not speak for this supposition.

Scheme 2. Proposed structures for the linkage region. Candidate arrangements indicated by i) and ii). Points susceptible to cleavage by N-bromosuccinimide (a) and proteolytic enzymes (b) indicated by arrows. Hatched areas designate regions bonded by non-covalent interactions. Subscripts represent arbitrary polymerization indices for chitin (y) and melanin (z).



The paucity of amino-termini reported in Table 4 together with the ability of proteolytic enzymes to cleave fraction P7ch into smaller fragments, the amino acid composition of which is identical to the parent material, (Table 2) makes possible further definition of the model previously offered for consideration (Lipke and

Geoghegan, 1971a). The glycosylated peptide backbone consists of subunits with identical amino acid composition (Scheme 2i). Arrangement a) or b) of Scheme 1 can be accommodated by this model. The amino acids in each subunit consist principally of aspartic and glutamic acids, serine, glycine and alanine. Whether the amino acid sequence within the subunits is also similar requires further study. An additional constituent of unknown structure which joins the peptide to the chitin chain has also been added based on the lack of chemical evidence favoring any of the bridges identified in other forms. In this respect, seryl or threonyl titers are unaffected by treatment of the complex with borohydride or sulfite in alkali, discounting glycosyl links to hydroxy amino acids (Tables 5 and 6). Neutral sugars were absent from these preparations, and hydroxylysine, if present, occurred in low levels, less than 1% of the total amino acid titer. This amount is insufficient to join the number of chitin chains present via glucosyl or galactosyl-hydroxlysine bridges (Kimura, 1972). Digestion with enzymes or dilute HCl, the conventional route to the isolation of aspartamido-N-acetyl-glucosamines from glycoproteins (Sinohara, *et al.*, 1971; Tarentino *et al.*, 1970) did not afford comparable products from puparial cuticle. In the model, the unknown bridge component is joined to the adjacent subunit by a bond which is sensitive to alkali, to nitrous acid, or to pepsin-thermolysin to a degree dependent on the length of the appended chitin chain. The amino acid sequence is not known, but differs from the Ser-Asn-Thr of sericin (Sinohara *et al.*, 1971) on the basis of the low level of threonine. The polysaccharide chain is shown with free reducing ends on the basis of the distribution of such groups following proteolysis (Table 2). The formation of significant levels of a sulfonic derivative strongly suggests the link to peptide is via carbon 3 of the amino sugar residue (Lipke and Geoghegan, 1971a). Since sclerotization of the puparium is independent of polysacchride or protein synthesis, formation of the -X- bridge may involve the addition of an activated precursor or free radical form of -X- to preformed chitin and subunit chains. In Scheme 2, i) and ii) are candidate structures which vary in the point of attachment of the polyphenol. In either arrangement, at least two different proteins are required for fabrication of the puparial integument.

REFERENCES

Attwood, M.M. and Zola, H. 1967. The association between chitin and protein in some chitinous tissues. Comp. Biochem. Physiol. *20:*993-998.

Constantopoulos, G., Dekaban, A.S. and Carroll, W.R. 1969. Determination of molecular weight distribution of acid mucopolysaccharides by Sephadex gel filtration. Anal. Biochem. *31:*59-70.

Corrigan, J.J. 1969. D-Amino acids in animals. Science *164:*142-149.

Dische, Z. and Borrenfreund, E. 1950. A spectrophotometric method for the micro-determination of hexosamines. J. Biol. Chem. *184:*517-522.

Dygert, S., Li. L.H., Florida, D. and Thoma. J.A. 1965. Determination of reducing sugar with improved precision. Anal. Biochem. *13:*367-374.

Fraenkel, G.S. and Rudall, K.M. 1940. Studies of the physical and chemical properties of the insect cuticle. Proc. Royal Soc. (London) B*129:*1-35.

Hackman, R.H. 1960. Studies on chitin. IV. The occurrence of complexes in which chitin and protein are covalently linked. Aust. J. Biol. Sci. *13:*568-577.

Kimura, S. 1972. Determination of the glycosylated hydroxylysines in several invertebrate collagens. J. Biochem. *71:*367-370.

Lipke, H. 1971. Conjugates of glucosamine in cockroach cuticle. Insect Biochem *1:*189-198.

Lipke, H. and Geoghegan, T. 1971a. The composition of peptidochitodextrins from sarcophagid puparial cases. Biochem. J. *125:*703-716.

Lipke, H. and Geoghegan, T. 1971b. Enzymolysis of sclerotized cuticle from *Periplaneta americana* and *Sarcophaga bullata.* J. Insect Physiol. *17:*415-425.

Lipke, H., Grainger, M. and Siakotos, A.N. 1965. Polysaccharide and glycoprotein formation in the cockroach. I. Identity and titer of bound monosaccharides. J. Biol. Chem. *240:*594-600.

Mayo, J.W. and Carlson, D.M. 1970. Effect of alkali and of sodium borohydride at alkaline pH on N-acetylchondrosine : reduction versus cleavage. Carbohydrate Res. *15:*300-303.

Pearson, F.G., Marchessault, R.H. and Liang, C.Y. 1960. Infrared spectra of crystalline polysaccharides. V. Chitin. J. Polym. Sci. *43:*101-116.

Pravdic, N. and Fletcher, H.G. Jr. 1971. The oxidation of 2-acetamido-2-deoxy-doses with aqueous bromine. Two diastereoisomeric 2-acetamido-2,3-dideoxyhex-2-enono-1,4-loctones from 2-acetamido-2-deoxy-D-glucose, -mannose, and -galactose. Carbohydrate Res. *19:*339-352.

Simpson, D.L. Hranisavljevic, J. and Davidson, E.A. 1972. β elimination and sulfite addition as a means of localization and identification of substituted seryl and threonyl residues in proteins and proteoglycans. Biochemistry *11:*1949-1856.

Sinohara, E. 1972. Molecular evolution of glycoproteins. Tohoku. J. Exp. Med. *106:*98-107.

Sinohara, H., Asano, Y. and Fukui, A. 1971. Glycopeptides from silk fibroin of *Bombyx mori*. Biochem. Biophys. Acta. *237:*273-279.

Spiro, R.G. and Fukushi, S. 1969. The lens capsule. Studies on the carbohydrate units. J. Biol. Chem. *244:*2049-2058.

Tarentino, A., Plummer, T.H. Jr. and Maley, F. 1970. Studies on the oligosaccharide sequence of ribonuclease B. J. Biol. Chem. *245:*4150-4157.

Weber, P. and Winzler, R.J. 1970. Sulfonated amino sugars derived from alkaline sulfite-treated glycoproteins. Arch. Biochem. Biophys. *137:*421-427.

Witkop, B. 1968. Chemical cleavage of proteins. Science *162:*318-326.