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The Covalent Linkage of Protein to Carbohydrate in the Extracellular Protein–Polysaccharide from the Red Alga *Porphyridium cruentum*

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The extracellular anionic polysaccharide isolated from cultures of a unicellular red alga, *Porphyridium cruentum*, contains a small amount of protein after extensive purification. The polysaccharide and protein are recovered in the same fraction after isopycnic CsCl-density-gradient centrifugation in 4M-guanidinium chloride, under conditions designed to separate proteins from polysaccharide. The peptide portion of the protein–polysaccharide is released from the polysaccharide by alkali under conditions for β-elimination. The released peptide is non-diffusible, but it can be separated from the polysaccharide by precipitation of the polysaccharide as the cetylpyridinium complex. Under conditions for β-elimination of certain *O*-glycosidic carbohydrate–protein linkages, selective destruction of serine and threonine occurs. The addition of a reducing agent to the alkali mixture produces a selective increase in alanine and α-aminobutyric acid. Addition of a tritiated reducing agent to the alkali mixture produces radioactive alanine and α-aminobutyric acid, and xylitol as the only sugar alcohol. Similar results are obtained from glycopeptides isolated from partial acid hydrolysates. A macromolecular structure of the protein–polysaccharide is suggested by a comparison of the intrinsic viscosity of material before and after treatment with alkali and proteolytic enzymes.

A carbohydrate–protein linkage in which serine or threonine residues in a protein are joined by *O*-glycosidic linkage to carbohydrate is particularly susceptible to alkali cleavage. The hydroxyl group of the serine or threonine residue is β to a carbonyl group, and alkali causes a β-elimination reaction which produces an unsaturated amino acid and a reducing sugar. These products may be identified after reduction.

The demonstration of a protein–carbohydrate linkage involving serine and threonine in an extracellular proteoglycan in a red alga is the first such report in the plant kingdom. The probable involvement of xylose in the linkage is also a new finding (Kieras, 1972; Kieras & Chapman, 1976).

Experimental

All chemicals and biochemicals were reagent grade. HCl used for amino acid anslysis was AristaR brand from BDH, Poole, Dorset, U.K. NaB^3H_4 (specific radioactivity 200mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Acetic anhydride was freshly distilled before use; pyridine was distilled over ninhydrin and stored at 4°C. The 2M-pyridine/acetate buffer which was used for the ion-exchange chromatography of glycopeptides was prepared by adjusting pyridine with acetic acid to pH 4.85 and diluting the solution with water to 2M-pyridine concentration. Xylitol was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Pfanstiehl Co. (Waukegan, IL, U.S.A.). The Pfanstiehl product gave two components on paper electrophoresis in 0.043M-NaOH/0.0125M-$Na_2B_4O_7$ buffer, pH 10.0 (Gomori, 1955).

Papain was crystallized from a crude preparation (type II, Sigma) by the method of Kimmel & Smith (1954). Pronase (Calbiochem, La Jolla, CA, U.S.A.) was dissolved in 0.02M-$CaCl_2$/0.05M-Tris/HCl buffer, pH 8.5, and 3vol. of acetone was added. The precipitate was redissolved in 0.02M-$CaCl_2$/0.05M-Tris/HCl buffer, pH 8.5, to a concentration of 7.5mg/ml; this constituted 75% of the original material. Guanidinium chloride was obtained from Mann Ultra-Pure Chemicals (Orangeburg, NY, U.S.A.) and CsCl (Sequenal grade) from Pierce Co. (Rockford, IL, U.S.A.). Cetylpyridinium chloride was purchased from K & K Laboratories (Plainview, NY, U.S.A.).

The culture conditions, isolation and purification procedures used in the preparation of the extra-

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cellular polysaccharide from *Porphyridium cruentum* (Ag.) Naegli (Indiana University Collection no. 161) have been described elsewhere (Kieras, 1972; Kieras *et al.*, 1976). Briefly, the algae were cultured in a chemically defined medium under sterile conditions. After the cultures reached stationary phase the medium was separated by centrifugation. The subsequent purification was similar to the procedure described below for the re-isolation of the polysaccharide after papain digestion.

The colorimetric analyses for sugar determinations have been described elsewhere (Kieras, 1972; Kieras *et al.*, 1976).

Automated neutral-sugar analysis (Technicon Sugar-Chromatography System) of the borate-complexed sugar on cation-exchange resins was performed on monosaccharides derived from acid hydrolysates (1M-HCl, 3h, 100°C, sealed tube) (Kieras, 1972). The neutral sugars were isolated from hydrolysates by passage through small (1cm×5cm) columns containing Dowex 50 (H^+ form) (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and Dowex 1 (formate form) (Bio-Rad Laboratories). The uronic acids were separated from the acid hydrolysates by adsorption on Dowex 1 (formate form) (contained in small columns) and then by elution from the resin with 0.5M-formic acid.

Samples were prepared for amino acid analysis by hydrolysis in 6M-HCl for 20h at 100°C in evacuated sealed tubes. Automated analysis was carried out on a Technicon Automatic Amino Acid Analyzer single-column system or on a Beckman model 120C two-column system. All glassware was heated to 554°C or washed with chromic acid before use.

Whatman 3MM paper was used for high-voltage electrophoresis of amino acid hydrolysates on a Gilson High-Voltage Electrophoretor model D. A Savant Flat-Plate Apparatus, model FP-30A, was used for electrophoresis of sugar hydrolysates on Whatman 1 or 3MM paper (23cm×57cm strips).

Sugar alcohols were detected on chromatograms by a periodate/benzidine method (Cifonelli & Smith, 1954). The periodate solution was buffered (0.015M-acetic acid/0.036M-sodium acetate, pH5) and the chromatogram was dipped twice in the oxidizing solution before being dipped in the benzidine reagent [use of this method is not advised, as benzidine is a carcinogen; an alkaline silver reagent (Smith, 1960) may be used instead]. Amino acids were detected on chromatograms by a ninhydrin reagent [1% ninhydrin (w/v) and 1% pyridine (v/v) in acetone].

Radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer as described by Stoolmiller & Dorfman (1969). The efficiency of counting under these conditions was found to be 40% by a control experiment in which a known amount of L-[^{3}H]proline was subjected to electrophoresis under the conditions described in the legend to Fig. 1.

Viscosity measurements were carried out in a Ubbelohde low-shear Cannon Semi-Micro Dilution Viscometer size 100 (Cannon Instruments, State College, PA, U.S.A.). The polymer was dissolved at 0.2mg/ml in several solvents (0.1M-sodium phosphate, pH7.0; 0.5M-guanidinium chloride, adjusted to pH7.0 with 0.1M-NaOH; 4.0M-guanidinium chloride, pH7.0; 0.1M-NaOH/0.6M-LiCl before and after 24h incubation and neutralization). Solutions were filtered through coarse glass filters, pipetted into the viscometer and equilibrated to 25.00±0.02°C in a water bath. Intrinsic viscosity was calculated by the procedure given by Mathews (1967).

The extracellular polysaccharide was subjected to isopycnic CsCl-density-gradient centrifugation in water solutions under the conditions described in Table 1. The polysaccharide was dissolved at 0.2–1.0mg/ml. The equations of Flamm *et al.* (1969) were used to calculate the amount of CsCl necessary to give a density at the bottom of the centrifuge tube of 1.86g/ml after centrifugation. This was calculated to require an initial density of CsCl–polysaccharide solution of 1.75g/ml; 12.24g CsCl was added to 9ml of polysaccharide solution.

A 1–2mg/ml solution of extracellular polysaccharide in 1M-NaCl containing 0.02M-EDTA (disodium salt) and 0.02M-cysteine hydrochloride (freshly prepared) was adjusted to pH6.5, and papain was added to give an initial enzyme/polymer (w/w) ratio of 1:5–1:10. After 24h at 65°C a second portion of disodium EDTA and cysteine hydrochloride (freshly prepared), equal to one-quarter to one-third of the original volume of the solution, and an amount of papain equal to the first portion were added; digestion was continued for a further 24h at 65°C. After the digest was diluted with water to 0.5M-NaCl and cooled to 37°C, the polysaccharide was precipitated by the slow addition with stirring of a 1% (w/v) solution of cetylpyridinium chloride. The cetylpyridinium–polysaccharide complex was recovered by centrifugation, and sufficient 2M-LiCl was added to give a final polysaccharide concentration of about 0.3–0.5mg/ml. After the polysaccharide was dissolved, 2vol. of ethanol was slowly added with stirring and the precipitated material was isolated by low-speed centrifugation (or decantation). The pellet after centrifugation was dissolved in 2M-LiCl, and the polysaccharide was precipitated with 2vol. of ethanol. After this precipitate had been dissolved in 2M-LiCl, the polysaccharide was once again precipitated with cetylpyridinium chloride and the subsequent isolation steps were repeated as outlined above. The final ethanol precipitate was dried in ether and stored over P_2O_5 and NaOH under partial vacuum. The yields of papain-digested material were usually 85% by weight.

Pronase digestion was carried out as described by Marks *et al.* (1962) on 2mg/ml solutions of material

in 0.5M-NaCl in 0.02M-$CaCl_2$/0.05M-Tris/HCl buffer, pH8.51. After the addition of Pronase to give an enzyme/polymer ratio of 1:6 (w/w), the mixture was adjusted to pH9.0 with 2M-Tris. Incubation for 20h at 52°C was followed by the addition of an equal amount of Pronase and a second 20h incubation period. The digest was then diluted to 0.25M-NaCl in 0.02M-$CaCl_2$/0.05M-Tris/HCl buffer, pH8.51, and after another addition of Pronase a third 20h period of incubation followed. The Pronase-digested material was reisolated by a series of steps similar to those described above for the papain-digested material. The yield of Pronase-digested material was 78% by weight.

Extracellular polysaccharide (50mg) was dissolved in 50ml of 0.5M-NaCl and an equal volume of 1M-NaOH was added. After stirring for 24h at room temperature (22°C), the solution was neutralized and dialysed against water (or against 0.5M-NaCl and then against water). (It may be noted that dialysis against salt solutions of high concentrations did not remove the peptide material.) Part of the alkali-treated material was reisolated by a cetylpyridinium chloride precipitation procedure similar to that described for papain-digested material; the rest was freeze-dried.

Protein–polysaccharide (30mg) was dissolved in 50ml of water, and an equal volume of 1.0M-NaOH/0.6M-KBH_4 was added. The solution was slowly stirred for 25.5h at room temperature. The pH was adjusted to 5 with 4M-acetic acid to destroy the borohydride. The solution was readjusted to pH7 with 0.1M-NaOH, dialysed against water and freeze-dried. The dry weight yield of non-diffusible material was 98%.

The palladium chloride catalyst was prepared as described by Tanaka & Pigman (1965). Protein–polysaccharide (30mg) was dissolved in 50ml of water vigorously stirred with 0.2ml of 0.8M-palladium chloride catalyst. The treatment with alkali and borohydride was performed as described above.

Preparations to be treated with alkali and NaB^3H_4 were first treated with KBH_4. Protein–polysaccharide (30mg) was dissolved in 0.02M-sodium phosphate buffer, pH7.5 (Gomori, 1955), and the solution was cooled to 4°C. After the addition of 0.2ml of 0.8M-palladium chloride catalyst, 9mg of KBH_4 was added. After 10min another 9mg of KBH_4 was added and the incubation was continued for an additional 10min at 4°C. Then the solution was warmed to room temperature and maintained at this temperature for an additional 40min. Next 50ml of 1M-NaOH and 3.8mg of NaB^3H_4 (final concentration 7mM in KBH_4 and NaB^3H_4) was added and the solution was slowly stirred for 24h at 22°C. The solution was adjusted to pH5 with 4M-acetic acid and readjusted to pH7 with 1M-NaOH. It was dialysed at 22°C until no further radioactivity was detectable in the solution outside the dialysis bag. The recovery of dried material was 96% by weight.

Preparation of glycopeptides by acid hydrolysis of proteolytically digested algal extracellular polysaccharide was carried out as described by Lindahl & Rodén (1966).

The glycopeptide fraction eluted by 2M-pyridine/acetate, pH4.85, from the Aminex A-5 column was acetylated by incubation of 0.1ml of its solution, containing about 30nmol of amino acids, with 10μl of acetic anhydride and 40μl of 5% (w/v) $NaHCO_3$ at pH7.5 for 1h; the incubation was repeated three times and the samples were evaporated to dryness with repeated additions of water.

Results and Discussion

The extracellular polysaccharide of the freshwater red alga *Porphyridium cruentum* is a high-mol.-wt. (0.5×10^6–10×10^6) heteropolymer consisting, by weight, of 42% hexose, 30% pentose, 8.5% uronic acid, 9% ester sulphate and 1–2% amino acids. The molar proportions of components relative to glucose are: galactose/xylose/glucose/uronic acid/sulphate, 2.12:2.42:1.00:1.22:2.61 (Kieras, 1972). The glucose and 15% of the galactose are in the D-configuration. The uronic acids are D-glucuronic acid and 2-*O*-methyl-D-glucuronic acid (Kieras *et al.*, 1976), and these are nearly all found in three aldobiuronic acids after acid hydrolysis of the polymer (Kieras, 1972; Heaney-Kieras & Chapman, 1976).

The extracellular polysaccharide always contained small amounts of residual protein (0.11–0.55μmol/mg of material) that persisted through extensive purification with cetylpyridinium chloride and repeated steps of ethanol precipitation followed by salt solubilization. To investigate whether or not the protein was covalently linked to polysaccharide, the preparations were subjected to isopycnic CsCl-density-gradient centrifugation in 4M-guanidinium chloride, under conditions designed to separate proteins from polysaccharides (Hascall & Sajdera, 1969). The distribution of carbohydrate and protein after centrifugation is displayed in Table 1. Under these conditions, nearly all the polysaccharide is found in the pellet fraction and virtually all the protein as well. When the pellet obtained from the first centrifugation was re-centrifuged under the same conditions, the result was the same as before: the polysaccharide and protein were both located in the pellet fraction. Alkali treatment of the material in the pellet fraction produced selective destruction of the amino acids serine (24%) and threonine (32%). When polysaccharide digested with papain and Pronase was subjected to centrifugation under these conditions in 4M-guanidinium chloride, the polysaccharide (97%) and protein (95%) were found in the pellet fraction.

Isopycnic CsCl-density-gradient centrifugation was carried out in higher-density CsCl gradients in

Table 1. *Isopycnic CsCl-density-gradient centrifugation of protein–polysaccharide in* 4M-*guanidinium chloride*
To samples dissolved at 1.0–2.0mg/ml in 4M-guanidinium chloride (pH7.0), solid CsCl was added to achieve a density of 1.5g/ml, as described by Hascall & Sajdera (1969). Centrifugation was carried out in 5ml cellulose nitrate tubes fitted with aluminium caps in a SW 50L rotor in a Beckman L2 65 Spinco ultracentrifuge at 21–23°C for 82h at 165300*g* (or for 110h at 105500*g*). After centrifugation, three 1.1ml supernatant fractions were aspirated off, dialysed against water and analysed for carbohydrate and amino acids (see the Experimental section for details). Some of the pellet fractions were analysed directly for these components and some were redissolved in 4M-LiCl, dialysed against 4M-LiCl and then against water, freeze-dried and dried to a constant weight before analysis. Some of the pellets (fraction 4 described in this Table) were dissolved in 4M-guanidinium chloride, pH7.0, and adjusted to a density of 1.5g/ml by the addition of solid CsCl and centrifuged as described above.

Fraction no.	Total hexose (μmol/fraction)	Total hexose Recovery (%)	Total uronic acid (μmol/fraction)	Total uronic acid Recovery (%)	Total amino acid (μmol/fraction)	Total amino acid Recovery (%)
1 (top)	0.1	0.1	0.00	0	0.34	14
2	0.6	0.1	0.04	0.5	0.26	10
3	1.2	2.6	0.16	2.1	—	—
4 (pellet)*	50.8	108	7.6	99	2.6	105
Composition of total starting material (20.6mg)	47.0	100	7.7	100	2.5	100

* Re-centrifugation of fraction 4 under the same conditions gave the same result, almost all carbohydrate and amino acids in fraction 4. Quantitative carbohydrate analyses (μg/mg): starting material: galactose, 140; xylose, 117; glucose, 59; re-centrifuged fraction 4: galactose, 138; xylose, 130; glucose, 64.

water. When a 1.0mg/ml polysaccharide solution was centrifuged under such conditions, 98% of the carbohydrate and 93% of the protein were found in the pellet fraction at a density of 1.86g/ml.

The identification of the amino acids in the peptide around the linkage of protein to polysaccharide was pursued by examining the amino acids remaining after proteolytic digestion. The total amount of amino acids is decreased by about 80% by proteolysis, and serine and threonine comprise about 27% of the residual amino acids (Table 2). Serine, glycine and alanine together make up about 39% of the amino acids in the starting material and between 50 and 64% after proteolytic digestion. In the glycopeptide fraction eluted by 2M-pyridine/acetate from the Aminex A-5 column these three amino acids represent 70% of the total (results not shown).

The peptide near the *O*-glycosidic linkage of protein to carbohydrate often contains residues of glycine and alanine. These amino acids predominate in the linkage-region peptide of two glycosaminoglycans (Stern, 1970; Johnson & Baker, 1973; Isemura & Ikenaka, 1975) and a variety of collagens (Isemura *et al.*, 1972; Morgan *et al.*, 1970). Alanine is present in the peptide of the linkage region of the freezing-point-depression protein of an antarctic fish (Shier *et al.*, 1972). In contrast, in another group of *O*-glycosidic carbohydrate–protein linkage regions that contain proline, glycine and alanine are either not major components or are absent altogether. Bovine *k*-casein, the A1 protein from myelin, and immunoglobulin G (Fiat *et al.*, 1972) are examples of this case.

A special characteristic of *O*-glycosidic carbohydrate–protein linkages involving serine and threonine is their susceptibility to alkali cleavage. This reaction, a β-elimination, destroys the linkage amino acid(s) and produces unsaturated products. The algal polysaccharide was treated with alkali and the amino acid composition was examined after dialysis, and after reisolation of the polysaccharide. After alkali treatment and dialysis, 89% of the total amino acids were recovered and about 76% of the serine and 69% of the threonine (Table 3). Although the alkali-released peptide is non-diffusible, it can be removed from the polysaccharide by a series of steps used to reisolate the polysaccharide (Table 3). Only 9% of the total original amino acids remain after the reisolation of the polysaccharide. Alkali treatment of the glycopeptide fraction eluted by 2M-pyridine/acetate destroyed 19% of the serine and 52% of the threonine (Table 4). *N*-Acetylation of this fraction increased the destruction of threonine to 67% but did not increase the destruction of serine.

Additional evidence for an *O*-glycosidic carbohydrate–protein linkage involving serine and threonine can be gained by demonstrating the conversion of the products of alkali treatment, presumably unsaturated amino acids, into saturated amino acids which are stable to acid hydrolysis. This conversion also helps to rule out the non-specific alkali destruction of amino acids in certain proteins (Marshall & Neuberger, 1970). The unsaturated amino acid produced by the β-elimination of serine would be reduced to alanine and the unsaturated amino acid derived from threonine would be reduced

Table 2. *Amino acid analyses of alkali-treated and proteolytically digested protein–polysaccharide*
Numbers in parentheses represent the percentages of amino acids destroyed by alkali. tr, Trace.

	Content (nmol/mg)			
Amino acid	Untreated	Alkali-treated	Papain-digested	Papain- and Pronase-digested
Asp	14.2	14.0	1.5	1.3
Thr	9.5	6.3 (34)	2.6	2.4
Ser	11.5	8.8 (25)	3.4	3.3
Glu	14.1	13.7	2.9	1.9
Pro	tr	tr	0.0	0.0
Gly	17.0	14.0	3.9	5.0
Ala	11.5	11.0	3.1	3.0
Val	9.3	9.2	1.0	0.0
Ile	5.7	6.0	0.0	0.0
Leu	7.9	7.4	1.4	1.9
Tyr	3.1	4.0	0.0	0.0
Phe	7.5	tr	0.0	0.0
His	tr	tr	0.0	0.0
Arg	tr	3.1	2.0	0.0
Lys	1.5	1.7	0.0	0.0
Total	112.8	99.2	21.8	17.8

Table 3. *Destruction of serine and threonine and increase in alanine and α-aminobutyric acid by alkali treatments of protein–polysaccharide*
Numbers in parentheses represent percentage recoveries.

Experiment	Total amino acids (nmol/mg)	Serine (nmol/mg)	Alanine (nmol/mg)	Percentage conversion Ser → Ala*	Threonine (nmol/mg)	α-Amino-butyric acid (nmol/mg)	Percentage conversion Thr → α-amino-butyric acid†
Analysis after dialysis of reaction mixture							
Protein–polysaccharide	113 (100)	11.5	11.5		9.5	0.0	
Protein–polysaccharide +alkali	101 (89)	8.8 (76)	11.5		6.3 (69)	0.0	
Protein–polysaccharide +alkali+reducing agent	122 (108)	7.6 (67)	13.2 (116)	45	7.5 (78)	0.4	22
Protein–polysaccharide +alkali+reducing agent+catalyst	110 (97)	7.2 (65)	13.8 (120)	54	7.3 (73)	0.8	30
Analysis after reisolation of polysaccharide							
Protein–polysaccharide +alkali+cetyl-pyridinium chloride precipitation	10 (9)	0.8 (6)			0.6 (6)		

* Ratio of alanine formed to serine destroyed as %.
† Ratio of α-aminobutyric acid formed to threonine destroyed as %.

to α-aminobutyric acid. Selective increases in alanine and α-aminobutyric acid are found in algal polysaccharide treated with alkali in the presence of $NaBH_4$ (Table 3). The increase in alanine corresponds to 45% of the amount of serine destroyed by alkali and the appearance of α-aminobutyric acid to 22% of the threonine destroyed.

The borohydride reduction of unsaturated amino acids produced by β-elimination is not usually quantitative. In the presence of alkali and borohydride,

86% of the serine and 15% of the threonine in sheep submaxillary glycoprotein were converted into their respective reduced amino acids (Marshall & Neuberger, 1970). β-Elimination and reduction of keratan sulphate from bovine nasal septum showed a conversion of serine into alanine of 50% and a conversion of threonine into α-aminobutyric acid of 11% (Kieras, 1974). In our case, the addition of a palladium chloride catalyst increased the amount of alanine formed by 9% (to 54%) and the amount of α-aminobutyric acid by 8% (to 30%) (Table 3). For a number of mucins the addition of a palladium chloride catalyst increased the transformation of serine into alanine to values ranging from 87 to 98% and the transformation of threonine into α-aminobutyric acid to values between 69 and 86% (Marshall & Neuberger, 1970).

The composition of the glycopeptide preparation fractionated on the Aminex A-5 column is shown in Table 4. The fraction eluted by 0.1 M-HCl contains nearly all the carbohydrate and 32% of the total recovered amino acids, but none of the serine or threonine. The fraction eluted by 2 M-pyridine/acetate, pH 4.85, has 68% of the amino acids and all of the serine and threonine; it will be referred to as the glycopeptide fraction. Glycine accounts for 44% of the total amino acids in this fraction, compared with 21% in the unfractionated glycopeptide material. Serine, threonine, glycine and alanine make up 80% of the amino acids in the glycopeptide fraction eluted by 2 M-pyridine/acetate buffer, but only 58% in the unfractionated glycopeptide preparation (results not shown). The ratio of serine and threonine to pentose increased from about 1:1000 in unfractionated glycopeptide material to about 1:2.5 in the glycopeptide fraction eluted by 2 M-pyridine/acetate buffer. The 2 M-pyridine/acetate fraction contains only neutral sugars. The monosaccharide components of this fraction are present in molar proportions relative to glucose of galactose/xylose/glucose of 2.90:3.52:1.00, compared with 2.12:2.42:1.00 in the starting material.

Confirmation of the amino acids involved in the carbohydrate–protein linkage and an examination of the sugar moiety in the linkage may be done by reducing the β-elimination products with a tritiated reducing agent. As shown in Figs. 1(*a*) and 1(*b*), treatment of either the protein–polysaccharide or the glycopeptide fraction eluted by 2 M-pyridine/acetate produced three main peaks of radioactivity. One of these is coincident with alanine. A second is coincident with α-aminobutyric acid, although the neighbouring portion of the peak is not. The sharp peak of radioactivity near the origin migrates far too slowly to be free amino acid(s), and no attempt was made to characterize it.

Since the four sugar components of the protein–polysaccharide are present in large amounts, it is

Table 4. *Composition of fractionated glycopeptides derived from proteolysed protein–polysaccharide*

Numbers in parentheses represent percentages of serine or threonine destroyed by alkali treatment. All values are expressed per g of starting (proteolysed) material. Preparation of glycopeptides by acid hydrolysis of proteolytically digested algal material was carried out as described by Lindahl & Rodén (1966). A suspension of 1 g of material in 1 litre of 0.02 M-HCl was adjusted with 1 M-HCl to pH 1.5 and heated for 4 h at 100°C. After concentration of the hydrolysate under reduced pressure at 20°C, the residual acid was removed by repeated freeze-dryings with water, and the sample was taken up in water and stored at −10°C. The glycopeptide preparation was fractionated on a column (0.9 cm × 19.5 cm) of Aminex A-5 (H^+ form; Bio-Rad Laboratories) equilibrated with 0.01 M-HCl. The sample (usually 400 mg of original material) was diluted 3-fold with water, adjusted to pH 2, and applied to the column under gravity flow. The components were eluted from the column with 0.01 M-HCl (400 ml), 2 M-pyridine/acetate buffer, pH 4.85 (140 ml), and 2 M-pyridine (140 ml) at a flow rate of 21 ml/h; at least 100 ml of column eluate containing no detectable carbohydrates was collected before the next solution was pumped into the column. Fractions (16 ml) were collected and assayed for carbohydrates and amino acids as described in the Experimental section. Pooled samples were evaporated several times with additions of small volumes of water and taken in a small amount of water.

	Total carbohydrate (μmol/g)	Colorimetric analysis (μmol/mg)			Total amino acids (nmol/g)	Serine (nmol/g)	Threonine (nmol/g)
		Hexose	Pentose	Uronic acid			
Hydrolysate of proteolysed protein–polysaccharide	5200	2800	1800	420	10400	660	620
0.01 M-HCl chromatographic fraction of hydrolysate	4730	2700	1600	360	2660	0	0
2 M-Pyridine/acetate, pH 4.85, fraction	5.2	3.0*	2.1*	0.0	5600	284 (19)	286 (52)
2 M-Pyridine fraction	0.0	0.0	0.0	0.0	0.0	0.0	0.0

* Quantitative carbohydrate analysis (μmol/g of starting material): galactose, 1.47; xylose, 1.80; glucose, 0.51. Details are given in the Experimental section.

rather difficult to measure directly the small amount of sugar involved in the linkage to protein. Instead, the sugar alcohol produced by reduction with NaB^3H_4 was examined by chromatography and electrophoresis. By paper chromatography xylitol was identified as the only sugar alcohol (Fig. 2). Some peaks of radioactivity on electrophoretograms or chromatograms could not be identified as either monosaccharides of the polysaccharide or the corresponding sugar alcohols. In the glycopeptide fraction eluted by 2M-pyridine/acetate buffer, these components persisted even after additional acid hydrolysis, at the expense of xylitol (Table 5). Since the polysaccharide contains moieties linked in 1,3-glycosidic linkages (Kieras, 1972), it is possible that β-elimination is followed by a 'peeling' reaction and rearrangement of these moieties to such compounds as saccharinic acids (Sowden, 1957; Baer, 1969).

Carbohydrate–protein linkages of the *O*-glycosidic type have been reviewed (Neuberger *et al.*, 1972; Lindahl & Rodén, 1972; Brimacombe, 1973; Marshall, 1974). The neutral sugars to which serine has been reported to be covalently linked are xylose in various glycosaminoglycans (Lindahl & Rodén, 1965, 1966; Helting & Rodén, 1968; Knecht *et al.*, 1967; Stern *et al.*, 1971) and mannose in enzymes (Lineback, 1968; Greiling *et al.*, 1969). Threonine has been shown to be covalently linked to galactose in earthworm cuticle collagen (Lee & Land, 1968) and to mannose in an enzyme (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) from *Aspergillus niger* (Lineback, 1968). An arabinose–hydroxyproline carbohydrate–protein linkage has been demonstrated in glycopeptides isolated from various plant cell walls (Lamport, 1967, 1969; Lamport & Miller, 1971) and from a unicellular green alga *Chlamydomonas reinhardi* (Miller *et al.*, 1972). The amino acid composition of red algae limits the possible choices for carbohydrate–protein linkage of *O*-glycosidic type, for these algae are exceptions to the general rule that algae contain hydroxyproline (Gotelli & Cleland, 1968).

Analysis of the effect of proteolysis and various alkali treatments on the intrinsic viscosity may provide some information about the macromolecular structure of the protein–polysaccharide. In one model, a long polysaccharide chain would be joined to a relatively short peptide at the non-reducing ternimus or at a short carbohydrate side chain. In this case, no change in intrinsic viscosity should occur after either alkali or proteolytic treatments. In another model, a

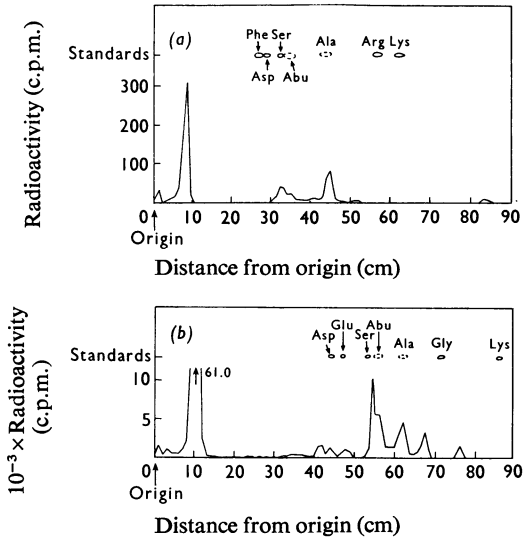

Fig. 1. *Distribution of radioactivity on paper electrophoretograms of products which were obtained by acid hydrolysis* (6M-*HCl*, 100°*C*, 20*h*) *of* (*a*) *alkali- and* NaB^3H_4*-treated protein–polysaccharide* (0.4*g*) *and* (*b*) *the glycopeptide fraction eluted from the Aminex column by* 2M-*pyridine/acetate buffer* (69 *nmol of amino acid*)

Electrophoresis was carried out in (*a*) 1.65M-formic acid, pH 1.6, at 66 V/cm for 4h, or, (*b*) in 2.5% formic acid/acetic acid, pH 2.0, at 71 V/cm for 4.6h. Ninhydrin location of external standards is represented by solid lines and of internal standards by dashed lines. After pieces (1 cm × 1 cm) of the electrophoretogram had been soaked in 0.5ml of water for 1h, liquid scintillant was added and the samples were counted for radioactivity. Abu, α-aminobutyric acid.

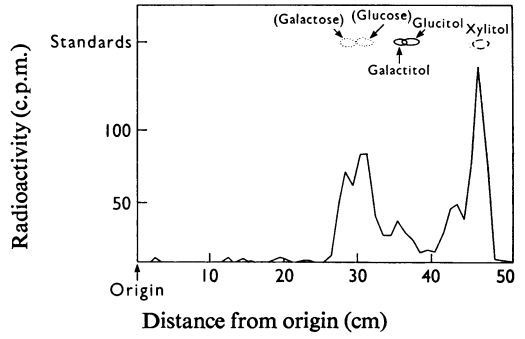

Fig. 2. *Distribution of radioactivity in the neutral sugar fraction of products which were obtained by alkali,* NaB^3H_4 *and* $PdCl_2$ *catalyst treatment of protein–polysaccharide* (0.8*g*) *after hydrolysis* (1M-*HCl*, 100°*C*, 3*h*)

Chromatography was carried out in ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.) for 51h on Whatman no. 1 paper. Carbohydrate components were identified by benzidine periodate or alkaline silver reagents (see the Experimental section for details). Dotted lines indicate carbohydrate components in the hydrolysate, dashed lines the xylitol added as an internal standard, and solid lines the external standards. Radioactivity was determined as described in Fig. 1.

Table 5. *Electrophoresis of neutral components of an acid hydrolysate of* 2M-*pyridine/acetate glycopeptide fraction after alkali and* NaB^3H_4 *treatment*

Values in parentheses represent percentages of the total radioactivity. Acid hydrolysis of a portion of the glycopeptide fraction containing 0.17 μmol of hexose was carried out in 1M-HCl for 3h at 100°C in a sealed tube. The neutral and acidic components of the hydrolysate were separated by ion-exchange chromatography as described in the Experimental section. Rehydrolysis in 1M-HCl of the neutral sugar fraction was carried out for 4h. High-voltage paper electrophoresis was performed in sodium borate buffer, pH 10.0, at 45 V/cm for 3h. The neutral monosaccharide components of the polysaccharide have a mobility greater than galactitol under these electrophoretic conditions; glucitol has a $R_{galactitol}$ of 0.74.

		Radioactivity (c.p.m./peak)				
Fraction	Peak ... $R_{galactitol}$ ...	A 0.22	B 0.51	Xylitol 0.67	Xylitol 0.81	Total c.p.m./ chromatogram
Neutral sugars		1700 (26)	2180 (34)	1715 (27)	870 (13)	6465
Neutral sugars after rehydrolysis		1800 (29)	2740 (44)	1049 (16)	706 (11)	6264

number of carbohydrate side chains would be joined by *O*-glycosidic linkage to a polypeptide backbone; a sharp non-reversible decrease in intrinsic viscosity would be observed after either treatment. The untreated algal polysaccharide had an intrinsic viscosity of about 21–23 dl/g, depending on the solvent. After alkali treatment, the intrinsic viscosity of the reisolated material was 22 dl/g. Proteolysis with papain and Pronase did not diminish the viscosity (24.3 dl/g). These results indicate that the molecule should have a structure of the first type, namely a long polysaccharide chain joined at its reducing terminus or at a short side chain to a peptide. This is in agreement with the (transmission) electron-microscopic observation that the isolated material is linear and that is overall length is little changed by proteolysis (Kieras, 1972; J. H. Kieras, unpublished work).

The extracellular protein polysaccharide from *P. cruentum* has a covalent linkage of protein to polysaccharide similar to that of the chondroitin sulphate portion of cartilage proteoglycan. The serine–xylose linkage is also found in a number of other glycosaminoglycans from various animal tissues. Additionally, in the algal material, threonine has been found to be joined in covalent linkage to polysaccharide; this is the first example of a threonine to xylose linkage. The predominance of glycine in the peptide near the linkage to carbohydrate in the alga material suggests that it is similar in composition to a number of other *O*-glycosidic carbohydrate–protein linkages involving serine and threonine.

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References

Baer, H. A. (1969) in *The Amino Sugars: the Chemistry and Biology of Compounds Containing Amino Sugars* (Jeanloz, R. W. & Balasz, E., eds.), vol. 1A, pp. 270–283, Academic Press, New York and London

Brimacombe, J. S. (1973) in *Carbohydrate Chemistry, a Specialist Periodical Report*, vol. 6, pp. 274–392, The Chemical Society, London

Cifonelli, J. A. & Smith, F. (1954) *Anal. Chem.* **26**, 1132–1134

Fiat, A.-M., Alais, C. & Jollès, P. (1972) *Eur. J. Biochem.* **27**, 408–412

Flamm, W. G., Bernstiel, M. L. & Walker, P. M. B. (1969) in *Subcellular Components, Preparation and Fractionation* (Birnie, G. D. & Fox, S. M., eds.), pp. 125–155, Plenum Press, New York

Gomori, G. (1955) *Methods Enzymol.* **1**, 138–146

Gotelli, I. B. & Cleland, R. (1968) *Am. J. Bot.* **55**, 907–914

Greiling, H., Vögele, P., Kisters, R. & Ohlenbusch, H.-D. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **250**, 517–518

Hascall, V. C. & Sajdera, S. W. (1969) *J. Biol. Chem.* **244**, 2384–2396

Heaney-Kieras, J. & Chapman, D. J. (1976) *Carbohydr. Res.* **52**, 169–177

Helting, T. & Rodén, L. (1968) *Biochim. Biophys. Acta* **170**, 301–308

Isemura, M. & Ikenaka, T. (1975) *Biochim. Biophys. Acta* **404**, 11–21

Isemura, M., Ikenaka, T. & Matsushima, Y. (1972) *Biochem. Biophys. Res. Commun.* **46**, 457–462

Johnson, A. H. & Baker, J. C. (1973) *Biochem. Soc. Trans.* **1**, 277–279

Kieras, F. J. (1974) *J. Biol. Chem.* **249**, 7506–7513

Kieras, J. H. (1972) Ph.D. Thesis, University of Chicago

Kieras, J. H. & Chapman, D. J. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1444

Kieras, J. H., Kieras, F. J. & Bowen, D. V. (1976) *Biochem. J.* **155**, 181–185

Kimmel, J. R. & Smith, E. L. (1954) *J. Biol. Chem.* **207**, 515–531

Knecht, J., Cifonelli, J. A. & Dorfman, A. (1967) *J. Biol. Chem.* **242**, 4652–4661

Lamport, D. T. A. (1967) *Nature (London)* **216**, 1322–1324
Lamport, D. T. A. (1969) *Biochemistry* **8**, 1155–1163
Lamport, D. T. A. & Miller, D. J. (1971) *Plant Physiol.* **48**, 454–456
Lee, Y. C. & Land, D. (1968) *J. Biol. Chem.* **243**, 677–680
Lindahl, U. & Rodén, L. (1965) *J. Biol. Chem.* **240**, 2821–2826
Lindahl, U. & Rodén, L. (1966) *J. Biol. Chem.* **241**, 2113–2119
Lindahl, U. & Rodén, L. (1972) in *The Glycoproteins* (Gottschalk, A., ed.), 2nd edn., pp. 491–517, Elsevier Publishing Co., Amsterdam
Lineback, D. R. (1968) *Carbohydr. Res.* **7**, 106–108
Marks, G. S., Marshall, R. D., Neuberger, A. & Papkoff, H. (1962) *Biochim. Biophys. Acta* **63**, 340–342
Marshall, R. D. (1974) in *Carbohydrate Chemistry, a Specialist Periodical Report*, vol. 7, pp. 294–355, The Chemical Society, London
Marshall, R. D. & Neuberger, A. (1970) *Adv. Carbohydr. Chem.* **25**, 431–478
Mathews, M. B. (1967) *Monogr. Pathol.* **7**, 309–311
Miller, D. H., Lamport, D. T. A. & Miller, M. (1972) *Science* **176**, 918–920
Morgan, P. H., Jacobs, H. G., Segrest, J. P. & Cunningham, L. W. (1970) *J. Biol. Chem.* **245**, 5042–5048
Neuberger, A., Gottschalk, A, Marshall, R. D. & Spiro, R. G. (1972) in *The Glycoproteins* (Gottschalk, A., ed.), 2nd edn., pp. 470–482, Elsevier Publishing Co., Amsterdam
Shier, W. T., Lin, Y. & DeVries, A. L. (1972) *Biochim. Biophys. Acta* **263**, 406–413
Smith, I. (1960) *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 252, Interscience Publishers, New York
Sowden, J. C. (1957) *Adv. Carbohydr. Chem.* **12**, 35–79
Stern, E. L. (1970) Ph.D. Thesis, University of Chicago
Stern, E. L., Lindahl, U. & Rodén, L. (1971) *J. Biol. Chem.* **246**, 5707–5715
Stoolmiller, A. C. & Dorfman, A. (1969) *J. Biol. Chem.* **244**, 236–246
Tanaka, K. & Pigman, W. (1965) *J. Biol. Chem.* **240**, 1487–1488