The Structure of a Glycopeptide Isolated from the Yeast Cell Wall

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1. Glycopeptides containing mannose were extracted from isolated yeast cell walls by ethylenediamine and purified by treatment with Pronase and fractionation on a Sephadex column. 2. A glycopeptide that appeared homogeneous on electrophoresis and ultracentrifugation had a molecular weight of 76 000, and contained a high-molecular-weight mannan and approx. 4% of amino acids. 3. The amino acid composition of the peptide was determined. It was rich in serine and threonine and also contained glucosamine. No cystine and methionine were detected. 4. The glycopeptide underwent a β -elimination reaction when treated with dilute alkali at low temperatures. The reaction resulted in the release of mannose, mannose disaccharides and possibly other low-molecular-weight mannose oligosaccharides. During the β -elimination reaction the dehydro derivatives of serine and threonine were formed. One of the linkages between carbohydrate and amino acids in the glycopeptide is an *O*-mannosyl bond from mannose and mannose oligosaccharides to serine and threonine. 5. After the β -elimination reaction the bulk of the mannose in the form of the large mannan component was still covalently linked to the peptide. This polysaccharide was therefore attached to the amino acids by a linkage different from the *O*-mannosyl bonds to serine and threonine that attach the low-molecular-weight sugars. 6. Mannan was prepared from the glycopeptide and from the yeast cell wall by treatment of the fractions with hot solutions of alkali. The mannan contained aspartic acid and glucosamine and some other amino acids. The aspartic acid and glucosamine were present in equimolar amounts; the aspartic acid was the only amino acid present in an amount equivalent to that of glucosamine. Thus there is the possibility of a linkage between the mannan and the peptide via glucosamine and aspartic acid. 7. Mannose 6-phosphate was shown to be part of the mannan structure. Information about the structure of the mannan and the linkage of the glucosamine was obtained by periodate oxidation studies. 8. The glucosamine present in the glycopeptide could not be released by treatment with an enzyme preparation obtained from the gut of *Helix pomatia*. This enzyme released glucosamine from the intact cell wall. Thus there are probably at least two polymers containing glucosamine in the cell wall. 9. The biosynthesis of the mannan polymer in the yeast cell wall is discussed with regard to the two types of carbohydrate-amino acid linkages found in the glycoprotein.

Since proteinaceous material was first found attached to the polysaccharides of the yeast cell wall (Northcote & Horne, 1952) several methods have been developed for the preparation of these glycoproteins. These methods include treatment of the purified isolated cell wall with sodium hydroxide (Falcone & Nickerson, 1956; Kessler & Nickerson, 1959), proteolytic enzymes (Eddy, 1958) and anhydrous ethylenediamine (Korn & Northcote, 1960).

Various types of linkage have been postulated between the macromolecules; Nickerson (1963) pointed out the possibility of ester linkages, and others suggested that at least part of the glucos-

amine present in the cell wall might constitute the bridge between the protein and the carbohydrate moieties.

The present work attempted to investigate the chemical nature of these linkages by using both chemical and enzymic methods on different glycopeptides isolated from the yeast cell wall.

MATERIALS AND METHODS

Extraction of polysaccharide complexes. These were extracted from commercial pressed baker's yeast by methods similar to those used by Korn & Northcote (1960).

Nomenclature of the preparations. The fractions obtained

Scheme 1. Extraction procedures for the preparation of glycopeptides and mannan from yeast.

by the procedure are designated A, B and C according to their solubility in ethylenediamine and water (Korn & Northcote, 1960). After purification of these fractions with Pronase digestion and fractionation on Sephadex columns,

the purified glycopeptides are called A₂, B₂ and C₂ respectively. Partial purification without Pronase treatment gave glycopeptides A, B and C respectively (Scheme 1).

Ultracentrifugation analyses. These were made in a

Spinco model E analytical ultracentrifuge. The schlieren optical system was used in all experiments. Samples were dissolved in 0.1 M-KH₂PO₄ (0.05–20%) and equilibrated with the same solution overnight.

Sedimentation analyses were performed at 59780 rev./min. in normal cells. Diffusion coefficients were calculated from the results of runs in a 12 mm. capillary-type synthetic-boundary cell at 12590 rev./min. by the method of Ehrenberg (1957). Pictures were taken every 16 min. and the areas under the curves and the distances travelled by the boundaries were obtained from enlarged photographic prints.

Partial specific volume. This was determined at 20° from density measurements in a pycnometer (volume 2.7 ml.). The partial specific volume was calculated as described by Schachman (1957).

Viscosity measurements. These determinations were performed at 21° in 0.1 M-KH₂PO₄ (0.1–2%) in a horizontal capillary viscometer (Tsuda, 1928; Oswald, 1933) (capillary radius 0.0385 cm.; length 25.50 cm.).

High-voltage paper electrophoresis. This was carried out in an apparatus similar to that described by Michl (1959) and Barrett & Northcote (1965). The buffers, coolants and times of experiments at pH 6.5, 3.5 and 2.0 were essentially as described by Ambler (1963). The electrophoretograms for polysaccharides were run in 0.05 M-sodium tetraborate, pH 9.2, on glass-fibre paper (Barrett & Northcote, 1965).

Paper chromatography. The samples were separated by descending paper chromatography on Whatman no. 1 paper in the following solvents: (a) neutral monosaccharides, ethyl acetate–pyridine–water (8:2:1, by vol.); (b) sugar phosphates, 2-methylpropan-2-ol–water (4:1, v/v) with 4 g. of picric acid added/100 ml. (Hanes & Isherwood, 1949); (c) hexosamines and cysteic acid peptides, butan-1-ol–acetic acid–water (2:1:1, by vol.).

Chromatographic sprays. Neutral sugars were detected and estimated by the aniline hydrogen phthalate method of Wilson (1959). Polysaccharide zones were coloured on glass-fibre paper with a reagent consisting of 96% ethanol (500 ml.) to which 25 ml. of conc. H₂SO₄ was added with 5 g. of α -naphthol (Barrett & Northcote, 1965). Sugar phosphates were run on Whatman no. 54 paper, and were located with the acid molybdate reagent described by Burrows, Grylls & Harrison (1952). Polyols were detected with the alkaline AgNO₃ reagent described by Trevelyan, Procter & Harrison (1950). Hexosamine spots were coloured with the reagent described by Partridge & Westall (1948). Protein zones were identified with the methanolic solution of Naphthalene Black described by Grassmann & Hannig (1952). Amino acid spots were coloured by spraying with a solution of 0.2% ninhydrin in acetone containing 2–3% collidine and 0.1% acetic acid, and heating at 60° for 5–10 min.

Carbohydrate analyses. These were made by a modification of the orcinol–H₂SO₄ method of Winzler (1955). Total reducing power was determined by the alkaline copper–arsenomolybdate technique of Nelson (1944).

Amino acids and amino sugar analyses. The acid hydrolyses of the samples were performed at lower concentrations than 1% (w/v). The samples in 3–6 N-HCl were frozen, sealed and then melted and heated at 100° for various times. Free amino acids in hydrolysates were estimated by the method of Cocking & Yemm (1954). Quantitative determinations were done by the chromatographic procedure of Moore, Spackman & Stein (1958) with a Technicon Auto-

Analyzer. Hexosamines were determined by the Auto-Analyzer, after hydrolysis of the polymers in 3 N-HCl for 14–16 hr. at 100°. Cysteine and methionine were determined by hydrolysis of the polymers after oxidation by performic acid. Amide nitrogen determinations were carried out on the AutoAnalyzer after hydrolysis of the polymers in 2 N-HCl at 100° for 3 hr. (Marks, Marshall & Neuberger, 1963).

Phosphorus determination. This was carried out by the method of Fiske & Subbarow (1925) as described by Umbreit, Burris & Stauffer (1957).

Measurement of periodate uptake. Samples (100 mg.) were dissolved in 0.015 M-NaIO₄ soln. (100% excess) that had been cooled previously to 4°, and kept at this temperature in the dark throughout the oxidation. A blank experiment was run at the same time under the same conditions. Samples (10 μ l.) were withdrawn and diluted 250-fold; the extinctions of the resulting solutions were measured at 223 m μ and compared with those of the original solution of periodate (Dixon & Lipkin, 1954; Aspinall & Ferrier, 1957).

Measurement of formic acid released. Samples (3 ml.) of the periodate reaction solution were treated with ethylene glycol (1 ml.) and made up to 10 ml. with water, and the mixture was shaken; after 20–30 min. at room temperature they were titrated with 0.01 N-NaOH with Methyl Red as indicator.

Measurement of mannose after periodate oxidation. Samples (1 ml.) of the periodate reaction solution treated with ethylene glycol were hydrolysed, after dialysis, by HNO₃ (3%, v/v) + urea (0.05%) (Barrett & Northcote, 1965), and mannose was estimated by the chromatographic method of Wilson (1959).

Reduction of the oxoglycopeptide A₂. After treatment with ethylene glycol the oxidized glycopeptide A₂ (94.6 mg.) was reduced with NaBH₄ (0.4 g.) at 4° for approx. 18 hr., and the residual polyalcohol was passed through a Sephadex G-25 column (190 cm. $\times$ 2 cm.), from which it was excluded, and hydrolysed with 0.25 N-HCl (150 ml.) at room temperature for 4 hr. The hydrolysate was neutralized by extracting the excess of acid with *N*-methyl-*NN*-di-*n*-octylamine in chloroform (10%, v/v) (Smith & Page, 1948); the aqueous layers plus the material at the interphase were collected and dried.

The hydrolyzed oxoglycopeptide A₂ was fractionated by elution with 0.1 N-acetic acid through a Sephadex G-10 column (55 cm. $\times$ 2 cm.). The eluted material was further analysed by paper and ion-exchange chromatography.

Partial hydrolysis of glycopeptide A₂. This was performed in 0.1 N-H₂SO₄ (10 mg. of peptide/ml.) for various times at 100° in sealed ampoules, and the hydrolysates were neutralized with BaCO₃ or Amberlite resin IRA-400 (CO₃²⁻ form).

Alkali treatment of glycopeptide A₂ and subsequent addition reactions. Glycopeptide A₂ (10 mg.) was treated with 0.1–2.0 N-NaOH (2.5 ml.) at 4° or approx. 21° for various times. The material was neutralized and treated with either KBH₄ (0.1 g.) at 4° for 12–16 hr. or 0.1 M-NaHSO₃ in 0.05 M-tris buffer, pH 9.0, for 24 hr. at 60°. The reduction with KBH₄ was terminated by acidifying the solution with acetic acid to pH 5.0. Samples, after being desalted by dialysis, were analysed for their amino acid constituents. After the treatment with NaHSO₃, and by the use of cysteic acid as a marker, two sulphonic acid derivatives were identified as

two separate but overlapping peaks at the beginning of the trace obtained from the amino acid analyser. The acids were formed from α -aminoacrylic acid and α -aminocrotonic acid.

Addition reactions were also carried out with $\text{Na}_2^{35}\text{SO}_3$ (27.1 mc/m-mole, 1.5 mg.; The Radiochemical Centre, Amersham, Bucks.) in 0.1 M- NaHSO_3 at pH 9.0 for 24 hr. at 60°. Radioactive material eluted from a Sephadex G-100 column (50 cm. $\times$ 2 cm.) was counted in a gas-flow Geiger counter and that on electrophoretograms was counted on the paper strips by liquid-scintillation spectrometry. Both methods were exactly as described by Stoddart & Northcote (1967).

Extraction of glycopeptides B_2 and C_2 . Fractions B and C (500 mg.) obtained from the ethylenediamine treatment of the cell wall were washed several times with water and partially hydrolysed with HCl (100 ml.) at pH 2.5–2.7 at 100° for 75 min. The solutions were evaporated to dryness in a rotary evaporator and digested twice with Pronase (Calbiochem, Los Angeles, Calif., U.S.A.) (1:50, w/w) in the presence of 0.5% NH_4HCO_3 , pH 8.0, at 37° for 24 hr. under toluene. After incubation, the precipitated material was removed by centrifugation and the solution was fractionated on a Sephadex G-50 column (40 cm. $\times$ 3 cm.) eluted with 0.1 N-acetic acid. The unretarded materials from the gel filtration were either glycoprotein B_2 or glycoprotein C_2 .

Purification of fraction A (Scheme 1). Fraction A (46.5 mg.) was purified by treatment with Pronase (2%, w/w) in 0.5% NH_4HCO_3 , pH 8.0, at 37° for 24 hr. under toluene and fractionation on a Sephadex G-50 column (50 cm. $\times$ 2 cm.). The material was eluted as two fractions with 0.1 N-acetic acid. The first, which was material excluded from the gel bed, contained all the carbohydrate and some protein material (glycopeptide A_2), whereas the retarded fraction showed only a strong extinction at 280 m μ and no colour with the orcinol- H_2SO_4 reagent (Fig. 1). Paper electrophoresis of the first fraction at pH 2.0, 6.5 and 9.2 gave a single, rather diffuse, spot that was coloured with the α -naphthol- H_2SO_4 reagent or with Naphthalene Black. Chromatography on a Sephadex G-200 column eluted with 0.1 N-acetic acid also showed a single broad retarded fraction. Fraction A, after purification by Sephadex G-50 filtration without Pronase treatment, was called glycopeptide A.

RESULTS

Sedimentation and diffusion analysis. A single boundary was observed when glycopeptide A_2 was sedimented at 20° in 0.1 M-potassium dihydrogen phosphate, pH 4.7, at 59780 rev./min. (Fig. 2). This boundary was shown to be asymmetrical when the result was plotted on a graph with $\log dn/dx$ and $(x_t - x_n)^2$ as co-ordinates (Hall & Ogston, 1956), where x_t is the distance from a position in the boundary measured to the centre of rotation and x_n is the distance from the maximum height of the schlieren diagram.

The dependence of the sedimentation coefficient of glycopeptide A_2 on concentration is shown in Fig. 3. The result indicated that the molecule was asymmetric. A value for S_{20} of 3.88×10^{-13} cm.g.⁻¹

Fig. 1. Purification of fraction A, after treatment with Pronase, on a column of Sephadex G-50 eluted with 0.1 N-acetic acid. Fractions (10 ml.) were collected. ▲, E_{280} ; ●, E_{505} of solutions treated with orcinol- H_2SO_4 reagent for carbohydrates.

sec.⁻¹ was obtained by extrapolation to zero concentration.

Diffusion measurements were performed at low angular speed at different concentrations of glycopeptide. The measured D_{20} of 3.6×10^{-7} cm.²sec.⁻¹ was not significantly different from that obtained by Korn & Northcote (1960) with the original fraction A measured in a Tiselius electrophoresis apparatus.

Partial specific volume. The value found for $\bar{v}$ was 0.66 ml./g.

Determination of molecular weight. The weight-average molecular weight of glycopeptide A_2 was calculated by means of the Svedberg equation (Svedberg, 1925). The number-average molecular weight was determined by estimating the *N*-terminal amino acid as the DNP derivative (Ghuysen, Tipper & Strominger, 1966), or by determining the glucosamine content in the molecule (Table 1).

Viscosity measurements. The viscosity values of glycopeptide A_2 at concentrations of 1% and upward were dependent on the rate of shear, probably because it is a polyelectrolyte; at lower concentrations down to 0.1% the viscosity was Newtonian. The intrinsic viscosity was found to be 0.32 dl./g., and the corrected viscosity increment calculated from the partial specific volume and the intrinsic viscosity was 32.5.

The axial ratio obtained from the viscosity increment by using Simha's equation (Simha, 1940; Mehl, Oncley & Simha, 1940), and assuming the molecule to be an unhydrated ellipsoid of revolution, was 20 for a prolate ellipsoid and 45 for an oblate ellipsoid.

Frictional coefficient. This value was calculated from S_{20} , D_{20} and $\bar{v}$ by the equation of Oncley (1941). The value of 2.19, obtained by assuming that the glycopeptide was a rigid unhydrated

Fig. 2. Boundary of glycopeptide A_2 (10 mg./ml.) sedimented at 20° in 0.1 M- KH_2PO_4 , pH 4.7, at 59780 rev./min. Movement was from right to left during a period of 144 min. Pictures were taken every 16 min.

Fig. 3. Dependence of the sedimentation coefficient (S_{20}) of glycopeptide A_2 on concentration. For experimental details see the text.

Table 1. Summary of physical and physicochemical characteristics of glycopeptide A_2 and mannan

Glycopeptide A_2	
S_{20} (cm.g. ⁻¹ sec. ⁻¹)	3.88×10^{-13}
D_{20} (cm. ² sec. ⁻¹)	3.60×10^{-7}
Partial sp. volume ($\bar{v}$)	0.66
Mol.wt. (weight-average) by sedimentation diffusion	
Glycopeptide A_2	7.6×10^4
Mannan	5.9×10^4 (Korn & Northcote, 1960)
Mol.wt. (number-average)	
Glucosamine content of glycopeptide A_2	8.0×10^4
DNP-mannan	7.3×10^4
Axial ratio of glycopeptide A_2	20 (prolate ellipsoid)

ellipsoid of revolution, corresponded to axial ratios of 25 for a prolate ellipsoid and 40 for an oblate ellipsoid (Svedberg & Pedersen, 1940).

By the application of the equations of Scheraga & Mandelkern (1953) to the physicochemical data for glycopeptide A_2 a value for β of 2.62×10^6 was obtained (β is defined as the parameter that is a function of the axial ratio of an ellipsoid of revolution). This value of β corresponded to a prolate ellipsoid with an axial ratio of 20 (Table 1).

Amino acid analysis. Acid hydrolysates were obtained from desalted freeze-dried samples of glycopeptide A_2 and two runs in the AutoAnalyzer were made on each hydrolysate. The recovery of the amino acids after different times of hydrolysis is shown in Table 2. The conditions of acid hydro-

lysis finally used were 6N-hydrochloric acid at 100° for 16 hr. The absolute amount in moles of each amino acid for 1 mole of glycopeptide A_2 was calculated (Table 3). One of the products of the acid hydrolysis of the glycopeptide was an acid that gave a deep-violet colour with ninhydrin and that appeared before cysteic acid and aspartic acid from the ion-exchange column of the amino acid analyser. The ratio of the extinctions of the coloured ninhydrin solutions of this acid at $440\text{m}\mu$ and $570\text{m}\mu$ was 1:3. The acid was therefore identified as laevulinic acid (Zacharius & Tolley, 1962). The protein content of the macromolecule was calculated as 3.6–3.9% from the amino acid analysis. Direct analysis by the method of Lowry, Rosebrough, Farr & Randall (1951) indicated that

Table 2. *Amino acid recoveries after hydrolysis of glycopeptide A₂ with 6N-hydrochloric acid at 100°*

The values given are μ moles recovered from 4.900 mg. of glycopeptide A₂. Neither cystine (cysteic acid) nor methionine (methionine sulphone) was detected before or after oxidation with performic acid.

Amino acid residue	Time of hydrolysis (hr.) ...	Amino acid recoveries					Extrapolated to zero time of hydrolysis or maximum value
		4	16	24	40	110	
Asp		133	134	135	139	129	134
Thr		390	380	306	239	118	430
Ser		254	201	150	101	37	250
Glu		121	120	119	123	118	121
Pro		122	133	115	119	122	122
Gly		80	85	90	93	116	116
Ala		190	185	187	178	183	190
Val		93	93	101	87	91	93
Leu		30		Very little change			30
Ile		31		Very little change			31
Tyr		14		Very little change			14
Phe		14		Very little change			14
Lys		31		Very little change			31
His		Traces		Traces	Traces	Traces	Traces
Arg		Traces		Traces	Traces	Traces	Traces
Laevulinic acid		Present					
Glucosamine		After hydrolysis for 16 hr. with 3N-HCl					61

Table 3. *Amino acid composition of glycoprotein A₂ and glycoprotein A*

Amino acid residue	Amount of each amino acid (μ g.) in 4.9 mg. of glycopeptide A ₂	Protein calc. in 4.9 mg. of glycopeptide A ₂ (%)	Amino acid (μ moles/ μ mole of glucosamine) (in 4.9 mg. of glycopeptide A ₂)	Nearest integral no. of moles of principal amino acids in 1 mole of the glycopeptide A ₂	Calc. wt. (μ g.) of each amino acid in 1 μ mole of glycopeptide A ₂	Protein calc. in 1 μ mole of glycopeptide A ₂ (%)	Nearest integral no. of moles of principal amino acids in 1 mole of glycopeptide A
Asp	17.8	3.7	2.19	2.0	266	3.9	3.0
Thr	51.2		7.05	7.0	833		7.0
Ser	26.2		4.09	4.0	420		4.0
Glu	17.8		1.98	2.0	294		3.0
Pro	14.0		2.00	2.0	230		3.0
Gly	8.7		1.90	2.0	150		3.0
Ala	16.0		2.95	3.0	267		4.0
Val	10.9		1.52	2.0	230		3.0
Ile	3.9		0.42	0.5	65		1.5
Leu	4.0		0.58	0.5	66		1.5
Tyr	2.5		0.23	0.2	45		0.5
Phe	2.3		0.23	0.2	41		0.5
Lys	4.5		0.58	0.5	73		1.5
His	Traces			Traces			Traces
Arg	Traces			Traces			Traces
Glucosamine				1.0			1.0

it contained 7% of protein. The latter result is probably too high, since it is known that although any peptide will yield some colour with the Folin-Ciocateu reagent some amino acid sequences are more chromogenic than others (Bailey, 1967).

Stability of glycopeptide A₂. Glycopeptide A₂ was easily degraded by both alkali and acid. Hydrolysis at low acid concentration was brought about by either methanolic 1% hydrogen chloride or 0.1N-sulphuric acid. When glycopeptide A₂ was refluxed

Fig. 4. Hydrolysis of glycopeptide A₂ with methanolic 1% (w/v) HCl during a 7 hr. period. The curves show the percentages of the total carbohydrate (●) and of the total protein (▲) of the glycopeptide that were progressively released into the solution.

Fig. 5. Production of free amino acids and sugars from the glycopeptide by hydrolysis with 0.1 N-H₂SO₄ at 100°. The percentage of amino acids released by hydrolysis (▲) was determined by the method of Cocking & Yemm (1954) and free sugars (●) by the ratio of total carbohydrate (measured in moles of mannose) to reducing power released (moles of mannose equivalent) during the hydrolysis.

with the former reagent at 100° and samples were withdrawn at different times, a progressive release of protein (determined by the method of Lowry *et al.* 1951) and carbohydrate (determined by the orcinol-sulphuric acid method) was shown to occur (Fig. 4). With 0.1 N-sulphuric acid the results were different (Fig. 5). The hydrolysis of amino acids (determined by the method of Cocking & Yemm, 1954) occurred after an initial period during which only carbohydrate was released (as shown by the ratio of total carbohydrate to reducing power released) from the glycopeptide. After a 3 hr. hydrolysis with sulphuric acid the neutralized solution was fractionated by elution with 0.1 N-acetic acid from a column of Sephadex G-15. The first fractions obtained from the column contained

material composed of all the amino acids and a little carbohydrate, whereas the bulk of the carbohydrate material was present in the more retarded fractions. Glycopeptides that had different molecular weights and were eluted by different volumes of 0.1 N-acetic acid from the Sephadex G-15 column, but almost a constant amino acid composition, were obtained by varying the hydrolysis times.

Isolation of sugar phosphates. Glycopeptide A₂ (100 mg.) was refluxed in 2 N-hydrochloric acid (100 ml.) at 100° for 75 min., and after neutralization the residual material was treated as indicated in Scheme 2. The products retained on the De-Acidite column were further fractionated by high-voltage paper electrophoresis at pH 3.5, and the different spots detected on a control strip of paper were

Scheme 2. Isolation of sugar phosphates from glycopeptide A₂.

eluted with 0.1N-acetic acid. The spots subsequently eluted were those that coloured both with the aniline phthalate and with the molybdate reagent. The fastest-moving sugar phosphate in the electrophoresis was eluted from the electrophoretogram and run on a paper chromatogram; it was a single component having the same position as authentic mannose 6-phosphate (R_{F1} 0.43).

The position of the phosphate residue was unambiguously established by periodate oxidation of the material eluted from the electrophoretogram. After the reaction the excess of periodate and iodate were removed as the relatively insoluble barium salts and the reaction mixture was run on Whatman no. 54 paper with solvent (b). Only glycollaldehyde phosphate (R_{F1} 0.63) and traces of mannose 6-phosphate were detected.

Alkali treatment of glycopeptide A₂. When glycopeptide A₂ was treated with sodium hydroxide at various concentrations and temperatures and then hydrolysed to liberate amino acids, a progressive loss of threonine and serine was detected. After 168 hr. in 0.1N-sodium hydroxide at 4°, 64% and 42% respectively of those amino acids were lost (Table 4). These results were explicable if it was assumed that β -elimination of the alkoxide moiety of glycopeptide A₂ occurred to give a dehydro derivative. The unmasking of reducing power during the process was determined indirectly by using the alkaline Somogyi-Nelson reagent, which would bring about β -elimination, and the increase

in reducing power was proportional to the incubation time of the glycopeptide with the reagent.

The formation of the dehydro derivatives of the amino acids in the glycopeptide during the alkali treatment was shown by measuring the extinction at 241m μ of the reaction mixture. This was recorded automatically in a spectrophotometer (Beckman DK 2A) and a steady increase in the extinction took place (Fig. 6), which was related to the formation of α -aminoacrylic acid.

Similar results were recorded from the alkali treatment of both glycopeptide B₂ and glycopeptide C₂. Glycopeptide B₂ contained 4% of protein and glycopeptide C₂ contained 3% (both values obtained by the method of Lowry *et al.* 1951).

Addition reactions of the alkali-treated glycopeptide A₂. When the residual product of the sodium hydroxide-treated glycopeptide was reduced with potassium borohydride and hydrolysed, an increase in the alanine concentration and the appearance of a new amino acid (α -aminobutyric acid) were observed, thus confirming the production of dehydro amino acids from serine and threonine during the β -elimination.

Reaction of the dehydro amino acids with sodium sulphite should yield the corresponding sulphonic acid derivatives by a nucleophilic addition reaction (Harbon, Herman, Rossignol, Jollès & Clauser, 1964).

After treatment with sodium sulphite for 24 hr. at 60°, 100% of the lost threonine and serine was

Table 4. *Changes in the concentration of certain amino acids present in 20 mg. of glycopeptide A₂ after treatment of the glycopeptide by 0.1 N-sodium hydroxide (β-elimination) at 4°*

Amino acid residue	Time of treatment (hr.) ...	Amino acid content (μmoles)			
		0	24	144	168
Aap		510	530	540	525
Thr		950	680 (−28%)	375 (−60%)	342 (−64%)
Ser		420	328 (−21%)	240 (−42%)	240 (−42%)
Glu		530	525	470	550
Pro		480	469	425	480
Gly		370	370	368	398
Ala		730	660	708	700
Val		430	411	400	435
Ile		160	169	160	160
Leu		Trace	Trace	Trace	Trace
Laevulic acid		Present	Present	Present	Present

Fig. 6. Formation of the dehydro derivatives of serine and threonine during the treatment of glycopeptide A₂ with 0.5 N-NaOH at 20° (β-elimination). ●, Glycopeptide in the presence of alkali; ▲, glycopeptide in the absence of alkali.

recovered as the corresponding sulphonic acid derivatives. When the addition reaction was performed with Na₂³⁵SO₃ on a neutral dialysed solution of the glycopeptide after β-elimination and the resulting mixture was desalted by passing it through a Sephadex G-100 column and eluting it with 0.1 N-acetic acid, all the radioactivity was found, unexpectedly, still associated with the fraction containing the carbohydrate; similar results were obtained when the radioactive sulphonic acid derivatives were run in high-voltage paper electrophoresis at pH 2.0, 6.5 and 9.2. The hydrolysis products of this radioactive glycopeptide (the hydrolysis was carried out with 12 N-hydrochloric acid at 37° for 3 days) were fractionated in a similar way to that described for sugar phosphates. The bulk of the radioactivity (80% of the total) was found to be retained on the De-Acidite column. On electrophoresis at pH 2.0 this material remained at the origin, whereas at pH 3.5 several radioactive spots were detected moving towards the anode.

Extent of the β-elimination reaction of glycopeptide A₂. β-Elimination of a solution of the glycopeptide

Table 5. *Carbohydrate material released by 0.1 N-sodium hydroxide at 21° for 24 hr. (β-elimination) from glycopeptide A*

Material	Diffusible sugars	
	Before β-elimination	After β-elimination
Glycopeptide A	None	Mannose and di- and probably other oligosaccharide; trace of glucose
Glycopeptide A previously treated with methanol-HCl	None	Mannose and traces of oligosaccharide; trace of glucose
Mannan	None	None
Mannose	—	Mannose and trace of glucose

was carried out in the normal manner, and the solution was neutralized and dialysed. The solution inside the dialysis sac was divided into two equal volumes, one of which was made 0.1 N and the other 0.25 N with respect to hydrochloric acid. The acidic solutions were heated at 95° for a period of 4 hr. During this time at half-hour intervals samples were taken from each solution and brought to 0.1 N with respect to sodium hydroxide and left at room temperature for 24 hr. There was no further increase in the extinction of the solution at 241 mμ. It was therefore assumed that the original β-elimination reaction had gone to completion.

Products released by alkali treatment of glycopeptide A. Solutions of the glycopeptide and of the glycopeptide that had been modified by treatment with methanolic 1% hydrogen chloride for 2 hr. at 100° (this latter treatment removed 20% of the original carbohydrate and 1.8% of the protein; Fig. 4)

Fig. 7. Stability of the *O*-glycosyl-hydroxy amino acid linkages in 0.5N-HCl (●) and 1.0N-HCl (▲) at 95°. The ordinate shows the percentage of β -elimination possible on the material recovered after hydrolysis.

Fig. 8. Behaviour of glycopeptide A_2 on treatment with cold 2N-NaOH (β -elimination) followed by hot 2N-NaOH, during which time the peptide was progressively degraded.

were dialysed against 20 vol. of water for 18 hr. at 4° and the solution inside the dialysis sac was made to a final concentration of 0.1N-sodium hydroxide. β -Elimination was carried out at 21° for 18 hr. During the reaction the change in extinction at 241m μ in the glycopeptide A solution was from 0.340 to 1.200 and for the solution of the modified material from 0.230 to 1.350. The alkaline solutions were neutralized with acetic acid and then dialysed against 20 vol. of water for 18 hr. at 4°. The diffusible fractions were desalted on a Zeo-Karb SRC 225 (H⁺ form) column, and samples were run on chromatograms to detect monosaccharides. A solution of mannan (0.1%) was treated in a similar manner to glycopeptide A. A solution of mannanose (0.1%) in 0.1N-sodium hydroxide was kept at 21°

Table 6. Amino acid composition of yeast mannan

The amino acid composition is given as μ mole of amino acid/ μ mole of mannan of mol.wt. 73000.

Amino acid residue	Content of amino acid (μ mole/ μ mole of glycopeptide)
Asp	1.00
Thr	0.14
Ser	0.15
Glu	0.45
Pro	0.38
Gly	0.84
Ala	0.76
Val	0.26
Ile	0.13
Leu	0.20
Tyr	Traces
Phe	Traces
Lys	0.18
Glucosamine	1.00

for 18 hr. and then dialysed and treated in the same manner as the other solutions. The results are shown in Table 5.

Stability of the *O*-glycosyl-hydroxy amino acid linkage. The stability of this link in various acid concentrations was tested by the ability of the residual material after acid hydrolysis to undergo β -elimination. After a fixed time of hydrolysis in the acid at 95° a sample was quickly evaporated in a rotary evaporator at room temperature and the residue subjected to β -elimination, which was measured spectrophotometrically. The results showed that the stability of the *O*-glycosyl bond was not only a function of the acid concentration (Fig. 7) but also depended on the anions present, since the hydrolysis occurred more readily with sulphuric acid than with hydrochloric acid.

Preparation of mannan from glycopeptide A_2 . Glycopeptide A_2 , if treated with 2N-sodium hydroxide at 21°, underwent β -elimination with a corresponding increment in the extinction of the solution at 241m μ . If the temperature of the alkaline solution was subsequently raised to 98° the extinction at 241m μ progressively decreased over a period of 2 hr. as the amino acids were degraded (Fig. 8). The solution after the hot alkaline digestion gave a precipitate with 75% ethanol. This precipitate on acid hydrolysis contained mannose, glucosamine and some amino acids that were identical with those found in yeast mannan prepared from intact yeast (Korn & Northcote, 1960) (Scheme 1).

Mannan (0.5g.) obtained by the method of Haworth, Heath & Peat (1941) (Scheme 1) was analysed for amino acids (Table 6).

Periodate oxidation of glycopeptide A_2 . The

Fig. 9. Uptake of periodate by glycopeptide A₂ during oxidation by 0.015 M-NaIO₄ at 4° in the dark.

Fig. 10. Production of formic acid during the oxidation of glycopeptide A₂ (4.25 mg) with 0.015 M-NaIO₄ at 4° in the dark.

amount of periodate reduced during the oxidation is shown in Fig. 9, and Fig. 10 shows the production of formic acid during the oxidation period. An initial rapid production of acid was followed by a second phase in which the production went on at a much lower rate.

The oxidation was continued for 12 days, and after reduction with borohydride the residual material was hydrolysed with 0.25 N-hydrochloric acid and fractionated on a Sephadex G-10 column eluted with 0.1 N-acetic acid. Two fractions were collected. The retarded material from the gel was shown to consist of glycerol and traces of mannose. The unretarded fraction was further fractionated on a Zeo-Karb SRC 225 (H⁺ form) ion-exchange column at pH 5.0. The material eluted from the resin with 1 N-hydrochloric acid was hydrolysed and was shown to contain mannose, glycerol and amino acids.

The oxoglycopeptide A₂ when hydrolysed with 3 N-hydrochloric acid released amino sugar, and this was estimated on the AutoAnalyzer; 90–95% of the original glucosamine was recovered. It had

hence not been destroyed during the oxidation of the glycopeptide.

Acetylglucosamine content of the mannan. When mannan (100 mg.) was hydrolysed in 0.5 N-hydrochloric acid at 100° for 5 hr. *N*-acetylglucosamine could be detected on paper chromatograms (Partridge & Westall, 1948; Johansen, Marshall & Neuberger, 1960). The residual amino sugars were recovered as glucosamine. The digestive juice of the *Helix pomatia* (Myers & Northcote, 1959) did not release *N*-acetylglucosamine when it was incubated with glycoprotein A₂ at several pH values (pH 3.3, 5.0, 7.0 and 8.2) (Myers & Northcote, 1958).

DISCUSSION

The water-soluble glycopeptide extracted from the cell wall of yeast by the method of Korn & Northcote (1960) was purified by proteolytic digestion and gel filtration. Before purification the fraction showed the presence of at least two components in the sedimentation diagram, whereas afterwards only one component was apparent. The material finally obtained was slightly polydisperse. It had a molecular weight of about 76 000, it was asymmetrical and resembled a prolate ellipsoid with an axial ratio of 20.

It has been reported that hydroxyl- and thiol-containing amino acids are destroyed by hot solutions of hydrochloric acid. We found that, in 6 N-hydrochloric acid at 100° after 24 hr. and 112 hr., 40% of serine and 29% of threonine, and 85% of serine and 72% of threonine, are lost respectively. These values are higher than those previously reported by Rees (1946), but his data referred to the free amino acids and not to the amino acids in a peptide in the presence of a high concentration of carbohydrate, which undoubtedly leads to humin formation. During the initial period of the hydrolysis the rate of decomposition of both the hydroxy amino acids was progressive and linear with time. One of the products formed from the carbohydrates during the hydrolysis was laevulinic acid. This acid was found and characterized in the eluates from the amino acid analysis and it was further identified by the ratio of its extinction, at 440 mμ and 570 mμ. The presence of laevulinic acid accounts for the unidentified spot found by previous workers in the chromatograms of hydrolysates of glycopeptide fractions obtained from yeast (Eddy, 1958).

Glycopeptide A₂ did not contain any sulphur-containing amino acids; neither cysteic acid nor methionine sulphone was detected in the hydrolysates of material oxidized by performic acid.

The amino acid analysis of the glycopeptide showed that serine and threonine accounted for over 40% of the total amino acid present in the

peptide and indicated that the carbohydrate might be linked to the peptide by an *O*-glycosyl bond to these hydroxy amino acids (Anderson, Hoffman & Meyer, 1963). After treatment with sodium hydroxide, glycopeptide A₂ lost 64% of its threonine and 45% of its serine. This would be expected if β -carbonyl elimination of an alkoxide bond occurred, and the alkali-treated glycopeptide was shown to contain α -aminoacrylic acid and α -aminocrotonic acid by spectrophotometric analysis (Price & Greenstein, 1948; Riley, Turnbull & Wilson, 1957; Carubelli, Bhavanandan & Gottschalk, 1965) and by their destruction with strong acids and alkalis (Bergman & Grafe, 1930). If the peptide after treatment with alkali was reduced with sodium borohydride and the peptide hydrolysed and analysed, an increase in the amount of alanine and α -aminobutyric acid occurred that corresponded to most of the loss of serine and threonine respectively (Tanaka, Bertolini & Pigman, 1964; Tanaka & Pigman, 1965). After treatment with alkali the sulphonic acid derivatives of serine and threonine could be prepared (Strumeyer, White & Koshland, 1963) in a quantitative yield from the dehydro derivatives. The expected yield was calculated from the loss of serine and threonine during the β -elimination reaction.

Therefore one type of linkage between the carbohydrate and peptide part of the molecules is an *O*-glycosyl bond between the sugar and the hydroxyl groups of serine and threonine. In spite of the evidence for such a linkage between the carbohydrate and peptide portions of the molecule it proved impossible after the completion of the β -elimination reaction to separate the high-molecular-weight polysaccharide from the peptide. If the peptide was labelled with radioactive sulphur after β -elimination by addition of sulphite to double bonds the radioactive peptide still remained with the carbohydrate fraction when the solution was passed through a Sephadex column or run on an electrophoretogram.

It was therefore possible that there were two types of linkage between the carbohydrate and protein parts of the molecules: bonds that involved the high-molecular-weight mannan component and the peptide, and others that, though they did not connect with the large mannan component, were nevertheless bonds between mannose and amino acids and constituted the *O*-glycosyl bonds with serine and threonine. These latter bonds were shown to attach low-molecular-weight oligosaccharides of mannose and mannose monosaccharides to the peptides, since these sugars were recovered from the dialysed glycopeptide after the β -elimination reaction.

The glycopeptide material that was obtained by hot alkaline treatment of yeast cell walls was

identical with yeast mannan, and contained glucosamine and amino acids. The amounts of glucosamine and aspartic acid in these preparations were equimolar and aspartic acid was the only amino acid present in an amount equivalent to the total concentration of the glucosamine. It was possible therefore that a linkage between these two monomers represented the main bridge between the high-molecular-weight mannan and the amino acids of the peptide. This kind of linkage has been demonstrated in a large number of glycoproteins (Fletcher, Marks, Marshall & Neuberger, 1963; Marks *et al.* 1963; Gottschalk, 1966). Though Eddy (1958) has shown that glucosamine can be liberated from the intact yeast cell wall by the digestive juice of *Helix pomatia*, this enzyme system did not remove it from the glycopeptide. It seems likely therefore that glucosamine occurs in more than one polymer in the cell wall, possibly in chitin (Bacon, Davidson, Jones & Taylor, 1966) as well as in the glycopeptides (Korn & Northcote, 1960).

The mannose phosphates, isolated from glycopeptide A₂ in which the phosphate is esterified on the primary hydroxyl groups of the mannose, may represent one part of the general structural linkage of this polysaccharide.

When the whole glycopeptide was oxidized by periodate, it was found that 1 mole of periodate was used/mole of anhydromannose present and a quantitative determination showed that 75–85% of the original mannose had been oxidized. During the oxidation, formic acid was released; rapid formation of this acid corresponded to the oxidation of non-reducing terminal mannosyl groups, and the slower release over a subsequent period of 12 days was probably because of the oxidation of amino acid residues. Similar slow progressive oxidations have been reported for glycopeptides of γ -globulin (Rothfus & Smith, 1962; Clamp & Hough, 1965) and for ovalbumin (Fletcher *et al.* 1963).

After oxidation of the mannan by periodate and subsequent reduction and hydrolysis of the oxopolysaccharide, glycerol was obtained but no erythritol, and thus no monomers linked only by (1 $\rightarrow$ 4)-bonds are present in the polysaccharide. This has been indicated by methylation studies (Cifonelli & Smith, 1955; Haworth *et al.* 1941; Northcote, 1954). Glucosamine was recovered quantitatively from the oxopolysaccharide and the amino sugars must be substituted in position 3, or, if acetylglucosamine is present, either in position 3 or 4 or both. Undegraded mannose was also recovered from the hydrolysed oxomannan. It occurred in the residual glycopeptides obtained from the hydrolysis that were isolated by ion-exchange chromatography. It was probable therefore that near the region of the linkage of the

mannan to the protein the polysaccharide was highly branched.

Our results indicate that the glycopeptide carrying the mannan component of the yeast cell wall has two types of peptide-carbohydrate linkages. One of these connects numerous small monosaccharide or oligosaccharide units to the peptide by glycosyl bonds on to the hydroxyl groups of serine and threonine, and the other connects the high-molecular-weight highly branched mannan to the peptide, and is probably a nitrogen glycosyl bond between *N*-acetylglucosamine and aspartamide [*N*-(β -aspartyl)- β -D-(*N*-acetyl)glucosaminide]. It is probable that all three glycopeptide fractions (fractions A, B and C) and yeast mannan that can be isolated from the yeast cell wall represent various degradative stages in the complex glycoprotein structure of the wall (Korn & Northcote, 1960).

Recently other glycoproteins have been shown to contain two types of interconnecting linkages between the protein and carbohydrate moieties (Dawson & Clamp, 1967; Spiro, 1967). In our preparations the two types of linkages carry mannose units at different degrees of polymerization, and it might be that these represent stages in a biosynthetic pathway for the formation of the large mannan molecule of the cell wall. The first stage could be thought of as a transfer of mannose monomers from the donor nucleotide (GDP-mannose) (Algranati, Behrens, Carminatti & Cabib, 1966; Behrens & Cabib, 1968) to serine or threonine in the peptide to give low-molecular-weight oligosaccharides with specific linkages, attached to the protein. A second stage would be the subsequent transfer of these units by a transglycosylation process to give the high-molecular-weight material attached to glucosamine and the protein. The different enzymes necessarily involved in these distinct synthetic steps could give the various linkages, i.e. (1 $\rightarrow$ 6), (1 $\rightarrow$ 2) and (1 $\rightarrow$ 3), present in the large mannan molecule.

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