The Protein Polysaccharides from the Nucleus Pulposus of Whale Intervertebral Disks. Effect of Disulfide Reducing Reagents*

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ABSTRACT: The amino acid and carbohydrate composition of the protein polysaccharides of the nucleus pulposus of the intervertebral disks of whale is reported. With the aid of a centrifugation method and a sucrose density gradient procedure, two major types of protein polysaccharides were separated. The first type, containing cystine, underwent certain changes upon reduction; the other type, containing no cystine, was not affected by reducing reagents. Viscosity, sedimentation, and diffusion measurements were used for describing the physical changes observed. From diffusion and sedimentation determinations, the molecular weight, M_w, of a protein polysaccharide, estimated at 8.5×10^6, changed to 6.6×10^5 after reduction with dithiothreitol. The reactive bonds were shown to be disulfide groups.

There is now much evidence, reviewed by Fitton Jackson (1964), to support the view that protein polysaccharides share a protein basal structure, or core, which is markedly different in its amino acid composition from that of collagen, on which side chains of an acid polysaccharide are attached. Results of physical measurements have shown that protein polysaccharides have molecular weights in the range of $4–50 \times 10^6$ (Mathews and Lozaityte, 1958). It has also been suggested that smaller size protein polysaccharides units, each with a molecular weight of 750,000, are part of the macromolecular aggregate (Partridge *et al.*, 1961). A fundamental structural unit for the protein core has been suggested to be a polypeptide with a molecular weight of 10,600 (Marler and Davidson, 1965).

Procedures for the preparation of protein polysaccharides differed in detail but in principle they consisted of aqueous extractions and subsequent fractionation in the centrifuge (Schubert, 1958; Pal *et al.*, 1966). These methods have been recently applied to the fractionation of the protein polysaccharides of bovine nucleus pulposus (Rosenberg *et al.*, 1967).

Experimental Procedure

The nucleus pulposus was removed from the intervertebral disks of migrant whales about 12-hr postmortem. The blood-free material removed from the terminal vertebrae of sei whales was kept under ice for about 4 hr before its stabilization as an acetone powder.

Preparation of Nucleus Pulposus Protein Polysaccharides (NPP).[1] Nucleus pulposus acetone powder (10 g) was gently stirred in distilled H_2O (800 ml) for 6 hr at 5°. The suspension was centrifuged at 13,000*g* in the Servall centrifuge (GSA Servall head) for 30 min. The residue was discarded and the supernatant was centrifuged again at 78,000*g* (30,000 rpm, Spinco head) for 16 hr.[2] The supernatant from the second centrifugation was precipitated with two volumes of 1% potassium acetate in ethanol, washed with ethanol and ether, and dried under vacuum. The product, NPP, weighed 7 g. For the fractionation, a solution of NPP at 1% concentration (600 ml) in 0.1 M KCl was centrifuged at 100,000*g* for 24 hr. The light fraction, NPP-L (2.9 g), was in the supernatant and the heavy fraction, NPP-H (2.6 g), was a gelatinous residue at the bottom of the tube. The supernatant and the residue, at a concentration of about 1% in 0.1 M KCl, were precipitated with two volumes of ethanol in the presence of potassium acetate, washed several times with ethanol and ether, and dried under vacuum. Centrifugation of NPP-H at 100,000*g* in 0.1 M KCl for 12 hr gave a heavier component, NPP-H_1 (0.76 g).

Sucrose Density Gradient Centrifugation. ISOLATION OF NPP-H_1 (F). Aliquots of 0.5 ml of 2.5% solution of NPP-H_1 in 0.6 M KCl were layered over 4.6-ml linear gradients of 10–40% sucrose in 0.6 M KCl and centrifuged at 5° for 6 hr at 38,000 rpm in a Spinco Model L ultracentrifuge using an SW-39 rotor. After centrifugation, the tubes were punctured at the bottom and 0.3-ml fractions were collected. Their light absorbance was determined at 280 mμ. Plots of absorbance at 280 mμ against cuvet number fraction showed the presence of

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[1] The abbreviations used that are not given in *Biochemistry 5*, 1445 (1966), are: NPP, nucleus pulposus protein polysaccharide; NPP-L and NPP-H, light and heavy sedimentation fractions of NPP.

[2] High-speed centrifugation of nucleus pulposus in distilled H_2O was useful for removal of impurities. Usually found were small residues, slightly brown and fibrous, containing small amounts of carbohydrate.

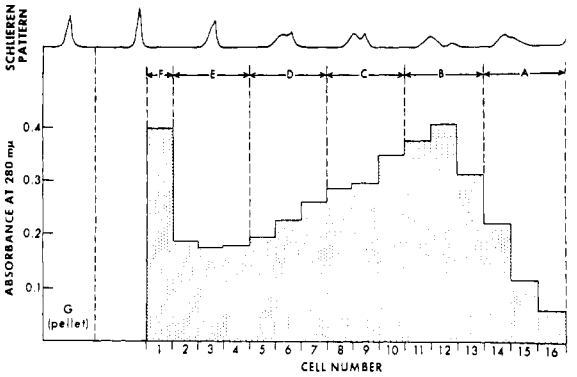

FIGURE 1: Sucrose gradient fractionation of NPP-H_1. Volume collected on each cell was about 0.3 ml, equivalent to 5 drops. By "pellet" is meant the material (about 5% of total) remaining attached to the centrifuge tube (bottom) after all fractions were collected. The upper part of the figure shows the sedimentation pattern of each of the subfractions isolated.

at least two major fractions (Figure 1). Several subfractions were arbitrarily separated; total recovery was about 90% from NPP-H_1 (Table I). In order to obtain fraction F, several preparations were pooled and dialyzed at 5° against 0.1 M KCl and distilled H_2O for 24 hr each. The solution was then lyophilized and the residue dissolved in 2.5×10^{-2} M EDTA, 2.5×10^{-2} M Tris, and 2.5×10^{-2} M tryglycollamate in 0.1 M KCl, final pH 7. The resulting solution was again dialyzed at 5° against the solvent for 24 hr; this procedure was followed by dialysis against double-distilled H_2O for 48 hr, with several changes. These operations were necessary, especially with fraction F, in order to remove heavy metals and sucrose from the preparation. Subfraction F was obtained in a powdered form by lyophilization.

Methods of Analysis. The relative proportion of glucosamine and galactosamine was determined by an ion-exchange method (Gardell, 1953) in samples hydrolyzed in 4 M HCl under nitrogen in sealed tubes for 14 hr at 100°. Total hexosamine was determined on these samples by the method of Boas (1953). Protein analyses were made with a Folin–Ciocalteu reagent using bovine serum albumin as the standard (Lowry *et al.*, 1951). Amino acids were analyzed on the Spinco Model 120B amino acid analyzer after hydrolysis in 6 M HCl in evacuated, sealed tubes at 110° for 22 hr (Spackman *et al.*, 1958). Hydroxyproline was estimated in these samples by the method of Woessner (1961). Glucuronic acid was estimated with a carbazole–sulfuric acid method (Dische, 1955a) and methylpentoses by the primary cysteine–sulfuric acid reaction (Dische, 1955b). Galactose was determined by the procedure of Trevelyan and Harrison (1952), using galactose as the standard. Total sialic acid was estimated according to the ion-exchange resin method of Svennerholm (1958) and free sialic acid by the thiobarbituric acid method of Warren (1959). Chemically bound neutral carbohydrates were identified after hydrolysis of the protein polysaccharides (0.1%) in 0.5 M HCl at 100° for 4 hr. This solution was then lyophilized, and the residue was dissolved in H_2O and passed through a small column containing 0.5 ml of AG-50W-X8 (H^+) (200–400 mesh) (Bio-Rad Laboratories, Richmond, Calif.). The eluate (5 ml) was again lyophilized and placed on paper for analysis, using descending chromatography. Solvents used were acetone–butanol–H_2O (7:2:1) and ethyl acetate–pyridine–H_2O (1.6:4:2) (Jermyn and Isherwood, 1949).

TABLE I: Recovery of Protein Polysaccharide Fractions from 100 mg of NPP-H_1 by a Sucrose Gradient Method.

NPP-H_1 (mg)	Fraction (%)						
	A	B	C	D	E	F	G
100	10.1	21.2	22.4	15.5	9.2	6.3	6.1

Reduction and Alkylation with [^{14}C]*ICH*$_2$–**CONH*$_2$. This procedure was designed for the qualitative identification of cystine (or cysteine) when other methods failed to reveal its presence. Protein polysaccharide solutions at 1% concentration (5 ml) in 0.1 M NaCl in 0.95 M Tris and 10^{-3} M disodium EDTA (final pH 8.5) were dialyzed at 5° against the solvent for 3 days with three changes. An aliquot of 1.0 ml was reduced with dithiothreitol and used at a final concentration of 5×10^{-3} M for 24 hr at 37° under vacuum followed by the addition of sixfold molar excess of [^{14}C]ICH_2–*$CONH_2$ (1.4 mCi/mg). After 2-hr incubation at room temperature, the reaction mixture was dialyzed against 0.6 M NaCl for 2 days and against distilled H_2O for 2 days at 5° with several changes of the solvent. The solution was lyophilized and the residue was dissolved in 1 ml of 3×10^{-3} M nonradioactive *S*-carboxymethyl-L-cysteine and hydrolyzed in 6 M HCl in evacuated, sealed tubes at 110° for 22 hr. This solution was then concentrated to dryness, and the residue was dissolved in 0.6 ml of H_2O and centrifuged to remove insoluble material. Two methods were used to identify *S*-[^{14}C]carboxymethyl-L-cysteine in the hydrolyzed sample. (A) About 0.2 ml of the solution was placed on a 0.9×150 cm column of AG-50W-X8 (H^+) (200–400 mesh) kept at constant temperature by circulating H_2O at 50° through its jacket. Elution was carried out with 0.2 M sodium citrate (pH 3.25) according to the procedure of Moore *et al.* (1958). The presence of *S*-carboxymethyl-L-cysteine and other amino acids was identified with ninhydrin. Radioactivity was measured in a scintillation counter. (B) Solution (0.2 ml) was subjected to high-voltage paper electrophoresis (Whatman No. 3MM) in 0.05 M ammonium formate (pH 3.6) at 1000 V (about 20 V/cm) for 1 hr. The distribution of radioactivity was determined by placing the paper on Kodak No-Screen Medical X-Ray film for 1 week. *S*-Carboxymethyl-L-cysteine (and other amino acids) was visualized by reaction with ninhydrin.

Physical Measurements. Ultracentrifuge analyses utilized a Spinco Model E analytical ultracentrifuge equipped with schlieren optical system at 20°. Sedimentation velocity measurements were made at 59,780 rpm with single-sector cells of 12-mm light-path length. In a

TABLE II: Chemical Composition of Protein Polysaccharides.

	NPP-L	NPP-H_1	NPP-H_1 (F)
Protein (%)[a]	14.6	38.4	44.3
Amino acids[b]			
Lys	39.2	28.6	35.0
His	14.3	17.0	18.5
Arg	33.8	53.1	51.4
Asp	68.4	91.6	92.6
Thr	75.3	77.2	76.6
Ser	100.8	66.9	70.9
Pro	90.5	92.5	83.5
Glu	127.9	126.0	123.4
Gly	150.2	93.5	88.4
Ala	68.9	68.6	78.5
Val	71.0	66.5	66.7
Cys ($^1/_2$)	0.0	25.7	20.5
Met	0.2	9.3	9.7
Ile	3.3	38.4	39.3
Leu	83.3	76.0	71.8
Tyr	1.0	32.7	36.5
Phe	3.1	35.6	36.7
Carbohydrates			
Total hexosamine (%)[a]	33.4	24.3	21.7
(mole ratio)[c]			
Glucosamine	0.205	1.400	1.460
Galactosamine	1.000	1.000	1.000
Uronic acid	0.868	1.012	1.150
Galactose	0.348	1.600	1.893

[a] Per cent by weight, corrected for H_2O content. [b] Amino acid residues/1000 amino acid residues. [c] Mole ratio with respect to galactosamine.

two-sedimentation components system, the Johnston–Ogston effect was taken into consideration and the pertinent corrections were made. Sedimentation coefficients, s, were obtained at four to five concentrations for each preparation. Extrapolation to infinite dilution was obtained by plotting $1/s$ against concentration. Diffusion measurements were made in the ultracentrifuge with a capillary-type synthetic boundary cell. The solvent was layered over the solution at about 10,000 rpm. The speed was then reduced to 1967 rpm and kept constant with the use of a low-speed attachment. Areas under the schlieren curves were measured with a planimeter on photographic enlargements (Schachman, 1959; Svedberg and Pedersen, 1940; Elias, 1961).

Viscosity determinations utilized a low-shear gradient capillary viscometer of the Ubbelohde type (Cannon-Ubbelohde, semimicrodilution viscometer, size 100, Cannon Instrument Co.). Outflow time was read to 0.1 sec. The temperature was maintained at 25 ± 0.02° (Schachman, 1957; Mathews and Dorfman, 1953).

Results

Chemical Composition. The results of analyses are shown in Table II. The amino acid composition of different fractions isolated from whale nucleus pulposus showed glycine and acidic amino acids to be major constituents. The fractions richer in protein contained increasing amounts of tyrosine and isoleucine. A salient feature of these analyses was, however, the finding that fraction NPP-H contained cystine while NPP-L had none.[3] No free sulfhydryl groups could be titrated in NPP-H and subsequent fractions using 2,2′-dithiodipyridine (Grassetti and Murray, 1967) either in dilute salt or in a denaturing solvent (5 M urea). Results obtained suggest that the nucleus pulposus has at least two

[3] Cystine cannot be categorically excluded in NPP-L. Detection of small amounts of cystine in protein polysaccharides is difficult even with independent and more selective methods of analysis. Thus, reductive alkylation with [^{14}C]ICH_2–*$CONH_2$ of NPP-L showed that this protein polysaccharide fraction (NPP-L) had trace amounts of cystine, detectable only after reduction with dithiothreitol. The cystine found in NPP-L could has come, however, from the free amino acid bound to the sample or from NPP-H, incompletely removed by the fractionation procedure. The presence of NPP-H in NPP-L was confirmed afterward when small amounts of NPP-H (0.04 g) were isolated from NPP-L (2.5 g) after this material (NPP-L) was subjected to a further and more extensive fractionation procedure.

protein polysaccharides with two different protein moieties.

The carbohydrate mole ratios with respect to galactosamine showed values to correspond well to the chemical composition of keratan sulfate and chondroitin sulfate. Glucosamine and galactosamine analyses seem to represent, then, the mucopolysaccharide composition of our samples. Since the fractionation procedure succeeded in separating protein polysaccharides with differing content of chondroitin sulfate and keratan sulfate (Table II), the existence of individual keratan sulfate and chondroitin sulfate–protein conjugates may be possible. With other means of purification, we have not been able, however, to isolate a fraction completely devoid of one of the two major polysaccharide components.

In most preparations, a neutral carbohydrate (galactose) was present in excess of the amount expected for the chemical composition of keratan sulfate (Table II). After mild acid hydrolysis of the protein polysaccharide, paper chromatography showed one carbohydrate spot identical in mobility with galactose. To some extent, then, galactose, and presumably glucosamine and galactosamine, might be glycoprotein components (Anderson, 1962). Other carbohydrate components were sialic acid and methylpentose, detected by colorimetric analysis (Table III).

TABLE III: Sialic Acid Methylpentose and Hydroxyproline Content of Nucleus Pulposus Protein Polysaccharides.

	Sialic Acid[a]	% Methylpentose	Hydroxyproline	Protein
NPP	2.4	0.6	0.065	20.6
NPP-L	1.9	0.7	0.050	14.6
NPP-H_1	4.3	0.5	0.092	38.4
NPP-H_1 (F)			0.109	44.3

[a] Bound sialic acid.

Several attempts were made to demonstrate the possible presence of carbohydrate-free proteins in NPP. Using a modification of the method of Sevag (Orrell *et al.*, 1964), small fractions were obtained in which the denatured protein enrichment was as high as 80%. None of these fractions, however, were found completely free of mucopolysaccharides. Centrifugation at 350,000*g* for 12 hr of NPP in distilled H_2O gave a component (*ca.* 2%) containing about 77% protein, 16% keratan sulfate, and 7% chondroitin sulfate, as inferred from results of colorimetric analysis.

The ultraviolet absorption spectra of NPP-H_1 (F) resemble other protein polysaccharide fractions (Campo and Dziewiatkowski, 1962). Solutions in 0.1 M NaOH were clear; however, initial absorbance measurements at 280 and 295 mμ increased rapidly with time, remaining essentially constant after 18 hr. The concentration of tyrosine and tryptophan in these solutions was computed in 18 hr from their average molar extinction coefficients as suggested by Beaven and Holiday (1952). Values were found to correspond to a tyrosine to tryptophan mole ratio of 3.5 for NPP-H_1 (F). The amount of tryptophan was estimated from this ratio and the content of tyrosine obtained by an ion-exchange column method (Table II). The amount of tryptophan in NPP-H_1 (F) was 10.6 residues/1000 amino acid residues.

Reduction of NPP-H_1. The protein polysaccharide NPP-H_1 had two sedimentation peaks. The two different sedimentation components of NPP-H_1, herein described as NPP-H_1 (I) and NPP-H_1 (II), corresponded to the fast- and slow-sedimentation peaks, respectively, observed in the analytical ultracentrifuge (Figure 2). Fraction NPP-H_1 was about 25% of NPP-H and 10% of the starting protein polysaccharide NPP. About 65% of NPP-H_1 was found to be NPP-H_1 (I). The proportion of the two sedimenting components of NPP-H_1 was computed from the relative area under the schlieren curve using appropriate corrections for the prominent Johnston–Ogston effect observed.

The effect of cysteine and dithiothreitol on the velocity sedimentation patterns of protein polysaccharides was first observed in NPP-H_1. The fast-sedimenting component NPP-H_1 (I) was found to disappear in the presence of these reducing agents.

The reduction of NPP-H_1 by cysteine and dithiothreitol as well as the reversibility of the reaction is shown in Figure 2. In view of the capacity of cystine to reverse the reduction to a large extent, the observed changes produced by cysteine in NPP-H_1 were certainly small. Significant changes were, however, observed with dithiothreitol. This reduction was partially reversed by $K_3Fe(CN)_6$. The effect of KBH_4, Na_2SO_3, and urea are also shown in Figure 2. Reductions with SO_3^{2} were possible only in the presence of *p*-hydroxymercuribenzoate. No significant changes in the protein polysaccharide sedimentation pattern were observed when NPP-H_1 was incubated in 1.0 M NH_2OH (pH 8.5) in evacuated tubes at 37° for 14 hr, or in the presence of ascorbic acid (10^{-2} M). A change of the sedimentation pattern of NPP-H_1 resembling that produced by dithiothreitol was seen when the sample was dissolved in 7 M urea. The urea effect was, however, not related to the effect produced by dithiothreitol or other reducing reagents since it was not reversed by cystine or $K_3Fe(CN)_6$. A two-component system was observed when NPP-H_1 was incubated in 7 M urea in the presence of dithiothreitol. Almost negligible was the change of the sedimentation pattern of NPP-H_1 produced after its dialysis against distilled H_2O.

The sedimentation coefficients of NPP-H_1 were dependent on the ionic strength of the solvent as expected for synthetic polyelectrolytes (Stern and Atlas, 1953). Thus, when the ultracentrifuge runs of NPP-H_1 were made in 0.15 M KCl, the sedimentation coefficients of the two peaks were in the range of about four-fifths of the value in 0.3 ionic strength. At an ionic strength of 0.6, the sedimentation coefficients were about the same as at 0.3 ionic strength. The ionic strength of 0.6 was

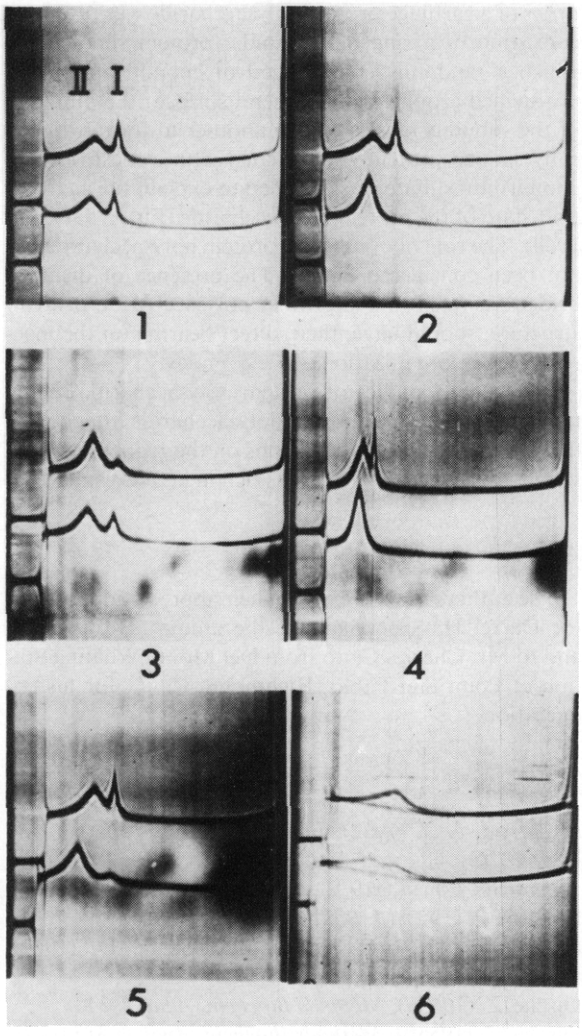

FIGURE 2: Changes in the sedimentation pattern of NPP-H_1 after reduction and oxidation. Solution mixtures, 0.5% in 0.1 M KCl, in 0.1 M Tris (pH 8.0), were incubated at 37° under vacuum for 14 hr. Lower row: (1) solution mixtures in 10^{-2} M cysteine; (2) solution mixture in 10^{-2} M dithiothreitol; (3) after reduction with dithiothreitol (10^{-3} M) and addition of cystine (10^{-2} M), incubation under vacuum at 37° for 14 hr. Upper row: solution mixtures in the absence of oxidoreduction reagents were controls for 1 and 2. Solution mixture in 10^{-3} M dithiothreitol was the control for 3. Changes in the sedimentation pattern of NPP-H_1 after addition of Na_2SO_3, KBH_4, and urea. Solution mixtures for the reduction: 0.5% in 0.15 M KCl incubated at 37° under vacuum for 14 hr. Lower row: (4) solution mixture in 10^{-2} M Na_2SO_3 in 10^{-3} M *p*-hydroxymercuribenzoate (pH 7.0); (5) solution mixture in 10^{-2} M KBH_4 (pH 9.3), and (6) solution mixture in 6 M urea in 10^{-2} M dithiothreitol (pH 8.0). Upper row: solution mixtures in the absence of reducing reagents were controls for 4–6. Control for 4 contained, in addition, about 10^{-4} M *p*-hydroxymercuribenzoate. All pictures but 6 were taken after 32 min at 59,780 rpm (20°), bar angle 70°. Picture of 6 was taken after 216 min at 42,040 rpm (20°).

then sufficiently high for considering the sedimentation rates independent of electrolyte concentration.

Reduction of NPP-H_1 (F). For the reduction, the sample dissolved in 0.6 M NaCl in 0.05 M Tris (pH 8) containing dithiothreitol at the final concentration of 10^{-2} M, was incubated in an evacuated, sealed tube for 14 hr at 37°, and dialyzed against the solvent for 18 hr at 5°. Control solutions containing no dithiothreitol were subjected to identical conditions. Protein polysaccharide concentrations were made by weight corrected for moisture.[4]

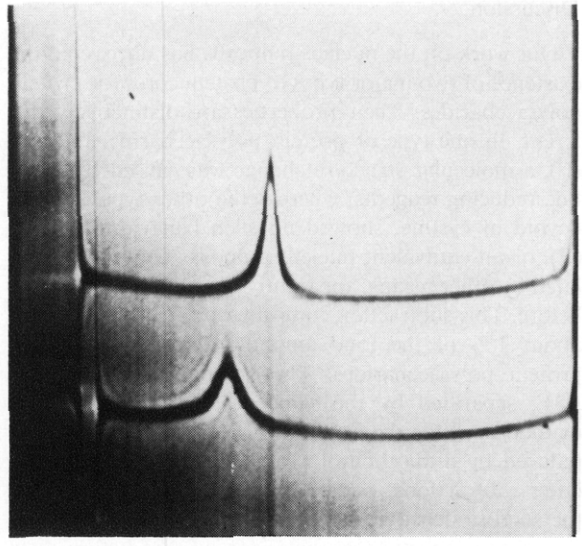

FIGURE 3: Sedimentation patterns of NPP-H_1(F). Upper row: before reduction. Lower row: after reduction; 0.5% concentrated, bar angle 70°; photographs taken after 40 min. Ultracentrifuge speed 59,780 rpm (20°).

In the ultracentrifuge, NPP-H_1 (F) showed a single sedimentation band before and after reduction (Figure 3). Plots of $1/s$ against concentration were linear. The sedimentation coefficient before reduction was 47.6 S and after reduction 9.1 S.

Throughout the diffusion experiments, no displacement of the boundary was observed at the operated speed of centrifugation (2000 rpm). The diffusion patterns showed no skewness. A linear relationship of D_{20} against protein polysaccharide concentration before and after its reduction was obtained. The concentration dependence of the function was very small. The diffusion coefficients of the original and reduced protein polysaccharide were 0.36 and 0.85×10^{-7} cm² per sec, respectively.

Upon reduction the intrinsic viscosity of NPP-H_1 (F) changed from 2.12 to 0.98 dl/g. No corrections were made for the effect of rate of shear on the viscosity.

Molecular weights were computed from sedimentation and diffusion coefficients by means of the Svedberg equation (Svedberg and Pedersen, 1940), using 0.6 ml/g for the partial specific volume of the protein polysaccharide. The apparent molecular weight of NPP-H_1 (F) estimated at 8.5×10^6 before reduction was changed to 6.6×10^5 after reduction, a net decrease of about 13-fold.

[4] The water content of protein polysaccharides was determined by weight difference before and after their drying under vacuum over P_2O_5 at 100° for 48 hr. Throughout all our analytical work, fresh preparations were used (nondried); weight corrections were made afterward. For the lyophilized preparation NPP-H_1(F) the weight loss upon drying was 10%.

Discussion

Our work on the nucleus pulposus has uncovered the existence of two major types of protein cores for protein polysaccharides. Their properties are distinctively different. In one type of protein polysaccharide, NPP-H_1 (F), a molecular structure change was caused by disulfide reducing reagents, whereas the other type, NPP-L, devoid of cystine, showed no such behavior. NPP-H_1 (F), or an equivalent nucleus pulposus component isolated by other means, apparently has not been described before. This subfraction comprises 10% of NPP-H_1 and about 1% of the total amount of nucleus pulposus protein polysaccharides. The remaining subfractions, A–E, separated by the same gradient centrifugation method used for the isolation of NPP-H_1(F), were also reduced by dithiothreitol (Table I and Figure 1). The latter subfractions, pooled together and centrifuged in the sucrose density gradient, were recovered in about the same proportion as they were present before their fractionation. A subfraction resembling NPP-H_1 (F) was isolated and was less than 2% of the pooled mixture. Subfractions A–E seem to be then different from each other and from NPP-H_1 (F).

The protein polysaccharide fraction NPP-H_1 shows a certain similarity to fractions PP-L4 and PP-L5 isolated from bovine nucleus pulposus (Rosenberg *et al.*, 1967). These bovine nucleus pulposus fractions show a sedimentation pattern having two boundaries, a glucosamine to galactosamine ratio, and an amount of cystine resembling the analytical values obtained for NPP-H_1. Since it is unlikely that bovine nucleus pulposus differs much from whale nucleus pulposus, fractions PP-L4 and PP-L5 are then expected to be susceptible to reduction in the same manner as fraction NPP-H_1.

The shape and molecular aggregation of the protein polysaccharides of the nucleus pulposus is not known; however, their chemical composition and physical properties show a resemblence to the protein polysaccharides of bovine nasal septum, a protein core along which are distributed chains of chondroitin sulfate (Mathews and and Lozaityte, 1958). The nature of the molecular model proposed for the cartilage macromolecule has suggested studies in which many of the results of the physicochemical changes observed in protein polysaccharides were to be interpreted primarily as interactions within the polysaccharide branches rather than as interactions involving the heavily shielded protein core. Disulfide linking, although a common reaction with proteins, was therefore not entirely evident with protein polysaccharides.

In view of the results obtained with the nucleus pulposus protein polysaccharides, it is reasonable to suggest that some of the cartilage complexes also may have some of their protein moieties cross-linked with disulfide groups.[5] A model, concerned with the macromolecular order of a cartilage protein polysaccharide was proposed consisting of a ring of ellipsoidal protein subunits on which a randomly coiled chain of chondroitin sulfate is attached around the peripheral surface; the piling up of the subunits on top of one another to form cylindrically shaped protein cores coated with a meshwork of chondroitin sulfate was intended to explain the aggregation state of the protein polysaccharide (Fitton Jackson, 1964). The role of cystine in a protein polysaccharide has not been considered before. The presence of disulfide bridges, as part of the protein polysaccharide primary structure, should have, then, direct bearing on the macromolecular organization of the complex.

Relevant to studies of protein polysaccharide reduction is also the nature of the polysaccharide attached to the peptide. All our observations on the reductions were shown to take place in samples rich in keratan sulfate.

[5] The light protein polysaccharide fraction from bovine nasal septa, PP-L5 (Pal *et al.*, 1966), showed a decrease of its sedimentation velocity and intrinsic viscosity after reduction with dithiothreitol. The reaction was carried out in 0.6 M NaCl under the conditions used for the reduction of NPP-H_1(F) (unpublished experiments by the authors).

Acknowledgment

The authors wish to express their appreciation to Doctor Darrel H. Spackman for the amino acid analysis, and to Mr. Charles Caito from Del Monte Whaling Station at Point San Pablo, Richmond, Calif., for his cooperation.

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The Use of Peptide Synthesis to Establish the Amino Acid Sequence of Tobacco Mosaic Virus Protein Tryptic Peptide 2*

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ABSTRACT: Peptides corresponding to two previously proposed sequences of a tryptic peptide representing residues 42–46 of the tobacco mosaic virus protein (TMVP) having a sequence interchange at residues 44 and 45 were synthesized using the Merrifield solid-phase method. (This peptide was designated as TMVP tryptic peptide 2 according to the nomenclature proposed by Tsugita *et al.* (Tsugita, A., Gish, D. T., Young, J., Fraenkel-Conrat, H., Knight, C. A., and Stanley, W. M. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 1463).) Comparison of the properties of these synthetic peptides with the peptide isolated from the protein revealed that the sequence in the protein is: Thr-Val-Val-Gln-Arg. (1) On paper chromatography the synthetic Thr-Val-Val-Gln-Arg and TMVP tryptic peptide 2 cochromatographed and migrated slower than the synthetic Thr-Val-Gln-Val-Arg-(2) The two synthetic peptides gave markedly different paper chromatographic patterns after partial acid hydrolysis with the pattern of TMVP tryptic peptide 2 being identical with that of synthetic Thr-Val-Val-Gln-Arg. (3) Synthetic Thr-Val-Gln-Val-Arg was completely hydrolyzed by overnight acid hydrolysis, but both the synthetic Thr-Val-Val-Gln-Arg and the TMVP tryptic peptide 2 required a longer hydrolysis time for complete hydrolysis. (4) Though difficulties were encountered in performing N-terminal stepwise degradation on the peptides, these analyses confirmed that the sequence of TMVP tryptic peptide 2 is Thr-Val-Val-Gln-Arg.

Since peptide synthesis has been greatly simplified by the use of the Merrifield (1964) solid-phase method, synthesis may soon be a common tool for use in sequence analysis. Recently, Bornstein (1967) utilized synthetic peptides in ascertaining that a peptide of collagen was α-L-glutamyl-L-prolyl-glycine and not α-L-glutamyl-L-prolyl-glycine. We have used synthesis in ascertaining the sequence of TMVP tryptic peptide 2[1] containing residues 42–46. This peptide was reported by both the Berkeley group and the Tubingen group in 1960 as Thr-Val-Gln-Val-Arg (Anderer *et al.*, 1960; Tsugita *et al.*, 1960) but in 1965 the Tubingen laboratory reinvestigated this peptide and found that the sequence was Thr-Val-Val-Gln-Arg (Anderer *et al.*, 1965). This only remaining point of difference between the two laboratories regarding the TMVP amino acid sequence has been utilized in this paper to ascertain whether synthesis of the two proposed sequences and comparison of their properties to that of TMVP tryptic peptide 2 can be used as a method for determining the correct sequence in naturally occurring peptides.

Experimental Procedures

TMVP was obtained from TMV[2] by treatment with 67% acetic acid (Fraenkel-Conrat, 1957). Amino acid

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[1] The tryptic peptide is numbered according to the nomenclature proposed by Tsugita *et al.* (1960).

[2] The tobacco mosaic virus was generously supplied both by Dr. C. A. Knight of the Virus Laboratory, University of California, Berkeley, and Dr. Stanley Mandeles of the Space Science Laboratory, University of California, Berkeley.