The Periodate Oxidation of Bovine Bone Sialoprotein, and some Observations on its Structure

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1. Bovine bone sialoprotein (mol.wt. 23 000) contains *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid, fucose, galactose, mannose, *N*-acetylglucosamine and *N*-acetylglucosamine residues in the form of a very small number, perhaps one, of highly branched oligosaccharide structures linked covalently to peptide. 2. Periodate oxidation of the sialoprotein results in quantitative destruction only of the sialic acid and fucose residue consistent with the earlier findings of their positions as terminal groups. 3. Terminal sialic acid residues are attached to galactopyranose residues by 2,3-linkages, and to some *N*-acetylglucosamine residues (at C-6). 4. Sequential Smith degradation indicates that *N*-acetylglucosamine residues may be present as points of branching (linked in C-1, C-3 and C-6) and *N*-acetylglucosamine residues are located in the inner part of the structure, adjacent to the carbohydrate-peptide bond(s). 5. Mannose residues appear to be linked in the 1,3-positions.

Repetitive periodate oxidation, reduction and selective partial hydrolysis (the Smith degradation), resulting in a stepwise degradation of polysaccharide chains (Abdel-Akher, Hamilton, Montgomery & Smith, 1952; Hamilton & Smith, 1956*a,b*; Smith & Unrau, 1959; Rothfus & Smith, 1963), is a means of obtaining useful structural data. Application of the procedure to a number of glycoproteins (Chatterjee, Durant, Hendrickson, Lee & Montgomery, 1961; Lee & Montgomery, 1961; Chatterjee & Montgomery, 1962; Bragg & Hough, 1961; Kamiyama & Schmid, 1961; Eylar & Jeanloz, 1962; Spiro, 1964; Jeanloz, 1964; Montgomery, Wu & Lee, 1965; Marshall & Porath, 1965) has given considerable information about some of the linkages and points of branching of the monosaccharide residues in the carbohydrate prosthetic groups.

The present paper is concerned with the application of periodate oxidation and the Smith degradation to bovine BSP,† a glycoprotein isolated from bovine cortical bone. The composition of this glycoprotein has been reported (Andrews & Herring, 1965; Andrews, Herring & Kent, 1967), and some evidence suggests that the carbohydrate components may be present as a single highly branched prosthetic group (Andrews *et al.* 1967).

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† Abbreviation: BSP, bone sialoprotein; BP, sialic acid-free BSP.

MATERIALS AND METHODS

Bovine BSP. BSP was prepared as the zinc salt according to the method described by Herring (1964). The molecular weight of BSP is taken as 23 000 (Andrews *et al.* 1967).

Sialic acids. Total sialic acid was determined by the direct Ehrlich procedure (Werner & Odin, 1952), or by the thiobarbituric acid procedure (Warren, 1959) after hydrolysis with 0.1 *N*-H₂SO₄ at 80° for 1 hr.

Acid hydrolysis of glycoprotein samples. An approx. 0.1% (w/v) solution of sample in 2 *N*-HCl was heated at 100° for 4 hr. Under these conditions, the recovery of hexose from BSP was 94.0% compared with direct determinations of hexose without prior hydrolysis, and the recovery of hexosamines from BSP was 90.0% as judged by the results of experiments in which internal standards of *N*-acetylhexosamine were included.

Monosaccharide analyses. Determinations of hexose, fucose and hexosamines, and the qualitative and quantitative paper chromatography of monosaccharide constituents were carried out as described by Andrews *et al.* (1967).

Periodate oxidation of BSP. BSP (3.7 mg.) was dissolved in 0.1 *M*-sodium acetate buffer (pH 5.0, 0.1 ml.) and 0.1 *M*-NaIO₄ (0.1 ml.) added. The progress of the oxidation at room temperature (21°) was followed by withdrawing portions (10 μ l.) of the mixture after suitable time-intervals, diluting to 7 ml. with water and measuring the extinction at 225 $m\mu$ (Aspinall & Ferrier, 1957). The extinction values of separate solutions of the acetate buffer, periodate, BSP and 0.1 *M*-sodium iodate were also measured after suitable dilution. Since the optimum time for oxidation was 40 min., a further sample of BSP (5.2 mg.) was dissolved in 0.1 *M*-sodium acetate buffer (pH 5.0, 0.15 ml.) and 0.1 *M*-NaIO₄ (0.15 ml.) added. After 40 min. excess of NaBH₄ (80 mg.)

was added to destroy the remaining periodate and to reduce aldehyde groups. The reduction was terminated after 65 min. by the addition of excess of acetic acid, added dropwise until effervescence ceased. The mixture was placed in cellophan tubing ($\frac{3}{8}$ in. diam. Visking tubing; Hudes Merchandising Corp.) that had previously been thoroughly washed and dialysed against distilled water (3×11 ., over 2 days). The solution of non-diffusible components, combined with the washing from the dialysis tubing, was made up to 10 ml. for analysis.

Preparation of sialic acid-free BSP. BSP was dissolved in 0.1 N-HCl to give a concentration of 10 mg./ml., and heated at $80^\circ \pm 2^\circ$ for 1 hr. The cooled solution was placed in cellophan tubing ($\frac{3}{8}$ in. diam.) and dialysed thoroughly against distilled water, until the diffusate was free of chloride and the pH had risen to 5.3. The solution of non-diffusible components was filtered, and the BP precipitated from the filtrate by addition of ethanol (4 vol.) containing zinc acetate (0.5 g./l.) and acetic acid (0.5 ml./l.). After standing overnight at 2° , the precipitate was collected by centrifugation (1870g), dried by suspension three times with absolute ethanol and twice with ether, and allowed to dry in air. No detectable sialic acid could be demonstrated in the product by the thiobarbituric acid method.

Smith degradation procedure. A curve showing the rate of reduction of periodate by BP (7.3 mg.) was obtained, as described above, to determine the optimum time and the total amount of periodate reduced. A further batch of BP (55.0 mg.) was then oxidized for this optimum time (30 min.) under identical conditions. This was followed by reduction with NaBH_4 and dialysis, as with BSP itself. The contents of the dialysis tubing and washings from the tubing were combined, rendered 0.05 N with respect to HCl and heated at 80° for 1 hr. before being dialysed again against distilled water. This dialysis was conducted initially against a small volume (30 ml.) of water, to give a concentrated solution of diffusate, and then twice more against a larger volume (11.). To the non-diffusible solution (about 10 ml.) were added zinc acetate (5 mg.) and acetic acid (5 μ l.), followed by ethanol (4 vol.) to precipitate the glycoprotein product (BP 1), which was collected by centrifugation (1870g) and dried with ethanol and ether.

A portion (7 ml.) of the 30 ml. of diffusate obtained above was evaporated to small bulk over P_2O_5 , and the residue analysed qualitatively by paper chromatography for identification of the polyols and any sugar components released by the mild acid hydrolysis.

For the second Smith degradation cycle, a sample of BP 1 (3.6 mg.) was reoxidized, as above, to determine the optimum time (17 hr.) for the oxidation. This was followed, as before, by the oxidation, reduction and mild acid hydrolysis of a larger batch of BP 1 (18.3 mg.) and precipitation of the product BP 2.

There appeared to be two phases to the oxidation reaction in this step (see the Results section), consisting of a rapid and a slow reaction. To study the destruction of components in these two phases, a further small batch of BP 1 (14.0 mg.) was subjected to the Smith degradation. Half the sample was oxidized for 60 min., and the other half for 20 hr. The oxidations were followed by reduction, mild acid hydrolysis and dialysis. In addition to the usual analyses, the glucosamine/galactosamine ratio was measured after separation on an ion-exchange column (0.6 cm. $\times$ 45 cm.) of

Dowex 50 (H^+ form; X8; 250–400 mesh) with a flow rate of 2.75 ml./hr. (Gardell, 1953).

A similar exploratory oxidation of a small portion of BP 2 (3.3 mg.) was followed by a third Smith degradation cycle on a larger batch of BP 2 material (6.5 mg.), with 21 hr. oxidation time, to yield BP 3. This product was not isolated by precipitation because of the small quantities involved, but the final dialysed solution was made up accurately to 6 ml.

Part of this solution of BP 3 (3.0 ml.) was rendered 50 mM with respect to sodium acetate and to NaIO_4 , by addition of the solid reagents (2 mg. and 32 mg. respectively) and the pH adjusted to 5.0 with acetic acid. Oxidation was allowed to continue for 18 hr., and followed by reduction, mild acid hydrolysis and dialysis to give a solution (6.2 ml.) of BP 4, the product of four cycles of the Smith degradation.

At the end of each cycle, analyses of total hexose and total hexosamine were carried out on the non-diffusible product, together with paper-chromatographic qualitative identification of hexose and hexosamine constituents and quantitative determination of the ratio of galactose to mannose. In each cycle, qualitative identification of the polyols, and any sugar components, released by the mild acid hydrolysis was made by paper chromatography.

Paper chromatography of polyols. Whatman no. 1 paper was washed for 48 hr. with 1.5 N-acetic acid and for 48 hr. with distilled water, and allowed to dry in air, before use. Chromatograms were developed with the organic phase of butan-1-ol-ethanol-water (4:1:5, by vol.) for 24 hr., or with ethyl acetate-pyridine-water (8:2:1, by vol.) for 16 hr. A sample of glycerol moved with $R_{\text{erythritol}}$ value 1.28 in the former solvent and 1.44 in the latter solvent. Samples of monosaccharides were also well resolved under these conditions, especially with the latter solvent. Polyols and monosaccharides were revealed by spraying the air-dried chromatograms either with a 1% solution of KMnO_4 in 2% Na_2CO_3 , or with the alkaline AgNO_3 reagent (Trevelyan, Procter & Harrison, 1950).

Reducing power. A small portion (0.2 ml.) of the solution of BP 4 obtained above, and a small volume of intact BSP solution (1 mg. in 20 μ l.), were applied as single spots (about 1 cm. diam.) to a piece of Whatman no. 1 filter paper. The paper was dried briefly in an oven at 100° and sprayed with alkaline AgNO_3 reagent (Trevelyan *et al.* 1950). Absence of reaction indicated that the compounds were non-reducing.

RESULTS

Periodate oxidation of BSP. The initial rate of periodate oxidation of BSP was very rapid, about 75% of the total oxidant being reduced within the first minute. After that the rate became slower, until the reaction was essentially complete after 40 min. There was a continued further small reduction of periodate for at least another 24 hr. attributed to slow over-oxidation. By extrapolation of the curve to zero time, it was found that 33.5 mol. of periodate were reduced/mol. of BSP during the rapid oxidation reaction. The total periodate initially present was equivalent to 62.6 mol./mol. of BSP.

Analysis of oxidized BSP gave a hexose content

of 11.2%, as determined by the anthrone procedure (Andrews *et al.* 1967), and 8.8% for the content of hexosamine. By comparison, intact BSP contained 11.7% and 9.2% respectively of these components (Andrews *et al.* 1967). Qualitative paper chromatography indicated the presence of glucosamine and galactosamine, which probably occurred as the *N*-acetyl derivatives (Andrews *et al.* 1967) in approximately equal proportions, and galactose and mannose, which were shown by quantitative paper chromatography to be present in the ratio 10:3. Thus it may be seen that the only carbohydrate components destroyed during the oxidation were the sialic acids and fucose, neither of which could be detected on analysis of oxidized BSP.

Sialic acid-free BSP. A sample of BSP (95.3 mg.) yielded 68.8 mg. of BP, which represented 90% of the theoretical yield. Fucose estimations on BP, and on the diffusate obtained after hydrolysis, indicated that the conditions required for removal of sialic acid also liberated an appreciable amount of the fucose from BSP. In view of the fact that the fucose content of the initial BSP was only about 0.7%, it was thought that the analytical procedure used was not sufficiently sensitive to give an accurate value for it in degraded BSP products. Chemical analysis of the remaining constituents of BSP showed that sialic acid and some of the fucose were the only components lost. In other respects the composition of BP closely resembled that of BSP.

The molecular weight of BP was calculated, from the loss of sialic acid residues, to be 18 200.

Smith degradation results. (a) First cycle. The time-course of periodate oxidation of BP resembled that for BSP itself, there being a rapid initial reaction, effectively complete within 30 min., accompanied by a much slower reaction lasting for more than 24 hr., and probably indicative of some over-oxidation. In the rapid reaction 21.5 mol. of periodate/mol. of BP were reduced. Utilization of all the periodate present in the reaction mixture would have required the reduction of 50.0 mol./mol.

Analysis of BP 1 showed that it contained 3.2% by weight of hexose, equivalent to 3.1 mol./mol. of BP 1 (see Table 1), if the molecular weight of BP 1 was taken to be 17 400 (calculated from the percentage loss of hexose in this first step of the Smith degradation and the molecular weight of BP). The ratio of galactose to mannose was found by quantitative paper chromatography to be 1:2, and thus approximately one residue of galactose and two of mannose survived the oxidation, nine residues of galactose and one of mannose being destroyed (Table 1). BP 1 contained 12.0% by weight of hexosamines and hence there was little attack on these constituents. Qualitative paper chromatography indicated the presence of glucosamine and galactosamine in approximately equal proportions. Glycerol was the only polyol liberated during the

Table 1. *Sequential Smith degradation of bone sialoprotein*

Monosaccharide composition (as mol./mol. of mol. wt. 23 000 and %, w/w) of non-diffusible products. Procedural details are given in the Materials and Methods section.

No. of oxidation- reduction cycles	Product	Sialic acid		Fucose		Galactose		Mannose		<i>N</i> -Acetyl- galactosamine		<i>N</i> -Acetyl- glucosamine	
		mol.	%	mol.	%	mol.	%	mol.	%	mol.	%	mol.	%
Expt. 1. Degradation of BSP													
—	BSP	15.2	20.7	1	0.7	10.5	7.9	3.1	2.4	5.9	4.4	5.8	4.4
						11.2%*				8.8%†			
1	Oxidized BSP	0	0	0	0	10.5		3.1		5.7		5.6	
Expt. 2. Sequential degradation of sialic acid-free BSP													
	BP	0	0	0.67	0.6	10.5		3.1		5.9		5.8	
						15.2%*				12.6%†			
						3.3%*				12.0%†			
1	BP 1			0	0	1.1		2.1		5.8		5.8	
2	BP 2					1.0	1.1	1.0	1.1	0.3	0.35	2.8	3.2
3	BP 3					1.1	1	0	0	0	0	1.0	1.2
4	BP 4					0.1	0.1					1.0	1.2

* Total hexose.

† Total hexosamine.

mild acid hydrolysis stage, no other components being released.

(b) Second cycle. In the time-course of periodate oxidation of BP 1 to give BP 2, it was observed that the curve had a marked inflexion, indicating again that oxidation occurred in two phases. As before, there was a rapid initial reaction, complete after about 60min., but there was also a slow reaction, probably complete in about 10–12hr., which was different from, and in addition to, the even slower over-oxidation reaction observed in previous experiments. It was calculated that the periodate reduction was approx. 9mol./mol. of BP 1 in the rapid phase (extrapolating from 2hr. to zero time) and about 12.0mol./mol. of BP 1 in the slow phase of the reaction (extrapolating from 12hr. to zero time). The total amount of periodate initially present was equivalent to 48.2mol./mol. of BP 1.

Analysis indicated that 2.21% by weight of hexose and 3.49% by weight of hexosamine remained in BP 2 (see Table 1). Allowing for a decrease in molecular weight to 15 600 for BP 2, due to further loss of components during the degradation, these percentages were equivalent to 1.9mol. of hexose/mol. of BP 2 and 3.0mol. of hexosamine/mol. of BP 2 respectively. Paper chromatography indicated the presence of galactose and mannose in equal proportions, and glucosamine with only a small trace of galactosamine. Glycerol and small amounts of erythritol were liberated by mild acid hydrolysis. Thus the second Smith degradation of BP destroyed one mannose, six *N*-acetylgalactosamine and three *N*-acetylglucosamine residues (Table 1).

Because of the inflexion in the periodate-reduction curve referred to above, it was considered of interest to study the destruction of hexosamine in this step of the degradation. The total hexosamine remaining after 60min. oxidation was 6.7% by weight or 5.8mol./mol. of BP 2. The ratio of glucosamine to galactosamine was found to be 8.7:1 after 60min. oxidation and 10:1 after 20hr. oxidation. Thus after 60min. oxidation 5.2mol. of glucosamine/mol. of BP 2 and 0.6mol. of galactosamine/mol. of BP 2 remained, and after 20hr. oxidation the 3.5% by weight of total hexosamine was equivalent to 3.0mol./mol. of BP 2, and hence there were 2.8mol. of glucosamine/mol. of BP 2 and 0.3mol. of galactosamine/mol. of BP 2. This indicated that one mannose residue and six *N*-acetylgalactosamine residues were rapidly attacked in the second Smith degradation cycle, followed by the slower attack on three *N*-acetylglucosamine units.

(c) Third cycle. In the third degradative cycle of BP 2 to BP 3 the periodate-reduction pattern was similar to that in previous stages, i.e. it consisted of a rapid initial reaction, complete in about

75min., with evidence of a slow over-oxidation reaction lasting for some hours. Total utilization of all the periodate present would have required the reduction of 47.0mol./mol. of BP 2, whereas the observed reduction was 16.0mol./mol. of BP 2. Analysis of BP 3 indicated that 1.3% by weight of hexosamine and 1.3% by weight of hexose survived this oxidation cycle. Allowing for a further small decrease in molecular weight to 14900 due to components lost on oxidation, it was calculated that BP 3 contained 1.0mol. of hexosamine/mol. and 1.1mol. of hexose/mol. Paper chromatography showed that glucosamine and galactose remained in BP 3. The only polyol liberated during the reaction cycle was glycerol. Thus this stage of the degradation comprised attack on two residues of glucosamine and one of mannose.

(d) Fourth cycle. The final Smith degradation yielded BP 4, which on analysis contained 1.21% by weight of hexosamine, shown by paper chromatography to be glucosamine. This was equivalent to 1.0mol./mol. of BP 4 if the molecular weight was taken to be 14700. There was also 0.14% by weight of hexose, or 0.1mol./mol. of BP 4, shown by paper chromatography to be a trace of galactose. Glycerol was again the only polyol released during the mild acid hydrolysis stage. Thus one residue of galactose was the only component destroyed in this final step of the degradation.

Both BP 4 and intact BSP were non-reducing.

DISCUSSION

Valuable information on the structure of carbohydrates may be gained from both measurements of the reduction of oxidant and the analysis of the oxidized material, usually after its reduction to a polyol (Smith & Montgomery, 1959). For meaningful results therefore it is essential that excess of periodate should be present at the start of the reaction, and it may be noted that in the experiments reported here only about 35–50% of the periodate initially present was utilized.

In the study of glycoproteins, however, the interpretation of periodate-reduction measurements is greatly complicated by the concomitant oxidation of protein components (Montgomery *et al.* 1965), and it has been reported that tryptophan, tyrosine, cysteine and methionine are particularly susceptible to attack (Schiffman, Kabat & Thompson, 1962). Because of the various oxidation reactions occurring it is difficult to determine the true end-point of the oxidation of the carbohydrate moiety, but in spite of the difficulty of avoiding considerable over-oxidation, useful information about the structure of the carbohydrate moiety of a glycoprotein may be obtained, especially from analysis of the components surviving the oxidation.

Periodate oxidation of BSP destroyed all 15 residues of sialic acid and the fucose residue, but no other components. These constituents therefore are most likely to occupy terminal positions in a branched carbohydrate chain (or possibly to be linked to one another in such a way as to remain susceptible to oxidation). The terminal locations of the sugars are supported by a reduction of 33.5 mol. of periodate/mol. of BSP (calculated value 32 mol./mol.).

The results of experiments with β -galactosidase (Andrews *et al.* 1967) suggested that much of the galactose occupied a subterminal position in BSP. This being so, after the hydrolytic removal of sialic acid, galactose should be oxidized in the first step of the Smith degradation of BP, as it would then occupy a terminal position. This was confirmed by the experimental results, which showed that nine residues of galactose and one of mannose were lost in this step. The identification of glycerol as the only polyol liberated indicated that these sugar residues had been cleaved between C-2 and C-3 and between C-3 and C-4, which was consistent with terminal locations in the polysaccharide chains. This would require the reduction of a total of 20 mol. of oxidant/mol. of BP, a figure in good agreement with the 21.3 mol./mol. of BP found experimentally.

The observation that neither galactose nor mannose was subject to attack by periodate in intact BSP strongly suggests that these hexoses were protected from this reagent by suitable linking of the sialic acid and fucose units. The fucose may be linked to mannose via a 1,3-bond, to preserve the latter from oxidative attack in the intact glycoprotein. In BSP there are 15 residues of sialic acid and some of these must be linked to nine residues of galactose in such a way as to protect them from the periodate. This can only be achieved if some of the sialic acid residues are joined to the C-3 position of the galactose units. It was considered probable that all nine subterminal galactose residues were protected by linkage to a sialic acid molecule through C-3 and that the remaining six sialic acid residues were linked to the C-4 or C-6 of *N*-acetylgalactosamine. However, in cervical mucins the 2,4-bond between sialic acid and *N*-acetylgalactosamine has been stated to be neuraminidase-resistant (Gibbons, 1963), whereas the bond linking sialic acid in BSP was found to be susceptible to cleavage by neuraminidase (Andrews *et al.* 1967). Thus on the basis of the results obtained, the glycosidic linkages of sialic acid in BSP, already considered to be α in configuration, may be to the C-3 of galactose and the C-6 of *N*-acetylgalactosamine residues. Both these types of bond have previously been reported in glycoproteins (Kuhn & Brossmer, 1958; Popenoe, 1959; Kamiyama &

Schmid, 1961; Egge, 1960; Gottschalk & Graham, 1959; Graham & Gottschalk, 1960).

The second cycle of the Smith degradation involved attack on one mannose, six *N*-acetylgalactosamine and three *N*-acetylglucosamine residues, about 9 mol. of oxidant/mol. of BP 1 being reduced rapidly and a further 12 mol./mol. of BP 1 more slowly. If all the component monosaccharide residues were cleaved between C-2 and C-3 and between C-3 and C-4, 20 molar proportions of periodate would be utilized, in good agreement with the 21 mol. found. If it is assumed also that the mannose lost in this step was protected from previous attack by the linking of the mannose unit destroyed in the first step to C-3, then the nine hexosamine units lost must have been protected until this step by the nine galactose residues removed in the previous degradation cycle. This can be explained if each of these hexosamine units in intact BSP was linked at C-3 (or possibly C-4) to galactose. Substitution of *N*-acetylhexosamine residues at C-4 affords protection towards periodate (Kamiyama & Schmid, 1961; Spiro, 1964; Jeanloz, 1964; Montgomery *et al.* 1965), indicating that *N*-acetylhexosamine residues are not susceptible to cleavage between C-2 and C-3. Although attack by periodate between C-2 and C-3 of an *N*-acetylhexosamine is improbable, it may arise nevertheless if loss of the *N*-acyl group occurs during the Smith degradation, e.g. at the acid-hydrolytic step. It is of interest that Nicolet & Shinn (1939) found that α -hydroxy-*N*-acylamines were susceptible to slow attack by periodate.

The identification of some erythritol together with larger amounts of glycerol during the mild acid hydrolysis of this degradation step indicates that some of the amino sugar residues may have been protected by substitution.

The oxidation reaction occurred in two phases, the galactosamine residues and the mannose residue being rapidly attacked, whereas the attack on the *N*-acetylglucosamine residues was much slower. The rapid oxidation is visualized as consisting of the reduction of 2 mol. of periodate/mol. of BP 1 for the mannose residue and a further 6 mol./mol. of BP 1 for the cleavage between C-3 and C-4 of all the galactosamine residues. Thus 8 mol. of periodate/mol. of BP 1 would be required in this phase of the reaction, in fair agreement with the 9 mol./mol. of BP actually found. The slow phase is envisaged as cleavage of all nine hexosamine residues requiring 9 mol. of periodate/mol. of BP 1, as against the observed reduction of 12 mol./mol. of BP 1.

The third cycle of the Smith degradation resulted in the destruction of two further *N*-acetylglucosamine residues and the remaining mannose residue, which must all have been preserved from previous

Scheme 1. Possible structure of carbohydrate prosthetic group, after removal of sialic acid residues, as suggested from the results of Smith degradation. Solid bars across the bonds indicate sites of rapid oxidation by periodate at the degradation step indicated. Open bars across the bonds indicate sites of slow oxidation. Ac, acetyl.

attack by suitable attachment of oligosaccharide chains destroyed in the earlier steps. If these three residues all occupied terminal positions, or were 1,6-linked in BP 2, which may be indicated by the identification of only glycerol after mild acid hydrolysis, the maximum amount of periodate reduced would be 6 mol./mol. of BP 2. The observed reduction of 16.0 mol./mol. of BP 2 is therefore difficult to explain. These two *N*-acetylglucosamine units and the mannose unit must be linked to the galactose and the *N*-acetylglucosamine units that survive this oxidation cycle in such a way as to confer protection on them.

After the fourth cycle of the Smith degradation a single residue of *N*-acetylglucosamine remained, and must have been protected from attack by the linkage to its C-3 position of the galactose residue still present after three cycles. The survival of one *N*-acetylglucosamine unit is strong evidence that BSP contains only one carbohydrate prosthetic group, which must be highly branched.

A tentative structure for the carbohydrate part of the BSP molecule may be suggested as three principal oligosaccharide chains (of the possible type shown in Scheme 1) in which sialic acids are terminal groups and one chain in which fucose may be terminal. These are envisaged as being linked to an inner 'core', consisting of three *N*-acetylglucosamine residues, one galactose and one mannose, which in turn links the prosthetic group to the polypeptide moiety. Asparagine or aspartic acid has already been implicated in a glycosylamine bond linking the carbohydrate and polypeptide moieties of BSP (Andrews *et al.* 1967), and the above results would indicate that the bond joins C-1 of *N*-acetylglucosamine to the β -amino group of asparagine. The type of bond proposed here to be present in BSP has been established in ovalbumin (Marshall & Neuberger, 1964) and has been suggested for many other glycoproteins, including orosomucoid (Kamiyama & Schmid, 1962; Eylar, 1962; Satake, Okuyama, Ishihara & Schmid, 1965), γ -globulins (Rothfus & Smith, 1963), fetuin (Spiro, 1963), transferrin (Spik, Monsigny & Montreuil, 1965) and ovomucoid (Montgomery & Wu, 1963; Montreuil, Biserte & Chosson, 1963).

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