Some Studies on the Composition of Bovine Cortical-Bone Sialoprotein

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1. An analysis of bovine bone sialoprotein, a homogeneous glycoprotein isolated from cortical bone, is presented. 2. Analytical results agree with earlier physical measurements indicating a molecular weight of about 23000. 3. Mild acid hydrolysis and treatment with neuraminidase showed that fucose and sialic acid occupy terminal positions on oligosaccharide chains. 4. Treatment of the sialic acid-free glycoprotein with β -galactosidase showed that much of the galactose occupies a sub-terminal location in the intact glycoprotein. 5. The polypeptide chain is rich in aspartic acid, glutamic acid, serine, threonine and glycine, and has no detectable free terminal amino group. 6. Glycopeptides were studied after proteolytic digestion. 7. It is considered that the carbohydrate moiety is highly branched and is probably linked by an acid- and alkali-stable glycosylamine bond involving aspartic acid.

Conjecture about the processes that occur during mineralization has stimulated interest in the nature of the organic matrices of tissues that have the ability to calcify (see reviews by Eastoe, 1956; Herring, 1964a; Balazs & Rogers, 1965). It has also been suggested, mainly on histochemical evidence, that carbohydrate-containing components of the ground substance may be responsible for the binding and localization in bone of certain radioisotopes such as ^{91}Y , ^{239}Pu and ^{241}Am (Vaughan, 1956; Herring, Vaughan & Williamson, 1962; Williamson & Vaughan, 1964).

With this in mind, studies have been made on the extraction and separation of glycoproteins and glycosaminoglycans from bovine cortical bone, and four main constituents have been isolated (Herring & Kent, 1963; Herring, 1964b, 1966). Of these, the sialoprotein, with strongly acidic properties and which reacted with the periodate-Schiff procedure, appeared to be of particular interest. The physical properties of this glycoprotein have been studied (Williams & Peacocke, 1965), and its binding capacity with Ca^{2+} , Y^{3+} and Th^{4+} ions has been demonstrated (Peacocke & Williams, 1966).

The present paper is concerned with further studies on the composition and structure of bovine BSP.† Some of this work has appeared in preliminary form (Andrews & Herring, 1965).

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† Abbreviation: BSP, bone sialoprotein.

MATERIALS AND METHODS

Bovine bone sialoprotein. BSP was prepared as the zinc salt according to the methods described by Herring (1964b).

Moving-boundary electrophoresis. An Antweiler micro-electrophoresis apparatus was used. BSP solutions (1%, w/v, dialysed thoroughly against the appropriate buffer) were examined in 0.1M-sodium acetate buffer, pH 5.0, or 0.12M-sodium diethylbarbiturate buffer, pH 8.6. Runs were performed at 80 v and at about 1.5 mA for 10 min. Photographs were taken at 2 min. intervals with the schlieren optical system, and at the end of runs measurements were made of the refractive index of the solution against distance travelled by the boundary.

Cellulose acetate electrophoresis. Electrophoresis was also carried out on cellulose acetate strips (5 cm. $\times$ 18 cm.) (Oxoid Division of Oxo Ltd., London, E.C. 4) run for 4 hr. at 50 v in a Shandon Universal Mark II apparatus. The same buffers used for moving-boundary electrophoresis were employed, and strips were subsequently air-dried and stained with aq. 1% (w/v) alcian blue solution in 1% (v/v) acetic acid for 10 min., or with 0.5% light green in methanol-acetic acid (9:1, v/v) (Smith, 1960).

Moisture content. Weighed samples of BSP were heated at 105° for 24 hr., cooled in a desiccator and reweighed to give the dry weight.

Zinc content. Samples of BSP were kindly analysed for zinc by Dr M. Webb of the Strangeways Research Laboratory, Cambridge, with a Perkin-Elmer model 303 atomic-absorption spectrophotometer.

Sialic acids. Quantitative estimation of the total sialic acid content was performed by the direct Ehrlich procedure (Werner & Odin, 1952), or by the thiobarbituric acid procedure (Warren, 1959) after hydrolysis with neuraminidase or 0.1 N- H_2SO_4 at 80° for 1 hr.

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Paper chromatography of sialic acids was carried out on Whatman no. 1 chromatographic paper that had been prewashed with 2*N*-acetic acid and water and dried. Chromatograms were developed with butan-1-ol-propan-2-ol-0.1*N*-HCl (1:2:1, by vol.) and ethylacetate-acetic acid-water (3:1:3, by vol.) (Gottschalk, 1960, p. 62) for 24 hr., and also with butan-1-ol-pyridine-water (6:4:3, by vol.) for 30 hr. The reagents described by Warren (1960) were used for detection of sialic acids.

It was found to be advantageous to remove cations from samples of sialic acid-containing solution (10–100 μ l.) before paper chromatographic analysis by passing them through a small pad (0.5 cm. $\times$ 1 cm.) of Zeo-Karb 225 SRC-24 (<200 mesh) ion-exchange resin (H^+ form). The pad was eluted with water (1 ml.), and the eluate collected on a watch-glass and evaporated to dryness over NaOH pellets. The residue was dissolved in a little water and applied to the paper strips.

For the quantitative determination of the proportion of sialic acid existing as the *N*-glycollyl derivative, a solution containing the mixed sialic acids obtained from hydrolysis of BSP was used. A portion (4 ml.), containing about 500 μ g. of total sialic acid as assayed by the direct Ehrlich procedure, was filtered through a small column (4.7 cm. $\times$ 0.5 cm.) of Dowex 2 (X8; 200–400 mesh) ion-exchange resin (formate form) to remove interfering substances. The column was washed with water (4 ml.) and sialic acids were eluted with 0.4*N*-formic acid (5 ml.). The eluate was evaporated to dryness over NaOH pellets and the residue dissolved in *N*-H₂SO₄ (1 ml.). This solution was heated on a boiling-water bath for 1 hr. and filtered through a small column (1.0 cm. $\times$ 0.5 cm.) of a mixture (1:1, w/w) of activated carbon (Ultrasorb S.C. 120/240; British Carbo-Norit Union Ltd., West Thurrock, Essex) and Celite 535 (Johns-Manville Co. Ltd., London, S.E. 1). The filtrate was evaporated to 0.45 ml. and glycollic acid assayed in 0.2 ml. portions as described by Gottschalk (1960, p. 60). From this the content of *N*-glycollylneuraminic acid in the initial solution was calculated and hence the proportion of *N*-glycollyl to *N*-acetyl derivative, as the colour yields of both these derivatives are similar in the direct Ehrlich procedure.

The rate of release of total sialic acids from BSP on acid hydrolysis was studied in both 0.1*N*-H₂SO₄ and 2*M*-H₂SO₄ at 80°. In each case the concentration of BSP was 1.13 mg./ml. and 0.2 ml. portions were withdrawn at intervals up to 2 hr. for assay of sialic acid by the thiobarbituric acid method (Warren, 1959). Similar experiments were used to study the enzymic release of sialic acid. BSP (2.7 mg.) dissolved in water (1.5 ml.) was incubated at 37° with 1 ml. of neuraminidase solution (L. Light and Co. Ltd., Colnbrook, Bucks.). At suitable intervals up to 6 hr., 0.1 ml. portions were withdrawn for sialic acid assay.

Acid hydrolysis of bone sialoprotein. The optimum yield of free hexose was obtained by heating a 0.1% solution of BSP in 3*N*-HCl at 100° for 4 hr. Optimum release of hexosamines was achieved after 4 hr. heating at 100° with 4*N*-HCl. In both cases recoveries of 95.0% were achieved, as judged by the results of direct determination of hexose without prior hydrolysis, or by the use of internal-standard mixtures during hydrolysis.

Hexose analyses. For the quantitative estimation of neutral hexose the anthrone method (Yemm & Willis, 1954) was used as a routine, both directly on BSP samples and after hydrolysis and separation of the neutral hexoses.

The conc. H₂SO₄/water ratio in the anthrone reagent was, however, increased from 5:2 (v/v) to 4:1 (v/v) to overcome the turbidity occasionally encountered after mixing the sample and reagent.

After hydrolysis and paper-chromatographic separation of the neutral monosaccharides followed by elution of the individual components with water, galactose, mannose and fucose were also estimated by the *p*-aminobenzoic acid method (Leopold, 1962) and the reducing method of Park & Johnson (1949).

Paper chromatograms of neutral monosaccharides on prewashed Whatman no. 1 paper were developed as a routine with ethyl acetate-pyridine-water (8:2:1, by vol.) (Leopold, 1962) for 16–24 hr. for qualitative separations or 40–48 hr. for quantitative work. The R_{Glc} values were 0.79 for galactose, 1.30 for mannose and 1.92 for fucose. For initial qualitative identification the organic phases of butan-1-ol-acetic acid-water (8:2:3, by vol.) and benzene-butan-1-ol-pyridine-water (1:5:3:3, by vol.) (Gaillard, 1953) were also used with 18 hr. development in both cases. For detection of monosaccharides the alkaline-AgNO₃ method (Trevelyan, Procter & Harrison, 1950), the aniline hydrogen phthalate method (Partridge, 1949) and the aniline hydrogen oxalate method (Horrocks & Manning, 1949) were employed. For quantitative paper chromatography it was found advantageous to desalt samples of hydrolysates (Herring & Kent, 1963) to avoid distortion of spots. The quantitative paper-chromatographic method of Wilson (1959) was also used.

Fucose estimation. To separate fucose liberated by acid hydrolysis from unhydrolysed material, the adsorptive properties of activated carbon (Ultrasorb) were utilized (Hughes & Whelan, 1958). Small columns of the 1:1 (w/w) activated-carbon-Celite mixture (0.1 g.), packed between two layers of Celite 3 mm. thick, were prepared in 0.5 cm. $\times$ 10 cm. tubes, the lower ends of which were closed by sintered-glass disks. The columns were washed with water (3 ml.) before use.

Samples (1 ml.) of a solution of BSP (9.2 mg.) in 0.1*N*-H₂SO₄ (11.2 ml.) were heated on a boiling-water bath for various times (0–8 hr.). The hydrolysates were passed through carbon-Celite columns that were each washed with water (1 ml.) and the monosaccharides were eluted with aq. 10% (v/v) ethanol (1 ml.). Fucose was estimated in the eluates by the thioglycollic acid method of Gibbons (1955). Standard fucose solutions of 2 μ g./ml., 5 μ g./ml. and 20 μ g./ml. were passed through this procedure and the overall recovery was found to be $97 \pm 2\%$.

Measurements of the total fucose content of BSP were also determined by quantitative paper chromatography and by direct application of the thioglycollic acid procedure (Gibbons, 1955) to BSP without prior hydrolysis, as well as after total hydrolysis followed by purification on carbon-Celite columns as above. Under the conditions of the method contributions to the fucose value by the presence of hexose and protein were negligible.

Hexosamine analysis. The modification of the Elson-Morgan reaction described by Boas (1953) was used as a routine. Galactosamine and glucosamine gave similar colour yields in this procedure.

Qualitative paper chromatography of hexosamines on prewashed Whatman no. 1 paper was performed with ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.) (Fischer & Nebel, 1955), butan-1-ol-pyridine-water (3:2:3,

by vol.) or butan-1-ol-pyridine-water (5:4:3, by vol.) for development. With the last-named solvent, which gave the best separation, galactosamine had $R_{\text{galactosamine}}$ 0.76, but with all solvents there was considerable elongation of spots.

The glucosamine/galactosamine ratio in hydrolysates was determined by chromatography on columns (0.6 cm. $\times$ 45 cm. of Dowex 50 (X8; H⁺ form; 250–400 mesh) with a flow rate of 2.75 ml./hr., as described by Gardell (1953).

Action of β -galactosidase. The enzyme was grade III (from liver; lot no. 22B-605) obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Sialic acid was removed from BSP by acid hydrolysis and a sample of the product (3.1 mg.) dissolved in 0.1 M-sodium phosphate buffer, pH 7.2 (0.5 ml.). β -Galactosidase (9.8 mg.) was added and the mixture incubated at 37° for 117 hr. under toluene to inhibit bacterial growth. The digest was passed through a carbon-Celite column (0.4 g., 0.5 cm. $\times$ 3.5 cm.) similar to that used for fucose estimations. The column was eluted consecutively with water (4 ml.), aq. 5% (v/v) ethanol (5 ml.), aq. 20% (v/v) ethanol (5 ml.) and aq. 50% (v/v) ethanol (5 ml.), each eluate being made up to 10 ml. Hexose was determined in 1 ml. portions by the anthrone procedure.

Action of alkali on bone sialoprotein. BSP (4.7 mg.) was dissolved in water (1 ml.), and 0.4 N-NaOH (1 ml.) was added. The mixture was heated at 100° for 2 hr., neutralized with 0.2 N-HCl (2 ml.) and dialysed three times against a small volume (6.5 ml.) of distilled water. The solution of non-diffusible components was made up to 10 ml., and the combined diffusates were made up to 20 ml. with water.

Ultraviolet spectra. The ultraviolet spectra of solutions of BSP in water and in 0.1 N-NaOH were measured, with 1 cm. silica cells, in a Unicam SP.500 spectrophotometer.

Amino acid analysis. Samples of BSP (5.2 mg.) were treated with performic acid (Fraenkel-Conrat, Harris & Levy, 1955) and the residues dissolved in 5.7 N-HCl (1 ml.). The sample tubes were sealed and heated at 100° for 24, 48 and 72 hr. The amino acids released were determined quantitatively with a Beckman automatic amino acid analyser. In three earlier experiments with 24 hr. hydrolysis time the pretreatment with performic acid was omitted. Amino acid analyses on glycopeptide fragments without prior performic acid treatment and also with 24 hr. hydrolysis times were carried out on a Technicon Autoanalyser.

For qualitative paper chromatography of amino acids butan-1-ol-acetic acid-water (4:1:5, by vol.) (Levy & Chung, 1953) was employed for development, chromatograms usually being run overnight. A 0.1% solution of ninhydrin in ethanol containing collidine (5%, v/v) (Levy & Chung, 1953) was used as the spray detection reagent.

Tyrosine was also estimated colorimetrically by a procedure with α -nitroso- β -naphthol (Cobbett, Kenchington & Ward, 1962) and the tyrosine/tryptophan ratio measured from the ultraviolet spectrum in 0.1 N-NaOH (Bencze & Schmid, 1957).

N-Terminal amino acid assay. Both the fluorodinitrobenzene procedure of Sanger (1952) and the formation of phenylthiohydantoin derivatives (Edman, 1950) were employed.

Paper chromatography of DNP-amino acids was performed on Whatman no. 1 paper buffered to pH 6.0 with phthalate (Blackburn & Lowther, 1951) by using buffer-saturated 2-methylbutan-2-ol for elution.

Starch-impregnated paper (Sjöquist, 1953) was used for the paper chromatography of phenylthiohydantoin deriva-

tives. Paper strips were developed with heptane-pyridine (7:3, v/v), heptane-butan-1-ol-90% formic acid (4:4:2, by vol.) or heptane-butan-1-ol-90% formic acid (40:30:9, by vol.) (Sjöquist, 1960) and the products revealed with the iodine-azide reagent (Sjöquist, 1953). In addition to the tentative identification of phenylthiohydantoin derivatives by their mobility in the above solvents, the derivatives were also hydrolysed with HI (Elliott & Peart, 1957) and the liberated amino acids examined by paper chromatography.

Phosphate analysis. BSP (1.6 mg.) was digested with conc. HNO₃ (0.5 ml.) and conc. H₂SO₄ (0.5 ml.) by heating until a clear solution was obtained. The digest was diluted with water to 4 ml. and phosphate assayed in 1 ml. portions by the method of Chen, Toribara & Warner (1956).

Identification of serine phosphate. For partial acid hydrolysis of the polypeptide chain, BSP (7.6 mg.) was dissolved in 2 N-HCl (1 ml.) and heated in a sealed tube at 100° for 16 hr. On cooling the hydrolysate was evaporated to dryness in the presence of NaOH pellets, and the residue dissolved in a buffer (0.06 ml.) 0.03 N with respect to both formic acid and acetic acid (pH 2.3), and applied as a line at the origin on a strip (40 cm. $\times$ 20 cm.) of Whatman 3MM chromatographic paper for high-voltage electrophoresis in a Pharograph apparatus. Electrophoresis for 90 min. at 3100 v and 20 ma was followed by spraying of the dried strips with 0.1% ninhydrin in ethanol containing collidine (5%, v/v) and heating at 100° for 5 min. to reveal amino acids, peptides and phosphoamino acids. Areas, on the anode side of the origin, from unstained strips, corresponding to areas containing ninhydrin-positive material, were eluted with 5.7 N-HCl (1 ml.). The eluates were heated at 104° for 24 hr. and amino acids released studied by paper chromatography.

Action of Pronase on bone sialoprotein. The protease from *Streptomyces griseus* (type V, purified; lot no. 53B-1530) was obtained from Sigma Chemical Co. and is referred to below as Pronase. The enzyme (75 mg.) was added to a solution of BSP (255 mg.) in 3 ml. of 0.1 M-tris-HCl buffer, pH 7.6, and incubation at 37° carried out for 8 days under toluene. Further portions of Pronase (15 mg.) were added after 2, 4 and 6 days' incubation. The mixture was then freed from salts and small peptides on a column (1.0 cm $\times$ 78 cm.) of Sephadex G-25 (fine grade, particle size 20–80 μ ; Pharmacia, Uppsala, Sweden) made up in water, and with water elution at a flow rate of 5–7 ml./hr. The void volume of the columns was 19.0 ml. Fractions of volume 1.5 ml. were collected and portions (50 μ l.) withdrawn for assay of hexose by the anthrone procedure and of sialic acid by the direct Ehrlich procedure. The presence of peptide material in fractions diluted with water (2 ml.) was indicated by E_{240} measurements.

Fractions containing glycopeptide material (sialic acid- and hexose-positive fractions) were pooled and evaporated to dryness, and the residue was dissolved in 3 mM-pyridine-acetate buffer, pH 5.1 (1.0 ml.), for study by chromatography on a column (2.0 cm. $\times$ 12 cm.) of DEAE-cellulose (Sigma Chemical Co.) made up in the 3 mM-buffer. After application of the sample to the column, the column was eluted with the 3 mM-buffer. This was followed by a linear elution gradient with the 3 mM-buffer (110 ml.) in the first reservoir and 0.6 M-pyridine-acetate buffer, pH 5.1 (110 ml.), in the second reservoir. After washing with 0.6 M-buffer (30 ml.) a second linear gradient was applied, with the 0.6 M-pyridine-acetate buffer (30 ml.) in the first reservoir and the same buffer (30 ml.) containing NaCl (0.5 M) in the

second. Fractions of volume 2.0 ml. were collected and portions (0.1 ml.) assayed for hexose and sialic acid. Fractions making up the individual hexose- and sialic acid-positive peaks were pooled, evaporated to dryness and dissolved in water (1 ml.).

RESULTS

Electrophoretic and ultracentrifugal analysis. The preparation of BSP used throughout this work behaved as a single homogeneous component on both moving-boundary and cellulose acetate electrophoresis at pH 5.0 and pH 8.6. The same batch of material has also been reported to be homogeneous on extensive ultracentrifugal analysis (Williams & Peacocke, 1965).

Carbohydrate constituents. Paper chromatograms of the sialic acids released by acid hydrolysis and neuraminidase treatment appeared to be identical, the major component moving identically with *N*-acetylneuraminic acid in all solvents. In addition there was a minor component identified as *N*-glycollylneuraminic acid from its mobility in each of the three solvents.

This was confirmed by colorimetric assay of the glycollic acid released on hydrolysis, which showed that 10.2% of the total sialic acid was present as the *N*-glycollyl derivative. This derivative gives a lower colour yield in the thiobarbituric acid assay than the *N*-acetyl derivative (Warren, 1959); if allowance is made for this, BSP contains 2.1% by weight of *N*-glycollylneuraminic acid and 18.4% of *N*-acetylneuraminic acid, giving a total sialic acid content of 20.5%, in close agreement with the value obtained by the direct Ehrlich procedure.

The graph showing the rate of release of sialic acid on hydrolysis in 0.1 *N*-sulphuric acid is shown in Fig. 1(a). After 30 min. 97% of the total sialic acid

was liberated. The curve obtained on hydrolysis with 2 *MN*-sulphuric acid was similar in general shape, and 94% was released after 50 min. Under the conditions described neuraminidase liberated 97% of the total sialic acid in 7 hr. (Fig. 1b).

Acid hydrolysis of BSP for the release of other monosaccharides was accompanied by considerable humin formation. This appeared to be due to the presence of sialic acid, since hydrolyses of BSP from which sialic acid had previously been removed, or of BSP that had been pretreated with sodium metaperiodate or performic acid, resulting in destruction of the sialic acid, were not accompanied by humin formation.

Total hexose content of BSP by the anthrone procedure was 11.7% by weight, but after taking into account the fucose value of 0.70% a corrected value of 10.7% was obtained (fucose gave a higher colour yield than galactose and mannose in the anthrone reaction). The aldoses galactose, mannose and fucose were identified by paper chromatography. The results of eight series of quantitative paper-chromatographic experiments showed that these components occurred in the proportions 10:3:1 respectively.

The total content of fucose in BSP measured colorimetrically by the thioglycollic acid method was 0.70% by weight. The rate of release of fucose on acid hydrolysis is illustrated in Fig. 2. After 8 hr. hydrolysis 82.3% of the total fucose had been liberated. The preferential release of fucose under these conditions of hydrolysis was shown by the finding that after 2 hr. hydrolysis 70% of the fucose had been liberated but only 16% of the total hexose had been released.

BSP was found to contain 9.16% by weight of hexosamine. There appeared to be approximately

Fig. 1. Rate of liberation of sialic acid. (a) BSP (1.13 mg./ml.) hydrolysed in 0.1 *N*-H₂SO₄ at 80°. (b) BSP (1.8 mg./ml.) incubated in neuraminidase solution at 37°. Sialic acid was estimated by the thiobarbituric acid method (Warren, 1959).

Fig. 2. Rate of liberation of fucose on mild acid hydrolysis. BSP (0.8 mg./ml.) was hydrolysed in 0.1 N-H₂SO₄ at 100°. Fucose was estimated in 1 ml. portions.

equal quantities of glucosamine and galactosamine present on qualitative paper-chromatographic analysis. The proportions of these two constituents were determined more accurately by ion-exchange chromatography (Gardell, 1953). Hydrolysis of 6.3 mg. of BSP and separation of the hexosamines in this manner yielded 239 μ g. of glucosamine and 243 μ g. of galactosamine. Allowing for a 90.0% recovery, as judged from experiments involving the use of internal standard of both hexosamines, during the hydrolysis (2 N-hydrochloric acid at 100° for 4 hr.), a recovery of 93.4% from the Dowex 50 column was obtained, and it was therefore concluded that BSP contained almost equal quantities of glucosamine and galactosamine. These were probably present as the *N*-acetyl derivatives, as indicated by the absence of DNP-hexosamine after treatment of BSP with fluorodinitrobenzene.

The carbohydrate composition of BSP is summarized in Table 1, molar proportions being calculated on the basis of a molecular weight of 23 000 for BSP (Andrews & Herring, 1965).

Action of β -galactosidase. Removal of sialic acid from BSP gave material that was only slowly attacked by β -galactosidase. Even under the very vigorous conditions described (see the Materials and Methods section) only 61.9% of the total galactose had been released after 117 hr. These conditions were not considered to represent the optimum, however, because less vigorous treatment released correspondingly less galactose, and it seems reasonable to suppose that longer incubation times might free further small quantities of galactose. The galactose was present in the aq.-5%-ethanol eluates from the charcoal-Celite columns and no evidence was obtained of release of any other constituents by this enzyme preparation.

Action of alkali on bone sialoprotein. Hexose analyses performed after treatment with alkali (see the Materials and Methods section) showed that, even under the vigorous conditions employed, 87.4%

Table 1. *Composition of the non-peptide portion of the bone sialoprotein molecule*

Percentages are expressed in terms of the dry weight of BSP, the molecular weight of which is taken to be 23 000, and are corrected for a moisture content of 13.4%.

	Composition	
	(%, w/w)	(mol./mol. of BSP)
Total sialic acids (Warren, 1959)	20.5	15.3
Total sialic acids (direct Ehrlich)	20.4	15.2
<i>N</i> -Acetylneuraminic acid	18.4	13.7
<i>N</i> -Glycolylneuraminic acid	2.1	1.6
Total hexose	10.7	13.6
Galactose	8.2	10.5
Mannose	2.5	3.1
Fucose	0.7	1.0
Total hexosamine	9.2	11.8
Glucosamine	4.6	5.9
Galactosamine	4.6	5.9
Zinc	1.4	5.0
Phosphate	1.4	3.4

of the initial hexose was present as non-diffusible material, presumably still linked to polypeptide. Since hexose was not detectable in the diffusate it was concluded that few hexose-containing oligosaccharides were released on treatment with alkali and that the carbohydrate residues of BSP were relatively alkali-stable. This was also indicated by the results of an earlier experiment in which, after alkaline hydrolysis at 80° for 1 hr. in 0.01 N-sodium hydroxide, both hexose and hexosamine were recovered quantitatively in the non-diffusible material.

Ultraviolet spectra of bone sialoprotein. (Fig. 3). From the ultraviolet spectrum in 0.1 N-sodium hydroxide the tyrosine/tryptophan ratio may be calculated (Bencze & Schmid, 1957), and for BSP this ratio was found to be 2.0:1.0. The total concentration of tyrosine and tryptophan may be calculated from the height of the maximum at 290 m μ . The initial weight of BSP taken was known, so, from the above ratio, the concentration of each of the two amino acids was calculated as a percentage of BSP by weight. There must, however, be at least 1 residue of tryptophan/mol. of BSP, so a minimum value for the molecular weight may be calculated. For BSP this was found to be 23 600, in close agreement with the values from ultracentrifugal analysis; this minimum value was therefore also the true value for the molecular weight.

Amino acid analysis. The results of amino acid analysis after the hydrolysis of performic acid-oxidized BSP are shown in Table 2. In other experiments without prior oxidation small quantities of cysteine were isolated and it was considered

Fig. 3. Ultraviolet spectra of BSP in water (1.5 mg./ml.) (○) and in 0.1N-NaOH (1.9 mg./ml.) (Δ).

Table 2. Amino acid composition of bone sialoprotein

	Amino acid composition	
	(%, w/w)	(mol./mol. of BSP)
Lysine	1.59	2.52
Histidine	0.74	1.07
Ammonia	0.93	12.55
Arginine	0.76	1.04
Cysteic acid	0.81	1.10
Aspartic acid	8.80	15.20
Threonine	5.02	9.70
Serine	3.20	7.00
Glutamic acid	12.80	20.09
Proline	2.15	4.27
Glycine	3.49	10.71
Alanine	1.36	3.51
Valine	1.38	2.71
Isoleucine	1.25	2.19
Leucine	1.43	2.53
Phenylalanine	0.72	1.00
Tyrosine*†	1.54	1.95
Tryptophan†	0.87	0.98

* Determined colorimetrically (Cobbett *et al.* 1962).

† Determined spectrographically (Bencze & Schmid, 1957).

that the cysteic acid shown in Table 2 occurred as cysteine in the native glycoprotein.

Calculation of a minimum value for the molecular weight of BSP, based on the assumption of at least 1 residue each of histidine, arginine, phenylalanine and tryptophan/mol., gave about 23 000. Thus this was also the true molecular weight and was in good agreement with the results of physical measurements.

No N-terminal amino acid could be detected by

either the fluorodinitrobenzene or phenyl isothiocyanate methods, either with or without prior treatment of the BSP with performic acid, and indeed BSP gave an almost colourless solution after treatment with fluorodinitrobenzene. It was therefore concluded that BSP had no free terminal amino group.

Phosphate groups. Total phosphate was found to be 1.4% by weight, which was equivalent to 3.4 mol./mol. of BSP. This was labile on treatment with 0.2N-sodium hydroxide at 100° for 2 hr., but less than 3% of the phosphate was liberated by mild acid treatment (0.1N-hydrochloric acid at 80° for 1 hr.). Stronger acid hydrolysis of BSP (2N-hydrochloric acid at 103° for 4.7 hr.), followed by removal of monosaccharide constituents on carbon-Celite columns and phosphate analysis on the eluate, showed that 43% of the total phosphate was present in the free state. The remaining 57% was liberated on further treatment of the eluate with 0.2N-sodium hydroxide at 100° for 2 hr. Thus 57% was still bound, presumably to polypeptide, after hydrolytic removal of all the carbohydrate constituents of BSP.

Partial acid hydrolysis of the polypeptide chain and high-voltage electrophoresis of the residue revealed two major ninhydrin-positive anodic components. A compact strongly stained zone had moved 4–5 cm. from the origin and a larger weakly stained zone had moved 9–17 cm. from the origin. A sample of standard serine phosphate moved 16 cm. on the same sheet. Areas of unstained electrophoretograms 2–7 cm. (X) and 7–17 cm. (Y) from the origin were cut out, eluted with 5.7N-hydrochloric acid and hydrolysed to release the amino acids present in these areas, which were considered to contain phosphoamino acids and phosphopeptides. On qualitative paper chromatography, although both X and Y contained traces of other amino acids including arginine, threonine, serine, glutamic acid, alanine, proline, valine and phenylalanine, serine was predominant in Y, and this was taken to be an indication that serine phosphate was initially present in the partial acid hydrolysate of BSP, and that at least part of the phosphate content of this substance was linked to the hydroxyl groups of serine in the intact glycoprotein.

Separation and analysis of glycopeptide fractions. Chromatography of the Pronase digest of BSP on Sephadex G-25 revealed that glycopeptide material was completely excluded from the Sephadex particles, and was therefore readily separated from salts and small peptide fragments. The results of further chromatography on DEAE-cellulose are shown in Fig. 4. Fractions were pooled as indicated on the diagram to give samples of the individual glycopeptide peaks and portions taken for suitable analyses (Table 3).

The three major peaks (*G*, *H* and *J*) were re-chromatographed on Sephadex G-50 (fine grade, particle size 20–80 μ), a column (1 cm. $\times$ 79 cm.) being made up in water and eluted with water. Peak *J* was resolved into two glycopeptide peaks (*J*₁ and *J*₂) and a further peak *J*₃, which did not contain sialic acid and was not further studied.

Peaks *A*, *B* and *C* contained largely polyglucose material resulting from the starch added to this particular preparation of Pronase during manufac-

ture as a stabilizing agent. On the basis of hexosamine analyses it was calculated that no more than 100 μ g. of glycopeptide material was present in these three peaks, which were therefore discarded.

The combined yield of all glycopeptide fractions amounted to 30.7% of the initial weight of BSP taken.

DISCUSSION

Gottschalk (1952) advanced the concept that glycoproteins should be visualized as conjugated proteins consisting of a polypeptide chain to which relatively small polysaccharides are linked covalently as individual prosthetic groups. Subsequent work has substantiated this view, though the size, number and composition of both the carbohydrate prosthetic groups and the polypeptide moiety vary considerably from one glycoprotein to another. The constituents of glycoproteins have been extensively reviewed (e.g. Gottschalk, 1962; Spiro, 1963; Schmid, 1964; Jeanloz, 1964), and, in the light of the analytical values reported, BSP emerges as a typical glycoprotein. Two notable features are the unusually high content of sialic acids, and the finding of both galactosamine and glucosamine in approximately equal proportions, as in general glycoproteins appear to contain predominantly only one hexosamine. However, a glycoprotein appearing somewhat similar to BSP has been reported to be present in

Fig. 4. Chromatography on a DEAE-cellulose column (2.0 cm. $\times$ 12 cm.) of a Pronase digest of BSP with a double gradient elution. Fractions 1–35 were eluted with 3 mM-pyridine-acetate buffer, pH 5.1, followed by 3 mM–0.6 M-pyridine-acetate buffer, pH 5.1, for fractions 35–150, and 0.05 M-NaCl in 0.6 M-pyridine-acetate buffer, pH 5.1, for fractions 150–200. —, Hexose (anthrone procedure); ----, sialic acid (direct Ehrlich).

Table 3. *Composition of glycopeptide fractions*

Components are expressed as mol./12 mol. of hexosamine, i.e. as mol./mol. of BSP.

Constituent	Fraction ...	Composition (mol./mol. of BSP)						
		<i>D</i>	<i>E</i>	<i>F</i>	<i>G</i>	<i>H</i>	<i>J</i> ₁	<i>J</i> ₂
Aspartic acid		1.1	1.5	1.0	1.0	1.1	2.5	2.6
Serine/threonine		4.1	1.5	2.7	6.6	6.7	9.1	7.1
Glutamic acid		0.6	1.2	0.5	0.5	0.5	2.3	2.9
Proline		1.8	0.1	0.4	3.9	3.2	3.8	2.2
Glycine		1.1	0.8	0.7	4.8	3.3	4.7	4.3
Alanine		0.7	0.4	0.2	0.4	0.4	0.7	1.0
Isoleucine		0.3	0.1	0.2	1.7	1.6	0.9	1.2
Leucine		0.4	0.2	0.2	0.4	0.7	0.6	0.2
Phenylalanine		0.2	0.1	0.1	0	0.7	0.5	0.2
Lysine		2.0	0.2	0.7	2.0	0.3	0.5	0.1
Histidine		1.1	0.2	0.6	0.4	0.2	0.4	0.3
Arginine		0.2	0.1	0	0.1	0.1	0.1	0.2
Valine		0.7	0	0.2	0.1	0	0	0
Tyrosine		0.1	0	0	0	0	0	0
Ammonia		6.0	3.2	1.8	5.8	4.6	5.2	4.0
Cysteic acid		0	0	0	0	0	0	0
Sialic acids		2.0	2.3	5.0	7.3	12.5	8.5	15.0
Hexosamine		12	12	12	12	12	12	12
Hexose		10.0	9.0	10.7	10.4	12.6	12.0	12.7
Galactose		7.0	5.0	7.4	7.2	9.6	9.0	9.7
Mannose		3	3	3	3	3	3	3

developing bovine teeth (Zamosciany & Veis, 1966).

The total release of the sialic acids of BSP by neuraminidase or by very mild acid hydrolysis, insufficiently vigorous to release any other components except for small amounts of fucose, strongly suggested a terminal location for sialic acid in the oligosaccharide chains. Neuraminidase is known to cleave the α -(2 $\rightarrow$ 6)-linkage of *N*-acetylneuraminic acid to *N*-acetylgalactosamine (Gottschalk & Graham, 1959), the α -(2 $\rightarrow$ 3)-linkage to galactose (Kuhn & Brossmer, 1958; Popenoe, 1959) and the α -(2 $\rightarrow$ 6)-linkage to galactose (Egge, 1960). Sialic acid has usually been found (Gottschalk, 1962) linked ketosidically to galactose or *N*-acetylgalactosamine, the neuraminidase-susceptible bond having the α -configuration. The presence of *O*-acetyl substituents has been reported to be responsible for the resistance of sialic acids towards neuraminidase (Faillard, 1957, 1959), and the complete release of sialic acids from BSP by this enzyme probably indicated that no such substituents were present on the sialic acid residues of this glycoprotein. This confirmed the results of paper chromatography, which failed to reveal the presence of any sialic acid derivative other than *N*-acetyl- and *N*-glycolylneuraminic acid. The absence of any other neuraminic acid derivatives may also be inferred from the close agreement between the direct Ehrlich and thiobarbituric acid assay procedures, because methoxy and many *O*-acetyl derivatives react in the former but not the latter method (Warren, 1959, 1960; Gibbons, 1963).

Fucose was liberated less readily than sialic acid. However, in 0.1*N*-sulphuric acid at 100° for 8 hr., 82% of the fucose but only 16% of the hexose was released. This suggested that fucose might occupy a terminal position, which has been confirmed by the results of periodate oxidation studies (Andrews, Herring & Kent, 1967).

After removal of sialic acid, β -galactosidase liberated 6 of the 10 galactose residues/mol. of BSP, but no other constituents. It was therefore concluded that at least 6 galactose residues/mol. occupy terminal positions in this material, or sub-terminal positions in the intact glycoprotein. From the results of periodate oxidation (Andrews *et al.* 1967), however, it was considered that 9 of the 10 galactose residues/mol. are terminal in sialic acid-free BSP, so that β -galactosidase might theoretically liberate up to 90% of the total galactose. The linkage of galactose in BSP may present considerable steric hindrance towards the enzyme, resulting in slow and incomplete release of this sugar, although the possibility of part of the galactose being linked by α -bonds, resistant to β -galactosidase, cannot be ruled out.

The composition of the polypeptide moiety of

BSP is remarkable for the high content of acidic amino acids, 35 of the 89 residues embodied in this portion of the molecule being either aspartic acid or glutamic acid. There are also large proportions of glycine, serine and threonine, but the remaining 12 other amino acids found together account for only a further 26 residues. The acidity of the polypeptide may be lowered to some extent by the occurrence of some of the acidic amino acids as the amides in the native glycoprotein. Ammonia (12.6 mol./mol. of BSP) was released on hydrolysis, but at least part of this was due to the destruction of sialic acid, for after removal of this constituent the amount of ammonia liberated fell to 9.3 mol./mol. under the same conditions. Decomposition of hexosamine residues may also have contributed to this total, and indeed titration results (Williams, 1966) indicated that there are 44.0 free carboxyl groups/mol. of mol.wt. 23000. Of these, 15.2 groups were present in the sialic acid, so that 28.8 were on the polypeptide portion. There are 35.3 acidic amino acid residues/mol., one of which was thought to be involved in linkage to the carbohydrate moiety, so the maximum number of amide groupings was calculated to be 5–6/mol.

Reaction with fluorodinitrobenzene or by the Edman procedure failed to reveal any free *N*-terminal amino group on the polypeptide chain of BSP. However, in preliminary experiments (A. T. de B. Andrews, G. M. Herring & P. W. Kent, unpublished observation) it was found that the glycopeptides, obtained from BSP by the action of Pronase, did react with fluorodinitrobenzene and hence possessed free *N*-terminal amino groups. This indicated that carbohydrate substitution of the *N*-terminal residue was very unlikely.

An alternative structure that would account for the lack of *N*-terminal groups is the involvement of the potential terminal group in an intramolecular ring to another part of the polypeptide chain. Perhaps the most probable substituent on the terminal amino group, however, is an acetyl group. Such a group has been reported on the *N*-terminus of several proteins and glycoproteins. e.g. orosomucoid (Bourrillon, Meyer & Dabat, 1965), tobacco-mosaic-virus protein (Tsugita, 1960), α -melanocyte-stimulating hormone (Harris, 1959), gonadotrophin (Bourrillon, Got, Bourguereau & Marcy, 1957) and human fibrinogen (Folk & Gladner, 1960).

The presence of phosphate groups attached to the polypeptide chain of BSP, and probably to serine, is also not without parallel, for in phosphoproteins such as phosvitin (Williams & Sanger, 1959), casein, pepsin, ovalbumin (Perlmann, 1955), collagen (Glimcher, Francois, Richards & Krane, 1964), the enamel proteins (Glimcher, & Krane, 1964) and human fibrinogen (Blombäck, Blombäck & Edman, 1962) the phosphate groups have been

reported to be linked chiefly to the hydroxyl group of serine. The possible role of phosphate groups in initiating the deposition of apatite crystals in calcifying organic matrices has been discussed with reference to the serine phosphate groups of the enamel proteins and collagen (Glimcher & Krane, 1964; Glimcher *et al.* 1964).

It has been shown (Sanger & Hoquard, 1962) that the serine residues of ovalbumin are phosphorylated after their incorporation into the polypeptide chains, rather than being incorporated as serine phosphate. In view of the isolation (Krane, Stone & Glimcher, 1965) from a number of sources, including diaphyseal bone, of protein phosphokinases that catalyse the phosphorylation of protein-bound serine by ATP, it is tempting to suggest that a similar mechanism may exist for introduction of phosphate into BSP.

Glycoproteins have been widely reported to be resistant to proteolytic enzymes, but this does not appear to be absolute but rather a question of degree (Weinfeld & Tunis, 1960), and it was found that, with a large enzyme/substrate ratio, frequent addition of further enzyme and digestion for several days, the potent proteolytic enzyme Pronase was capable of causing considerable hydrolysis of BSP. In agreement with findings on transferrin and caeruloplasmin (Jamieson, 1964, 1965), removal of sialic acid from BSP did not markedly affect the rate or extent of hydrolysis by Pronase.

Analysis of the glycopeptide fractions (Table 3) revealed that even under the vigorous conditions employed the extent of proteolytic hydrolysis was comparatively modest, and quite large glycopeptides (*G*, *H*, *J*₁ and *J*₂), each containing 20–25 amino acid residues/mol., were the major products formed. However, quantities of the small glycopeptides *D*, *E* and *F*, representing respectively 0.8%, 1.0% and 3.6% of the original weight of BSP taken, were also obtained. The amino acid composition of glycopeptide *E* was principally rather more than 1 residue each of aspartic acid, serine or threonine and glutamic acid and rather less than 1 residue of glycine/mol. Glycopeptide *F*, on the other hand, contained nearly 3 residues of serine or threonine, 1 of aspartic acid, and rather less than 1 each of glycine, lysine and histidine/mol. Glutamic acid seemed to represent only about half a residue in glycopeptides *D*, *F*, *G* and *H*, and therefore presumably was unlikely to be involved in the bond uniting the carbohydrate and polypeptide moieties. Thus, from glycopeptide *E*, the carbohydrate was linked either to aspartic acid or to serine or threonine.

The hexose present in glycopeptides *D*, *E*, *F* and *G* was somewhat decreased with respect to intact BSP, apparently by some loss of galactose. The proportion of galactose shown in Table 3 was given

by making measurements of the galactose/mannose ratio by quantitative paper chromatography, and calculating the amount of galactose/3 residues of mannose. The sialic acid content of glycopeptides was also lowered, and this agreed with the findings of Izumi, Makino & Yamashina (1962), who reported partial release of sialic acid on treatment of orosomucoid with Pronase. It seemed possible that the decrease in the galactose content of glycopeptides *D*, *E*, *F* and *G* might also be the result of attack by this enzyme, or a contaminating enzyme in this preparation; it may be noted that these four glycopeptides were also those from which much of the sialic acid had been removed, thence exposing galactose as terminal residues.

The analytical results on all the glycopeptide fragments were consistent with the presence of only 1 carbohydrate prosthetic group/mol. of BSP. The calculated molecular weight of such a group was 9220, or 40.0% of the total weight of the BSP molecule, and was considerably larger than is usual in glycoproteins. A study of the literature showed that the prosthetic groups of glycoproteins, though they may occur as simple disaccharides, e.g. as in submaxillary mucins, usually fall within the molecular-weight range of about 1200, as in caeruloplasmin, to about 3500, as in fetuin.

Another feature of the BSP molecule arose from the glycopeptide analyses, namely the apparent concentration of non-acidic amino acids in the vicinity of the carbohydrate-polypeptide bond. In the intact BSP molecule aspartic acid and glutamic acid together accounted for almost 40% of the total amino acids, whereas in the large glycopeptides *G* and *H*, each containing almost a quarter of the polypeptide chain, they comprised only 7% and 9% respectively of the total amino acids. Similar, but less marked, percentage decreases of the acidic amino acids were observed in glycopeptides *D*, *J*₁ and *J*₂. This decrease appeared to be accompanied by a slight increase in the proportion of hydroxy-amino acids in the region of the prosthetic group attachment. The net result was that the acidic amino acids were generally congregated in regions of the polypeptide chain furthest away from the prosthetic group, with its attendant sialic acid residues, thus spreading the total charge more evenly over the whole molecule.

The carbohydrate-polypeptide link in many glycoproteins (Grant & Simkin, 1964) involves a hexosamine and an asparagine residue in a glycosylamine bond, but in the submaxillary mucins a glycosidic bond between the reducing group of *N*-acetylgalactosamine and the hydroxyl group of serine or threonine has been reported, although the possibility of some bonds occurring as an ester type has also been suggested (Bhavanandan, Buddecke, Carubelli & Gottschalk, 1964).

From the glycopeptide analyses, bonds of any of these three types could be present in BSP. The linking of a monosaccharide residue via its reducing group to the β -amido group of an asparagine residue would give rise to a glycosylamine bond. A bond between the sugar residues and aspartic acid would also result from the joining of a hexosamine residue via its amino group to a carboxyl group of aspartic acid. Glycosidic bonds would arise from the junction of a reducing group of a monosaccharide to the hydroxyl group of serine or threonine, whereas an ester bond would be the result of combination of the monosaccharide reducing group to the β -carboxyl group of aspartic acid or a C-terminal carboxyl group. From the analyses of the glycopeptides *E* and *F*, no other types of bond were likely to be relevant to BSP.

Glycosidic bonds to serine or threonine and ester bonds are very sensitive towards alkaline hydrolysis (Harbon, Herman, Rossignol, Jolles & Clauser, 1964; Bhavanandan *et al.* 1964). The glycosylamine bond, however, has been stated (Neuberger, 1964) to be comparatively stable to both acid and alkali. The observation that no prosthetic groups were released from BSP even on treatment with 0.2N-sodium hydroxide at 100° was taken to be definite evidence in favour of a glycosylamine bond in this molecule.

Thus BSP is a glycoprotein, of molecular weight 23 000, with a single large carbohydrate prosthetic group, of molecular weight 9220, probably joined by a glycosylamine bond to an asparagine residue of the polypeptide chain. There is probably only one polypeptide chain in the molecule and there is no evidence that it is of a branched structure, but the carbohydrate portion of the molecule must be considerably branched, there being 15 terminal sialic acid residues and 1 terminal fucose unit/mol. (Andrews *et al.* 1967).

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