Preparation and Characterization of Armadillo Submandibular Glycoproteins

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The nine-banded armadillo (*Dasypus novemcinctus mexicanus* Peters) was chosen for this study so that a comparison could be made of the salivary mucus glycoproteins of an ancient mammalian species with those derived from previously studied, more highly evolved, species. Two mucus glycoproteins, armadillo submandibular glycoprotein A and armadillo submandibular glycoprotein B, were prepared from the armadillo submandibular gland by a modification of the method of Tettamanti & Pigman (1968) (*Arch. Biochem. Biophys.* 124, 41–50). The composition of glycoprotein A is the simplest one among the known mucus glycoproteins. Six amino acids constitute 98.5 mol/100 mol of the protein of glycoprotein A and 82 mol/100 mol of that of glycoprotein B. These are serine and threonine (which make up 40–50% of the molar amino acid composition), glutamic acid, glycine, alanine and valine. Proline is absent from glycoprotein A and comprises only 2.3% of glycoprotein B. For both glycoproteins, the protein content, as determined by the method of Lowry, Rosebrough, Farr & Randall (1951) (*J. Biol. Chem.* 193, 265–275), with bovine serum albumin as standard, was nearly 60% higher than when determined by the sum of the amino acids. The ratios of total mol of amino acid/total mol of carbohydrate are 1:0.63 for glycoprotein A and 1:0.68 for glycoprotein B. *N*-Acetylneuraminic acid and *N*-acetylgalactosamine, in a molar ratio of about 0.35:1.00, are the principal carbohydrates present in both glycoproteins. Neutral sugars seem to be absent from glycoprotein A, but galactose and fucose are present in glycoprotein B. The carbohydrate side chains in glycoprotein A are composed of about two-thirds monosaccharide and one-third disaccharide residues, whereas those of glycoprotein B are more complex. For both glycoproteins, essentially all of the *N*-acetylgalactosamine was attached *O*-glycosidically to the hydroxyamino acid residues of the protein core. The linkage of *N*-acetylneuraminic acid in glycoprotein A was extremely sensitive to dilute acid and neuraminidase. Glycoprotein B has chemical properties similar to those of glycoprotein A. However, whereas glycoprotein A was susceptible to both *Clostridium perfringens* and *Vibrio cholerae* neuraminidases, only the latter enzyme had an effect on glycoprotein B at pH 4.75. Both glycoproteins were homogeneous by cellulose acetate electrophoresis and ultracentrifugal analyses. The apparent mol.wts. of glycoprotein A and glycoprotein B were 7.8×10^4 and 3.1×10^4 respectively.

The mammalian mucus glycoproteins are among the principal components of secretion of the epithelial cells lining the oral, gastrointestinal, respiratory and reproductive tracts. These glycoproteins, which form viscous solutions, are usually of high molecular weight and function as lubricants and surface coatings. They include the so-called blood-group-type substances and frequently show blood-group activity (Horowitz *et al.*, 1964; Carlson, 1968; Payza *et al.*, 1970a; Braig & Aminoff, 1972). Many have been purified and well characterized. Their chemical compositions vary not only among species, but also among individual glands in the same species.

The nine-banded armadillo (*Dasypus novemcinctus mexicanus* Peters) which was used for this study has a long protrusible tongue which secretes a sticky mucous substance that aids in the gathering of small

insects. The major salivary gland (submandibular) of this species consists of one gland on each side of the mandible. Compared with the size of the animal's head, it is very large. An unusual feature of the armadillo is its salivary bladder (vesica salivaria), which is used probably as a temporary storage organ for saliva. The armadillo was chosen for this work, in order to compare the composition of its salivary glycoprotein with that of more highly evolved species. It was also desired to compare the chemical composition of individually isolated submandibular glycoproteins from monozygous quadruplet armadillos for which variations in organ weight and metabolic parameters have been reported (Storrs & Williams, 1968).

The purpose of the present paper is to describe a method for the isolation and purification of the

armadillo submandibular glycoprotein from pooled glands and the characterization of the resultant material by chemical, enzymic and physical methods.

Materials and Methods

N-Acetylneuraminic acid and *N*-glycolylneuraminic acid were products of Sigma Chemical Co., St. Louis, MO, U.S.A. *Clostridium perfringens* neuraminidase was supplied by Worthington Biochemical, Freehold, NJ, *Vibrio cholerae* was from Behring Diagnostic, Woodburg, NY, U.S.A., and *N*-acetyl-galactosamine was from Cyclo Chemical Co., Los Angeles, CA, U.S.A.; hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad, Richmond, CA, U.S.A.; Vicor digestion tubes, catalogue no. 7900, were from Corning Glass Works, Corning, NY, U.S.A.

Submandibular glands of armadillos, captured on the Gulf coast of Mexico, were provided by Dr. J. Shackleford, University of South Alabama (Mobile, AL, U.S.A.) and Dr. E. Storrs of Gulf South Research Institute (New Iberia, LA, U.S.A.). Six glands were used in this work; the glands measured about 5 cm × 1.5 cm and weighed about 4–6 g each. They were kept frozen at –20°C until used.

Isolation and purification

The frozen glands were thawed, cut into pieces, freed of connective and fatty tissues, washed with cold water and homogenized for 2 min in a glass grinder with 3 vol. of ice-cold 0.01 M-NaCl. The extract was collected by low-speed centrifugation, and the residue was rehomogenized with the same volume of 0.01 M-NaCl. This operation was repeated about six times. The combined extracts were heated at 68°C for 20 min and centrifuged for 30 min at 27000g. The supernatant was diluted with 0.01 M-NaCl to a final volume of 60 ml/g of trimmed gland, acidified to pH 5.25 with 0.2 M-acetic acid and finally centrifuged at 27000g for 2.5 h. The sediment was discarded. The supernatant was stirred with a glass rod, and a 30% (w/v) cetyltrimethylammonium bromide solution was added dropwise until a heavy precipitate resulted, or until a final concentration of 5% cetyltrimethylammonium bromide was reached. The solution was left overnight and the precipitate collected by centrifugation at 27000g for 2 h at 4°C. The precipitate was washed with 10 × 10 vol. of cold water, and then dissolved in a minimum volume of 50% (w/v) aq. CaCl₂. Ethanol was added slowly to a concentration of 62% (v/v), while stirring. Any precipitate was removed by centrifugation at the same speed as above for 2 h. Ethanol was added to the supernatant solution to a concentration of 75% (v/v). After standing for at least 1 h, the precipitate was collected by centri-

fugation at the above speed for 30 min, dissolved in a minimum volume of cold water, dialysed for 2 days against about 20 vol. of distilled water, changed three or four times daily, and freeze-dried.

The freeze-dried material was suspended in 0.01 M-phosphate buffer, pH 6.8, (5 mg of freeze-dried material/ml of buffer) and left to dissolve overnight. After the addition of hydroxyapatite, the glycoprotein suspension (60–70 mg of hydroxyapatite/mg of glycoprotein) was stirred for 2 h and centrifuged at 12000g for 20 min. The first supernatant was discarded, since it contained mainly contaminating proteins and only traces of glycoprotein. The residue (hydroxyapatite–glycoprotein) was resuspended in the same volume of phosphate buffer, stirred for 2 h, and centrifuged as before. The supernatant was saved, and the operation repeated until the content of sialic acid in the supernatant fell suddenly to a low value, usually after seven to ten extractions. The supernatants were pooled and dialysed for 2 days against 20–25 vol. of water, with several changes each day. Any insoluble material contained in the non-diffusible fluid was removed by centrifugation. Finally, the clear fluid was freeze-dried. This product was designated glycoprotein A. The glycoprotein, which remained adsorbed to the hydroxyapatite, was subjected to four additional 1 h washings with 8 vol. each of the 0.01 M-phosphate buffer (pH 6.8) to eliminate residual glycoprotein A. These washings were discarded. The precipitate was then suspended in 5 vol. of 0.15 M-phosphate buffer, pH 6.8, stirred for 2 h and centrifuged. This treatment was repeated four times. The supernatant fraction was dialysed for 3 days against 20–25 vol. of distilled water. Insoluble material in the non-diffusible fluid was removed by centrifugation and discarded. The clear supernatant was freeze-dried and referred to as glycoprotein B.

Analytical procedures

The moisture content of the product was determined by heating to a constant weight at 110°C *in vacuo* in an Abderhalden drier containing P₂O₅. Protein content was measured by the procedure of Lowry *et al.* (1951), with crystalline bovine serum albumin as the standard, and by summation of the amino acids, as determined by amino acid analysis. Unless otherwise stated, the latter method was used. Sialic acid was determined by the resorcinol (Svennerholm, 1957) and the thiobarbituric acid (Aminoff, 1961) methods. *N*-Acetylneuraminic acid was used as the standard. Total hexosamine was determined by the Elson–Morgan method, as described by Boas (1953), after hydrolysis of the samples in 6 M-HCl for 4 h at 110°C. The ratio galactosamine/glucosamine was determined by means of the amino acid analyser (Moschera & Pigman, 1975) under the same hydrolytic conditions as those used for total hexosamine

analysis. For the determination of fucose, the cysteine/ H_2SO_4 reaction of Dische & Shettles (1948) was used, with a heating period of 10 min. Galactose was analysed by the method of Dische & Danilchenko (1967). Uronic acid was analysed by the carbazole procedure (Bitter & Muir, 1962). Sulphate was determined by the benzidine method of Antonopoulos (1962), by using hydrolysis in 25% formic acid at 100°C for 24 h or more and using K_2SO_4 as the reference compound. The absence of nucleic acids and tryptophan was demonstrated by u.v. absorption at 260 and 280 nm, with yeast RNA and tryptophan as the reference materials respectively.

Paper chromatography for identification of neutral sugars. The samples were hydrolysed in Pyrex tubes with 2M-HCl at 100°C for 2 h. The solution was neutralized by the addition of dry AG 1W-X8 resin (HCO_3^- ; 100–200 mesh), filtered and passed through a column (0.5 cm $\times$ 10 cm) of Dowex 50 ($\times 8$; 100–200 mesh; H^+ form). The eluate was evaporated to dryness and dissolved in a minimum amount of water. Samples of the hydrolysates were applied to Whatman no. 1 paper sheets and developed by descending chromatography for 72 h, with butan-1-ol/acetic acid/water (4:1:5, by vol.) as the solvent system; L-fucose, D-mannose, D-xylose and D-galactose, which served as reference compounds, were run on the same paper strips, in addition to the hydrolysates of bovine submaxillary glycoprotein and rat sublingual glycoprotein. Alkaline AgNO_3 (Trevelyan *et al.*, 1950) was used to detect the spots.

Amino acid analysis. For acid hydrolysis, 3 ml of conc. HCl was added to an equal volume of a 1 mg/ml sample in a screw-capped Pyrex tube. To determine the optimum time for hydrolysis, the solution was hydrolysed at 110°C from 6 to 144 h, cooled and evaporated to dryness under vacuum. The residues were dissolved in 15 ml of 0.2M-sodium citrate buffer, pH 2.2, and filtered.

To check for the presence of tryptophan or other acid-sensitive amino acids, alkaline hydrolysis was carried out as follows. A portion (3 ml) of a 1 mg/ml sample was pipetted into the bottom of a Vicor digestion tube, by the technique of Noltmann *et al.* (1962). Solid $\text{Ba}(\text{OH})_2$ was added to a final concentration of 2M- $\text{Ba}(\text{OH})_2$. The sample was frozen and sealed under vacuum, and incubated at 110°C for 70 h. After hydrolysis, the barium was precipitated as BaCO_3 by the addition of CO_2 gas. The barium-free sample was concentrated under reduced pressure, filtered and freeze-dried. The residue was dissolved in 15 ml of 0.2M-citrate buffer, pH 2.2.

The hydrolysed samples were analysed with a Beckman model 120B amino acid analyser, under the conditions of Noltmann *et al.* (1962) and Downs & Pigman (1969).

Mild acid hydrolysis. Samples were dissolved in 0.01M-HCl and hydrolysed at 80°C over a period of

3 h. Sialic acid released from the samples was determined by the thiobarbituric acid method.

Isolation of sialic acid and preparation of desialized armadillo submandibular glycoproteins. Samples of armadillo submandibular glycoproteins were dissolved in 0.01M-HCl and hydrolysed at 80°C for 30 min, neutralized with 0.05M-NaOH and dialysed at 4°C against 50 vol. of water for 24 h with frequent changes of water. The first two diffusates were pooled and concentrated in a rotary vacuum evaporator at 30°C. The sialic acid was separated from the concentrated diffusates, as described by Spiro (1966). The non-diffusible material was collected, freeze-dried and designated desialized armadillo submandibular glycoprotein.

Identification of sialic acid. For paper chromatography, the sialic acid was dissolved in a small amount of water and chromatographed on Whatman no. 1 paper by the descending technique for 60 h with butan-1-ol/pyridine/water (6:4:3, by vol.) as the solvent system. *N*-Acetylneuraminic acid and sialic acid isolated from bovine submaxillary mucin were used as reference standards. The positions of the sialic acids were located by using Ehrlich's reagent (Svennerholm, 1958) and thiobarbituric acid (Warren, 1960).

Studies on the types of sialic acids by g.c./m.s. (gas chromatography/mass spectrometry) were carried out by Dr. C. C. Sweeley and Dr. J. N. Gerber, Department of Biochemistry, Michigan State University. The samples were converted into trimethylsilyl derivatives by the method of Yu & Ledeen (1970). The g.c./m.s. system was a Varian 2100 gas chromatograph connected through a Watson-Biemann all-gas separator to a Varian MAT CH-5 double-focusing mass spectrometer, interfaced to a PDP 11/40-based computer data system, developed in the Michigan State University Mass Spectrometry Facility. A glass U-shaped column (2 m $\times$ 3 mm), packed with 1% SP-2100 on Chromosorb W, was maintained isothermally at 215°C for these analyses. Mass-spectrometric conditions were as follows: resolution, 1000; ion-source temperature, 240°C; separator temperature, 250°C.

The *O*-acetyl content of the glycoproteins was measured by the alkaline hydroxylamine procedure of Hestrin (1949), with D-glucose penta-acetate as the reference compound. The amount of *N*-glycolyl groups in the isolated sialic acid was determined by the method of Klenk & Uhlenbruck (1957).

Neuraminidase treatment. The experimental procedure for *Cl. perfringens* neuraminidase is described in Fig. 3; for *V. cholerae* neuraminidase the same procedure was used except for the composition of the acetate buffer (pH 5.6), which contained 5 mequiv. of Ca^{2+} ion; the concentration of *V. cholerae* enzyme was 0.05 unit/ml.

***N*-Acetyl- β -hexosaminidase treatment.** Highly purified *N*-acetyl- β -hexosaminidase from jack-bean meal

was a gift from Dr. Y. Li (Delta Regional Primate Research Center, Tulane University, New Orleans, LA, U.S.A.). For enzyme treatment, 0.2–0.4 mg each of desialized glycoproteins A and B was dissolved in 0.4 ml of 0.05 M-sodium citrate buffer, pH 4.5, and incubated with 1 unit of *N*-acetyl- β -hexosaminidase at 37°C for times varying from 1 to 48 h. The reaction mixtures were assayed by the Morgan–Elson method (Reissig *et al.*, 1955), with *N*-acetylgalactosamine as the reference standard.

Alkaline borohydride treatment. The alkaline β -elimination reaction was performed by the method of Downs *et al.* (1973). For the determination of hexosaminols, the alkali-treated samples were analysed as described by Moschera & Pigman (1975).

Physical methods. The intrinsic viscosity at zero concentration was determined in 1 M-NaCl at 30°C in a 0.5 ml Cannon–Fenske glass viscometer, size 100.

Electrophoresis was carried out on cellulose acetate sheets by using the Beckman microzone system with sodium borate buffer (pH 9.0). Analyses were run in duplicate and stained with 1% Alcian Blue at pH 1.5 and with periodic acid/Schiff reagent (Pearce *et al.*, 1964).

Molecular-weight determinations were made by equilibrium-sedimentation measurements (Schachmann, 1957) at 25°C in an analytical ultracentrifuge (Beckman model E), by using a 1 mg/ml solution of armadillo submandibular glycoprotein in 1 M-NaCl. The partial specific volume was taken as 0.633 cm³/g (Creeth & Knight, 1967). The homogeneity of the isolated glycoproteins was also examined at a 10 mg/ml concentration by sedimentation-velocity measurements.

Results

Isolation and purification

For the preparation of armadillo submaxillary glycoproteins, the procedure of Tettamanti & Pigman (1968) for isolation and purification of bovine and ovine submaxillary glands was modified, as outlined above.

The salivary glycoprotein was easily extractable with 0.01 M-NaCl from the homogenized armadillo submaxillary gland. However, during acidification, the solutions became gel-like at pH 5.5 and could not be centrifuged. For this reason, the glandular extract was heated at 68°C for 20 min, and diluted fourfold before acidification with 0.01 M-NaCl to a final concentration of 1 g of trimmed gland/60 ml. Adjustment of this solution to pH 5.25 with dilute acetic acid resulted in the formation of a precipitate, which contained mostly extraneous protein. At this pH, only 10% of the total sialic acid was lost, whereas the total loss of protein was about 30%. Stronger acidification is not desirable because of a sharp increase in the loss of sialic acid in the precipitate.

At the stage of ethanol fractionation, the sialic acid-containing material was precipitated at an ethanol concentration between 63 and 75% (v/v).

The recovery of total sialic acid at this stage was 55% of that of the initial extract and the sialic acid-containing material represented 1.0% of the initial wet gland weight.

For further purification, the ethanol-fractionated glycoprotein was suspended in 0.01 M-sodium phosphate buffer, pH 6.8, and solid hydroxyapatite was added. Glycoprotein A was obtained by batchwise extraction with 0.01 M-sodium phosphate buffer from hydroxyapatite. This was followed by treatment with 0.15 M-sodium phosphate buffer, which yielded a fraction designated glycoprotein B. Both glycoproteins were soluble in water. The yield of glycoprotein A was three times higher than that of glycoprotein B and the total recovery of sialic acid for both fractions represented about 80% of that of the ethanol-fractionated material.

The amino acid content of the glycoprotein fraction before hydroxyapatite treatment was about 60% by weight. This value is higher than that for either glycoprotein (Table 1). Thus the treatment with hydroxyapatite removes some extraneous protein.

Glycoproteins A and B correspond to the major and minor salivary glycoproteins respectively of other species as reported previously in this laboratory (Tettamanti & Pigman, 1968; de Salegui & Plonska, 1969; Moschera & Pigman, 1975).

Chemical composition

The chemical compositions of the armadillo submandibular glycoproteins are given in Table 1.

Glycoproteins A and B show significant differences in chemical composition. The carbohydrate moiety of glycoprotein A consists almost entirely of *N*-acetylgalactosamine and *N*-acetylneuraminic acid, whereas that of glycoprotein B is more complicated. Less than 1% of fucose was found in glycoprotein A; about 3% of galactose and 3% of fucose were found in glycoprotein B. Paper chromatography of the neutral sugars suggested that the only neutral sugar present in glycoprotein A is fucose, whereas glycoprotein B contains fucose and galactose. In addition, traces of mannose and glucose appear to be present in glycoprotein B. As in ovine submaxillary glycoprotein (Tettamanti & Pigman, 1968) and porcine submaxillary glycoprotein (de Salegui & Plonska, 1969), galactosamine is the principal hexosamine in both of these salivary glycoproteins.

The quantitative and qualitative analyses of sialic acids are summarized in Table 2. None or only trace amounts of *O*-acetyl and *N*-glycolyl groups were found in the sialic acid of the purified glycoproteins. This was confirmed by g.l.c./m.s. studies as well as by

Table 1. *Chemical composition of purified armadillo submandibular glycoproteins*

Sugars and amino acids were calculated on the basis of the anhydrous molecular weight. Each value represents the mean of four separate analyses $\pm$ s.d. *N*-Acetylneuraminic acid was determined by the method of Svennerholm (1957); for other analyses, see in the Materials and Methods section. AAA, Amino acid analyser; LP, protein by method of Lowry *et al.* (1951).

	Composition			
	(g/100 g)		(mmol/100 g)	
	Glycoprotein A	Glycoprotein B	Glycoprotein A	Glycoprotein B
<i>N</i> -Acetylneuraminic acid	13.3 $\pm$ 0.4	7.9 $\pm$ 0.2	43.0 $\pm$ 1.3	25.6 $\pm$ 0.7
<i>N</i> -Acetylhexosamine	26.6 $\pm$ 1.1	15.0 $\pm$ 0.9	131.5 $\pm$ 6.4	73.9 $\pm$ 4.4
Glc/NAc/GalNAc	1/100	1/14	—	—
Galactose	Trace	2.8	—	19.3
Fucose	0.8	3.0	5.5	20.5
Protein (LP)	87.2 $\pm$ 4.0	71.0 $\pm$ 2.9	—	—
Protein (AAA)	53.5 $\pm$ 0.56	44.9 $\pm$ 0.5	573.2 $\pm$ 9.7	465.7 $\pm$ 4.3
LP/AAA	1.63 $\pm$ 0.06	1.58 $\pm$ 0.03	—	—
Ash	2	5	—	—
Total recovery (LP)	129.9	99.6	—	—
Total recovery (AAA)	94.2	73.6	—	—

Table 2. *Determination and identification of sialic acid in armadillo submaxillary glycoproteins*

Details of methods are described in the text. Each value is the mean $\pm$ s.d. from four separate analyses. T, Trace or none.

Analytical method	Composition (mmol/100 g)	
	Glycoprotein A	Glycoprotein B
Resorcinol	43.0 $\pm$ 1.3	26.5 $\pm$ 0.7
Mild acid hydrolysis and thiobarbituric acid assay	42.0 $\pm$ 0.9	20.9 $\pm$ 0.8
<i>Cl. perfringens</i> neuraminidase, pH 5.6	42.0 $\pm$ 2.0	20.0 $\pm$ 1.0
pH 4.7	42.0 $\pm$ 1.8	5.6
Glycolyl group	T	T
<i>O</i> -Acetyl group	T	T
G.c./m.s.	AcNeu	AcNeu
Paper chromatography	AcNeu	AcNeu

paper chromatography, both of which revealed only *N*-acetylneuraminic acid in these materials.

N-Acetylneuraminic acid was identified as its trimethylsilyl derivative by g.c./m.s. Gas chromatograms of modified glycoproteins A and B each showed one major and one minor component. The larger component was identified as the heptamethylsilyl derivative by the presence in the mass spectrum of an ion at *m/e* 798 for loss of a methyl group from the molecular ion (mol.wt. 813) along with other significant ions at *m/e* 117 ($[\text{CO}_2\text{SiMe}_3]^+$), 245 ($[\text{Me}_3\text{SiOCHCHN}(\text{SiMe}_3)\text{COCH}_3]^+$) and 258 ($[\text{CH}_2\text{CH}(\text{OSiMe}_3)\text{CHN}(\text{SiMe}_3)\text{COCH}_3]^+$). The mass spectrum was identical with that of a reference sample of *N*-acetylneuraminic acid on the basis of the ions at *m/e* 726, 173 and 186 that represent *M*-15, $[\text{CH}_3\text{CONHCHCHOSiMe}_3]^+$ and $[\text{CH}_3\text{CONHCHCH}(\text{OSiMe}_3)\text{CH}_2]^+$ respectively. Both of the peaks

on the gas chromatograms were derived from *N*-acetylneuraminic acid therefore, and differed only in the presence or the absence of methylsilyl group on the amide nitrogen.

On a molar basis, the amount of galactosamine contained in the purified materials, as in ovine and porcine submaxillary glycoprotein, exceeded that of sialic acid; for glycoprotein A the molar amount of galactosamine was about three times higher than the molar sum of sialic acid and neutral sugars.

The total recovery (sum of carbohydrate and protein contents) of glycoproteins A and B (Table 1) was 94.2 and 73.6% of the dry weight respectively. No sulphate, nucleic acid, tryptophan (or other unusual amino acids) or uronic acid was detected in these materials.

The ash contents were 2 and 5% for glycoprotein A and B respectively.

Table 3. *Amino acid composition of armadillo submaxillary glycoproteins after hydroxyapatite treatment*
 Samples were hydrolysed in 6M-HCl at 110°C for 22h. The means $\pm$ s.d. from four separate analyses are shown. His, Met, Tyr and Trp were absent from both samples. Thr and Ser values were obtained by extrapolation to zero time from 22, 48, 72 and 96h hydrolysis data. Val values are averages for 72 and 96h hydrolysis, as described in the text.

Amino acid	Composition			
	(mmol/100g)		(mol/100mol)	
	Glycoprotein A	Glycoprotein B	Glycoprotein A	Glycoprotein B
Lys	0.5	13.0	0.1	2.8
Arg	1.4	6.5	0.2	1.4
Asp	4.0	23.3	0.7	5.0
Thr	203.2 $\pm$ 3.0	113.2 $\pm$ 2.2	35.5 $\pm$ 0.20	24.3 $\pm$ 0.52
Ser	112.1 $\pm$ 2.5	73.6 $\pm$ 1.2	19.6 $\pm$ 0.44	15.8 $\pm$ 0.21
Glu	56.8 $\pm$ 0.7	53.1 $\pm$ 1.0	9.9 $\pm$ 0.15	11.4 $\pm$ 0.20
Pro	0	10.7 $\pm$ 1.1	0	2.3 $\pm$ 0.28
Gly	82.6 $\pm$ 2.0	66.1 $\pm$ 1.9	14.4 $\pm$ 0.30	14.2 $\pm$ 0.20
Ala	34.3 $\pm$ 0.4	32.1 $\pm$ 0.9	6.0 $\pm$ 0.10	6.9 $\pm$ 0.15
Cys	0	9.8	0	2.1
Val	75.4 $\pm$ 1.7	43.8 $\pm$ 1.9	13.2 $\pm$ 0.27	9.4 $\pm$ 0.28
Ile	1.4	7.0	0.2	1.5
Leu	1.5	11.6	0.3	2.5
Phe	0	1.9	0	0.4

Table 4. *Physical data of armadillo submaxillary glycoproteins*

For methods, see the text. Plot of η_{sp}/c versus c was linear over the range of 0.1 g per 100ml to 0.5 g per 100ml in 1 M-NaCl at 30°C.

Glycoprotein	Intrinsic viscosity (dl/g)	Molecular weight	$10^{-13} \times$ Sedimentation coefficient (s)
A	0.3	7.8×10^4	1.5
B	0.2	3.1×10^4	1.8

Amino acid analysis

The rate of release and destruction of amino acids during acid hydrolysis of armadillo submandibular glycoproteins has been studied by using 6M-HCl at 110°C for times varying from 1 to 144h. The optimum time for acid hydrolysis was 22h (with corrections for the destruction of hydroxyamino acids and for the incomplete release of valine). The correction factors for the hydroxyamino acids were obtained by determining the ratio between the extrapolated values corresponding to 22, 48, 72 and 96h hydrolysis data to the zero time and the 22h hydrolysis values, and were 1.06 for threonine and 1.13 for serine, for both glycoproteins. For valine, the correction factor was obtained by determining the ratio of the mean values for 72h and 96h hydrolysis to the 22h hydrolysis values and was 1.20 for glycoprotein A and 1.08 for glycoprotein B.

The amino acid composition of armadillo submaxillary glycoproteins is given in Table 3. Six amino acids (threonine, serine, glutamate, glycine,

alanine and valine) constitute 98 mol/100mol of the protein of glycoprotein A and 82 mol/100mol of that of glycoprotein B. The hydroxyamino acids represent 40–50 mol/100mol of the total amino acids. Proline, histidine, cysteine, methionine, tryptophan, tyrosine and phenylalanine are absent from glycoprotein A. However, glycoprotein B contains 2.5% of proline, but no histidine, tyrosine, methionine and tryptophan. The protein core of glycoprotein A differs from that of glycoprotein B in that it has a higher proportion of threonine, but is significantly lower in lysine, arginine and aspartic acid and contains no proline and cysteine.

Physical properties

The physical properties of armadillo submaxillary glycoproteins are summarized in Table 4. Compared with other salivary glycoproteins (Payza *et al.*, 1970b; Pigman *et al.*, 1973; Moschera & Pigman, 1975), the armadillo submandibular glycoproteins

Fig. 1. Sedimentation pattern of the purified armadillo submandibular glycoprotein A

A sample of glycoprotein (10 mg/ml) in 1 M-NaCl was centrifuged at a constant speed of 56100 rev./min; phase-plate angle of 60°. (a) and (b) are taken after 64 and 224 min respectively.

Fig. 2. Cellulose acetate electrophoresis of glycoproteins A and B

Experimental conditions: Beckman Microzone cell model R-101 with Beckman no. 324330 membrane (56 mm × 144 mm) and borate buffer, pH 9.2, at 250 V for 50 min.

show lower molecular weights, lower intrinsic viscosities and lower sedimentation coefficients. The apparent molecular weight is about 7.8×10^4 and 3.1×10^4 for glycoproteins A and B respectively. The ultracentrifugal-sedimentation-velocity pattern of glycoprotein A is shown in Fig. 1. A single symmetrical peak was observed in 1 M-NaCl throughout the run. A similar pattern, but with somewhat more diffusion, was found for glycoprotein B, indicating a higher degree of polydispersity for this material than for glycoprotein A. On cellulose acetate electrophoresis in borate buffer, at pH 9.2, both glycoproteins stained with Alcian Blue, each giving a single band and showing the same mobility (Fig. 2). However, the glycoproteins did not stain with the periodic acid/Schiff reagent under the conditions used (Pearce *et al.*, 1964).

Mild acid hydrolysis

The release and destruction of sialic acid from the purified armadillo submandibular glycoproteins, as a function of time, is illustrated in Fig. 3. The pattern for glycoprotein A shows two phases: a rapid release

Fig. 3. Effect of neuraminidase and mild acid hydrolysis on armadillo submandibular glycoproteins

Neuraminidase treatment: 0.4 ml of 1 mg/ml glycoprotein solution was added to 0.4 ml of 0.1 M-acetate buffer (pH 4.75 or 5.6) and incubated with 0.2 ml of 0.01 unit of *Cl. perfringens* neuraminidase/ml at 37°C for the times indicated. Mild acid hydrolysis: samples were dissolved in 0.01 M-HCl and incubated at 80°C for the times indicated. Both reaction mixtures were assayed for free sialic acid by the thiobarbituric acid method (Aminoff, 1961) and the results expressed as percentage of total sialic acid residues in glycoproteins A and B respectively. At zero time, the thiobarbituric assay gave values of 18 and 15% respectively for glycoproteins A and B. ○, Glycoprotein B, neuraminidase-treated at pH 4.7; ●, glycoprotein A, neuraminidase-treated at pH 4.7; □, glycoprotein B, neuraminidase-treated at pH 5.6; ■, glycoprotein A, neuraminidase-treated at pH 5.6; △, glycoprotein B after acid hydrolysis (pH 2, 80°C); ▲, glycoprotein A after acid hydrolysis (pH 2, 80°C).

of almost all of the sialic acid within 30 min, followed by destruction. The hydrolysis of sialic acid for glycoprotein B gave a curve composed of three phases: rapid release of 80% of sialic acid within 30 min, release and destruction at a similar rate between 30 and 120 min and more rapid destruction after 120 min.

After mild acid treatment for 30 min, almost all of the sialic acid and fucose of glycoprotein A as well as 80% of sialic acid and over 60% of the fucose and galactose of glycoprotein B were removed. The mild acid-treated armadillo submandibular glycoproteins, like the untreated ones, reacted strongly in the Lowry *et al.* (1951) protein assay.

Enzyme treatment

Neuraminidase. The results of the treatment of glycoproteins A and B with *Cl. perfringens* neuraminidase and *V. cholerae* neuraminidase are illustrated in Fig. 3 and Table 2. Glycoprotein A was extremely sensitive to both neuraminidases. About 70% of its

Fig. 4. Disappearance of hydroxyamino acids and formation of α -aminobutyric acid and alanine during alkaline β -elimination and reduction

A solution of glycoprotein (3 mg/ml) in 0.1 M-NaOH and 0.3 M-NaBH₄ was incubated at 45°C for 0 and 35 h. The treatment was followed by the simultaneous additions of 0.66 M-NaBH₄ and 0.016 M-PdCl₂ in 0.8 M-HCl. For analysis of amino acids, the alkaline-treated samples were hydrolysed with 6 M-HCl. ○, Production of alanine from glycoprotein A (—) or glycoprotein B (---). △, Loss of serine from glycoprotein A (—) or glycoprotein B (---). ●, Production of α -aminobutyric acid (—) or loss of threonine (---) from glycoprotein B. ▲, Production of α -aminobutyric acid (—) and loss of threonine (---) from glycoprotein A.

sialic acid was rapidly released within 15 min. Glycoprotein exhibited similar activity to *V. cholerae* neuraminidase. However, with *Cl. perfringens* neuraminidase, the release of sialic acid from glycoprotein B was very sensitive to pH. Complete hydrolysis occurred at pH 5.6 but no release occurred at pH 4.75.

N- β -Acetylhexosaminidase. When the desialized glycoproteins A and B were incubated with *N*-acetyl- β -hexosaminidase for 48 h, no detectable *N*-acetyl-galactosamine was liberated.

Alkaline borohydride treatment

The disappearance of threonine and serine residues and formation of α -aminobutyric acid and alanine as a function of time during alkaline β -elimination are shown in Fig. 4. The glycosylated threonine residues of both glycoproteins were more labile to alkali than has been observed for other submandibular glycoproteins (Bertolini & Pigman, 1967; Moschera & Pigman, 1975). The maximum extent of lability was attained after 5 h, at which time about 70 and 64% respectively of the total threonine glycoproteins A and B had disappeared. The disappearance of serine was somewhat more complex in both glycoproteins and probably involves two stages; namely, a rapid

loss within about 5 h followed by a slow secondary rate of disappearance. The maximum destruction of serine residues was obtained after alkaline treatment for 30 h, at which time only 20% of the total amount of serine was lost. Over 75% of the threonine and serine lost was recovered as α -aminobutyric acid and alanine respectively.

The molar changes of hexosamine, serine and threonine of the armadillo submandibular glycoproteins, after alkaline β -elimination and reduction, are shown in Table 5. The glycoproteins exhibited an almost complete loss of galactosamine, 85% of which was recovered as galactosaminitol; the loss of galactosamine was 90–95% of that of the hydroxyamino acids and none of the glucosamine, present in small amounts in glycoproteins A and B, was lost by this treatment.

To eliminate the possibility of the existence of a direct *O*-glycosidic linkage of sialic acid to the hydroxyamino acids of the protein core, the β -elimination reaction was carried out on mild acid-treated glycoproteins A and B, which still contained approx. 2–5 mol/100 mol of the original sialic acid respectively. No difference was observed in the extent of destruction of the hydroxyamino acids whether this or the original material was subjected to alkaline treatment.

Discussion

The nine-banded armadillo is an inhabitant of the coastal regions of the Gulf of Mexico. For biochemical work, this species provides several interesting features. It is, along with the opossum, one of the oldest known mammalian species. It is unusual in that four homozygous quadruplets are found in each litter. This characteristic provides an opportunity to investigate possible differences in biochemical and other parameters of each quadruplet. A preliminary study of this type has been reported by Storrs & Williams (1968). Since the submandibular glands from such quadruplets are relatively large, biochemical differences among them can be studied in a precise manner.

The salivary gland in the mandible of armadillo has been studied histochemically by Shackleford (1963), who found it rich in mucus secretions. No marked distinction into submaxillary and sublingual glands could be defined. Hence the term submandibular is appropriate for this gland.

The present work describes the preparation and partial characterization of two glycoproteins (A and B) from pooled glands.

Homogeneity

Since only small amounts of the two armadillo submaxillary glycoproteins were available, the use of

Table 5. *Effect of alkaline β -elimination and reduction on armadillo submaxillary glycoproteins*

For experimental details, see Fig. 4. Hexosaminitols were determined as described by Moschera & Pigman (1975).

Component	Composition (mmol/100 g dry wt.)	
	Glycoprotein A	Glycoprotein B
Galactosamine (remaining)	3.0	3.5
Galactosamine (lost)	127.6	63.8
Recovery as galactosaminitol	109.7	57.4
Threonine (remaining)	52.1	35.9
Threonine (lost)	126.9	62.9
Recovery as 2-aminobutanoic acid	103.5	47.2
Serine (remaining)	86.0	58.4
Serine (lost)	19.0	8.9
Recovery as alanine	15.4	8.5
Thr + Ser (lost)	146	72

immunological methods to demonstrate homogeneity was not possible. Consequently, the occurrence of a single symmetrical peak during sedimentation velocity and a linear plot of $\ln c$ versus $(x^2 - m^2)$ in the ultracentrifugal studies, a single spot in cellulose acetate electrophoresis and the low content of basic and aromatic amino acids are used as evidence for the absence of extraneous protein from our purified preparations.

Chemical composition

For glycoprotein A, *N*-acetylneuraminic acid, *N*-acetylgalactosamine and protein constitute about 95% of dry weight (Table 1). Glycoprotein B has the same components but contains, in addition, small amounts of *N*-acetylglucosamine, galactose and fucose.

The carbohydrate composition of glycoproteins A and B differs appreciably in several respects from that of other major salivary glycoproteins (Pigman *et al.*, 1970). The carbohydrate content in the armadillo salivary glycoproteins is only about 30–40% (g/100g), as opposed to 60–70% for other known 'major' mucus glycoproteins. The sialic acid content in both glycoproteins and the hexosamine content in glycoprotein B are much lower than those observed for other 'major' mucus glycoproteins. *N*-Acetylneuraminic acid was the only type of sialic acid identified in the armadillo submandibular glycoproteins.

The total amino acid content of glycoprotein A is higher than for the other mucus glycoproteins studied in this laboratory. Some striking features with respect to amino acid composition are found in glycoprotein A. It contains no proline, whereas mucus glycoproteins from other animal sources usually have proline as a major component. The

amino acid composition of glycoprotein A is the simplest one of all of the known mucus glycoproteins (Pigman *et al.*, 1970). Five aliphatic amino acids and glutamic acid account for 98% of all of the amino acids present. Further, threonine and serine represent 50% of the total amino acids (mol/100mol).

It has been assumed that glycoprotein B corresponds to the 'minor' salivary glycoproteins of other species. Usually the 'minor' glycoproteins contain a larger amount of aromatic amino acids and of histidine than the 'major' glycoproteins (Pigman *et al.*, 1973), but actually, the amino acid composition of glycoprotein B seems to be similar to that of the 'major' mucus glycoprotein of other species, which contains larger amounts of the two hydroxyamino acids (which make up 30–50% in molar composition), and less histidine and aromatic amino acids than are usually encountered in the 'minor' mucus glycoproteins.

Analysis of protein by the Lowry *et al.* (1951) method

The protein content in both armadillo submandibular glycoproteins, as determined by amino acid analysis, is much lower than that found by the Lowry *et al.* (1951) method with bovine serum albumin as the standard. The protein ratio between the Lowry *et al.* (1951) procedure and amino acid analysis is about 1.6 (Table 1). The desialized products show the same ratio. The phenol method of Lowry *et al.* (1951) gives a combination of colours arising from the presence of such amino acids as tyrosine, cysteine and tryptophan, whereas the biuret reaction is based on the presence of peptide linkages (Chou & Goldstein, 1960). The amino acid composition of the armadillo submandibular glycoproteins suggests that the biuret reaction is the main feature in the Lowry *et al.* (1951) assay. The colour value varies

widely, depending on the nature of the amino acids involved in the peptide bonds. This is confirmed by the observation that an acid hydrolysate of armadillo submandibular glycoprotein gave no colour with the Lowry *et al.* (1951) method. Sialic acids had no influence on the colour yield, since the desialized material gave the same ratio of protein content obtained by the Lowry *et al.* (1951) method to that obtained in the amino acid analyser. Sulphated glycoproteins from dog gastric mucosa, which contain a considerable amount of proline, show low reactivity in the Lowry *et al.* (1951) assay (Pamer *et al.*, 1968). Consequently, the negligible amount of proline in armadillo submandibular glycoprotein might be a major reason for the high reactivity in the Lowry *et al.* (1951) assay.

Recovery of protein and carbohydrates

Although the recovery of total constituents in glycoprotein A was good (94%), that of glycoprotein B was poorer. Hence glycoprotein B was examined for other possible components. Total ash was 5% and no sulphate was found. Thus inorganic constituents do not make a significant contribution. Further, no tryptophan was detected by either direct chemical analysis or u.v. spectroscopy. On the basis of the above observations, the reason for the lack of quantitative recovery of constituents in glycoprotein B is not clear.

Susceptibility to enzyme activity

Glycoprotein A is more sensitive to the action of *C. perfringens* neuraminidase than are other salivary glycoproteins (Cassidy *et al.*, 1965).

When armadillo submandibular glycoproteins, dissolved in 0.04M-acetate buffer at pH4.75, were incubated with *Cl. perfringens* neuraminidase at 37°C, glycoprotein A was completely desialized within 24h (Table 2), whereas no removal of sialic acid from glycoprotein B was observed even after 24h incubation at this pH, which is the optimum pH for this enzyme.

Since for both glycoproteins A and B, the sialic acid was exclusively *N*-acetylneuraminic acid (Table 2), and resistance to enzymic removal of sialic acid is usually due to the presence of *O*-acetyl groups, the resistance of glycoprotein B to *Cl. perfringens* enzyme at pH4.75 seems to be anomalous. However, at pH5.6, the hydrolysis by this enzyme was complete. Thus pH is a critical factor that must be considered when resistance to the action of this enzyme is observed.

Alkaline borohydride treatment

The number and nature of the oligosaccharide side chains were determined by the use of the alkaline

borohydride reaction of Bertolini & Pigman (1967), followed by reduction in the presence of purified PdCl₂, as described by Downs *et al.* (1973).

As shown in Fig. 4, the loss of threonine is rapid and is completed in 5h, then slowing down. The secondary phase of the reaction might be the result of a slow reverse aldol condensation to produce glycine (Vercelotti *et al.*, 1967). However, since some alanine is formed during this phase of the reaction, a slow secondary β -elimination seems to be a more plausible explanation.

Carbohydrate side-chain-protein core linkage

On alkaline borohydride treatment, the armadillo submandibular glycoproteins and their desialized products gave identical results. For these materials, the loss of galactosamine was 90–95mol/100mol of the loss of the hydroxyamino acids. None of the glucosamine, present in small amounts, was destroyed in any of the samples. These results indicate that all of the *N*-acetylgalactosamine in both glycoprotein A and B appear to be *O*-glycosidically linked to the hydroxyamino acid residues of the protein core, in which threonine residues must be the principal points of attachment. Consequently, *N*-acetylgalactosamine can be used as the basis for the calculation of the number and the size of the carbohydrate side chains (Moschera & Pigman, 1975). To obtain such information, the analytical data are examined in terms of mol of sugars/per 100mol of *N*-acetylgalactosamine (or *O*-glycosidic linkages) and are shown in Table 6.

On the basis of these molar ratios, the oligosaccharide chains of glycoprotein A are composed of one-third of AcNeu $\rightarrow$ GalNAc $\rightarrow$ Thr(Ser) and two-thirds of GalNAc $\rightarrow$ Thr(Ser). Other chains could be present, but only in small amounts.

The oligosaccharide side chains of glycoprotein B seem to be more complex, such as:

This structure seems likely, in view of the fact that fucose and sialic acid have always been found as non-reducing terminal groups in such glycoconjugates (Pamer *et al.*, 1968). This is confirmed by the rapid release of the sialic acid residues from armadillo submandibular glycoproteins by mild acid hydrolysis. Since nearly all of the *N*-acetylgalactosamine is *O*-glycosidically linked to the protein core in glycoprotein B, and on the assumption that all of the fucose and sialic acid occupy end positions, the oligosaccharides appear to consist of mono-, di- and tri-saccharides.

The molar ratio of total amino acids to total *O*-glycosidic linkages indicates that at least one out of every 4.4 and 6.8 amino acids for glycoproteins A and

Table 6. *Relative oligosaccharide content of armadillo submandibular glycoproteins*

Content is calculated assuming that all of the galactosamine residues (130.2mmol/100g in glycoprotein A and 68.7mmol/100g in glycoprotein B) are involved in an O-glycosidic linkage between protein core and carbohydrate side chains.

Component	Content (mol/100mol of galactosamine)	
	Glycoprotein A	Glycoprotein B
GalNAc	100	100
GlcNAc	1.0	8
AcNeu	31	30
Gal	Trace	25
Fuc	4	30
Amino acid/GalNAc	4.4	6.8
GalNAc/Thr + Ser	0.41	0.42

B respectively, carries an oligosaccharide unit and that only about 40% of the hydroxyamino acid residues participate in carbohydrate linkages.

Note Added in Proof (Received 1 November 1976)

We now believe that glycoproteins A and B are not major and minor mucins. Instead, the gland has not differentiated into separate submandibular and sublingual glands.

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