USE OF GAS–LIQUID CHROMATOGRAPHY IN THE ANALYSIS OF HYDROLYSATES OF BOVINE-OESTRUS CERVICAL GLYCOPROTEIN*

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ABSTRACT

Gas–liquid chromatography has been used to determine the composition of the non-protein part of an immunologically pure glycoprotein from the uterine, cervical secretion of cows in oestrus. The results obtained for fucose, galactose, and sulphate are in good agreement with previous determinations. Some discrepancies were observed for sialic acid, 2-amino-2-deoxyglucose, and 2-amino-2-deoxygalactose. These differences are explainable by the lack of specificity of the non-chromatographic methods used in earlier investigations. The data suggest the presence of fucose, galactose, 2-amino-2-deoxyglucose, 2-amino-2-deoxygalactose, sialic acid, and sulphate in the molar proportions 1:6.23:1.80:1.31:2.28:0.35.

INTRODUCTION

Variations in carbohydrate content have been reported in bovine[1] and human[2] cervical secretions in different states of ovarian function. Such chemical changes may have effects upon the physical properties of the cervical mucus and be of significance in reproductive physiology.

However, the identification and estimation of carbohydrate components of the epithelial glycoprotein from the uterine cervix have been based on the use of colour reactions that are rather non-specific. Therefore a study of the composition of the bovine, cervical mucin was undertaken, using gas–liquid chromatography, to assess the accuracy of its assumed composition.

The determination of neuraminic acid, neutral sugars, and amino sugars in a polymer, whether by chromatography, colorimetry, or other methods, must be preceded by hydrolytic cleavage of all glycosidic bonds. For this purpose, acid hydrolysis is the method of choice[3], but the conditions required to complete the hydrolysis of hexosaminide bonds are so severe that all of the sialic acid and much of the neutral sugar is destroyed. On the other hand, sialic acid is also destroyed under

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conditions that ensure liberation of all the neutral sugars. As there is no single method that is ideal for the analysis of every glycoprotein, a suitable set of conditions has to be selected in each case.

MATERIALS

Cervical secretion. — Bovine, cervical mucus was obtained from cows in oestrus, and pooled. The main cervical glycoprotein was prepared by gel-filtration as described elsewhere[4], and was found to be immunologically pure.

Reagents. — Neuraminidase, *N*-acetylneuraminic acid, *N*-glycoloylneuraminic acid, D-galactose, D-mannose, L-fucose, 2-amino-2-deoxy-D-glucose hydrochloride, and 2-amino-2-deoxy-D-galactose hydrochloride, erythritol, and perseitol were commercial samples.

The ion-exchange resins, Dowex-50W x8 (20–50 mesh), Dowex-2 x8 (100–200 mesh) used in the carbonate form, and Dowex-50W x12 (200–400 mesh) were obtained from Fluka. The *N*-ethoxycarbonyl derivatives of amino sugars were prepared by using ethyl chloroformate. Trimethylsilylation was effected with a mixture of dried, redistilled pyridine, hexamethyldisilazane, and chlorotrimethylsilane (5:1.5:1).

PROCEDURES

Preparation of neuraminic-free glycoprotein. — Sialic acid was removed from purified glycoprotein by means of neuraminidase, using 100 μl of the enzyme solution (50 U) for 1 mg of pure glycoprotein dissolved in 2 ml of acetate buffer (0.1M, pH 5.5). Hydrolysis was carried out for 4 h at 37°, following which the solution was dialysed for 24 h at 0–4°. The contents of the dialysis tubing were washed out quantitatively and made up to 10 ml with distilled water.

Hydrolysis of bovine, cervical glycoprotein. — Various conditions were tested for the hydrolytic removal of neuraminic acids and neutral and amino sugars. The bovine glycoprotein was dissolved at different concentrations, ranging from 0.25 to 0.50%, in hydrochloric acid of the appropriate molarity.

(*a*) *Sialic acid* was cleaved from the bovine glycoprotein solution by hydrolysis in 0.1M HCl for 60 min at 80°, and in 0.01M HCl for 30, 45, 60, 90, 120, and 150 min, at 100°.

(*b*) *Neutral sugars* were split off either in 0.5M HCl for 4 and 6 h, respectively, as described by Gibbons[1], or in 2M HCl for 1, 1.5, 2, and 3 h, respectively.

(*c*) *Amino sugars* were split off either by 0.5M HCl at 100° for 4 and 6 h, as described by Gibbons[1], or in 4M HCl at 100° for 3, 4, 5, 6, and 8 h, respectively. Hexosamine was also removed from neuraminic acid-free glycoprotein by treatment with 4M HCl at 100° for 4 and 6 h, and with 0.5M HCl at 100° for 4 h.

Deacidification of hydrolysates and addition of internal standards. — (*a*) *Sialic acid.* After hydrolysis, 100 μl of 15% aqueous perseitol was added as the internal standard, and the hydrolysate was neutralised by sodium hydroxide. The solution was then made up to 5 ml with distilled water.

(*b*) *Neutral sugars.* After hydrolysis, 100 μl of 30% aqueous erythritol was added, and the solution was deacidified by passing through columns (1 × 8 cm) of Dowex-2 x8 in the carbonate form, followed by washing with distilled water. The first 4 ml of effluent were discarded and the next 5-ml portion was collected.

(*c*) *Amino sugars.* The hydrolysates were evaporated *in vacuo* over pellets of sodium hydroxide. Erythritol was then added in the same amount as for neutral sugars, and the solution was made up to 5 ml with distilled water.

N-*Ethoxycarbonylation of amino sugars and preparation of the trimethylsilyl derivatives.* — The deacidified or neutralised hydrolysates were buffered by adding 0.3 ml of saturated, aqueous sodium hydrogen carbonate, after which 0.025 ml of ethyl chloroformate was added. The solutions were then allowed to stand at room temperature for 1 h. The product was deionised by passing through a column (i.d. 1 cm) containing a 2-cm layer of Dowex-2 x8 resin over which was laid a 2-cm layer of Dowex 50-W x12 resin. The hexosamines were thus obtained as their *N*-ethoxycarbonylated derivatives. The retention times of these products are higher than those of the parent compounds, so that any interference with neutral sugars could be avoided, as demonstrated by Oates and Schrager[5]. This treatment also had the advantage of permitting a reproducible determination of sialic acids. The effluents were then lyophilised and trimethylsilylated as described above.

Sulphate determinations. — Sulphate was determined by the method of Srinivasan *et al.*[6].

G.l.c. analyses. — These were effected with a Packard chromatograph equipped with a hydrogen-flame detector and a Westronics recorder, using the following coiled glass-columns: (*a*) for sialic acid, an 8 ft × 2 mm (i.d.) column packed with Chromosorb Q (80–100 mesh) and 2% of Silicone GE X E60 (Applied Science Lab. Inc.); (*b*) for neutral sugars, a 6 ft × 2 mm (i.d.) column packed with ABS (100–110 mesh) and 3% SE 30 (Hewlett Packard); (*c*) for amino sugars, a 6 ft × 2 mm (i.d.) column packed with Chromosorb G (80–100 mesh), 2% Apiezon L (Hewlett Packard), and 4% cyclohexane–dimethanol-adipate (Phase Separation Ltd.), as described by Oates and Schrager[5]; (*d*) for sulphate, a 2 ft × 4 mm (i.d.) column packed with Chromosorb Q (80–100 mesh) and 20% Silicone GE XE60. The following flow-rates were used: nitrogen, 20; hydrogen, 25; and air, 300 ml/min. The detector temperature was 260°, and the injection temperature 240°; the temperature was kept at 110° for chromatography of butylamine in the procedure for sulphate determination. Linear temperature programming at 1.5°/min, from 135–230°, was used for neutral and amino sugars. For *N*-acetyl- and *N*-glycoloyl-neuraminic acids, which were obtained together in a single peak, the initial temperature of 150° was maintained for 5 min, and then increased at 4°/min to 230°. The areas of the various peaks were measured by planimetry. The determination of total hexosamines by the Rondle–Morgan technique[7] was carried out, under the conditions described by Gibbons, both on intact glycoprotein and on neuraminic acid-free glycoprotein.

RESULTS

The release of neutral monosaccharides from cervical glycoprotein during acid hydrolysis under various conditions of acid strength and time, at 100°, is presented in Table I. Four neutral monosaccharides were released, with optimum liberation after hydrolysis for 2 h with 2M hydrochloric acid. Galactose and fucose appeared as two major components, and mannose and glucose only in trace amounts.

TABLE I

THE RELEASE OF NEUTRAL MONOSACCHARIDES[a] FROM BOVINE, CERVICAL GLYCOPROTEIN DURING ACID HYDROLYSIS AT 100°

HCl (M)	*Reaction time* (*h*)	*Fucose* (%)	*Galactose* (%)	*Mannose* (%)	*Glucose* (%)
0.5	4	2.71	18.78	0.20	0.10
0.5	6	3.23	19.14	0.35	0.15
2.0	1	3.81	24.23	0.62	0.30
2.0	1.5	4.23	25.79	0.69	0.30
2.0	2	4.41	30.28	0.69	0.35
2.0	3	3.10	22.15	0.44	0.30

[a] Expressed as % of dry glycoprotein.

TABLE II

THE RELEASE OF SIALIC ACIDS FROM CERVICAL GLYCOPROTEIN DURING ACID HYDROLYSIS AT 100°

HCl (M)	*Reaction time* (*min*)	*Sialic acids* (*% of dry glycoprotein*)
0.10	60	12.08
0.01	30	18.47
0.01	45	18.50
0.01	60	19.04
0.01	90	17.19
0.01	120	16.09
0.01	150	15.14

TABLE III

THE RELEASE OF AMINO SUGARS[a] FROM CERVICAL GLYCOPROTEIN DURING ACID HYDROLYSIS AT 100°

HCl (M)	*Reaction time* (*h*)	*2-Amino-2-deoxyglucose* (%)	*2-Amino-2-deoxygalactose* (%)
0.5	4	8.89	4.58
0.5	6	8.99	4.43
4.0	3	8.14	5.39
4.0	4	8.12	5.61
4.0	5	8.66	6.19
4.0	6	8.65	6.35
4.0	8	8.67	6.35

[a] Expressed as % of dry glycoprotein.

TABLE IV

COMPARISON OF DETERMINATIONS OF HEXOSAMINES[a] IN INTACT (I) AND NEURAMINIC ACID-FREE (NF) GLYCOPROTEINS

Glycoprotein	*HCl* (M)	*Reaction time* (*h*)	*G.l.c.*			*Rondle and Morgan method* *Hexosamines*
			2-Amino-2-deoxyglucose	*2-Amino-2-deoxygalactose*	*Hexosamines*	
NF	0.5	4	8.72	4.43	13.15	14.02
I	0.5	4	8.89	4.58	13.47	22.50
NF	4.0	4	8.23	5.60	13.83	n.d.[b]
I	4.0	4	8.12	5.61	13.73	n.d.
NF	4.0	6	8.73	6.33	15.06	n.d.
I	4.0	6	8.65	6.35	15.00	n.d.

[a] Expressed as % of dry glycoprotein. [b] n.d. = not determined.

The kinetics of sialic acid release are shown in Table II. It will be seen that sialic acids are particularly abundant in bovine oestrus, cervical glycoprotein.

The release of hexosamines from cervical glycoprotein is described in Table III; 2-amino-2-deoxyglucose appeared to be released more readily than the *galacto* analogue.

In Table IV, comparison data are included for hexosamines as determined by g.l.c. and by Rondle–Morgan techniques, both on intact, pure glycoprotein and on the same material after neuraminidase treatment. It should be noted that, with glycoprotein not pretreated by neuraminidase, the total hexosamine values determined by the Rondle–Morgan method were almost twice as high as those found by the chromatographic method.

Bovine, cervical mucus was found to contain 0.92% of sulphate.

DISCUSSION

The method of preparation of pure, oestrus glycoprotein and some of its properties have been discussed elsewhere[4]. As far as the preparation of neuraminic acid-free glycoprotein is concerned, it must be pointed out that the material obtained after neuraminidase treatment may not be entirely free of sialic acid, since some types of neuraminic acid are known to be resistant to this enzyme[9].

TABLE V

COMPOSITION[a] OF BOVINE, CERVICAL GLYCOPROTEIN

	Present findings		*Gibbons*[1]	*Gibbons*[8]
	%	*Ratio*	%	%
Neutral sugars				
Fucose	4.41	1	5.1	3.2
Galactose	30.28	6.23	27.5	32.0
(Mannose)[b]	0.69	0.14		
(Glucose)[b]	0.35	0.07		
Total	35.83		32.6	35.2
Amino sugars				
2-Amino-2-deoxyglucose	8.67	1.80	15.3	
2-Amino-2-deoxygalactose	6.35	1.31	11.4	25.8
Total	15.02		26.7	
Sialic acid	19.04	2.98	13.8	14.5
Total carbohydrate	69.89		73.1	75.5
Sulphate	0.92	0.35		±0.5

[a]Expressed as molar ratios and % of dry glycoprotein. [b]Probably a contaminant.

When comparing our results with those of Gibbons (Table V), some discrepancies become apparent. Good agreement was obtained for the determination of fucose and galactose, but we observed the presence of some mannose and glucose;

the latter, but probably not the former, sugar may be a contaminant. Their detection was presumably due to the greater sensitivity and specificity of the g.l.c. procedure. Differences in methods may also explain the higher sialic content observed. Our values are indeed closer to the data reported by Gibbons for bovine, pregnancy mucus. On the other hand, our hexosamine values are much lower than those reported by Gibbons. However, using similar hydrolysis conditions and the same method of analysis (Rondle–Morgan) employed by Gibbons on intact glycoprotein, all discrepancies disappear. With neuraminic acid-free glycoprotein, the results given by the Rondle–Morgan method are not different from the g.l.c. data for intact or neuraminic acid-free glycoprotein. More-drastic hydrolysis conditions increase, in a similar fashion, the release of hexosamine from intact and neuraminic acid-free glycoprotein. It seems likely that the conditions used by Gibbons to release hexosamines were inadequate. Pretreatment with neuraminidase affected only the sialic acid content, without modifying the other components of bovine, cervical glycoprotein. The Rondle–Morgan method over-estimates hexosamine in glycoprotein having a high content of sialic acid, particularly when the release of hexosamines is performed under mild conditions which do not destroy all of the neuraminic acid. The colour then observed should be produced both by the indirect Ehrlich reaction due to hexosamines and by the direct Ehrlich reaction due to sialic acid. Gibbons himself reported that mild pretreatment with acid decreased the (apparent?) hexosamine content[1].

It appears that 2-amino-2-deoxyglucose is split off much faster than the *galacto* analogue, perhaps because the latter is involved in the linkage with the polypeptide core of the molecule.

The values obtained for the sulphate content are in good agreement with published data for the cow[8] and human[2].

In conclusion, it seems that the carbohydrate moiety of bovine, cervical mucus from oestrogenised cows contains fucose, galactose, 2-amino-2-deoxy-glucose and -galactose, sialic acid, and sulphate in the molar proportions indicated in Table V.

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