Chemical Properties of Two Mucoids from Bovine Cervical Mucin

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Our understanding of the chemistry and physical chemistry of epithelial mucin has in the past been handicapped by the lack of methods of fractionation which did not, at the same time, destroy the essential physical properties of the original secretion. Earlier work has, in the main, been confined to the analysis of the crude material (Boyland, 1946; Folkes, Grant & Jones, 1950; Bergman & Werner, 1951; Werner, 1952; Bacila & Ronkin, 1952), or, where purification has been carried out, no effort has been made to preserve the viscoelastic gel-like properties of the mucin. In the latter case, a particular biological activity has usually been the object of the purification procedure, e.g. virulence-enhancing activity (Smith, 1951; Smith, Gallop & Stanley, 1952) or blood-group serological activity (see Kabat, 1956). Indeed a primary object in purification has usually been the destruction of the physical properties of the mucin to facilitate fractionation. To this end, enzymic digestion (Kabat, 1956; Lawton, McLoughlin & Morgan, 1956) and alkali-treatment (Satoh, 1949; Yosizawa, 1950) have frequently been employed. An alternative approach has been the use, as starting material, of a secretion in which a substantial proportion of the mucoid is readily soluble, e.g. urine (Freudenberg & Molter, 1939), ovarian-cyst fluid (Gibbons, Morgan & Gibbons, 1955) and meconium (Rappoport & Buchanan, 1950). A recent review (Bettleheim-Jevons, 1958) summarizes the present state of our knowledge of the chemistry of these substances.

The object of this work was to obtain the constituent responsible for the physical properties of native mucin as an essentially homogeneous preparation in a condition as near to the native state as possible in order to study the factors which underlie these physical properties. Bovine cervical mucin has been used as source material since this secretion shows marked physical changes during the oestrous cycle (Scott-Blair, Folley, Malpress & Coppen, 1941). Both the thick plasto-elastic gel secreted during pregnancy and the thinner viscoelastic 'spinnbar' material secreted at oestrus can be conveniently obtained and studied. A study of this secretion may throw some light on the mechanism of fertilization in mammals; furthermore, it is reasonable to suppose that the physical properties of the secretion are controlled by the circulating sex hormones. There is already some experimental evidence in support of this expectation (F. A. Glover, unpublished observations), so that this study may also have some interest for the endocrinologist. The secretion appears to be a fairly simple one, containing little extraneous material other than protein, and although some contamination with urinary and faecal matter is inevitable, this can, with care, be relatively slight. In this report the chemical properties of two mucoids, isolated from oestrous mucin and pregnancy mucin respectively, are described. The physicochemical properties of these preparations are described in the next paper (Gibbons & Glover, 1959).

EXPERIMENTAL

Materials and methods

Fractionation. The figures quoted for g refer to the force exerted at the centre of the centrifuge tube.

Mucin from the external os of the uterus of the cow was collected and stored frozen until used. The mucin from a number of cows was pooled, but pregnancy and oestrus samples were kept separate. Oestrous mucin was thawed at room temperature and centrifuged at 44 000 g at 0° for 90 min. in a Spinco preparative ultracentrifuge; the aqueous phase was removed and the gel phase allowed to stand with an equal volume of water with occasional gentle shaking for 12–24 hr. at 4°. The centrifuging was then repeated and the gel phase again allowed to stand with water. This procedure was repeated once more, the supernatants being pooled. The gel phase was allowed to stand with five times its volume of aqueous saturated $CaCl_2$ soln.–ethanol (9:1, v/v). This solvent has been found more effective than saturated $CaCl_2$ alone. The mucin usually dissolves in 1–2 days at 4°, but it is sometimes necessary to bring the mucin to room temperature to effect solution. Sufficient ethanol is then added at 4° to make the solution 20% (v/v) with respect to ethanol, when solid debris may be removed by centrifuging at about 2000 g. Further ethanol is then added at 4° with gentle agitation by hand, to 50% (v/v), when a fraction is removed by sedimentation at 44 000 g. Further fractions may be recovered between 50 and 60% (v/v) of ethanol, and between 60 and 90% (v/v) of ethanol. The first fraction is again dissolved in saturated $CaCl_2$ soln.–ethanol (9:1, v/v) and reprecipitated at 50% (v/v) of ethanol; this procedure is repeated once more if the addition of excess of ethanol to the supernatant produces anything more than a slight opalescence. Table 1 shows the analysis of fractions separated during this procedure.

Pregnancy mucin was allowed to stand with ten times its volume of water before the initial centrifuging, when considerable swelling usually occurs. Subsequently the material was worked up in exactly the same way as the oestrus material. The fractions precipitated below 50% ethanol concentration from both materials were found to sediment with a single boundary in the ultracentrifuge and were considered worthy of detailed study. Their chemical properties are described herein and their physical properties in the next paper (Gibbons & Glover, 1959), where the ultracentrifuge schlieren diagrams are given (Fig. 8). They were either dissolved in saturated $CaCl_2$ soln.–ethanol (9:1, v/v) and kept at $-10°$ or dialysed free of Cl ion at 4° (when the solution becomes a gel) and stored frozen until required. Samples required for ash determinations were dialysed free of Cl ion, then dialysed for 48 hr. against 0·5% sodium ethylenediaminetetra-acetate solution which had been brought to pH 6·0 (indicator paper) by addition of 3 N-NaOH, and finally dialysed against several changes of distilled water.

Materials in the supernatants obtained during preparative ultracentrifuging of the mucin could be further split into two fractions by centrifuging at 103 000 *g* for 5 hr. (see Table 1).

Fractionation for sialoprotein. The following experiments were performed in order to detect the presence of a possible sialoprotein component similar to that isolated from salivary mucin by McCrea (1953) and Heimer & Meyer (1956). (i) Oestrous mucin (80 ml.) was made 6 M with respect to urea and further treated as described by Heimer (1957). (ii) A specimen of the supernatant after ultracentrifuging of the crude oestrous mucin (fraction 2, Table 1) was fractionated with ethanol in the presence of Ba^{2+} ions, as described by Curtain & Pye (1955). (iii) A specimen of freeze-dried oestrous mucin (1 g.) was extracted with phenol–water (9:1, v/v) (Morgan & King, 1943) and the insoluble portion again extracted with phenol–ethylene glycol (7:3, v/v) (Annison & Morgan, 1952*a*). (iv) A further specimen (1 g.) of dried oestrous mucin was treated with ficin for 16 hr. at 37° in the presence of toluene (Lawton *et al.* 1956) and the digest after freeze-drying was extracted with phenol–water (9:1, v/v).

Mild acid hydrolysis. A sample of mucoid prepared from oestrous mucin (about 50 mg.) was allowed to stand at room temperature for 48 hr. in 10 ml. of 0·1 N-HCl. This solution was then fractionated by addition of ethanol, material being removed at 60 and 80% (v/v) of ethanol. The final supernatant was neutralized with 1 ml. of N-NaOH, evaporated at reduced pressure to small volume and freeze-dried. Material for chromatography was extracted with ethanol from the dried solids.

Analytical methods

Solutions for analysis were prepared by dissolving the dried material in 0·5 N-H_2SO_4 or 0·5 N-HCl; where this was undesirable a portion of the gel was dissolved in a little water with a few drops of N-NaOH and subsequently neutralized with N-H_2SO_4. In the latter case the concentration of the solution was determined by estimating the nitrogen separately on a dried sample of the same fraction. Aqueous solutions of the untreated mucoids are unsuitable for volumetric analysis.

Samples were prepared for reducing-sugar and hexosamine analyses by hydrolysis in a sealed Pyrex test tube for 16 hr. in 0·5 N-HCl at 100°; under these conditions hydrolysis is complete, and destruction of monosaccharides, other than sialic acid, is negligible (see Fig. 1). A portion of the hydrolysate (1 ml.) was then diluted, neutralized with

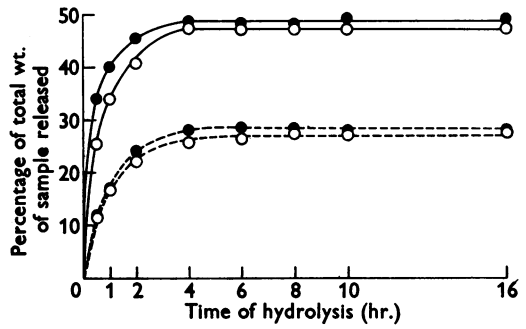

Fig. 1. Rate of hydrolysis of oestrous mucoid (○) and pregnancy mucoid (●) in 0·5 N-HCl at 100°. - - - -, Hexosamine released (as glucosamine base); —, reducing sugars released (as glucose).

Table 1. *Analysis of materials obtained during fractionation of bovine cervical mucin*

Fraction	Mucin	N* (%)	Hexosamine† (%)	Reducing sugar‡ (%)	Galactose (%)	Fucose (%)	Sialic acid§ (%)
1. Material not sedimenting at 103 000 *g*, 5 hr.	Oestrus	12·6	7·5	16·0	13·8	1·2	6·5
	Pregnancy	13·5	11·4	16·0	14·1	1·0	7·7
2. Material sedimenting at 103 000 *g*, 5 hr., but not sedimenting at 44 000 *g*, 1½ hr.	Oestrus	8·5	16·7	31·5	21·4	3·8	11·0
	Pregnancy	7·4	15·1	33·1	20·2	4·1	12·4
3. Material sedimenting at 44 000 *g*, 1½ hr., precipitated from aq. sat. $CaCl_2$ between 50 and 60% of ethanol	Oestrus	6·5	24·7	45·0	28·0	5·0	13·4
	Pregnancy	6·0	26·8	48·8	29·6	5·0	15·3
4. As 3, material precipitated above 60% (v/v) of ethanol	Oestrus	10·2	5·1	11·2	7·5	0·8	7·3
	Pregnancy	8·9	16·4	29·5	18·9	2·0	9·8

* Kjeldahl. † As base. ‡ As glucose. § As the *N*-acetylated compound.

0·5 ml. of N-NaOH and made up to known volume (usually 10 ml.). For paper chromatography, the hydrolysate was passed down a small (0·5 cm. × 8 cm.) column of Amberlite IR 4B (British Drug Houses Ltd.) in its acetate form, washed through with about 10 vol. of water and the effluent freeze-dried. For amino acid analysis and paper chromatography a similar hydrolysate in 6 N-HCl was prepared, which was taken to dryness *in vacuo* over silica gel and solid NaOH. Other estimations were made on the intact materials.

Nitrogen. Kjeldahl nitrogen was estimated in the apparatus of Markham (1942).

Fucose. The method of Gibbons (1955) was used; with the low fucose values encountered, interference from galactose was serious; this was allowed for by making up standards containing appropriate amounts of galactose.

Hexosamine. The method given by Rondle & Morgan (1955) was followed. Results are expressed in terms of glucosamine base.

Reducing sugars. The colorimetric technique of Nelson (1944) was used. Results are expressed in terms of glucose.

Galactose. The anthrone method described by Yemm & Willis (1954) was employed, the extinction at 625 mμ being corrected for the absorption due to the fucose content. This is not a wholly valid procedure since fucose and galactose interact on heating in strong H_2SO_4, but in view of the small amounts of fucose present the error involved is likely to be within experimental error. Sialic acid gives no colour with anthrone.

Sialic acid. The acid *p*-dimethylaminobenzaldehyde reagent of Werner & Odin (1952) was employed. Authentic *o*-sialic acid (*N*-acetylneuraminic acid) was used as standard, in terms of which sialic acid analyses are given.

'N-*Acetylhexosamine*' *colour.* The *N*-acetylhexosamine colour–time of heating relationship was obtained under the standard conditions recommended by Aminoff, Morgan & Watkins (1952).

Acetyl group. A weighed sample (20–25 mg.) in 4–5 ml. of N-H_2SO_4 was hydrolysed in a sealed ampoule at 100° for 16 hr. When cool, the contents and washings were transferred quantitatively to a Markham still and 10 ml. of conc. H_2SO_4 was added. Volatile acid was then steam-distilled for 3 min. into 3 ml. of boiled water. The distillate was titrated with 11·7 mN-$Ba(OH)_2$ solution with methyl red–bromothymol blue indicator. *N*-Acetylglucosamine and acetanilide both yield acetic acid quantitatively under these conditions.

Amino acid analysis. The amino acids in hydrolysates (6 N-HCl; 16 hr. at 100°) of both mucoids were separated and estimated on two resin columns (Moore & Stein, 1951, 1954). The peak effluent volumes were used to identify the amino acids present by comparison with analyses of known mixtures. Confirmation by paper chromatography was also obtained. Under the conditions used the two hexosamines emerge between phenylalanine and lysine as a single very large component. The absence of hydroxyproline was confirmed by using the specific reaction of Newman & Logan (1950) on tubes in the appropriate region.

Hexosamine ratios. The ratio of glucosamine: galactosamine was determined on the same hydrolysates as the amino acids by the method of Gardell (1953) as modified by Crumpton (1958).

Lysozyme. Activity was estimated approximately by observing the initial rate at which turbidity decreased, with a standard suspension of *Micrococcus lysodeikticus* in 0·066 M-phosphate buffer, pH 6·8. A photoelectric colorimeter (Morris, 1944) with a red filter was employed.

Ash. The residue remaining after igniting 100–150 mg. at 500° for 5 hr. in an electric furnace was weighed. Owing to shortage of material, these estimations were not duplicated.

Lipids. These were determined by extraction of the dried material (about 50 mg.) with ether in a Soxhlet apparatus, the soluble material being subsequently estimated as described by Paul (1958) with tristearin as standard. Chloroform after ether did not extract appreciable amounts of additional material.

Paper chromatography. The usual one-dimensional descending technique was employed with the following solvents and spray reagents.

(i) Sugars and amino sugars (0·5 N-HCl hydrolysates): solvent 1. Ethyl acetate–pyridine–acetic acid–water (Fischer & Nebel, 1955); solvent 2. Butanol–acetic acid–water (Partridge, 1948); spray reagent 1. *o*-Aminodiphenyl (Timmel, Glaudemans & Currie, 1956); spray reagent 2. Hexosamine reagents (Partridge, 1948).

(ii) Amino acids (6 N-HCl hydrolysates): solvent 1. Amyl alcohol–pyridine–water–diethylamine (Giri & Rao, 1953); solvent 2. Butanol–benzyl alcohol–water (Block & Bolling, 1951); spray reagent 1. 0·2% Ninhydrin (Meyer, 1957); spray reagent 2. Pauly diazo reagents for histidine (Sanger & Tuppy, 1951); spray reagent 3. Iodoplatinate reagent for cysteine and methionine (Toennies & Kolb, 1951).

(iii) Sialic acid: Solvent. Butanol–acetic acid–water; spray reagent. $AgNO_3$ in acetone followed by ethanolic KOH (Heimer, 1957).

Physical examinations

Absorption spectra. The ultraviolet absorptions of both materials were measured at 0·1% concentrations in saturated $CaCl_2$ soln.–ethanol (9:1, v/v) in a 1 cm. cell and a Unicam SP. 500 spectrophotometer. Infrared spectra were obtained with a Grubb–Parsons double-beam instrument having a rock-salt prism, by the KBr disk technique.

Electrophoresis. Some experiments were performed with the Tiselius apparatus (Hilger and Watts Ltd.); in acetate buffer, pH 5·8, $I = 0·1$. The mucin gives an intractable gel in this solvent and at best a semi-opaque gel at a concentration of about 0·5% was induced with difficulty to form suitable boundaries. Electrophoresis was carried out for from 3 to 5 hr. at 15 mA.

RESULTS

Physical properties. Both mucoids are, in water, clear gelatinous cohesive materials forming two phases. The oestrus preparation is physically indistinguishable from the initial secretion to the eye and the gel phase shows, like the native secretion, 100% elastic recoil [measured as described by Scott-Blair *et al.* (1941)]. The pregnancy mucoid is less firm and thick than the native mucous plug of pregnancy, but if dehydrated somewhat it becomes closely similar physically to the starting material.

A study of the sedimentation, viscosity and flow birefringence of these two purified specimens is reported in the next paper (Gibbons & Glover, 1959). Electrophoretic analysis was, as indicated, unsatisfactory, but with the oestrous mucoid the absence of material migrating more rapidly than the main component could be demonstrated. The absence of material migrating more slowly is not so certain, owing to the opacity of the specimen and the tendency of the mucin to adhere to the sides of the cell, giving a poorly defined descending boundary. The mucoid migrates anodically. The heterogeneity of crude oestrous mucin is readily detectable by electrophoresis, three or sometimes four components being visible.

The ultraviolet-absorption spectra showed no specific absorption maxima between 220 and 400 mμ. There was large absorption below 220 mμ and the pregnancy mucoid showed a small inflexion in the region of 270 mμ. The infrared-absorption spectra are shown in Fig. 2. They are closely similar, the main difference being in the slightly higher absorptions in the region of 1600 and 1400 cm.$^{-1}$ in the oestrous mucoid, probably attributable to carboxyl groups.

Chemical properties. Both mucoids in approximately 1 mg. amounts gave negative tests for tryptophan (glyoxylic acid test), ketoses (Seliwanoff test), heptoses (Dische, 1953) and uronic acids (Dische, 1947). After hydrolysis in 0·5 N-acid for 16 hr. neither sulphate nor phosphate (Allen, 1940) could be detected. The ferric hydroxamate test for *O*-acyl groups (McComb & McCreedy, 1957) was strongly positive for both mucoids. Both mucoids are completely precipitated at zero ionic strength by very low concentrations of cetyltrimethylammonium chloride and also by 0·01 M-flavianic acid. The mucoids are rapidly liquified at room temperature by dilute neutral sodium hypochlorite, as observed by Boyland (1946).

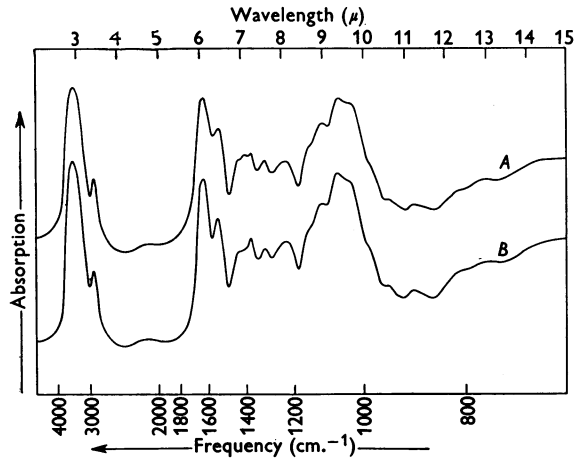

Fig. 2. Infrared-absorption spectra of *A*, oestrous mucoid, and *B*, pregnancy mucoid.

No qualitative difference between the oestrous and pregnancy mucoids was found. The sugars fucose, galactose, glucosamine and galactosamine were identified chromatographically. The following amino acids were likewise identified: serine, threonine, aspartic acid, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, phenylalanine, lysine and arginine. Histidine, cysteine and methionine were shown to be absent by the use of specific spray reagents. Less than 0·2 % of lipid material could be extracted, though the crude mucins contain approximately 0·5–1·0 % of ether-extractable material. The identity of this lipid was not established, but it does not contain nitrogen, phosphorus, sugar or steroid in detectable amounts. It may be related to the material described by Lindahl & Nilsson (1957).

Chromatographic analysis of hydrolysates of the crude mucins reveals the presence of small amounts of glucose and mannose in addition to the sugars already mentioned. These two sugars are found in increased amount in the supernatants from the preparative ultracentrifuging procedure. The nature of the materials containing these two components is being investigated. The final supernatant after centrifuging at 103 000 *g* contains largely protein material. A sample of this material was precipitated with sodium sulphate at 20 % (w/v) concentration, and the resultant protein precipitate after dialysis was subjected to ultracentrifugal and electrophoretic examination, on the basis of which bovine serum albumin and globulin are tentatively identified.

Quantitatively two marked differences were found between the oestrus and pregnancy preparations. The amount of sialic acid in the pregnancy sample is significantly higher than in the oestrus material. A sufficient number of pregnancy (three) and oestrus (six) materials have been partially purified and examined to indicate that this difference is probably not fortuitous. The two mucoids contain about the same total amount of hexosamine but the glucosamine:galactosamine ratios differ. Hexosamine ratios have been obtained only for the two samples described here and it is uncertain whether this difference will be found generally between oestrus and pregnancy materials.

The complete analysis of the two mucoids is given in Table 2.

Both mucoids give a positive Morgan–Elson test for *N*-acetylhexosamine (Aminoff *et al.* 1952). The amount of colour developed increases with time of heating in 0·05 N-Na_2CO_3 to a rather indefinite maximum around 25–30 min., decreasing slowly thereafter (Fig. 3). The absorption spectrum of the

chromophore was found to be closely similar to that of authentic *N*-acetylglucosamine heated for 4 min. with 0·05 N-Na_2CO_3.

The rate at which free hexosamines and reducing sugars are produced by hydrolysis with 0·5 N-HCl at 100° is shown in Fig. 1.

Both mucins in their native state have weak but readily demonstrable lysozyme activity, equivalent to between 0·05 and 0·1 % of that of crystalline egg-white lysozyme (The Armour Laboratories, Eastbourne, Sussex). The purified preparations do not possess this enzymic activity, which can be found in appropriately increased concentration in fraction 1 (see Table 1). Egg-white lysozyme has no observable effect on the mucin (cf. Lawton *et al.* 1956).

Fractionation of crude oestrous mucin according to Heimer (1957) gave three fractions from the clot formed at pH 3·7: (i) material insoluble in acetate buffer (180 mg.; N, 8·2, galactose, 20·9, sialic acid, 11·1 %); (ii) material removed from solution in acetate buffer by the Sevag procedure (50 mg.; N, 12·6, galactose, 11·5, sialic acid, 6·3 %); (iii) material remaining in the final supernatant (6 mg.; N, 7·7, galactose, 14·9, sialic acid, 3·0 %). Two fractions were isolated from material fractionated by Curtain & Pye's (1955) procedure: (i) precipitated with ethanol, between 40 and 50 % (v/v) of ethanol (35 mg.; N, 6·4, galactose, 23·1, sialic acid, 12·5 %); (ii) precipitated at above 50 % (v/v) of ethanol (75 mg.; N, 6·5, galactose, 23·3, sialic acid, 12·8 %). Phenol-extraction of dried crude mucin yielded a phenol-insoluble material, part of which was water-soluble; analysis of this latter fraction gave N, 6·9, galactose 20·8, sialic acid, 13·5 %. Negligible amounts of sialic acid could be found in the fractions soluble in phenol. Enzymic digestion followed by phenol extraction gave a phenol-insoluble material (the whole of which was water-soluble), which contained: N, 5·8, galactose, 28·8, sialic acid, 12·6 %. Some sialic acid-containing material was in this instance soluble in phenol but almost the whole of it could be precipitated by addition of ethanol to the phenol supernatant to 12·5 % (v/v).

Hydrolysis of an oestrous mucoid preparation in 0·1 N-HCl at 20° for 48 hr. results in the material, initially a two-phase gel, slowly dispersing to give ultimately a clear viscous solution, rather like gastric mucin after peptic autolysis. The results of subsequent fractionation are shown in Table 3. It can be seen that the only component showing a marked decrease in amount is sialic acid, and in confirmation of this a chromatogram of the material not precipitated at 80 % (v/v) of ethanol concentration showed two spots only, both attributable, by comparison with an authentic sample, to sialic acid. No sugar or oligosaccharide was detected.

Table 2. *Analysis of oestrous and pregnancy mucoids*

Figures are given as g./100 g. of dry mucoid.

	Oestrous mucoid	Pregnancy mucoid
Galactose	27·5	28·1
Fucose	5·1	5·0
Glucosamine*	15·3	12·6
Galactosamine*	11·4	15·7
Sialic acid†	13·8	17·5
Acetyl‡	10·0	11·1
Ash	3·1	3·7
Nitrogen §	6·45	6·04
Serine	3·20	2·46
Threonine	6·28	5·45
Aspartic acid	1·85	0·73
Glutamic acid	2·63	1·77
Glycine	1·25	0·88
Alanine	2·02	1·70
Proline	3·98	2·40
Valine	1·59	1·77
Isoleucine	0·46	0·40
Leucine	1·19	0·86
Phenylalanine	1·12	1·14
Lysine	0·77	1·02
Arginine	1·38	1·38

* As base. † As the *N*-acetyl compound.
‡ As CH_3·CO. § Kjeldahl.

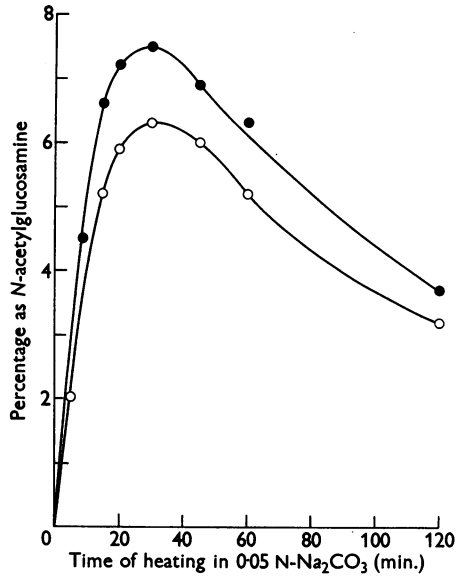

Fig. 3. *N*-Acetylhexosamine reaction. Oestrous mucoid (○) and pregnancy mucoid (●). The amount of colour formed is expressed in terms of an equal weight of *N*-acetylglucosamine heated in 0·05 N-Na_2CO_3 for its optimum time (4 min.).

Table 3. *Analysis of a sample of oestrous mucoid treated with 0·1 N-hydrochloric acid at room temperature for 48 hr. and subsequently fractionated with ethanol*

	N* (%)	Hexosamine† (%)	Galactose (%)	Reducing sugars‡ (%)	Fucose (%)	Sialic acid§ (%)
Initial material	6·62	20·0	21·7	39·8	5·1	13·4
Material after acid hydrolysis, fractionated with ethanol						
(*a*) 0–60% (v/v) ethanol precipitate	6·76	22·0	22·8	42·0	5·2	8·4
(*b*) 60–80% (v/v) ethanol precipitate	6·88	22·8	21·1	38·6	5·0	7·7

* Kjeldahl. † As base. ‡ As glucose. § As the *N*-acetyl comound.

DISCUSSION

It can be seen that the two preparations isolated are chemically mucoids of the 'blood-group substance' type or, according to Werner (1952), fucomucans. The analyses of Shettles, Dische & Osnos (1951) have already suggested the presence of this type of material in human mid-cycle cervical mucin. From the appearance of the mucoids isolated, there is every reason to believe that it is these materials which are responsible for the characteristic physical properties of epithelial mucin. The fractionation procedure has been designed to avoid physical or chemical damage to labile structures, for it has been found that such apparently harmless procedures as vigorous stirring or drying change the material physically. Shaking the mucin with amyl alcohol–chloroform mixtures, for example, results in the formation of a dense white gel in the organic layer from which mucin cannot be recovered in its native state; the aqueous layer is essentially devoid of mucoid. The purified mucoid may be reconstituted after careful freeze-drying over silica gel, to give apparently unchanged material, but the native secretion when freeze-dried becomes completely insoluble. It is suggested that the long thread-like mucoid molecules are extensively intertwined with one another in the gel phase and that any violent shearing results in their being torn apart and irreversibly broken by the applied physical force. This has been reported to occur to some other long-chain molecules (Tsvetkov & Frisman, 1945). In the native state, contaminating protein is probably enmeshed within the matrix of entangled mucoid threads, and part of the protein becomes denatured on drying. This would account for the behaviour of the freeze-dried native secretion.

The homogeneity of these two mucoid preparations cannot be regarded as established. It has been shown that they both sediment as one component and that the oestrous mucoid is probably electrophoretically homogeneous also; furthermore, known contaminants in the crude mucin (albumin- and globulin-like proteins, lysozyme, glucose- and mannose-containing material) are not detectable in the purified preparations. It is probable that extraneous material has been largely eliminated therefore, but it is possible that the preparations consist of a mixture of closely similar mucoids; indeed, since the starting material was collected from a number of different individual cows, this is probable. Despite the application of physical and immunological techniques, the establishment of homogeneity of mucoid preparations is not entirely satisfactory at present, and if the recent genetical theory due to Watkins & Morgan (1959) is correct it would seem that strict homogeneity in such preparations is likely to be unattainable. The use of individual animals with some immunological control would be a considerable improvement, however, and experiments along these lines are projected.

The amount of sialic acid in both these preparations is considerably higher than has been previously reported for this type of mucoid (Gibbons *et al.* 1955). It is unlikely, however, that this is due to contamination with sialoprotein since none of the procedures employed gave any indication of a separation of the galactose and sialic acid into different fractions. McCrea (1953) has shown that sialoprotein is readily soluble in phenol and the experiments described would almost certainly have revealed its presence. It is considered that the sialic acid is an integral part of the mucoid molecule, as isolated, and this view is supported by the rapid anodic electrophoretic migration of the oestrous mucoid. It is probably unwise to infer the presence of sialoprotein from the occurrence of large amounts of sialic acid in a mucous secretion.

The nature and distribution of the amino acids in these two mucoids do not suggest any extensive contamination with extraneous protein, for they are rather unlike that of proteins generally. The same amino acids are present as are found in the human blood-group mucoids (Aminoff, Morgan & Watkins, 1950; Annison & Morgan, 1952*a*, *b*; Gibbons & Morgan, 1954), with the single exception of phenylalanine, which these workers found to be absent and which is present in the two mucoids

examined here. The striking feature in the amino acid analysis (Table 2) is the large amount of the hydroxyamino acids, amounting together to about one-third of the total amino acids; the rather high proline content of these materials is also noteworthy. There is somewhat less of the acidic and somewhat more of the basic amino acids in the pregnancy sample than in the oestrus sample; otherwise the analyses of the two mucoids are quite similar. Threonine has been recognized as a major constituent of this type of mucoid since its isolation from a urinary mucoid in 1942 (Freudenberg, Walch & Molter, 1942); moreover Aminoff & Morgan (1951) find hydroxyamino acids to account for 34% of the amino acid nitrogen in their thoroughly purified blood-group A mucoid. Carsten & Kabat (1956) give amino acid analyses for samples of blood-group active mucoids isolated from hog gastric mucin and from human saliva, which are very similar to the analyses in Table 2; thus the distribution of amino acids reported here may be fairly typical of mammalian epithelial fucomucan. Two other published amino acid analyses of mucin, however, that of Folkes *et al.* (1950) (frog-spawn mucin) and Brand & Saidel (1946) (hog gastric mucin), do not show any marked resemblance to that of these two bovine cervical mucoids.

These two mucoids also resemble the human blood-group substances in that they contain the same sugar components, including sialic acid. Furthermore, the course of the *N*-acetylhexosamine reaction (Fig. 2) and the rate of hydrolysis of hexosamine in 0·5N-acid at 100° (Fig. 3) are similar to the behaviour of blood-group mucoids under the same conditions; free reducing groups, on the other hand, appear in 0·5N-acid hydrolysates somewhat more rapidly because of the relatively large amount of the very acid-labile component, sialic acid. This substance is rapidly destroyed in 0·5N-acid at 100°. The human substances also contain more fucose than the bovine mucoids, but Beiser & Kabat (1952) find that their blood-group active mucoids isolated from bovine gastric mucin are similarly low in fucose, so that fucose content is likely to be related to an inter-species difference in mucin structure. The lability of the fucose towards acids has not yet been investigated, but unlike the sialic acid it is not labile at pH 1 at room temperature. The greater part of the fucose in blood-group substances is labile at pH 3·0 at 100°.

The two bovine cervical mucoids are of considerably higher molecular weight (4×10^6) than any blood-group mucoid so far described (Gibbons & Glover, 1959).

It has been suggested (Gibbons, 1959) that the differing physical properties of epithelial mucin are related to the expansion factor of the constituent thread-like random-coil molecules. The two mucoids described here have been shown to have markedly different expansion factors (Gibbons & Glover, 1959), and it is probable that this in turn is related to the sialic acid component, since the sialic acid content constitutes the only major chemical distinction between the two mucoids so far as is known at present. Some confirmation of this view is given by the result of the 0·1N-acid hydrolysis experiment, since here the mucoid is converted from a two-phase gel into a viscous solution, which implies a marked increase in expansion factor, and there is concomitant release of some of the sialic acid residues. However, it is quite likely that some depolymerization may also be occurring, in which case the physical properties of the solution would change from this cause also. This point is being investigated. This experiment suggests that a major portion of the sialic acid is present as end group. It should be observed that Odin (1958) has concluded that the physical properties of the contents of human ovarian cysts are related, in the same way as cervical secretions, to their sialic acid contents.

The amount of acetyl group present in these mucoids is considerably greater than that required for acetylation of the amino groups of the hexosamine and the sialic acid, but is approximately correct if the sialic acid is mono-*O*-acetylated in addition. The positive ferric hydroxamate test supports this suggestion, but, as has been pointed out by Armstrong, Baddiley, Buchanan, Carss & Greenberg (1958), the test is not specific for *O*-acetyl groups; amino acid esters, which may well be present, also react.

The analyses in Table 2 account, within experimental error, for the whole of the material, and it is unlikely that a major constituent remains to be identified. The amino acids, sialic acid and hexosamines account for almost the whole of the nitrogen so that amide nitrogen is likely to be small. Quite large amounts of NH_4^+ ion are produced by acid hydrolysis but it is probable that most of this is due to the destruction of the sialic acid. The determination of hexosamine ratio gave a somewhat low overall recovery of hexosamine (88 and 90% respectively for the oestrous and the pregnancy mucoids), so that there is the possibility of an unidentified hexosamine being present. However, it is considered more probable that slight destruction of the hexosamines in 6N-hydrochloric acid accounts for the low yields.

Cervical mucin appears to be a mucous secretion containing mucoid relatively uncontaminated with extraneous material. The major foreign material appears to be protein, which may be eliminated relatively easily. Buccal and gastrointestinal mucin, in addition to contamination with food

particles, contains also acidic mucopolysaccharides (Werner, 1952; Glass, 1953; Pasternak, Kent & Davies, 1958); in salivary mucin a sialoprotein is present as a major constituent (McCrea, 1953; Curtain & Pye, 1955; Heimer & Meyer, 1956; Heimer, 1957). Urinary mucin contains a closely similar sialoprotein (Tamm & Horsfall, 1952). Cervical mucin does not contain appreciable amounts of uronic acid or ester sulphate; and experiments designed to isolate a sialoprotein constituent similar to that described by the above-quoted authors suggest that this material, if present, is a very minor constituent. It is possible that the small amount of mannose detected in the crude mucin originates from a material of this type (Gottschalk & Ada, 1956). Two other important sources of mucin, namely ovarian-cyst fluids and meconium, suffer from the disadvantage that they have been incubated at body temperature, often for long periods, a circumstance which may alter the physical and chemical properties of these secretions; in fact the ease with which soluble, relatively low-molecular-weight mucoid may be isolated from them rather suggests this. For these reasons, cervical mucin, apart from its intrinsic interest from the standpoint of fertility and of endocrinology, may well be a useful secretion upon which to study the biochemistry of mammalian mucin.

SUMMARY

1. A method for the isolation of undegraded mucoid from bovine cervical mucin is described.

2. Two samples of mucoid, one isolated from mucin obtained at oestrus and the other from pregnancy mucin, are shown to be largely free from known contaminants in the crude secretion, and to be the materials chiefly responsible for the physical properties of the native mucin.

3. The mucoids were found to consist of 75–80% of carbohydrate and 20–25% of amino acid residues, and to behave chemically in a manner similar to the human blood-group substances.

4. Fucose, galactose, glucosamine, galactosamine, sialic acid, threonine, serine, aspartic acid, glutamic acid, proline, glycine, alanine, valine, leucine, isoleucine, plenylalanine, lysine and arginine were identified chromatographically in both mucoids and estimated. The sialic acid content of the pregnancy mucoid (17·5%) is higher than that of the oestrous mucoid (14%).

5. The results of hydrolysis of oestrous mucoid in 0·1 N-hydrochloric acid at 20° suggest that part of the sialic acid is present as end group and that the sialic acid content is one of the factors affecting the physical properties of mucin.

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The Physicochemical Properties of Two Mucoids from Bovine Cervical Mucin

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A study of the phenomenon of flow-birefringence in solutions of macromolecules can yield information relevant to the size, shape and flexibility of the molecules in solution. Essentially this involves observation of the optical properties of a solution under shear, conveniently in a concentric-cylinder viscometer having a transparent base, fitted to a vertical optical bench. Consider a small segment of the annulus with the inner or outer cylinder rotating at constant speed: the flow lines will be along the annulus in a horizontal plane, the direction of the velocity gradient will lie radially across the annulus, also horizontally, and the line of sight will be vertical and at right angles to both. A schematic diagram of a suitable apparatus is given by Glover & Suffolk (1957).

Two factors are involved in the phenomenon of flow-birefringence: the optical and the hydrodynamic.

(1) *Optical factors.* A birefringent body possesses different refractive indices for light polarized in two directions at right angles. Linearly polarized light passed normally through such a body, in general, emerges elliptically polarized, and cannot be extinguished by rotation of an analyser. If the direction of polarization of incident light is coincident with, or at right angles to, the optic axis of the body, it emerges unchanged and can be extinguished by an analyser. Lines defining the direction of the optic axis are, when applied to a solution under shear, termed neutral lines. The factors which determine the position of the neutral lines in a flowing solution of birefringent molecules will be considered below. Their direction may be located by examining the flowing solution between crossed Nicol prisms. The smaller angle between the neutral lines and the flow lines is called the angle of isocline (χ). The amount of double refraction, that is the difference of refractive indices of the flowing solution for light with electric vectors parallel with and at right angles to the neutral lines, may be measured by means of a quarter-wave plate. It is referred to as the birefringence (Δn) and may be positive or negative.