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Studies on the Mucin Derived from Human Colloid Breast Carcinoma

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1. A non-diffusible mucoid, showing a single peak in the ultracentrifuge, was isolated from human colloid breast carcinoma by treatment with trypsin and pepsin. The material contained threonine, leucine (isoleucine), valine, proline, glycine and glutamic acid in the approximate molar proportions 5:1:1:2:1:1. Smaller amounts of aspartic acid and serine were also found. For each 5 threonine residues, 6 *N*-acetylgalactosamine and 3–4 galactose residues were present. 2. The mucoid possessed reducing properties by the Park & Johnson (1949) procedure; these were attributable to the action of mild alkali, as employed in this procedure. Mild alkaline treatment by the Aminoff, Morgan & Watkins (1952) procedure gave rise to a diffusible *N*-acetylgalactosamine chromophore that gave an enhanced colour with Ehrlich's reagent. That galactosyl-(1→3)-*N*-acetylgalactosamine residues were liberated was supported by periodate studies. 3. Alkaline liberation of hexosamine residues was accompanied by a specific destruction of threonine. After 40 min. at 100° in 0·18 N-lithium hydroxide, both moieties had almost completely disappeared from the ninhydrin-positive components formed on subsequent acid hydrolysis. Glycine and α-oxobutyric acid were present in the acid hydrolysate, showing that both possible pathways of a β-elimination reaction were involved. Formation of diffusible peptide on very mild alkaline treatment was attributable to the rupture of the original peptide core, necessitated by the second of these two pathways. 4. Hydroxamate formation on treatment with hydroxylamine showed the presence of carbohydrate linkage to glutamic acid or aspartic acid residues or both. This could account for the single *N*-acetylgalactosamine residue not linked to threonine. 5. The native mucin contained sialic acid, which was cleaved by the acid environment used in the treatment with pepsin. A statistical model of the mucin would require each prosthetic group to be linked, via *N*-acetylgalactosamine, to threonine, which would occupy every alternate position among the amino acids in the peptide core.

The term colloid carcinoma of the breast is usually reserved for those neoplasms that exhibit a markedly gelatinous character. The mucin is produced by malignant epithelial cells and can be observed both within the cell and, usually in large amounts, in the stroma. A strongly positive reaction with periodic acid–Schiff reagent is a feature of this type of tumour (Evans, 1956). Histochemical studies led Johnson & Helwig (1963) to conclude that the secretion was a sialomucin containing a protein moiety. Hyaluronic acid was excluded on the basis of lack of digestion by hyaluronidase, and negative reactions with Alcian blue at pH 0·4 and toluidine blue at pH 1·0 were interpreted as indicating an absence of appreciable amounts of sulphated acid mucopolysaccharides. Positive reactions with these latter reagents, at higher pH values, suggested the presence of sialic acid.

As part of a study on the influence of the stroma on tumour spread and invasion, a specimen of a colloid breast carcinoma was examined for constituent acid mucopolysaccharides after digestion with proteolytic enzymes. Fractionation with ethanol from calcium acetate buffer (Meyer, Davidson, Linker & Hoffman, 1956) failed to yield any hyaluronic acid or chondroitin sulphate, but instead a product with a higher solubility in ethanol was obtained. On examination, this material was found to contain high concentrations of threonine, *N*-acetylgalactosamine and galactose. The properties of the mucoid and the mode of attachment of the large numbers of carbohydrate prosthetic groups are described in the present paper. (Mucoid is the term used in the present paper to denote the non-diffusible mucopolysaccharide isolated from the tissue by treatment with proteolytic enzymes.)

MATERIALS AND METHODS

Materials

Carbohydrates. D-Glucosamine hydrochloride was purchased from British Drug Houses Ltd., Poole, Dorset, and *N*-acetylglucosamine from L. Light and Co. Ltd., Colnbrook, Bucks. D-Galactosamine hydrochloride was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and *N*-acetylgalactosamine from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. *N*-Acetylneuraminic acid was purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. Other carbohydrates were Laboratory Reagent grade from British Drug Houses Ltd.

Analytical methods

Hexosamine. Samples were hydrolysed in 5 N-HCl for 16 hr. at 105° and analysed by the method of Rondle & Morgan (1955). Consistently higher colour yields were obtained, however, by use of a Na_2CO_3–$NaHCO_3$ buffer, pH 9·5 (Immers & Vasseur, 1950), rather than the 0·5 N-Na_2CO_3 normally employed. Galactosamine was preferentially determined by use of the distillation technique of Cessi & Serafini-Cessi (1962).

Sialic acid. The acid diphenylamine reaction as employed by Anderson & Maclagan (1955) was used, and measurements were made in a Unicam SP.600 spectrophotometer at 525 mμ.

Galactose. The orcinol–H_2SO_4 reagent of Winzler (1955) was employed, and confirmatory analyses were made with cysteine–H_2SO_4 reagent (Dische, 1955). In experiments where dialysis was employed, well-washed cellulose casings were used and blanks were carried out by using the same amount of dialysis tubing, minus the addition of mucoid.

Reducing sugar. The method of Park & Johnson (1949) was used with heating times of 15 min. Samples of a standard solution of the mucoid were chosen so that the amount of reducing sugar, as glucose, fell to within the range 1–9 μg.

Uronic acid. The method of Dische (1947) was used.

Sulphate. This was determined on samples hydrolysed for 16 hr. with 5 N-HCl by the method of Fromageot (1955).

Amino acids. Samples of mucoid (usually 300–400 μg.) were hydrolysed in 3 ml. of 5 N-HCl for 16 hr. The acid was removed by a stream of air, and the process repeated twice after the addition of water. After being left overnight in a desiccator over H_2SO_4 and NaOH, the residue was taken up in water and applied to sheets (23 cm. × 20 cm.) of Whatman 3MM paper. Two-dimensional chromatography was carried out in solvent system *A* for 7 hr., and then in solvent system *B* for 3 hr. (Salton & Mulhaud, 1959). Hexosamine and amino acids were detected by spraying with ninhydrin. For quantitative analyses, the dried chromatograms were heated for 15 min. at 110° and the fluorescent areas detected by viewing in ultraviolet light from a Wood's glass filter. These areas were marked and cut out, and the amino acid contents determined by the method of Rosen (1957). Known amounts of amino acids were subjected to two-dimensional chromatography and eluted for construction of a standard curve. Proline was determined by measurement at 440 mμ and by direct visual comparison with standards that were chromatographed and sprayed with ninhydrin under identical conditions.

Paper chromatography. The following solvent systems were used, and Whatman no. 1 paper was employed, unless otherwise stated: *A*, butan-1-ol–acetic acid–water (4 :1:1, by vol.); *B*, redistilled A.R. pyridine–water (4:1, v/v); *C*, ethyl acetate–pyridine–acetic acid–water (15:10:2:6, by vol.); *D*, butan-1-ol–pyridine–water (6:4:3, by vol.); *E*, butan-1-ol–ethanol–water (4:1:1, by vol.). Reducing sugars were detected by silver nitrate–NaOH (Trevelyan, Procter & Harrison, 1950), and amino sugars by ninhydrin or by the Elson–Morgan test (Partridge, 1949).

Paper electrophoresis. Paper electrophoresis of the mucoid was carried out in sodium barbiturate buffer, pH 8·6 and *I* 0·08, at 10 v/cm. for 5 hr. The position of the mucoid was determined by the periodic acid–Schiff reagent as used by Varley (1958).

Ultracentrifuge studies. A Spinco model E ultracentrifuge was employed in a run kindly performed by Mr M. Smith, C.S.I.R.O. Biochemical Unit, University of Sydney.

Periodate oxidation. Oxidation conditions were based on the methods of Popenoe (1959) and Graham & Gottschalk (1960). First, 2 ml. of 0·01 M-KIO_4 was added to a solution of mucoid (1 mg.) in 1 ml. of 0·1 M-sodium acetate buffer, pH 5·0. The mixture was cooled in ice and left at 5° in the dark for 5 hr. 1,2-Propylene glycol was then added in excess, and, after standing for a further 1 hr., the solution was dialysed against distilled water overnight in the cold room. The solution was concentrated to dryness and then taken up in water, and samples were used for the determination of galactose and hexosamine (after acid hydrolysis in the latter case).

Treatment with hydroxylamine. Conditions were adapted from those of Graham, Murphy & Gottschalk (1963). Hydroxylamine hydrochloride (1 M) was adjusted to pH 12 with conc. NaOH. To 0·05 ml. of an aqueous solution of mucoid (approx. 11 mg./ml.), 0·05 ml. of the basic hydroxylamine was added and the mixture left at 37° for 7 hr. The solution was diluted with water (3 ml.) and dialysed against running tap water overnight. It was then evaporated to dryness by a stream of air at 40°, and dissolved in water (0·5 ml.). Hydroxamate was measured by oxidation with iodine to nitrite, followed by diazotization of sulphanilic acid and coupling the diazo salt with α-naphthylamine (Yashphe, Halpern & Grossowicz, 1960). The method was scaled down by one-half and extinctions were measured at 530 mμ.

Treatment with dilute sodium carbonate. Aminoff *et al.* (1952) established conditions for obtaining the maximum yield of chromophore from *N*-acetylhexosamines on heating with weak base. The chromophore produced a colour on addition of Ehrlich's reagent, with absorption maxima at 550 and 590 mμ. The conditions chosen for mild alkaline treatment of the mucoid, and subsequent analysis of the chromophore produced, were as described by these authors. However, the heating time was arbitrarily increased to 20 min., since heating times longer than 4 min. were necessary when blood-group substances were used. In addition, the method was scaled down by one-half. *N*-Acetylgalactosamine samples were heated for 4 min. only to obtain maximum colour yield for construction of a standard curve. Extinctions were measured at 590 mμ.

Dialysis of the chromophore, obtained from the mucoid on heating in Na_2CO_3 solution, was carried out as follows.

A 0·05 ml. sample of mucoid solution (containing 114 μg. of bound galactose) was diluted to 0·5 ml. with water. Then 0·05 ml. of 0·5 N-Na_2CO_3 was added and the tube sealed with a polythene stopper. Heating was carried out in a boiling-water bath for 20 min., and then the solution was chilled in an ice bath and adjusted to pH 5·5–6·0 with dilute acetic acid. Dialysis was carried out with agitation, against 30 ml. of water at 4°, for 2·5 hr. As a control, 265 μg. of *N*-acetylgalactosamine was treated in a similar manner, but with a heating time of only 4 min. Both diffusates were evaporated to dryness *in vacuo* at 40°, and the colour was measured after treatment with Ehrlich's reagent. The recovery of *N*-acetylgalactosamine in the diffusate was 87%, and this value was used as a correction factor for expressing the recovery of the diffusible chromophore liberated from the mucoid.

Treatment with lithium hydroxide. Mucoid (280–560 μg.) was heated with 0·5 ml. of 0·18 N-LiOH in a sealed tube in a boiling-water bath for 20 min. The solution was then neutralized with HCl, evaporated to dryness in an air stream at 40°, and the residue extracted with ethanol (2 × 4 ml.) and then acetone (2 × 4 ml.). The remaining material was hydrolysed overnight in 5 N-HCl at 110° and the amino acids and hexosamine were separated by two-dimensional chromatography in solvents *A* and *B*.

α-Oxo acid formation. To 620 μg. of mucoid in 1 ml. of water was added 0·15 ml. of 0·5 N-Na_2CO_3 and the solution heated in a sealed tube for 2 hr. at 100°. Then 0·02 ml. of a 0·6% solution of *o*-phenylenediamine (Hopkin and Williams Ltd., Chadwell Heath, Essex) in 5 N-HCl was added, and the concentration of HCl then adjusted to 4 N by the addition of conc. HCl. The solution was hydrolysed at 110° overnight, cooled, adjusted to pH 8·0 with conc. NaOH solution and extracted with chloroform (3 ml.). The chloroform extract was evaporated to dryness and then redissolved in chloroform, and samples were used for paper chromatography (Hockenhull & Floodgate, 1952).

α-Oxobutyric acid was prepared from DL-α-aminobutyric acid (British Drug Houses Ltd., Laboratory Reagent grade) by diazotization followed by a short heat treatment at 50° to form the α-hydroxy acid. The latter was isolated as an oil by ether extraction of the concentrated aqueous solution and oxidized by addition of $KMnO_4$ in alkaline solution, at 0°, until oxidation was complete as judged by the persistence of a pink colour when a drop of the mixture was placed on filter paper. These conditions were essentially those used for the oxidation of mandelic acid by Corson, Dodge, Harris & Hazen (1951). The MnO_2 was removed and the solution neutralized, concentrated, acidified and extracted with ether, which yielded the oily oxo acid. The *p*-nitrophenylhydrazone had m.p. 206° [Vogel & Schinz (1950) give m.p. 194°] (Found: C, 50·5; H, 4·8; N, 17·2%. Calc. for $C_{10}H_{11}N_3O_4$: C, 50·7; H, 4·7; N, 17·7%). Heilbron & Bunbury (1953) list the m.p. as 232°. It is likely that the *p*-nitrophenylhydrazone with m.p. 206° is a geometric isomer, since two isomeric forms of the *o*-nitrophenylhydrazone of ethyl α-oxobutyrate have been isolated (Hughes, Lions & Ritchie, 1939) and two melting points recorded for the phenylhydrazone of α-oxobutyric acid. Both this oxo acid and pyruvic acid were treated with *o*-phenylenediamine in 4 N-HCl at 110° in a similar manner to the mucoid. Paper chromatography of the chloroform extracts yielded zones that had a blue fluorescence in ultraviolet light. This was in contrast with Hockenhull & Floodgate (1952), who obtained derivatives from oxo acids and *o*-phenylenediamine, by treatment with cold HCl, that gave a yellow fluorescence in ultraviolet light. In the present work the *o*-phenylenediamine, either alone or after heating in 4 N-HCl, did not give rise to blue-fluorescent zones, and the addition of oxo acid was necessary for their formation.

Isolation of the mucoid

The extremely gelatinous tumour was diagnosed as a tubular adenocarcinoma. A 5 g. sample of tumour was homogenized in water (40 ml.) and adjusted to pH 1–2 with conc. HCl. Pepsin (10 mg. of Difco pepsin 1:10000) was added, and the solution layered with toluene and incubated overnight at 37°. Adjustment to pH 7·0 was then made with NaOH, and trypsin (10 mg. of Difco trypsin 1:250) added and the incubation continued for a further 2 days. After centrifuging, the supernatant was stirred for 2 hr. with 5 g. of fuller's earth, then filtered and dialysed overnight against running tap water. The dialysis residue was evaporated to dryness *in vacuo* at 40° and dissolved in 10 ml. of 5% (w/v) calcium acetate in 0·5 N-acetic acid. Ethanol fractionation was then carried out; fractions at 31% (v/v) and 40% (v/v), collected after standing overnight at 4°, only amounted to traces. Precipitation did not occur until the ethanol concentration was 65% (v/v). This fraction was collected, redissolved in buffer and reprecipitated with ethanol. The fine white precipitate was washed with ethanol and then with ether. The yield was 52 mg., and an aqueous solution clarified by spinning at 20000*g* had $[\alpha]_D^{20} + 12{\cdot}5°$.

When a sample of the above mucoid was treated with papain and then carboxypeptidase (Fletcher, Marks, Marshall & Neuberger, 1963) an 86% recovery of non-diffusible material was obtained. The molar proportions of the constituent amino acids and carbohydrates were similar to those of the starting material.

RESULTS

Chemical composition. The isolated mucoid contained neither uronic acid nor acid-hydrolysable sulphate. The products of acid hydrolysis, carried out with a variety of acid strengths, were galactose, hexosamine and a number of amino acids. Galactose was identified by comparison of its R_F with standards in solvents *C*, *D* and *E*. Galactosamine was identified as the amino sugar by chromatography in solvent *C*, with barium chloride-impregnated paper (Heyworth, Perkins & Walker, 1961). This was confirmed when the amino sugar produced on acid hydrolysis was found to be quantitatively converted into volatile chromogen by the method of Cessi & Serafini-Cessi (1962).

Quantitative analysis. Table 1 lists the quantitative composition of the mucoid. The amino acids leucine (isoleucine), valine, proline, threonine, glycine and glutamic acid were always observed in two-dimensional chromatograms. Aspartic acid and serine occurred in smaller amounts and alanine

Table 1. *Quantitative composition of the mucoid*

Component	Composition (μmole/400 μg. of mucoid)	Composition (%)
Leu (Ile)	0·136	4·5
Val	0·094	2·8
Pro	0·252	7·2
Thr	0·610	18·2
Gly	0·163	3·6
Glu	0·110	4·0
Asp*	0·031	1·0
Ser*	0·042	1·1
Galactosamine	0·740	33·0
(expressed as *N*-acetylgalactosamine)	(0·740)	(39·6)
Galactose	0·445	20·0
Sialic acid	0·023	1·7

* Approximate values only.

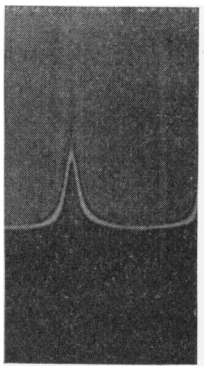


Fig. 1. Sedimentation pattern obtained on a 1% (w/v) solution of the mucoid in 0·01 M-sodium phosphate buffer, pH 7·4, containing KCl (0·15 M) and EDTA (1 mM). The time was 140 min. after reaching the maximum speed of 59780 rev./min.

only in traces. Sometimes these amino acids were not observed when smaller amounts of hydrolysed mucoid were chromatographed. Because of these small amounts, and also because of their proximity to other amino acids on the paper chromatograms, they could not be assayed with any degree of accuracy. The accuracy for the determination of the other amino acids was about $\pm 10\%$ from three separate analyses.

Treatment with acid. Liberation of small amounts of galactose and free galactosamine was observed on treatment with 0·1 N-hydrochloric acid at 100° for 10 min. With 5 N-hydrochloric acid at 100°, all the hexosamine contained in the mucoid was liberated within 15 min. Samples removed at intervals up to 7 hr. all contained identical amounts of hexosamine as measured by the method of Rondle & Morgan (1955). By using paper chromatography (solvent *C*) to examine the progress of hydrolysis in 5 N-hydrochloric acid, the concentration of galactose was maximum at 15 min. Subsequently, its concentration was decreased, presumably by destruction under the strongly acid conditions.

Sialic acid. Although sections of the tumour were strongly metachromatic and deeply staining with the periodic acid–Schiff reagent, no acid mucopolysaccharides similar to hyaluronic acid or chondroitin sulphate were isolated. This suggested that the original mucin probably contained sialic acid. The latter usually occurs in mucins as a terminal group that is extremely sensitive to acid. The low pH, as used in the 37° incubation of the tumour with pepsin, would in all probability lead to a splitting of any sialic acid present. Assay of the mucoid fraction showed 1·7% of sialic acid, again suggesting that this may have been a constituent of the native mucin. Further supplies of this tumour were unavailable, and as yet it has not been possible to secure any further specimens. However, there was available a small ampoule of a high-speed supernatant of a saline extract of the tumour, originally prepared for enzyme studies, and stored frozen. A small quantity of crude mucin was prepared from this by the addition of an excess of ethanol. The resultant precipitate was taken up in 0·2 N-sodium hydroxide and centrifuged, and galactose and sialic acid were determined in samples of the supernatant. The sialic acid/galactose molar ratio was 0·6.

Physical properties. The mucoid did not move from the origin when subjected to electrophoresis on paper in barbiturate buffer, pH 8·6. Fig. 1 shows the results of an ultracentrifugal analysis. A single peak with $S_{20,w}$ 2·35 s was obtained under the conditions outlined in the Figure. The molecular weight would fall into the range 10000–15000 if the molecule were spherical and 20000–50000 if it were linear. This aspect could not be studied further owing to lack of material.

Reducing properties. As an initial step, designed to determine both the nature and method of linkage of the carbohydrate component, or components, to the peptide portion of the molecule, the presence of reducing groups was sought. Aminoff & Morgan (1951) presented evidence that blood-group A substance contained reducing *N*-acetylgalactosamine end groups. For the study of the breast mucoid, the semi-micro method of Park & Johnson (1949) was used. This procedure involves heating the test substance for 15 min. at 100° in 0·03 N-sodium carbonate containing potassium ferricyanide. At the end of this time, Fe^{3+} ions are added, and the reduced ferrocyanide is measured as Prussian blue. Under these conditions the yield of

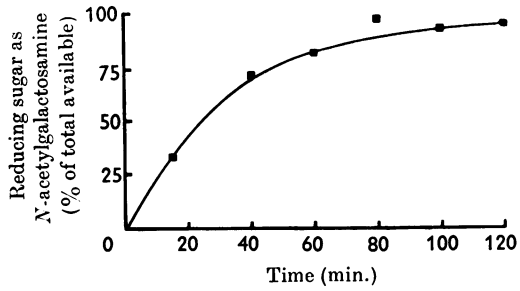


Fig. 2. Liberation of reducing sugar, expressed as *N*-acetylgalactosamine, from a 20 μg. sample of the mucoid heated under the conditions of the Park & Johnson (1949) procedure. Various amounts of *N*-acetylglucosamine, heated for 2 hr., were employed as standards.

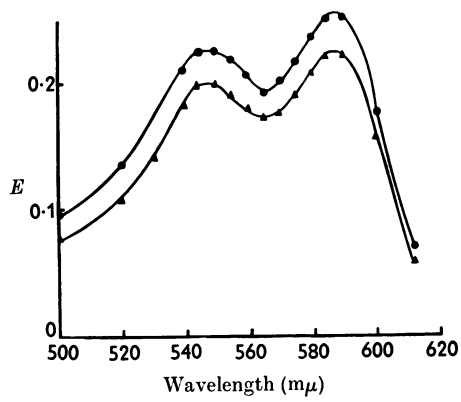


Fig. 3. Comparison of the absorption spectra of the products from the mucoid (500 μg.) and from *N*-acetylgalactosamine (approx. 200 μg.) by the procedure of Aminoff *et al.* (1952). A heating time of 20 min. was employed with the mucoid (●) and 4 min. with the *N*-acetylgalactosamine (▲).

reducing sugar, as glucose, was 6·5 μg./50 μg. of mucoid. When this amount of mucoid was applied to filter paper and tested for reducing properties by dipping into an aqueous-acetone solution of silver nitrate, followed by spraying with aqueous-ethanolic 0·5 N-sodium hydroxide (Trevelyan *et al.* 1950), a negative result was obtained. However, 6·5 μg. of glucose gave a distinct brown zone under these conditions. This suggested that the reducing power may have resulted from the release of carbohydrate groups by the weak alkali. Extending the heating time gave the results shown in Fig. 2. Both *N*-acetylgalactosamine and *N*-acetylglucosamine were found to have equivalent reducing power to glucose on a molar basis. Results, quoted below, demonstrated that *N*-acetylhexosamine was released from the mucoid under similar treatment with mild alkali. The reducing sugar liberated is expressed as *N*-acetylgalactosamine in Fig. 2; on this basis 96% of the available acetylhexosamine is liberated by the Park & Johnson (1949) procedure if longer heating times are employed.

Liberation of chromophore under the conditions of Aminoff et al. (1952). With the blood-group substances a minimum heating time of 12 min. in 0·05 N-sodium carbonate at 100° was necessary for maximum colour production on the addition of Ehrlich's reagent. An arbitrary time of 20 min. was used for investigations with the breast mucoid. The colour produced from 500 μg. of mucoid was equivalent to 240 μg. of *N*-acetylgalactosamine. Assuming all the amino sugar in the mucoid to be acetylated, this corresponded to 117% of the amount available based on a 33% hexosamine analysis. Further, the liberated chromophore appeared to be completely diffusible, since the yield in the diffusate, from a parallel experiment, amounted to 106% of the potential amount. Fig. 3 compares the absorption characteristics of the final coloured complex with that obtained from *N*-acetylgalactosamine. When the non-diffusible residue was examined for its galactose content, a fall to 50% of the intial value was obtained.

Detailed investigation of the effects of alkalis. After treatment of the mucoid with 0·1 N-sodium carbonate for 60 min. at 100° followed by dialysis, the amount of hexosamine present after acid hydrolysis of the non-diffusible fraction was decreased to 6% of its initial value. Examination of the acid hydrolysate of samples of mucoid subjected to dilute carbonate for 20 min. under the conditions of Aminoff *et al.* (1952) revealed that the concentration of threonine relative to the other amino acids had been specifically decreased. In addition, hexosamine was still present, but in diminished concentration, even though the action of sodium carbonate under these conditions was shown to yield an acetylhexosamine chromophore in excess of 100% of the theoretical. Dialysis of a sample of mucoid heated for 20 min. in sodium carbonate revealed substantial amounts of peptide in the diffusate, the amount of threonine being extremely low compared with those of the other amino acids.

The use of a stronger alkali, such as 0·18 N-lithium hydroxide for 20 min., led to almost complete elimination of threonine and hexosamine from the acid-hydrolysable constituents of the mucoid. A significant observation was a rise in the relative concentration of glycine. Analysis of the non-diffusible material remaining after treatment with sodium carbonate and dialysis, followed by lithium hydroxide and dialysis, is given in Table 2. The recovery of ninhydrin-positive peptide in the non-diffusible fraction was 28%. Though most of

Table 2. *Effect of alkali on the amino acid composition of the mucoid*

A sample was heated in 0·04 N-Na_2CO_3 for 20 min., then neutralized and dialysed. The non-diffusible material was concentrated and heated for 20 min. in 0·18 N-LiOH, followed by neutralization and dialysis. The material remaining in the sac was then subjected to acid hydrolysis. Only those amino acids listed were determined, and the molar proportions are given to the nearest whole number.

Amino acid	Molar proportions (Leu = 1)	
	Control	Alkali-treated
Leu	1	1
Val	1	1
Thr	5	1
Gly	1	2
Glu	1	1

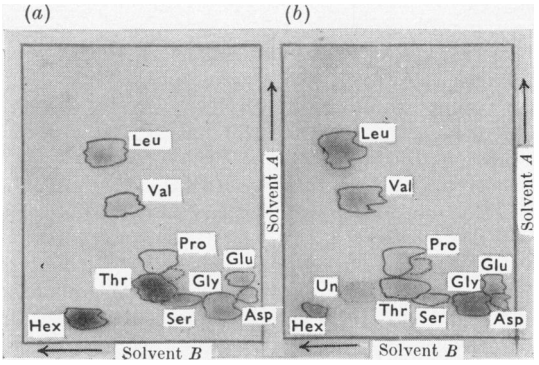


Fig. 4. Illustration of the action of alkali on the mucoid. A sample in 0·05 ml. of 0·18 N-LiOH was heated in a sealed capillary for 40 min., then hydrolysed with 4 N-HCl overnight. The hydrolysate was chromatographed in two dimensions and sprayed with ninhydrin. There was a great decrease in the relative concentrations of threonine and galactosamine and an increase in the glycine concentration. (*a*) Control; (*b*) alkali-treated. Hex, hexosamine; Un, unknown component that gave an orange colour with ninhydrin.

the peptide was evidently rendered diffusible by such treatment, removal of lithium chloride was achieved by ethanol and acetone extraction of the dried residue after neutralization of the lithium hydroxide with hydrochloric acid. It was unlikely, however, that this would lead to loss of non-diffusible material.

By increasing the time of heating in alkali and examining the acid-hydrolysable components by two-dimensional paper chromatography, a parallel decrease in both hexosamine and threonine was evident (Fig. 4). After 40 min. in 0·18 N-lithium hydroxide, the threonine/leucine ratio was decreased from 5:1 to approx. 0·5:1, showing that about 90% of the threonine had been lost.

Table 3. *R_F values of derivatives formed from α-oxo acids and o-phenylenediamine*

The mucoid was heated in dilute Na_2CO_3 before hydrolysis with 4 N-HCl containing *o*-phenylenediamine.

Substance	R_F		
Solvent ...	Methanol	Ethanol	Butan-1-ol satd. with 1·5 N-NH_3
Pyruvic acid derivative	0·68	0·72	0·78
α-Oxobutyric acid derivative	0·68	0·84	0·91
Mucin	0·68	0·84	0·91

Tentative identification of α-oxobutyric acid. Formation of diffusible peptide by mild alkali suggested that the destruction of threonine may have resulted in rupture of the peptide core. A β-elimination reaction would lead to two possible degradation products of threonine: glycine and α-oxobutyric acid (see the Discussion section). The formation of glycine has already been mentioned, and demonstration of the production of α-oxobutyric acid was attempted by trapping with *o*-phenylenediamine as the oxo acid was gradually liberated by acid hydrolysis of the alkali-treated mucoid. This reagent was chosen since the resulting 2-quinoxalinol should have been more stable to heating in the presence of strong acid than, say, the phenylhydrazone derivative (Hockenhull & Floodgate, 1952). Table 3 lists the R_F of the derivative obtained in this manner together with the R_F of the product resulting from identical treatment of authentic α-oxobutyric acid. Identification had to be limited to an experiment of this type since only traces of material were available at this stage of the investigation.

Periodate oxidation. Analysis of galactose and hexosamine was undertaken after oxidation of the mucoid with 7 mM-periodate for 5 hr. The results were (percentages): galactose, 3·5 (after oxidation), 20·0 (control), loss 82·5; galactosamine, 33·0 (no loss).

Treatment with hydroxylamine. Murphy & Gottschalk (1961) have provided evidence that 83% of the α-D-sialyl-(2→6)-*N*-acetylgalactosamine prosthetic groups of bovine submaxillary-gland mucoprotein are joined by glycosidic ester linkages to the carboxyl groups of aspartic acid and glutamic acid residues. Treatment of ovine submaxillary-gland mucoprotein (which contains a similar number of such linkages) with alkaline hydroxylamine led to the formation of hydroxamates (Graham *et al.* 1963).

Owing to the sensitivity of hydroxamates of amino acids to alkali (Raacke, 1958), the yield of hydroxamate is not quantitative. A yield 50% of the theoretical was obtained with ovine submaxillary-gland mucoprotein. This type of linkage was sought in the breast mucoid by application of the hydroxylamine technique. A positive result was obtained, the final coupled diazo salt having $\lambda_{max.}$ 530 mμ (Yashphe *et al.* 1960). The yield was 22% of the available glutamic acid.

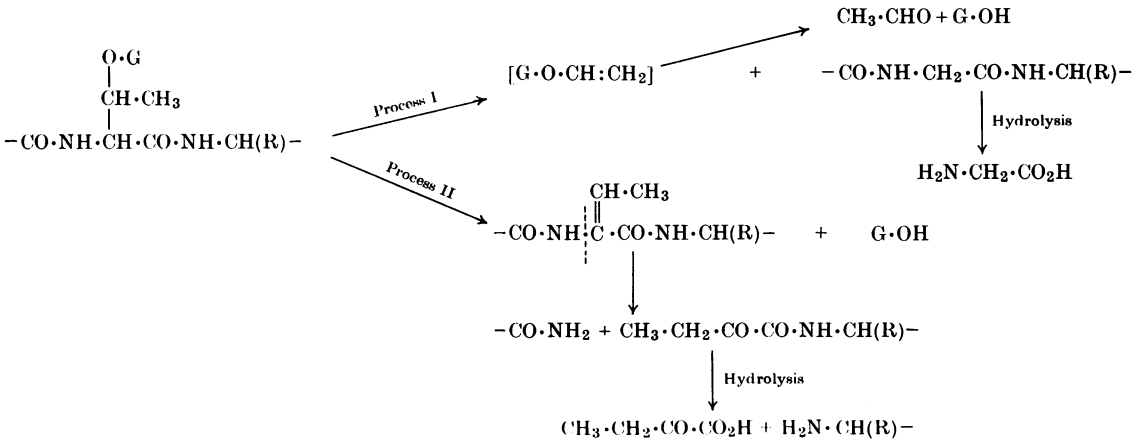
DISCUSSION

One of the most striking features of the chemistry of mucins is the amino acid composition of the peptide moiety. Threonine, serine, proline, alanine and glycine predominate. The first three amino acids of this group make up 50% of the peptide of blood-group substances (Morgan, 1963), and reach a high percentage in bovine and ovine submaxillary-gland mucoproteins and also in bovine cervical mucin. Glutamic acid and aspartic acid are invariably present but, together, they total only 30–40% of the combined hydroxyamino acids (Hashimoto, Hashimoto & Pigman, 1964). Glutamic acid is always found in higher concentration than aspartic acid. Serine and threonine usually occur in approximately equal molar amounts in the submaxillary-gland mucoproteins from different species, but the threonine/serine ratio is about 2:1 in bovine sublingual-gland and cervical mucoproteins (Hashimoto *et al.* 1964). The mucoid from the colloid breast carcinoma possesses this distinctive amino acid composition but differs in the very low alanine content and in the high content of threonine. Serine, though present, is of minor significance. The absence of basic amino acids, coupled with the failure of the mucoid to move from the origin on electrophoresis at pH 8·6, suggested that the carboxyl groups of glutamic acid and aspartic acid may have been joined in ester or amide linkages to carbohydrate. An *N*-(β-aspartyl)glycosylamine linkage has been shown to occur in egg albumin (Fletcher, Marshall & Neuberger, 1963), and linkage of carbohydrate to an aspartyl residue has been demonstrated in human γ-globulin (Rosevear & Smith, 1961). In addition, Graham *et al.* (1963) have provided evidence for the ester type of linkage for most of the carbohydrate prosthetic groups of ovine submaxillary-gland mucoprotein. Thus linkage of a polysaccharide, composed of *N*-acetylgalactosamine and galactose, to glutamic acid or aspartic acid residues or both was a possibility to be considered in the case of the breast-carcinoma mucoid. However, liberation of a reducing sugar, under the extremely mild alkaline conditions of the Park & Johnson (1949) procedure, which, when expressed as *N*-acetylgalactosamine, accounted for almost all the amino sugar contained in the mucoid, suggested a ready release from individual linkages. Near quantitative release from a polysaccharide unit would be extremely unlikely under the feebly alkaline conditions employed. Strong alkalis (e.g. 2 N-sodium hydroxide) will liberate reducing sugar from certain oligosaccharides, but this is accompanied by extensive degradation (Gehman, Kreider & Evans, 1936). The presence of ferricyanide in the Park & Johnson (1949) procedure evidently caused complete oxidation of the reducing group of the liberated *N*-acetylhexosamine, thus preventing formation of an acetamidofuran chromophore (Kuhn & Krüger, 1957). By contrast, heating the mucoid with sodium carbonate of similar strength for 20 min. by the Aminoff *et al.* (1952) procedure led to the formation of diffusible chromophore. Analysis of the non-diffusible residue after such treatment showed that 50% of the available galactose was still present, although apparently all the hexosamine had been removed. Such results seemed to suggest that the galactose was combined with peptide at sites independent of amino sugar linkages. However, two-dimensional paper-chromatographic examination of hydrolysates of mucoid, which had been heated for 20 min. under the conditions of Aminoff *et al.* (1952), revealed substantial amounts of free galactosamine, despite the fact that chromophore in excess of the theoretical had been liberated. It was thus apparent that *N*-acetylgalactosamine, carrying a substituent in the 3-position, had been formed by the alkaline treatment. *N*-Acetylhexosamines substituted in this position are known to give enhanced colour production in the Morgan–Elson procedure, substituents at the 4-position causing inhibition of chromophore production and substituents at position 6 having no effect (Jeanloz & Trémège, 1956; Strominger, Park & Thompson, 1959). That galactose was the substituent at position 3 was provided by two pieces of evidence. Periodate destroyed most of the galactose but left the amino sugar unattacked. This showed that the latter was not present as a reducing end group and was probably substituted in position 3 to account for its stability to oxidation. At the same time galactose was very probably in terminal linkage. The presence of galactosyl-(1$\rightarrow$3)-*N*-acetylgalactosamine groups in the mucoid, which were capable of being liberated by mild base, was thus indicated. Further evidence for the linkage of galactose to *N*-acetylgalactosamine was provided by the results shown in Fig. 2: after 20 min. in 0·03 N-sodium carbonate [Park & Johnson (1949) conditions], only 46% of the available *N*-acetylhexosamine had been liberated. This is in good agreement with the retention of 50% of the available galactose in the non-diffusible fraction on heating the mucoid in

0·04 N-sodium carbonate for 20 min. [Aminoff *et al.* (1952) conditions].

Reduction in the relative concentrations of threonine and hexosamine on treatment with alkalis strongly suggested a β-elimination mechanism (Witkop, 1961). This was supported by the formation of glycine and rupture of the peptide chain, as witnessed by the liberation of diffusible peptide material (Scheme 1). Glycosides, containing a negative substituent in the β-position, undergo an elimination reaction under the influence of mild base. Such a group is provided by the carbonyl group of the threonine residues, and glycosides linked to this hydroxyamino acid could be expected to undergo elimination by either process I or II. By process I, the vinyl glycoside would be formed, which itself would be unstable in alkali and decompose to free sugar and acetaldehyde (Ballou, 1954). The threonine residue is converted into glycine. By process II, a dehydropeptide is initially formed. Dehydropeptides are known to be unstable to mild alkali and acid (Witkop, 1961), and would undergo further breakdown to yield an amide with rupture of the peptide chain at the position indicated. Threonine in this case is converted into α-oxobutyric acid. The virtual elimination of threonine and *N*-acetylgalactosamine from the mucoid on prolonged heating in alkali must mean that all, or nearly all, the threonine residues are joined to *N*-acetylgalactosamine. Rupture of the molecule by process II would quickly lead to diffusible material being formed. Gibbons & Roberts (1963) found that bovine cervical mucin was rapidly fragmented into diffusible material on heating in 0·05 N-sodium carbonate for 20 min. Since the peptide linkage in proteins is relatively alkali-stable (Warner, 1942), it is therefore likely that similar linkages of prosthetic groups to threonine, and perhaps serine, are present in this mucin and also in blood-group substances, which also suffer extensive disintegration in mild alkali (Knox & Morgan, 1954). By contrast, Murphy & Gottschalk (1961) found that only 0·5% of the protein of bovine submaxillary-gland mucoprotein was rendered diffusible by heating in 0·01 N-sodium hydroxide at 80° for 4 hr. In later studies, however (Graham *et al.* 1963), ovine submaxillary-gland mucoprotein, when heated in 0·05 N-sodium hydroxide for 30 min., was found to be rendered diffusible to the extent of one-third of the peptide material.

Since sialic acid was lost from the native breast-carcinoma mucin by mild acid, the sialic acid residues were probably terminal and linked to either *N*-acetylgalactosamine or galactose. From the approximate value of 3:2 for the galactose/sialic acid molar ratio, obtained with the small amount of native mucin available, two types of prosthetic groups could occur. For each five hexosamine–threonine linkages, three could be linked in turn to galactose and two to sialic acid. α-D-N-Acetylneuraminyl-(2→6)-*N*-acetylgalactosamine is the prosthetic group contained in submaxillary-gland mucoproteins (Graham & Gottschalk, 1960). β-D-Galactosyl-(1→3)-*N*-acetylgalactosamine occurs in brain gangliosides (Klenk, Hendricks & Gielen, 1962) and in ovarian-cyst mucopolysaccharides (Morgan, 1963). The possibility remains, however, that some galactose, as well as sialic acid, may have been lost from the native mucin during the treatment with pepsin at the low pH employed in the isolation of the mucoid. Thus a single prosthetic



Scheme 1. Possible processes by which the mucoid is degraded on treatment with alkalis. G, Carbohydrate prosthetic group.

group composed of *N*-acetylhexosamine, galactose and sialic acid may be attached to the threonine residues in the native mucin. Clarification of this point must wait until a further specimen of colloid breast carcinoma can be obtained.

Formation of a hydroxamic acid on treatment of the mucoid with alkaline hydroxylamine provides evidence for the linkage of carbohydrate to the carboxyl groups of glutamic acid or aspartic acid. The *N*-acetylgalactosamine/threonine ratio was 6:5, so that the extra hexosamine residue could be linked via a carboxyl group. A statistical model would require the attachment of prosthetic groups to virtually every alternate amino acid (threonine) residue in the peptide chain. Molecular models were capable of being constructed with, for example, the sequence Thr-Gly-Thr-Val and galactosyl-(1→3)-*N*-acetylgalactosamine prosthetic groups on each threonine moiety.

The distribution of carbohydrate linkages to hydroxyamino acids and dicarboxylic acids in the breast mucoid may be compared with those occurring in submaxillary-gland mucin. Murphy & Gottschalk (1961) have indicated that the bulk of the prosthetic groups in ovine (83%) and bovine (89%) submaxillary-gland mucin are joined by glycosidic ester linkages to carboxyl groups. The remaining prosthetic groups were thought to be linked by an *O*-glycosidic bond to serine or threonine residues or both. Evidence for this type of linkage was obtained by the isolation from ovine mucin of a glycopeptide that contained glycine, serine, alanine, galactosamine and valine in the proportions 1:1:3:1:1 (Graham *et al.* 1963). Linkages of this type were regarded as being more resistant to alkali than are those of the glycosidic ester type. Hashimoto, Tsuiki, Nisizawa & Pigman (1963) found that, although bovine submaxillary-gland mucin was resistant to attack by pepsin and trypsin, it was degraded by a protease from *Streptomyces griseus*. These workers isolated a diffusible glycopeptide containing glycine, serine, proline, threonine, sialic acid and galactosamine in the proportions 1:1:1:1:2:2, together with small amounts of other amino acids. Blix (1963) isolated a glycopeptide from acid-treated ovine submaxillary-gland mucin by incubation with papain. Serine plus threonine occurred in equimolar ratio to *N*-acetylgalactosamine and to the extent of twice that of the glutamic acid, again indicating the involvement of the hydroxyamino acids in glycosidic linkage. Apparent discrepancies in the relative abundance of both types of linkages in the submaxillary-gland mucins is probably explained by somewhat different species of mucin being studied by the various workers, as suggested by Graham *et al.* (1963).

The possible linkage of carbohydrate to serine and threonine residues in other mucopolysaccharides and mucoproteins is being examined by similar techniques.

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REFERENCES

Aminoff, D. & Morgan, W. T. J. (1951). *Biochem. J.* **48**, 74.
Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1952). *Biochem. J.* **51**, 379.
Anderson, A. J. & Maclagan, N. F. (1955). *Biochem. J.* **59**, 638.
Ballou, C. E. (1954). *Advanc. Carbohyd. Chem.* **9**, 59.
Blix, G. (1963). *Ann. N.Y. Acad. Sci.* **106**, 164.
Cessi, C. & Serafini-Cessi, F. (1962). *Biochem. J.* **82**, 43P.
Corson, B. B., Dodge, R. A., Harris, S. A. & Hazen, R. K. (1951). In *Organic Syntheses, Collective Vol.* 1, p. 241. Ed. by Gilman, H. & Blatt, A. H. New York: John Wiley and Sons Inc.
Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Dische, Z. (1955). *Meth. biochem. Anal.* **2**, 313.
Evans, R. (1956). *Histological Appearances of Tumours*, p. 353. Edinburgh and London: E. and S. Livingstone Ltd.
Fletcher, A. P., Marks, G. S., Marshall, R. D. & Neuberger, A. (1963). *Biochem. J.* **87**, 265.
Fletcher, A. P., Marshall, R. D. & Neuberger, A. (1963). *Biochim. biophys. Acta*, **71**, 505.
Fromageot, C. (1955). In *Methods in Enzymology*, p. 324. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Gehman, H., Kreider, L. C. & Evans, W. L. (1936). *J. Amer. chem. Soc.* **58**, 2388.
Gibbons, R. A. & Roberts, G. P. (1963). *Ann. N.Y. Acad. Sci.* **106**, 218.
Graham, E. R. B. & Gottschalk, A. (1960). *Biochim. biophys. Acta*, **38**, 513.
Graham, E. R. B., Murphy, W. H. & Gottschalk, A. (1963). *Biochim. biophys. Acta*, **74**, 222.
Hashimoto, Y., Hashimoto, S. & Pigman, W. (1964). *Arch. Biochem. Biophys.* **104**, 282
Hashimoto, Y., Tsuiki, S., Nisizawa, K. & Pigman, W. (1963). *Ann. N.Y. Acad. Sci.* **106**, 233.
Heilbron, I. & Bunbury, H. M. (1953). *Dictionary of Organic Compounds*, revised ed., vol. 3, p. 145. London: Eyre and Spottiswoode Ltd.
Heyworth, R., Perkins, H. R. & Walker, P. G. (1961). *Nature, Lond.*, **190**, 261.
Hockenhull, D. J. D. & Floodgate, G. D. (1952). *Biochem. J.* **52**, 38.
Hughes, G. K., Lions, F. & Ritchie, E. (1939). *J. Roy. Soc. N.S.W.* **72**, 209.
Immers, J. & Vasseur, E. (1950). *Nature, Lond.*, **165**, 898.
Jeanloz, R. W. & Trémège, M. (1956). *Fed. Proc.* **15**, 282.
Johnson, W. C. & Helwig, E. B. (1963). *Ann. N.Y. Acad. Sci.* **106**, 794.
Klenk, E., Hendricks, U. W. & Gielen, W. (1962). *Hoppe-Seyl. Z.* **330**, 140.
Knox, K. W. & Morgan, W. T. J. (1954). *Biochem. J.* **58**, v.
Kuhn, R. & Krüger, G. (1957). *Chem. Ber.*, **90**, 264.

Meyer, K., Davidson, E., Linker, A. & Hoffman, P. (1956). *Biochim. biophys. Acta*, **21**, 506.
Morgan, W. T. J. (1963). *Ann. N.Y. Acad. Sci.* **106**, 177.
Murphy, W. H. & Gottschalk, A. (1961). *Biochim. biophys. Acta*, **52**, 349.
Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Popenoe, E. A. (1959). *Biochim. biophys. Acta*, **32**, 584.
Raacke, I. D. (1958). *Biochim. biophys. Acta*, **27**, 416.
Rondle, C. M. J. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.
Rosen, H. (1957). *Arch. Biochem. Biophys.* **67**, 10.
Rosevear, J. W. & Smith, E. L. (1961). *J. biol. Chem.* **236**, 425.
Salton, M. R. J. & Mulhaud, C. (1959). *Biochim. biophys. Acta*, **35**, 254.
Strominger, J. L., Park, J. T. & Thompson, R. E. (1959). *J. biol. Chem.* **234**, 3263.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Varley, H. (1958). *Practical Clinical Biochemistry*, 2nd ed. London: William Heinemann Ltd.
Vogel, E. & Schinz, H. (1950). *Helv. chim. acta*, **33**, 116.
Warner, R. C. (1942). *J. biol. Chem.* **142**, 741.
Winzler, R. J. (1955). *Meth. biochem. Anal.* **2**, 279.
Witkop, B. (1961). *Advanc. Protein Chem.* **16**, 232.
Yashphe, J., Halpern, Y. S. & Grossowicz, N. (1960). *Analyt. Chem.* **32**, 518.