

Biosynthesis of Intestinal Mucins

4. UTILIZATION OF [1-¹⁴C]GLUCOSE BY SHEEP COLONIC MUCOSA *IN VITRO**

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(Received 9 May 1962)

The mucins of sheep colonic mucosa, like other epithelial secretions, are members of the group of 'neutral' mucosubstances (Werner, 1953; Kent, 1962). These characteristically contain L-fucose, D-galactose, N-acetyl amino sugars and frequently sialic acids and mannose (Gottschalk, 1960). Their chemical structure and the nature of the sugar-peptide links involved have not yet been established. Previous investigations (Pasternak, Kent & Davies, 1958; Pasternak & Kent, 1958) have shown that mucosal cells, as scrapings *in vitro*, were highly active in incorporating [³⁵S]sulphate into the mucins of various gastrointestinal tissues. Wolf & Varandani (1960) and Wolf, Varandani & Johnson (1961) described partly purified enzyme systems from rat and pig colonic tissues, which catalysed the incorporation of isotopically labelled sulphate. In addition, homogenates incubated with [¹⁴C]glucose gave radioactive mucins, from which [¹⁴C]glucosamine could be isolated after acidic hydrolysis. A detailed investigation of the conversion of glucose into glucosamine in this tissue has been made by Pasternak (1961). Evidence was presented by Wolf & Varandani (1960) and by Wolf *et al.* (1961) that the sulphated constituent of their mucin preparations resembled chondroitin sulphate. This acidic mucosubstance had been reported to occur in stomach tissue by Smith, Gallop, Harris-Smith & Stanley (1952) and by Smith & Gallop (1953).

That the mucosubstances of intestinal secretions are synthesized from sugar nucleoside diphosphates may be inferred from knowledge of other carbohydrate-synthesizing systems in mammals. Foster & Ginsburg (1961) have demonstrated the enzymic conversion of GDP-mannose into GDP-fucose in rabbit-lung preparations, suggesting that the pathway that has been more fully established for the bacterial conversion of D-glucose into L-fucose (Ginsburg, 1961) operates also in mammals. The presence of UDP-glucuronic acid in gastric and intestinal mucosal tissues has been demonstrated by Dutton (1959) and by Stevenson & Dutton (1962). The mammalian biosynthetic route to sialic acid from N-acetylmannosamine (or its phosphate) and phosphoenolpyruvate was demon-

strated by Roseman, Jourdain, Watson & Rood (1961) and by Warren & Felsenfeld (1961). Synthesis of sialic acid from glucose or glucose 6-phosphate, pyruvate and glutamine has been observed by Lind (1961). There is as yet little information on the mechanism of assembly of the sugar units into mucopolysaccharides.

The present experiments are concerned with an investigation of the uptake of [1-¹⁴C]glucose by sheep colonic mucosal cells, and its possible conversion into the monosaccharide constituents of the mucosubstances present. Incorporation of [³⁵S]sulphate was also examined to compare the present experiments with those of previous workers.

METHODS AND MATERIALS

Preparation and incubation of tissue. Sheep colons were collected immediately after slaughter of the animals and were kept in ice-cold iso-osmotic salt soln. Scrapings were prepared according to the procedure of Pasternak *et al.* (1958). The colons, 5–25 cm. from the anus, were cut into sections and washed in cold water and ice-cold salt soln. The scrapings from them were placed in chilled Krebs medium III (Krebs, 1950), with glucose omitted. In some experiments, glucose (40 μmoles) or L-fucose (40 μmoles) was added per 4 ml. of incubation medium. The scrapings (15–50 mg. dry wt.) were incubated manometrically at 37° in an atmosphere of O₂, CO₂ being absorbed by 3 drops of 2N-NaOH in the centre well. In some experiments, Polybactrin (Calmic Ltd.) was added at final concentrations of 0.01 and 0.1%.

Dry weights. Dry weights of tissues were determined by removing the contents of manometer vessels after incubation, centrifuging and drying the sedimented tissue at 105° for 18 hr.

Radioactivity experiments. In isotopic experiments, [1-¹⁴C]glucose of high specific activity (supplied by The Radiochemical Centre, Amersham, Bucks.) was added at a final amount of about 10 000 counts/min./flask. The incubations were carried out in stoppered O₂-filled flasks with 60–200 mg. of tissue (dry wt.) in 20 ml. of medium III and were terminated after 3 hr. by addition of 95% ethanol (3 vol.). Control experiments were carried out similarly except that ethanol was added without incubation. In [³⁵S]sulphate experiments, about 10 μC of carrier-free ³⁵SO₄²⁻ ions at pH 7 (sample SJS1, The Radiochemical Centre) and 40 μmoles of glucose were included in the incubation mixtures. In some experiments, Polybactrin (0.01% final concn.) was added.

* Part 3: Kent & Pasternak (1958).

The [^{14}C]glucose was purified before use by chromatographic separation on Whatman no. 3 paper with butan-1-ol-ethanol-water. The glucose band was eluted with water. No radioactive impurity could be detected in the product.

Extraction and preparation of mucin products. The ethanol-treated incubation mixtures were centrifuged at 800g for 5 min., and the sedimented material was treated with crystalline papain (obtained from British Drug Houses Ltd., Poole, Dorset), approx. 6 mg./200 mg. dry wt. of tissue, in 0.04M-sodium citrate-citric acid buffer (pH 5.5, final vol. 20 ml.), containing (final concn.) 5 mM-cysteine and 1 mM-disodium ethylenediamine tetraacetate (EDTANa_2) (Kimmel & Smith, 1954). A few crystals of benzoic acid were added to inhibit bacterial growth. The tissue and mucin, digested for 24 hr. at 37°, was subsequently dialysed (cellophan) for 4 days at 2° against distilled water (4 × 4l.). The resulting digested mucin was precipitated from the filtered dialysed preparation by addition of ethanol to a final concentration of 70% (v/v). Finally the product was dried in ethanol and *in vacuo* over P_2O_5 .

In another experiment, ^{35}S -labelled mucin was treated with alkali at room temperature (Pasternak & Kent, 1958), 0.5N-NaOH being used, for 16 hr. After centrifuging, a small quantity of material was precipitated by addition of acetic acid (3 vol.). Further material was precipitated with ethanol (3 vol.), extracted again with 6.25 mM-sodium tetraborate and dialysed for 3 days against distilled water at 2°. Finally the mucin was precipitated and dried as for the papain-digested products.

Fractionation of digested mucin preparation with rivanol. The digested mucin preparations were fractionated by precipitation in ammonium formate-formic acid by 6,9-diamino-2-ethoxyacridine lactate monohydrate (rivanol; Whitehouse & Boström, 1961). Four portions (2 ml.) each containing 3.9 mg. of mucin in 0.1M-formic acid and varying amounts of ammonium formate were precipitated by addition of 1% (w/v) of rivanol. In this procedure, complexes formed with more acidic polysaccharides are soluble only in concentrated ammonium formate. In contrast, neutral and less acidic polymers form complexes soluble at lower concentrations of ammonium formate. Rivanol-soluble materials were recovered by addition of ethanol. The precipitated mucin-rivanol complexes were taken up in 5M-ammonium formate (1 ml.) and butan-1-ol (1 ml.). The mixture was shaken, centrifuged and the organic layer, containing dissolved rivanol, was discarded. The mucosubstances were then precipitated from the aqueous layer with ethanol.

Radioactive assays. ^{14}C -Labelled mucins were counted as thin films on alkali-etched aluminium planchets (2.1 cm. diam.) with a mica end-window counter EHM2, operating at 1680v. Electrophoresis strips and paper chromatograms were scanned in 1 cm. or 4 mm. strips with a plastic end-window counter (6 cm. diam.) operating at 1550v. Counts were corrected for background (approx. 7.8 and 58 counts/min. respectively). Over 1000 counts were collected for each measurement of radioactivity.

Zone electrophoresis. Mucin preparations were submitted to electrophoresis on cellulose acetate strips (20 cm. × 5 cm.; Oxo Ltd., London, E.C. 4) in 12.5 mM-borate buffer (pH 9.3). In some experiments, 0.01M- EDTANa_2 (final concn.) was present. Protein constituents were detected with Azocarmine B (Plückthun & Götting,

1951). Neutral and acidic polysaccharides were detected by mucicarmine (Hamerman, 1955) and Alcian Blue (Heremans & Vaerman, 1958). A concentration of 1% of the latter in 0.05M-sodium acetate buffer, pH 4.5, was used. The treatment was for 10 min., followed by washes in 2% acetic acid.

Cationic fractionation on paper. This was carried out by upward elution of cetylpyridinium complexes of digested mucin on Whatman no. 4 paper with a concentration gradient of MgCl_2 (Marsden & Kent, 1962).

Hydrolysis and chromatography. Digested mucins were hydrolysed for 3 hr. in 0.5N- H_2SO_4 in sealed tubes at 100°. The acid was removed by passage through a column of (7 cm. × 1 cm.) Dowex 1 (acetate form) and the effluents were evaporated to dryness at 40° under vacuum. The following solvents were used for chromatography (Whatman no. 1 paper, descending method): butan-1-ol-ethanol-water (5:1:4, by vol.) and ethyl acetate-pyridine-water (2:1:2, by vol.). Reducing sugars were detected by spraying chromatograms with aniline hydrogen phthalate or with alkaline AgNO_3 (Trevelyan, Procter & Harrison, 1950). Amino sugars were detected by means of ninhydrin and by acetylacetone-*p*-dimethylaminobenzaldehyde (Partridge, 1948).

For analysis of amino acids and amino sugars mucins were hydrolysed for 16 hr. in 2N-HCl in sealed tubes at 102° (Blix, 1948). Hydrolysates were evaporated to dryness after removal of 'humin' by centrifuging. Amino acids were separated by the paper-chromatographic method of Mizell & Simpson (1961) and detected with ninhydrin. Amino sugars were separated according to Gardell (1958). To release ester sulphate groups, hydrolysis was for 5 or 16 hr. with 4N-HCl in sealed tubes at 102°. Hydrolysed components were separated in the ethanol-ammonium acetate solvent of Pasternak & Kent (1958), after removal of the acid *in vacuo*. Acidic mucosubstances were meta-chromatically stained with Azur A (0.1% in 70% ethanol; Kramer & Windrum, 1955). Oxidizable products were detected by the periodate-Schiff reaction (Björnesjö, 1955).

RESULTS

The average Q_{0_2} of five mucosal preparations (ten experiments) was -4.4 ± 1.4 (s.d.) in the absence of added sugars. Values of -3.7 ± 1.2 (s.d.) were found in the presence of glucose, whereas with added fucose Q_{0_2} was -3.5 ± 1.1 (s.d.). All values refer to the first hour of incubation. After 3 hr. the rate of oxygen uptake diminished considerably, to between 20 and 70% of the initial value. Addition of Polybaectrin did not influence Q_{0_2} .

Properties of digested mucin preparations. The papain-digested preparation was a white powder readily soluble in water, giving opalescent and, at high concentrations, viscous solutions. The yield ranged from 10 to 50% of the dry weight of scrapings used. A typical preparation had P, 0.67% (Chen, Toribara & Warner, 1956), N (Kjeldahl), 7.3%, and ash, 7.4% (values corrected for moisture, 10.6%).

Zone electrophoresis. Examination of papain-digested specimens revealed the presence of two components, I (faster) and II (slower). Both were stainable with Alcian Blue, mucicarmine and, non-metachromatically, with Azur A. The slower component II consisted of two closely similar substances which have not yet been separated in larger quantities for individual examination. Free protein was not detected in these preparations. Electrophoresis of the ^{14}C -labelled mucin from the [^{14}C]-glucose experiments showed that only the slower-moving peak, II, was radioactive. There was also present in incubated and in inactive control experiments a 'non-specific' radioactive peak, which showed no staining properties with any of the reagents used. It was considered not to be related to the mucosubstance under investigation since on fractionation with rivanol the peak could successfully be removed without diminution of the ^{14}C -labelled mucin product. The same pattern after electrophoresis was observed when either carbonate-bicarbonate buffer (pH 10) or sodium diethylbarbiturate-barbitone (pH 8.6) was used. That the movement was truly electrophoretic and not merely electro-osmotic was demonstrated by the movement of glycogen for a short distance in the opposite direction under the same conditions.

Fractionation of isotopically labelled mucins. The separated more- and less-acidic substances, obtained by rivanol fractionation, were examined by electrophoresis and analysed for fucose, amino sugar and sialic acid contents.

The results are shown in Table 1, in which it will be seen that the precipitation of fractions containing amino sugar, fucose and sialic acid by rivanol is prevented by the presence of ammonium formate in a final concentration of 0.5 M or greater. Subsequent electrophoresis of the material soluble in rivanol at 0.5 M-ammonium formate showed that it no longer contained component I, whereas the insoluble fraction at this concentration of ammonium formate contained principally I with only traces of II.

In a large-scale fractionation with rivanol, digested mucin (62 mg.) was fractionated in 0.5 M-

ammonium formate, and 74% (w/w) was found to be rivanol-soluble. The soluble fraction was found to contain 6.3% of fucose.

Evidence for the presence of nucleic acids. The appearance of ribose in the acidic hydrolysates of unfractionated mucin suggested the presence of nucleic acids, which was further indicated by the finding of 0.67% of P in the material. Component I migrated in the same position as a known specimen of RNA and had similar staining properties with Alcian Blue, mucicarmine and Azur A. The electrophoretic behaviour was distinct from that of chondroitin sulphate or hyaluronic acid. Spectrophotometric examination of rivanol-insoluble fraction containing component I showed that it had λ_{max} 260 m μ in aqueous solution. The nucleic acid content was found to vary between 3.4 and 27% in the different preparations of digested mucin, measured as RNA by u.v. absorption. The fraction also gave a positive Dische diphenylamine reaction for DNA. Addition of protamine sulphate (Eli Lilly and Co., Indianapolis, Minn., U.S.A.) to the original digested mucin resulted in the precipitation of 83% of the organic phosphate it contained.

Distribution of radioactivity in the ^{14}C -labelled mucin. The most radioactively labelled sample of mucin so far obtained contained 62.2 ± 0.7 (s.e.) counts/min./mg., and the ethanol-treated control contained 48.2 ± 0.7 (s.e.) counts/min./mg. After rivanol fractionation in 0.5 M-ammonium formate the soluble fraction contained 91 ± 2 counts/min./mg. and the control none. These values are of the order of 10% of added radioactivity. An attempt was made to increase the specific activity by freeing the mucosal scrapings from pre-existing mucin during incubation for 30 min. in Krebs medium III, in oxygen. The tissue was removed and transferred to fresh medium containing [^{14}C]glucose. The digested mucin from this 'pre-incubated' experiment gave 31.7 counts/min./mg. for rivanol-fractionated material. Without pre-incubation the same tissue gave digested rivanol-fractionated mucin having 56.9 counts/min./mg. with [^{14}C]glucose and 43.8 counts/min./mg. with L-fucose (40 μ moles in 20 ml.) added as well as [^{14}C]glucose.

Table 1. *Composition of rivanol-mucin complexes in different concentrations of ammonium formate*

Analytical details are given in the Methods and Materials section. Samples (3.9 mg.) of mucin were taken at each concentration of formate.

Final concn. of ammonium formate (M) ...	0		0.5		1.0		2.0	
	Sol.	Insol.	Sol.	Insol.	Sol.	Insol.	Sol.	Insol.
Fucose ($\mu\text{g.}$)	12	135	137	19	159	19	143	2
Sialic acids* ($\mu\text{g.}$)	7	105	72	5	93	3	96	4
Hexosamine† ($\mu\text{g.}$)	252	640	660	139	650	213	685	142
'Humin' present in hydrolysates	-	++	++	-	++	-	++	-

* As *N*-acetylneuraminic acid.

† As glucosamine hydrochloride.

Acidic hydrolysis (0.5N-sulphuric acid; 3 hr.; 100°) and paper chromatography of the most active fractionated digested mucin described above showed the presence of four principal monosaccharide constituents: galactose 9.8, fucose 5.9, amino sugars 17.7 and mannose (or xylose, or arabinose) 0.4 counts/min./mg. of mucin. Ribose possibly arising from RNA was detected, but was on no occasion found to be radioactive. These values were not substantially changed when Polybactrin was included in the original incubation mixture. The 'humin' produced during acidic hydrolysis was found to be radioactive, containing in one instance 2.1 counts/min./mg. of mucin from an incubated preparation and none from an inactivated control preparation. Considerable amounts of radioactivity were found to remain stationary at the origins of chromatograms of 3 hr. hydrolysates.

The hydrolysis (2N-hydrochloric acid; 16 hr.; 102°) of the rivanol-fractionated material (91 counts/min./mg.) for amino sugars gave 'humin', which had 10.7 ± 0.1 counts/min./mg. of mucin. Amino sugars separated by paper chromatography had a radioactivity of 15.7 counts/min./mg. of mucin. Amino acid chromatography indicated the presence of at least 13 amino acids, some of which were radioactively labelled.

From the 2N-hydrochloric acid hydrolysate, two Elson-Morgan-positive materials were eluted by 0.3N-hydrochloric acid from a Dowex 50 (X 8) resin column, by the method of Gardell (1958). These appeared between 58 and 68 ml. (peak 1), and 70 and 80 ml. (peak 2). Material from alternate tubes was used in the colorimetric reaction, and the contents of the remaining tubes corresponding to peak 1 and peak 2 were pooled and dried *in vacuo*. In the Stoffyn & Jeanloz (1954) method for identification of amino sugars peak 1 gave arabinose and peak 2 gave lyxose. Chromatography in ethyl acetate-pyridine-acetic acid-water (Fischer & Nebel, 1955) showed that peak 1 moved with glucosamine and peak 2 with galactosamine. These amino sugars were present in the ratio 3.25:1 respectively and had radioactivities in the ratio 0.69:1. Analysis of mucin for total hexosamine (Blix, 1948) gave 21.6 ± 3.6 (s.d.)% calculated as glucosamine hydrochloride (six experiments). Results for analysis and incorporation of ^{14}C from labelled glucose are shown in Table 2.

Sialic acids in the mucin. Sialic acids were detected in digested-mucin preparations by the alkaline Ehrlich reaction (Aminoff, 1961) and by the thiobarbituric acid method of Warren (1959).

A sample of ^{14}C -labelled mucin (sialic acid content, 5.5%) soluble in rivanol in 0.5M-ammonium formate, and having about 76 counts/min./mg., was hydrolysed for 20 min. at 80° in

0.1N-sulphuric acid. The acid was neutralized with barium carbonate, and ethanol was added to a concentration of 70% (v/v). The clear supernatant after centrifuging was evaporated at 40° *in vacuo*. It contained sialic acid detectable by the methods given above. Chromatography in butan-2-ol-acetic acid-water (4:1:5, by vol.) (Gottschalk, 1960) demonstrated a spot reacting with alkaline silver nitrate, *p*-anisidine hydrochloride, orcinol and thiobarbituric acid, having R_f 0.72, slightly greater than fucose, R_f 0.68.

This component was radioactive. In other solvents [butan-2-ol-acetone-acetic acid-water (6:6:3:5) and butan-1-ol-propan-1-ol-0.1N-hydrochloric acid (1:2:1)] a similar radioactive component giving reactions of sialic acid was present. This separated clearly from the other radioactive components (fucose and an immobile substance). The sialic acid contained 84 counts/min./mg. of the acid, i.e. 4.6 counts/min./mg. of mucin (see Table 2).

The sialic acid-free mucin was recovered by water extraction of the barium sulphate-mucin mixture obtained after precipitation of the hydrolysis products with ethanol. The modified mucin was precipitated and dried. Mild acidic hydrolysis gave 9.8 mg. of modified mucin and 200 μg . of *N*-acetylneuraminic acid. Direct estimation of sialic acid in another mucin sample gave 5.0%. Removal of sialic acid resulted in a modification of the electrophoretic properties of the mucin, and its diminished reaction with sodium metaperiodate.

Incorporation of [^{35}S]sulphate. Incorporation of sulphate into mucin led to a radioactivity of 878 counts/min./mg. In five different experiments, values were in the range 654-1260. This accounts for approximately 0.1% of the added sulphate. Papain-digested mucin when subjected to electrophoresis was radioactive only in component II, and subsequent digestion with alkali and electrophoresis in the presence of added chondroitin

Table 2. *Radioactivity and content of some mucin components*

Results for all the components except for sialic acid are for a rivanol-fractionated mucin having 91 counts/min./mg. The sialic acid result is for a rivanol-fractionated mucin having 44 counts/min./mg. Details of hydrolyses and separations are given in the text.

Component	Radioactivity (% of total counts incorporated)	Content in mucin (% dry wt.)
Galactose	10.8	—
Fucose	6.5	6.1
Mannose	0.4	—
Humin	11.8	—
Glucosamine HCl	8.4	20.2
Galactosamine HCl	3.7	6.1
Sialic acid	6.1	5.5

sulphate failed to alter the position of the radioactivity. Radioautography showed that the radioactivity corresponded very closely with the main Alcian Blue-staining component of component II. Precipitation with cetylpyridinium chloride in the presence of added chondroitin sulphate according to the conditions of Wolf & Varandani (1960) showed that the radioactivity remained mainly in the cetylpyridinium chloride-soluble mucin fraction and not in the cetylpyridinium chloride-insoluble chondroitin sulphate.

Hydrolysis of the ^{35}S -labelled mucin followed by chromatography showed that the radioactivity released travelled in the same place as $^{35}\text{SO}_4^{2-}$ ions and not as cysteine or methionine. However, radioactivity was not fully released under conditions that sufficed for the liberation of sulphate from chondroitin sulphate. Some radioactivity remained at the origin.

The alkali digestion of ^{35}S -labelled scrapings yielded very little acetic acid-insoluble material and this consisted only of protein and mucin (component II). The ethanol-precipitated material on electrophoresis gave a broad band of radioactive material (component II) and also a smaller amount of radioactivity in a position just behind that of component I. The mucin was examined, together with chondroitin sulphate, by salt-gradient chromatography on Whatman no. 4 paper of the cetylpyridinium chloride complex (Marsden & Kent, 1962). This method separated at least three radioactive components, of which one migrated in approximately the same position as chondroitin sulphate, whereas the others ran at greater speeds, as expected for mucin.

Action of papain. It was thought desirable to examine the possibility that papain may modify the mucin by transferring amino acids or peptides to it from proteins or by reversal of proteolysis. Radioactive ^{14}C -labelled protein (kindly given by Dr J. R. Quayle) was isolated from *Escherichia coli* that had been grown on ^{14}C acetate, by the method of Roberts, Abelson, Cowie, Bolton & Britten (1955). The protein had an activity of $49\,600 \pm 240$ counts/min./mg. of protein. In the present experiments 3.07 mg. was incubated with about 5 mg. of papain, with and without 8.45 mg. of mucin, in the buffer described above. The digest was dialysed and filtered and was treated with ethanol (3 vol.). From the mucin digests, 7.5 mg. of solid product was obtained but only 0.4 mg. in the absence of mucin. The mucin product had an activity of about 450 counts/min./mg. This and the mucin-free controls were subjected to electrophoresis in borate buffer, pH 9.3, and in acetate buffer, pH 4.5. The majority of the counts were present in an anodic band in borate and moved towards the cathode in acetate. A broad peak of

low radioactivity moved towards the anode in both buffers, and in borate overlapped the mucin bands. In acetate buffer, however, distinct separation was observed. The same broad peak was present both in control (no mucin) and mucin-containing preparations.

DISCUSSION

The wide scatter of values found for the Q_{O_2} of different preparations and different samples of the same preparation of mucosal tissue arises probably from varying proportions of very viscous mucin 'solution' and solid tissue. It appears, however, that the addition of sugars such as glucose or fucose does not affect the rate of oxygen uptake (Mah, 1962). This suggests that endogenous substances or added organic acids are used for oxidative purposes and that glucose, not being metabolized primarily through an oxidative pathway, may be available for biosynthetic purposes.

In earlier investigations (Pasternak *et al.* 1958) mucin preparations were submitted to alkali digestion before electrophoretic examination. The present use of papain is considered a desirable alternative. The mucin on electrophoresis moves as a more compact band after enzyme treatment than when digested with alkali. Moreover, considerable stainable protein is retained in the latter preparation and the mucopolysaccharide-staining reactions are less strong. Pusztai & Morgan (1961) have shown that papain degrades human-blood-group substances only to a slight extent, some four peptide links being cleaved in each molecule. The experiments with radioactive protein suggest that artifacts produced by transferase activity of papain do not constitute a serious source of error.

The results indicate that the mucosal tissue was highly active in taking up ^{14}C glucose and maintained a relatively high Q_{O_2} over 3 hr. The fate of the incorporated radioactivity has been studied and the monosaccharide constituents with significant radioactivity that have been identified include galactose, fucose, amino sugars and sialic acids and possibly mannose (Table 2). Sialic acid(s) is present in a readily hydrolysable form as in the α_1 -seroglycoproteins (orosomucoids) and may, similarly, constitute terminal units of the mucoprotein structure.

Attempts to increase the amount of radioactive incorporation by removal of the existing mucin from the tissue by a process of preincubation were not successful, probably because the mucin in its native form is not soluble in water. ^{35}S Sulphate was incorporated by mucosal scrapings into the same substance that becomes labelled with ^{14}C glucose, although only to a low degree. Papain digestion liberated the radioactive mucin, but there was no evidence for the presence of chondroitin

sulphate or similar acidic polysaccharides. Alkali extraction instead of enzymic digestion gave several electrophoretically separable components, of which one was of the mucin type and another, in small quantity only, of a more acidic nature. Alkali digestion of papain-treated mucin did not give this acidic component, which may therefore be an artifact of the alkali treatment. Alternatively some of the papain-digested material may be lost in dialysis, or may remain in an insoluble form not affected by proteolysis.

Werner (1953) obtained evidence for sulphated mucopolysaccharides in pig colon, and Wolf *et al.* (1961) classified their rat colonic mucin as similar to chondroitin sulphate. This was on the basis of its failure to move in chromatography in m -ammonium acetate-ethanol, a property shared with the present mucin, its precipitation by 1% cetylpyridinium chloride in 0.25 M -sodium chloride, and its electrophoresis properties. In these last-mentioned properties, the present papain-digested material differs clearly from acidic mucosubstances.

An apparent incorporation of radioactivity occurred when [^{14}C]glucose was added to ethanol-denatured preparations of mucosal tissue. The radioactivity did not persist after treatment with rivanol, though hydrolysis of unfractionated mucin preparations gave a spurious radioactive zone in chromatograms, not present in [^{14}C]glucose itself. The zone showed no staining properties with any of the reagents employed.

The addition to scrapings of L-fucose together with [^{14}C]glucose caused little variation in the amount of [^{14}C]glucose used by the tissue, and it appears therefore unlikely that fucose is incorporated directly into the mucin. The observed utilization of L-fucose (Kent & Mah, 1961) by that tissue may well be a step in the degradation of the sugar rather than a preliminary to its incorporation.

SUMMARY

1. Incubation of surviving sheep colonic mucosal scrapings in oxygen with [^{14}C]glucose results in labelling of the mucosubstances. L-Fucose did not inhibit this incorporation.

2. The papain-digested ^{14}C -labelled mucosubstances, analysed by zone electrophoresis, consist of two nearly-related ^{14}C -labelled mucopolysaccharides and unlabelled nucleic acids (RNA and DNA). The nucleic acids can be removed by fractionation in rivanol-ammonium formate.

3. Acidic hydrolysis of the ^{14}C -labelled mucopolysaccharides gave ^{14}C -labelled sialic acid, galactose, glucosamine and galactosamine, fucose and possibly mannose, containing 6, 11, 8, 4, 7 and < 1% of the total incorporated radioactivity respectively.

4. Scrapings incubated with [^{35}S]sulphate gave, on digestion with papain, ^{35}S -labelled mucin and no other non-diffusible radioactive components. Alkali digestion gave a mixture of radioactive products, including one more acidic than the mucin. Alkali treatment of the papain-digested mucin did not yield this acidic component.

5. Papain digestion with non-radioactive mucin and ^{14}C -labelled protein from *Escherichia coli* did not produce a radioactive mucin product.

The authors thank Dr M. W. Whitehouse and Dr C. A. Pasternak for helpful discussions and for the gift of rivanol, and Dr J. R. Quayle and Dr G. W. Manley for generously providing materials. This work was carried out with the help of Departmental grants from the Rockefeller Foundation and the U.S. Public Health Service and with an award (to P. D.) of a Medical Research Council Scholarship for training in research methods.

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The Determination of Galactosamine in the Presence of Glucosamine

APPLICATION TO *ASPERGILLUS NIGER* CELL WALLS

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(Received 16 August 1962)

Methods for the estimation of galactosamine in the presence of glucosamine involve modification of the galactosamine (Scott, 1962) or become increasingly inaccurate for the minor component when it represents less than one-fifth of the total hexosamine (Gardell, 1958). Cessi & Serafini-Cessi (1962), by a distillation method, have used borate to release selectively the 2-methylpyrrole (the most important Elson-Morgan chromogen), due to D-galactosamine, from the mixture of the acetylacetone condensation products with D-galactosamine and D-glucosamine. Slein (1952) estimated galactosamine directly. Using yeast hexokinase, he showed that glucosamine but not galactosamine was phosphorylated in the presence of ATP. The phosphorylation of glucosamine under these conditions is well authenticated (Brown, 1951; Grant & Long, 1952). Glucosamine 6-phosphate was then removed by precipitation with barium hydroxide-zinc sulphate, leaving galactosamine to be determined in the supernatant. Slein suggested that the method would be useful when only small amounts of galactosamine were present in a mixture with glucosamine. The present paper extends Slein's (1952) observations and improves the method of separating galactosamine from other products in the reaction mixture. The method is applied to *Aspergillus niger*-cell-wall hydrolysates.

MATERIALS

D-Glucosamine hydrochloride (W. Kerfoot and Sons, Burnley, Lancs.) was recrystallized from 3N-HCl, water and finally by a methanol-acetone-water procedure (Roseman

& Ludowieg, 1954) to give $[\alpha]_D^{25} + 71.8^\circ$ (*c* 2.0 in water). D-Galactosamine hydrochloride (L. Light and Co. Ltd., Colnbrook, Bucks.) had $[\alpha]_D^{25} + 96.0^\circ$ (*c* 2.4 in water); another sample (California Corp. for Biochemical Research), recrystallized once from methanol-acetone-water, had $[\alpha]_D^{25} + 96.4^\circ$ (*c* 3.2 in water). All samples were chromatographically pure with respect to other amino sugars, non-nitrogenous sugars and amino acids. Dowex 50 (minus 400 mesh, 200-325 wet-mesh range; X 5) (California Corp. for Biochemical Research) was used in the ion-exchange columns. ATP was the disodium salt (98% pure) (Sigma Chemical Co.); solutions were adjusted to pH 6.5-7.0 with NaOH before use.

Yeast hexokinase was Sigma grade 3 (150 000 Kunitz-MacDonald units/g.). It had no phosphatase activity; the use of such a grade of enzyme is highly important in the assay described in this paper.

CO₂-free NaOH (0.01N) was prepared by 'method B' of Vogel (1951). All other chemicals were of analytical reagent quality.

Phosphate buffer (0.25M) was made by dissolving 39 g. of NaH₂PO₄·2H₂O in ion-free water, adjusting the pH to 7.62 with 2N-NaOH and making up to 1 l. Acetate buffers (0.1 and 1.0M) were made by adjusting solutions, containing 0.1 and 1.0 mole of NaOH respectively in 900 ml. of ion-free water, to pH 5.2 with acetic acid and then making up to 1 l. Tris buffer was the buffer at pH 7.6 and 1.02 described by Datta & Grzybowski (1961).

METHODS

Assay methods. Total hexosamine was estimated by the method of Boas (1953), with glucosamine hydrochloride as standard.

Titrimetric experiments were done in the Radiometer titrator (Type TTT/C) with a Titrograph assembly (Type