

Studies on Gastric Mucoproteins

THE PRODUCTION OF RADIOACTIVE MUCOPROTEINS BY PIG GASTRIC MUCOSAL SCRAPINGS *IN VITRO*

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1. Optimum conditions, including the effect of media of different pH values, were determined for the incorporation of radioactive precursors into mucoproteins by pig gastric mucosa *in vitro*. 2. Mucosal scrapings incorporated radioactivity from [U-¹⁴C]-glucose and from [G-³H]threonine or [G-³H]serine solely into the carbohydrate and protein portions respectively of the mucoprotein molecules. 3. Of the radioactive mucoprotein 22% was water-soluble and up to 80% of the remainder was soluble in other solvents. 4. Pronase was the most successful proteolytic enzyme tested for making the mucoprotein water-soluble, up to 94% dissolving after digestion. 5. The Pronase digestion products of the mucoproteins were separated from protein by equilibrium-density-gradient centrifugation in a CsCl gradient. 6. These Pronase-digested mucoproteins were further fractionated on Sepharose 4B and the isolated fractions analysed by chemical and sedimentation-velocity methods. 7. Pronase digestion and solvent extraction of mucosal scrapings labelled with ¹⁴C in the carbohydrate and ³H in the protein showed that one type of mucoprotein was the only non-diffusible biosynthetic product of the scrapings *in vitro*, and that this mucoprotein was the only mucoprotein constituent of the water-soluble and water-insoluble mucus.

This paper describes the initial experiments in a systematic study of the components of gastric mucus. Because of the known complexity of gastric secretions (Glass, 1965) attempts were made to simplify the system by studying only washed mucosal scrapings. To identify the components of mucous secretions it was considered necessary first to study the metabolism of the mucosal cells and, in particular, to label radioactively the macromolecules in their secretions.

Preparations from a number of mucus-secreting tissues have been shown to be metabolically active *in vitro* and to incorporate radioactivity from suitable radioactive precursors into the mucoprotein of their mucous secretions. Such preparations include mucosal scrapings from sheep colon (Draper & Kent, 1963) and human stomach (Kent & Allen, 1968), and slices of sheep and ox submaxillary glands (Schauer *et al.*, 1968). The experiments in the present paper describe the further development of these techniques and their application to the study of the metabolism and production of radioactive mucoproteins by gastric mucosal scrapings *in vitro*. Further, Pronase digestion followed by solvent extraction has been used to solubilize the mucoproteins of the insoluble mucus for comparison with the previously characterized mucoproteins of the water-soluble mucus (Snary & Allen,

1971). A preliminary report of this work has been published (Snary & Allen, 1969).

Experimental

Materials

Enzymes. The following enzyme preparations were supplied by Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.: ficin (EC 3.4.4.12; crude freeze-dried powder), papain (EC 3.4.4.10; crystalline suspension in sodium acetate buffer, pH 4.5) and pepsin (EC 3.4.4.1; freeze-dried powder from pig stomach). Calbiochem Ltd., London W.1, U.K., supplied Pronase B (freeze-dried material).

Radioactive isotopes. All radioactive compounds were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The specific radioactivities of the isotopes were as follows: DL-[G-³H]threonine, 150 mCi/mmol; DL-[G-³H]serine, 20 mCi/mmol; D-[U-¹⁴C]glucose, 250 mCi/mmol; Na₂³⁵SO₄, carrier-free. All radioisotopes used were checked for purity by chromatography. Radioactivity was measured in a Beckman LS 200 liquid-scintillation counter with scintillation fluid prepared as described by Herberg (1960). Insoluble samples were digested for about

20 min with 2M-NaOH (10 mg/ml) at 85°C until solubilized. All samples were counted for radioactivity until there was a less than 5% error in the counts registered. Radioactivity on chromatograms was measured by eluting sections of chromatograms with up to 0.4 ml of water directly into scintillation vials and adding 10 ml of scintillation fluid.

Methods

Analytical methods. The methods used for hydrolysis and chromatography of the neutral sugars, hexosamines and amino acids were those of Allen & Kent (1968). Ester sulphate was liberated by hydrolysis in 6M-HCl for 18 h at 110°C. The hydrolysate was evaporated to dryness under vacuum [4.0 kN/m² (30 mmHg)] at 40°C, and the residue dissolved in 0.5 ml of water and chromatographed with ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.) (Fischer & Nebel, 1955).

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Carbohydrate-positive material eluted from columns was determined by the anthrone method of Seifter *et al.* (1950). Quantitative values of hexose were determined by the orcinol method of Weimer & Moshin (1953) with galactose as a standard. Values obtained for total hexose by the orcinol determination were corrected for interference by fucose, which gave 63% by wt. of the E_{540} value obtained with galactose. Fucose was measured by the method of Gibbons (1955) and hexosamines by the method of Ludoweig & Benmaman (1967).

Preparation of tissue. Stomachs were collected in ice immediately after slaughter of the animals. The stomachs were opened from the cardio-oesophageal entrance along the lesser curvature to the pyloric sphincter. The contents of the stomach were removed and the stomach was washed thoroughly with cold water. Where obvious anatomical differences were seen between the pyloric, fundic and cardiac regions the stomach was sectioned accordingly. The mucosal surface of the sections was scraped with a scalpel and the scrapings were placed in ice-cold modified Krebs-Ringer medium III (Draper & Kent, 1963). In all experiments pooled samples of scrapings from four stomachs were used.

Respiration and incorporation of radioactivity by the tissue. (a) Measurement of respiration and incorporation of radioactivity. Mucosal scrapings were incubated in manometer flasks with simultaneous measurement of respiration. To the Krebs-Ringer medium used for these incubations was added 20 μ Ci of Na₂³⁵SO₄ or 1 μ Ci of D-[U-¹⁴C]glucose. Experiments were terminated by the addition of trichloroacetic acid (final concn. 5%).

(b) Preparation of radioactive material. Mucosal scrapings were incubated in stoppered flasks at 37°C

for 2½ h. The incubation mixture contained 15 ml of Krebs-Ringer medium and 5 ml of scrapings, together with 10 μ Ci of D-[U-¹⁴C]glucose, 60 μ Ci of DL-[G-³H]serine or 60 μ Ci of DL-[G-³H]threonine. In many experiments [U-¹⁴C]glucose was added to the incubation medium together with one of the tritiated amino acids. The incubations were terminated by the addition of 3 vol. of ethanol.

The products of incubation were dialysed at 4°C against 2 litres of water for 4 days, with three changes of water a day. No radioactivity was incorporated by the control incubation mixtures containing tissue previously killed by treatment with trichloroacetic acid (final concn. 5%). Samples containing Na₂³⁵SO₄ were first dialysed against 2 × 2 litres of 5% (w/v) non-radioactive Na₂SO₄ for 24 h at 4°C before dialysis against water.

Dry weights of tissues and mucins were measured from freeze-dried materials.

Solvent extractions. After dialysis of the mucosal scrapings, the non-diffusible material was centrifuged at 5000g for 20 min to separate a water-soluble fraction from a water-insoluble residue. In some experiments the water-insoluble residue was further extracted with 100 ml of one or more of the following solvents: 8M-urea, 0.2M-2-mercaptoethanol, 2M-NaCl, 2% (w/v) sodium deoxycholate or 0.5M-formic acid. Each solvent extraction was carried out for 4 days with gentle shaking at 4°C. The soluble fractions were removed after centrifuging the extract at 5000g for 20 min.

Assay of blood-group activity. Blood-group A activity was assayed by the method of Kabat & Bezer (1945). The dilution of antiserum that just gave complete agglutination of a 4% (v/v) suspension of erythrocytes was used. The blood-group activity of the material was expressed as the amount of material (μ g/0.1 ml) that just inhibited complete agglutination of the erythrocytes by the antiserum.

Enzyme digestions. Pepsin digestions were carried out by the method of Kabat (1956). Mucosal scrapings (5 ml; about 500 mg freeze-dried wt.) were incubated with 100 mg of pepsin in 15 ml of 0.5M-sodium citrate, pH 2.3. Papain digestions were carried out by the method of Kimmel & Smith (1954). The digestion medium contained 3.5 ml of water, 1 ml of 0.2M-sodium citrate, pH 5.0, 0.5 ml of papain (3.0 mg/ml) and 30 mg of freeze-dried mucosal scrapings. The papain was prepared as described by Kimmel & Smith (1954). Pronase digestions were carried out by the method of Ambler (1963). The incubation medium contained 30 mg of freeze-dried scrapings and 5 mg of Pronase in 5.0 ml of 0.2M-ammonium acetate, pH 6.5. Ficin digestions were by the method of Pusztai & Morgan (1961). The incubation mixture contained 30 mg of scrapings, 5 mg of ficin and 5 ml of 0.2M-sodium phosphate-0.1M-sodium citrate-0.01M-KCN at pH 4. All incubations were continued

for 3 days, with further amounts of enzyme added after 24 and 48 h. Two or three crystals of thymol were added to prevent bacterial growth. Incubations were performed at 37° and 60°C and, in some experiments, scrapings were incubated at 100°C for 15 min before enzymic digestion.

The products from Pronase digestion were fractionated by using CsCl equilibrium density-gradient centrifugation (Creeth & Denborough, 1970). The freeze-dried digestion products were dissolved in CsCl soln., density 1.42 g/ml, and centrifuged at 43000 rev./min for 48 h in an MSE Superspeed 50 preparative ultracentrifuge, in 10 ml polycarbonate tubes. Complete separation of protein (at the top of the tube, final CsCl density 1.38 g/ml) from the mucoproteins (at the base of the tube, final CsCl density 1.56 g/ml) was obtained after centrifuging for 48 h (D. Snary & A. Allen, unpublished work). The mucoprotein was further fractionated by using Sephadex G-100 (25 cm × 1 cm) and Sepharose 4B (25 cm × 2 cm) columns eluted by water. Sedimentation-velocity studies were performed by using a Beckman model E analytical ultracentrifuge with the mucoprotein dissolved in a buffer of 0.18 M-KCl-0.02 M-potassium acetate-0.02% NaN₃, pH 5.5, as described by Snary & Allen (1971).

Results

Metabolism of pig gastric mucosal tissue in vitro

Surface scrapings from the different anatomical regions of the pig stomach were incubated manometrically in the presence of 1 μCi of D-[U-¹⁴C]-glucose for 2½ h at 37°C. The metabolic activity of the scrapings was measured by three parameters: oxygen

absorption, radioactivity from the [U-¹⁴C]glucose liberated as ¹⁴CO₂, and radioactivity incorporated into the non-diffusible material from [U-¹⁴C]glucose (Table 1). In all preparations the metabolic activity, as measured by oxygen uptake, ceased within 2½ h of the start of the incubation (Fig. 1). Mucosal scrapings from the cardiac region of pig stomach incorporated the most radioactivity from [U-¹⁴C]-glucose into the non-diffusible material and this source of tissue was therefore used for all further experiments.

The metabolic activity of the scrapings was not affected by the addition of penicillin G (final concn. 500 units/ml) and of streptomycin (final concn. 0.1 mg/ml). The specific radioactivity of the non-diffusible material from incubations with [U-¹⁴C]-glucose was 440 d.p.m./mg in the presence of antibiotics and 440 d.p.m./mg in the absence of antibiotics (average of two experiments). Similarly, the oxygen uptake by the mucosal scrapings incubated in the presence and absence of antibiotic was 1.4 and 1.1 μl of oxygen/mg dry wt. of tissue (average of two experiments) respectively.

The optimum pH for metabolic activity of mucosal scrapings was studied by incubating scrapings manometrically in modified Krebs-Ringer medium containing [U-¹⁴C]glucose and buffered with sodium phosphate or 3,3-dimethylglutaric acid at different pH values in the range pH 3.2-8.9. The pH optimum for oxygen absorption, ¹⁴CO₂ release and incorporation of radioactivity into the non-diffusible material was found in all three cases to be pH 7.4 (Table 2). To compare these results with the pH of the stomach contents of pigs, ten stomachs were collected from two separate batches of slaughtered animals and the pH of the stomach contents was measured. The pH

Table 1. *Metabolism of pig gastric mucosal scrapings incubated in vitro with D-[U-¹⁴C]glucose*

Incubations were performed in manometer flasks containing in the main compartment 3 ml of modified Krebs-Ringer medium III, 1 ml of tissue (approx. 60 mg dry wt.) and 1 μCi of D-[U-¹⁴C]glucose; 0.2 ml of 2 M-NaOH was included in the centre well of the flasks to trap released CO₂. Incubations were for 2½ h at 37°C, and were terminated by the addition of 0.5 ml of 30% (w/v) trichloroacetic acid from the side arms of the flasks. The results are average values from at least three experiments; numbers in parentheses represent the range of values obtained.

Anatomical region of stomach	Radioactivity incorporated into non-diffusible material		Radioactivity released as ¹⁴ CO ₂ (d.p.m./mg dry wt. of tissue)	Oxygen absorbed (μl/mg dry wt. of tissue)
	Sp. radioactivity (d.p.m./mg dry wt. of tissue)	% of added radioactivity		
Cardiac	5500 (±2500)	18.7	1890 (±120)	4.0 (±1.7)
Fundic	3070 (±2000)	7.0	820 (±150)	2.9 (±1.0)
Pyloric	1600 (± 890)	4.8	104 (± 40)	1.3 (±0.5)

values were found to vary between 2.6 and 6.9. In all cases the measured pH of the stomach contents was below pH 7.4, the optimum pH for the metabolic activity of the scrapings *in vitro*.

The distribution of radioactivity incorporated from [U-¹⁴C]glucose into the sugars of the non-diffusible material was examined by acid hydrolysis and paper chromatography. After hydrolysis for 3 h at 105°C in 0.25 M-H₂SO₄, chromatography showed the following sugars to be present: glucose, galactose, mannose, fucose, glucosamine and galactosamine, and a trace of ribose. Hydrolysis for 16 h at 105°C in 2 M-HCl, primarily for the release of hexosamines, followed by

chromatography, indicated that the material contained glucosamine and galactosamine as well as a wide spectrum of amino acids. The radioactivity in the non-diffusible material was distributed amongst the component sugars as follows: galactose 57%, fucose 8%, glucosamine and galactosamine 20%. No radioactivity was found in any of the other sugars or in the amino acids.

The metabolism of different tissue sections from the stomach wall, namely surface mucosal scrapings, pieces of mucosal layer removed from the underlying tissue and slices of whole stomach wall, was studied (Table 3). The surface mucosal scrapings had the highest oxygen absorption, ¹⁴CO₂ release and incorporation of radioactivity from [U-¹⁴C]glucose into the non-diffusible material of the three tissue sections tried. In contrast the specific radioactivity of the non-diffusible material labelled from Na₂³⁵SO₄ was the highest for pieces of mucosal sheet. Acid hydrolysis and chromatography showed that all the radioactivity incorporated from Na₂³⁵SO₄ into the mucosal sheet was present as ester sulphate; a single radioactive spot co-chromatographed with standard radioactive sulphate (*R*_{methionine} 0.8), and no radioactivity was found in cystine and cysteine (*R*_{methionine} 0.1) or methionine.

The incorporation of radioactivity from [G-³H]-threonine and [G-³H]serine into the non-diffusible material is shown in Table 4. Acid hydrolysis and chromatography in both ethyl acetate-pyridine-acetic acid-water (5:5:3:1, by vol.) and ethanol-NH₃ soln. (sp.gr. 0.88)-water (18:1:1, by vol), showed that [G-³H]serine was incorporated solely as serine. By using the same solvents [G-³H]threonine was shown to be incorporated as glycine and threonine containing 50 and 47% of the total radioactivity respectively.

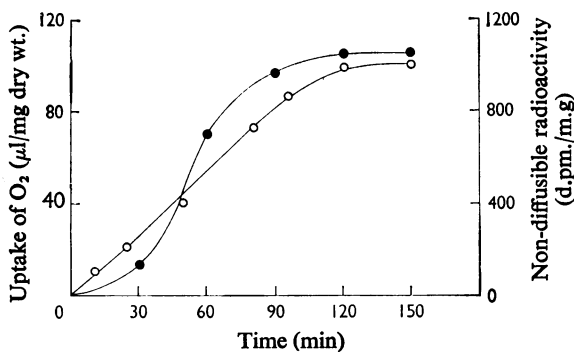


Fig. 1. Oxygen uptake by and incorporation of radioactivity into the non-diffusible fraction by pig stomach cardiac mucosal scrapings

Incubations were performed as described in Table 1. ○, Uptake of oxygen (μl/mg dry wt. of tissue); ●, radioactivity in the non-diffusible fraction.

Table 2. Metabolism of pig stomach cardiac mucosal scrapings incubated *in vitro* in media of different pH values with D-[U-¹⁴C]glucose

Incubations were performed as described in Table 1. The pH 7.4 medium was modified Krebs-Ringer medium III, other pH values were achieved by replacing the sodium phosphate buffer in modified Krebs-Ringer medium III with 0.154 M-3,3-dimethylglutaric acid adjusted to the required pH with 0.154 M-NaOH (final concn. of 3,3-dimethylglutaric acid, 4 mmol/l). The pH of the whole medium was adjusted to the required value where necessary with 0.2 M-HCl or 0.2 M-NaOH. The results are average values from four experiments; numbers in parentheses represent the range of values obtained.

pH of medium	Sp. radioactivity of non-diffusible material (d.p.m./mg dry wt. of tissue)	Radioactivity released as ¹⁴ CO ₂ (d.p.m./mg dry wt. of tissue)	Oxygen absorbed (μl/mg dry wt. of tissue)
3.2	31 (±4)	35 (±13)	0.5 (±0.1)
5.0	1730 (±370)	1830 (±150)	1.8 (±0.3)
6.9	4480 (±180)	2750 (±130)	3.5 (±0.5)
7.4	5340 (±600)	3380 (±100)	4.4 (±0.2)
8.9	1360 (±700)	1410 (±400)	2.7 (±1.3)

Table 3. *Metabolism of different tissue sections from the wall of the cardiac region of pig stomach incubated in vitro with D-[U-¹⁴C]glucose or Na₂³⁵SO₄*

Incubations were performed as described in Table 1, with either 1 μCi of D-[U-¹⁴C]glucose or 10 μCi of Na₂³⁵SO₄ added to the incubation medium. Incubations with added Na₂³⁵SO₄ had MgSO₄ omitted from the modified Krebs-Ringer medium III and MgCl₂ added (final concn. 2mmol/l). After incubation with Na₂³⁵SO₄ samples were dialysed at 4°C against 2 × 2 litres of 5% (w/v) Na₂SO₄ for 24h before dialysis against water. The results are average values from three experiments; numbers in parentheses represent the range of values obtained.

Tissue section	Dry wt. of tissue (mg)	Sp. radioactivity of non-diffusible material labelled from Na ₂ ³⁵ SO ₄ (d.p.m./mg dry wt. of tissue)	Sp. radioactivity of non-diffusible material labelled from D-[U- ¹⁴ C]glucose (d.p.m./mg dry wt. of tissue)	Radioactivity released as ¹⁴ CO ₂ (d.p.m./mg dry wt. of tissue)	Oxygen absorbed (μl/mg dry wt. of tissue)
Surface mucosal scrapings	74	60 (±60)	12700 (±1000)	1440 (±700)	3.1 (±0.9)
Pieces of mucosal sheet	150	1270 (±500)	1200 (±220)	910 (±400)	0.8 (±0.2)
Slices of stomach wall	332	310 (±100)	520 (±168)	740 (±250)	0.4 (±0.1)

Table 4. *Water-soluble non-diffusible radioactivity present in pig stomach cardiac mucosal scrapings previously incubated with either D-[U-¹⁴C]glucose, DL-[G-³H]threonine or DL-[G-³H]serine*

The methods are described in the Experimental section. The incubation mixture (total volume 20ml) contained 10 μCi of D-[U-¹⁴C]glucose, 60 μCi of DL-[G-³H]serine or 60 μCi of DL-[G-³H]threonine. The results are average values from six experiments; numbers in parentheses represent the range of results obtained.

Radioactive isotope	Sp. radioactivity of non-diffusible material (d.p.m./mg dry wt. of tissue)	10 ⁻⁶ × Total non-diffusible radioactivity (d.p.m.)	Water-soluble non-diffusible radioactivity (% of total)
D-[U- ¹⁴ C]Glucose	5500	1.42	21.8 (±12.4)
DL-[G- ³ H]Threonine	22800	4.58	23.5 (±1.9)
DL-[G- ³ H]Serine	10280	3.32	21.8 (±6.6)

Fractionation of the non-diffusible material

The distribution of the radioactivity between the water-soluble and water-insoluble fractions of the non-diffusible radioactivity incorporated into the mucosal scrapings from [U-¹⁴C]glucose, [G-³H]-threonine and [G-³H]serine is shown in Table 4. An average of about 22% of the radioactivity in the non-diffusible material was found to be water-soluble for all three radioactive labels used. The water-soluble fraction constituted between 4.2 and 15.1% (average 8.2%, four experiments) by weight of the freeze-dried non-diffusible material. With material labelled from [U-¹⁴C]glucose the specific radioactivity of the water-soluble fraction was 10400 (±3000) d.p.m./mg dry wt., compared with 3900 (±500) d.p.m./mg dry wt. for the total non-diffusible fraction. Only a further 0.2-3.0% (average 1.2%, from 12 experiments) of the total radioactivity from [U-¹⁴C]glucose in the non-

diffusible material was solubilized by shaking the water-insoluble residue with another 100ml of water for 4 days at 4°C. The possibility that 3 vol. of ethanol added at the end of the incubation *in vitro* may affect the amount of radioactivity that was water-soluble was studied by omitting the addition of ethanol to some incubations. No significant difference was found between the percentage of water-soluble radioactivity in the non-diffusible material with ethanol (9.8-16.7%; average 12.5%, four experiments) or without ethanol (7.0-17.8%; average 12.0%, four experiments).

The water-insoluble radioactivity of the non-diffusible material labelled from [U-¹⁴C]glucose and [G-³H]threonine was further solubilized by treatment with 0.2M-mercaptoethanol, 8M-urea or 0.5M-formic acid (Table 5). 2M-Sodium chloride solubilized only a further 12% of the water-insoluble radio-

Table 5. *Solubility in different solvents of radioactive non-diffusible material from pig stomach cardiac mucosal scrapings previously incubated with D-[U-¹⁴C]glucose and DL-[G-³H]threonine*

The methods are described in the Experimental section. The results are average values from three experiments; numbers in parentheses represent the range of values obtained.

Radioactive isotope	% of total radioactivity solubilized by solvent			% of total radioactivity insoluble	
	First extraction	Second extraction	Third extraction		
Expt. 1	Water	0.2M-Mercaptoethanol	8M-Urea	—	
	³ H	27.4 (±7.4)	7.1 (±3.2)	42.6 (±10.1)	22.9 (±3.4)
Expt. 2	¹⁴ C	26.3 (±4.1)	7.9 (±1.9)	43.0 (±5.0)	22.8 (±6.8)
	Water	8M-Urea	0.2M-Mercaptoethanol	—	
Expt. 3	³ H	20.5 (±5.3)	33.4 (±5.1)	14.1 (±2.1)	32.0 (±3.4)
	¹⁴ C	20.9 (±6.1)	34.0 (±9.2)	13.4 (±3.3)	31.7 (±5.9)
Expt. 3	Water	0.5M-Formic acid	—	—	
	³ H	11.1 (±0.9)	5.1 (±2.1)	—	83.8 (±0.8)
Expt. 3	¹⁴ C	11.1 (±2.1)	4.8 (±0.9)	—	84.1 (±1.7)

Table 6. *Solubility after proteolytic digestion of the non-diffusible material radioactively labelled from D-[U-¹⁴C]glucose*

The experimental conditions are described in the Experimental section. The results are average values from three experiments; numbers in parentheses represent the range of values obtained.

Enzyme	Incubation temperature (°C)	Total non-diffusible radioactivity present (d.p.m.)	Soluble radioactivity	
			d.p.m.	% of total radioactivity
Pronase	37	105 000	98 000	93.5 (±1.0)
	37*	99 000	85 000	86.0 (±1.5)
	60	108 000	101 000	93.7 (±1.5)
Papain	37	89 000	78 500	88.2 (±0.8)
	37*	103 000	78 000	76.2 (±4.1)
	60	86 000	73 000	85.0 (±2.1)
Ficin	37	80 000	65 000	81.2 (±1.0)
	37*	72 000	52 000	65.0 (±3.1)
	60	81 000	64 000	79.1 (±3.0)
Pepsin	37	887 000	346 000	39.0 (±2.0)

* Tissue previously denatured by heating at 100°C for 15 min.

activity. A second extraction with 100 ml of a solvent solubilized only a further 0.2–0.8% of the radioactivity present in the non-diffusible material. Between 94 and 98% of the radioactivity solubilized by these solvents was found to be soluble after dialysis against water. Sodium deoxycholate (0.2%, w/v) solubilized 37.7% of the water-insoluble radioactivity in the non-diffusible fraction; however, after dialysis against water, only 2.4% of the radioactivity was found to be soluble in water. Table 5 shows that the maximum amount of radioactivity solubilized by any of the combinations of solvents tried was still only 78% of the total non-diffusible radioactivity in the mucosal scrapings. Table 5 also shows that the

same percentage of ³H-labelled protein and ¹⁴C-labelled carbohydrate were solubilized by treatment of the non-diffusible material with any one of the solvents used.

Enzymic digestion of the non-diffusible material

In an effort to solubilize the whole of the radioactivity incorporated from [U-¹⁴C]glucose into the non-diffusible fraction, digestion with proteolytic enzymes was tried (Table 6). Papain, ficin and Pronase solubilized a similar percentage of radioactivity under all conditions used. Pepsin was less

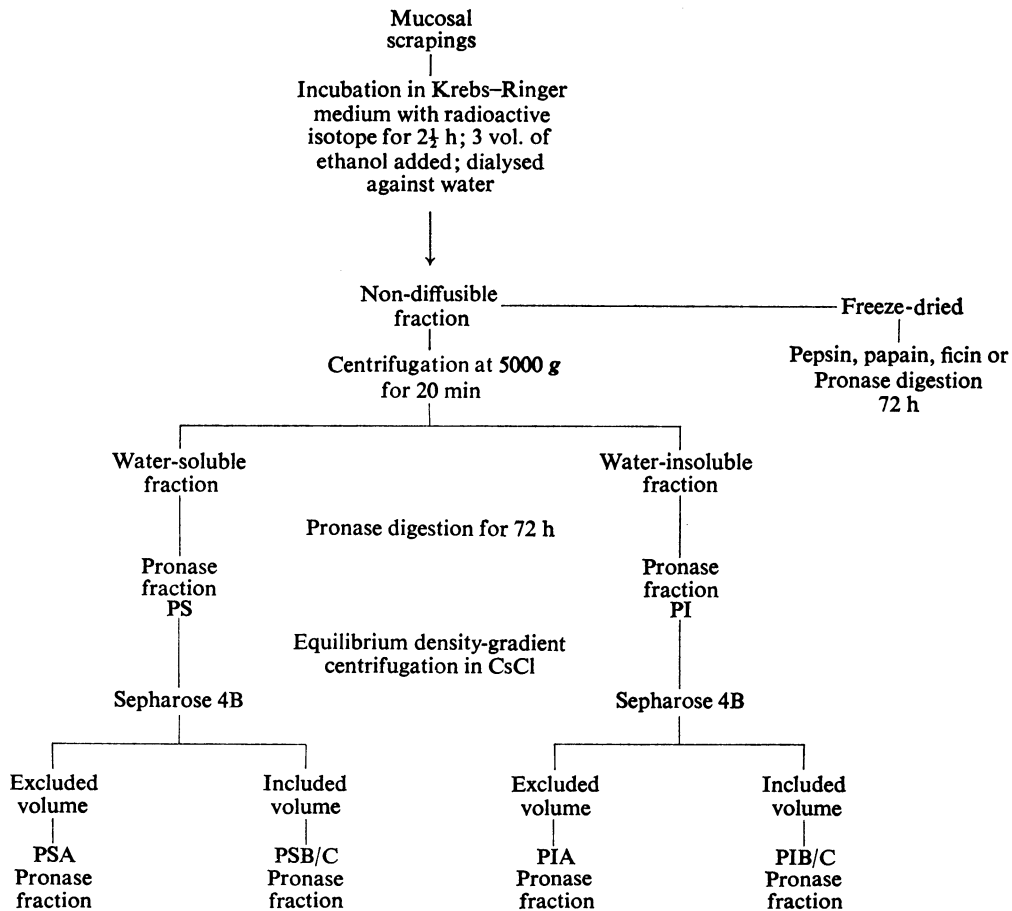
effective, only 39% of the total radioactivity in the sample being solubilized after digestion.

Pronase-digestion products (37°C incubation) were chosen for further study and a summary of the procedures used is shown in Scheme 1. The identities of the Pronase-digestion products from the water-soluble fraction (fraction PS) and the water-insoluble fraction (fraction PI) were investigated as follows. Gel filtration on Sephadex G-100 of both fractions PS and PI gave identical elution profiles, with all the hexose-positive material (anthrone method) in the excluded volume. Further, the freeze-dried excluded peaks from gel filtration on Sephadex G-100 of fractions PS and PI had very similar blood-group A activities: 0.5 µg/0.1 ml and 0.6 µg/0.1 ml respectively.

The Pronase-digestion products from the water-soluble (fraction PS) and the water-insoluble (fraction PI) fractions were further compared by fractionation

with CsCl equilibrium density-gradient centrifugation. The fractions from the bottom third of the tube, which contained after centrifugation all the carbohydrate-containing material, were then dialysed against water (six changes of water for 48 h) at 4°C and freeze-dried. These fractions obtained from the water-soluble fraction (PS) and the water-insoluble fraction (PI) were examined by sedimentation-velocity analysis in 0.2M-KCl buffer, pH 5.5, at concentrations of 5 and 2.5 mg/ml. Two boundaries were found to be present in each fraction. The measured $s_{25,w}$ values for each of the two components in the fractionated material from the water-soluble fraction (PS) and the material from the water-insoluble fraction (PI) respectively were closely similar (Table 7).

The freeze-dried carbohydrate-containing fractions isolated by density-gradient centrifugation from the



Scheme 1. Procedures used during enzymic digestion studies on pig stomach cardiac mucosal scrapings

Table 7. Sedimentation-velocity coefficients for the fractionated Pronase-digestion products of the water-soluble and water-insoluble mucus

The methods are described in the Experimental section.

	Fraction	Concn. of sample ...	$s_{25,w}$ (S)	
			5 mg/ml	2.5 mg/ml
Water-soluble mucus	PSA		14.1	21.4
	PSB/C		4.72	5.49
Water-insoluble mucus	PIA		14.0	20.9
	PSB/C		4.66	5.44

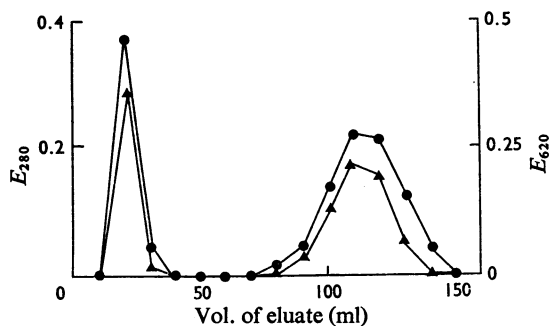


Fig. 2. Fractionation on Sepharose 4B of the carbohydrate-containing fraction from the Pronase digest of the water-insoluble mucus (fraction PI)

●, E_{280} ; ▲, anthrone-positive material (E_{620}). A sample (2 ml; approx. 5 mg dry wt./ml) was loaded on a Sepharose 4B column (25 cm × 2 cm), eluted with water by downward flow and 6 ml fractions were collected. An identical result was obtained for the Pronase digest of the water-soluble mucus.

Pronase digests of the water-soluble fraction (PS) and water-insoluble fraction (PI) were dissolved in water (5 mg/ml) and further fractionated by gel filtration on Sepharose 4B. Similar elution profiles as measured by E_{280} and total hexose were given by both the water-soluble and water-insoluble fractions (Fig. 2). In both cases 45% by wt. of the mucoprotein was excluded on Sepharose 4B, the remaining 55% being in the included volume. The water-soluble fraction (PS) was separated into two fractions on Sepharose 4B, fraction PSA in the excluded volume and fraction PSB/C in the included volume; similarly the water-insoluble fraction (PI) was separated into fractions PIA and PIB/C (Scheme 1 and Fig. 2). The overall chemical compositions of the four fractions PSA, PSB/C, PIA and PIB/C were found to be closely similar (Table 8).

Discussion

The mucosal scrapings survived in Krebs-Ringer medium for up to 2 h, as shown by the oxygen absorption and the incorporation of radioactivity (Fig. 1). The radioactivity incorporated into the combined water-soluble and water-insoluble mucus by the mucosal scrapings from the cardiac region of pig stomach, 3000–8000 d.p.m./mg dry wt., was comparable with that found for sheep colonic mucosal scrapings and human gastric scrapings (Allen, 1966; Kent & Allen, 1968). The pH optimum of the metabolic activity was pH 7.4, i.e. the pH of the Krebs-Ringer medium (Table 2). In contrast the pH of pig stomach contents varied between 2.6 and 6.9. Further, studies by Marcussen (1970) using electrodes *in vivo* have shown that a pH gradient exists across the stomach contents, values as high as pH 7.0 being recorded at the centre of the contents and values as low as pH 1.0 being recorded near the mucosal surface. At pH 3.2 the metabolic activity of the mucosal scrapings was only 4% of the value at pH 7.4, and even at pH 5.0 the metabolic activity was only 47% of the activity at pH 7.4. This supports the idea of a protective mechanism, presumably involving the mucous layer (Florey, 1962), between the stomach contents and the mucosal cells.

The percentage of radioactivity incorporated into the water-soluble and water-insoluble mucus from [^{14}C]glucose into different sugars was as follows: galactose, 57%; glucosamine and galactosamine, 20%; fucose, 8%. The remaining 15% of the radioactivity unaccounted for could be explained by losses during the hydrolysis procedures; also, the radioactivity, if any, in the sialic acid residues was not measured. These sugars are the constituents of the water-soluble mucoproteins in pig gastric mucus (Snary & Allen, 1971). Traces of mannose were present in the mucosal scrapings. However, the mannose was not radioactively labelled and it is not a constituent of the purified mucus (Snary & Allen, 1971). It is quite possible that the mannose comes from material made elsewhere than in the cardiac

Table 8. Chemical analysis of the fractionated Pronase-digestion products of the water-soluble and water-insoluble mucus

The methods are described in the Experimental section. Each value is an average of at least two determinations.

Fraction	Water-soluble mucus		Water-insoluble mucus	
	PSA (%, by wt.)	PSB/C (%, by wt.)	PIA (%, by wt.)	PIB/C (%, by wt.)
Hexose	27.7	27.2	27.8	26.8
Hexosamine	28.6	27.2	28.3	26.3
Fucose	13.0	10.5	12.2	10.5
Protein	9.2	11.6	8.4	10.3

region of the stomach and in this connexion it is noteworthy that the mannose-containing serum mucoproteins have been found in gastric secretions (Glass, 1965). In the pig cardiac preparation no radioactivity from [U-¹⁴C]glucose was found in the amino acids, ribose, uronic acids or glucose, showing that no detectable incorporation of radioactivity had taken place into nucleic acids, acid mucopolysaccharides or glycogen. This is evidence of restricted utilization of glucose during the incubation *in vitro* of mucosal scrapings, although radioactive glucose was also metabolized by the respiratory enzymes of the cells, since ¹⁴CO₂ was released during the incubations. The absence of radioactive labelling in ribose and deoxyribose, together with the lack of effect of antibiotics on the incorporation of radioactivity into the non-diffusible material, showed that in these experiments no radioactivity was incorporated into bacterial material. The pathways by which glucose can be converted into galactose, fucose and hexosamines and the incorporation of the sugars from their nucleotide derivatives into mucoproteins have been well documented for other tissues (Ginsberg, 1964), including gastrointestinal mucosal tissues (Kent & Allen, 1968; Race *et al.*, 1968). Both [G-³H]serine and [G-³H]threonine were incorporated into the non-diffusible material by the scrapings *in vitro*. Whereas added [G-³H]serine was incorporated into the non-diffusible material solely as serine, radioactivity from [G-³H]threonine was incorporated into both threonine and glycine in approximately equal proportions. The [G-³H]threonine used in these experiments was chromatographically pure, and amino acid analysis (Snary & Allen, 1971) rules out the unlikely degradation of threonine to glycine during acid hydrolysis. It appears therefore that a metabolic pathway exists in pig gastric mucosal scrapings for the conversion of threonine into glycine. An enzyme capable of converting threonine into glycine, threonine aldolase, has been found in the

liver and kidneys of a number of species (Braunstein & Vilenkina, 1949).

Radioactivity was not incorporated from Na₂³⁵SO₄ into the mucosal scrapings to a significant extent. This is notable because of the isolation from gastrointestinal tissue of sulphated mucoproteins, e.g. sheep colonic mucoprotein (Kent & Marsden, 1963) and a dog stomach mucoprotein (Pamer *et al.*, 1968), as well as the sulphated mucopolysaccharides isolated from pig stomach (Meyer *et al.*, 1937; Kimura *et al.*, 1964). The surface mucosal scrapings incorporated the largest amount of radioactivity from [U-¹⁴C]glucose into the non-diffusible material and had the highest O₂ uptake and CO₂ output of the different tissue sections (Table 3). The deeper tissue sections across the stomach wall did, however, incorporate significant amounts of radioactivity from Na₂³⁵SO₄. The radioactivity from Na₂³⁵SO₄ (1270 d.p.m./mg dry wt.) incorporated into the mucosal sheet was shown to be present as ester sulphate, and no radioactivity was incorporated into the sulphur-containing amino acids. In keeping with these results are radioautographic studies, which have shown that the cells that incorporated Na₂³⁵SO₄ were the mucous neck cells in the gastric glands and not the surface epithelial mucus-secreting cells (Cornet *et al.*, 1964). The absence of Na₂³⁵SO₄ incorporation by the surface mucosal scrapings shows that they did not contain significant amounts of the deeper secretory cells.

Since only 22% of the radioactive carbohydrate from [U-¹⁴C]glucose and 22% of the radioactive protein from [G-³H]serine or [G-³H]threonine was found to be water-soluble (Table 4), attempts were made to solubilize the radioactive water-insoluble mucus. The water-insoluble radioactivity was further solubilized by treatment with one or more different solvents (Table 5), but in no case was more than 80% of the total radioactivity solubilized. When mucosal scrapings, containing both ¹⁴C-labelled carbohydrate and ³H-labelled protein, were extracted with different

solvents, including water, the $^3\text{H}/^{14}\text{C}$ ratio in the different fractions was constant for one experiment. Thus the same percentage of the total radioactive carbohydrate and radioactive protein was soluble in each of the different solvents used. This suggests that the same spectrum of radioactive mucoproteins are solubilized by all the different solvents used and that the radioactive products in the insoluble non-diffusibile material are closely related to the water-soluble radioactive material.

Since solvents were only partly successful in solubilizing all the water-insoluble radioactivity in the mucosal scrapings, enzymic methods were tried (Table 6). Various enzymic methods have been used to isolate mucoproteins from mucous secretions, e.g. pepsin digestion of pig stomach mucosa (Kabat, 1956), papain digestion of sheep colonic mucosa (Draper & Kent, 1963) and dog stomach mucosa (Pamer *et al.*, 1968), and ficin digestion of ovarian cysts (Pusztai & Morgan, 1958). Pepsin digestion of pig mucosal scrapings from the cardiac region solubilized only 39% of the radioactivity from $[\text{U}-^{14}\text{C}]$ -glucose. However, approx. 22% of this radioactivity would have been water-soluble anyway, so enzymic digestion was responsible for solubilizing only a further 17% of the total radioactivity. Skoryna & Waldron-Edwards (1967) found that pepsin solubilized 20% of the carbohydrate of the insoluble mucus from human stomach. In contrast to pepsin, the three proteases ficin, papain and Pronase were very effective in solubilizing the radioactivity incorporated from $[\text{U}-^{14}\text{C}]$ -glucose into the water-insoluble material. Since Pronase was the most effective, 94% of the total radioactivity being solubilized, the Pronase-digestion products were studied further.

The products of Pronase digestion of the water-soluble mucus (fraction PS) and the water-insoluble mucus (fraction PI) were shown to be very similar. In both fractions PS and PI (Scheme 1), all the carbohydrate-positive material was excluded on Sephadex G-100 and had very similar blood-group A activities. Removal of the protein by CsCl density-gradient centrifugation gave a carbohydrate-containing fraction, which, on sedimentation analysis, was shown to possess two peaks of closely similar $s_{25,w}$ values for fractions PS and PI, at two different mucoprotein concentrations (Table 7). Corresponding to these two boundaries observed by sedimentation analysis, two mucoprotein fractions, A and B/C, were isolated by gel filtration on Sepharose 4B of the carbohydrate-containing fraction from the CsCl density-gradient centrifugation. The Pronase digests of the water-soluble and water-insoluble mucus gave identical profiles on Sepharose 4B after density-gradient fractionation, and the two mucoprotein fractions accounted for all the carbohydrate-containing material in the Pronase digests in each case (Fig. 2). The fractions PSA and PSB/C isolated from

the water-soluble mucus and the fractions PIA and PIB/C isolated from the water-insoluble mucus (Scheme 1) had, within the limits of the analyses, identical fucose, hexosamine and hexose contents (Table 8). Similarly, the analysis of the protein content of all these fractions, as measured by the method of Lowry *et al.* (1951), is very similar, although nothing can be deduced about the composition of the different amino acids in the respective mucoprotein fractions. Also, this method has been shown to give low protein values for mucoproteins (Pamer *et al.*, 1968). It has been suggested for human stomach that the water-soluble mucus is of similar carbohydrate composition to the water-insoluble mucus (Schrager & Oates, 1971).

Fractionation and characterization of the non-diffusible water-soluble fraction from pig cardiac scrapings has shown that all the radioactivity in ^{14}C -labelled carbohydrate and ^3H -labelled protein resides in one type of mucoprotein (Snary & Allen, 1971). This mucoprotein, which comprises 84% of the water-soluble fraction, consists of two distinct fractions of different molecular weights (1.9×10^6 and 1.1×10^5), but both fractions have closely similar chemical and immunological properties (Snary *et al.*, 1970). The identical patterns of radioactive material obtained from the solvent-extraction studies, and more particularly the identical nature of the mucoproteins released by Pronase digestion of the water-soluble and water-insoluble mucus, indicate that the same mucoprotein type is in both fractions. These results also show that gastric scrapings *in vitro* are incorporating radioactivity from $[\text{U}-^{14}\text{C}]$ -glucose, $[\text{G}-^3\text{H}]$ -serine and $[\text{G}-^3\text{H}]$ -threonine into a single type of mucoprotein, which is the principal non-diffusible constituent of the water-soluble mucus, the only mucoprotein constituent of the water-insoluble mucus and, further, the only non-diffusible radioactive material produced by the gastric mucosa *in vitro*.

The reason why 22% of the radioactive mucoprotein is water-soluble whereas the remainder is in the water-insoluble gel is unknown. It is possible that all the mucoprotein was water-soluble but that a large percentage was made insoluble owing to interactions between the mucoprotein molecules or between the mucoprotein and other cellular material. Thus the water-insoluble radioactive mucoprotein when extracted with 2M-NaCl, 0.2M-mercaptoethanol or 8M-urea was found to be water-soluble when the solvent was dialysed away. Further, the high-molecular-weight component of the water-soluble mucoprotein will gel reversibly above 3 mg/ml (Snary *et al.*, 1971). Alternatively, the mucoprotein may be made *in vivo* in an intrinsically insoluble form, which is partly or completely solubilized by proteolytic digestion or solvent extraction. Since solvents such as deoxycholate and urea, which are not known to break covalent bonds, solubilize a substantial part of the

water-insoluble gel it would seem unlikely that covalent bonding is involved in gel formation.

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