The metabolism of sialic acids in isolated rat colonic mucosal cells

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The activities of ten enzymes involved in sialic acid metabolism were measured in colonic mucosal cells from rats and compared with those in liver. A methodology was devised that enabled all ten enzyme activities to be evaluated in a single rat colon preparation. Enzyme assays with radioactively labelled substrates were developed for maximum sensitivity, and the identification of substrates and products was carefully checked to assess the contribution of contaminants to enzyme reactions with low activity. The activities of most enzymes involved in the biosynthesis of *N*-acetyl-D-neuraminic acid (NeuAc) from UDP-*N*-acetyl-D-glucosamine were found to be more than 20-fold lower than those in liver. The activities of CMP-NeuAc synthase, *N*-acetyl-D-glucosamine 2-epimerase, *N*-acetyl-D-glucosamine kinase, sialyltransferase and sialidase were similar to or 2–4-fold lower than in liver. The biosynthesis of NeuAc via its 9-phosphate was demonstrated in the 100 000g supernatant of colonic-cell homogenates by enzymic assay and precursor experiments with *N*-acetyl[¹⁴C]-mannosamine. No alternative route for NeuAc formation could be detected. The 100 000g supernatant fractions of liver, kidney and colonic mucosal cells utilized *N*-acetyl[¹⁴C]mannosamine with differing efficiencies. Radioactive products identified as sialic acid biosynthetic intermediates amounted to 49%, 0.04% and 5.6% of added precursor in liver, kidney and colon respectively. Catabolism of labelled precursor to non-hexosamine products was high in kidney and colonic mucosal-cell fractions.

The sialic acids occur widely as terminal negatively charged monosaccharide components of mucus glycoproteins (Corfield & Schauer, 1982a). The sialoglycoproteins are secreted by epithelial tissues, including those of the gastrointestinal tract, where the content of sialic acids in glycoproteins is characteristic for each region. The mucus glycoproteins secreted by the colonic mucosa are rich in sialic acids, present predominantly as the *O*-acetylated form in man (Rogers *et al.*, 1979). Histochemical analysis of the glycoproteins in colonic mucosal cells during disease suggests changes in the type and amount of sialic acids (Culling *et al.*, 1979; Ehsanullah *et al.*, 1982).

Most investigations of the biosynthesis of sialic acids have been carried out in liver (Warren, 1972; Corfield & Schauer, 1982b), whereas in colonic mucosa only Kent & Draper (1968) have presented data, and these did not include detailed enzymic analysis. The enzymes responsible for NeuAc formation, its activation to CMP-NeuAc, sialyl transfer and the catabolic reactions (CMP-NeuAc

hydrolase, sialidase and acylneuraminate pyruvate-lyase) have been well established (Corfield & Schauer, 1982b). Rat liver was until recently the only tissue where the complete enzymic pathway to NeuAc had been established. Evidence has now been presented that many other tissues synthesize sialic acid but contain very low or negligible activities of some key enzymes catalysing NeuAc formation from UDP-GlcNAc (Van Rinsum *et al.*, 1983). In contrast, the activation of NeuAc to CMP-NeuAc is detectable in many tissues (Kean, 1970), together with sialyltransferase and sialidase activities (Corfield & Schauer, 1982b).

The biosynthesis of NeuAc takes place in the cytosol (Van Rinsum *et al.*, 1983), whereas the CMP-NeuAc synthase is located in the nucleus (Kean, 1970; Coates *et al.*, 1980), and sialyl transfer occurs predominantly in Golgi membranes (Schachter, 1978), CMP-NeuAc hydrolysis in the plasma membranes (Kean & Bighouse, 1974) and sialidase in the cytosol or in lysosomal membranes (Corfield *et al.*, 1981a). The significance of this topographical distribution in sialic acid metabolism remains unclear. The demonstra-

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tion of large variations in enzyme activity among tissues (Van Rinsum *et al.*, 1983) suggests that the route for sialic acid biosynthesis established in liver may differ in other tissues and that alternative regulatory mechanisms apply.

In order to study sialic acid metabolism and the changes responsible for the observed histochemical differences in colonic disease, enzymic analysis of all levels of sialic acid metabolism was carried out in rat colonic mucosal cells. Part of this work has been presented in preliminary form (Corfield *et al.*, 1983a).

Materials and methods

Materials

The following radiochemicals were purchased from Amersham International (Amersham, Bucks., U.K.): *N*-acetyl-D-[1-¹⁴C]glucosamine (2.25 GBq/mmol), *N*-acetyl-D-[U-¹⁴C]mannosamine (9.4 GBq/mmol), D-[1-¹⁴C]mannosamine hydrochloride (2.2 GBq/mmol), *N*-acetyl-D-[4,5,6,7,8,9-¹⁴C]neuraminic acid (8.95 GBq/mmol), UDP-*N*-acetyl-D-[U-¹⁴C]glucosamine ammonium salt (9.95 GBq/mmol) and NaB³H₄ (9.4 and 22.0 GBq/mmol). CMP-*N*-acetyl-D-[4-¹⁴C]neuraminic acid (59.2 MBq/mmol) was from New England Nuclear, Dreieich, Germany.

The Sigma Chemical Co. (Poole, Dorset, U.K.) supplied the following materials: *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, D-mannosamine, acetoacetic acid, phosphoenolpyruvate cyclohexylammonium salt, dithiothreitol, NaBH₄, penicillin, streptomycin sulphate, *N*-acetylneuraminate pyruvate-lyase (aldolase) from *Clostridium perfringens*, potato acid phosphatase (type II) and snake-venom phosphodiesterase (type II). UDP-*N*-acetyl-D-glucosamine disodium salt, UDP dipotassium salt and yeast hexokinase were from the Boehringer Corp. (Lewes, East Sussex, U.K.). The sodium salts of ATP, CTP and UTP were obtained from BDH Chemicals (Poole, Dorset, U.K.). *N*-Acetyl-D-neuraminic acid was from the Kantoishi Chemical Co. (Kyoto, Japan), and α₁-acid glycoprotein from the Scottish National Blood Transfusion Centre (Edinburgh, Scotland, U.K.). Micellar scintillation cocktail NE 260 was a product of Nuclear Enterprises (Edinburgh, Scotland, U.K.). All ion-exchange resins were purchased from Bio-Rad Laboratories (Watford, Herts., U.K.). Silica gel 60 and cellulose precoated thin-layer plates (0.2 mm thickness) were from E. Merck (Darmstadt, West Germany). All other chemicals were of the highest purity available from BDH Chemicals.

D-Mannosamine 6-phosphate, D-[1-¹⁴C]mannosamine 6-phosphate (18.1 MBq/mmol), *N*-acetyl-D-mannosamine 6-phosphate and *N*-acetyl-D-[1-

¹⁴C]mannosamine 6-phosphate (18.1 MBq/mmol) were prepared by using hexokinase, purified and analysed as described by Jourdan & Roseman (1962). *N*-Acetyl-D-[4-¹⁴C]neuraminic acid 9-phosphate (10.5 MBq/mmol) was prepared by using pig salivary-gland enzyme and analysed by the method of Watson *et al.* (1966). *N*-Acetyl-D-[1-¹⁴C]glucosamine 1-phosphate (9.95 GBq/mmol) was synthesized from UDP-*N*-acetyl-D-[1-¹⁴C]glucosamine by using snake-venom phosphodiesterase (Sela *et al.*, 1972).

Bovine brain gangliosides (Veh *et al.*, 1977), bovine submandibular-gland mucus glycoprotein (Corfield *et al.*, 1981b) and *N*-acetylneuraminyl-α(2-3)-lactose (Veh *et al.*, 1981) were prepared as described previously.

Gangliosides, mucus glycoprotein and α₁-acid glycoprotein were radiolabelled in their sialic acid moieties (Veh *et al.*, 1977). Sialyl-α(2-3)-lactitol [³H]ol was synthesized as detailed by Frisch & Neufeld (1979).

The specific radioactivities of the sialidase substrates were 0.85, 1.22, 1.28 and 2.63 GBq/mmol of sialic acid for ganglioside, mucus glycoprotein, α₁-acid glycoprotein and sialyl-α(2-3)-lactitol [³H]ol respectively.

Sialyltransferase substrates were prepared by mild acid hydrolysis (0.1 M-HCl at 80°C for 60 min) of α₁-acid glycoprotein and mucus glycoprotein, which contained 220 and 700 nmol of theoretical acceptor sites/mg dry wt. respectively, calculated as the total concentration of D-galactose, *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine minus the concentration of sialic acid. These concentrations were determined by g.l.c. (Clamp *et al.*, 1971).

Separation of substrates and products

Substrates and products, individually or from enzyme incubations, were applied to columns containing 1 ml of Dowex AG1 (X8; 200-400 mesh; HCO₃⁻ form) at room temperature. After a washing with 10 ml of water, components were eluted by different concentrations of NH₄HCO₃ (10 ml) as follows: NeuAc, 70 mM; *N*-acetylhexosamine monophosphate esters, 120 mM; CMP-NeuAc, 200 mM; UDP-GlcNAc and NeuAc-9-phosphate, 300 mM. Effluent and eluents were collected directly into scintillation vials as two 5 ml fractions and dried in an oven at 80°C. After dissolution of the dried material in 200 μl of water, 10 ml of scintillation fluid was added and the radioactivity measured.

Products from incubations of tissue homogenates with [¹⁴C]ManNAc were separated at 4°C on columns (1.2 cm × 8 cm) of Dowex AG1 (X8; 200-400 mesh; HCO₃⁻ form), which were washed with 75 ml of water and eluted with a gradient

(2 × 150 ml) of 0–300 mM-NH₄HCO₃ followed by 50 ml of 1 M-NH₄HCO₃. Fractions of 5 ml volume were collected.

T.l.c. separation of GlcNAc and ManNAc (system 1) was on silica gel as described by Günther & Schweiger (1965). Radioactive samples were evaporated to dryness and dissolved in 30 µl of 23 mM-GlcNAc plus 23 mM-ManNAc in water. Radioactivity was determined in silica gel scraped from the plate, suspended in 500 µl of water plus 10 ml of scintillation cocktail. Free sialic acids and the C-7 and C-8 analogues were separated on cellulose thin-layer plates (0.2 mm thickness) in butan-1-ol/propan-1-ol/0.1 M-HCl (1:2:1, by vol.) (system 2). Sialyl-lactitol and lactitol were chromatographed on silica gel 60 plates in ethanol/butan-1-ol/pyridine/water/acetic acid (100:10:10:30:3, by vol.) (system 3) as described by Veh *et al.* (1981). Radioactivity was determined in scrapings as above.

G.l.c. of GlcNAc and ManNAc was carried out on 4% SE 30 Gas-Chrom Q in glass columns (2 m × 2 mm internal diam.) run isothermally at 160°C.

Samples were also analysed after de-*N*-acetylation by using an automated ion-exchange method by Dr. P. J. Winterburn, Department of Biochemistry, University College, Cardiff (Voisey & Winterburn, 1982).

Characterization of substrates and products

Combinations of acid hydrolysis, enzymic cleavage, ion-exchange chromatography and t.l.c. were employed to confirm the identity of all substrates and products from enzymic reactions.

Phosphorylated monosaccharides were dephosphorylated with potato acid phosphatase (Kundig *et al.*, 1966). Hexosamines and *N*-acetylhexosamines were separated on Dowex 50 (X8; 200–400 mesh; H⁺ form), the hexosamines being eluted with 2 M-HCl. GlcNAc-1-phosphate was dephosphorylated by mild acid hydrolysis (0.1 M-HCl for 10 min at 100°C), and the remaining *N*-acetylhexosamine 6-phosphate was separated on Dowex AG1 and dephosphorylated as above with acid phosphatase.

UDP-GlcNAc was identified as GlcNAc after mild acid hydrolysis (0.1 M-HCl, for 10 min at 100°C) or as GlcNAc-1-phosphate after incubation with snake-venom phosphodiesterase (Sela *et al.*, 1972).

Sialic acids were incubated with acylneuraminate pyruvate-lyase (Brunetti *et al.*, 1962), and the products were applied to Dowex AG1 columns for quantitative determination of ManNAc or analysed by t.l.c. after purification on Dowex AG2 (X8; 200–400 mesh; formate form) with elution with 800 mM-formic acid.

CMP-NeuAc was identified as NeuAc, after mild acid hydrolysis (0.1 M-HCl for 10 min at 100°C), by using acylneuraminate pyruvate-lyase.

Preparation of tissue, cells and homogenates

Male Sprague-Dawley rats were obtained from Olac (Bicester, Oxon, U.K.) and maintained on Oxoid SE1 diet and water *ad libitum*. Animals were killed by CO₂ suffocation. The liver and kidneys were removed, cooled on ice, chopped with scissors and homogenized in 0.15 M-NaCl (1:3, w/v) by using 20 strokes of a glass/Teflon Potter-Elvehjem homogenizer (clearance 0.3 mm). Each homogenate was centrifuged at 1000g for 5 min, the pellet discarded and the supernatant centrifuged at 100 000g for 60 min to yield high-speed supernatant fractions.

Colonic mucosal cells were prepared by a modification of the method of Bull & Bookman (1977) and correspond to the crypt epithelial fraction described by those authors. After the colon was removed and washed through with phosphate-buffered saline (0.15 M-NaCl/0.1 M-sodium phosphate buffer, pH 7.0), it was opened longitudinally, washed, blotted, weighed and placed in 20 ml of 1 mM-dithiothreitol/phosphate-buffered saline containing 100 units of penicillin and 100 µg of streptomycin sulphate/ml. After 20 min at room temperature the tissue was transferred to 20 ml of fresh solution as before and incubated for a further 20 min. The colonic tissue was transferred into 20 ml of 0.75 mM-EDTA/phosphate-buffered saline containing antibiotics as above for 90 min at 37°C. Gentle mechanical agitation was given with blunt forceps. The tissue was transferred to fresh solution and incubated under the same conditions, and the combined cell suspensions were collected by centrifugation. The cell pellet was washed three times in phosphate-buffered saline/antibiotic solution. This preparation was homogenized after suspension in 0.15 M-NaCl (0.9 ml/g wet wt. of colonic tissue).

Homogenization was for 10 s in a Polytron homogenizer at setting 6 with ice cooling. The homogenate was centrifuged at 100 000g for 60 min, and the membrane pellet was resuspended in 0.15 M-NaCl with a small Potter-Elvehjem homogenizer to yield a protein concentration of 15–20 mg/ml. The supernatant contained approx. 1 mg of protein/ml. These fractions were used immediately for enzymic assay or precursor experiments. In preliminary experiments the cell suspension was made 0.5% (w/v) with respect to Triton X-100, and disrupted by 25 passages in an all-glass hand-held Potter-Elvehjem homogenizer or sonicated in an MSE sonicator fitted with a small probe, set at amplitude 4, for 10 s at maximum energy with ice-bath cooling. The

resulting homogenate was centrifuged at 1000g for 10 min and the resulting supernatant at 100000g as described above.

Enzyme assays

Incubations were carried out at 37°C in Eppendorf reaction tubes (1.5 ml volume). In those cases where the products were to be separated on ion-exchange columns, the reaction was stopped by the addition of 0.5 ml of 95% ethanol at 4°C, cooled at 4°C for 15 min and centrifuged at 12000g for 5 min. The supernatant was applied to a Dowex AG1 (HCO₃⁻ form) column as described above. Blank incubations included tissue fraction heated at 96°C for 5 min unless otherwise detailed.

UDP-GlcNAc 2-epimerase (UDP-2-acetamido-2-deoxy-D-glucose 2-epimerase, EC 5.1.3.14). The assay mixture contained 20 µmol of Tris/HCl, pH 7.5, 0.925 kBq of UDP-[¹⁴C]GlcNAc (8.95 GBq/mmol), 0.1 µmol of UDP-GlcNAc, 5 µmol of MgSO₄, 0.1 µmol of dithiothreitol, 1 nmol of EDTA, 2 nmol of UDP and 50 µl of tissue extract in a total volume of 100 µl. Assay was for 20 min. After ion chromatography the water wash containing ManNAc was assayed for radioactivity.

NeuAc-9-phosphate synthase (N-acetylneuraminic acid-9-phosphate pyruvate-lyase, EC 4.1.3.20). The assay mixture consisted of 10 µmol of Tris/HCl, pH 7.8, 0.37 kBq (0.02 µmol) of [¹⁴C]-ManNAc-6-phosphate (18.3 MBq/mmol), 0.2 µmol of phosphoenolpyruvate, 2 µmol of MgCl₂, 0.4 µmol of dithiothreitol, 0.54 µmol of acetoacetate, 2.5 µmol of NaF and 50 µl of tissue extract in a total volume of 100 µl. Assay was for 45 min. Sialic acid (70 mM-NH₄HCO₃) and NeuAc-9-phosphate (300 mM-NH₄HCO₃) eluates were measured.

CMP-sialic acid hydrolase (CMP-N-acetylneuraminic acid N-acetylneuraminohydrolase, EC 3.1.4.40). assay was modified from Kean (1970). The incubation mixture contained 20 µmol of Tris/HCl, pH 9.0, 0.66 kBq of [¹⁴C]NeuAc (8.95 GBq/mmol), 0.25 µmol of NeuAc, 0.25 µmol of CTP, 2 µmol of MgCl₂, 0.03 µmol of 2-mercaptoethanol and 25 µl of tissue extract in a total volume of 50 µl. CMP-NeuAc was measured after 20 min.

CMP-sialic acid hydrolase (CMP-N-acetylneuraminic acid N-acetylneuraminohydrolase, EC 3.1.4.40). The incubation was as described by Kean & Bighouse (1974), with 3.4 kBq (0.06 µmol) of CMP-[¹⁴C]NeuAc (59.2 MBq/mmol). The reaction products were separated on Dowex AG1 columns as above.

GlcNAc 2-epimerase (2-acetamido-2-deoxy-D-glucose 2-epimerase, EC 5.1.3.8). The assay was modified from Van Rinsum *et al.* (1983), and the incubation mixture contained 10 µmol of Tris/HCl, pH 8.0, 1.5 kBq of [¹⁴C]ManNAc (9.4 GBq/mmol), 0.5 µmol of ManNAc, 1.5 µmol of ATP, 1.5 µmol of

MgCl₂, 2.5 µmol of NaF and 50 µl of tissue extract in a total volume of 100 µl. The incubation was terminated after 20 min with 500 µl of ethanol and applied to a Dowex AG1 column as described above. The water wash (10 ml) was passed through 2 ml of Dowex 50 (20–50 mesh; H⁺ form), washed with 5 ml of water and freeze-dried. GlcNAc and ManNAc were separated by silica-gel t.l.c. as detailed above. The 120 mM-NH₄HCO₃ eluate (10 ml) from Dowex AG1 was freeze-dried and NH₄HCO₃ was removed by using Dowex 50 (H⁺ form). The 6-phosphate fraction was dephosphorylated with acid phosphatase, repurified on Dowex AG1 and Dowex 50 (H⁺ form) and analysed by t.l.c.

ManNAc kinase (ATP:2-acetylamin-2-deoxy-D-mannose 6-phosphotransferase, EC 2.7.1.60). The incubation mixture contained 10 µmol of Tris/HCl, pH 8.1, 2.1 kBq of [¹⁴C]ManNAc (9.4 GBq/mmol), 0.5 µmol of ManNAc, 1.2 µmol of ATP, 1 µmol of MgCl₂, 2.5 µmol of NaF and 50 µl of tissue extract in a total volume of 100 µl. Assay was for 30 min. The 120 mM-NH₄HCO₃ fraction from Dowex AG1 chromatography was freeze-dried, dephosphorylated and analysed by t.l.c. as described above for GlcNAc 2-epimerase.

GlcNAc kinase (ATP:2-acetamido-2-deoxy-D-glucose 6-phosphotransferase, EC 2.7.1.59). The incubation mixture contained 10 µmol of Tris/HCl, pH 7.5, 1.1 kBq of [¹⁴C]GlcNAc (2.25 GBq/mmol), 0.2 µmol of GlcNAc, 0.5 µmol of ATP, 1.0 µmol of MgCl₂, 2.5 µmol of NaF and 50 µl of tissue extract in a final volume of 100 µl. Incubation was for 30 min. The 120 mM-NH₄HCO₃ fraction from Dowex AG1 chromatography, containing GlcNAc-6-phosphate, was assayed for radioactivity.

Sialyltransferase (CMP-N-acetylneuraminic acid: D-galactosyl-glycoprotein N-acetylneuraminyltransferase, EC 2.4.99.1). Assay of endogenous and exogenous activity with desialylated α₁-acid glycoprotein or desialylated mucus glycoprotein was as described by Corfield *et al.* (1983b).

Sialidase (acetylneuraminyl hydrolase, EC 3.2.1.18). Assays for sialidase activity were carried out with four substrates. Incubation mixtures contained 20 µmol of sodium acetate, pH 4.0, and each substrate at a final concentration of 0.2 mM-sialic acid [0.24 MBq of α₁-acid glycoprotein (0.71 GBq/mmol of sialic acid), 0.24 MBq of mucus glycoprotein (1.22 GBq/mmol of sialic acid), 0.7 MBq of ganglioside (0.85 GBq/mmol of sialic acid) or 0.52 MBq of sialyl-α(2-3)-lactitol [³H]ol (2.63 GBq/mmol of sialic acid)] and 50 µl of tissue extract in a final volume of 100 µl. Incubation mixtures with α₁-acid glycoprotein and mucus glycoprotein contained 0.1 mg of Triton X-100. After 120 min 100 µl of ovalbumin (80 mg/ml in

0.1 M-sodium phosphate buffer, pH 7.0, at 4°C) was added for α_1 -acid glycoprotein and mucus glycoprotein incubations, 500 μ l of the same solution for the ganglioside incubations. The reaction was terminated by precipitation with 500 μ l of 5% (w/v) phosphotungstic acid/15% (w/v) trichloroacetic acid mixture (α_1 -acid glycoprotein and mucus glycoprotein) or 200 μ l of 10% (w/v) trichloroacetic acid (ganglioside). After centrifugation at 12000g for 5 min a 500 μ l sample of supernatant fluid was removed from each incubation and mixed with 10 ml of NE 260 scintillation fluid. Incubations with sialyl- α (2-3)-lactil[3 H]ol were terminated by the addition of 1 ml of ice-cold water, each mixture was centrifuged at 12000g for 5 min, and the supernatant was applied to a Dowex AG1 (HCO₃⁻ form) column and washed with 2 $\times$ 5 ml of water, dried down and the radioactivity measured.

N-Acylneuraminate pyruvate-lyase (EC 4.1.3.3). The assay was as described previously (Corfield *et al.*, 1976), with 1.55 kBq of [14 C]NeuAc (8.95 GBq/mmol) with 50 μ l of tissue extract in a final volume of 100 μ l. Products of the reaction were separated on Dowex AG1 columns as described above. Incubation was for 60 min.

Lactate dehydrogenase (EC 1.1.1.27). The method of Bergmeyer *et al.* (1963) was used to measure lactate dehydrogenase activity.

Incubations of tissue homogenates with [14 C] ManNAc

Tissue homogenates of rat liver, rat kidney and rat colonic mucosal cells prepared as above were incubated with [14 C]ManNAc as follows: 500 μ l of 10000g supernatant, 37 kBq of [14 C]ManNAc (9.4 GBq/mmol) 120 μ mol of Tris/HCl, pH 7.4, 1.2 μ mol of MgCl₂, 15 μ mol of NaF, 2 μ mol of ATP, 2 μ mol of phosphoenolpyruvate and 2 μ mol of UTP in a total volume of 1.5 ml were incubated for 40 min (liver and kidney) or for 60 min (colonic mucosa) at 37°C.

Incubations were terminated by addition of 1.5 ml of 95% ethanol at 4°C. Incubations were mixed and kept at 4°C for 30 min before centrifugation at 12000g for 5 min. The pellets were washed with three 1 ml portions of ethanol at 4°C and the supernatants pooled. Ion-exchange chromatography on Dowex AG1 (HCO₃⁻ form) was as described above. The pellets were dissolved in 0.2 M-NaOH and the radioactivity was determined.

Analytical methods

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Sialic acids were measured by micro-adaptations of the orcinol/Fe³⁺ (Böhm *et al.*, 1954) and periodate/resorcinol (Jourdan *et al.*, 1971) colorimetric assays. Radioactivity was measured on a

Searle Isocap 300 liquid-scintillation counter. Corrections for quench were made routinely by using external-standardization curves for 14 C and 3 H in NE 260 scintillation cocktail.

Results and discussion

Substrate purity and product identification in enzymic assays

The presence of contaminants in radioactive substrates, even though in very small amounts, may be sufficient to give false results in enzyme assays. The product in such cases must be characterised. This situation is especially important in enzyme assays utilizing GlcNAc and ManNAc where low degrees of cross-contamination do occur in commercial radioactive samples (Table 1). The low enzyme activities found in colonic mucosal cells relative to liver (Table 2) further emphasize the necessity for substrate and product identification.

Enzyme assays

The assay of enzymes participating in sialic acid biosynthesis in all homogenates is complicated by the instability of key enzymes such as UDP-GlcNAc 2-epimerase and ManNAc kinase (Ellis *et al.*, 1972; Kikuchi & Tsuiki, 1973; Van Rinsum *et al.*, 1983), by the action of phosphatases, pyrophosphatases and ATPases (Watson *et al.*, 1966; Van Rinsum *et al.*, 1983) and by competition of several enzymes for the same substrate, notably the inter-conversions of GlcNAc and ManNAc by kinases and epimerase activities (Corfield & Schauer, 1982b; Van Rinsum *et al.*, 1983). Furthermore colonic-cell homogenates contain substantially lower enzyme activities relative to liver (Table 2).

Membranes and supernatant (100000g) demonstrated product formation that was linear with time and protein concentration for all supernatant enzymes (results not shown), including those susceptible to phosphatase, pyrophosphatase and ATPase action. These interfering activities were localized essentially in the membrane pellet. Membrane-associated enzyme activities gave linear responses with the total-membrane pellet. This system was developed so that all the enzyme activities listed in Table 2 could be measured in a single tissue sample.

Homogenization of the mixed colonic cells by freezing and thawing, ultrasonication, Potter-Elvehjem homogenizer and Polytron homogenizer methods showed that Polytron homogenization was the most effective for cell disruption and resulted in the best recovery of enzyme activities (Fig. 1). The assay of enzymes was carried out on the same day as homogenization.

Table 1. *Purity of radioactive substrates and products of enzymic reaction*

Samples were analysed by the techniques listed in the Table and given in detail in the Materials and methods sections. Dowex AG1 was eluted with water or NH_4HCO_3 as indicated in parentheses. The recovery of radioactivity was calculated from the d.p.m. (>7500 in each case for products and >25000 in each case for substrates) at the start of each experiment. The ratio of ManNAc to GlcNAc was derived from t.l.c. in system 1 (see the Materials and methods section) expressed as percentages of the total d.p.m. scraped from the plates. Sialic acids (system 2) and lactitol (system 3) were similarly expressed as percentages of the d.p.m. scraped from the plate.

Substrate	Sequence of treatment	System 1		
		ManNAc (%)	GlcNAc (%)	Recovery (%)
UDP-GlcNAc	Mild acid; Dowex AG1 (H ₂ O)	3	97	97
	Phosphodiesterase; Dowex AG1 (120mM-NH ₄ HCO ₃); acid phosphatase; Dowex AG1 (H ₂ O)	4	96	95
ManNAc-6-phosphate	Acid phosphatase; Dowex AG1 (H ₂ O)	96	4	99
ManNAc	T.l.c. only	98	2	99
GlcNAc	T.l.c. only	1	99	100
CMP-sialic acid	Mild acid; Dowex AG1 (70mM-NH ₄ HCO ₃); pyruvate-lyase; Dowex AG1 (H ₂ O)	100	0	97
Sialidase glycoconjugates	Mild acid; Dowex AG2 (formate form) (800mM-formic acid); t.l.c.	(System 2: 92% as C-7/C-8-sialic acid)		93
Sialyl-lactit[³ H]ol	<i>Vibrio cholerae</i> sialidase; Dowex AG1 (H ₂ O); t.l.c.	(System 3: 95% as lactit[³ H]ol)		94
Enzyme assay (product)				
UDP-GlcNAc 2-epimerase (ManNAc)	Dowex AG1 (H ₂ O)	94	6	91
ManNAc kinase (ManNAc-6-phosphate)	Acid phosphatase; Dowex AG1 (H ₂ O)	89	11	93
Sialic acid-9-phosphate synthase (NeuAc-9-phosphate)	Acid phosphatase; Dowex AG1 (70mM-NH ₄ HCO ₃); pyruvate-lyase; Dowex AG1 (H ₂ O)	96	4	89
CMP-sialic acid synthase (CMP-NeuAc)	Mild acid; Dowex AG1 (70mM-NH ₄ HCO ₃); pyruvate-lyase; Dowex AG1 (H ₂ O)	95	5	96
	CMP-sialic acid hydrolase pyruvate lyase; Dowex AG1 (H ₂ O) (NeuAc)	96	4	97
GlcNAc-2-epimerase (GlcNAc)	Dowex AG1 (H ₂ O); Dowex 50 (H ⁺ form) (H ₂ O)	0	100	95
GlcNAc kinase (GlcNAc-6-phosphate)	Acid phosphatase; Dowex AG1 (H ₂ O)	3	97	96
Sialyltransferase (R-NeuAc)	<i>Vibrio cholerae</i> sialidase; Dowex AG2 (formate form) (800mM-formic acid)	(System 2: 95% as [¹⁴ C]NeuAc)		98
Sialidase (C-7/C-8-NeuAc, lactit[³ H]ol)	Dowex AG2 (formate form) (800mM-formic acid)	(System 2: 91% as C-7/C-8-sialic acid)		91
	Dowex AG1 (H ₂ O)	(System 3: 97% as lactit[³ H]ol)		88

UDP-GlcNAc 2-epimerase is known to be a regulatory enzyme in sialic acid biosynthesis in liver (Kornfeld *et al.*, 1964; Kikuchi & Tsuiki, 1973) and tracheal mucosa (Ellis *et al.*, 1972) as a consequence of the feedback inhibition by CMP-NeuAc. The enzyme is unstable in both liver (Kikuchi *et al.*, 1971; Kikuchi & Tsuiki, 1973) and trachea (Ellis *et al.*, 1972). Low activities have been reported in tissues other than liver and salivary gland (Ellis *et al.*, 1972; Van Rinsum *et al.*, 1983). UDP-GlcNAc 2-epimerase activity is 20-fold lower in colonic mucosa than in liver (Table 2). Characterization of the product of enzyme activity

(Table 1) is important, as UDP-GlcNAc may be metabolized by several enzymes (Van Rinsum *et al.*, 1983), leading to the formation of GlcNAc-1-phosphate, GlcNAc or transfer of GlcNAc to glycoconjugates.

Owing to the presence of GlcNAc 2-epimerase and GlcNAc kinase converting ManNAc into GlcNAc and then into GlcNAc-6-phosphate, it was necessary to analyse and correct for the concentration of GlcNAc-6-phosphate when measuring ManNAc kinase activity (Van Rinsum *et al.*, 1983). In liver the activity of both kinases was similar (Table 2; Van Rinsum *et al.*, 1983), but in

Table 2. Activity of sialic acid-metabolic enzymes in liver and colonic-mucosal-cell homogenate fractions
The results are given as the means $\pm$ S.E.M. The subcellular fraction used to assay enzyme activity is indicated by S, 100000g supernatant, or M, 100000g membrane pellet.

Enzyme	Subcellular fraction	Enzyme activity (nmol of product/h per mg of protein)			
		Liver	n	Colon	n
UDP-GlcNAc 2-epimerase	S	162 $\pm$ 41	4	8.2 $\pm$ 4.7	8
ManNAc kinase	S	320 $\pm$ 74	4	6.5 $\pm$ 2.1	7
Sialic acid-9-phosphate synthase	S	51 $\pm$ 9	4	4.8 $\pm$ 2.4	8
CMP-NeuAc synthase	S, liver; M, colon	130 $\pm$ 27	4	64.2 $\pm$ 3.0	7
CMP-NeuAc hydrolase	M	650 $\pm$ 107	4	1.2 $\pm$ 0.33	9
GlcNAc 2-epimerase	S	141 $\pm$ 25	4	23.6 $\pm$ 2.6	6
GlcNAc kinase	S	390 $\pm$ 76	4	180 $\pm$ 19.8	7
Acylneuraminate pyruvate-lyase	S	56 $\pm$ 11	4	0	8
Sialyltransferase					
Asialo mucus glycoprotein	M	—	—	0.17 $\pm$ 0.02	6
Asialo α_1 -acid glycoprotein	M	0.36 $\pm$ 0.03	4	0.76 $\pm$ 0.09	6
Sialidase					
Sialyl-lactitol	M	3.5 $\pm$ 0.7	4	0.85 $\pm$ 0.12	6
α_1 -Acid glycoprotein	M	0.02 $\pm$ 0.006	4	0.11 $\pm$ 0.02	6
Mucus glycoprotein	M	0	4	0.03 $\pm$ 0.008	6
Ganglioside	S	0.12 $\pm$ 0.03	4	0.94 $\pm$ 0.12	6

Fig. 1. Disruption of colonic mucosal cells

Rat colonic mucosal cells were disrupted by 1, Triton X-100 treatment, 2, Potter-Elvehjem homogenization, 3, sonication, or 4, Polytron homogenization, as described in the Materials and methods section. The disrupted cells were centrifuged to yield: ■, 1000g pellet; □, 100000g pellet; ▨, 100000g supernatant. Enzyme activities and protein were assayed as given in the Materials and methods section. The results for each experiment are expressed as a percentage of the total activity found in each experiment (pellets and supernatant).

colonic tissue the GlcNAc kinase and 2-epimerase activities were 25-fold and 3.6-fold greater respectively, and approx. 10% of the monophosphate formed in the ManNAc kinase assay could be identified as GlcNAc-6-phosphate (Table 1).

The activities of both kinases and the GlcNAc 2-epimerase have been reported in various rat tissues (Kikuchi *et al.*, 1971; Ellis *et al.*, 1972; Kikuchi & Tsuiki, 1973, 1979; Allen *et al.*, 1980; Van Rinsum *et al.*, 1983). GlcNAc kinase and 2-epimerase were found in most tissues investigated, but ManNAc kinase was frequently not detectable (Kundig *et al.*, 1966), or at very low activities (Ellis *et al.*, 1972; Van Rinsum *et al.*, 1983), in tissues other than liver, small intestine and salivary gland. Colonic tissue also shows low activity, but is an active sialoglycoprotein-secreting tissue. This suggests that the regulation associated with low activities of the key enzymes UDP-GlcNAc 2-epimerase and ManNAc kinase proposed for 'non-sialoglycoprotein-secreting' tissues (Van Rinsum *et al.*, 1983) may not hold in all cases.

The formation of NeuAc-9-phosphate from ManNAc-6-phosphate and phosphoenolpyruvate could be detected in liver and colonic tissue (Table 2). The identification of NeuAc-9-phosphate as product of the reaction in colonic mucosa was aided by the use of pyruvate kinase to regenerate phosphoenolpyruvate in large-scale assays (Watson *et al.*, 1966). Alternative routes to sialic acid were investigated as detailed below, but could not be detected.

The activity of NeuAc-9-phosphate synthase has been reported in rat liver and pig salivary glands (Watson *et al.*, 1966; Kikuchi *et al.*, 1971; Warren, 1972) and in a number of rat tissues by using a radioactivity assay (W. Ferwerda, personal communication). Previous assays of this enzyme relied on the colorimetric detection of sialic acid, which did not discriminate between NeuAc and NeuAc-9-phosphate (Watson *et al.*, 1966; Kikuchi *et al.*, 1971). The radioactivity assay system described in the present paper allows detection of both these components. No assay for the NeuAc-9-phosphate phosphatase was carried out, although the formation of NeuAc in assays without NaF indicated the presence of an activity sufficient to dephosphorylate all NeuAc-9-phosphate formed in these assays. The specific NeuAc-9-phosphate phosphatase detected in liver (Watson *et al.*, 1966) has also been confirmed in many rat tissues (W. Ferwerda, personal communication).

The formation of free *N*-acetylneuraminic acid by the NeuAc-9-phosphate phosphatase reaction provides a substrate for CMP-NeuAc production and also for the catabolic route to ManNAc via acylneuraminate pyruvate-lyase. This latter enzyme was active in liver, but could not be detected

in the high-speed supernatant or membrane fraction of colonic-cell homogenates (Table 2). The lyase has been associated with the catabolism of sialic acid and is present in kidney tissue (Brunetti *et al.*, 1962) and in mouse intestine (Nöhle & Schauer, 1981).

The formation of CMP-NeuAc is a central feature of sialic acid metabolism in tissues (Kornfeld *et al.*, 1964; Warren, 1972) since it is the substrate for many sialyltransferase reactions (Corfield & Schauer, 1982b). CMP-sialic acid synthase has been detected in many animal tissues (Kean, 1970; Coates *et al.*, 1980) and is usually present at high activities relative to other enzymes in the sialic acid pathway. The enzyme has been found associated with the nucleus (Kean, 1970; Coates *et al.*, 1980). CMP-sialic acid synthase activity in rat liver was found to be in the range reported by others (Table 2; Kean, 1970; Kikuchi *et al.*, 1971; Van Rinsum *et al.*, 1983), and colonic mucosal cell activity was only 2-fold lower. In keeping with the nuclear location of the enzyme, over 95% of the activity was found associated with the 1000g pellet for colon-cell homogenates, and in liver the enzyme is released from the nucleus in 0.15M-NaCl (Table 2). Routine assay of the 100000g pellet from colon, without separation of nuclei, gave similar results and indicates the absence of significant nuclear disruption during the homogenization technique.

The metabolic pool of CMP-NeuAc may be held in balance by CMP-sialic acid hydrolase (Ferwerda *et al.*, 1983; Van Dijk *et al.*, 1983), localized in the plasma membranes (Kean & Bighouse, 1974). In the liver the ratio of CMP-sialic acid hydrolase activity to CMP-sialic acid synthase activity is high (approx. 5:1, Table 2), but in mucosal cells much lower activities of the hydrolase are found and the ratio is reversed (1:54). The sialyltransferase activity for glycoprotein substrates (Table 2) appears to be well below the metabolic capacity of the CMP-NeuAc synthase reaction in both liver and colonic cells. Interaction of these enzymes to regulate CMP-NeuAc concentrations has clearly been demonstrated in regenerating rat liver (Van Dijk *et al.*, 1983).

Sialidase activity was assayed with four substrates representative of different classes of glycoconjugates. Activity against mucus glycoprotein was very low or absent, that against α_1 -acid glycoprotein and sialyl- α -(2-3)-lactitol could be detected in the membranes, and activity against ganglioside was predominantly in the high-speed supernatant (Table 2). Sialidase activity was found in the same low range as that for the sialyltransferases. The properties of the sialidase activities have been further characterized and are reported elsewhere (Corfield & Clamp, 1984).

Alternative routes to sialic acid in colonic mucosal cells

Because of the low concentrations of NeuAc-9-phosphate detected in preliminary experiments the existence of alternative routes of NeuAc formation was investigated. Bacteria are known to synthesize NeuAc from ManNAc and phosphoenolpyruvate with no formation of NeuAc-9-phosphate (Warren, 1972; Brossmer *et al.*, 1980), but no conversion of *N*-acetyl[U - ^{14}C]mannosamine into sialic acid could be detected in standard or large-scale assays,

with colonic-cell homogenate, high-speed supernatant or membranes. Inclusion of ATP in the assay led to the formation of ManNAc-6-phosphate and low concentrations of NeuAc and NeuAc-9-phosphate. Replacement of ManNAc by UDP-[^{14}C]GlcNAc, [^{14}C]GlcNAc-1-phosphate or [^{14}C]GlcNAc-6-phosphate gave no detectable NeuAc or NeuAc-9-phosphate as product. The absence of NeuAc and ManNAc-6-phosphate formation from GlcNAc-6-phosphate rules out the existence of a GlcNAc-6-phosphate 2-epimerase similar to that described in bacterial systems

Fig. 2. Metabolism of [U - ^{14}C]ManNAc by rat tissue high-speed supernatant fractions

Rat tissue homogenate 100000g supernatants were incubated with 37 kBq of [U - ^{14}C]ManNAc under conditions given in the Materials and methods section. Liver and kidney fractions were incubated for 40 min and colonic mucosa for 60 min at 37°C. The reaction was stopped by addition of 95% ethanol, and the precipitate was removed and washed. The combined supernatants were applied to Dowex AG1 (HCO_3^- form) columns, washed with distilled water and eluted with a gradient of 0–0.3 M NH_4HCO_3 . Fractions of 5 ml volume were collected. Further details are given in the Materials and methods section. The peaks 1 to 4 were analysed as described in the Materials and methods section, and the data for these analyses are shown in Table 3. Each column was calibrated with radioactive markers indicated by the arrows: a, ManNAc; b, NeuAc; c, ManNAc-6-phosphate; d, NeuAc-9-phosphate.

(Ghosh & Roseman, 1965). Formation of NeuAc-9-phosphate could only be demonstrated as described above with [^{14}C]ManNAc-6-phosphate as substrate.

In order to assess the possibility of a colonic-cell inhibitor, a preparation of sialic acid-9-phosphate synthase was made from pig submandibular glands (Watson *et al.*, 1966). Measurement of NeuAc-9-phosphate formation in experiments with and without colonic-cell high-speed supernatant showed no inhibitory action. The pig synthase was also inactive with ManNAc, UDP-GlcNAc, GlcNAc-1-phosphate and GlcNAc-6-phosphate as substrates in the absence of ATP.

Routes to sialic acid other than via ManNAc-6-phosphate do not appear to be present in colonic mucosal cells.

Formation of low- M_r intermediates from [$U\text{-}^{14}\text{C}$]ManNAc

The incorporation of radioactive label from [^{14}C]ManNAc into soluble intermediates in tissues as demonstrated by others (Harms *et al.*, 1973; Ferwerda *et al.*, 1981, 1983) was evaluated by using ion-exchange chromatography (Fig. 2). Liver and kidney were chosen for comparison with colonic mucosal cells as they represent active sialic acid-synthesizing and -catabolizing tissues respectively.

With 100000g supernatant fractions demonstration of NeuAc formation via the NeuAc-9-phosphate was possible (Fig. 2 and Table 3). Conversion of [^{14}C]ManNAc into ethanol-soluble intermediates was most efficient in rat liver (65% utilization of added radioactivity), although the protein-bound radioactivity recovered was lower than that from in either kidney or colon (Table 3). Analysis of the peaks for each experiment (Fig. 2 and Table 3) showed that kidney and colon contained large amounts of radioactive products in peak 3 behaving as non-hexosamine material, and evidence that GlcNAc 2-epimerase was active, as peak 1 contained significant amounts of GlcNAc (Table 3). The presence of fructose phosphates, glucose phosphates, lactate and pyruvate due to catabolism of ManNAc to glycolytic intermediates may account for the non-hexosamine material in peak 3 and the non-sialic acid products in peak 4 (Kikuchi & Tsuiki, 1979; O'Connor & Davidson, 1982). Analysis of peak-4 material for hexosamine was negative and rules out the presence of UDP-GlcNAc, which would be eluted close to this position in the gradient applied to the Dowex AG1 column (Fig. 2 and Table 3).

The conversion of [^{14}C]ManNAc into the sialic acid-biosynthetic intermediates ManNAc-6-phosphate, NeuAc and NeuAc-9-phosphate amounted

Table 3. *Identification of sialic acid and N-acetylhexosamine components from precursor experiments with N-acetyl- $[U\text{-}^{14}\text{C}]$ mannosamine*

The material pooled in peaks 1–4 shown in Fig. 2 together with the radioactivity associated with the precipitated protein pellet is expressed as a percentage of the total radioactivity used in each experiment. The analysis of each individual peak was carried out as described in the Materials and methods section and is expressed as a percentage of the total radioactivity used in each experiment.

Fraction (from Fig. 2)	Radioactivity (% of total)		
	Liver	Kidney	Colonic mucosa
Peak 1	35	88	80
Peak 2	4	<0.1	0.2
Peak 3	52	11	11
Peak 4	9	–	6
Protein pellet radioactivity	0.5	1.1	1.1
Peak 1			
ManNAc	83	68	75
GlcNAc	5	15	10
Non-hexosamine	9	15	12
Peak 2			
NeuAc	93	–	87
Non-hexosamine	5	–	10
Peak 3			
ManNAc-6-phosphate	72	0.4	6.6
GlcNAc-6-phosphate	11	18	0.6
GlcNAc-1-phosphate	<0.5	<0.5	0
Non-hexosamine	15	80	88
Peak 4			
NeuAc-9-phosphate	85	–	79
Non-hexosamine	12	–	19

to 49%, 0.04% and 5.6% of added precursor in liver, kidney and colonic mucosal cells respectively (Table 3).

The use of radioactive-tracer methods demonstrates the biosynthesis of sialic acid through its 9-phosphate in the 100000g supernatant fraction of colonic-mucosal-cell homogenates. The mucosal-cell population contains mainly cuboid and columnar epithelial cells (80–85%), with goblet cells making up the remainder (Brasitus, 1982). The goblet cells are responsible for the production and secretion of the mucus glycoproteins, which would be expected to account for the largest part of sialic acid biosynthesis in the colonic mucosa. Separation of colonic goblet cells from absorptive cells can be achieved by using density gradients of Percoll (A. P. Corfield & J. R. Clamp, unpublished work) and should allow a closer analysis of ManNAc metabolism than in the mixed cell population used in the present studies.

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