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Studies on the Enzymic *N*-Acylation of Amino Sugars in the Sheep Colonic Mucosa

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1. D-[2-^{14}C]Glucose, [2-^{14}C]acetate, hydroxy[3-^{14}C]pyruvate, [3-^{14}C]pyruvate and [U-^{14}C]glycine were incorporated by surviving scrapings of sheep colonic mucosal tissue into glycoprotein. 2. D-[2-^{14}C]Glucose, [2-^{14}C]acetate, incorporated hydroxy-[3-^{14}C]pyruvate and [3-^{14}C]pyruvate resulted in labelling of each of the monosaccharide residues of the glycoprotein, namely *N*-glycollylneuraminic acid, *N*-acetylneuraminic acid, galactose, fucose, glucosamine and galactosamine. [U-^{14}C]Glycine was incorporated as glycyl and seryl residues of the glycoprotein. 3. Despite *N*-glycollylneuraminic acid being quantitatively the predominant sialic acid (*N*-glycollylneuraminic acid and *N*-acetylneuraminic acid were 8·5 and 5·2% by weight of the glycoprotein respectively) the corresponding ratio of the radioactive labelling from D-[2-^{14}C]glucose in *N*-glycollylneuraminic acid to that in *N*-acetylneuraminic acid was 1·00:7·27 (expressed as percentages of the total radioactivity in the glycoprotein). Neutral sugar, hexosamine and *N*-acetylneuraminic acid residues of the mucoprotein were each labelled to a similar extent. 4. Similarly, the ratio of the radioactivity in *N*-glycollylneuraminic acid to that in *N*-acetylneuraminic acid in the mucoprotein from tissue incubations with [2-^{14}C]-acetate was 1·0:4·0. 5. Both [2-^{14}C]acetate and [2-^{14}C]glucose with whole tissue led to labelling of the *N*-glycollyl substituent and of the main nonose skeleton of the *N*-glycollylneuraminic acid. In whole-tissue incubations, [3-^{14}C]pyruvate was also a precursor of radioactive *N*-glycollylneuraminic acid. 6. Hydroxy[3-^{14}C]-pyruvate and [U-^{14}C]glycine caused labelling of the carbohydrate and peptide residues of the glycoprotein, but did not give rise to labelling in the *N*-glycollylneuraminic acid residues. 7. With a wide variety of possible *N*-glycollyl precursors (fructose 6-phosphate, hydroxypyruvate, glycollate and chemically synthesized glycollyl-CoA) biosynthesis of *N*-glycollylglucosamine was not observed in cell-free preparations.

Studies by Draper & Kent (1963) and Allen & Kent (1968) have shown that surviving sheep colonic tissue (as scrapings) is metabolically active and incorporates ^{14}C-labelled precursors into both the peptide and the carbohydrate residues of a well-characterized glycoprotein fraction (Kent, Ackers & Marsden, 1967). With D-[U-^{14}C]glucose and D-[2-^{14}C]glucose as substrates, radioactivity was incorporated into all the monosaccharide residues of the glycoprotein fraction, and [U-^{14}C]-threonine and [U-^{14}C]serine were incorporated as threonyl and glycyl or seryl residues respectively of the peptide portion. $^{35}SO_4^{2-}$ was incorporated solely as hydrolysable sulphate. 'Cell-free' preparations of sheep colonic scrapings contained the full complement of enzymes for the *N*-acetylation of glucosamine and the biosynthesis of *N*-glycollylneuraminic acid and *N*-acetylneuraminic acid from the respective *N*-acylglucosamines (Kent & Draper, 1968). In the present work the biosynthetic studies of the glycoprotein were extended by using D-[2-^{14}C]glucose and [2-^{14}C]acetate, acetate being of particular importance in the metabolism of ruminant tissues (e.g. Annison & Lewis, 1959), with a view to finding the origin and mechanism of enzymic *N*-glycollylation by this tissue.

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METHODS AND MATERIALS

The preparation of the tissue, the measurement of the Q_{O_2}, the isolation of the glycoprotein fraction and the measurement of radioactivity were performed by the methods of Draper & Kent (1963). Incubation of the tissue with radioactive substrates, and acidic hydrolysis of the isolated glycoprotein and chromatographic separation of the resulting sugar residues were described by Allen & Kent (1968).

Materials. All radioactive chemicals were obtained from The Radiochemical Centre, Amersham, Bucks. *N*-Acetylneuraminic acid was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., and Sigma (London) Chemical Co., London, S.W. 6, *N*-glycollylneuraminic acid was from Sigma (London) Chemical Co., and hydroxypyruvic acid (lithium salt) was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. The following enzymes were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany: D-glucose 6-phosphate dehydrogenase (EC 1.1.1.49), hexokinase (EC 2.7.1.1), L-lactate dehydrogenase (EC 1.1.1.27), glucose phosphate isomerase (EC 5.3.1.9) and phosphate acetyltransferase (EC 2.3.1.8). Sigma (London) Chemical Co. supplied D-amino acid oxidase (EC 1.4.3.3), and Koch–Light Laboratories Ltd. supplied acid phosphatase (EC 3.1.3.2).

Preparation of D-[U-^{14}C]*fructose 6-phosphate.* This was prepared enzymically with the following incubation medium (total volume 3·0ml.): 2·72ml. of 0·5M-tris–HCl buffer, pH 7·4, 0·1ml. of 0·017M-ATP, 0·1ml. of 0·1M-$MgCl_2$, 0·02ml. of hexokinase (1mg. of protein/ml., used as supplied) and 25μc (in 0·06ml. of water) of D-[U-^{14}C]fructose (479μc/mg.). The preparation was gently shaken at 37° for 20min., the reaction stopped with 3vol. of ethanol, the precipitated protein centrifuged and the supernatant concentrated to dryness at 15mm. Hg and 30°. The residue, redissolved in 2·5ml. of water, was used as the D-[U-^{14}C]fructose 6-phosphate preparation. Chromatography of this preparation in propan-1-ol–aq. NH_3 (sp.gr. 0·88)–water (6:3:1, by vol.) (Hanes & Isherwood, 1949) on washed Whatman no. 4 paper gave a single radioactive peak running identically with fructose 6-phosphate, detected by alkaline $AgNO_3$ (Trevelyan, Procter & Harrison, 1950) and by ammonium molybdate–$HClO_4$ (Bandurski & Axelrod, 1951). A simultaneous incubation of 60μg. of unlabelled D-fructose with hexokinase under the above conditions resulted in a quantitative yield of D-fructose 6-phosphate, determined enzymically by the method of Hohorst (1963).

Preparation of hydroxy[3-^{14}C]*pyruvate.* This was prepared enzymically with the following incubation medium (total volume 0·96ml.): 0·16ml. of 0·1M-sodium pyrophosphate–HCl buffer, pH 8·5, 0·4ml. of D-amino acid oxidase solution and 0·4ml. of a solution containing 6μmoles of unlabelled DL-serine and 2·2μmoles (50μc) of DL-[3-^{14}C]serine. The D-amino acid oxidase solution was prepared by dissolving 5mg. of enzyme preparation, as supplied, in 1ml. of 0·02M-sodium pyrophosphate–HCl buffer, pH 8·5. The mixture was incubated for 1hr. at 37°, after which time there was no further hydroxypyruvate formation (determined by the method of Bücher, Czok, Lamprecht & Latzko, 1963) in a control containing 8·2μmoles of unlabelled DL-serine. After incubation the mixture was placed on an Amberlite IR-120 (H^+ form) column (10cm. × 1cm.) and eluted with water. The first 50ml. of the eluate was collected, dried, redissolved in water (0·5ml.) and used without further purification as hydroxy[3-^{14}C]pyruvate. Chromatography of this product in ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.) (Fischer & Nebel, 1955) showed that 90% of the total radioactivity moved identically with standard hydroxypyruvate, the remaining 10% being serine. Hydroxypyruvate was detected with alkaline $AgNO_3$, and serine with ninhydrin (Consden & Gordon, 1948). Of the total radioactivity added as DL-serine, 42% was recovered as hydroxypyruvate (giving a specific radioactivity of 6·25μc/mole).

Chemical synthesis of N-*glycollyl*-D-*glucosamine.* This compound was prepared by the method of Jourdian & Roseman (1962). The *N*-glycollyl derivative had m.p. 187–188°; Jourdian & Roseman (1962) give m.p. 184–189°: $[\alpha]_D^{20}+53{\cdot}1° \rightarrow +29{\cdot}2°$ const. (24hr., c 1·63 in water) (Found: C, 40·8; H, 6·5; N, 5·9. Calc. for $C_8H_{15}N_1$: C, 40·5; H, 6·4; N, 5·9%).

On chromatography in butan-1-ol–ethanol–water (5:1:4, by vol.) (Hirst & Jones, 1949) *N*-glycollylglucosamine ran as a single spot, staining with acetylacetone–*p*-dimethylaminobenzaldehyde (Partridge, 1948).

Acyl-CoA derivatives. Acetyl-CoA was prepared and determined by the method of Stadtman (1957). Glycollyl-CoA was prepared by the method of Jourdian & Roseman (1962) and assayed by the following methods. Assay of free thiol groups by the nitroprusside reaction (Stadtman, 1957) showed that 49% of the thiol group of CoA had become esterified. The product had a u.v.-absorbing peak at 232mμ, in contrast with free CoA. The hydroxamic acid test with $FeCl_3$ was positive after addition of alkaline hydroxylamine, and gave a total of 1·84μmoles of glycollohydroxamic acid, equivalent to 46% conversion of the total CoA present.

Analysis of acetyl and glycollyl substituents of the glycoprotein fraction. These groups were released as methyl acetate and methyl glycollate by methanolysis of the glycoprotein for 4hr. at 105° in a sealed tube in methanolic 2N-HCl. The methyl esters were distilled with the methanol solvent *in vacuo* (15mm. Hg) and were collected at −70° (Ludowieg & Dorfman, 1960). Distillation was carried out at 90° to ensure quantitative yields of the methyl glycollate and acetate. For quantitative estimation of the total *N*-acetyl groups of mucin, the distilled methyl esters were determined directly by the method of Ludowieg & Dorfman (1960), by using methyl glycollate and ethyl acetate as standards. For measurement of the specific radioactivities of the acyl groups, the distilled methyl esters were separated chromatographically as the hydroxamic acids. Paper chromatography of the hydroxamic acids (approx. 50μg.) proved difficult because of their great sensitivity towards traces of inorganic salts. Satisfactory results were obtained as follows: the distillate and combined washings (total vol. 2·0ml.) were treated with 2ml. of a freshly prepared solution of saturated $Ba(OH)_2$ (3vol.) and 0·3M-hydroxylamine hydrochloride (2vol.) and the mixture was incubated at 25° for 1hr. The excess of Ba^{2+} ions was then precipitated by the addition of solid CO_2 and the solution was centrifuged. The supernatant was evaporated to dryness (40° at 15mm. Hg), redissolved in ethanol (200μl.) and chromatographed. The following solvents were used for chromatography of the hydroxamic acids by upward development on Whatman no. 1 paper that had been washed with 2N-acetic acid, dried, washed with water and dried: butan-1-ol–acetic acid–water (4:1:5, by vol.) (Wainfan & Van Bruggen, 1957); isobutyric acid–water (9:1, v/v) (Fink & Fink, 1949); phenol–water (9:1, w/v) (Fink & Fink, 1949). Hydroxamic acids were detected by the $FeCl_3$ spray (Fink & Fink, 1949). The hydroxamic acid content of eluates from chromatograms was determined by the method of Ueno (1960).

Cell-free preparations. Scrapings of the colonic mucosal tissue were homogenized in buffer by two strokes in a chilled loose-fitting Teflon-in-glass Potter–Elvehjem homogenizer. The following homogenizing media were used: 0·3M-sucrose containing 2mM-EDTA adjusted to pH 7·4 with $KHCO_3$ (Sherratt & Hübscher, 1963); 20mM-tris–HCl buffer,

pH 7·4, containing 0·125M-KCl and 2mM-EDTA (Senior & Isselbacher, 1960); 0·1M-KH_2PO_4 buffer, pH 7·4, containing 2mM-EDTA (Moretti & Wolf, 1961). The ratio of scrapings to homogenizing medium was 3 vol. of scrapings to 2 vol. of medium. The 'dilute particle preparation' was prepared by homogenizing 1 vol. of scrapings with 3 vol. of medium followed by centrifugation at 4000g for 10min. The supernatant was used as the 'dilute particle preparation' (14·7–16·8mg. of protein/ml.). The 'particle-free preparation' (10·7–13·7mg. of protein/ml.) was the supernatant formed after centrifugation of a homogenate of 3 vol. of scrapings to 2 vol. of medium at 105000g for 60min. at 4°.

Isolation of N-acylhexosamines from 'cell-free' incubation mixtures. After incubation the samples were cooled to 4°, 200 µg. each of carrier N-acetylglucosamine and N-glycollylglucosamine were added, and the pH was adjusted to pH 4·8 with 0·5N-HCl. Formic acid–sodium formate buffer, pH 4·8 (1ml.), was added together with wheat-germ acid phosphatase (300 units), and the mixture was digested for 30min. at 37°. The reaction was terminated by the addition of ethanol (3 vol.) and the precipitated tissue with mucin was separated centrifugally. The supernatant was placed on a mixed-bed resin (Amberlite Monobed 3) column (20cm. × 1cm.) and eluted with water. The first 100ml. of aqueous eluate was concentrated to dryness at 30° at 15mm. Hg, redissolved in water and analysed by paper chromatography for the individual amino sugars. N-Acylated amino sugars were separated from each other and from the added radioactive substrates by chromatography in butan-1-ol–pyridine–water (6:4:3, by vol.) on Whatman no. 4 paper impregnated with saturated sodium metaborate solution (Cardini & Leloir, 1957). N-Acylated amino sugars were detected by the acetylacetone–p-dimethylaminobenzaldehyde spray (Partridge, 1948). N-Acylated hexosamines were also separated by electrophoresis in 1% sodium tetraborate, pH 9·5, on strips of Whatman 3MM paper in a water-cooled Kohn Universal electrophoresis tank (Shandon Scientific Co. Ltd., London, N.W. 10) (Jourdian & Roseman, 1962). A voltage of 25v/cm. was applied for 30min. N-Acylated hexosamines in solution were determined quantitatively by the method of Reissig, Strominger & Leloir (1955).

Assay of glucosamine acyltransferase activity. The incubation mixture (total volume 1·0ml.) contained: potassium dihydrogen phosphate buffer, pH 7·4 (50 µmoles), KCl (33·5 µmoles), $MgCl_2$ (20 µmoles), $MnCl_2$ (1·25 µmoles, 2-mercaptoethanol (7·5 µmoles), ATP (5 µmoles), NAD (0·075 µmole), NADP (0·075 µmole), thiamine pyrophosphate (0·2 µmole), CoA (0·2 µmole), D-glucosamine hydrochloride (2·5 µmoles), N-acyl precursor (25 µmoles) and 0·5ml. of a cell-free preparation. Incubation was for 1hr. at 37°, and the reaction was terminated by addition of 0·5ml. of trichloroacetic acid (12%, w/v). The N-acylhexosamine content was determined as N-acetylhexosamine by the method of Reissig *et al.* (1955).

RESULTS

Utilization of D-[2-^{14}C]glucose by mucosal tissue. Glycoprotein preparations isolated by papain digestion of sheep colonic scrapings from incubation with D-[2-^{14}C]glucose had specific radioactivities ranging from 4280 to 16820 counts/min./mg. dry wt. of glycoprotein (mean 10226 counts/min./mg. dry wt. of glycoprotein, 6 experiments). This represented 7·7–36·4% (mean 22·9%, 6 experiments) of the total radioactivity added. Electrophoresis of the glycoprotein preparation in borate buffer at pH 9·3 (Draper & Kent, 1963) showed that only the major glycoprotein component (fraction H) had become ^{14}C-labelled. The distribution of the

Table 1. *Distribution of radioactivity in constituents of the glycoprotein preparation isolated from incubation of sheep colonic tissue with D-[2-^{14}C]glucose*

The incubation mixture contained (total vol. 20·1 ml.): 5ml. of scrapings (approx. 500mg. dry wt. of tissue), 15ml. of Krebs medium III (D-glucose omitted, 3·2mM-L-glutamine added) and 0·1ml. of D-[2-^{14}C]glucose (100 µc/ml.; high specific radioactivity, 28·4–31·3 µc/µmole). Incubation was for 2·4hr. at 37° in O_2. In all cases the controls, which had 3 vol. of ethanol added at zero time, had no significant incorporation of radioactivity above a background of 8–9 counts/min. Similar results were obtained in four other experiments.

Product	10^{-3} × Sp. radioactivity (counts/min./µmole) (±S.D.)	Total radioactivity (counts/mg. of glycoprotein)	Percentage of total radioactivity in glycoprotein
Glycoprotein	—	16800	100
Sialic acids			
N-Acetyl	7·60 ± 0·034	800	4·8
N-Glycollyl	1·05 ± 0·027	143	0·8
Hexosamines			
Galactosamine	8·86 ± 0·180	2530	15·1
Glucosamine	8·10 ± 0·060	7620	45·3
Neutral sugars			
Fucose	7·38 ± 0·250	1670	17·1
Galactose	6·04 ± 0·210	2980	9·5
Total		15743	92·6

Table 2. *Summary of incorporation of* D-[2-^{14}C]*glucose into the sialic acids of the glycoprotein*

Sialic acid	$10^{-3}\times$ Sp. radioactivity (counts/min./μmole)		Percentage of total radioactivity in glycoprotein found in sialic acid residues	
	Range	Mean (9 expts.)	Range	Mean (9 expts.)
N-Acetyl	1·90 –8·26	4·38	2·07 –8·34	4·68
N-Glycollyl	0·206–1·05	0·405	0·328–1·18	0·64
Ratio *N*-acetyl/*N*-glycollyl	7·7 –17·0	10·8	4·4 –12·8	7·27

Table 3. *Incorporation of radioactivity from* D-[2-^{14}C]*glucose into the* O/N-*acyl groups of the glycoprotein isolated from sheep colonic tissue*

Conditions of incubation were as described in Table 1

	Sample 1		Sample 2	
	Sp. radioactivity (counts/min./mg.) (±S.D.)	Percentage of total radioactivity in glycoprotein	Sp. radioactivity (counts/min./mg.) (±S.D.)	Percentage of total radioactivity in glycoprotein
Glycoprotein	8900		8950	
	(counts/min./μmole) (±S.D.)		(counts/min./μmole) (±S.D.)	
N-Acetylneuraminic acid				
Total	3440±20	3·73	3540±20	4·28
O/*N*-Acetyl	106±13	0·115	118± 5	0·144
N-Glycollylneuraminic acid				
Total	208±17	0·328	222± 5	0·349
O/*N*-Glycollyl	36·2±11·4	0·057	33·4± 9·2	0·052
Hexosamines				
N-Acetyl	157± 4	2·27	148± 2	2·14

radioactivity among the monosaccharide constituents of the glycoprotein preparation, after a typical incubation of the tissue with D-[2-^{14}C]-glucose, is given in Table 1. It was found that D-[2-^{14}C]glucose readily led to labelling in all the known sugar residues of the glycoprotein, namely sialic acids, galactose, fucose, glucosamine and galactosamine. The labelling in the amino acid residues was less than 0·001%; small radioactive peaks were observed co-chromatographing with aspartic acid and glycine–glutamic acid (8±4 and 5±3counts/min. respectively, corrected for background). Thus the labelling of the whole glycoprotein obtained under the conditions described could be accounted for almost wholly in the sugar constituents.

Further consideration has been given to the question of the labelling of the sialic acid constituents. The sialic acid content of the glycoprotein preparations ranged from 3·8 to 8·5% (mean 6·3%), measured by the thiobarbituric acid method (Aminoff, 1961), and from 6·4 to 10·1% (mean 8·1%), measured by the resorcinol method (Svennerholm, 1957). These values are corrected for the different extinctions of *N*-acetylneuraminic acid and *N*-glycollylneuraminic acid. Previous work has shown that the sialic acids are located solely in the major H component of the glycoprotein (Kent & Marsden, 1963). Over 90% of the total sialic acid present is released on acidic hydrolysis in 0·1N-sulphuric acid at 80° for 40min. (Allen & Kent, 1968). The molar ratio of *N*-glycollylneuraminic acid to *N*-acetylneuraminic acid, eluted from paper chromatograms, varied from 1·10:1·00 to 2·28:1·00 (mean value 1·65:1·00, 15 samples) in different preparations of the glycoprotein. Closely comparable values were obtained with the thiobarbituric acid and the resorcinol methods. A summary of the incorporation of radioactivity from D-[2-^{14}C]glucose into the sialic acid residues of the glycoprotein preparation is given in Table 2. The sialic acids together accounted for 5·32% of the total radioactivity in the glycoprotein fraction. With D-[U-^{14}C]glucose, similar labelling patterns were obtained, the ratios (in two experiments) of the specific radioactivity of the total labelling in the *N*-glycollylneuraminic acid to that in the *N*-acetylneuraminic acid being 1·0:6·2 and 1·0:10·2. Thus though radioactivity from both forms of D-[^{14}C]-glucose was incorporated into *N*-glycollylneur-

Table 4. *Utilization of sodium* [2-^{14}C]*acetate* (*specific radioactivity* 403μc/*mg.*), *sodium* [3-^{14}C]*pyruvate* (*specific radioactivity* 120μc/*mg.*) *and sodium hydroxy*[3-^{14}C]*pyruvate* (*specific radioactivity* 60μc/*mg.*) *by sheep colonic mucosal tissue*

The incubation medium (total volume 10·1 ml.) contained: mucosal tissue (2·5 ml., approx. 250 mg. dry wt. of tissue), Krebs medium III (D-glucose omitted, 3·2 mM-L-glutamine added) (7·5 ml.) and radioactive substrate (0·1 ml.). Incubation was for 2·5 hr. at 37° in O_2. In all cases the radioactivity in the zero-time controls was less than 5% of these values.

Incubation medium		Glycoprotein isolated		CO_2 evolved	
Radioactive substrate	10^{-5} × Radioactivity added (counts/min.)	Sp. radioactivity (counts/min./mg.) (± S.D.)	Incorporation (% of total radioactivity (added)	Total radioactivity isolated (counts/min.) (± S.D.)	Incorporation (% of total radioactivity added)
Expt. I					
Sodium [2-^{14}C]acetate	8·11	5056 ± 60	14·6	154722 ± 290	18·3
Sodium [3-^{14}C]pyruvate	8·11	381 ± 4·4	0·9	31252 ± 210	3·80
Sodium hydroxy[3-^{14}C]pyruvate	3·24	695 ± 5·9	3·5	2372 ± 66	0·72
Expt. II					
Sodium [2-^{14}C]acetate	8·11	3955 ± 49·6	10·9	234000 ± 246	28·45
Sodium [3-^{14}C]pyruvate	8·11	590 ± 1·5	1·09	30920 ± 81·0	3·76
Sodium hydroxy[3-^{14}C]pyruvate	4·22	107 ± 8·0	0·76	926 ± 13·4	0·35

Table 5. *Incorporation of radioactivity from sodium* [2-^{14}C]*acetate by surviving mucosal tissue into the glycoprotein fraction isolated from sheep colonic tissue*

Conditions were as described in Table 4. Hexosamines were determined by the method of Levvy & McAllan (1959).

	Sample 1	Sample 2	Sample 3
	Sp. radioactivity (counts/min./mg.) (± S.D.)		
Glycoprotein	1930 ± 4	3070 ± 5	1710 ± 6
	Sp. radioactivity (counts/min./μmole) (± S.D.)		
N-Acetylneuraminic acid			
Total	936 ± 2	990 ± 3	–
O/*N*-Acetyl	825 ± 98	763 ± 53	69 ± 4
N-Glycollylneuraminic acid			
Total	115 ± 5	160 ± 9	–
N-Glycollyl	–	165 ± 19	58 ± 3
Hexosamines			
Carbohydrate skeleton	415 ± 25	516 ± 26	–
N-Acetyl	650 ± 12	498 ± 20	108 ± 6
Galactose	94 ± 7	120 ± 8	–
Fucose	27 ± 4	38 ± 4	–

aminic acid and *N*-acetylneuraminic acid, there was considerably less incorporation (only 14·2% of that with *N*-acetylneuraminic acid) into the former despite its being present in the greater amounts, the molar ratio of *N*-acetylneuraminic acid to *N*-glycollylneuraminic acid being 1·00:1·65.

Analysis of the labelling in the *O*/*N*-acyl groups of the sialic acids by methanolysis and separation of the distilled glycollyl and acetyl methyl esters as their hydroxamic acid derivatives is shown in Table 3. With both *N*-acetylneuraminic acid and *N*-glycollylneuraminic acid the main proportion of the radioactivity was in the carbohydrate skeleton of the sialic acid and the difference in the overall labelling between these acids lay mainly in the difference in the labelling of their carbohydrate skeletons. However, significant labelling did occur in both the *N*-acetyl and the *N*-glycollyl substituents, indicating their biosynthesis from D-[2-^{14}C]-glucose under the conditions of the experiment.

Utilization of [2-^{14}C]*acetate,* [3-^{14}C]*pyruvate and hydroxy*[3-^{14}C]*pyruvate by mucosal tissue.* The results given in Table 4 show the incorporation of ^{14}C from [2-^{14}C]acetate, [3-^{14}C]pyruvate and hydroxy[3-^{14}C]pyruvate into the glycoprotein preparation isolated by papain digestion. Incubations

Table 6. *Distribution of radioactivity from sodium* [3-^{14}C]*pyruvate and hydroxy*[3-^{14}C]*pyruvate in the glycoprotein from sheep colonic tissue*

Glycoprotein	Hydroxy[3-^{14}C]pyruvate	[3-^{14}C]Pyruvate
	Sp. radioactivity (counts/min./mg.) ($\pm$ S.D.)	
	695 $\pm$ 5·9	381 $\pm$ 1·5
	Radioactivity on chromatogram (counts/min. above background)	
Sialic acid and fucose hydrolysate (8mg. of mucoprotein with 0·1N-H_2SO_4 at 80° for 40min.)		
N-Acetylneuraminic acid	24 $\pm$ 5	66 $\pm$ 7
N-Glycollylneuraminic acid	Nil	31 $\pm$ 7
Fucose	89 $\pm$ 8	46 $\pm$ 7
Hexosamines and amino acid hydrolysate (2·4mg. of sialic acid-free glycoprotein with 2N-HCl at 105° for 16hr.)		
Galactose	307 $\pm$ 22	72 $\pm$ 8
Hexosamines	276 $\pm$ 19	156 $\pm$ 15

of the different labelled substrates with samples of the same tissue preparation were performed in parallel. The carbon dioxide production was in each case measured simultaneously.

Radioactivity from [2-^{14}C]acetate was incorporated readily into all the monosaccharide residues of the glycoprotein preparation (Table 5). The ratios of the relative specific radioactivity of *N*-acetylneuraminic acid to that of *N*-glycollylneuraminic acid were 8·2:1·0 and 6·2:1·0. Further, analysis of the *O*/*N*-acyl groups of the sialic acids from glycoprotein from the [2-^{14}C]acetate incubation showed that the major portion of the labelling resided in the *N*-acetyl and *N*-glycollyl groups, in contrast with that from D-[2-^{14}C]glucose, where the carbohydrate skeleton of the glucose was far more extensively labelled.

The results of acid hydrolysis and chromatography of radioactive glycoprotein obtained from incubation of the tissue with either [3-^{14}C]pyruvate or hydroxy[3-^{14}C]pyruvate are shown in Table 6. Radioactivity from [3-^{14}C]pyruvate appeared in all the known monosaccharide residues of the glycoprotein fraction, showing a similar labelling pattern to that found with [2-^{14}C]acetate (Table 5). Radioactivity from hydroxy[3-^{14}C]pyruvate was incorporated into the neutral sugars (galactose and fucose) and into the hexosamine constituents of the glycoprotein preparation. Only very low labelling occurred in the *N*-acetylneuraminic acid residues and no peak of radioactivity corresponding to *N*-glycollylneuraminic acid was detectable on chromatography. No labelling was observed in serine or in any other amino acid liberated by hydrolysis of glycoprotein from hydroxy[3-^{14}C]-pyruvate incubations. Draper & Kent (1967) have shown that, though sheep colonic tissue incorporated L-[^{14}C]serine into seryl and glycyl residues of the glycoprotein, no labelling of the carbohydrate constituents was observed. Thus it would seem unlikely that the labelling in the carbohydrate portion of the mucoprotein from the hydroxy-[3-^{14}C]pyruvate incubations was due to utilization of the 10% DL-serine radioactive impurity present.

Electrophoresis of the glycoprotein preparation from incubations of the tissue with [2-^{14}C]acetate, [3-^{14}C]pyruvate or hydroxy[3-^{14}C]pyruvate in borate buffer, pH 9·3, again showed that only the major glycoprotein component, fraction H, was radioactive.

Incorporation of [1-^{14}C]*glycollate into the glycoprotein preparation by surviving tissue.* Two incubations of the tissue with large amounts of [1-^{14}C]-glycollate (200μC) produced, after papain digestion, glycoprotein containing 2042 and 750counts/min./mg. of glycoprotein respectively. The commercial sample of [1-^{14}C]glycollate was found to contain radioactive impurities, 2% of the total radioactivity as acetate and 23% of the total radioactivity as formate (determined by formation of the hydroxamic acid derivatives). The [^{14}C]acetate impurity (2%), equivalent to 4μC/incubation mixture, was sufficient to account for the labelling found in this experiment. Further analysis of the radioactive glycoprotein obtained showed a pattern of radioactive labelling similar to that with [2-^{14}C]acetate. In particular there was low labelling (59·4 and 32·5counts/min./μmole) in the *N*-glycollylneuraminic acid residues, and when compared with labelling (2600 and 372counts/min./μmole) in the *N*-acetylneuraminic acid residues this gave a ratio of the specific radioactivities of 1:44 and 1:12 in favour of *N*-acetylneuraminic acid.

Incorporation of [U-^{14}C]*glycine into the glycoprotein by the surviving tissue.* A summary of the incorporation experiments with [U-^{14}C]glycine is given in Table 7. Chromatography of the hydrolysed glycoprotein showed no incorporation of radio-

Table 7. *Incorporation of* [U-^{14}C]*glycine into the glycoprotein from sheep colonic mucosal tissue*

The incubation medium (total volume 10·1 ml.) contained: mucosal tissue (2·5 ml., approx. 250 mg. dry wt. of tissue), Krebs III medium (D-glucose omitted, 3·2 mM-L-glutamine added) (7·5 ml.) and [U-^{14}C]glycine (specific radioactivity 67 μc/μmole) (5 μc in 0·1 ml.). Incubation was for 2·5 hr. at 37° in O_2. No radioactivity was incorporated above background in the zero-time controls.

	Glycoprotein isolated	
	Sp. radioactivity (counts/min./mg.) (± S.D.)	Incorporation (% of total radioactivity added)
Expt. I	81 ± 5·3	0·137
Expt. II	40 ± 6·7	0·132

activity into the sugar residues of glycoprotein by this preparation, and in particular no incorporation into the *N*-glycollylneuraminic acid residues. Chromatographic analysis of the amino acids revealed two radioactive peaks, one running with glycine, accounting for 53% of the total radioactivity applied, and the other with serine, accounting for 37% of the total radioactivity applied. Electrophoretic studies showed that only the major glycoprotein component, was radioactive.

Assay of glucosamine acyltransferase activity in cell-free preparations of sheep mucosal tissue. The quantitative formation of *N*-acylglucosamine from glucosamine in the presence of various *N*-acyl precursors was investigated. With all three tissue preparations (namely homogenate, dilute particle preparation and particle-free supernatant) only acetate increased the acylation of glucosamine (30 mμmoles of *N*-acetylglucosamine/hr./mg. of protein for the particle-free supernatant) above the endogenous rate by the tissue preparation (12·8 mμmoles of *N*-acetylglucosamine/hr./mg. of protein for the particle-free supernatant). Sodium pyruvate and the possible *N*-glycollyl precursors, sodium glycollate, lithium hydroxypyruvate and sodium fructose 6-phosphate, produced no observable increase above the control in which *N*-acyl precursor was omitted. The enzyme was unstable at 4°, losing all its activity within 5 hr. of preparation, though activity was retained if the preparation was frozen immediately at −70° and kept below 0°.

With the particle-free supernatant preparation, acetyl-CoA (0·16 μmole) could replace the requirement for acetate in the above system, and the overall rate of formation of *N*-acylhexosamine was higher than it was with acetate. During 30 min. 93% of the acetyl groups of acetyl-CoA (0·16 μmole) were transferred to glucosamine. The enzyme preparation used to acylate glucosamine with acetyl-CoA was stable at 4°. Four experiments with glycollyl-CoA (0·13 μmole) with the particle-free enzyme preparation gave a net formation of 5·7, 3·1, 0·5 and 5·7 mμmoles of *N*-acylhexosamine (measured as *N*-acetylglucosamine) after 30 min. incubation at 37°. In the same experiment a control sample (without glycollyl-CoA) and a sample with acetyl-CoA (0·16 μmole) yielded a net formation of 2·6 mμmoles and 131 mμmoles of *N*-acylhexosamine respectively. The formation of *N*-acylhexosamine with glycollyl-CoA was considered not to be significant, especially since the differences in E_{580} were very low at these concentrations.

In some experiments unlabelled *N*-acyl precursor was replaced by one of the following radioactive substrates: 7·9 μM-D-[U-^{14}C]fructose 6-phosphate (3·4 μc; glucosamine hydrochloride was omitted in this experiment), 4·5 μM- and 91·5 μM-sodium [2-^{14}C]acetate (0·625 and 12·5 μc), 19·0 μM-sodium [3-^{14}C]pyruvate (1·0 μc) or 25 μM-DL-[U-^{14}C]serine (10 μc). Incubation was at 37° for 90 min., after which the contents were digested with acid phosphatase and the radioactive *N*-acylhexosamines were isolated with added carrier. In none of these experiments was evidence found for formation of radioactive *N*-glycollylglucosamine on chromatography in butan-1-ol–pyridine–water (6:4:3, by vol.) on borate-impregnated Whatman no. 4 paper of the acid phosphatase-digested mixture. Further, there was no radioactivity above that of the control in the *N*-glycollylglucosamine eluted from the paper. Though there was no incorporation of radioactivity into *N*-glycollylglucosamine by the particle-free preparation, D-[U-^{14}C]fructose 6-phosphate (*N*-acetylglucosamine peaks of 76·0 and 54·5 counts/min. above background) and [2-^{14}C]acetate (*N*-acetylglucosamine peaks of 227 and 1810 counts/min. above background) gave well-defined peaks, absent from the controls, which chromatographed with standard *N*-acetylglucosamine. The identity of the *N*-[^{14}C]acetylglucosamine formed in these experiments was further confirmed by electrophoresis in borate buffer at pH 9·3. [3-^{14}C]Pyruvate and DL-[U-^{14}C]serine did not give rise to any peaks of radioactivity running with the *N*-acylhexosamines after incubation with the cell-free tissue preparations.

DISCUSSION

Previous studies (Draper & Kent, 1963; Allen & Kent, 1968) have shown that surviving scrapings of sheep colonic mucosal tissue are metabolically active in glycoprotein biosynthesis. Scrapings incubated in Krebs medium III (glucose omitted, 3·2 mM-L-glutamine added) in an atmosphere of

oxygen incorporated radioactivity from D-[1-^{14}C]-glucose, [U-^{14}C]threonine and $^{35}SO_4^{2-}$ into the monosaccharide, threonine and ester sulphate residues respectively of a glycoprotein. The glycoprotein fraction obtained by papain digestion has been shown to consist of a major glycoprotein component, fraction H, and two minor components, fraction LG, together with some nucleic acid (Kent *et al.* 1967).

In the present work, extension of the studies of the biosynthesis of the glycoprotein has shown that radioactivity from D-[2-^{14}C]glucose, [2-^{14}C]acetate, [3-^{14}C]pyruvate and hydroxy[3-^{14}C]pyruvate is incorporated solely into all the monosaccharide constituents of glycoprotein fraction H, whereas that from [U-^{14}C]glycine is incorporated into the protein part of the glycoprotein. The metabolism of acetate by the tissue is of special interest in relation to ruminant metabolism (Annison, Leng, Lindsay & White, 1963). The results showed that acetate is utilized as a source of energy by the tissue (28·5 and 18·3% of total radioactivity added was released as $^{14}CO_2$) and for incorporation of acetate carbon into glycoprotein (10·9 and 14·6% of total added radioactivity was incorporated). The demonstration of acetyl-CoA synthetase activity in a cell-free preparation of this tissue (Kent & Allen, 1966, 1968) is consistent with these results.

N-Glycollylneuraminic acid is the major sialic acid of glycoprotein H; it makes up 8·54% of the glycoprotein, whereas *N*-acetylneuraminic acid makes up 5·2%, measured by the resorcinol method. The presence of this sialic acid leads to the question of the biochemical origin of glycollyl groups. Although of common occurrence in plants (e.g. see Zelitch, 1965), glycollate is comparatively rare in animals (Jourdian & Roseman, 1962).

Both *N*-acetyl-D-glucosamine and *N*-glycollyl-D-glucosamine have been shown to be substrates in mammalian tissues for the biosynthetic pathway leading to the corresponding sialic acids (Jourdian & Roseman, 1963). Similar enzymic activities have been demonstrated by Kent & Draper (1968), and confirmed in the present studies, for sialic acid biosynthesis in the particle-free supernatant from sheep colonic tissue. These results suggest that the *N*-glycollylation step may involve glucosamine 6-phosphate in a manner analogous to the formation of *N*-acetyl-D-glucosamine 6-phosphate (Davidson, Blumenthal & Roseman, 1957). Despite *N*-glycollylneuraminic acid being a major monosaccharide component of the glycoprotein, the incorporation of radioactivity into this sialic acid from D-[2-^{14}C]-glucose is markedly lower than that into the other sugar components of the glycoprotein (Table 1), in particular *N*-acetylneuraminic acid. Distribution of the labelling in the *O*- and *N*-acyl substituents of the sialic acid residues after incubation with D-[2-^{14}C]glucose shows that the radioactivity is incorporated into both the *N*-glycollyl and the *N*-acetyl substituents, but that in both sialic acid residues the major portion of radioactive labelling resides in the nonose skeleton of the sialic acid structures. The low level of radioactive labelling from D-[2-^{14}C]glucose in the *N*-glycollylneuraminic acid compared with the *N*-acetylneuraminic acid residues may be a reflection of differences in the biosynthetic pathways of these sialic acids, or could be due to a larger pool concentration of *N*-glycollylneuraminic acid precursor or to a lower rate of *N*-glycollylneuraminic acid biosynthesis.

With [2-^{14}C]acetate, labelling favours the *N*-acetylneuraminic acid, compared with *N*-glycollylneuraminic acid (Table 5), but [2-^{14}C]acetate incorporation into the corresponding nonose structures is less than the incorporation into the respective *N*-acetyl and *N*-glycollyl substituents.

At least three biosynthetic pathways leading to glycollate derivatives have been described in mammalian systems. First, attempts were made to demonstrate that involving the active thiamine pyrophosphate – glycollaldehyde intermediate 2-(1,2-dihydroxyethyl)thiamine pyrophosphate in the transketolase reaction (EC 2.2.1.1) (e.g. Datta & Racker, 1961; Holzer, Kattermann & Busch, 1962). Incubation of substrate amounts of D-fructose 6-phosphate (25 μmoles), a donor in the transketolase reaction, and D-glucosamine (2·5 μmoles) in the presence of added cofactors (including ATP and thiamine pyrophosphate) with cell-free preparations of sheep colonic mucosa produced no notable increase over the endogenous rate of amino sugar acylation. Similarly incubations with substrate amounts of D-glucosamine and tracer amounts of D-[U-^{14}C]fructose 6-phosphate with cell-free preparations of colonic mucosal tissue failed to produce labelling in the *N*-glycollyl-D-glucosamine isolated with added carrier from the incubation medium. In these experiments with D-[U-^{14}C]fructose 6-phosphate and in similar experiments with [2-^{14}C]acetate, radioactivity was incorporated into *N*-acetylglucosamine isolated with unlabelled carrier from the incubation medium. *N*-Acetylation of glucosamine with substrate amounts of acetate has been shown in this system to involve glucosamine 6-phosphate as an intermediate (Kent & Draper, 1968).

Another alternative possible source of glycollate investigated was that arising as the active thiamine pyrophosphate–glycollaldehyde intermediate or as glycollyl-CoA from hydroxypyruvate via the hydroxypyruvate dehydrogenase system (da Fonseca-Wollheim, Heesen & Holzer, 1964). Radioactivity from hydroxy[3-^{14}C]pyruvate was incorporated into the glycoprotein by whole sheep colonic mucosal tissue, total incorporation of added radio-

activity being 3·5% and 0·76% in two experiments. Galactose and the hexosamine residues in the glycoprotein contained the major amount of radioactive labelling, with smaller amounts in fucose and *N*-acetylneuraminic acid. In particular, the absence of labelling in *N*-glycollylneuraminic acid makes it unlikely that under these conditions hydroxypyruvate is a precursor of the *N*-glycollyl substituent. Besides hydroxypyruvate being a substrate for the hydroxypyruvate dehydrogenase and the transketolase reaction (Racker, de la Haba & Leder, 1953), other pathways of hydroxypyruvate metabolism have been described that would account for its conversion into hexose (e.g. Dawkins & Dickens, 1965). The results with hydroxy[3-^{14}C]-pyruvate would be consistent with such a pathway of hydroxypyruvate metabolism via D-glycerate and thence to 2-phosphoglyceric acid and so into the gluconeogenic pathway. It is unlikely that the hydroxypyruvate was extensively transaminated to L-serine (Meister, Fraser & Tice, 1954; Sallach, 1956) in the colonic tissue, since there was no labelling from hydroxy[3-^{14}C]pyruvate in the serine residues of the glycoprotein. Kent & Draper (1968) showed that radioactive serine was incorporated as seryl and glycyl residues into the glycoprotein.

In cell-free preparations, substrate amounts of hydroxypyruvate (25 μmoles) in the presence of various cofactors (e.g. CoA, ATP and thiamine pyrophosphate) failed to increase the rate of *N*-acylation of glucosamine (2·5 μmoles) above the rate of the control without hydroxypyruvate. Further, though the acyl group from acetyl-CoA was transferred almost quantitatively (93%) to glucosamine by a cell-free preparation, the same preparation did not promote acyl transfer from chemically synthesized glycollyl-CoA. This is consistent with differences between the enzymes involved in *N*-glycollylneuraminic acid and *N*-acetylneuraminic acid biosynthesis.

Glycine may be a further alternative source of oxidized C_2 fragments, e.g. glyoxylate and glycollate (e.g. Meister, 1965). [U-^{14}C]Glycine was poorly incorporated into the glycoprotein by colonic tissue; no radioactive labelling occurred in the sugar residues, and of the amino acids only serine and glycine were labelled. The conversion of glycine into serine in other tissues is well documented (Meister, 1965).

No evidence for the direct use of free glycollic acid for *N*-glycollylation by the tissue could be found. [1-^{14}C]Glycollate was not selectively incorporated into the *N*-glycollyl groups of the glycoprotein by whole tissue. Further incubation of substrate amounts of glycollate (25 μmoles) with glucosamine (2·5 μmoles) and cell-free preparations failed to produce any net *N*-acylhexosamine synthesis above that of the control. These results with glycollate further emphasize the apparent absence of an activating enzyme system for *N*-glycollylation of glucosamine in this tissue.

In summary, surviving scrapings of sheep colonic tissue can utilize glucose and acetate for the biosynthesis of the *N*-acetyl and *N*-glycollyl moieties of sialic acids. However, though the enzymic system involved in *N*-acetylation of amino sugars in this tissue is readily demonstrable, no similar system for *N*-glycollylation could be found. Further experiments, possibly involving amino sugar acceptors other than glucosamine 6-phosphate and the known mammalian sources of glycollate discussed above, will be necessary to demonstrate the pathway of *N*-glycollylation in mammalian tissues.

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