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Note

Use of radioactively labeled *N*-acetylmannosamine to monitor monooxygenase activity*

ANNE P. SHERBLOM
Department of Biochemistry, University of Maine, Orono, Maine 04469 (U.S.A.)
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Although *N*-glycolylneuraminic acid (NeuGl) is a common constituent of almost all animal cells, human tissue does not normally contain NeuGl, and glycolipids containing NeuGl are antigenic in humans[1]. Recent interest in this sialic acid derivative has been generated by findings that patients with lymphoma[2], leukemia[2], and colon cancer[3] have serum glycolipids which contain NeuGl as well as antibodies to these glycolipids. The formation of *N*-glycolyl groups is thought to occur by hydroxylation of *N*-acetyl groups, a reaction that is catalyzed by *N*-acetylneuraminate (NeuAc) monooxygenase[4] (EC 1.14.99.18). The porcine submaxillary-gland monooxygenase is active[4] both with free and bound *N*-acetylneuraminic acid but not with CMP-NeuAc.

Study of the monooxygenase has been hampered by lack of a simple reliable assay. The published assay for NeuAc monooxygenase[4] is technically impractical, since it requires the determination of a small proportion of radioactive NeuGl in the presence of a large excess of the reactant NeuAc. Previous studies have demonstrated that the determination of glycolyl and acetyl groups is facilitated by methanolysis and hydroxamate formation[5,6]. Thus, we have investigated the use of the substrate 2-[1-^{14}C]acetamido-2-deoxy-D-mannose to monitor *N*-glycolyl formation by monitoring the incorporation of ^{14}C into *N*-glycolyl groups. Since ManNAc has not been used previously in studies of sialic acid synthesis, the products formed by incubation of this compound with whole cells and cell homogenates were characterized. This report describes (a) a comparison of the incorporation of ManN[1-^{14}C]Ac and [6-^{3}H]ManNAc by cells, (b) the analysis of the ^{14}C products after incubation of crude homogenates with ManN[1-^{14}C]Ac, and (c) an estimate of *N*-glycolyl formation in both the entire sample and in various fractions by a crude homogenate of rat mammary-tumor ascites cells.

In order to ensure that the acetyl group of ManN[1-^{14}C]Ac was not hydrolyzed or transferred during sialic acid biosynthesis and incorporation, a dual-

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TABLE I

INCORPORATION OF 2-[1-^{14}C]ACETAMIDO-2-DEOXY-D-MANNOSE AND 2-ACETAMIDO-2-DEOXY-D-[6-^{3}H]-MANNOSE BY MAT-C1 CELLS[a]

Time (min)	*$^{3}H/^{14}C$ Ratio*		
	Medium	*Incorporated NeuAc*	*Incorporated NeuGl*
30	8.1	6.3	5.5
60	8.0	7.3	6.6
120	8.0	6.5	5.9
180	8.3	6.9	6.7

[a]Cells were incubated for various times with a mixture of the two isotopes, and the $^{3}H/^{14}C$ ratio of the medium and of NeuAc and NeuGl in the ethanol-precipitable fraction was determined.

label study was performed. Intact cells were incubated with ManN[1-^{14}C]Ac and [6-^{3}H]ManNAc, and the ^{3}H-to-^{14}C ratio was monitored for incorporated NeuAc and NeuGl as a function of time. The ^{3}H-to-^{14}C ratio determined for incorporated NeuAc was not significantly different from that for incorporated NeuGl, and the values did not appear to change with time (Table I). The variability observed, particularly at short times, is due to the low value of c.p.m. recovered after thin-layer chromatography. These results support previous work[7] which indicated that NeuGl is formed by hydroxylation of the *N*-acetyl group rather than through transfer of a glycolyl group. The difference in ^{3}H-to-^{14}C ratio between the medium and incorporated values is not large and may be due to differences which arise in comparing soluble samples *vs.* those extracted from thin-layer fractions. The ratio clearly did not increase on incorporation, thus indicating that significant hydrolysis or transfer of the acetyl group of ManNAc did not occur.

Ascites-cell crude homogenate was incubated with ManN[1-^{14}C]Ac under conditions which have been shown previously to be favorable for sialic acid synthesis and monooxygenase activity[4,6]. The products obtained after a 10-minute incubation were analyzed by liquid chromatography. Two radioactive peaks were obtained corresponding to standards of ManNAc and NeuAc, whereas significant proportions of radioactively labeled CMP-NeuAc were not produced (Fig. 1). The amount of radioactivity recovered in the NeuAc peak increased as the amount of cell homogenate increased. Up to 10% of the total radioactivity recovered from the column was associated with the NeuAc peak.

Samples of crude homogenate were incubated with ManN[1-^{14}C]Ac as described above and the formation of the [^{14}C]glycolyl group was monitored by thin-layer chromatography after methanolysis and hydroxamate formation. The thin-layer separation of glycolyl and acetyl hydroxamate is shown in Fig. 2, and the radioactivity recovered in the glycolyl peak was taken as an estimate of *N*-glycolyl formation. The incorporation achieved within a 10-min incubation time was not exceeded by longer times of 1- and 2-h incubation. *N*-Glycolyl formation was

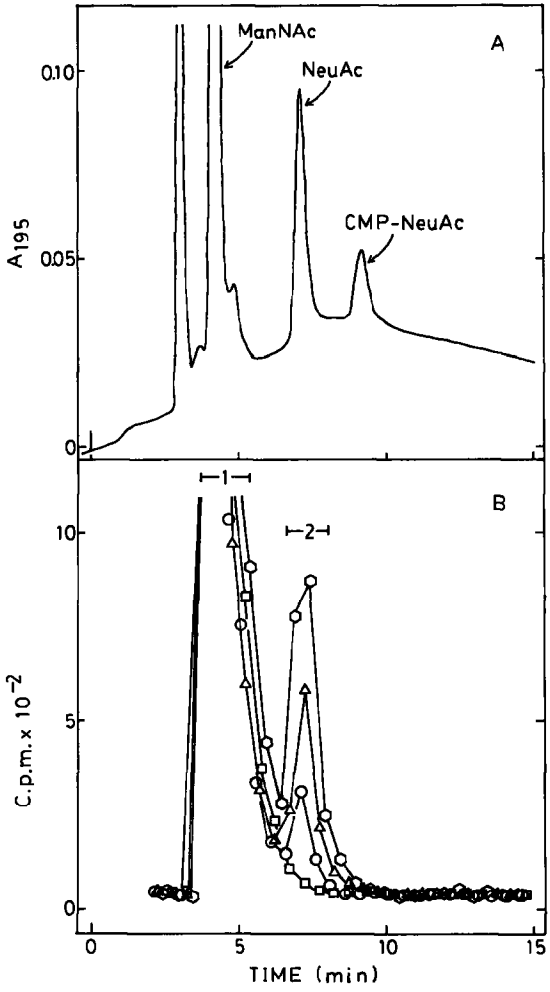

Fig. 1. Separation of products by l.c. under elevated pressure after incubation of ManN[1-^{14}C]Ac with: (□) Heat-inactivated control, or (○) 10 μL, (△) 50 μL, or (◇) 100 μL of MAT-C1 crude cell homogenate. (A) Pattern produced by a standard mixture of ManNAc, NeuAc, and CMP-NeuAc.

dependent upon the amount of cell homogenate added, although this dependence was not linear above ~0.2 mg of total protein. Under the conditions of 10-min incubation and <0.2 mg of protein, less than 5% of reactant was converted into a glycolyl product. Based on three separate experiments, the rate of formation of *N*-glycolyl group by MAT-C1 crude homogenate was 940 ±160 pmol·h^{-1}mg^{-1} protein. This value is within an order of magnitude of the specific activity reported[4] for *N*-acetylneuraminate monooxygenase in a crude homogenate of pig submaxillary gland (210 pmol·h^{-1}mg^{-1})[4].

The products formed by incubation of MAT-C1 crude homogenate with ManN[1-^{14}C]Ac were also tested for the formation of glycolyl content in peaks 1

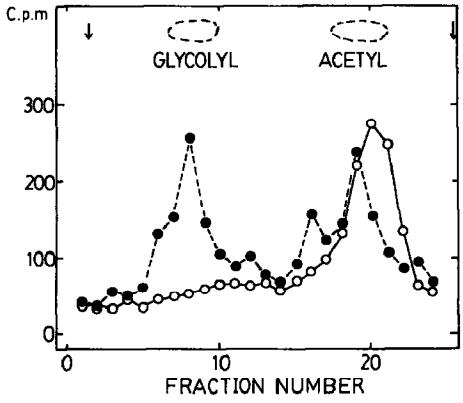

Fig. 2. Thin-layer chromatography of products formed by incubation of MAT-C1 crude homogenate (●) or heat-inactivated crude homogenate (○), for 10 min, with ManN[1-^{14}C]Ac, followed by methanolysis and hydroxamate formation.

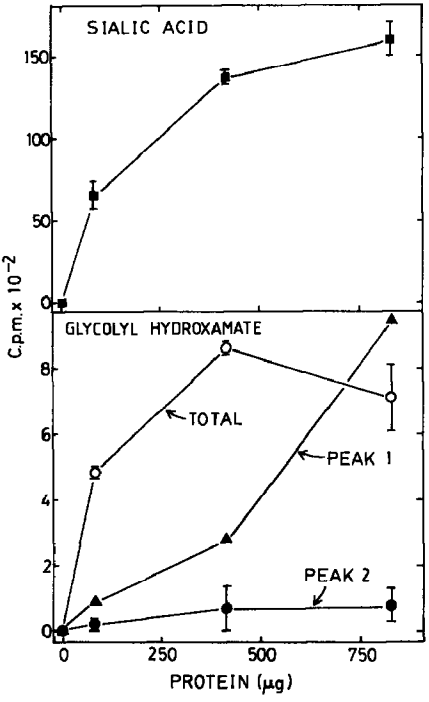

Fig. 3. Incorporation of radioactivity from ManN[1-^{14}C]Ac into sialic acid (upper panel) and glycolyl-containing compounds (lower panel) by a crude homogenate of MAT-C1 cells. The amount of radioactive glycolate was determined for the entire sample (○) as well as for peak 1 (▲) and peak 2 (●), by l.c. under elevated pressure (Fig. 1).

and 2 by l.c. (Fig. 1). The recovery of radioactivity in sialic acid, total glycolyl content, and glycolyl content of peaks 1 and 2, after incubation of samples containing ~300 000 c.p.m., is shown in Fig. 3. These data suggest that of the total glycolyl content formed, <10% was associated with a compound that migrated similar to NeuAc. Significantly higher levels of glycolyl were detected in the mannosamine peak, as compared with the sialic acid peak. As an alternative to the l.c. separation, samples were also hydrolyzed under mild conditions (0.1M hydrochloric acid) and separated into neutral and acidic fractions by ion-exchange chromatography. In a typical experiment involving a 10-min incubation with 0.3 mg of crude homogenate protein, 17 000 c.p.m. were recovered in the acidic fraction by ion-exchange chromatography. Total and acidic glycolyl content formed were estimated from three separate experiments yielding an average of 880 ±150 c.p.m. recovered in the total glycolyl content and 150 ±50 c.p.m. recovered in the acidic glycolyl content. These results are very similar to those obtained by l.c. analysis (Fig. 3), indicating that no more than 10–20% of the total glycolyl content was associated with an acidic component. Thus, despite the observation that net sialic acid synthesis proceeds at 15–20 times the rate of *N*-glycolyl formation, the glycolyl product is preponderantly neutral.

2-[1-^{14}C]Acetamido-2-deoxy-D-mannose was examined as a potential substrate for monitoring monooxygenase activity. The procedures described herein for monitoring *N*-glycolyl formation have two advantages over the previous estimation of *N*-glycolyl formation by separation of NeuAc and NeuGl: (a) The radioactively labeled acetate from the substrate is selectively removed during evaporation by incubation with methanolic HCl; the boiling point of methyl acetate is 57° whereas that of methyl glycolate is 151°. Thus, the radioactivity associated with acetyl hydroxamate (Fig. 2) is typically low, even though ~300 000 c.p.m. were added to the assay mixture. (b) The acetyl and glycolyl hydroxamates are separated better by t.l.c. than are *N*-acetyl- and *N*-glycolylneuraminic acid. As shown above, the glycolyl produced in acidic fractions may be taken as a measure of the formation of *N*-glycolylneuraminic acid. However, 80–90% of the total glycolyl groups formed by a crude homogenate of rat mammary tumor ascites cells is associated with a neutral component. Thus, 2-[1-^{14}C]acetamido-2-deoxy-D-mannose may be useful as a substrate to monitor monooxygenase, provided the products are separated by either l.c. or ion-exchange chromatography prior to determination of [^{14}C]glycolyl groups.

EXPERIMENTAL

Cells and crude homogenate. — Fresh rat-mammary tumor ascites cells (13762 MAT-C1) were used for all the studies. The properties of these cells have been described previously[8]. The cells contain a major mucin-type glycoprotein which accounts for >80% of the bound sialic acid[9]. The structures of the *O*-linked oligosaccharides of the major glycoprotein have been determined[10], as well as the

distribution of NeuGl, which accounts for 56% of the sialic acid[11]. A crude homogenate was prepared by suspending cells (which had been washed in phosphate-buffered saline solution, pH 7.4, and swollen in 10mM Tris, pH 8.0) in 0.2M Tris, pH 7.4 (1 vol./vol. of packed cells) and homogenizing with the tight pestle of a dounce homogenizer. The supernatant obtained by centrifugation at 1000 *g* (1 min) was recentrifuged at 10 000 *g* (10 min) to produce a supernatant referred to as "crude homogenate".

Monooxygenase assay. — 2-[1-^{14}C]Acetamido-2-deoxy-D-mannose at a specific activity of 0.74 GBq/mmol was obtained from ICN Biochemicals Inc., and 2-acetamido-2-deoxy-D-[6-^{3}H]mannose, 370 GBq/mmol, from New England Nuclear. The monooxygenase assay contained the following components in a total volume of 200 μL: 0.18M Tris (pH 7.4), NADPH (0.8 μmol), $FeSO_4$ (0.2 μmol), ascorbic acid (1 μmol), phosphoenolpyruvate (0.2 μmol), ATP (2.4 μmol), glutathione (0.4 μmol), 2-[1-^{14}C]acetamido-2-deoxy-D-mannose (18.5 KBq, 30 nmol), and enzyme source (≤0.3 mg protein). Controls contained heat-inactivated enzyme source. Oxygen was blown into the tube, and the sample was capped tightly, incubated at 37° for various times (standard assay was 10 min), and heat-inactivated by immersion in a boiling-water bath for 2 min. The sample was transferred to a glass ampule, lyophilized, and methanolyzed in the sealed ampule with 1.5M methanolic HCl (0.7 mL) at 80° for 16 h. After cooling and evaporation under N_2, a KOH–hydroxylamine solution in methanol (100 μL) was added[5], the sample centrifuged to remove any precipitate, and 20 μL were applied to a cellulose thin-layer plate (160 μ). The plate was developed in 1:2:1 butanol–propanol–0.1M HCl. Standards were prepared by treatment of acetic and glycolic acid with methanolic HCl and KOH–hydroxylamine, and the spots were detected with $FeCl_3$ in 1-butanol, while the radioactive lanes were covered with aluminum foil. The radioactive lanes were divided into 0.5-cm fractions, and the fractions were mixed with water (200 μL) and Instagel (5 mL) prior to scintillation counting.

Characterization of the product after the monooxygenase assay. — The samples were characterized both by l.c. under elevated pressure and by ion-exchange chromatography after mild acid hydrolysis. For l.c., the heat-inactivated sample was centrifuged and the supernatant (60 μL) was applied to a Whatman Partisil-10 PAC column as described by Brown *et al.*[12], except that 25mM KH_2PO_4 (pH 5.2) was substituted for 25mM sodium acetate–mM NaH_2PO_4 (pH 4.5) to permit monitoring at a wavelength of 195 nm. The radioactively labeled fractions corresponding to ManNAc and NeuAc (Fig. 1) were pooled separately and lyophilized. For ion-exchange chromatography, the samples were treated with 0.1M HCl at 80° for 1 h, made neutral, and chromatographed on columns[11] (1 mL) of Dowex 50 (H^+) and AG 1-X8 (AcO^-). The acidic components were eluted from AG 1-X8 with 0.5M pyridinium acetate (pH 5.0; 3 mL). After lyophilization, the content of glycolyl was determined as described above.

Dual-label study. — Freshly washed MAT-C1 cells were incubated with 2-[1-^{14}C]acetamido-2-deoxy-D-mannose (15 KBq/mL) and 2-acetamido-2-deoxy-D-

[6-^{3}H]mannose exactly as described previously[11]. At various times, the cells were washed and extracted with 70% ethanol. The ethanol-precipitable material was hydrolyzed under mild conditions and, after ion-exchange chromatography, the acidic component was separated by t.l.c. to determine[11] the $^3H/^{14}C$ ratio of incorporated NeuAc and NeuGl.

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