Metabolism of *N*-Acetylneuraminic Acid in Mammals: Isolation and Characterization of CMP-*N*-acetylneuraminic Acid†

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ABSTRACT: Radiolabeled sialoglycoproteins were detected upon analysis of liver and kidneys of mice that had been injected with *N*-acetyl[4,5,6,7,8,9-^{3}H]neuraminic acid. Similar results were also found upon incubation of this radiolabel with liver slices. CMP-*N*-acetylneuraminic acid (CMP-NeuNAc), a postulated intermediate in the conversion of free NeuNAc to NeuNAc bound to glycoproteins and glycolipids, was detected and characterized for the first time from a mammalian tissue; neither the nucleotide moiety nor the biological activity of this compound, when derived from a mammalian tissue, has been previously reported. Identification of CMP-NeuNAc, labeled in both the nucleotide and sugar moieties, was based on (1) comigration with authentic standard in several chromatographic systems, (2) detection of labeled CMP and NeuNAc after mild acid hydrolysis, (3) determination of its susceptibility to $NaBH_4$ reduction and NeuNAc aldolase cleavage before and after mild acid hydrolysis, and (4) demonstration of its ability to transfer NeuNAc in an assay in vitro. The concentration of CMP-NeuNAc in mouse liver is 37 ± 10 nmol/g of wet tissue. After labeling with a mixture of *N*-acetyl[1-^{14}C]- and -[4,5,6,7,8,9-^{3}H]neuraminic acid, we found that the differences in isotope ratio between free and covalent-bound NeuNAc (in CMP-NeuNAc and proteins) were less than 12%. This suggests that NeuNAc had remained intact during the above experiments and that aldolase-catalyzed cleavage to pyruvate and *N*-acetyl[^{3}H]mannosamine and subsequent condensation of this latter compound with cellular phosphoenolpyruvate had not occurred.

N-Acetylneuraminic acid is a terminal sugar in many glycoproteins and glycolipids. Recent studies in our laboratory showed that *N*-acetyl[4,5,6,7,8,9-^{3}H]neuraminic acid is taken up by mammalian and avian cells grown in tissue culture, and subsequently incorporated into glycoproteins and glycolipids (Hirschberg et al., 1976; Hirschberg & Goodman, 1976; Hirschberg & Yeh, 1977). These observations stimulated our interest in determining whether CMP-NeuNAc, a postulated intermediate in this incorporation, could be detected. Synthesis of this sugar nucleotide has been well documented in bacteria in vivo (Comb et al., 1966); in addition, many mammalian tissues possess enzymatic activities that catalyze its synthesis from CTP and NeuNAc in vitro (Kean & Roseman, 1966; Kean, 1970) and numerous studies have shown that sugar nucleotide synthesized in vitro can transfer its sugar moiety to appropriate acceptors (Roseman, 1970); however, characterization of this compound derived from mammalian tissues has been incomplete: specifically neither its nucleotide moiety nor its activity as NeuNAc donor in vitro has been determined. The latter two points are particularly relevant in view of the nuclear localization of most CMP-NeuNAc synthetase activity (Kean, 1970; Van Dijk et al., 1973; Coates et al., 1978), while other sugar nucleotide synthetases are cytoplasmic (Coates et al., 1978).

We were also interested in determining whether NeuNAc had remained intact during our previous experiments with *N*-acetyl[4,5,6,7,8,9-^{3}H]neuraminic acid or whether aldolase-catalyzed cleavage to pyruvate and *N*-acetyl[^{3}H]-mannosamine and subsequent condensation of this latter compound with cellular phosphoenolpyruvate had occurred. Previously Brunetti et al. (1962) had reported the occurrence of NeuNAc aldolase activity in several mammalian tissues. Our earlier studies (Hirschberg et al., 1976; Hirschberg & Goodman, 1976; Hirschberg & Yeh, 1977), though different from those of Brunetti et al. (1962), showed no evidence for such a reaction; however, the position of the radiolabel in the NeuNAc acid molecule used in these studies did not allow us to rule out such a mechanism. A preliminary report of this work has been published (Carey & Hirschberg, 1978).

Materials and Methods.

Animals. Adult male mice, ICR strain (average weight 25 g), were purchased from National Laboratories, O'Fallon, MO, and from Hilltop Laboratories, Pittsburgh, PA.

Preparation of N-Acetyl[4,5,6,7,8,9-^{3}H]neuraminic Acid. *N*-Acetyl[4,5,6,7,8,9-^{3}H]neuraminic acid was synthesized enzymatically from *N*-acetyl[G-^{3}H]-D-mannosamine (New England Nuclear; 2.3 Ci/mmol) and phosphoenolpyruvate as previously described by Warren & Glick (1966). The specific activity was 2.3 Ci/mmol. Radiochemical purity was at least 99.5% based on the criteria previously described (Hirschberg et al., 1976).

Preparation of N-Acetyl[1-^{14}C]neuraminic Acid. *N*-Acetyl[1-^{14}C]neuraminic acid was synthesized enzymatically from [1-^{14}C]phosphoenolpyruvate cyclohexylammonium salt (Amersham/Searle; 12.4 mCi/mmol) and *N*-acetyl-D-mannosamine (Sigma Chemical Co.) by a modification of the procedure used for the synthesis of [^{3}H]NeuNAc (Warren & Glick, 1966). To the 100000*g* rat liver supernatant solution, solid ammonium sulfate was added to a final concentration of 30%. The precipitate was separated by centrifugation at 30000*g* for 10 min, redissolved in 2 mL of water, and desalted by passing through a Sephadex G-25 column (10 × 1 cm). The material eluted in the void volume was used for the enzymatic synthesis. The incubation mixture contained in a final volume of 10.0 mL: Tris–acetate pH 7.6 (1.0 mmol); *N*-acetylmannosamine (25.0 μmol); [1-^{14}C]phosphoenolpyruvate (50 μCi; 4.0 μmol); ATP (25.0 μmol); NAD (2.5 μmol); NADP (2.5 μmol); magnesium acetate (300.0 μmol), and enzyme extract (2–3 mg of protein). The mixture was incubated for 1 h at 37 °C and subsequently applied to a Dowex 1–formate column (15 × 1 cm) and eluted with a linear gradient of formic acid (0–1.0 M). The radioactive material that eluted in the region of NeuNAc was further purified by preparative paper chromatography in systems A and B. The

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final *N*-acetyl[1-^{14}C]neuraminic acid had a radiochemical purity greater than 99.5% based on the criteria previously described (Hirschberg et al., 1976). The overall yield was approximately 2%.

Chromatography. The following solvent systems were used for ascending chromatography on Whatman 3 MM paper or cellulose thin-layer sheets (Brinkman): (solvent system A) ethanol–1 M ammonium acetate, pH 7.5 (6:4); (solvent system B) pyridine–ethyl acetate–acetic acid–water (5:5:1:3); (solvent system C) 1-butanol–pyridine–water (1:1:1); (solvent system D) ethanol–1 M Tris-HCl, pH 7.5 (7:3); (solvent system E) 5 M ammonium acetate (pH 9.5)–saturated $Na_2B_4O_7$–water–ethanol–0.5 M EDTA (40:160:100:300:1); (solvent F) isobutyric acid–water–ammonium hydroxide (8:7:1); (solvent system G) 1-propanol–ammonium hydroxide (3:2); and (system H) 2-propanol–water–HCl (76:18:17). Descending chromatography on Whatman DE-81 paper was performed using solvent system I: 0.2 M ammonium acetate (pH 7.5)–ethanol (7:3). Radioactivity was monitored either by using a Packard strip counter or by cutting the paper into 1-cm strips, eluting with 1 mL of H_2O, adding 9 mL of Aquasol-2 (New England Nuclear), and counting in a Packard liquid scintillation spectrometer.

Injection of Labeled Compounds into Mice. For short term studies with [4,5,6,7,8,9-^{3}H]NeuNAc, mice were injected in the tail vein with 13.6 μCi in NaCl (0.2 mL; 0.9%). After 5, 10, 25, and 60 min the animals were sacrificed by decapitation, the liver and kidneys were quickly removed and washed with cold NaCl (0.9%), and the tissues frozen in liquid nitrogen. For long-term studies with [^{3}H]NeuNAc, 209 μCi were injected. For the double-label experiment, a mixture of [1-^{14}C]- and [4,5,6,7,8,9-^{3}H]NeuNAc (0.9 μCi of ^{14}C; 10.5 μCi of ^{3}H; specific activities 12.4 mCi/mmol for ^{14}C and 143 mCi/mmol for ^{3}H) was injected in NaCl (0.2 mL; 0.9%) as described above. [5-^{3}H]Cytidine (200 μCi; 27.6 Ci/mmol, New England Nuclear) was injected as described for NeuNAc.

Incubations with Liver Slices. Mouse liver slices (approximately 1.5 × 4 mm) were incubated in either Krebs–bicarbonate buffer supplemented with minimal essential medium amino acids (Gibco) or Eagle's minimal essential medium (KC Biological) at 37 °C in an atmosphere of 5% CO_2/95% O_2 in a shaking water bath. Under these conditions incorporation of [^{3}H]NeuNAc or [^{3}H]leucine into phosphotungstic acid-insoluble material is linear for at least 1 h. In a typical experiment approximately 20 slices (about 20 mg of protein) were incubated in 1 mL of medium containing either [^{3}H]NeuNAc (7.4 μCi) or [5-^{3}H]cytidine (100–200 μCi). At the end of the incubation (30 to 60 min), the slices were washed extensively with cold Krebs–bicarbonate buffer and processed as described below.

Preparation of Phosphotungstic Acid Soluble and Insoluble Fractions. Frozen tissue was thawed and homogenized in cold water (10 mL/g of tissue) with a motor-driven Potter-Elvehjem tissue homogenizer. Liver slices were homogenized by hand in a ground-glass tissue homogenizer. Proteins and lipids were precipitated by adding an equal volume of phosphotungstic acid (2% in 1 N HCl) and chilling the mixture on ice for 20 min prior to centrifugation. The phosphotungstic acid insoluble pellet was washed two times with phosphotungstic acid (3 mL; 1% in 0.5 N HCl) and four times with water (3 mL each). The washed pellet was solubilized in either Protosol (New England Nuclear) for liquid scintillation counting in toluene–Omnifluor (New England Nuclear), 1 N NaOH (for protein determination), or 1% sodium dodecyl sulfate ($NaDodSO_4$) (for gel filtration and protease digestion). NeuNAc was released from the pellet by acid hydrolysis as previously described (Hirschberg & Yeh, 1977).

Gel Filtration and Protease Digestion. A phosphotungstic acid insoluble pellet containing approximately 3 mg of protein was solubilized in 1 mL of 1% $NaDodSO_4$ in 0.1 M Tris-HCl, pH 8.4, by brief heating. One-half of the sample was applied to a Bio-Gel P-10 column (10 × 1 cm) packed in the above $NaDodSO_4$-containing buffer. This same buffer was used to elute the column (Hirschberg & Yeh, 1977). The other half of the sample was digested with protease (Sigma Chemical Co. type VI, from *S. griseus*) for 48 h at 50 °C by adding three portions of enzyme (0.5 mg each) at 0, 8, and 24 h of incubation. The sample was subsequently applied to the above column (Hirschberg & Yeh, 1977).

Scintillation Spectrometry. Radiolabeled samples were counted in a Packard liquid scintillation spectrometer. For determinations of "spill over" and absolute efficiencies, standards of [^{14}C]- and [^{3}H]toluene (New England Nuclear) were added to the scintillation vials that were being measured.

Protein Determination. Protein was determined by the method of Lowry et al. (1951).

Isolation and Characterization of Labeled CMP-NeuNAc from Liver. To fresh homogenates obtained as described above was added 2 volumes (v/v) of cold ethanol. The sample was chilled on ice for 20 min and then centrifuged at 30000*g* for 20 min. The supernatant solution was removed and the pellet was washed with another 2 volumes of 60% ethanol. The supernatant solutions were combined and dried under reduced pressure. For whole animal experiments, the dried ethanol extract was dissolved in water (1000 mL) and applied to a DEAE-cellulose column (15 × 3 cm) equilibrated in water. The column was first washed with 400 mL of water and then with 400 mL of 0.3 M ammonium acetate, pH 7.3. This latter fraction was lyophilized, dissolved in 50 mL of water, and applied to a DEAE-cellulose column (15 × 1 cm) equilibrated in water. The column was eluted with a linear gradient of ammonium acetate, pH 7.3 (0–0.5 M). Fractions of 3–4 mL were collected. Aliquots were counted in Aquasol-2 (New England Nuclear). For isolation of CMP-NeuNAc from liver slices, the ethanol extract (after drying and redissolving in water) was added directly to the 15 × 1 cm DEAE-cellulose column and eluted with a linear gradient of ammonium acetate.

In experiments in which [^{3}H]cytidine was used, the fractions containing CMP-NeuNAc from the DEAE-cellulose column were pooled, lyophilized, and subjected to preparative paper chromatography in solvent system F. Upon removal from the chromatography tank, the paper was immediately sprayed (before drying) with 1 M $NaHCO_3$ to avoid breakdown of CMP-NeuNAc due to residual acid. The CMP-NeuNAc-containing region was cut out, eluted with water, lyophilized, and subjected to preparative paper chromatography in solvent system A.

Phosphatase Digestion of [^{3}H]CMP-NeuNAc Obtained from Liver Slices. [^{3}H]CMP-NeuNAc was dissolved in 100 μL of 0.1 N HCl and incubated at 37 °C for 20 min; after this time 10 μL of 1 N NaOH and 10 μL of 0.5 M Tris base were added (final pH 10.7); 2.5 units of alkaline phosphatase (Sigma type III) were added and the mixture was incubated at 37 °C for 20 min; the mixture was dried, redissolved in 20 μL of H_2O, and applied as a small streak to a cellulose thin-layer plate and chromatographed in solvent system H; the plate was scraped and counted in Aquasol-2.

Assay for Biological Activity of CMP-[^{3}H]NeuNAc Synthesized in Vivo. A Golgi-rich fraction obtained from rat

Table I: *N*-Acetylneuraminic Acid Incorporation Following Incubation with Liver Slices and Intravenous Injection into Mice[a]

liver slices		intravenous injections				
		liver			kidney	
time (min)	sp act. (insol cpm/mg of protein)	time (min)	phosphotungstic acid insoluble, radioact. (cpm)	sp act. (insol cpm/mg of protein)	phosphotungstic acid insoluble, radioact. (cpm)	sp act. (insol cpm/mg of protein)
0	370	5	13 680	78	3420	91
30	5620	10	11 620	58	5690	78
60	11000	25	11 780	53	4960	165
		60	13 400	81	9470	222

[a] Mouse liver slices were incubated with [^{3}H]NeuNAc (7.4 μCi). At the end of the incubation, the slices were washed with cold buffer and phosphotungstic acid insoluble fractions were obtained as described in Materials and Methods. In whole animal experiments, four mice were injected each with 13.6 μCi of [^{3}H]NeuNAc. Phosphotungstic acid insoluble fractions from liver and kidneys were obtained as described in Materials and Methods.

liver as described by Schacter et al. (1970) or by Fleischer (1974) was used as a source of CMP-NeuNAc:glycoprotein sialyltransferase activity. The assay mixture, described by Briles et al. (1977), contained in a final volume of 0.15 mL: Triton X-100 (0.3 mg), asialofetuin (0.5 mg, a gift of Dr. Michael Green), $NaPO_4$ (5 μmol), NaCl (15 μmol), Golgi-rich protein (0.2 mg), and CMP-[^{3}H]NeuNAc obtained from liver after a 10-min injection with 100 μCi of [^{3}H]NeuNAc or from liver slices incubated for 1 h with 75 μCi of [^{3}H]NeuNAc. The final pH was 7.4. Duplicate samples were incubated for 60 min at 37 °C. The reaction was stopped by addition of 1 mL of phosphotungstic acid (1% in 0.5 N HCl). The pellets were washed four times with water (2 mL each), solubilized in Protosol, and counted in toluene–Omnifluor. Rat liver Golgi fraction isolated by the method of Fleischer (1974) was used in the lactose:CMP-NeuNAc sialyltransferase assay described by Paulson et al. (1977). The assay contained in a final volume of 0.2 mL: lactose (40 μmol), $NaPO_4$ (20 μmol), Triton X-100 (0.4 mg), Golgi protein (20 μg), and CMP-[^{3}H]NeuNAc (from liver slices). The final pH was 6.8. The mixture was incubated at 37 °C for 30 min. One milliliter of cold 5 mM $NaPO_4$, pH 6.8, was added and the mixture was applied to a 4-cm column of Dowex 1–PO_4 (packed in a Pasteur pipet). The column was washed with 1 mL of cold 5 mM $NaPO_4$, pH 6.8. The effluent was collected and dried, and an aliquot was counted by liquid scintillation spectrometry. The remainder was applied to cellulose thin-layer sheets for chromatography in solvent systems A and G. The plates were scraped and counted in Aquasol-2. Standard sialyllactose (Sigma) and lactose (Eastman) were visualized by spraying with silver nitrate (Trevelan et al., 1950).

Results

Our previous studies on the metabolism of NeuNAc had been done with cells grown in tissue culture. We reasoned that such a system would not be practical for the isolation and characterization of CMP-NeuNAc if the sugar nucleotide concentration turned out to be low. We therefore chose mouse liver slices and livers of intact mice for this purpose.

To determine whether mouse liver slices could incorporate NeuNAc into glycoproteins and to help choose appropriate time points for detection of CMP-NeuNAc, incubations with [^{3}H]NeuNAc were performed for different periods. As shown in Table I there was a linear increase of phosphotungstic acid insoluble radioactivity with time (up to 1 h), suggesting incorporation into macromolecules. Additional proof that the radioactivity was covalently attached to macromolecules was obtained by dissolving the insoluble pellet in sodium dodecyl sulfate and examining its behavior before and after Pronase treatment on a Bio-Gel P 10 column (Hirschberg & Yeh, 1977). Over 90% of phosphotungstic acid insoluble radioactivity was in NeuNAc as determined by Dowex 1–formate chromatography and paper chromatography in solvent system B (Hirschberg & Yeh, 1977). Incubation of liver slices with [^{3}H]leucine also showed a linear incorporation into macromolecules for at least 1 h (not shown).

NeuNAc, administered intravenously into mice, was also incorporated into macromolecules of liver and kidney, although the specific activities were lower than those with liver slices (Table I). At different times after injection, the specific activity was constant in liver (within experimental error) but increased in kidney. One hour after injection, 0.04% of the radioactivity remained in blood and 88% was recovered in urine.

Previous studies with cells grown in tissue culture suggested that, even after relatively long times of labeling (i.e., 12–24 h), virtually no metabolism of the radiolabel in NeuNAc had occurred (Hirschberg & Goodman, 1976; Hirschberg & Yeh, 1977). We wanted to determine whether or not this was also true in mice. A phosphotungstic acid insoluble fraction from liver and kidney was obtained 12 and 24 h after injection. NeuNAc was the only radioactive compound detected in this fraction and between 60 and 80% of the radioactivity was sensitive to Pronase treatment.

Biosynthesis, Isolation, and Characterization of CMP-[^{3}H]NeuNAc. To determine whether CMP-NeuNAc could be detected in mouse liver slices after incubation with [^{3}H]NeuNAc, ethanol–water extracts were obtained after 30 and 60 min of labeling and were applied to a DEAE-cellulose column and eluted with a gradient of ammonium acetate as described in Materials and Methods. Two peaks were detected in the eluates, containing a total of 99% of the applied radioactivity. Peak A which eluted at approximately 0.1 M ammonium acetate contained only NeuNAc based on the behavior of the radioactivity on Dowex 1–formate column chromatography (Hirschberg et al., 1976) and ascending paper chromatography in solvent systems A and B. Peak B, which eluted at approximately 0.2 M ammonium acetate, contained 230 and 440 cpm/mg of protein for the 30- and 60-min samples, respectively. The radioactivity of this peak comigrated with standard CMP-NeuNAc on a DEAE-cellulose column (not shown), on Whatman 3 MM paper in solvent systems A (Figure 1A), C, D (not shown) and Whatman DE-81 (Figure 1B).

Treatment of peak B material with 0.1 N hydrochloric acid and subsequent analyses by paper chromatography showed that radioactivity no longer comigrated with CMP-NeuNAc but with authentic NeuNAc (Figures 1C and 1D). After incu-

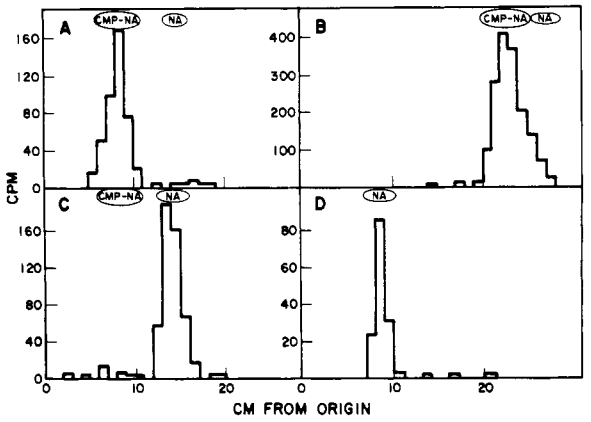

FIGURE 1: Characterization of CMP-[^{3}H]NeuNAc acid isolated from liver slices after incubation with [^{3}H]NeuNAc for 60 min. (A) Paper chromatography in solvent system A; (B) DE-81 paper chromatography in solvent system I; (C) paper chromatography in solvent system A following incubation with HCl (0.1 N, 37 °C, 20 min); (D) paper chromatography in solvent system C following incubation with HCl (0.1 N, 37 °C, 20 min). NA, NeuNAc; CMP-NA, CMP-NeuNAc.

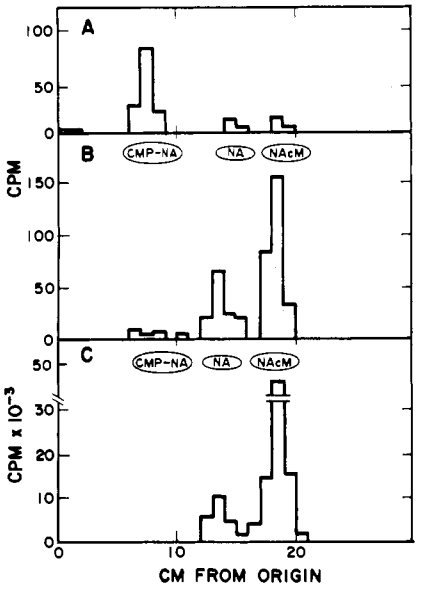

FIGURE 2: Susceptibility to sialic acid aldolase of CMP-[^{3}H]NeuNAc isolated from liver slices. CMP-[^{3}H]NeuNAc (2500 cpm) before or after incubation with HCl (0.1 N, 37 °C, 20 min) was digested with sialic acid aldolase (0.1 unit, Sigma Chemical Co., grade III) in 0.1 mL of 20 mM $NaPO_4$, pH 7.4, for 1 h at 37 °C. The mixture was then dried under a stream of nitrogen, redissolved in 20 μL of water and chromatographed. (A) Paper chromatography in solvent system A of CMP-[^{3}H]NeuNAc incubated with sialic acid aldolase. (B) Paper chromatography in solvent system A of CMP-[^{3}H]NeuNAc incubated with HCl followed by sialic acid aldolase. (C) Paper chromatography in solvent system A of standard [^{3}H]NeuNAc after incubation with sialic acid aldolase. NA, NeuNAc; CMP-NA, CMP-NeuNAc; NAcM, *N*-acetylmannosamine.

bation of radioactivity from peak B with NeuNAc aldolase and subsequent analysis by paper chromatography, approximately 75% of the radioactivity still comigrated with authentic CMP-NeuNAc (Figure 2A). However, pretreatment with 0.1 N hydrochloric acid followed by incubation with aldolase showed that virtually no radioactivity comigrated with CMP-NeuNAc. Seventy-five percent comigrated with standard *N*-acetylmannosamine (R_f 0.63) and the remainder with NeuNAc (Figure 2B). Treatment of peak B with $NaBH_4$ followed by acid hydrolysis and paper chromatography yielded a single radioactive peak which comigrated with standard NeuNAc (Figure 3A). However, treatment with acid prior

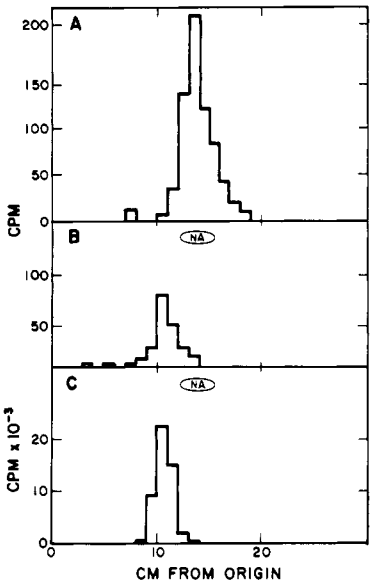

FIGURE 3: Susceptibility of CMP-[^{3}H]NeuNAc isolated from liver slices to $NaBH_4$ reduction. CMP-[^{3}H]sialic acid (2000 cpm) before or after treatment with HCl (0.1 N, 37 °C, 20 min) was incubated with $NaBH_4$ (0.2% in 0.1 mL of 1 mM NaOH) at room temperature for 30 min. The mixture was then dried under a stream of nitrogen, redissolved in 20 μL of water, and chromatographed. (A) Paper chromatography in solvent system A of CMP-[^{3}H]NeuNAc incubated with $NaBH_4$ followed by HCl. (B) Paper chromatography in solvent system A of CMP-[^{3}H]NeuNAc incubated with HCl followed by $NaBH_4$. (C) Paper chromatography in solvent system A of standard [^{3}H]NeuNAc after incubation with $NaBH_4$. NA, NeuNAc.

to $NaBH_4$ yielded a radioactive peak with mobility slower than that with NeuNAc (R_f 0.37). This peak comigrated with the $NaBH_4$ reduction product of standard [^{3}H]NeuNAc (Figures 3B and 3C). The biological activity of this CMP-[^{3}H]-NeuNAc will be shown in a later section.

CMP-[^{3}H]NeuNAc was also detected in livers of mice that had been injected intravenously with [^{3}H]NeuNAc. A radioactive peak which eluted in the same region as peak B of the experiments with liver slices was detected in animals at 5, 10, 25, and 60 min after injection and contained 2200, 4000, 3500, and 2700 cpm, respectively. Aliquots of this material comigrated with standard CMP-NeuNAc on DEAE-cellulose columns eluted with a gradient of ammonium acetate and on paper chromatography in solvent systems A, C, and D. Its instability toward mild acid, as monitored by paper chromatography in solvent systems A and B, was the same as for standard CMP-NeuNAc.

Biosynthesis, Isolation, and Characterization of [^{3}H]-CMP-NeuNAc. To characterize the nucleotide moiety of the putative CMP-[^{3}H]NeuNAc, liver slices were incubated for 30 min with [5-^{3}H]cytidine. An ethanol–water extract was obtained, applied to a DEAE-cellulose column, and eluted as described above. Radioactivity which eluted in the peak B region was pooled. Since only a portion of this material (approximately 40%) was susceptible to mild acid hydrolysis, suggesting heterogeneity, it was further purified by preparative paper chromatography in solvent systems F and G as described under Materials and Methods. Characterization of this material as [^{3}H]CMP-NeuNAc was based on (1) comigration with authentic standard on DEAE-cellulose column chromatography (not shown) and chromatography on 3 MM paper in solvent systems A (Figure 4A), C, and F (not shown) and DE-81 paper in solvent system I (not shown); (2) treatment with 0.1 N HCl for 20 min at 37 °C and subsequent analyses by paper chromatography which showed that all the radio-

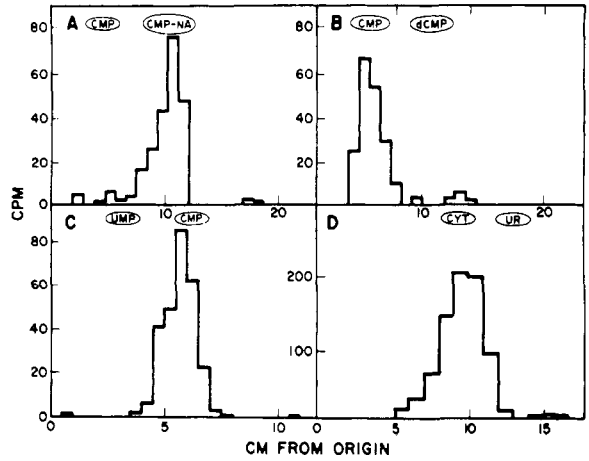

FIGURE 4: Characterization of [^{3}H]CMP-NeuNAc isolated from liver slices after incubation with [5-^{3}H]cytidine. (A) Paper chromatography in solvent system A; (B) paper chromatography in solvent system E after incubation with HCl (0.1 N, 37 °C, 20 min); (C) paper chromatography in solvent system F after incubation with HCl (0.1 N, 37 °C, 20 min); (D) paper chromatography in solvent system H of material shown in panels B and C after treatment with alkaline phosphatase. CMP-NA, CMP-NeuNAc; CYT, cytidine; UR, uridine.

Table II: Ability of CMP-[^{3}H]NeuNAc from Mouse Liver to Transfer NeuNAc in Vitro[a]

expt	time (min)	acceptor	PTA-insoluble radioact. (cpm)
		asialofetuin	
1	0	+	17.6 ± 0.7
	60	-	30.0 ± 1.2
	60	+	106.0 ± 2.1
2	60	-	47.0 ± 3.7
	60	+	321.0 ± 10.5
		lactose	
3	0	+	48
	15	+	1250
	30	+	2380
	30	-	210

[a] Experiment 1: CMP-[^{3}H]NeuNAc (5000 cpm for each assay) isolated from mouse liver was assayed with a Golgi fraction prepared according to Schacter et al. (1970). Reaction conditions have been described under Material and Methods. Experiments 2 and 3: CMP-[^{3}H]NeuNAc (16 000 cpm for each assay of experiment 2, 10 000 cpm for each assay of experiment 3) isolated from liver slices was assayed with a Golgi fraction prepared according to Fleischer (1974). Assay conditions and product identification have been described under Materials and Methods. Values in experiments 1 and 2 are those from duplicate determinations.

activity comigrated with CMP and not with dCMP (Figure 4B) or with UMP (Figure 4C); and (3) reaction of what had been tentatively identified as CMP (derived from CMP-NeuNAc) with alkaline phosphatase yielding radiolabeled cytidine and not uridine (Figure 4D).

[^{3}H]CMP-NeuNAc was also isolated from mouse liver after intravenous injection of [5-^{3}H]cytidine. In a typical experiment, from an animal sacrificed after 20 min, a radioactive peak containing approximately 5000 cpm (out of a total of 1 million) was observed to elute from a DEAE-cellulose column in the CMP-NeuNAc region. Following further purification by preparative paper chromatography in solvent systems F and G a radioactive compound was obtained which comigrated with CMP-NeuNAc on DEAE-cellulose column chromatography and paper chromatography in solvent systems A and D. Upon treatment with mild acid, all the radioactivity comigrated with CMP and none with dCMP.

Biological Activity of Isolated CMP-[^{3}H]NeuNAc. The ability of what tentatively had been identified as CMP-NeuNAc to transfer its sialic acid moiety to appropriate acceptors was tested using a rat liver Golgi fraction as a source of CMP-NeuNAc glycosyl transferase. As shown in Table II, the addition of asialofetuin to the assay system caused a 3.5–7-fold increase in the phosphotungstic acid insoluble radioactivity over endogenous values. Control incubations containing only free [^{3}H]NeuNAc did not incorporate radioactivity into phosphotungstic acid material above background levels (not shown). In experiment 3, with lactose as acceptor, there was a 10-fold increase over controls lacking acceptor. Analysis of the product by chromatography showed essentially one radioactive peak which comigrated with standard α(2–6)-sialyllactose (R_f 0.45 in system A). In some incubations 10% of the radioactivity comigrated with α(2–3)-sialyllactose.

Determination of the Amount of CMP-NeuNAc in Mouse Liver. Three separate analyses were done to determine the concentration of CMP-NeuNAc in mouse liver using tissues from 4, 5, and 6 animals, respectively. Standard CMP-[^{14}C]NeuNAc was added as tracer to either a tissue homogenate or to an ethanol–water extract which was further purified by DEAE-cellulose and paper chromatography as previously described for [^{3}H]CMP-NeuNAc. The amount of CMP-NeuNAc was calculated based on the chemical determination of the NeuNAc content (Warren, 1959) in the purified fraction after correction for recovery of the tracer (5–20%). The concentration was 37 ± 10 nmol/g of wet tissue.

Does the NeuNAc Skeleton Remain Intact during Its Uptake by Cells and Subsequent Incorporation into Glycoproteins? To determine whether the skeleton of NeuNAc had remained intact during the previous experiments or whether aldolase-catalyzed cleavage to pyruvate and *N*-acetyl[^{3}H]-mannosamine and subsequent condensation of this latter compound with cellular phosphoenolpyruvate had occurred, a mixture of *N*-acetyl[1-^{14}C]- and -[4,5,6,7,8,9-^{3}H]neuraminic acid was injected intravenously into a mouse or incubated with liver slices. In the whole animal experiment (Table III), the phosphotungstic acid soluble (free NeuNAc) and insoluble radioactivity from liver had a ^{14}C to ^{3}H ratio very similar to that of the injected sugar. Acid hydrolysis of the phosphotungstic acid insoluble pellet yielded NeuNAc of an isotope ratio very similar to that injected.

Since previous studies in vitro (Brunetti et al., 1962) had found NeuNAc aldolase activity to be particularly high in kidney, an ethanol–water extract of this tissue was obtained following intravenous injection of doubly labeled NeuNAc. Upon paper chromatography of this extract, two peaks, containing both tritium and ^{14}C, could be seen (not shown). The larger peak comigrated with standard NeuNAc (R_f 0.50) and the smaller with CMP-NeuNAc (R_f 0.30). Both peaks had an isotope ratio very similar to that of the injected NeuNAc (Table III). Phosphotungstic acid soluble and insoluble fractions of kidney were also obtained, in a manner similar to liver, and the isotope ratios of these fractions were again similar to that of the injected sugar. The same results were also obtained when a similar fractionation procedure was used with liver slices. After incubation with double-labeled NeuNAc, the ^{14}C to ^{3}H ratio was constant over the range 0.089–0.108.

Discussion

A major objective of this study was to provide evidence for the occurrence of CMP-NeuNAc in mammalian tissues. Previous studies in eukaryotes had characterized this sugar nucleotide (labeled in the NeuNAc moiety) by cochroma-

Table III: Isotope Ratio of *N*-Acetylneuraminic Acid Isolated from a Mouse 30 min after Intravenous Injection of a Mixture of *N*-Acetyl[4,5,6,7,8,9-^{3}H]- and *N*-Acetyl[1-^{14}C]neuraminic Acid[a]

fraction analyzed	^{14}C (dpm)	^{3}H (dpm)	^{14}C/^{3}H
NeuNAc injected			0.087
liver PTA-soluble fraction	170	1170	0.078
liver PTA-insoluble fraction	100	1160	0.090
liver PTA-insoluble fraction after acid hydrolysis and Dowex chromatography	150	1930	0.078
kidney ethanol–water soluble fraction after paper chromatography (CMP-NeuNAc fraction)	100	1230	0.081
kidney ethanol–water soluble fraction after paper chromatography (NeuNAc fraction)	1330	16290	0.082
kidney PTA-soluble fraction	360	4620	0.078
kidney PTA-insoluble fraction after acid hydrolysis and Dowex chromatography	220	2400	0.092

[a] One mouse was injected with a mixture of [^{14}C]- and [^{3}H]-NeuNAc (0.9 μCi of ^{14}C and 10.5 μCi of ^{3}H) and sacrificed 30 min later. Phosphotungstic acid soluble and insoluble fractions of liver were obtained as described under Materials and Methods. The phosphotungstic acid soluble radioactivity was characterized as NeuNAc based on its behavior on paper chromatography in system A and on a Dowex 1-formate column. Subjecting the phosphotungstic acid insoluble pellet to hydrolysis in H_2SO_4 (0.1 N, 80 °C, 1 h) solubilized 95% of the radioactivity of both isotopes which behaved as sialic acid on a Dowex 1-formate column. An aqueous homogenate of kidney was also obtained and two-thirds of it was extracted with ethanol as described for the isolation of CMP-NeuNAc. To the remaining one-third of the homogenate, phosphotungstic acid (2% in 1 N HCl) was added and acid-soluble and -insoluble fractions were obtained and characterized as described for liver. To determine the isotope ratio, all samples were counted for 50 min.

tography with standard and by isotope dilution techniques (Harms et al., 1973; Corfield et al., 1976; Briles et al., 1977). However, surprisingly, the nucleotide moiety and biological activity had never been determined.

In the present study we report the isolation, following radiolabeling with [^{3}H]cytidine or [^{3}H]NeuNAc, of a radiolabeled compound which is chromatographically identical with authentic CMP-NeuNAc and yields upon mild acid hydrolysis radiolabeled CMP or NeuNAc, respectively. In addition, the isolated CMP-NeuNAc was active as a NeuNAc donor in our system in vitro in which free NeuNAc was inactive. Upon incubation or injection with [^{3}H]NeuNAc, only free NeuNAc and CMP-NeuNAc could be detected in the water–ethanol soluble extract of liver and kidney. The chromatographic and chemical behavior of the isolated CMP-NeuNAc was different from that of any radioactive compound detected after injections with [^{3}H]deoxycytidine and [^{3}H]uridine. These results, although consistent with the specificity for CTP of CMP-NeuNAc synthetase in vitro (Kean & Roseman, 1966), do not rule out the occurrence of other NeuNAc-containing nucleotides. Our results also do not rule out modification of the injected *N*-acetylneuramic acid by additional acetylation or hydroxylation. However, studies of Schauer et al. (1972) suggest that such modifications could have been detected by the chromatographic systems used in our studies.

Another objective of this study was to determine whether in eukaryotes NeuNAc aldolase catalyzed cleavage of NeuNAc occurred during uptake and subsequent incorporation of the sugar into macromolecules. Although in previous experiments with mammalian and avian cells grown in tissue culture no evidence for such cleavage was obtained, this mechanism could not be eliminated as it was possible that the concentration of *N*-acetyl-[G-^{3}H]mannosamine, derived from [4,5,6,7,8,9-^{3}H]NeuNAc, was too low to be detected. The design of the experiment shown in the present study, using a mixture of [1-^{14}C]- and -[4,5,6,7,8,9-^{3}H]NeuNAc, assumes that, if aldolase-catalyzed cleavage occurs, the cellular pools and turnover rates of the products, [1-^{14}C]pyruvate and *N*-acetyl[^{3}H]mannosamine, are such that the isotope ratio of the resynthesized NeuNAc would be different from the initial one. We have found that the isotope ratio of NeuNAc in the various cellular fractions from whole animals and liver slices differed from that used initially by less than 12%, strongly suggesting that NeuNAc uptake and incorporation into macromolecules occurs in the absence of detectable aldolase activity.

Brunetti et al. (1962) have reported that NeuNAc aldolase activity is particularly prominent in kidney homogenates. Although we did not find such activity in these studies, our results are not necessarily contradictory, as conditions used in our experiments were very different from those of Brunetti et al. (1962).

We had previously observed with mammalian and avian cells grown in tissue culture the absence of metabolism of labeled NeuNAc even after relatively long periods (12–14 h). This suggested that such a labeling procedure could be used for specific labeling of sialoglycoproteins in vivo (and perhaps sialoglycolipids). We have found similar results here with mice, although the specific activity of the NeuNAc bound to macromolecules was rather low. Considerably higher values could be obtained with liver slices.

Previous efforts to label cellular sialoglycoproteins and sialoglycolipids have made extensive use of labeled glucosamine as precursor of NeuNAc. However, this procedure is not specific as glucosamine (as the *N*-acetyl derivative) is also a component of glycoproteins and can also be converted to sugars other than NeuNAc, particularly after relatively long periods. Labeled *N*-acetylmannosamine has been shown by Harms et al. (1973) to be a more specific precursor for labeling NeuNAc moieties; however, significant metabolism to other compounds was also reported.

While the physiological significance of the above results with NeuNAc is not clear at this time, several possibilities come to mind: this may be a mechanism for utilization of dietary NeuNAc (bound to glycoproteins and glycolipids) in a manner similar to that suggested for dietary fucose-containing macromolecules (Ishihara & Heath, 1968). Another possibility is that neuraminidase activity in serum (Schauer et al., 1976) may act upon circulating sialoglycoproteins yielding free NeuNAc and asialoglycoproteins. Recent studies of Ashwell & Morell (1974) have shown that these proteins are rapidly removed from circulation by the liver. Our results suggest that (at least part) of the free NeuNAc could be reutilized by cells.

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Isolation and Identification of 4-Hydroxy- and 4-Oxoretinoic Acid. In Vitro Metabolites of *all-trans*-Retinoic Acid in Hamster Trachea and Liver†

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ABSTRACT: Incubation of [^{3}H]retinoic acid in the presence of hamster liver 10000*g* supernatant produces several metabolites that are more polar than the parent compound. Two of these metabolites are identical with synthetic *all-trans*-4-hydroxyretinoic acid and *all-trans*-4-oxoretinoic acid both in ultraviolet absorption and mass spectral characteristics and in migration rates on two different reverse-phase high-pressure liquid chromatographic systems. The metabolites produced in a cell-free liver incubation reaction also migrate on a high-pressure liquid chromatography column together with metabolites isolated from a tracheal organ culture system. Both the metabolites and the synthetic standards show less biological activity than the parent *all-trans*-retinoic acid in a tracheal organ culture assay.

Retinoic acid has been shown to be a normal intermediate in the metabolism of retinol and its esters (Crain et al., 1967; Deshmukh et al., 1965; Dunagin et al., 1964; Emerick et al., 1967; Ito et al., 1974; Kleiner-Bössaler & DeLuca, 1971). It is known to be active in supporting growth (Krishnamurthy et al., 1963; Malathi et al., 1963; Zile & DeLuca, 1968) and in maintaining epithelial differentiation (Dowling & Wald, 1960). The metabolism of retinoic acid has been actively studied in the past [for example, DeLuca & Roberts (1969), Dunagin et al. (1965, 1966), Ito et al. (1974), Lippel & Olson (1968), Nath & Olson (1967), Nelson et al. (1971), Sundaresan & Therriault (1968)], but it is not yet apparent as to whether it is the parent compound itself or a further metabolite that is the final active form of retinoic acid in controlling epithelial differentitation.

With the application of high-pressure liquid chromatography (LC)[1] to the separation of retinoids (Frolik et al., 1978a; McCormick et al., 1978a), there has been a renewed interest in the search for a possible active metabolite of *all-trans*-retinoic acid. This resurgence began with the identification of several rat fecal and urinary metabolites by Hänni and co-workers (Hänni et al., 1976; Hänni & Bigler, 1977). After a single intraperitoneal dose of 27.2 mg of *all-trans*-retinoic acid into vitamin A normal rats, these investigators isolated and identified 4-oxoretinoic acid as well as the all-trans and 9-cis isomers of 5′-hydroxyretinoic acid from the feces. They have also found several decarboxylated metabolites in the urine. These compounds, however, being found in the feces and urine, are most likely excretion products and do not represent an "active" form of retinoic acid.

More recently, 5,8-oxyretinoic acid has been implicated as a possible in vivo metabolite of retinoic acid in the rat intestinal mucosa (Napoli et al., 1978). However, since this compound is readily obtained from 5,6-epoxide under the acidic conditions employed in the isolation procedure (John et al., 1967; Morgan

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[1] Abbreviations used: LC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.