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Metabolic Labeling of Sialic Acids in Tissue Culture Cell Lines: Methods to Identify Substituted and Modified Radioactive Neuraminic Acids[1]

SANDRA DIAZ AND AJIT VARKI[2]

University of California at San Diego, The UCSD Cancer Center, Department of Medicine, T-011, San Diego, California 92093

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The parent sialic acid *N*-acetylneuraminic acid can be modified or substituted in various ways, giving rise to a family of more than 25 compounds. The definitive identification of these compounds has previously required isolation of nanomole amounts for mass spectrometry or NMR. We have explored the possibility of using the known metabolic precursors of the sialic acids, particularly *N*-acetyl-[6-^{3}H]mannosamine, to label and identify various forms of sialic acids in tissue culture cells. Firstly, we defined several variables that affect the labeling of sialic acids with *N*-acetyl-[6-^{3}H]mannosamine. Secondly, we have devised a simple screening method to identify cell lines that synthesize substituted or modified sialic acids. We next demonstrate that it is possible to definitively identify the natures of the various labeled sialic acids without the use of mass spectrometry, even though they are present only in tracer amounts. The methods used include paper chromatography, analytical de-*O*-acetylation, periodate release of the 9-^{3}H as [^{3}H]formaldehyde (which is subsequently converted to a specific ^{3}H-labeled chromophore), acylneuraminate pyruvate lyase treatment with identification of [^{3}H]acylmannosamines, gas–liquid chromatography with radioactive detection, and two new high-pressure liquid chromatography methods utilizing the amine-adsorption:ion suppression and ion-pair principles. The use of an internal *N*-acetyl-[4-^{14}C]neuraminic acid standard in each of these methods assures precision and accuracy. The combined use of these methods now allows the identification of radioactive tracer amounts of the various types of sialic acids in well-defined populations of tissue culture cells; it may also allow the identification of hitherto unknown forms of sialic acids.

KEY WORDS: sialic acids; *O*-acetyl groups; *N*-glycolyl groups; metabolic labeling; acetyl-[^{3}H]mannosamine.

The sialic acids are a family of derivatives of neuraminic acid that have been implicated in many biological roles (1–3). The classic work of Blix, Klenk, Gottschalk and others [reviewed in Ref. (1)] identified this class of sugars and also showed that *N*- and *O*- substitutions of the parent molecule could result in significant diversity. Following up on these findings, the elegant studies of Schauer and others have to date resulted in the positive identification of more than 30 different free and glycosidically bound sialic acids in nature (1–3). The most commonly found sialic acid is *N*-acetylneuraminic acid (Neu5Ac).[3] The other sialic acids are all believed to be derived

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[2] To whom all correspondence should be addressed.

[3] Abbreviations used: The various sialic acids are designated by combinations of Neu = neuraminic acid; Ac = acetyl; Gc = glycolyl; and Lac = lactyl. The amino group at the 5 position is always substituted with an acetyl or a glycolyl group. Other substitution positions are indicated by numerals. For example, *N*-acetyl-9-mono-*O*-acetylneuraminic acid may be written as Neu5,9Ac$_2$ [after R. Schauer and others (2)]. Other abbreviations used include FEL, Friend murine erythroleukemia cells; Me_3Si, trimethylsilyl-; LDH, lactate dehydrogenase; and TMSI, trimethylsilylimidazole.

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from this molecule by different modifications and substitutions. The most common type of modification is the substitution of *O*-acetyl esters at the hydroxyl groups of the 4, 7, 8, or 9 positions (2,3). These substitutions are known to have effects on neuraminidase (sialidase) (3–6), acylneuraminate pyruvate-lyase (3–7), CMPsialate synthase (8–10), and sialyltransferase (11) action, on alternate pathway complement activation (12), on bacterial antigenicity and pathogenicity (13), and on antibody recognition of gangliosides (14,15). Another common modification is that of an *N*-glycolyl group in place of the *N*-acetyl group at the 5-position (1,16,17). This modification also has effects on neuraminidase action (5), and is believed to be antigenic to the human species (18,19). Very little else is known about the biological significance of these modifications in the sialic acids.

The work of Kean, Roseman, Warren and others (20–23) clearly defined the pathway of biosynthesis of the parent molecule. Schauer and others subsequently demonstrated the basic enzymatic mechanisms involved in some of the substitution and modification reactions (16,24–27). Such modifications are highly tissue and species specific (2,3), suggesting that the enzymatic reactions responsible must be under very careful control. To further study the biosynthesis and regulation of these reactions in detail, it is necessary to use model systems of monoclonal populations of cells in culture. Such systems have not been yet identified. Furthermore, the conventional approach to the positive identification of the various sialic acids, which requires purification of nanomole amounts of these compounds and mass spectrometric analysis (2,3) is not applicable to studies of radioactive tracer labeling, pulse-chase experiments, and identification of biosynthetic intermediates in tissue-culture cell lines. In this study, we have utilized known principles of sialic acid biochemistry, modified preexisting methods, and developed some new methods that allow the detailed study of sialic acid diversity in metabolically labeled tissue culture cells.

MATERIALS

The following materials were obtained from the sources indicated: trimethylsilylimidazole (TSMI), Pierce Chemical Company; Dowex 1 [AG 1-X8 (formate form)] and Dowex 50 [AG 1-X2 (H^+ form)], Dowex 3X4A (100–200 mesh, chloride form), Bio-Rad; 3% OV-17 on Gas ChromQ (100/120 mesh), Applied Sciences Laboratories. Chemically synthesized Neu5Ac (>99% purity), Kantoishi Pharmaceutical Company, Tokyo, Japan; Neu5Gc, *Clostridium perfringens* *N*-acetylneuraminic acid aldolase (acylneuraminate pyruvate-lyase) and *N*-glycolylneuraminic acid (Neu5Gc), Sigma Chemical Company; NADH and lactate dehydrogenase (LDH) (from pig's heart), Boehringer–Mannheim; *Vibrio cholerae* and *Arthrobacter ureafaciens* neuraminidases, Calbiochem. *N*-Glycolylmannosamine was prepared by isolating the neutral product of the action of acylneuraminate pyruvate-lyase on Neu5Gc. Neuraminidase from *Streptococcus sanguis* was prepared as previously described (6). Authentic 9-*O*-acetyl-*N*-acetylneuraminic acid ($Neu5,9Ac_2$) was kindly provided by Professor Roland Schauer, Kiel, Federal Republic of Germany. Other substituted sialic acids were prepared from bovine submaxillary gland mucin using previously described methods (12,28). [4-^{14}C]Neu5Ac and [6-^{3}H]ManNAc were from New England Nuclear. All other chemicals were of reagent grade and were purchased from commercial sources. The Dowex 3X4A resin was converted to the formate form by the procedure recommended by the manufacturer.

Cells. The murine myeloma cell line P3×63Ag8 (29) and hybridoma cell lines KS1/4 (30) were provided by Dr. Nissi Varki and Dr. Ralph Reisfeld of the Research Institute of Scripps Clinic, La Jolla, California. The murine myeloma cell line MOPC-11 (31) was provided by Dr. Mark Glassy of the UCSD Cancer Center. A subclone of the Friend murine erythroleukemia cell line (FEL745.6) (32) and a similar line of Balb/c origin (BB88.8) were kindly provided by Dr. Bob Hyman and

Dr. Jayne Leslie of the Salk Institute, La Jolla, California. Another Friend erythroleukemia line (DS-19) was provided by Dr. John Yu, Scripps Clinic and Research Foundation. All cells were grown in RPMI 1640 with 10% fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin, in a humidified incubator at 5% CO_2, and 37°C.

METHODS

Screening procedure for identifying tissue culture cell lines that synthesize different types of sialic acids. Cells (10^7) were "equilibrium" labeled for 3 days with 100 μCi of [6-^{3}H]ManNAc, exactly as described below. Membranes were isolated (see below), and sialic acids were released with acid and purified exactly as described below. *N*-Acetyl-[4-^{14}C]neuraminic acid was added as an internal standard (equal to approximately $\frac{1}{4}$ of the ^{3}H cpm). After evaporation, the sample was split into two equal portions. One half was applied directly to Whatman 3MM paper; the other was de-*O*-acetylated as described below, evaporated to remove the ammonia, and then spotted on the paper. The chromatogram was developed for 15 h in system A, dried, and cut into 1-cm strips that were monitored for ^{3}H and ^{14}C radioactivity.

Isolation of labeled membranes. The labeled cells were chilled on ice, pelleted at 1000*g*, and washed three times in 10 ml of ice-cold phosphate-buffered saline, pH 7.0. The pellet was sonicated in 1 ml of 20 mM sodium phosphate buffer, pH 7.5, using 4 × 15-s pulses of a Model W-185-F Heat Systems sonicator cell disrupter with a probe setting of 30. The sonicate was centrifuged at 75*g* for 15 min. The pellet thus obtained consisted primarily of nuclei and unbroken cells, and typically contained less than 10% of the total radioactivity; it was discarded. The supernate was centrifuged at 100,000*g* for 30 min to pellet the labeled membranes, which were washed once in 5 mM pyridinium acetate, pH 5.5, and resuspended in 1 ml of the same buffer.

Release of sialic acids. In some experiments, membrane-bound sialic acids were released by heating in 2 M acetic acid at 80°C for 3 h (33). Alternatively, the membranes were suspended in 1 ml of 100 mM sodium acetate 4 mM calcium acetate, pH 6.0, and treated with 20 mU each of neuraminidases from *S. sanguis* and *V. cholerae* for 24–48 h under a toluene atmosphere. (Because of the very small amounts of actual bound sialic acids in the samples, the enzyme:substrate ratio is not of concern.) In more recent experiments, we have found that *A. ureafaciens* neuraminidase can be used in place of the *S. sanguis* enzyme. The enzymatic and acid hydrolysis conditions were chosen to maximize release of Neu5Ac and Neu5Gc, with or without *O*-acetylation, and to minimize loss of the ester groups (6,33).

Purification of sialic acids. Radioactive sialic acids released from glycosidic linkage by enzymatic or acid hydrolysis were purified in a manner similar to that described in Ref. (33), with some modifications and with exclusion of the ether-extraction step. All steps were carried out at room temperature. The acid hydrolysis reactions were chilled and spun at 10,000*g* for 15 min, the supernate was evaporated to remove the acid, and the residue was brought up in 0.5 ml of water for application to Dowex 50. The enzyme reactions were centrifuged similarly, and the supernate was applied directly to the Dowex 50 column. The samples were loaded onto a 1-ml column of Dowex 50 (hydrogen form). The column effluent and 4 ml of water washings were collected and the pH of the pooled washings was checked with pH paper to ensure that it was greater than 3. The washings were passed over a 1-ml column of Dowex 3X4A (formate form) equilibrated in 10 mM sodium formate, pH 5.5. The column was washed with 7 ml of 10 mM formic acid. The sialic acids were eluted from the column with 10 ml of 1 M formic acid, and the acid was removed by evaporation. All evaporations of aqueous samples were performed on a Buchler Shaker-Evaporator with the water bath set at 35°C or a Savant centrifuge evaporator with the heating element turned on (35–40°C). This pro-

cedure results in >90% recovery with minimal loss of *O*-acetyl groups. However, some migration of *O*-acetyl groups from the 7 to the 9 position can occur during the purification (33).

De-O-acetylation of sialic acids. Purified radioactive sialic acids were heated in 2 ml of 2 M ammonium hydroxide at 60°C for 1 h, and the ammonia was removed by evaporation (33). If a visible residue was left behind, the sample was brought up in 0.5 ml of water and passed over Dowex 50 exactly as described above. This was done because the residue caused interference with optimal paper chromatography. In some cases de-*O*-acetylation with 0.1 N NaOH was performed (33).

Enzymatic cleavage of sialic acids with acylneuraminate pyruvate-lyase. Purified sialic acids were mixed with 50 nmol of cold carrier Neu5Ac and a [4-^{14}C]Neu5Ac internal standard and treated with 5 mU of *C. perfringens* acylneuraminate pyruvate-lyase in 250 μl of 0.1 M potassium phosphate, pH 7.2. To drive the reaction, 2 mM NADH and 7 mU of LDH were also included in the reaction, which was allowed to proceed for 12–24 h under a toluene atmosphere. The reaction mixture was then diluted to 500 μl with water and applied to tandem 1-ml columns of Dowex 50 (hydrogen form) and Dowex 1 (formate form) at room temperature. The columns were washed with 4 ml of water and the neutral run-through was collected and monitored for radioactivity.

Descending paper chromatography was carried out on Whatman 3MM paper in System A, *n*-butanol:acetic acid:water (4:1:5, upper phase); System B, *n*-butanol:*n*-propanol:0.1 N HCl (1:2:1); or System C, 95% ethanol:1 M ammonium acetate, pH 5.3 (79:26).

Gas–liquid chromatography with radioactive detection. Purified sialic acids were mixed with 100-nmol amounts of cold-carrier standards, derivatized with trimethylsilylimidazole, and analyzed by gas–liquid chromatography on OV-17 (3%) at 200°C (isothermal) exactly as described by Schauer (28). A first run was carried out with the flame-ionization detector on, to identify the retention times of the internal unlabeled standard sialic acids. The flame was then turned off and an identical injection performed. The effluent gases were condensed *directly* onto the walls of 6-ml plastic scintillation vials that were prechilled on ice and then inverted over the detector outlet. The vials were changed at 20-s intervals, based on the movement of the same chart recorder paper. Scintillation fluid (4 ml) was *immediately* added to each vial.

Periodate treatment and acetylacetone reactions. Purified sialic acids were brought up in 10 μl of water and incubated at 4°C for 45 min in either 0.1 N NaOH or 0.1 N NaCl. The base-treated reactions were then neutralized with HCl. To these reactions, 20 μl of 2.5 mM sodium periodate in phosphate-buffered saline (at various pH values, as indicated) was added, and then incubated for 15 min at 4°C in the dark. The excess periodate was destroyed with 12.5 μl of 2% sodium arsenite in 0.5 M HCl. Acetylacetone reagent {37.5 μl [0.2% acetylacetone (v/v), 0.3% glacial acetic acid (v/v), 15% ammonium acetate (w/v), in water]} was then added, and the mixture was heated at 60°C for 10 min (28). The reaction volume was then reduced to about 10 μl by evaporation, in preparation for paper chromatography.

High-pressure liquid chromatography. Various types of siliac acids were separated from each other by two new HPLC methods. System A employed a Varian Micropak AX-5 column eluted in the isocratic mode with acetonitrile:water:0.5 M NaH_2PO_4 (64:26:10) at 1 ml/min. This method is based on the ion-suppression:amine absorption principle used for separation of acidic oligosaccharides by Mellis and Baenziger (34). Slightly shorter retention times but somewhat sharper peaks were obtained when a gradient from 64:26:10 to 54:36:10 was employed over 20 min. System B was a reverse-phase-ion-pair method, using a 4 × 300-mm Waters μBondapak C_{18} column and en eluting buffer of 0.4% tetrabutylammonium formate, pH 4.5, at 1.0 ml/min. Fractions of 0.4–0.6 ml were collected and monitored for radioactivity. In system A, optimal counting efficiency was obtained if the

acetonitrile was first evaporated under a current of warm air. These methods can also be used preparatively. In the case of System A, the pooled fractions are diluted 10-fold in distilled water and passed directly over a 1-ml Dowex 3X4A column (formate form) equilibrated in 10 mM sodium formate, pH 5.5. The sialic acids are then eluted with 10 × 1 ml of 1 M formic acid. The eluate is then taken to dryness. For System B the pooled fractions are passed directly over a 1-ml Dowex 50 (H^+ form) column in water, washed through with 4 ml of water, and taken to dryness to remove the formic acid generated.

System A can also be monitored for uv absorbance at 195–205 nM. The various sialic acids can be easily detected in the 0.5 to 5-nmol range with the detector at range 0.08 AUFS (not shown). System B cannot be monitored in this manner because of the high absorbance of the buffer. System A could also be used to separate the different acylmannosamines.

RESULTS AND DISCUSSION

Metabolic labeling of sialic acids in tissue culture cells. Previous studies have shown that sialic acids can be labeled with the precursors [^{3}H]glucosamine or *N*-acetyl-[^{3}H]mannosamine (35–40), or with radioactive sialic acid itself (41). While [^{3}H]glucosamine is the most efficiently incorporated of the three, the percentage of the label that enters the sialic acids is low [8–35%, see Ref. (40)]. [^{3}H]Sialic acid itself, while highly specific, suffers from two problems: first, incorporation is extremely poor, because it is a low efficiency process with a K_m of 10 mM (41); second, to obtain sufficient uptake for meaningful studies highly expensive amounts of this label have to be used. For example, pulse labeling of 10^7 cells with 1 mCi of [^{3}H]Neu5Ac for 30 min resulted in uptake of only about 10,000 cpm (A. Varki and S. Diaz, unpublished observations). We have therefore investigated in detail the use of the relatively specific precursor, *N*-acetyl-[6-^{3}H]mannosamine. Previous studies using this labeled sugar have included either direct administration to animals (35,37) or pulse labelings of tissues (38) or tissue culture cells (39,40) for varying periods of time. In these studies incorporation of label into sialic acids was studied, but specific identification of the different types of sialic acids was not attempted. Furthermore, we were unable to find any published analysis of the variables affecting this labeling method. We therefore first carried out a series of control experiments to study the variables in the labeling of tissue culture cells. We found that the uptake of *N*-acetylmannosamine by the cells is a very-low-efficiency process that is linear and nonsaturable up to concentrations as high as 20 mM. The percentage efficiency of uptake was essentially unchanged by manipulation of the concentrations of the glucose, serum, amino acids, vitamins, or glutamine in the medium. Thus, it is very different from other radioactive sugars such as [2-^{3}H]mannose and [6-^{3}H]glucosamine which compete with glucose for uptake (40). Removal of pyruvate from the labeling medium caused a slight loss of efficiency of labeling, but additional pyruvate above 1 mM did not improve it. Even with prolonged labeling, a very minor fraction of the label was utilized, and the rest was left unchanged in the media. Many previous studies assume that [^{3}H]ManNAc is incorporated exclusively into sialic acid and its precursors (42–44). However, in more than 40 different tissue culture cell lines that we have labeled to date, we found a highly variable amount (30–80%) of the membrane-bound label was in sialic acids (whether analyzed by acid hydrolysis or neuraminidase treatment). This problem with the specificity of this labeling technique has not been previously addressed, and is best explained by the existence of the mammalian 2′-epimerase that can convert ManNAc to GlcNAc (45). We have also found that the half-life of the intracellular [6-^{3}H]ManNAc pool is very long (greater than 2 h, not shown). Therefore, there is ample opportunity for the label to be incorporated into the many met-

abolic products that can arise from [^{3}H]-GlcNAc (44).

Screening for the synthesis of substituted sialic acids in tissue culture cell lines. There are to date no well-established examples of continuous tissue culture cell lines that synthesize a major amount of substituted or modified sialic acids. To identify such cell lines, we have developed a simple screening procedure that is described under Methods. The basic principle is to purify ^{3}H-sialic acids from cell membranes that are equilibrium labeled with [6-^{3}H]ManNAc, mix them with an internal standard of [4-^{14}C]Neu5Ac, and chromatograph the mixture on paper. In cell lines with no substitution of sialic acids, there is a perfect comigration of the ^{3}H and ^{14}C labels (not shown). As shown in Fig. 1 (left), such an analysis of labeled membranes from the Friend murine erythroleukemia cell line 745.6 showed a ^{3}H peak that migrated ahead of the [^{14}C]Neu5Ac standard, in the position expected for a mono-*O*-acylated sialic acid. Upon treatment of the mixture with mild alkali under conditions that would cause de-*O*-acylation (33), this peak was eliminated, further suggesting that it was an *O*-acylated sialic acid. Similar results were obtained in other murine erythroleukemia cells such as DS-19 and BB88.8, and in human melanoma cells (not shown), suggesting that all of these cells may contain *O*-acylated sialic acids. When a similar analysis was performed on labeled cell membranes from the P3×63AG8 mouse myeloma cell line more than 90% of the label migrated about 1 cm behind the ^{14}C standard in the position expected for *N*-glycolylneuraminic acid (see Fig. 1, right). In this case, base

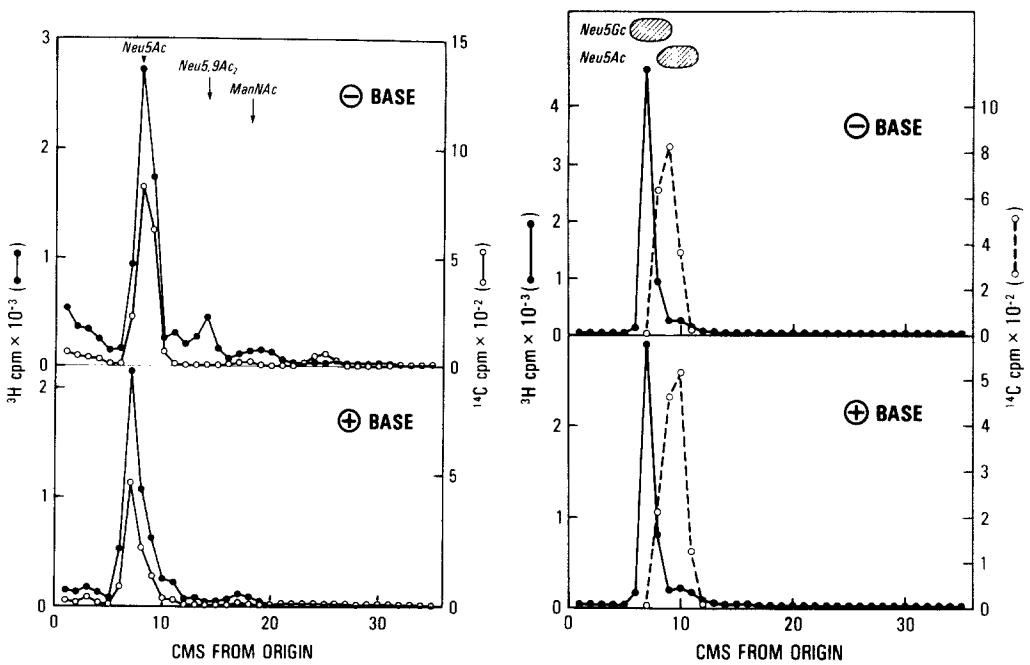

FIG. 1. Paper chromatography of ^{3}H-sialic acids released by acid hydrolysis from cell membranes. Cell membranes were isolated from 745.6 erythroleukemia cells (left) or P3×63Ag8 myeloma cells (right) that had been labeled for three days with [6-^{3}H]ManNAc. The sialic acids released by acid hydrolysis were purified as described under Methods. A [^{14}C]Neu5Ac internal marker was added, and aliquots were chromatographed on Whatman 3MM paper in System A for 14 h with (lower) or without (upper) intermediate base treatment with ammonia. The standards stained by the silver nitrate/alkali method are Neu5Ac, *N*-acetylneuraminic acid; Neu5,9Ac$_2$, *N*-acetyl-9-*O*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid, and ManNAc, *N*-acetylmannosamine.

treatment had no effect on this profile, suggesting the absence of base-labile ester groups. Similar analysis of several other murine myeloma cell lines such as MOPC-11, and murine monoclonal antibody-producing hybridomas such as KS 1/4.3, gave almost identical results (data not shown), suggesting that many murine myeloma cells may have a very high proportion of Neu5Gc. Thus, substituted or modified sialic acids with even the slightest degree of difference in migration from that of the internal [^{14}C]Neu5Ac standard will be easily picked up by this simple screening method.

Preparative analysis of sialic acids released by enzymatic means. Further proof of the identity of these labeled sialic acids required the isolation of larger amounts of radioactivity. This was done by labeling 10^7 cells for 3 days with 1–2 mCi of [6-^{3}H]ManNAc in 20 ml of complete media. The membrane fractions were isolated and treated with a combination of neuraminidases chosen to maximize release of substituted and nonsubstituted sialic acid species (6,33). The released radioactivity was purified as described under Methods and fractionated by preparative paper chromatography in System A. In the case of FEL 745.6, the radioactivity migrated in five peaks, named A through E (See Fig. 2). The sialic acids from the murine myeloma cell line were purified by paper chromatography in a different system (System B) that more completely separates Neu5Ac from Neu5Gc. As shown in the upper panel of Fig. 3, >90% of the radioactivity again comigrated with authentic Neu5Gc. These peaks (Figs. 2 and 3 upper) were pooled as indicated, passed through a 45-μM filter, taken to dryness, repurified on Dowex 3×4A, and stored frozen in 10 mM formic acid.

Analysis of individual peaks by de-O-acylation. Aliquots of each peak from FEL 745.6 were de-*O*-acetylated with base in the presence of a [^{14}C]Neu5Ac standard and then rechromatographed as shown in Fig. 4. Peak A, which represented 87.5% of the total counts released by neuraminidase, comigrated with a [^{14}C]Neu5Ac standard and was unaffected by de-*O*-acetylation, suggesting that it was

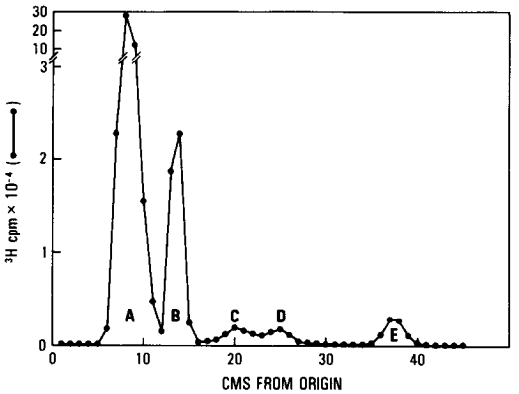

FIG. 2. Preparative paper chromatography of ^{3}H-sialic acids released by neuraminidases from cell membranes. Cell membranes from FEL745.6 cells labeled for three days with [6-^{3}H]ManNAc were treated with neuraminidases as described under Methods. The released sialic acids were further purified, spotted on Whatman 3MM paper and chromatographed in System A for 20 h. Strips of 1 cm were cut and soaked in 2 ml 10 mM HCOOH, and 20 μl of each was monitored for radioactivity. The various peaks were pooled as follows: A, 7–10; B, 13–15; C, 18–22; D, 25–28; and E, 36–39 cm.

Neu5Ac. Prior to base treatment, 90% of peak B continued to migrate in its original position. After base treatment, all of the radioactivity comigrated with the internal standard. This suggests that the parent compound of peak B is Neu5Ac, and that the base treatment removed an *O*-acyl group. Treatment of peaks C and D resulted in more than 50% of the radioactivity in each case shifting under the ^{14}C standard. This suggests that at least a portion of these peaks had *O*-acyl groups on the parent compound Neu5Ac. Their position of migration suggests that they have more than one *O*-acyl group, and they presumably represent higher-side-chain *O*-acylated sialic acids, e.g., Neu5,7,9Ac$_3$ or Neu5,8,9,Ac$_3$. Together, these two peaks represented only 1.7% of the total counts released by neuraminidase. It was therefore not feasible to carry out further studies to conclusively establish their identity. Peak E remained completely resistant to base treatment. We are uncertain of the identity of this unknown compound. Since it was labeled by [6-^{3}H]ManNAc, released by neuramini-

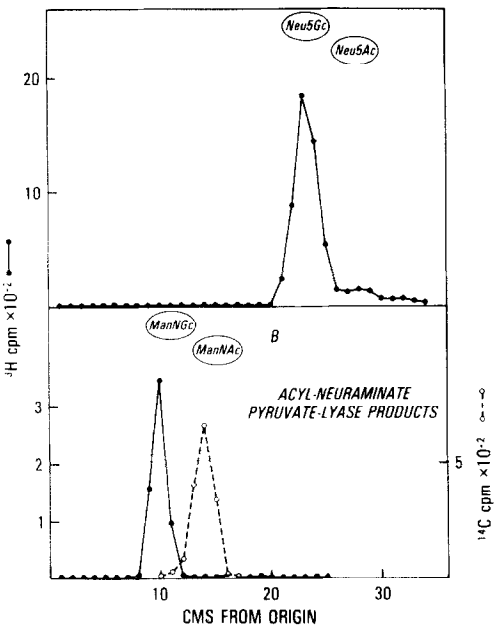

FIG. 3. Analysis of ^{3}H-sialic acids released by neuraminidases from cell membranes. Cell membranes from P3×63Ag8 cells labeled for 3 days with [6-^{3}H]ManNAc were treated with neuraminidases, and the sialic acids were purified as described under Methods. One aliquot of the purified sialic acids was spotted directly on Whatman 3MM paper and chromatographed in System B for 24 h (upper). Another aliquot was treated with acylneuraminate pyruvate-lyase after addition of a [4-^{14}C]Neu5Ac internal standard. The neutral products of this reaction were isolated as described under Methods, spotted on Whatman 3MM paper, and chromatographed in System A for 14 h (lower). The standards, which were run in parallel in each case (and stained with the silver nitrate/alkali method) are Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; ManNAc, *N*-acetylmannosamine; and ManGc, *N*-glycolylmannosamine.

dase, and copurified with sialic acids through several steps, it could represent a hitherto unidentified sialic acid. Similarly, we have found an unidentified base-resistant sialic acid in human melanoma cells (A. Varki and S. Diaz, unpublished observations). Further studies of these unknown sialic acids are in progress.

The ^{3}H label is in the 9 (or 8) position of the metabolically labeled sialic acids. Since the amounts of each peak available were insufficient to allow their definitive identification by GLC/MS (2,3), it was necessary to devise alternate approaches for their positive identification. Based on the well-established pathways for the biosynthesis of sialic acids from *N*-acetylmannosamine (20–23), we predicted that the ^{3}H label from [6-^{3}H]ManNAc should be incorporated into the 9 position of the biosynthetic sialic acids (see Fig. 5). It is also well known that treatment of unsubstituted sialic acids with mild periodate results in the conversion of the 9-carbon to formaldehyde, and the 8-carbon to formic acid, leaving behind the C-7 aldehyde analog (46–49). We reasoned that if the prediction regarding the position of the ^{3}H label were true, treatment with mild periodate should result in the generation of [^{3}H]formaldehyde from [9-^{3}H]Neu5Ac (see Fig. 5). To test this hypothesis, we made use of the acetylacetone ("Hantzsch") reaction of formaldehyde, which results in incorporation of the formaldehyde molecule into the bright

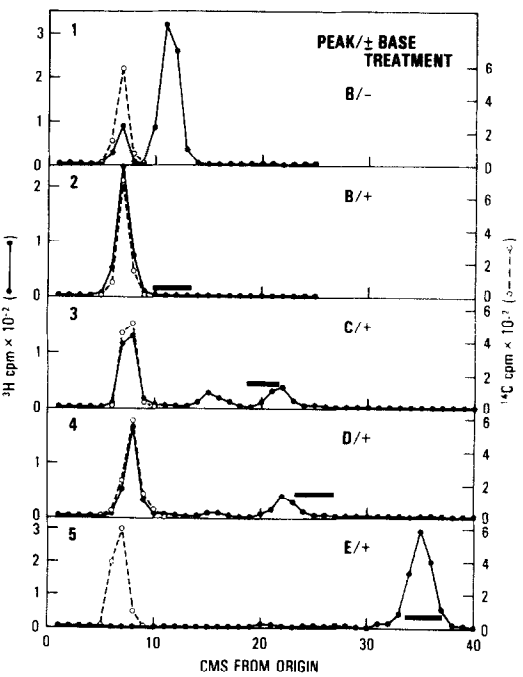

FIG. 4. Paper chromatography of neuraminidase-released peaks. Aliquots of the radioactive peaks purified from the neuraminidase-released material (see Fig. 2) were chromatographed on Whatmann 3MM paper in System A for 14 h, with or without prior base treatment, as indicated. In each case a [^{14}C]Neu5Ac internal standard was included. The solid bars indicate the position of migration of each peak prior to base treatment.

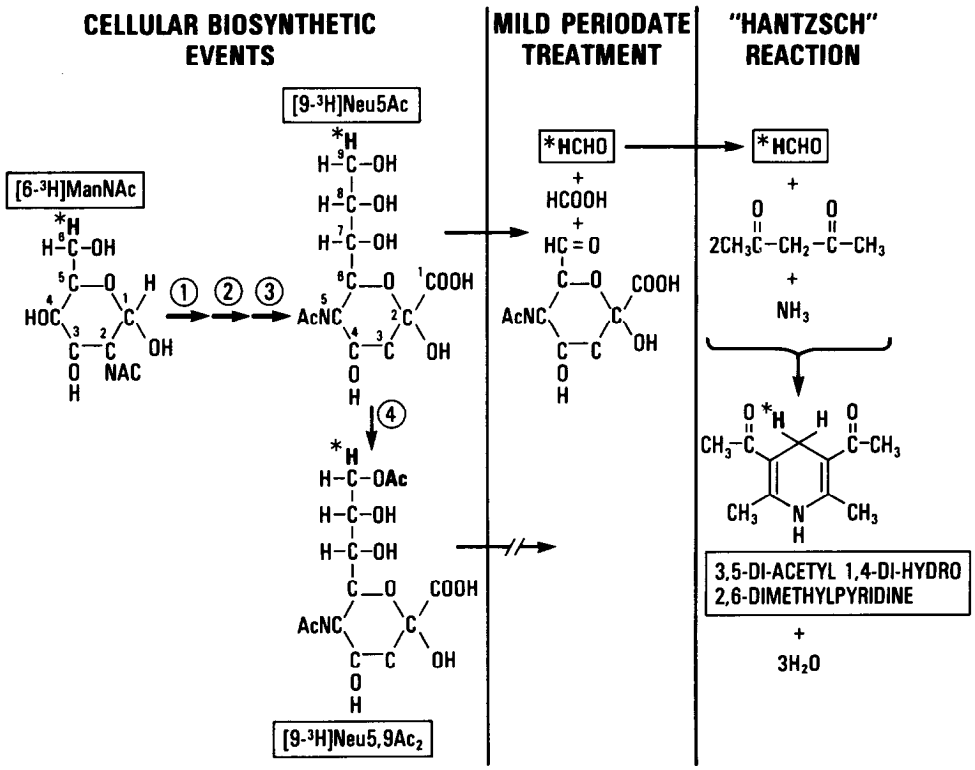

FIG. 5. Rationale for the analysis of ^{3}H-sialic acids by mild periodate/acetylacetone reaction. The cellular biosynthetic events predicted include the biosynthesis of *N*-acetyl-[9-^{3}H]neuraminic acid from *N*-acetyl-[6-^{3}H]mannosamine, and the 9-*O*-acetylation of this sialic acid. Mild periodate treatment would result in the release of [^{3}H]formaldehyde from [9-^{3}H]Neu5Ac, but not from [9-^{3}H]Neu5,9Ac$_2$. The [^{3}H]formaldehyde would be condensed into a yellow chromophore by reaction with two molecules of acetylacetone and one molecule of ammonia. The ^{3}H in question is indicated by an asterisk.

yellow chromophore 3,5,-diacetyl-1,4-dihydro-2,6-dimethyl pyridine (50). Therefore, if [^{3}H]formaldehyde is generated by periodate treatment, the label should be subsequently incorporated into this chromophore if the acetyl acetone reaction is carried out (see Fig. 5). This in fact proved to be the case. As shown in Fig. 6, the radioactivity from a [^{14}C]-formaldehyde standard comigrated on paper chromatography in System C with the yellow chromophore. When peak A (predicted to be [9-^{3}H]Neu5Ac) was treated with mild periodate and the products were subjected to the acetylacetone reaction, most of the radioactivity then comigrated with the yellow chromophore. Without periodate treatment the radioactivity comigrated perfectly with [4-^{14}C]Neu5Ac (not shown). As shown in Fig. 6, the reaction could be made to go to completion by adjustment of the pH of the periodate reaction to 6.4. Thus, peak A, which comigrates in two chromatography systems with [^{14}C]-Neu5Ac, is proven to be [9-^{3}H]Neu5Ac. Similar findings were obtained with the putative [9-^{3}H]Neu5Gc from myeloma cells (not shown).

Peak B has an O-acyl group in the 9 or 8 position. The data presented so far suggest that peak B is an *O*-acylated sialic acid. The fact that it is released by neuraminidase suggests that the *O*-acyl group is not in the 4 position, since such sialic acids are completely resistant to the neuraminidases used (3,6). To further identify the position of the *O*-acyl group, we

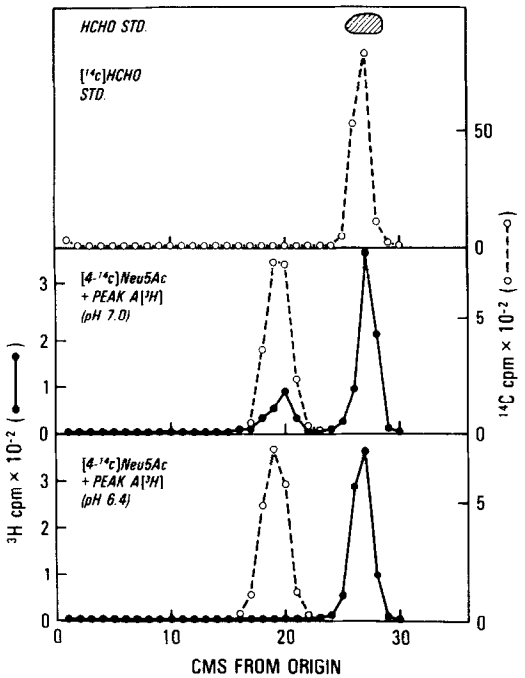

FIG. 6. Paper chromatography of the products of periodate treatment and/or acetylacetone reaction. Reaction mixtures were spotted on Whatman 3MM paper and chromatographed in System C until the front reached 30 cm. The upper panel shows the product of an acetylacetone reaction carried out on 10,000 cpm of [^{14}C]formaldehyde. The spot marks the position of migration of the yellow chromophore (see Fig. 5) generated by a similar reaction containing 500 nmol of unlabeled formaldehyde. The middle and lower panels are the products of treatment of aliquots of peak A (see Fig. 2) with mild periodate, followed by the acetylacetone reaction. The pH values indicate the conditions of the periodate treatment. In each case, 2000 cpm of [^{14}C]Neu5Ac was added *after* the addition of the arsenite reagent.

used the fact that the presence of such a group at the 8 or 9 position is known to hinder the action of mild periodate, and prevent the generation of [^{3}H]formaldehyde, whereas an *O*-acyl group at the 7 position should not block this reaction (see Fig. 5) (12,50). As shown in Fig. 7, the ^{3}H in peak B indeed could only be released as [^{3}H]formaldehyde after de-*O*-acylation. *O*-Acyl groups in the 8 position are extremely rare, presumably because they rapidly migrate to the 9 position. Thus, the *O*-acyl group in peak B is very likely to be in the 9 position. Of course, since *O*-acyl groups at the 7 position can also migrate (more slowly) to the 9 position (3,33,51), we cannot be certain that all of the *O*-acyl groups of peak B were in the 9 position originally. Since the periodate reaction can also be carried out on intact bound sialic acids with and without de-*O*-acetylation (12,50), it should be possible to use this approach to directly identify the existence of side-chain *O*-acetylated sialic acids on freshly isolated membranes, before any artifactual migration could have occurred. We are currently exploring this possibility.

Action of acyl neuraminate pyruvate-lyase. As an alternate approach to their identification, the radiolabeled compounds were treated with acylneuraminate pyruvate-lyase, which specifically cleaves sialic acids into acylman-

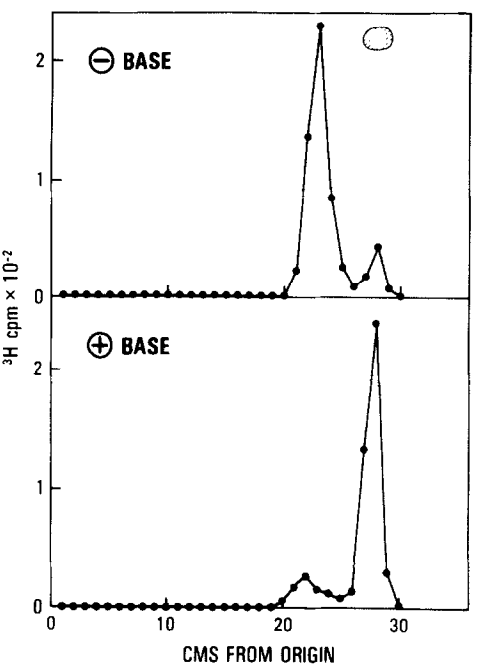

FIG. 7. Paper chromatography of products of periodate/acetylacetone reactions. Aliquots of peak B (see Fig. 2) were treated with mild periodate and the acetylacetone reaction with or without prior de-*O*-acetylation as indicated. The reactions were spotted directly on Whatman 3MM paper and chromatographed in System B until the front reached 30 cm. The spot indicates the position of migration of the yellow chromophore arising from a separate acetylacetone reaction utilizing 500 nmol of formaldehyde.

nosamines and pyruvate (7). These reactions were carried out with an internal [4-^{14}C]Neu5Ac standard; the expected products of the reaction would be [1-^{14}C]- or [6-^{3}H]acylmannosamines, and unlabeled pyruvate. As shown in Fig. 8, the neutral products of the reaction carried out with peak A comigrated with the ^{14}C product as expected. When the reaction was carried out with peak B about 30% of the radioactivity was converted to a neutral species under the conditions used whereas 90% of the ^{14}C standard became neutral. Since reduced reactivity with the acylneuraminate pyruvate-lyase (relative to Neu5Ac) is typical of *O*-acylated sialic acids (7). With more prolonged treatment and addition of more enzyme, further conversion could be obtained (data not shown). As shown in the lower panel of Fig. 8, the ^{3}H-labeled neutral product of this reaction consisted predominantly of a peak that ran ahead of the *N*-acetyl-[^{14}C]mannosamine standard. When this peak was isolated preparatively and treated with mild base, it shifted almost completely under the ^{14}C standard (not shown). This proves that the ^{3}H product is an *O*-acylated mannosamine, and provides further evidence that peak B is an *O*-acylated sialic acid.

The putative [9-^{3}H]Neu5Gc from the murine myeloma cells was also studied in this manner. Under the conditions used for the reaction, 31% of the ^{3}H radioactivity was converted to a neutral species whereas 80% of the ^{14}C standard became neutral. Such reduced reactivity with the acylneuraminate pyruvate-lyase (relative to Neu5Ac) is also known for Neu5Gc (7). In spite of this, as shown in the lower panel of Fig. 3, the neutral ^{3}H product of this reaction consisted almost exclusively of a peak that ran in the position of the *N*-glycolylmannosamine standard; none of the ^{3}H radioactivity comigrated with [^{14}C]-ManNAc product. This suggests that the 10% of ^{3}H radioactivity comigrating with Neu5Ac (see upper panel of Fig. 3) is not [^{3}H]Neu5Ac but some other unknown sialic acid resistant to the acylneuraminate pyruvate-lyase.

The O-acyl group is an O-acetyl group. More

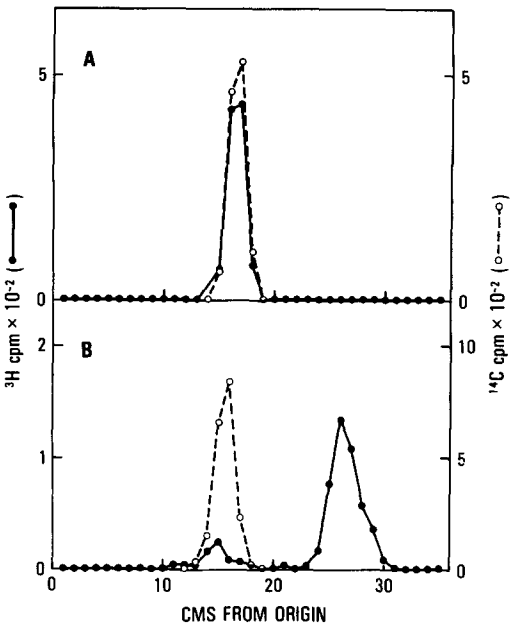

FIG. 8. Paper chromatography of the neutral products of acylneuraminate pyruvate-lyase reactions. Aliquots of peaks A and B (see Fig. 2) were treated with acylneuraminate pyruvate-lyase, and the neutral products were isolated as described under Methods. In each case an internal standard of [^{14}C]Neu5Ac was added prior to the addition of the enzyme. The products were spotted on Whatman 3MM paper and chromatographed in System A for 13 h. Strips of 1 cm were cut and monitored for ^{3}H and ^{14}C radioactivity. The ^{14}C peak is the expected [1-^{14}C]ManNAc product. The ^{3}H represents the neutral products of peak A (upper) and peak B (lower).

than one kind of *O*-acyl ester has been reported in the sialic acids (2,3). To further characterize peak B, it was studied by gas–liquid chromatography. Since the quantities of material were quite insufficient for positive identification by mass spectrometry, we carried out radioactive detection in the presence of an internal [^{14}C]Neu5Ac standard. Previous studies of this kind used collection by bubbling into scintillation fluid (52), or into plastic tubes that had to be attached and detached (53). Besides being cumbersome and posing a risk of finger burns, these methods gave a very low recovery of radioactivity. We have devised a simple method of collection that allows recovery of 40–60% of the radioactivity from the column (see Methods). As shown in Fig. 9 (left), the Me_3Si-

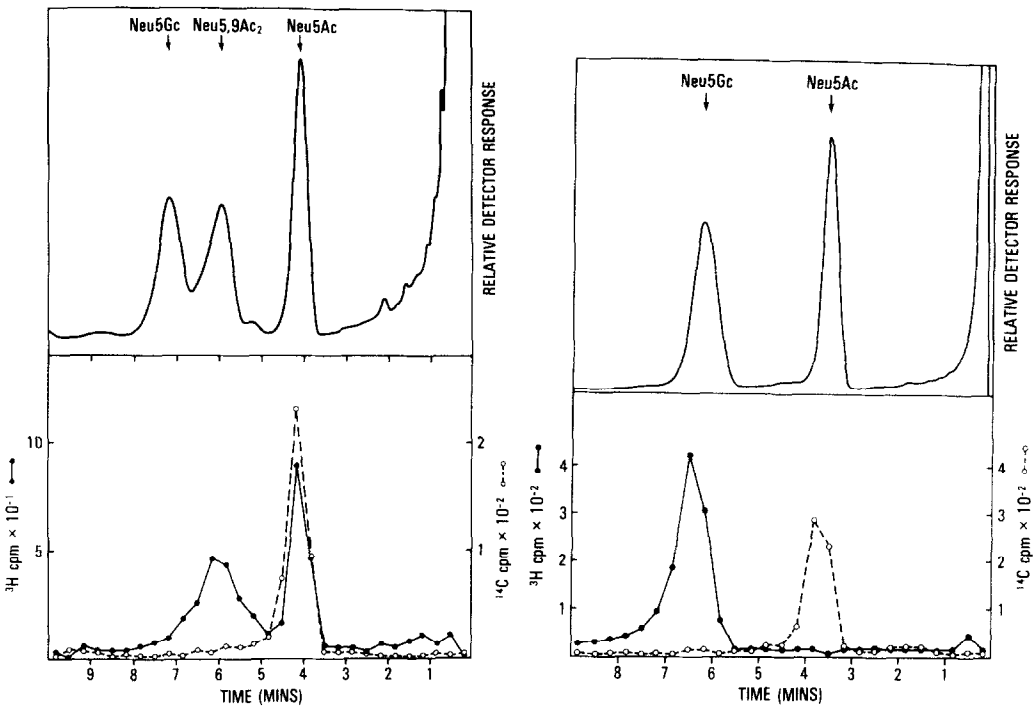

FIG. 9. Gas–liquid chromatography of sialic acids. 5000 cpm of peak B (see Fig. 2), 5000 cpm of [^{14}C]Neu5Ac, and 300 nmol of purified sialic acids from bovine submaxillary mucin were taken to dryness and derivatized in 10 μl of TSMI, and 3-μl aliquots were studied by gas–liquid chromatography with radioactive detection on a column of OV-17 (3%) as described under Methods (left). 5000 cpm of ^{3}H-sialic acids released from [6-^{3}H]ManNAc-labeled membranes of murine myeloma cells by neuraminidases, 5000 cpm of [^{14}C]Neu5Ac, and 150 nmol each of Neu5Ac and Neu5Gc were analyzed in exactly the same way (right).

ester, Me_3Si-ethers derivative of peak B coelutes with 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac$_2$). Since other *O*-acyl groups such as *O*-lactyl groups have marked effects on the retention time of the sialic acid in this system (28), it appears very likely that peak B contains an *O*-acetyl group. As shown in Fig. 9 (right), the major Me_3Si-ester, Me_3Si-ethers of the sialic acid from the murine myeloma cells coelutes with the Neu5Gc standard.

Development of high-pressure liquid chromatography methods for the analysis of sialic acids. Some HPLC methods for the separation of different sialic acids have been recently described (54–56). Since on-line detection of the sialic acids in the nanomole range is done by measuring uv absorbance in the 190–205 nM range, this requires extreme purity of the samples and reagents under study. Furthermore, methods that utilize buffer salts with high uv absorbance cannot be used to develop any new system. The availability of these various labeled sialic acid compounds has allowed us to devise two new HPLC methods for the separation of sialic acids. The first utilizes the principle of amine adsorption/ion suppression (34). An example of the separation of a complex mixture of labeled sialic acids (utilizing the pure compounds prepared from the labeled cells) is shown in Fig. 10. Internal standards such as [^{14}]Neu5Ac, [^{14}C]ManNAc, and [^{14}C]acetate (not shown) provide markers in case of slight variations from run to run [see also Ref. (57)]. The second method utilizes the ion-pair method between sialic acid and tetrabutylammonium formate to allow hydrophobic chromatography. An example is shown in Fig. 11. This method does not sep-

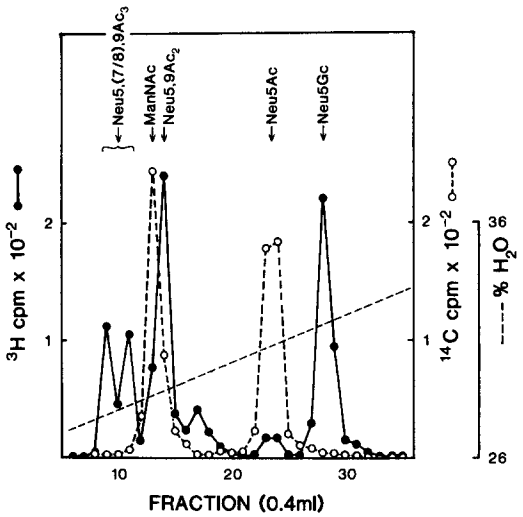

FIG. 10. Amine-adsorption:ion-suppression HPLC for separation of various sialic acids. Aliquots of Peaks B ($Neu5,9Ac_2$) and C [$Neu5(7/8)9Ac_3$] from the erythroleukemia cells, the major peak (Neu5Gc) from myeloma cells, and ^{14}C standards of Neu5Ac and ManNAc were mixed and separated on a Varian AX-5 Micropak column (30 × 0.4 cm) in System A as described under Materials and under Methods. The elution position of each was previously determined by separate runs of each labeled compound.

arate *N*-glycolyl- and *N*-acetylsialic acids, but allows separation according to the number of *O*-acetyl groups (i.e., increasing hydrophobicity). Because the basic principle of the method includes ion-pairing and hydrophobic interactions, we are hopeful that molecules such as *O*-methyl- and *O*-sulfate sialic acids will also be separable by this method. We are currently searching for such molecules in labeled tissue culture cell lines.

CONCLUSION

In this study, we have described an approach to the identification of various types of substituted and modified sialic acids in continuous tissue culture cell lines. This has provided model systems for the study of these compounds. Furthermore, it allows the identification of new types of sialic acids (for example, see peak E in FEL 745.6 cells and the minor sialic acid of the myeloma cells). Such unknown sialic acids can then be prepared in larger amounts for identification by more conventional means. We also show here that many of these labeled compounds can be identified by a combination of several different indirect methods, some of which make use of the fact that the ^3H label from [6-^3H]ManNAc is incorporated into the 9 position of the sialic acids. Of course, none of the individual methods can stand on their own in comparison with mass spectrometry or NMR for the positive identification of these radioactive tracer amounts of these compounds. However, we believe the combination of methods presented provides reasonably convincing proof of their identity. This then allows the use of such cell lines to study complex questions about the metabolism of the various types of sialic acids that cannot be studied in more conventional ways. For example, the murine myeloma, human melanoma, and murine erythroleukemia cells can now be used to carry out pulse-chase experiments. This will permit us to identify more directly the subcellular sites of sialic acid modification and substitution in monoclonal defined populations of cells. The cells with *O*-acetylation can also be used to directly study the question of whether *O*-acetylation of sialic

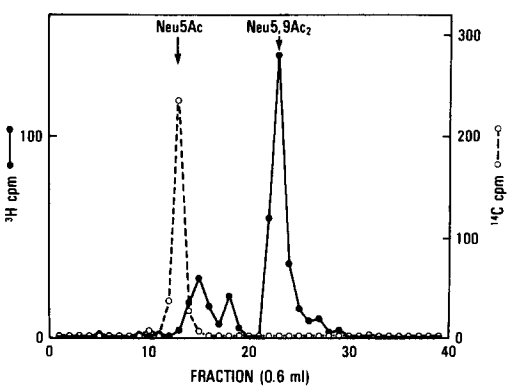

FIG. 11. Reverse-phase-ion-pair HPLC for separation of sialic acids. Aliquots of [4-^{14}C]Neu5Ac (99% pure), and peak B (shown in this study to be 90% [9-^3H]$Neu5,9Ac_2$) were mixed, dried down, brought up in 10 μl of 0.4% tetrabutylammonium formate, pH 4.5, and separated by HPLC in System B as described under Methods. Fractions of 0.6 ml were monitored for radioactivity.

acids might initially occur at the 7 position, with subsequent migration to the 9 position at the surface of the cells. Questions of gene regulation can be addressed by studying hybridomas between such cell lines that synthesize different types of sialic acids. The effects of induced differentiation of leukemia cells on the expression of *O*-acetylation can be explored. Such studies are currently under way.

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