Identification and Characterization of *N*-Acetyl-2,3-didehydro-2-deoxyneuraminic Acid as a Metabolite in Mammalian Brain†

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ABSTRACT: We have identified *N*-acetyl-2,3-didehydro-2-deoxyneuraminic acid (NADNA) in bovine and in rat brain. Identification was made by mass spectrometric and gas–liquid chromatographic analysis of the per(trimethylsilyl) derivative of the purified brain compound. Central nervous system NADNA hitherto has escaped detection; it behaves chromogenically and chromatographically during purification on ion-exchange chromatography as free *N*-acetylneuraminic acid (NANA) that also occurs in brain. Although NADNA is a dehydro analogue of NANA, we have ascertained that brain NANA does not give rise to NADNA as an artifact during its purification from brain. Three hours after intracranial injection of [^{14}C]-*N*-acetylmannosamine ([^{14}C]ManNAc), we detected [^{14}C]NANA but no [^{14}C]NADNA in rat brain. ManNAc is a brain NANA precursor, and at this time, formation of cytidine 5′-phosphate (CMP)–[^{14}C]NANA from [^{14}C]ManNAc is at a maximum. This finding precludes decomposition of CMP–NANA as a source of brain NADNA. Upon intracranial injection of [^{14}C]ManNAc, [^{14}C]NADNA became detectable at 19 h and reached a maximum level around 40 h later; this maximum of labeling of NADNA coincides with the maximum label in brain sialo conjugate–NANA. These findings clearly demonstrate the occurrence of NADNA in mammalian brain. From the evidence, NADNA may derive enzymatically from brain sialo conjugates.

N-Acetyl-2,3-didehydro-2-deoxyneuraminic acid (NADNA),[1] a dehydro analogue of *N*-acetylneuraminic acid (sialic acid; NANA), originally was known purely as a synthetic compound with a potent inhibitory effect on various sialidases (Meindl & Tuppy, 1969a,b; Miller et al., 1978; Corfield et al., 1981; Veh & Sander, 1981). Subsequently, NADNA was demonstrated to occur, along with NANA, in the urine of an unusual sialic acid excreting subject with sialuria; recently, NADNA has been found in nonnervous extracellular body fluids, e.g., urine, serum, and saliva of normal human subjects (Kamerling & Vliegenthart, 19755; Haverkamp et al., 1976). Thus far, NADNA has been observed only in these extracellular fluids. We demonstrate in this report that free NADNA occurs in bovine brain, a vertebrate organ rich in membrane sialo conjugates. We have found that NADNA is labeled metabolically in vivo upon intracerebral injection into rat of radioactive *N*-acetylmannosamine (ManNAc), the obligate metabolic precursor of NANA in rat brain (Quarles & Brady, 1971; Yohe & Rosenberg, 1977), and therefore, NADNA apparently is derived metabolically from NANA or NANA-containing compounds in vivo. These findings may help open the way to further study of the biological role of NADNA in the mammalian organism.

Materials and Methods

Animals and Materials. Twenty-day-old Sprague-Dawley rats were used for brain labeling experiments. Bovine brains 30 min after slaughter were obtained from the Aurora Packing Co., North Aurora, IL. The brains were brought to the laboratory on ice and kept frozen until use. [1-^{14}C]ManNAc (54.5 mCi/mmol) and [4-^{14}C]NANA (50 mCi/mmol) were purchased from New England Nuclear Corp., Boston, MA. NADNA was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. All other chemicals were reagent-grade quality.

Purification of Brain NADNA. Bovine brains (56.3 g) were homogenized in 170 mL of dioxane containing 20% water (v/v) at 0 °C in a Dounce homogenizer. The homogenate was filtered through glass wool. The residue on the glass wool was washed with 80 mL of dioxane containing 20% water (v/v). The filtrate was combined with the wash and filtered under suction. The precipitates were discarded. The filtrate was mixed with 250 mL of ethyl acetate. The water phase separating from the organic phase was collected and extracted twice with 100 mL of ethyl acetate. A white interphase between the lower, water and upper, organic phase was discarded. Turbidity that formed in the water phase was clarified by filtration through a sintered glass filter. The resulting clear solution was made up to 1 L by adding water. All procedures until this step were done on an ice bath. [4-^{14}C]NANA (0.3 μCi) was added to this solution as a marker for identifying the NANA fraction nondestructively and to monitor possible chemical change in the subsequent anion-exchange chromatography step. The solution was passed through a Dowex 1 column (carbonate form, 1.2 × 25 cm). This column was washed with 10 mL of water. A linear gradient of concentration of triethylammonium bicarbonate (pH 7.2, 0–0.05 M) was applied as the eluant. The total elution volume was 400 mL. Fractions of 6.55 mL of elution buffer were collected, and radioactivity of 200 μL of each fraction was determined by liquid scintillation spectrometry on a Beckman Model LS 7500 spectrometer. The fractions that contained more than 90 cpm of radioactivity were collected. The recovery of radioactivity from this column was 91.6%. The combined eluate was evaporated to dryness, and the dried residue was dissolved in 1 mL of 2-propanol–methyl acetate–water (2:2:1 v/v/v) and

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[1] Abbreviations: NADNA, *N*-acetyl-2,3-didehydro-2-deoxyneuraminic acid; NANA, *N*-acetylneuraminic acid; H_2-NADNA, *N*-acetyl-2-deoxyneuraminic acid; HPTLC, high-performance silica gel G thin-layer chromatography.

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chromatographed on a cellulose column (1.2 × 25 cm) previously equilibrated with this same solvent mixture used for dissolving the sample and for elution from the column. Each 1.3 mL of eluate was collected, and the radioactivity of 20 μL of each fraction was determined as described above. Aliquots (400 μL) of every second fraction were evaporated to dryness and subjected to silica gel G high-performance thin-layer chromatography (HPTLC). The HPTLC chromatograms were run along with authentic NANA and NADNA by using 2-propanol–methyl acetate–0.25% $CaCl_2$ in water (2:2:1 v/v/v) as the developing solvent. Orcinol–Fe^{3+}–HCl reagent (Bohm, et al., 1954) was used for detecting NANA and NADNA on the plate. Those fractions containing both NANA and NADNA by HPTLC were combined, evaporated to dryness, and further dried over P_2O_5 under reduced pressure overnight at room temperature. A part of the dried sample was trimethylsilylated with (trimethylsilyl)imidazole and subjected to gas–liquid chromatography using an OV-17 packed column as described by Kamerling & Vliegenthart (1975). Final identification of NADNA in the sample from bovine brain was done by gas–liquid chromatography/mass spectrometry analysis. A part of the sample after the cellulose chromatography was dissolved in 1 mL of water and applied on a charcoal column (0.5 × 1 cm). After the column was washed with 2 mL of water, NADNA and NANA were eluted with 2 mL of water containing 10% acetone (v/v) and 5% methanol (v/v). The latter fraction was dried under a continuous flow of dry N_2 gas in a tapped vial sealed with a Teflon-coated cap. (Trimethylsilyl)imidazole (50 μL) was added to the dried sample. After sonication of the sample in a sonicating water bath, it was incubated at 60 °C for 10 min. The derivatized sample was analyzed in a Finnigan Automated GC–MS Model 1020 mass spectrometer using a capillary column (OV-1701, 10 m, i.d. 0.25 mm, film thickness 0.2 μm). The mass spectrum was scanned from 60 to 700 amu, and the extracted ion profile for three characteristic mass fragments (186, 285, and 399) was obtained. The purification procedures used for bovine cerebrum and rat brain were identical.

Metabolic Labeling of NANA and NADNA in Rat Brains. Radioactive [1-^{14}C]ManNAc (10 μCi) was injected intracerebrally into 21-day-old rat pups according to the method described by Yohe & Rosenberg (1977). Two animals were decapitated 3 h after the injection. The brains were removed immediately and frozen at −80 °C until used. Other pairs of rats were decapitated at 19 and 40 h after the injection, and the brains were stored at −80 °C. The brains were thawed in ice-cooled dioxane containing 20% water (v/v). The procedure for extraction was the same as described for bovine brain except that the amounts of solvent were reduced in proportion to the relative wet weights of rat and bovine brain and filtrations were replaced by centrifugations (10000 rpm for 20 min at 2 °C in a Sorvall SS34 rotor). The distribution of radioactive label in each fraction is listed in Table I. The water phase was evacuated briefly to remove ethyl acetate and diluted to 10 mL with water. The diluted sample was chromatographed on a Dowex 1 column (carbonate form, 0.8 × 4 cm). After the column was washed with 30 mL of water, a linear concentration gradient of triethylammonium bicarbonate (pH 7.2) was applied as eluant (0–0.1 M). The total volume of elution buffer used for the linear gradient was 100 mL. After elution with the linear gradient, the column was eluted further with 1.0 M triethylammonium bicarbonate solution. Each 2 mL of eluate was collected. The radioactivity of 20 μL of each fraction was determined. The fractions in the second radioactive peak that were found to contain NANA by thin-layer chromatography were collected and evaporated to dryness for further analysis. The dried sample was dissolved in 50 μL of water. An aliquot of this solution (10 μL) was spotted on HPTLC plates with authentic NANA and NADNA as reference compounds. The chromatogram was developed in 2-propanol–methyl acetate–0.25% $CaCl_2$ (2:2:1 v/v/v). After detection of the authentic compounds on the plate by orcinol–Fe^{3+}–HCl spray reagent, the distribution of radioactivity in each 1 mm of silica gel scraped from the layer was determined. The thin-layer plate was divided into 1 mm wide strips from the origin to the solvent front. Each strip was extracted with 1 mL of water in a scintillation vial before adding 10 mL of scintillation mixture (Aquasol, New England Nuclear, Boston, MA).

Table I: Distribution of Radioactivity in Rat Brain Extract 3 and 40 h after Intracerebral Injection of [1-^{14}C]-*N*-Acetylmannosamine[a]

fractions	total radioact. (cpm) per fraction	
	3 h	40 h
total homogenate	5270000	2960000
fractions after removing the precipitate		
water phase	1890000	220000
interphase[b]	60000	29000
organic solvent phase	39000	2600

[a] Brains isolated at 3 and 40 h after injection were homogenized with dioxane containing 20% H_2O (v/v). After removal of the precipitate by centrifugation, an equal volume of ethyl acetate was added to the supernatant to separate the water phase from the organic phase containing sialoglycosphingolipids. [b] Thin film that formed between the phases.

Further purification was done by paper chromatography before subjecting the sample to hydrogenation. The chromatogram was developed in 1-propanol–methyl acetate–water (2:2:1 v/v/v) on Whatman No. 1 paper with authentic NADNA. The UV-absorbing spot comigrating with authentic NADNA on the chromatogram was extracted with water and divided. These two water solutions were dried under N_2 separately.

Hydrogenation. A dried sample was dissolved in 50 μL of methanol, and 1 mg of platinum oxide was added to this solution. Hydrogenation was done under hydrogen gas at high pressure (20 psi) for 7 h at 23 °C.

After the hydrogenation, the catalyst was sedimented by centrifugation and washed with 50 μL of methanol 5 times. The methanol solution of the product and the washes were combined and spotted on a HPTLC plate after newly adding authentic NADNA. The chromatogram was developed in 1-propanol–methyl acetate–0.125% $CaCl_2$ and 2.5% boric acid solution in water (2:2:1 v/v/v).

Results

Detection of NADNA in Bovine and Rat Brain. Since extracts prepared from bovine brain contained a great amount of material that makes direct analysis by gas–liquid chromatography impractical, ion-exchange chromatography and cellulose chromatography were required before detection of NADNA by gas–liquid chromatography. The fractions containing [^{14}C]NANA from Dowex 1 anion-exchange column chromatography (Figure 1A) were combined and subjected to cellulose column chromatography. As shown in Figure 1B, the fractions from cellulose chromatography contain two orcinol-positive (lilac) bands on high-performance thin-layer chromatography (fractions 30–48) plus another (blue) band. The upper and lower lilac bands corresponded to authentic NADNA and NANA, respectively. We did not try to identify the blue band. We could not separate NADNA from NANA

Table II: Interpretation of Some Important Fragment Ions Present in the Mass Spectrum of Trimethylsilylated NADNA Trimethylsilyl Ester

m/e	atomic formula	structural formula
651 (2.2)[a]	$C_{26}H_{57}NO_8Si_5$	$M^{+}\cdot$
399 (7.2)	$C_{17}H_{31}O_5Si_3$	$M^{+}\cdot - HOSi(CH_3)_3$, $\cdot CH_2OSi(CH_3)_3$, and NH_2COCH_3
358 (8.6)	$C_{14}H_{26}O_5Si_3$	$M^{+}\cdot - \cdot CHOSi(CH_3)_3 - CH_2OSi(CH_3)_3$, $\cdot NHCOCH_3$, and $2\cdot CH_3$
285 (100.0)	$C_{12}H_{21}O_4Si_2$	$M^{+}\cdot - \cdot CHOSi(CH_3)_3 - CHOSi(CH_3)_3 - CH_2OSi(CH_3)_3$ and NH_2COCH_3
217 (13.0)	$C_9H_{21}O_2Si_2$	$CH_2{=}COSi(CH_3)_3{-}CH{=}O^{+}Si(CH_3)_3$
205 (8.0)	$C_8H_{21}O_2Si_2$	$CH_2OSi(CH_3)_3{-}CH{=}O^{+}Si(CH_3)_3$
186 (37.0)	$C_8H_{16}NO_2Si$	$CH_3CO{-}{}^{+}NH{=}CH{-}CH{=}CHOSi(CH_3)_3$, $CH_3CO{-}{}^{+}NH{=}CH{-}COSi(CH_3)_3{=}CH_2$
147 (80.0)	$C_5H_{15}OSi$	$(CH_3)_2{}^{+}Si{-}OSi(CH_3)_3$
117 (18.2)	$C_4H_9O_2Si$	$(CH_3)_3Si{-}O{-}C(=O)^{+}$

[a] Numbers in parentheses are the intensities of the ions, relative to that of *m/e* 285.

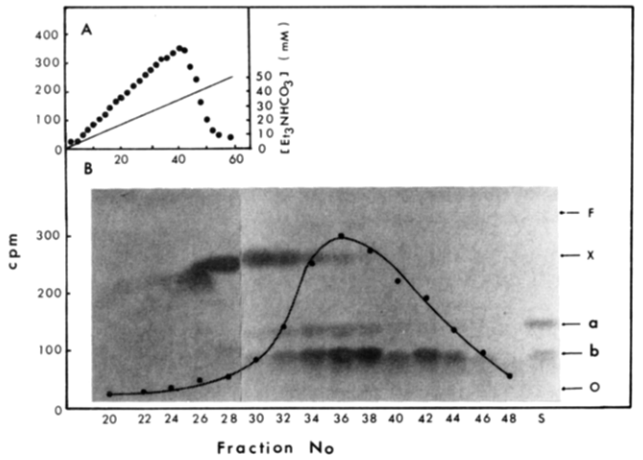

FIGURE 1: Elution profiles of bovine brain extract on Dowex 1 column chromatography and cellulose chromatography. (A) The brain extract was subjected to Dowex 1 column chromatography after mixing with [4-^{14}C]NANA for identifying the NANA fraction. A linear gradient of concentration of triethylammonium bicarbonate (Et_3NHCO_3, pH 7.20) was applied as the eluant. (B) The NANA fraction obtained from (A) was subjected to cellulose column chromatography as described under Materials and Methods. Aliquots of every second fraction were subjected to silica gel G HPTLC. Orcinol–Fe^{3+}–HCl reagent was used for detecting NANA and NADNA on the thin-layer plate. Chromatograms of materials from cellulose column chromatography and HPTLC were superimposed. The arrows F, X, a, b, and O show the positions of the solvent front, an unknown blue spot, NADNA, NANA, and the origin, respectively. Column S represents the authentic reference samples. The amounts of NANA and NADNA determined by densitometric analysis of this thin-layer plate were 34.2 and 10.1 nmol/g wet weight of bovine brain, respectively.

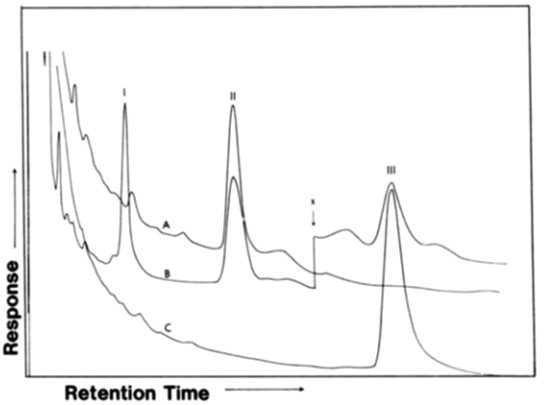

FIGURE 2: Gas–liquid chromatography on 3% OV-17 of the orcinol-positive fraction from cellulose column chromatography. Curves A and C show the gas–liquid chromatograms of trimethylsilyl derivatives of authentic NANA and NADNA, respectively. The chromatogram of the experimental sample (curve B) shows three main peaks. Peaks II and III correspond to the authentic trimethylsilyl derivatives and NANA and NADNA, respectively. Peak I is unknown. Arrow X shows the time point of a 4-fold decrease of attenuation.

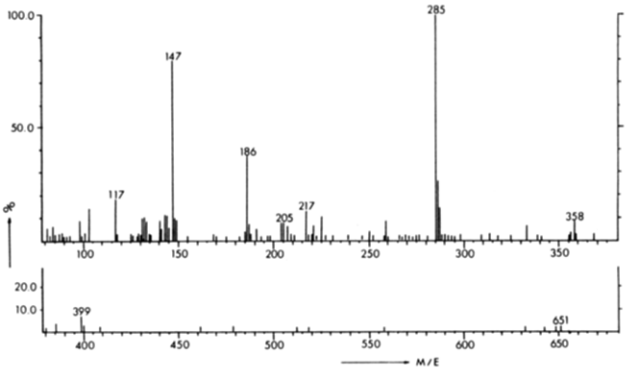

FIGURE 3: Mass spectrum at 65 eV NADNA trimethylsilyl ester. Only *m/e* values from 80 to 680 amu are given.

sufficiently by cellulose column chromatography. However, it enabled us to analyze the sample from bovine brain by gas–liquid chromatography by isolating NADNA and NANA from a large amount of interfering materials. All fractions that contained NANA or NADNA and both NANA and NADNA (fractions 30–48) were combined and dried under N_2. The dried residue was derivatized to the trimethylsilyl ether–trimethylsilyl ester as described under Materials and Methods. As shown in Figure 2, three main peaks were observed. Peak II and peak III correspond to the authentic trimethylsilyl derivatives of NANA and NADNA, respectively. Further identification of NADNA in the sample from bovine extract has been done by combined capillary gas–liquid chromatography–mass spectrometry after purification by charcoal column chromatography as described under Materials and Methods. The derivatized sample gave a main peak at the same retention time as the authentic per(trimethylsilyl) derivative NADNA. The mass spectrum (65 eV) of this peak is shown in Figure 3. The most characteristic mass values of the spectrum of this peak are shown in Table II with intensity and chemical assignments. Although there was a low intensity of fragmentation at a high mass range (over *m/e* 400), we observed a molecular mass at *m/e* 651 whose spectrum was identical with that given by the authentic derivative. Like bovine brain, we could detect NADNA also in samples of rat brain.

Formation of NADNA from [1-^{14}C]ManNAc. Radioactive ManNAc has been used as an obligatory precursor for radioisotopic labeling of NANA in sialo compounds in rat brain. Although a metabolic pathway leading to NADNA is not known, it appeared reasonable to employ [^{14}C]ManNAc for isotopic precursor labeling of this compound because of its structural relationship to NANA and the consequent probability that it is derived metabolically from the latter. It has been shown that the time sequence for maximum labeling of NANA by radioactive ManNAc in rat brain in vivo is different for various sialo compounds (Ferwerda et al., 1981; Yohe et al., 1980). The migration of radioactivity from ManNAc to CMP–NANA and then to polysialogangliosides (G_{T1b}, G_Q) has

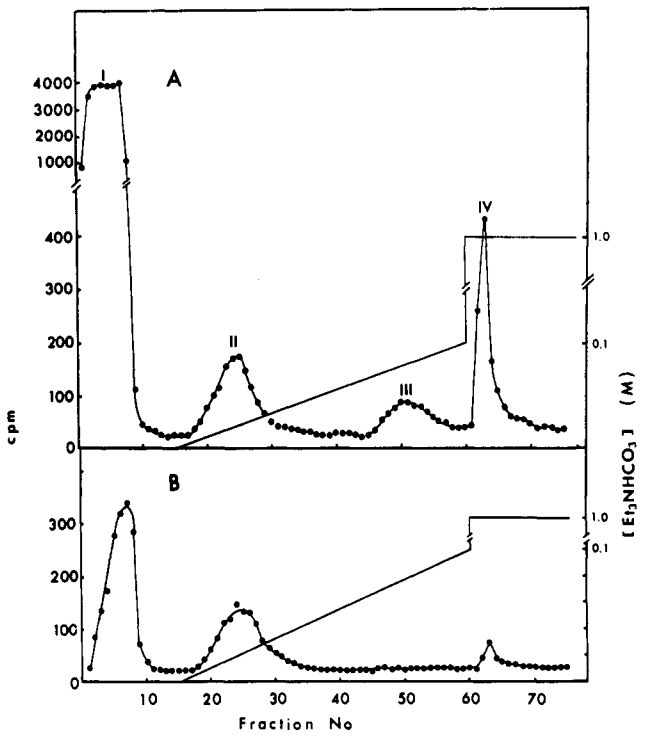

FIGURE 4: (A) Chromatogram on Dowex 1 of the extract from rat brain 3 h after injection of [1-^{14}C]ManNAc. (B) Chromatogram of the extract from rat brain 40 h after the injection of [1-^{14}C]ManNAc. [Peak I: (A) 1 658 000 cpm; (B) 110 000 cpm. Peak II: (A) 106 000 cpm; (B) 110 000 cpm. Peak III: (A) 56 000 cpm; (B) no peak. Peak IV: (A) 87 000 cpm; (B) 12 000 cpm.]

been reported to reach a maximum within a few hours after the injection of labeled ManNAc into rat brain (Yohe et al., 1980). In contrast, it requires over 30 h for maximum labeling of other gangliosides and sialoglycoproteins. We analyzed brain extracts obtained from 3 to 40 h after the injection of [^{14}C]ManNAc intracerebrally into rat brains. Anion-exchange chromatograms of the key extracts are shown in Figure 4. Peak II was found to be the fraction containing free NANA. The radioactivities in this peak obtained from both brain extracts were very similar for these two kinds of samples. However, the radioactivities in peaks I, III, and IV were reduced greatly in the brain extract obtained 40 h after injection, suggesting that the pool of free sialic acid in brain is characteristically in a more steady state than its related metabolites. Peak II was analyzed by HPTLC after adding authentic NANA and NADNA as carriers. In the chromatogram of peak II from the brain labeled for 40 h, radioactivity was observed in the area corresponding to the position of carrier NADNA, in addition to NANA (Figure 5). However, radioactivity was not observed in the area of NADNA on HPTLC of peak II from rat brain labeled for 3 h (Figure 6).

Hydrogenation of Metabolically Labeled NADNA. The double bond between carbons 2 and 3 of NADNA is known to be reduced by catalytic hydrogenation (Meindl & Tuppy, 1969). We employed this reaction to confirm further the identification of metabolically labeled NADNA. Peak II from the brain extract labeled for 40 h was purified further on paper chromatography and divided to two equal parts. One of them was hydrogenated as described under Materials and Methods. The reaction mixture was analyzed by HPTLC using an improved solvent system that gives good separation between NANA and NADNA. The other part was analyzed directly in the same HPTLC system at the same time. Although the sample purified on paper chromatography still showed some radioactivity in carrier NANA, the main radioactivity was

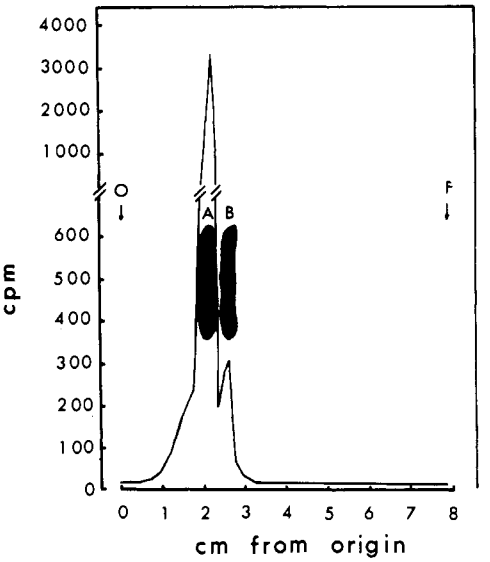

FIGURE 5: Silica gel high-performance thin-layer chromatograms of peak II in Figure 4B. The chromatograms were run along with (A) authentic NANA and (B) NADNA by using 2-propanol–ethyl acetate–0.25% $CaCl_2$ in water (2:2:1 v/v/v) as the developing solvent. O and F represent the origin and solvent front, respectively. Orcinol–Fe^{3+}–HCl reagent was used for detecting NANA.

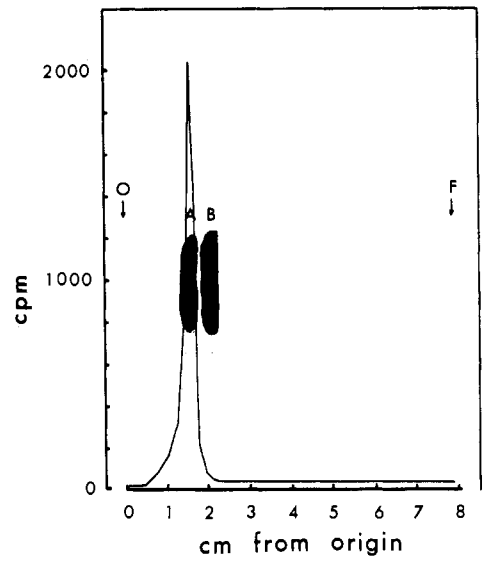

FIGURE 6: Silica gel high-performance thin-layer chromatograms of peak II in Figure 4A. The procedures, conditions, and symbols are the same as described in the legend to Figure 4.

localized with authentic NADNA. After being subjected to the hydrogenation reaction, the main radioactive peak shifted to the position below that of NANA where the reduced authentic NADNA also shifted (Figure 7).

Discussion

Detection of metabolic NADNA in mammalian organs that normally are rich in sialo compounds must be done carefully; otherwise, it is possible that NADNA may be formed as an artifact during the isolation process. It has been reported that CMP–NANA can degrade in vitro to this unsaturated sialic acid derivative (Beau et al., 1978). The procedure used in this study showed no artifactual production of this compound during the isolation procedure. Radioactive NADNA was not detected in rat brain labeled with radioactive ManNAc after 3 h, at which time the radioactivity of CMP–NANA is at a maximum (Ferwerda et al., 1981). We detected and chemically identified radioactive NADNA in rat brain 40 h after

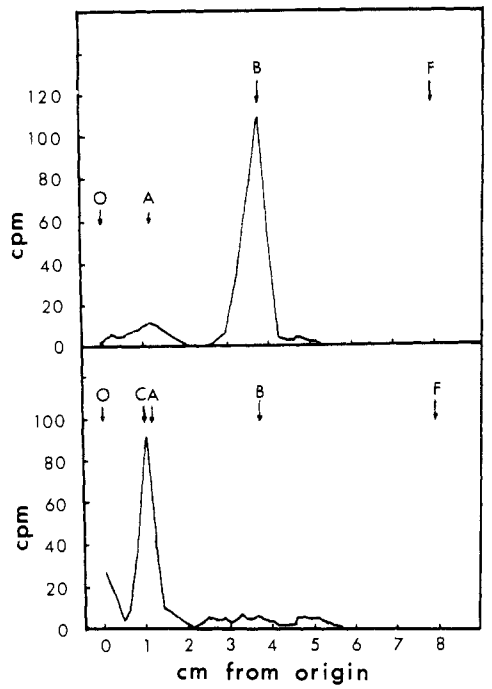

FIGURE 7: Silica gel high-performance thin-layer chromatograms of metabolically labeled NADNA before (top) and after (bottom) catalytic hydrogenation. Letters A, B, and C represent the positions of authentic NANA, NADNA, and hydrogenated authentic NADNA (H_2-NADNA), respectively. O and F represent the origin and solvent front, respectively. The chromatograms were developed in 1-propanol–methyl acetate–0.125% $CaCl_2$ and 2.5% boric acid in water (2:2:1 v/v/v).

the injection of radioactive ManNAc, at which time the specific activity of CMP–NANA is low and has been reported to be approximately one-tenth of that in brain labeled for a few hours (Ferwerda et al., 1981). We also found trace amounts of radioactivity in NADNA fractions 19 h after the injection (data not shown). The amount of radioactivity found in NADNA in this present study showed no correlation with the specific radioactivity of CMP–NANA. This finding suggests that, in vivo, NADNA is not formed directly from CMP–NANA in rat brain. Concerning the reported occurrence of NADNA in human body fluids, it has been postulated that the compound may be formed from CMP–NANA by an enzymatic or else a spontaneous reaction (Kamerling & Vliegenthart, 1975; Schauer, 1982). For the reasons stated above, it is unlikely that NADNA is formed from CMP–NANA by a spontaneous reaction in brain. It is still possible that the compound is formed enzymatically from CMP–NANA. However, in this case, a special metabolic pool of CMP–NANA must be hypothesized that is different from the pool into which the radioactive ManNAc precursor migrates directly after administration. The observations described in this report, coupled with the finding that NADNA is a potent competitive inhibitor for mammalian sialidases (Corfield et al., 1981; Veh & Sander, 1981) as well as bacterial sialidases (Meindl & Tuppy, 1969; Miller et al., 1978), lead us to speculate that this unsaturated neuraminic acid derivative may be a transition-state intermediate formed and released during the catabolism of gangliosides and sialic acid containing glycoproteins by sialidases. This speculation is supported by our preliminary observation that injected radioactive NADNA is incorporated directly into gangliosides and glycoprotein as NANA in rat brain (Saito & Rosenberg, 1983). The finding that free NADNA exists in brain, which is rich in synaptic membrane gangliosides and other sialo conjugates, makes it possible that NADNA may function as a biological inhibitor for intrinsic synaptic membrane sialidases (Yohe & Rosenberg, 1977), controlling the metabolism of membrane sialo conjugates.

Registry No. NADNA, 24967-27-9; NANA, 131-48-6.

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