Synthesis of 2′-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic Acid and Detection of Skin Fibroblast Neuraminidase in Normal Humans and in Sialidosis†

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ABSTRACT: A procedure for the synthesis of the fluorogenic substrate analogue 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid for the human acid neuraminidase has been developed. The substrate was employed for the characterization of the enzyme in sonicates of cultured human skin fibroblasts and for enzymatic detection of the neuraminidase deficiency in the neurological storage disorder, sialidosis. Synthesis was accomplished by reacting 2-deoxy-2-chloro-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminic acid methyl ester with the sodium salt of 4-methylumbelliferone in acetonitrile at room temperature. The coupled product was purified on silicic acid chromatography, followed by base-catalyzed removal of the *O*-acetyl and methoxy blocking groups, and with additional purification of the hydrolyzed product on silicic acid. The overall yield, based on *N*-acetylneuraminic acid, was 37%. Under linear assay conditions, at pH 4.3, the apparent maximal velocities (nmol (mg of protein)$^{-1}$ h^{-1}) for normal fibroblasts were 58–115, 0.2–1.8 for sialidosis fibroblasts, and 28–38 for obligate heterozygotes. The apparent K_m for normals was 0.13 mM.

Sialidosis is a hereditary lysosomal storage disease transmitted as an autosomal recessive trait, resulting from a profound deficiency of acid α-D-*N*-acetylneuraminidase (EC 3.2.1.18) (Cantz et al., 1977; Durand et al., 1977; Kelly & Graetz, 1977; O'Brien, 1978; Thomas et al., 1978a; Wenger et al., 1978). Near absence of neuraminidase activity accounts for storage in tissues and urinary excretion of massive amounts of sialyloligosaccharides (Cantz et al., 1977; Durand et al., 1977; Spranger et al., 1977; Strecker et al., 1977; O'Brien, 1978; Lowden & O'Brien, 1979). A detailed review summarizing many of the clinical and biochemical aspects of sialidosis has appeared (Lowden & O'Brien, 1979).

In addition to its involvement in sialidosis, human neuraminidases are of great interest because of their potential involvement in ganglioside metabolism (Venerando et al., 1975; Sandhoff & Pallmann, 1978), their elevated levels in viral transformed cells (Santer et al., 1978), and the presence of naturally occurring antibodies against desialated human lymphocytes (Rogentine, 1975). Detection and assays of human neuraminidase activity have been difficult due to the low levels of enzymatic activity and the lack of a suitable chromophoric substrate whose cleavage product can be sensitively detected with low background values. Most assays currently employed require long incubation times, which deviate from linearity or are extremely involved and complex (Ohman et al., 1970; Schauer et al., 1976). For this reason the diagnosis of sialidosis and the detection of carriers of the trait have been difficult. This has also limited the detailed characterization and analysis of the human enzyme.

We report here the preparation of the fluorogenic substrate, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid. This substrate was employed for the assay of the acid neuraminidase from cultured human skin fibroblasts. Conditions are given for the diagnosis of homozygotes and heterozygotes of sialidosis using this substrate.

Experimental Procedure

Reagents. Triton X-100 was obtained from Sigma Chemical Co., Zwittergent 314, an amphoteric surfactant, from Calbiochem-Behring Corp. and sodium taurocholate from Koch-Light Laboratories, Ltd. All other solvents were of reagent grade and were thoroughly dried and freshly distilled before use. Thin-layer chromatography (TLC) was carried out on silica gel 60, precoated-glass TLC plates (E. M. Laboratories, Inc.).

Fibroblast Culture. Skin fibroblasts were available from normal adults and children, sialidosis patients, and obligate heterozygotes and were grown as previously described (Okada et al., 1971). The clinical description of each patient has been reported previously and recently summarized (Lowden & O'Brien, 1979). Cells were harvested for assays 21–35 days after the second or third subculture.

Synthesis of 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic Acid. 2′-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4MU-NANA) was prepared by reacting 2-deoxy-2-chloro-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminic acid methyl ester with the sodium salt of 4-methylumbelliferone (4MU) in acetonitrile in the presence of silver carbonate followed by base-catalyzed removal of the acetyl and methoxy blocking groups.

(A) Preparation of 2-Deoxy-2-chloro-4,7,8,9-tetra-O-acetyl-N-acetylneuraminic Acid Methyl Ester. The 2-chloro derivative of acetylated NANA methyl ester was synthesized from natural α-D-*N*-acetylneuraminic acid (NANA) according to the following modification of the method of Kuhn et al. (1966). In a typical preparation, NANA (Sigma Chemical Co., Grade VI, *E. coli*; 0.97 g, 3.15 mmol) was esterified in dry methanol (100 mL) in the presence of a cation-exchange resin (Bio-Rad Laboratories, AG 50W-X8; 3.0 g, H^+ form) for 3 h at room temperature. The resin was removed with filtration and washed with additional methanol. The washes and filtrate were pooled, and the solvent was removed under vacuum. The isolated white solid, about 0.93 g or 91% yield, was estimated to be about 95% pure, giving a single major spot on TLC analysis in butanol/acetic acid/water (3/1/1, by vol.; solvent system A) when visualized with resorcinol–HCl reagent (Svennerholm, 1963).

Acetylation of the NANA methyl ester was carried out in 25 mL of acetic anhydride with 25 μL of $HClO_4$ as catalyst,

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[1] Abbreviations used: 4MU-NANA, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid; 4MU, 4-methylumbelliferone; NANA, α-D-*N*-acetylneuraminic acid; 4MU-peracetyl-NANA methyl ester, 2′-(4-methylumbelliferyl)-4′,7′,8′,9′-tetra-*O*-acetyl-α-D-*N*-acetylneuraminic acid methyl ester; TLC, thin-layer chromatography.

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at 45 °C for 3 h. Excess acetic anhydride was removed under vacuum and traces of anhydride in the crude product were destroyed by the addition of 50 mL of saturated NH_4Cl solution. The acetylated NANA methyl ester was extracted five times with 50 mL of chloroform. The chloroform extracts were pooled and washed twice with 25 mL of saturated $NaHCO_3$ solution and then dried over Na_2SO_4. The solvent was removed under vacuum and the residue dried over phosphorus pentoxide under vacuum overnight. Yield of the resulting colorless, viscous syrup was 1.54 g.

The crude acetylated methyl ester of NANA was converted to 2-deoxy-2-chloro-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminic acid methyl ester in 25 mL of acetyl chloride saturated (at −40 °C) with dry HCl by reacting in a sealed vessel at room temperature for 20 h. After careful removal of the excess solvent under N_2, the residue was dried under vacuum over phosphorus pentoxide for 2 h. Due to the extreme reactivity of this material, the following coupling reaction was carried out without delay.

(*B*) *Synthesis of 2′-(4-Methylumbelliferyl)-4′,7′,8′,9′-tetra-O-acetyl-α-*D*-N-acetylneuraminic Acid Methyl Ester (4MU-peracetyl-NANA Methyl Ester).* 4MU-peracetyl-NANA methyl ester was synthesized by reacting 2-deoxy-2-chloro-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminic acid methyl ester with 1.7 g (8.5 mmol, a threefold molar theoretical excess) of the sodium salt of 4MU and 2.0 g of Ag_2CO_3 in 35 mL of dry acetonitrile at room temperature overnight. The progress of the reaction was monitored by TLC in solvent system B, containing ethyl ether/methanol/ammonium hydroxide (100/10/1, by vol). TLC of the coupled product gave a major spot at R_f 0.26 and two minor spots at R_f 0.41 and 0.59, respectively, with the resorcinol–HCl spray. The reaction mixture was diluted with an equal volume of acetone; the insoluble material was removed with filtration and washed with additional acetone. The filtrate and washes were pooled and solvent was removed under vacuum.

The 4MU-peracetyl-NANA methyl ester was dissolved in a small amount of acetone and purified on a silicic acid column (2.5 × 55 cm, Unisil, 100–200 mesh, Clarkson Chemical Co.). Unreacted 4MU was eluted from the column with 400 mL of diethyl ether. The column was then eluted with a gradient of 550 mL of diethyl ether containing increasing amounts of acetone (550 mL). The progress of the column was monitored by TLC in solvent system B. The fractions containing the desired product were pooled and solvent was removed under vacuum. The yield of purified product was about 1.02 g or 50% based on NANA.

(*C*) *Removal of Blocking Groups and Final Purification.* Removal of the *O*-acetyl and methoxy groups was accomplished by methanolysis in dry methanol with sodium methoxide (6.3 mmol) for 24 h at room temperature. After removal of the excess methanol under vacuum, aqueous base hydrolysis at pH 12.0 was carried out by the addition of 5 mL of water. After 24 h at room temperature, the hydrolysate was adjusted to pH 6.0 with AG 50W-X8 resin, H^+ form and, after removal of the resin, the solvent was removed under vacuum.

The hydrolyzed material was purified on a silicic acid column (2.5 × 23 cm), eluted with a gradient of 250 mL of acetone containing increasing amounts of water (200 mL). The fractions containing the desired product (R_f 0.28 in solvent system A) were pooled and solvent was removed under vacuum. The product was suspended in 5.0 mL of water, and residual 4MU was removed by repeated extraction with an equal volume of freshly distilled ethyl acetate. The aqueous layer was adjusted to pH 8.0 with dilute NH_4OH and lyophilized. The yield was 0.57 g or 37% based on NANA as the ammonium salt. This material gave a single major spot on TLC in solvent system A. It gave the following optical rotation: $[\alpha]_D^{22}$ °C +59.8° (H_2O, pH 5.0). The compound was nearly quantitatively cleaved (greater than 96% of the theoretical) by *Clostridium perfringens* neuraminidase (Sigma Chemical Co.) indicating the α configuration of the ketosidic linkage.

Neuraminidase Assays. The fibroblast monolayer was washed three times with isotonic saline and scraped from the flask in isotonic saline. The fibroblast pellet was obtained by centrifugation at 1000*g* for 5 min at 4 °C. The pellet was suspended in distilled water (10 μL of water/mg of wet tissue weight) and then disrupted by sonication for 1 s by using a Technic International Sonifier (Technic International Co.).

Assays were conducted in 0.17 M sodium acetate buffer, pH 4.3, 1.3 mM 4MU-NANA, and 0.06–0.2 mg of sonicate protein in a total volume of 30 μL. Incubations were carried out at 37 °C in a shaking water bath for 15 min. The reaction was terminated with 1 mL of 0.085 M glycine–carbonate buffer, pH 9.3 and tubes were kept at 4 °C before reading fluorescence. Fluorescence was determined on a Turner fluorometer with excitation at 365 nm and emission at 450 nm (G. K. Turner Associates), by using 4MU (MCB Chemical Co.) as standard. Protein concentration was determined by the method of Lowry et al. (1951).

The average of duplicates is reported here with an average error between duplicates of ±5%. Assays were carried out on fibroblast sonicates within 6 h of harvesting. Freezing of pellets or sonicates and frozen storage at −20 °C resulted in losses of activity between 50 and 90%. Substrate could be stored in water containing 0.02% NaN_3 (to prevent growth of microorganisms) at 4 °C, at a concentration of 4 mM without increase in blank value over a 1-month period. When blank values for 4MU increased significantly, they were reduced by extracting aqueous solutions with freshly distilled ethyl acetate (three extractions of 3 volumes each). TLC revealed no losses of 4MU-NANA in the ethyl acetate extracts and nearly quantitative removal of 4MU. In one instance, ethyl acetate was used which apparently contained traces of acetic acid and water, produced upon long storage. This resulted in hydrolysis of 4MU-NANA with rapid release of 4MU, pointing to the extreme acid lability of 4MU-NANA.

Results

Characterization of 4MU-NANA. When 4MU-NANA, prepared as summarized in Figure 1, was analyzed by TLC, a weakly fluorescent spot at R_f 0.28 was detected when the plate was visualized under ultraviolet light (Figure 2). Small amounts of residual 4MU (R_f 0.9) and NANA (R_f 0.09) were detected as contaminants in the final product. Acid hydrolysis of 4MU-NANA was accomplished by spraying plates with sulfuric acid (9 N) spray at room temperature. The resultant cleavage of the ketosidic bond liberated the 4MU which greatly enhanced the fluorescence of the 4MU-NANA spot (Figure 2, lane 2). The 4MU-NANA spot also gave the heaviest stain when the chromatograms were sprayed with the resorcinol–HCl reagent (Figure 2, lane 3).

The amount of residual 4MU in the purified 4MU-NANA was quantitated by measuring fluorescence directly and comparing this to fluorescence obtained after complete acid hydrolysis of 4MU-NANA (0.1 N HCl, 80 °C, 15 min). The final product contained 0.2 mol % residual 4MU. Complete acid hydrolysis gave 93 mol % of the theoretical amount of 4MU released. The NANA content of the final product was

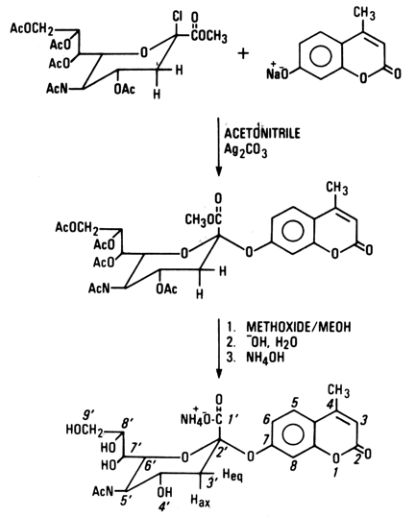

FIGURE 1: Reaction scheme for the preparation of 4MU-NANA. (Step 1) Coupling of 2-deoxy-2-chloro-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminic acid methyl ester with the sodium salt of 4MU. (Step 2) Methanolysis and aqueous hydrolysis of coupled product.

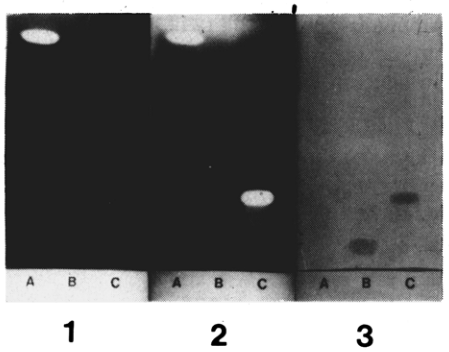

FIGURE 2: TLC analysis of purified reaction product. Solvent system A: butanol/acetic acid/water (3/1/1, by vol). (Panel A) 4MU; (panel B) NANA; (panel C) 4MU-NANA. (Lane 1) Plate visualized under ultraviolet light; (lane 2) plate visualized under ultraviolet light after treatment with sulfuric acid; (lane 3) plate visualized with resorcinol–HCl reagent.

Table I: NMR Assignments for 4MU-NANA[a]

assignment	chemical shift (ppm)	integration intensity (theor/exptl protons)
C_3'–H_{axial}[b]	2.04	not resolved
CH_3–*N*-acetyl	2.06	3.0/3.5
C_4–CH_3	2.38	3.0/3.1
C_3'–$H_{equitorial}$[b]	2.89	1.0/1.0
$C_{4'-9'}$–H	3.59–4.14	7.0/8.8
C_3–H	6.19	1.0/1.0
C_8; C_6–H[c]	7.13; 7.15	2.0/2.0
C_5–H	7.64	1.0/1.0

[a] Signal assignments were made relative to sodium 3-(trimethylsilyl)tetradeuteriopropionate in ^2H_2O at 22 °C. Due to the extreme acid lability of 4MU-NANA, TSP was added immediately prior to acquiring spectrum. Spectra were taken on a Varian HR-220 spectrometer. [b] Assignments were made on the basis of similar NANA derivatives previously assigned (Lutz et al., 1968). [c] Assignments were made on the basis of those of 4MU (Sadtler Standard Spectra, 1976).

98 mol % of the theoretical expected by using the thiobarbituric assay (Warren et al., 1959).

The NMR spectrum of 4MU-NANA was consistent with the expected structure. The assignments for the major signals were made by comparison with those of authentic NANA and 4MU (Sadtler Standard Spectra, 1976) and of other derivatives of NANA as assigned by others (Lutz et al., 1968).

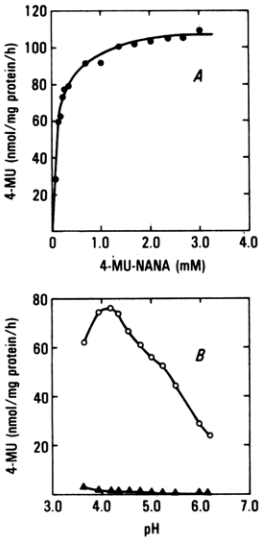

FIGURE 3: (A) Substrate dependence on activity. Standard assay conditions were employed except for variation in substrate concentration. (B) pH dependency of acid neuraminidase in sodium acetate buffer. (●) Normal fibroblasts; (▲) sialidosis, type 1, fibroblasts.

Table II: Neuraminidase V_{max} Values for Normal, Sialidosis, and Sialidosis Heterozygotes Fibroblasts

fibroblast type	fibroblast source	V_{max} (nmol (mg of protein)$^{-1}$ h^{-1})
normal	L.P.	75
normal	K.W.	75
normal	K.C.	58
normal	R.P.	72
normal	J.T.	67
normal	H.K.	67
normal	A.K.	115
sialidosis, type 1[a]	S.O.	1.8
sialidosis, type 1	De.B.	0.8
sialidosis, type 1	Do.B.	1.1
sialidosis, type 1	T.S.	0.6
sialidosis, type 1	F.G.	1.7
sialidosis, type 2[a]	J.R.	0.2
heterozygote	H.O.	31
heterozygote	E.O.	38
heterozygote	S.G.	28

[a] The phenotypic differences between sialidosis types 1 and 2 have been summarized by Lowden & O'Brien (1979).

These results are summarized in Table I.

Characterization of Fibroblast Neuraminidase, Normal and Sialidosis. The activity of the normal fibroblast acid neuraminidase was linear with sonicate protein (2–8 mg/mL) and with time for 30 min. The substrate dependence on activity was determined (Figure 3A) and saturation kinetics were observed with an apparent K_m of 0.13 mM.

The enzyme had an acid pH optimum at pH 4.4 (Figure 3B). There was an indication of a second optimum in normal subjects at pH 5.5. In sialidosis patients' cells, activity was strikingly deficient throughout the entire pH range (Figure 3B). In citrate–phosphate buffer, neuraminidase activity was one-half that in acetate buffer.

Routine assays were carried out at 1.3 mM 4MU-NANA for 15 min in 0.17 M acetate buffer, pH 4.3. The range of V_{max} values for normal fibroblasts was 58–115 nmol (mg of protein)$^{-1}$ h^{-1}. In contrast, the V_{max} values obtained for the sialidosis patients' cells were considerably lower, in the range of 0.2–1.8 nmol (mg protein)$^{-1}$ h^{-1} or about 0.3–2.4% of the normal mean. Values for obligate heterozygotes (parents of

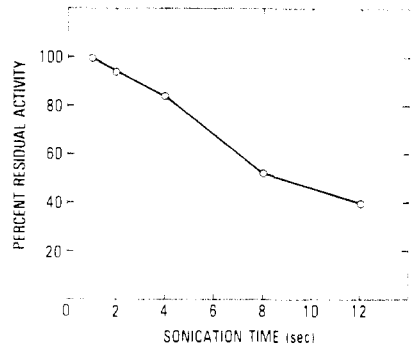

FIGURE 4: Effect of sonication on neuraminidase activity in normal skin fibroblasts. Fibroblasts were suspended in distilled water (10 μL/mg cultured cell weight) at 4 °C and irradiated as indicated. Standard assay conditions were employed.

probands) were intermediate (Table II).

The effect of various cell disruption techniques on the level of enzyme activity was evaluated. Brief sonic irradiation provided for optimal release of enzyme activity; activity obtained by freeze–thawing cell pellets in water three times in a dry ice bath or by hand homogenization with 20 strokes in a ground-glass Toenbrock homogenizer was 32% and 48%, respectively, of that obtained by sonication. However, activity was lost with prolonged sonication (Figure 4), leading to a 50% reduction in activity after 7–12 s.

The nonionic surfactant Triton X-100 has previously been shown to stimulate neuraminidase activity in bovine brain (Sandhoff & Pallmann, 1978) toward ganglioside G_{D1a}. In our hands, Triton X-100 at a concentration of 1 mg/mL inhibited by 50% the neuraminidase activity toward 4MU-NANA when present in the sonicate or when added to the incubation mixture after sonication. Zwittergent 314 and sodium taucholate were without effect upon activity at concentrations of 2–6 mg/mL when added at the sonication step.

Discussion

Preliminary reports of the utilization of 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid for the detection of neuraminidase activity have appeared (Potier et al., 1977; Mameli et al., 1978; Thomas et al., 1978b). However, the detailed procedure for the synthesis of this fluorogenic substrate analogue and its use in the detection of sialidosis has not been reported. The apparent synthesis of 4MU-NANA by a different approach from that employed here has recently become available (Thomas et al., 1978b). The reported physical characteristics for 4MU-NANA prepared by this method differed from that of the material obtained by the method described here. Most notably different were the specific optical rotation of the final product and its behavior on thin-layer chromatography. Unfortunately, due to the limited physical data available on this material, a more direct comparison of the two methods or the two products is difficult. It should be noted, however, that the 4MU-NANA prepared by Thomas et al. (1978b) was not tested as a substrate for the acid neuraminidase from human skin fibroblasts.

In general, the synthesis of ketosidic derivatives of α-D-*N*-acetylneuraminic acid has been limited to the coupling of 2-deoxy-2-chloro-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminic acid with Ag_2CO_3 to alcohols which were also employed as solvents (Tuppy & Gottschalk, 1972). Coupling with 4MU, here, was enhanced by its conversion to the sodium salt and by use of acetonitrile as solvent. The overall yield and product purity were greatly reduced when 4MU was employed or Ag_2CO_3 was omitted. Other solid chromophoric or fluorogenic alcohols which lack the required reactivity may be coupled by this approach.

The detection and diagnosis of sialidosis heterozygotes and homozygotes are facilitated by assay of acid neuraminidase with 4MU-NANA as substrate. Previously the primary analytical method for detection of sialidosis has been the identification of the excreted sialyloligosaccharides with TLC (O'Brien, 1978) combined with neuraminidase assay by using oligosaccharides containing NANA or with 3-methoxyphenyl-NANA (Lowden & O'Brien, 1979). The 4MU-NANA assay for acid neuraminidase is 10–50-fold more sensitive than prior assays. Our results demonstrate that the fluorogenic substrate analogue is highly specific for the acid neuraminidase which is deficient in sialidosis since the mutant cells are nearly totally deficient. Furthermore, heterozygote detection is made more accurate since activities can be measured when kinetics are still linear rather than under nonlinear conditions which are required for the less sensitive substrates. In addition, the availability of 4MU-NANA makes possible the detection of low levels of activity which are present in leucocytes and amniotic cells (J. S. O'Brien, unpublished data), and which would be obtained in purification of the enzyme. All in all, the availability of 4MU-NANA will make it possible to explore in more detail the molecular genetics of human acid neuraminidase.

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Proton Nuclear Magnetic Resonance Study of the Conformation and Configuration of the Cyclized Pyridine Nucleotide Adducts†

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ABSTRACT: We have closely examined by high-frequency ^{1}H nuclear magnetic resonance spectroscopy the structure of the adducts which form when various carbonyl compounds react with pyridine nucleotides at elevated pH. These studies show that the adducts of *N*-(2,6-dichlorobenzyl)nicotinamide–acetone, *N*-(2,6-dichlorobenzyl)nicotinamide–pyruvate, NMN–pyruvate, NAD–pyruvate, NAD–acetaldehyde, and NAD–oxaloacetate form with identical structural features as well as configuration. The following structural features are observed: (1) the adducts are pyridine N-4-substituted compounds; (2) a second six-membered ring forms by addition of the nicotinamide amido to the carbonyl group of the compound forming the addition complex; (3) cyclization occurs stereospecifically, indicating that the stereochemistry is predetermined by the initial attack at the N-4 position; (4) two diastereomeric forms are observed for each nucleotide adduct. Finally, the determination of configuration at all symmetric carbon atoms in these adducts will be discussed.

The nucleophilic addition of carbonyl compounds to NAD$^+$ has been extensively studied because of the important biochemical properties of the resulting adducts. These adducts are specific inhibitors of various dehydrogenases (Long & Kaplan, 1973; Everse et al., 1971, 1972), and they have been used as specific eluants for the purification of dehydrogenases by affinity chromatography (Lee et al., 1974; Kaplan et al., 1974). Furthermore, they are thought to be related to the abortive ternary complex of NAD$^+$, pyruvate, and heart type lactate dehydrogenase which serves a regulatory function in heart muscle (Everse et al., 1972; Arnold & Kaplan, 1974). Investigations into the chemical properties of these adducts by Burton & Kaplan (1954) have led to the proposal that the adducts originate from the nucleophilic attack by the α carbon of a carbonyl compound on the N-4 position of NAD$^+$. Subsequent studies of NAD$^+$ adducts (Burton et al., 1957; Dolin & Jacobson, 1964) and nicotinamide derivatives (Ludowieg et al., 1964) have substantiated this proposal.

In order to understand the origins of substrate specificity reflected in the specificity of inhibition by the NAD adducts, detailed knowledge of their chemistry and conformation is required. ^{1}H NMR provides a unique tool for such investigations since resonances of diastereomers are in principle nonequivalent (Mislow & Raban, 1966) and conformations can be determined from the angular dependence of vicinal coupling constants (Karplus, 1963; Gutowsky et al., 1959). Thus at high magnetic fields it is possible to resolve and assign the absorptions for specific diastereomeric forms of the adducts. From this information it is possible to determine the stereoselectivity of the reactions and the populations of the resulting forms, as well as the conformation and configuration of the adducts. In this study we provide ^{1}H NMR data for a number of biologically important NAD$^+$ adducts and related model compounds and determine their conformations as well as configurations.

Experimental Section

Materials

N-(2,6-Dichlorobenzyl)nicotinamide–Acetone Adduct. One gram of *N*-(2,6-dichlorobenzyl)nicotinamide (DCB-Nic[1]), prepared according to Krohnke & Ellegast (1956), was dissolved in 60 mL of acetone:water (1:1). To this solution was added 3 mL of a saturated solution of sodium carbonate. After 5 min, 1 volume of water was added and the reaction mixture was placed in the freezer at −20 °C, whereupon the DCB–Nic–acetone adduct crystallized to give a yield of 800 mg (66%).

N-(2,6-Dichlorobenzyl)nicotinamide–Pyruvate Adduct. Column purification of the DCB-Nic–pyruvate adduct was

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[1] Abbreviations used: NAD$^+$, nicotinamide adenine dinucleotide; NAD–pyruvate, NAD–acetaldehyde, and NAD–oxaloacetate are the pyruvate, acetaldehyde, and oxaloacetate adducts of NAD$^+$, respectively; Nic, nicotinamide, DCB, *N*-(2,6-dichlorobenzyl); DCB-nicotinamide–acetone and DCB-nicotinamide–pyruvate are the acetone and pyruvate adducts of DCB-nicotinamide, respectively; N-2, N-4, N-5, N-6, N-9, N-10ax, and N-10eq are the nicotinamide 2, 4, 5, 6, 9, 10 axial, and 10 equatorial protons, respectively, and A-2 and A-8 refer to the adenine 2 and 8 protons; TSP, sodium trimethylsilylpropionate-*2,2,3,3-d*$_4$; DSS, 4,4-dimethyl-4-silapentane-5-sulfonate; TMAC, tetramethylammonium chloride; Me_4Si, tetramethylsilane; EDTA, (ethylenedinitrilo)tetraacetic acid; forms *R* and *S* are the forms with an *R* and *S* configuration at N-4 of the adducts.

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