Interaction of *N*-Acetyl-4-epi-D-neuraminic Acid with Key Enzymes of Sialic Acid Metabolism†

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ABSTRACT: In spite of the axially orientated hydroxy group at C-4, the benzyl α-glycoside of *N*-acetyl-4-epi-D-neuraminic acid (4-epi-NeuAc) is a substrate for sialidases from *Vibrio cholerae*, *Clostridium perfringens*, and *Arthrobacter ureafaciens*, although to an extent which differs depending on the enzyme. Surprisingly, *V. cholerae* sialidase is by far the slowest acting enzyme; this is in contrast to its usual behavior. Fowl plague virus sialidase and bovine testis sialidase also cleave this glycoside slowly. 4-Epi-NeuAc is not a substrate for *N*-acetylneuraminic acid aldolase from *C. perfringens* but reversibly inhibits the enzyme with a K_i = 2.3 mM. The *N*-acetylneuraminic acid analogue is not converted to the corresponding CMP-glycoside by CMP-sialic acid synthase from bovine brain; however, it is an effective reversible inhibitor of the enzyme. The kinetic properties were analyzed with an assay system at pH 9 as well as an assay system at pH 7.5. The results from Dixon and Hanes plots did not agree. Therefore, no conclusions about the mechanism of the inhibition could be reached. This is the first reported sialic acid analogue which can act as an inhibitor of CMP-sialic acid synthase.

Sialic acids are a family of carbohydrates which are important for a multitude of biological recognition phenomena (Schauer, 1985). Studies on the correlation between chemical structure and biochemical function for the sialic acids are, therefore, of special interest. Synthetic sialic acid analogues have been used to probe the substrate specificity of the key enzymes involved in sialic acid metabolism (Corfield & Schauer, 1982; Gross et al., 1987). These results may finally lead to the tailored synthesis of inhibitors influencing specific steps in the metabolic pathway.

In sialic acids, C-4 near the anomeric center seems to play an important role in enzyme–substrate interactions. Thus, *N*-acetyl-4-*O*-acetylneuraminic acid, *N*-acetyl-4-*O*-methylneuraminic acid, *N*-acetyl-4-deoxyneuraminic acid, and *N*-acetyl-4-oxoneuraminic acid showed greatly altered properties toward sialidases,[1] NeuAc aldolase, and CMP-sialic acid synthase (Corfield & Schauer, 1982; Higa & Paulson, 1985; Corfield et al., 1986; Shukla & Schauer, 1986; Hagedorn & Brossmer, 1986; Gross & Brossmer, 1987; our unpublished results).

N-Acetyl-4-epineuraminic acid represents an additional analogue modified at C-4 which resembles neuraminic acid in that it contains all the essential parts such as the carboxy group, the equatorial acetamido group, and the trihydroxypropyl side chain. It only differs in having an axially oriented hydroxy group at C-4.

In this paper we report on the influence of a modification at C-4 for the action of several sialidases, NeuAc aldolase, and CMP-sialic acid synthase. 4-Epi-NeuAc proved to be a reversible inhibitor of NeuAc aldolase and the first reversible inhibitor of CMP-sialic acid synthase to be reported.

Materials and Methods

Materials

All chemicals used were of analytical grade and were purchased from Merck (Darmstadt) or Serva (Heidelberg). Crystalline *N*-acetylneuraminic acid was prepared in this laboratory (Czarniecki & Thornton, 1977). Cytidine 5′-monophosphate (CMP) was obtained from Boehringer (Mannheim), cytidine 5′-triphosphate from Biomol (Ilvesheim), and bovine serum albumin from Serva (Heidelberg). Grade E acetonitrile was obtained from Zinsser (Frankfurt). Cytidine 5′-(monophospho-*N*-acetylneuraminic acid) (CMP-NeuAc) was prepared enzymatically as described previously (Gross et al., 1987). 4-Methylumbelliferyl-α-NeuAc was synthesized in our laboratory. NeuAc aldolase (*Clostridium perfringens*, 5.5 units/mg) was obtained from Sigma (München), *Vibrio cholerae* neuraminidase (1 unit/mL) was from Behringwerke (Marburg), and *C. perfringens* sialidase (1 unit/mg), *Arthrobacter ureafaciens* sialidase (0.02 unit/mg), and lactate dehydrogenase (500 units/mg) were from Boehringer (Mannheim). Fowl plague virus (sialidase activity 5 units/mg) was kindly supplied by Prof. Rott (Giessen). Bovine testis sialidase was partially purified 500-fold, yielding an enzyme preparation (500 milliunits/mg) free of proteolytic activity (unpublished results).

Methods

Synthesis of 4-Epi-NeuAc (5-Acetamido-3,5-dideoxy-D-glycero-D-talo-nonulopyranosonic Acid). Benzyl α-glycoside of *N*-acetylneuraminic acid was converted to the diphenylmethyl ester and then to the 8,9-*O*-isopropylidene derivative. Reaction with trifluoromethanesulfonic anhydride then produced the 4-*O*-triflate; this rearranged spontaneously via the

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[1] Abbreviations: BSA, bovine serum albumin; DTE, dithioerythritol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; NeuAc, *N*-acetyl-D-neuraminic acid (5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid); benzyl-α-NeuAc, 2-benzyl-*N*-acetyl-α-D-neuraminic acid; 4-epi-NeuAc, 5-acetamido-3,5-dideoxy-D-*glycero*-D-*talo*-2-nonulosonic acid; benzyl-4-epi-α-NeuAc, 2-benzyl-5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*talo*-2-nonulosonic acid; 4-deoxy-NeuAc, *N*-acetyl-4-deoxy-D-neuraminic acid; 4-*O*-methyl-NeuAc, *N*-acetyl-4-*O*-methylneuraminic acid; 4-*O*-acetyl-NeuAc, *N*-acetyl-4-*O*-acetylneuraminic acid; CMP-NeuAc, cytidine 5′-(monophospho-*N*-acetylneuraminic acid); VCN, *Vibrio cholerae* neuraminidase. The following enzymes were used: CMP-sialic acid synthase (EC 2.7.7.43); sialidase, acetylneuraminyl hydrolase (EC 3.2.1.18); NeuAc aldolase, *N*-acetylneuraminate pyruvate-lyase (EC 4.1.3.3); lactate dehydrogenase (EC 1.1.1.27).

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4,5-oxazoline and afforded, after removal of the protecting groups, *N*-acetyl-4-epineuraminic acid. The 300-MHz ^1H NMR spectrum showed an unusually high α/β ratio (20/80) compared to that of the corresponding epimer at C-4 (6/94) (Villalva, 1987). The detailed synthesis will be described elsewhere. The same compound has been prepared by a different route (Baumberger & Vasella, 1986).

Protein Determination. Protein content was determined by the Bio-Rad protein assay with BSA as standard.

Analytical HPLC System. The HPLC system used was as described previously (Gross et al., 1987). CMP and CMP-NeuAc were measured at 275 nm (Gross et al., 1987) with an aminopropyl-phase column (0.4 cm × 8.0 cm, Serva).

Preparation of CMP-Sialic Acid Synthase. CMP-Sialic acid synthase from bovine brain was partially purified as described (Gross et al., 1987).

CMP-Sialic Acid Synthase Assay. The enzyme preparation used for kinetic studies contained a CMP-sialic acid synthase activity of approximately 200 milliunits/mg of protein. One unit is defined as the amount of enzyme producing 1 μmol of CMP-NeuAc/min at pH 9.0, under the assay conditions described previously (Gross et al., 1987); the synthase assay was also performed as reported in this paper.

The reaction mixture contained 1.5–1.8 milliunits of synthase, varying amounts of NeuAc or 4-epi-NeuAc, and routinely 10 mM CTP. Assays with various concentrations of CTP were performed as above with 10 mM NeuAc. Initial rates for kinetic studies were determined after 30-min incubation time at 37 °C. Samples (15–40 μL) of the final assay supernatant were analyzed by HPLC for the amount of CMP-NeuAc synthesized as described above. The extent of synthase inhibition by 4-epi-NeuAc was studied as follows. NeuAc was varied (0.6–10 mM) without inhibitor and in the presence of 0.25, 0.4, 2.0, 3.75, 7.5, or 15.0 mM 4-epi-NeuAc. In further experiments, concentrations of 4-epi-NeuAc were varied (0.1–10 mM) at fixed concentrations of NeuAc (0.6, 2.0, 5.0, and 10 mM). Additionally, CTP was varied (0.6–5 mM) in the absence and in the presence of 2 mM 4-epi-NeuAc.

Inhibition of CMP-sialic acid synthase by 4-epi-NeuAc at pH 7.5 was studied with a reaction mixture (200 μL) containing 160 mM Tris/HCl, pH 7.5, 7 mM $MnCl_2$, 1 mM DTE, 0.5 mg/mL BSA, 3.0–3.5 milliunits of synthase, 5 mM CTP, and various concentrations of NeuAc or 4-epi-NeuAc. Initial rates were determined after 30-min incubation at 37 °C. The extent of synthase inhibition by 4-epi-NeuAc was studied by varying the NeuAc concentration (0.6–5.0 mM), without inhibitor and in the presence of 0.5, 2.0, and 3.75 mM 4-epi-NeuAc. Additionally, concentrations of 4-epi-NeuAc were varied (0.5–6 mM) at fixed concentrations of NeuAc (1.0 and 2.0 mM). CTP was varied (0.6–3.0 mM) in the absence and in the presence of 2 mM 4-epi-NeuAc at 10 mM NeuAc. The K_m values of the enzyme for NeuAc or CTP, in the absence of inhibitor, were calculated from Hanes plots with an average of four to six kinetic measurements. Assays for the kinetics were performed in duplicate with at least five concentrations of substrate, or inhibitor, respectively.

Sialidase Experiments. The following buffers were used for the sialidase assay: *C. perfringens* sialidase and *A. ureafaciens* sialidase, 0.1 M sodium acetate, pH 5.5; VCN, 0.1 M sodium acetate/9 mM $CaCl_2$, pH 5.5; fowl plague virus sialidase, 0.1 M Tris/maleate, pH 5.4; bovine testis sialidase, 0.1 M sodium citrate/phosphate, pH 4.3.

Fluorometric Sialidase Assay. Sialidase activity was determined essentially as described by Potier et al. using the 4-methylumbelliferyl α-glycoside of NeuAc (Potier et al., 1979). The amount of 4-methylumbelliferone released was determined with an external standard (1 nmol). One unit is defined as the amount of enzyme cleaving 1 μmol of substrate/min. Inhibition of sialidases was investigated at 1 mM concentration of 4-methylumbelliferyl α-glycoside with or without 10 mM 4-epi-NeuAc and NeuAc, respectively.

Sialidase Incubation. Time course of sialidase action was followed in incubations (1 mL) containing 0.1 mmol of the appropriate buffer (see above), 1 μmol of benzyl α-glycoside of NeuAc or 4-epi-NeuAc, and the respective sialidase: VCN (200 milliunits), fowl plague virus sialidase (200 milliunits), *C. perfringens* sialidase (200 milliunits), or *A. ureafaciens* sialidase (100 milliunits). Assays with bovine testis sialidase (300 μL) contained 30 milliunits of enzyme and 1 mg/mL BSA. After appropriate times at 37 °C, aliquots (30–50 μL) were removed, and the amounts of NeuAc or 4-epi-NeuAc released were quantified by the thiobarbituric acid method (Warren, 1959). Corresponding controls were done in the absence of enzyme.

Spectrophotometric Assays. (*1*) *Coupled Enzymatic Assay.* The assay (1 mL) contained 200 μmol of KH_2PO_4, pH 7.2, 200 nmol of NADH, 5.5 units of lactate dehydrogenase, and 5 μmol of NeuAc or 10 μmol of 4-epi-NeuAc. The reaction was started by the addition of 20 milliunits of NeuAc aldolase; the absorption at 340 nm was measured for 30 min at room temperature.

(*2*) *NeuAc Aldolase Assay.* The assay was performed essentially as described (Elson & Morgan, 1933); the reaction mixture (200 μL) contained 20 μmol of KH_2PO_4/K_2HPO_4, pH 7.2, 24 μg of BSA, and differing amounts of NeuAc and 4-epi-NeuAc. After addition of 10 milliunits of NeuAc aldolase, tubes were incubated for an appropriate time at 37 °C. The reaction was terminated by the addition of 10 μL of 1 N NaOH and 40 μL of 0.8 M sodium tetraborate, pH 11; subsequently, tubes were heated at 95 °C for 3 min. After addition of 1.2 mL of Ehrlich reagent, the tubes were incubated for 20 min at 37 °C, and the absorption at 578 nm was measured. Corresponding controls were lacking enzyme. One unit is defined as the amount of enzyme producing 1 μmol of *N*-acetylmannosamine/min at 12 mM NeuAc concentration under the assay conditions described above. The amount of *N*-acetylmannosamine formed was calculated in reference to an external standard. Absorption was linear with *N*-acetylmannosamine from 10 to 40 μg per 200-μL assay mixture.

Initial rates for the kinetics were determined after 15-min incubation. The extent of enzyme inhibition by 4-epi-NeuAc was studied as follows: first, NeuAc was varied between 1.25 and 10 mM without inhibitor and in the presence of 1.8 and 4.5 mM inhibitor; second, the concentration of the inhibitor was varied between 0.9 and 6.5 mM at fixed concentrations of NeuAc (1.5, 2, 3, 4, and 6 mM). Assays were performed in duplicate.

RESULTS

Studies with Sialidases. The susceptibility of benzyl α-glycosides of 4-epi-NeuAc to different sialidases was investigated with the thiobarbituric acid method. Figure 1 shows the time course for cleavage of benzyl α-glycosides of NeuAc and 4-epi-NeuAc by several bacterial sialidases. Surprisingly, the α-glycoside of 4-epi-NeuAc was slowly attacked by these enzymes. After 22 h, cleavage by *C. perfringens* sialidase was complete, by *A. ureafaciens* sialidase about 50%, and by *V. cholerae* sialidase only by about 11%. In contrast, NeuAc was completely liberated from its benzyl α-glycoside by each enzyme after 1 h (Figure 1). The sialidase from fowl plague virus

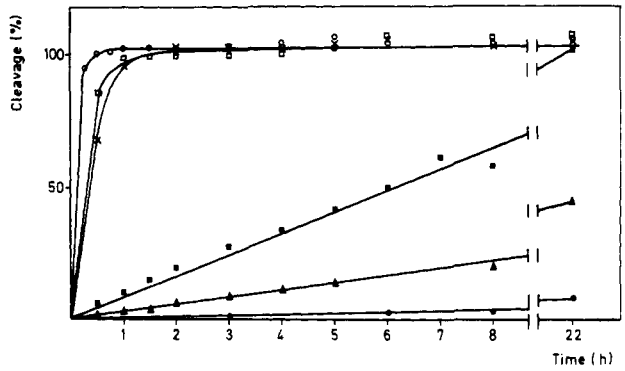

FIGURE 1: Time courses for release of NeuAc or 4-epi-NeuAc from the corresponding benzyl α-glycoside by bacterial sialidases. *C. perfringens* sialidase (200 milliunits): benzyl-α-NeuAc (○); benzyl-α-4-epi-NeuAc (■). *V. cholerae* sialidase (200 milliunits): benzyl-α-NeuAc (×); benzyl-α-4-epi-NeuAc (●). *A. ureafaciens* sialidase (100 milliunits): benzyl-α-NeuAc (□); benzyl-α-4-epi-NeuAc (▲). Assays were performed as described under Methods.

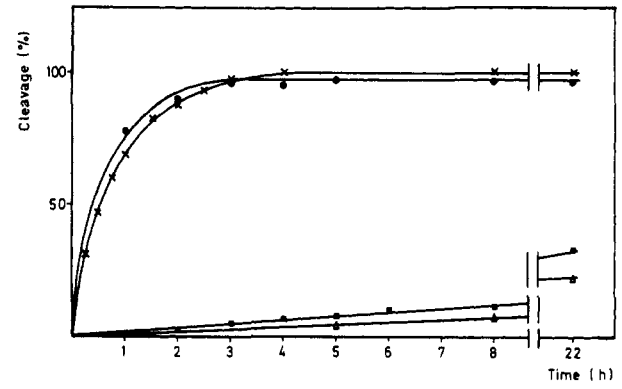

FIGURE 2: Time courses for release of NeuAc or 4-epi-NeuAc from the corresponding benzyl α-glycoside by fowl plague virus and bovine testis sialidase. Fowl plague virus sialidase (200 milliunits): benzyl-α-NeuAc (×); benzyl-α-4-epi-NeuAc (■). Bovine testis sialidase (30 milliunits): benzyl-α-NeuAc (●); benzyl-α-4-epi-NeuAc (△). Assays were performed as described under Methods.

cleaved benzyl-4-epi-NeuAc by about 35% after 22h, whereas NeuAc was completely released from the respective glycoside after 3 h (Figure 2).

Figure 2 shows the time course for interaction of bovine testis sialidase with both substrates. After 22 h about 20% of the 4-epi-NeuAc was liberated, whereas release of NeuAc was complete after 4 h.

Inhibition of different sialidases by 4-epi-NeuAc was investigated with the 4-methylumbelliferyl α-glycoside of NeuAc as substrate. With a 10-fold excess of 4-epi-NeuAc compared to the fluorescent substrate (1 mM), *V. cholerae* sialidase was not inhibited. The sialidases from *C. perfringens* and fowl plague virus, however, were inhibited by 36% and 40%, respectively. At the same concentration, inhibition by NeuAc was about 15–20%.

Studies with NeuAc Aldolase. 4-Epi-NeuAc was not a substrate for NeuAc aldolase from *C. perfringens* as determined by the coupled enzymatic assay. However, this NeuAc analogue proved to be a reversible inhibitor of NeuAc aldolase. After dialysis of an enzyme–inhibitor mixture, activity was completely restored.

For determination of inhibition pattern, and K_i value, the reaction rate was obtained from the amount of *N*-acetylmannosamine formed by a modified Morgan-Elson assay. The effect of incubation time on the reaction rate was measured both in the presence of 4.5 mM 4-epi-NeuAc and in the absence of inhibitor. Product formation was linear with time

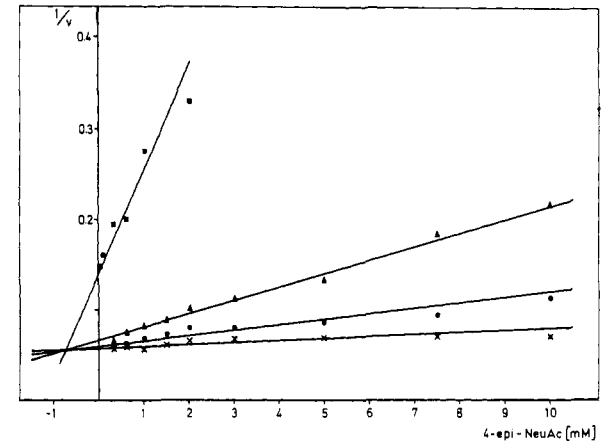

FIGURE 3: Initial rate kinetics for inhibition of CMP-sialic acid synthase from bovine brain by 4-epi-NeuAc plotted according to Dixon (Dixon & Webb, 1964). Assays were performed as described under Methods. Concentrations of 4-epi-NeuAc (0.2–10 mM) were varied at four fixed concentrations of NeuAc: 0.6 (■), 2.0 (▲), 5.0 (●), and 10.0 mM (×).

for at least 20 min in assays containing 10 milliunits of aldolase (2.4 μg of protein) and 1.0 or 10 mM NeuAc. Initial rates were determined after 15-min incubation as described under Methods.

Initial rate data for inhibition of NeuAc aldolase by 4-epi-NeuAc were plotted according to Lineweaver–Burk and Dixon (Dixon & Webb, 1964). As expected, a competitive inhibition pattern was obtained in both plots. The K_i value obtained from the Dixon plots was 2.3 mM; a comparable K_i value of about 2.4 mM was calculated from Lineweaver–Burk plots. The K_m value of NeuAc aldolase for NeuAc was calculated from an average of five Lineweaver–Burk plots as 4.2 mM, similar to the previously published values (Comb & Roseman, 1960; Nees et al., 1976). The K_i value agreed with the inhibition data obtained by the coupled enzymatic assay.

Studies with CMP-Sialic Acid Synthase. Assay conditions for CMP-sialic acid synthase were those described by Kean and Roseman (1966). 4-Epi-NeuAc was not activated to a corresponding CMP glycoside by CMP-sialic acid synthase from bovine brain, even though amounts of enzyme (3 milliunits) and incubation times (2 h) were increased compared to those in the standard assay described under Methods.

In contrast, this analogue reversibly inhibited the synthase. After dialysis of an enzyme–inhibitor mixture, activity was restored compared to that of the enzyme dialyzed without inhibitor. Preincubation of the synthase with inhibitor did not increase inhibition. Activity of the enzyme at pH 9 was stable during preincubation at 37 °C for up to 4 h in the presence of 1 mM 4-epi-NeuAc and 10 mM CTP. In contrast, activity decreased strongly without inhibitor, reaching 12% after 4 h though CTP (10 mM) was present at saturating concentrations.

Synthase activity at 10 mM NeuAc was linear with protein concentration over a wide range (Gross et al., 1987). Formation of CMP-NeuAc was linear also with incubation time for at least 45 min in assays containing 0.6 mM or 5.0 mM NeuAc and 1.8 milliunits of enzyme. This was additionally measured in the presence of 0.5 or 5.0 mM 4-epi-NeuAc.

The Dixon plots of initial rate data show straight lines intersecting in accordance with competitive inhibition (K_i value about 0.8 mM) (Figure 3). Hanes plots, however, obtained with various concentrations of NeuAc at fixed inhibitor concentrations (Figure 4), were not in accordance with a competitive mechanism, or any other common inhibitory pattern. The apparent K_m value of the synthase for NeuAc calculated

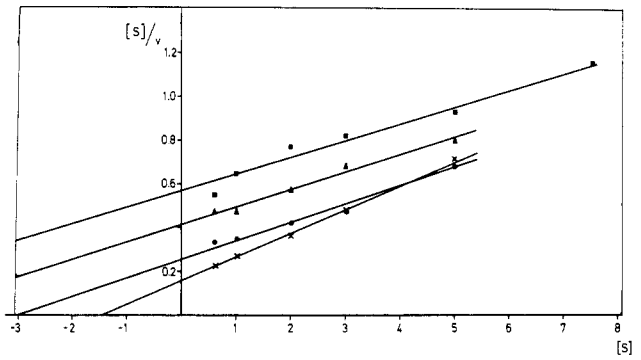

FIGURE 4: Initial rate kinetics for inhibition of CMP-sialic acid synthase from bovine brain by 4-epi-NeuAc plotted according to Hanes (Dixon & Webb, 1964). Assays were performed as described under Methods. Concentrations of NeuAc (0.6–8 mM) were varied without inhibitor (×) and at three fixed concentrations of 4-epi-NeuAc: 0.4 (●), 7.5 (▲), and 15 mM (■). Each point represents the average of duplicate assays. The kinetics were calculated by linear and nonlinear regression analysis. Both gave identical results.

Table I: Apparent K_m Values of CMP-Sialic Acid Synthase for NeuAc at pH 9.0 and 7.5 in the Presence of Fixed Concentrations of 4-Epi-NeuAc

	apparent K_m value (mM)[a]	
[4-epi-NeuAc] (mM)	at pH 9.0	at pH 7.5
	1.4	0.80
0.25	2.4	
0.5	3.15	1.0
2.0	5.6	2.0
3.75	6.7	2.9
5.0	7.15	
7.5	14.55	
15.0	29.65	

[a] K_m values were calculated from Hanes plots by linear and nonlinear regression analysis.

from Hanes plots increased significantly with the concentration of 4-epi-NeuAc (Table I). From the Hanes plots alone, the mechanism of inhibition cannot be deduced although kinetics were calculated by linear and nonlinear regression analysis.

Hanes plots obtained with various concentrations of CTP at 10 mM fixed NeuAc concentration, with or without inhibitor (2 mM), yielded an identical apparent K_m value for CTP. The concentration of $MgCl_2$ (5–60 mM) did not influence the extent of synthase inhibition by 4-epi-NeuAc.

Additionally, inhibition studies with 4-epi-NeuAc were performed at a more physiological pH of 7.5. The assay system was based on that described by Higa and Paulson for calf brain synthase (Higa & Paulson, 1985). At pH 7.5, CMP-sialic acid synthase from bovine brain required manganese ions for optimal activity, with maximal stimulation between 5.5 and 8.5 mM. With the assay conditions described, formation of CMP-NeuAc at 10 mM NeuAc was linear with enzyme concentration up to 7.6 milliunits/0.2 mL. Formation of CMP-NeuAc was also linear with time for at least 45 min in assays containing 0.6 or 5.0 mM NeuAc and 3.5 milliunits of synthase. The K_m value of the CMP-sialic acid synthase for CTP, determined at saturating concentrations of NeuAc (10 mM), was found to be 0.5 mM. The kinetic plot showed significant substrate inhibition above 6.0 mM CTP. A K_m value of 0.8 mM was measured for NeuAc at saturating concentrations of CTP (5.0 mM), which is slightly lower than the value of 1.4 mM measured at pH 9 (Gross et al., 1987). The V_{max} value at pH 7.5 was only 40% of the value obtained at pH 9.0. Inhibition kinetics with 4-epi-NeuAc at pH 7.5 were performed at saturating concentrations of CTP (5.0 mM). The kinetic data at pH 7.5 were comparable to those obtained at pH 9 (Table I). In contrast to pH 9, the synthase was stable during preincubation at pH 7.5 for up to 4 h in the absence or in presence of 4-epi-NeuAc.

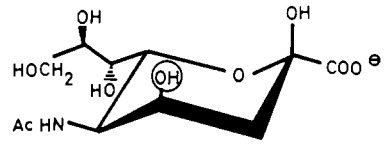

FIGURE 5: Structure of *N*-acetyl-4-epineuraminic acid.

Discussion

The enzymatic action of the sialidases is strongly influenced by the substituent at C-4 of the sialic acid molecule. Bacterial sialidases, especially, require an unsubstituted hydroxy group at C-4 since they are inactive toward the α-glycosides of 4-*O*-acetyl-, 4-*O*-methyl-, 4-deoxy-, and 4-oxo-NeuAc (Corfield & Schauer, 1982; Corfield et al., 1986; Baumberger et al., 1986; Gross & Brossmer, 1987; unpublished results). The viral and mammalian enzymes, on the other hand, release 4-*O*-methyl-NeuAc and 4-deoxy-NeuAc from α-glycosidic linkage (Corfield & Schauer, 1982; Hagedorn & Brossmer, 1986).

N-Acetyl-4-epineuraminic acid was also found to be suitable as a compound for exploring substrate properties of NeuAc analogues modified at C-4, as the only difference to the parent molecule is an axial hydroxy group instead of an equatorial one (Figure 5). Surprisingly, studies performed with the benzyl α-glycoside of 4-epi-NeuAc showed for the first time significant differences among bacterial sialidases with respect to the steric environment of C-4. The very slow release of 4-epi-NeuAc from its benzyl glycoside by fowl plague virus and bovine testis sialidases is in contrast to the results obtained for α-glycosides of 4-deoxy- and 4-*O*-methyl-NeuAc (Beau & Schauer, 1980; Hagedorn & Brossmer, 1986). Thus, an axially orientated hydroxy group at C-4 strongly influences the enzyme–substrate interaction of each sialidase, reducing the cleavage rate to a different extent for each.

Interestingly, sialidases of fowl plague virus and *C. perfringens* are more strongly inhibited by 4-epi-NeuAc than by NeuAc itself. This observation may be explained by the higher α/β anomer ratio of 4-epi-NeuAc (20/80). It has been shown that the first product from the action of sialidases is the α-anomer of NeuAc, which then mutarotates to give the final equilibrium mixture with an α/β ratio of about 6/94 (Friebolin et al., 1980a,b). For NeuAc aldolase from *C. perfringens*, a mechanism of action has been proposed (DeVries & Binkley, 1972a; Nees et al., 1976; Barnett et al., 1971). The catalytic process is initiated by the transfer of a proton from the hydroxy group at C-4 of NeuAc to an imidazole residue at the active center. Such a step is not possible with 4-epi-NeuAc due to the axial orientation of this hydroxy group. In contrast to 4-*O*-acetyl-NeuAc and 4-*O*-methyl-NeuAc, which are neither substrates nor inhibitors of NeuAc aldolase (Corfield et al., 1982), significant competitive inhibition was observed with 4-epi-NeuAc. The K_i value (2.3 mM) was slightly lower than that for pyruvate (3.0 mM), indicating a contribution of the C-4 to C-9 portion of 4-epi-NeuAc to substrate recognition (Nees et al., 1976; Barnett et al., 1971). Whereas 3-fluoro- and 3-hydroxy-NeuAc were described as irreversible inhibitors of NeuAc aldolase (Gantt et al., 1964; DeVries & Binkley, 1972), 4-epi-NeuAc is the first reversible inhibitor to be described which is based on the structure of NeuAc. Until now no inhibitors of CMP-sialic acid synthase have been described which are structurally related to NeuAc. Compounds capable of blocking the attachment of terminal sialic acid to a nascent glycoconjugate would be of especially great value in investi-

gating the influence on cell surface structures which are involved in signal transfer and tumor metastasis. Thus in this context, blocking or decreasing the synthesis of CMP-activated sialic acids in vivo would represent a suitable approach.

In contrast to 4-deoxy-NeuAc and 4-*O*-methyl-NeuAc, the axially orientated hydroxy group in 4-epi-NeuAc apparently does not allow a spatial interaction with CMP-sialic acid synthase, and therefore, the derivative is not converted to its CMP glycoside. To our surprise, however, this NeuAc analogue proved to be a reversible inhibitor of the enzyme from bovine brain.

The kinetics were first performed by application of an assay system at pH 9 based on that described by Kean and Roseman (1966) to relate the results to earlier studies. Whereas a K_i value of about 0.8 mM was obtained from Dixon plots, a comparable inhibitor constant could not be calculated from the Hanes plots. Though the chemical structure would favor a competitive mechanism with respect to NeuAc, the Hanes plots suggested an unusual inhibition pattern. From these results, an effect on the CTP binding site does not seem to be likely. The influence on the NeuAc binding site cannot be described kinetically by a simple competitive mechanism. A direct interaction, however, of 4-epi-NeuAc with the NeuAc binding site was supported by the observation that the enzyme is protected against denaturation in presence of this analogue. For comparison, the K_i value of CMP-sialic acid synthase from bovine submaxillary glands for CMP was found to be 9.5 mM, as determined from a Dixon plot, indicating that a high concentration of CMP was necessary for significant inhibition (Schauer & Wember, 1973). For methyl-β-NeuAc, which was previously reported to inhibit bovine brain synthase (Korytnyk et al., 1984), an even higher K_i value of about 20 mM was measured in our laboratory.

Aimed at inhibitors to influence sialic acid metabolism in vivo, the assay system for CMP-sialic acid synthase described by Kean and Roseman suffers from the use of an unphysiological pH of 9.0 (Kean & Roseman, 1966); to our knowledge, the present paper is the first report which uses in addition an assay system at pH 7.5 to describe the inhibition kinetics of NeuAc analogues toward CMP-sialic acid synthase. The kinetic patterns obtained with 4-epi-NeuAc at pH 9 and pH 7.5 were essentially identical. The assay system at pH 7.5 will be useful for further studies on synthetic substrates or inhibitors of CMP-sialic acid synthase in order to approach the physiological milieu.

In this laboratory a number of structurally different NeuAc analogues modified at C-3, C-4, C-6, C-7, and C-9 have been synthesized (Villalva Basabe & Brossmer, 1984; Hagedorn & Brossmer, 1986; Gross & Brossmer, 1987, Gross et al., 1984, 1987; Mack & Brossmer, 1987; unpublished results). So far only 4-epi-NeuAc has been found to inhibit CMP-sialic acid synthase.

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