J. Biochem. **96**, 507–522 (1984)

Purification and Properties of *N*-Acetylneuraminate Lyase from *Escherichia coli*[1]

Yoshihiro UCHIDA, Yoji TSUKADA, and Tsunetake SUGIMORI

Kyoto Research Laboratories, Marukin Shoyu Co., Ltd.,
Uji, Kyoto 611

Received for publication, March 23, 1984

N-Acetylneuraminate lyase [*N*-acetylneuraminic acid aldolase EC 4.1.3.3] from *Escherichia coli* was purified by protamine sulfate treatment, fractionation with ammonium sulfate, column chromatography on DEAE-Sephacel, gel filtration on Ultrogel AcA 44, and preparative polyacrylamide gel electrophoresis. The purified enzyme preparation was homogeneous on analytical polyacrylamide gel electrophoresis, and was free from contaminating enzymes including NADH oxidase and NADH dehydrogenase.

The enzyme catalyzed the cleavage of *N*-acetylneuraminic acid to *N*-acetylmannosamine and pyruvate in a reversible reaction. Both cleavage and synthesis of *N*-acetylneuraminic acid had the same pH optimum around 7.7. The enzyme was stable between pH 6.0 to 9.0, and was thermostable up to 60°C. The thermal stability increased up to 75°C in the presence of pyruvate.

No metal ion was required for the enzyme activity, but heavy metal ions such as Ag^+ and Hg^{2+} were potent inhibitors. Oxidizing agents such as *N*-bromosuccinimide, iodine, and hydrogen peroxide, and SH-inhibitors such as *p*-chloromercuribenzoic acid and mercuric chloride were also potent inhibitors.

The K_m values for *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid were 3.6 mM and 4.3 mM, respectively. Pyruvate inhibited the cleavage reaction competitively; K_I was calculated to be 1.0 mM. In the condensation reaction, *N*-acetylglucosamine, *N*-acetylgalactosamine, glucosamine, and galactosamine could not replace *N*-acetylmannosamine as substrate, and phosphoenolpyruvate, lactate, β-hydroxypyruvate, and other pyruvate derivatives could not replace pyruvate as substrate.

The molecular weight of the native enzyme was estimated to be 98,000 by gel filtration methods. After denaturation in sodium dodecyl sulfate or in 6 M guanidine-HCl, the molecular weight was reduced to 33,000, indicating the existence of 3 identical subunits.

The enzyme could be used for the enzymatic determination of sialic acid; reaction conditions were devised for determining the bound form of sialic acid by coupling neuraminidase from *Arthrobacter ureafaciens*, lactate dehydrogenase, and NADH.

[1] A part of this paper was presented at the Annual Meeting of the Agricultural Chemical Society of Japan, Fukuoka, April, 1980.

N-Acetylneuraminate lyase [*N*-acetylneuraminate pyruvate lyase, *N*-acetylneuraminic acid aldolase, EC 4.1.3.3] cleaves *N*-acylneuraminic acid in a reversible reaction to yield pyruvate and *N*-acyl-D-mannosamine. This enzyme was originally found in some neuraminidase-producing bacteria such as *Vibrio cholerae* (*1*, *2*) and *Clostridium perfringens* (*3–5*). The enzyme was also found to be widely distributed in animal tissues such as rat liver and brain (*5*), pig kidney (*6*), and bovine kidney (*7*). Among these sources of the enzyme, bacterial *N*-acetylneuraminate lyase from *C. perfringens* has been purified and well characterized by several groups of workers (*4*, *5*, *8–16*). Although the occurrence of the enzyme was also indicated in *Escherichia coli* K 235 (*5*), no report has appeared dealing with the purification of the enzyme to a homogeneous state and its characterization. This is mainly because the enzyme from *E. coli* is very feeble in activity and is difficult to purify. We have devised a culture medium for producing *N*-acetylneuraminate lyase in high yields, and have found that *E. coli* produces potent *N*-acetylneuraminate lyase activities under these culture conditions (*17*).

The present paper describes the purification of *N*-acetylneuraminate lyase from *E. coli* and the enzymatic characteristics of the enzyme.

MATERIALS AND METHODS

Cultivation—E. coli AKU 0007, one of the strains preserved in the Laboratory of Applied Microbiology, Kyoto University, was inoculated into 10 2-liter Erlenmeyer flasks each containing 500 ml of liquid medium consisting of 0.5% *N*-acetylneuraminic acid, 0.2% $(NH_4)_2HPO_4$, 0.3% NaCl, 0.1% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, and 0.05% yeast extract, pH 7.0 (before autoclaving), and then incubated at 28°C for 40 h on a rotary shaker at 200 rpm. The cells were harvested by centrifugation at 15,000 × *g* for 20 min.

Assay of N-Acetylneuraminate Lyase Activity—The standard assay system contained the following components in a total volume of 200 μl : 50 μl of substrate solution [*N*-acetylneuraminic acid (Na salt), 20 μmol/ml], 50 μl of 200 mM phosphate buffer (pH 7.5), and 100 μl of enzyme preparation. The reaction was conducted at 37°C for 10 min, and the amount of *N*-acetylmannosamine produced was compared with that of a control containing the same components except for the enzyme. *N*-Acetylmannosamine was determined with the Morgan-Elson color reaction (*18*) modified by Brunetti *et al.* (*6*). One unit of *N*-acetylneuraminate lyase activity is defined as the amount that releases 1 μmol of *N*-acetylmannosamine per min under the reaction conditions used.

Assay of Other Enzyme Activities—Glycosidase activities such as neuraminidase, galactosidase, mannosidase, fucosidase, and *N*-acetylhexosaminidase were assayed as described previously (*19*). Protease and phospholipase C activities were also determined as described previously (*19*). NADH oxidase and NADH dehydrogenase activities were determined by Mackler's method (*20*), and King and Howard's method (*21*), respectively.

Protein Determination—Protein concentration was determined by the method of Lowry *et al.* with bovine serum albumin as a standard (*22*).

Polyacrylamide Gel Electrophoresis—Analytical disc gel electrophoresis was performed according to the method of Ornstein (*23*) and Davis (*24*) by using 7.5% polyacrylamide gel at a constant current of 3 mA per gel in Tris-glycine buffer (pH 8.3). Sodium dodecyl sulfate (SDS)-gel electrophoresis was carried out according to the method of Weber *et al.* (*25*) by using 7.5% polyacrylamide gel at a constant current of 8 mA per gel. Proteins were visualized with Coomassie Brilliant Blue G 250 (*26*).

Molecular Weight Determination—The molecular weight of the native enzyme was estimated by gel filtration based on the method of Andrews (*27*). A column of Sephacryl S-300 (2.6 × 100 cm) was equilibrated with 100 mM phosphate buffer (pH 7.3) and eluted with the same buffer at a flow rate of 50 ml per h. The following standard proteins were used for calibration: cytochrome *c*, myoglobin, chymotrypsinogen A, ovalbumin, albumin (bovine), lactate dehydrogenase (pig heart), and γ-globulin. The partition coefficient, K_{av}, was calculated by the equation of Laurent and Killander (*28*).

The subunit size of *N*-acetylneuraminate lyase was estimated by SDS-gel electrophoresis as described by Weber *et al.* (*25*) with the following molecular weight markers: cytochrome *c*, myoglobin, chymotrypsinogen A, ovalbumin, and albu-

min (bovine). Prior to electrophoresis, the samples were dissolved in 10 mM phosphate buffer (pH 7.2) containing 25% glycerol, 1.0% SDS, and 1.0% 2-mercaptoethanol, and incubated for 3 h at 37°C. Electrophoresis was carried out with 7.5% polyacrylamide gel at a constant current of 8 mA per gel.

The subunit size was also determined by gel filtration on Sephacryl S-300 in guanidine-HCl according to the modified procedure of Mann and Fish (*29*). Each protein (5 mg) was dissolved in 0.5 ml of 6 M guanidine-HCl containing 20 mM dithiothreitol. The pH was maintained at 8.5 for 1 h to complete the reduction of disulfide bonds and then alkylated with 40 mM iodoacetic acid, pH 8.5. The samples were applied to a column of Sephacryl S-300 (2.6×45 cm) previously equilibrated with 6 M guanidine-HCl, and eluted with the same buffer at a flow rate of 50 ml per h.

Chemicals—*N*-Acetylneuraminic acid is a product of our company (*30*). *N*-Glycolylneuraminic acid was a generous gift from Dr. K. Hotta, Kitasato University. The calibration proteins were obtained from both Boehringer Mannheim and Mann Research Laboratories. Other chemicals were either from Nakarai Chemicals Ltd., Kyoto, or from Sigma Chemical Company, U.S.A.

RESULTS

Isolation and Purification of N-Acetylneuraminate Lyase—Since the enzyme is an intracellular enzyme, the harvested cells were disrupted to extract the enzyme. The isolation and purification were carried out by the following procedures. Unless otherwise indicated, all purification steps were conducted between 0 and 5°C.

Step 1. Cell disruption: The harvested cells (wet weight, 72 g) were washed twice with 2.0 liters of 10 mM phosphate buffer (pH 7.3) and suspended in 500 ml of the same buffer, and then disrupted by 60 min sonication with a 20 kHz ultrasonic oscillator (Tomy Model UR-200P, Tomy Seiko Co., Ltd., Tokyo). The sonicated suspension was centrifuged at 20,000×*g* for 30 min. The precipitate was discarded.

Step 2. Protamine sulfate treatment: To the supernatant solution was slowly added under continuous stirring 35 ml of 1% protamine sulfate solution. After centrifuging at 15,000×*g* for 20 min, the precipitate was removed. The supernatant solution was adjusted to 800 ml by addition of 10 mM phosphate buffer (pH 7.3).

Step 3. Ammonium sulfate fractionation: Solid ammonium sulfate (312 g) was slowly added under strirring to the supernatant fluid to give a final saturation of 60%. After stirring for an additional 30 min, the mixture was centrifuged at 12,000×*g* for 20 min, and the supernatant was brought to 80% saturation by further addition of ammonium sulfate (137 g). After standing for 1 h, the precipitate was collected by centrifugation at 12,000×*g* for 20 min, and dissolved in 100 ml of 10 mM Tris-HCl buffer (pH 7.3) followed by dialysis for 16 h against the same buffer.

Step 4. DEAE-Sephacel column chromatography: The dialyzed enzyme preparation was applied to a DEAE-Sephacel column (4.4×20 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.3). The column was washed with the same buffer, and was developed by linear gradient elution with the mixing chamber containing 2.0 liters of 10 mM Tris-HCl buffer (pH 7.3) and the reservoir containing 2.0 liters of 1.0 M NaCl in 10 mM Tris-HCl buffer (pH 7.3). Fractions were collected at a rate of 20 ml/tube/6 min. *N*-Acetylneuraminate lyase activities were eluted in the fractions of about 0.35 M NaCl. The active fractions were pooled and concentrated by ultrafiltration with a Diaflo UM-10 membrane (Amicon Corporation), and then salted out by the addition of ammonium sulfate to 80% saturation.

Step 5. Gel filtration: The precipitate collected by centrifugation at 15,000×*g* for 20 min was dissolved in 100 mM phosphate buffer (pH 7.3), and applied to a column of Ultrogel AcA 44 (2.6×100 cm) previously equilibrated with the same buffer. Elution was performed with the same buffer at a flow rate of 3 ml/tube/6 min. The active fractions were pooled and salted out by the addition of ammonium sulfate to 80% saturation. After standing for 1 h, the precipitate was collected by centrifugation at 12,000×*g* for 20 min, and dissolved in 20 ml of 50 mM Tris-H_3PO_4 buffer (pH 7.2), followed by dialysis for 16 h against the same buffer. The *N*-acetylneuraminate lyase preparation obtained here still contained impurities and gave several protein bands on polyacrylamide gel electrophoresis. Further

purification of the enzyme was carried out by preparative polyacrylamide gel electrophoresis.

Step 6. Preparative polyacrylamide gel electrophoresis: The enzyme preparation from Step 5 was subjected to preparative polyacrylamide gel electrophoresis by using the Toyo CD-50 apparatus. The concentration of polyacrylamide was 7.5% and the electrophoresis was carried out according to the method described by Kikutani (*31*). The elution buffer (100 mM Tris-HCl, pH 8.1) was pumped at a speed of 60 ml/h and was collected in 10 ml fractions. The *N*-acetylneuraminate lyase activity appeared in fractions 57 to 66 as shown in Fig. 1. Fractions giving a single protein band by analytical disc electrophoresis (fractions 59 to 62) were collected as the purified enzyme preparation.

A summary of the purification procedure is presented in Table I. The enzyme preparation obtained here was purified approximately 73-fold in about 42% yield.

Purity of N-Acetylneuraminate Lyase—To check the homogeneity of the enzyme, the purified enzyme preparation was subjected to polyacrylamide gel electrophoresis. As shown in Fig. 2, the enzyme preparation gave a single protein band on both disc and SDS-disc gel electrophoreses.

As shown in Table II, the purified *N*-acetylneuraminate lyase preparation was found to be

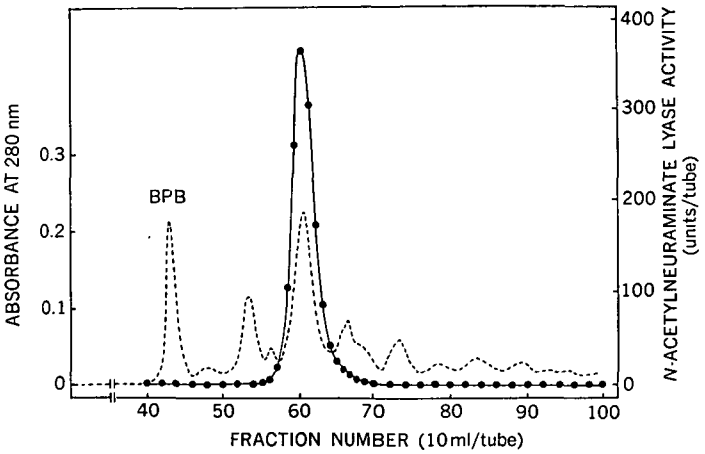

Fig. 1. Preparative polyacrylamide gel electrophoresis of *N*-acetylneuraminate lyase from *E. coli*. The enzyme preparation purified by gel filtration on Ultrogel AcA 44 was subjected to preparative gel electrophoresis in 7.5% polyacrylamide gel. The electrophoretic conditions are given in the text. ----, absorbance at 280 nm; —●—, *N*-acetylneuraminate lyase activity. BPB, bromophenol blue (tracking dye).

TABLE I. Summary of the purification of *N*-acetylneuraminate lyase from *E. coli*.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification	Yield (%)
Cell-free extract	2,736	3,508	0.78	1.0	100
Protamine sulfate	2,723	3,204	0.85	1.1	99.5
Ammonium sulfate (60–80%)	2,460	1,557	1.58	2.0	89.9
DEAE-Sephacel	1,829	171	10.7	13.7	66.8
Ultrogel AcA 44	1,637	65.4	25.0	32.1	59.8
Preparative PAGE	1,141	20.1	56.8	72.8	41.7

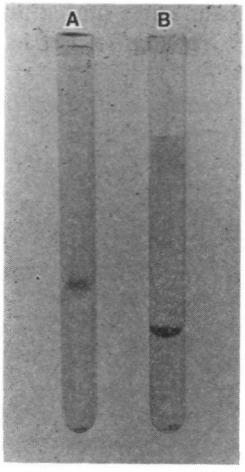

Fig. 2

completely free from protease, phospholipase C, and glycosidases such as α- and β-glucosidases, α- and β-galactosidases, α-mannosidase, α-L-fucosidase, α- and β-*N*-acetylglucosaminidases, α- and β-*N*-acetylgalactosaminidases, α- and β-*N*-acetylmannosaminidases, and neuraminidase, and was also proved to be free from NADH oxidase and NADH dehydrogenase.

Fig. 2. Polyacrylamide gel electrophoresis of purified *N*-acetylneuraminate lyase from *E. coli*. The purified *N*-acetylneuraminate lyase preparation (25 μg) was applied to a column of 7.5% polyacrylamide gel in the presence (A) or absence (B) of SDS. The experimental details are given in " MATERIALS AND METHODS."

TABLE II. Detection of various enzyme activities in the *N*-acetylneuraminate lyase preparation from *E. coli*. Both crude (cell-free extract) and purified enzyme were used for the assay of contaminating enzymes. The activities of protease, phospholipase C, and glycosidases were assayed as described previously (*19*), and those of NADH oxidase and NADH dehydrogenase were measured by Mackler's method (*20*), and King and Howard's method (*21*), respectively.

Enzyme	Substrate	Enzyme activity (units/ml enzyme)	
		Crude	Purified
N-Acetylneuraminate lyase	*N*-Acetylneuraminic acid	10.00	80.00
Protease	Casein	0.039	0.000
Phospholipase C	L-α-Phosphatidyl choline	N.D.[a]	0.00
Neuraminidase	*N*-Acetylneuraminosyl-lactose	0.00	0.00
Neuraminidase	Colominic acid	0.00	0.00
α-Glucosidase	Phenyl-α-D-glucoside	0.00	0.00
β-Glucosidase	Phenyl-β-D-glucoside	0.00	0.00
α-Galactosidase	Phenyl-α-D-galactoside	0.00	0.00
β-Galactosidase	Phenyl-β-D-galactoside	0.04	0.00
α-Mannosidase	Phenyl-α-D-mannoside	0.00	0.00
α-Fucosidase	Phenyl-α-L-fucoside	0.00	0.00
α-*N*-Acetylglucosaminidase	Phenyl-*N*-acetyl-α-D-glucosaminide	0.00	0.00
β-*N*-Acetylglucosaminidase	Phenyl-*N*-acetyl-β-D-glucosaminide	0.00	0.00
α-*N*-Acetylgalactosaminidase	Phenyl-*N*-acetyl-α-D-galactosaminide	0.00	0.00
β-*N*-Acetylgalactosaminidase	Phenyl-*N*-acetyl-β-D-galactosaminide	0.00	0.00
α-*N*-Acetylmannosaminidase	Phenyl-*N*-acetyl-α-D-mannosaminide	0.00	0.00
β-*N*-Acetylmannosaminidase	Phenyl-*N*-acetyl-β-D-mannosaminide	0.00	0.00
NADH oxidase	NADH	7.61	0.00
NADH dehydrogenase	NADH : 2,6-dichlorophenol indophenol[b]	3.67	0.00

[a] N.D., not determined. [b] 2,6-Dichlorophenol indophenol was used as an electron acceptor.

pH Optimum and Stability—The effects of pHs on the rate of cleavage of *N*-acetylneuraminic acid or synthesis of *N*-acetylneuraminic acid from *N*-acetylmannosamine and pyruvate are illustrated in Fig. 3. Both reactions had a pH optimum around 7.7. In the cleavage reaction no difference was observed between phosphate buffer and Tris-HCl buffer (Fig. 3A), but the synthetic reaction was depressed by Tris-HCl buffer as shown in Fig. 3B.

As shown in Fig. 4, *N*-acetylneuraminate lyase from *E. coli* is stable at 37°C for 24 h in buffers from pH 6.0 to 9.0. The result indicates that this enzyme can remain active during prolonged incubation when the reaction is conducted at around neutral pH.

Thermal Reactivity and Stability—The standard reaction mixture was allowed to react at various temperatures for 10 min and the amount of

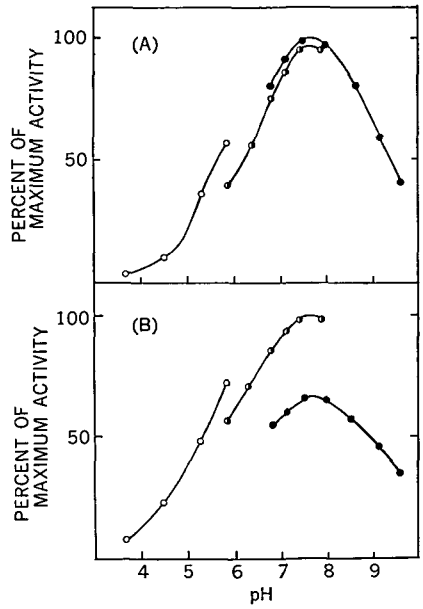

Fig. 3. Effect of pH on the reaction rate. Cleavage reaction (A) was performed at 37°C for 10 min with the following components in a total volume of 200 μl: 50 μl of 20 mM *N*-acetylneuraminate solution, 50 μl of 200 mM buffer solution, and 100 μl of enzyme preparation (0.01 unit). Synthetic reaction (B) was performed at 37°C for 60 min with the following components in a total volume of 200 μl: 25 μl of 40 mM *N*-acetylmannosamine solution, 25 μl of 40 mM pyruvate solution, 50 μl of 200 mM buffer solution, and 100 μl of enzyme preparation (0.01 unit). The buffer solutions used were acetate buffer (○, pH 3.6–5.9), phosphate buffer (◑, pH 5.9–7.9), and Tris-HCl buffer (●, pH 6.8–9.6). In the cleavage reaction, the reaction product (*N*-acetylmannosamine) was determined with the Morgan-Elson reaction modified by Brunetti *et al.* (*6*), and in the synthetic reaction, the reaction product (*N*-acetylneuraminic acid) was assayed by a thiobarbituric acid procedure (*19*).

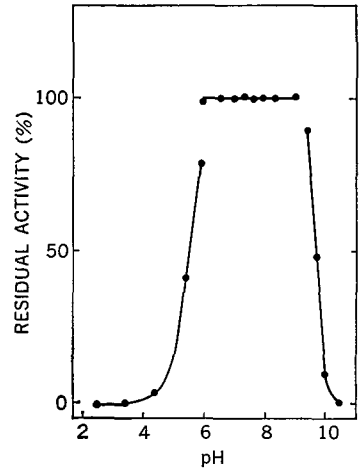

Fig. 4. Effect of pH on the stability of *N*-acetylneuraminate lyase. *N*-Acetylneuraminate lyase was incubated at 37°C for 24 h in 20 mM buffers of pH 2.2 to 10.2; HCl-CH_3COONa and CH_3COOH-CH_3COONa buffers for pH 2.2–5.9, NaH_2PO_4-K_2HPO_4 buffer for pH 5.9–8.0, Tris-HCl buffer for 6.5–9.1, and K_2HPO_4-NaOH buffer for 9.5–10.2. The remaining *N*-acetylneuraminate lyase activities were determined by the standard assay procedure.

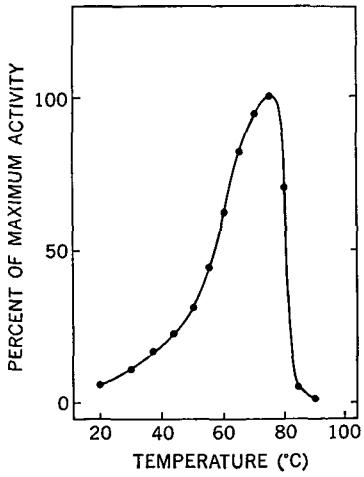

Fig. 5. Effect of temperature on the reaction rate. The reaction conditions (with the exception of reaction temperature) were the same as described as the standard assay procedure.

N-acetylmannosamine produced was compared with that of a control containing the same components except for the enzyme. The results are illustrated in Fig. 5. *N*-Acetylneuraminate lyase from *E. coli* had its optimum at a relatively high temperature, around 75°C. The enzyme was then incubated at pH 7.7 in a water bath maintained at various temperatures for 10 min, followed by chilling in an ice bath. An aliquot of the treated enzymes was assayed for remaining *N*-acetylneuraminate lyase activity by the standard procedure, and the activity was compared with that of a control kept at 5°C. As shown in Fig. 6A, *N*-acetylneuraminate lyase from *E. coli* was found to be thermostable up to 60°C, and the thermal stability was found to be markedly enhanced up to 75°C in the presence of pyruvate. The enzyme seems to have a moderate heat stability.

The time courses of thermal inactivation of the enzyme at 80°C were followed as shown in Fig. 6B. In the absence of pyruvate, 10 min of incubation resulted in 77% inactivation of the enzyme, whereas in the presence of pyruvate, the inactivation was only 8%. As is also shown in Fig. 6B, *N*-acetylmannosamine, sucrose, and Ca^{2+} were somewhat protective against heat inactivation, although not as significantly as pyruvate.

Effects of Metal Ions on the Enzyme Activity —The enzyme was preincubated with metal ions, and the remaining activities were determined by the standard assay procedure. The results are summarized in Table III. No metal ions stimulated the enzyme activity. The enzyme, however,

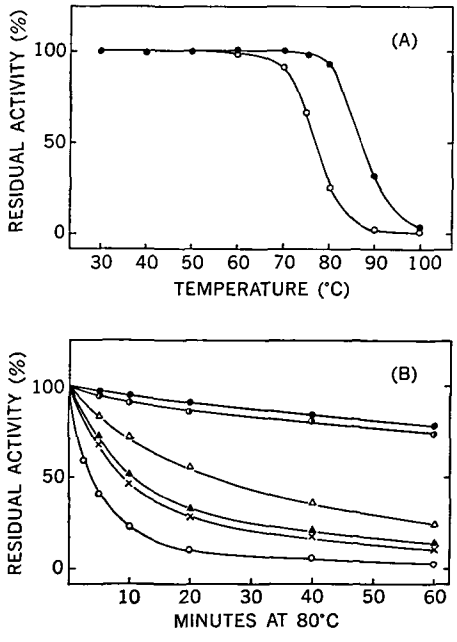

Fig. 6. Thermal stability of *N*-acetylneuraminate lyase. *N*-Acetylneuraminate lyase was heated in 20 mM phosphate buffer (pH 7.7), and the remaining activities were determined by the standard assay procedure. (A) Thermal inactivation of *N*-acetylneuraminate lyase incubated at the indicated temperatures for 10 min in the presence (●) or absence (○) of 5 mM pyruvate. (B) Time course of the thermal inactivation of *N*-acetylneuraminate lyase incubated at 80°C in the presence of the following compounds: 20 mM pyruvate (●), 5 mM pyruvate (◑), 5 mM *N*-acetylmannosamine (△), 100 mM sucrose (▲), 2 mM $CaCl_2$ (×), and none (○).

TABLE III. Effects of metals on *N*-acetylneuraminate lyase from *E. coli*. *N*-Acetylneuraminate lyase (250 μl) was preincubated with 250 μl of sample solution (40 mM, 4 mM, 0.4 mM, or 0.04 mM) at 37°C for 10 min, then 100 μl aliquots were taken and the activities were assayed as described in the text.

Metal	Final conc. (mM)	Relative activity (%)
None	—	100
LiCl	10	99
NaCl	10	95
KCl	10	96
$MgCl_2$	1	100
$CaCl_2$	1	99
$BaCl_2$	1	98
$CuCl_2$	1	37
	0.1	89
$AgNO_3$	0.1	0
	0.01	6
$ZnSO_4$	1	100
$CdCl_2$	1	96
$HgCl_2$	0.1	0
	0.01	22
$MnCl_2$	1	94
$FeSO_4$	1	95
$CoCl_2$	1	97
$NiCl_2$	1	96
$Pb(CH_3COO)_2$	1	99

is markedly inhibited by heavy metal ions: Ag^{+} and Hg^{2+} at the concentration of 0.1 mM repressed the enzyme activity completely, and Cu^{2+} at the concentration of 1.0 mM inhibited the activity by 63%.

Effects of Various Compounds on Enzyme Activity—Various types of compounds including chelating agents, SH-inhibitors, SH-compounds, carbonyl group inhibitors and oxidizing agents were examined for inhibitory or stimulatory action on the enzyme. The results are summarized in Table IV. Chelating agents such as α,α'-dipyridyl, 8-hydroxyquinoline, *o*-phenanthroline, sodium diethyldithiocarbamate, thiourea, sodium pyrophos-

TABLE IV. Effects of various compounds on *N*-acetylneuraminate lyase from *E. coli*. Enzyme activities were assayed after preincubation as described in Table III.

Compound	Final conc. (mM)	Relative activity (%)
None	—	100
EDTA	1	99
α,α'-Dipyridyl	1	100
8-Hydroxyquinoline	1	100
o-Phenanthroline	1	100
Sodium diethyldithiocarbamate	1	99
Thiourea	1	100
Sodium pyrophosphate	1	100
Monoiodoacetic acid	1	100
Sodium arsenite	1	100
Sodium arsenate	1	99
p-Chloromercuribenzoic acid	0.1	0
	0.01	9
L-Ascorbic acid	1	83
2-Mercaptoethanol	1	92
Glutathione	1	95
L-Cysteine HCl	1	94
2,3-Dimercapto-1-propanol	1	100
Sodium thioglycollate	1	99
Dithiothreitol	1	99
Sodium bisulfate	1	97
Hydroxylamine HCl	1	98
Semicarbazide HCl	1	100
Phenylhydrazine HCl	1	98
Thiosemicarbazide HCl	1	100
Sodium fluoride	1	100
Sodium azide	1	100
Phenylmethylsulfonyl fluoride	1	99
N-Bromosuccinimide	0.01	0
	0.001	36
Iodine	0.01	0
	0.001	45
Hydrogen peroxide	10	0
	1	16

phate, and EDTA; SH-compounds (S-S dissociating agents) such as 2-mercaptoethanol, glutathione, L-cysteine, sodium thioglycollate, and dithiothreitol; and carbonyl group inhibitors such as sodium bisulfite, hydroxylamine, semicarbazide, phenylhydrazine, and thiosemicarbazide were neither inhibitors nor activators of the enzyme. However, SH-inhibitors such as *p*-chloromercuribenzoic acid and mercuric chloride (Table III), and oxidizing agents such as *N*-bromosuccinimide, iodine, and hydrogen peroxide were shown to be highly inhibitory.

Michaelis Constant and Maximum Velocity—Studies on the rate of cleavage reaction as a function of substrate concentration were carried out with *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid as substrates. From the double reciprocal plots of the activity against the concentrations of substrates, the apparent K_m values for *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid were calculated to be 3.6 mM and 4.3 mM, and V_{max} values for *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid were 154.5 μmol/mg enzyme/min, and 40.0 μmol/mg enzyme/min, respectively (Table V).

Pyruvate inhibited the cleavage reaction of *N*-acetylneuraminic acid competitively; the apparent inhibition constant, K_I, obtained from Dixon's plots was 1.0 mM (data not shown).

Substrate Specificity—The substrate specificity was studied *via* the condensation reaction. The reaction was followed by measuring the reaction products as well as the substrate consumption. The reaction products were determined by the thiobarbituric acid procedure, and were also detected on thin layer chromatograms. The consumed substrates were calculated by determining the residual amino sugars either by the Morgan-Elson test for *N*-acetylhexosamines or by an amino acid analyzer for hexosamines.

As summarized in Table VI, the following sugars could not replace *N*-acetylmannosamine as substrate: *N*-acetylglucosamine, *N*-acetylgalactosamine, glucosamine, and galactosamine. When mannosamine was used as the substrate, no significant consumption of the substrate was occurred, but a small amount of thiobarbituric acid-positive product seemed to be formed. On thin layer chromatogram, a new spot giving the same color as *N*-acetylneuraminic acid but of higher R_f value was detected by spraying with diphenylamine-aniline reagents. This spot could not be detected if the enzyme is omitted from the reaction mixture.

The substrate specificity was then studied with *N*-acetylmannosamine and pyruvate analogues. The following compounds could not replace pyruvate as substrate: phosphoenolpyruvate, lactate, α-ketobutyrate, acetoacetate, acetopyruvate, β-

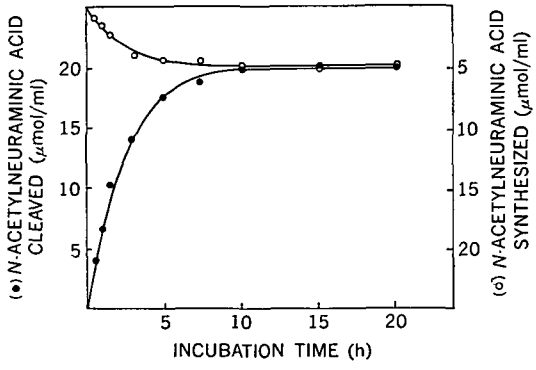

Fig. 7. Equilibrium of *N*-acetylneuraminate lyase reaction. The reaction mixture consisted of substrate solution (either 500 μl of 50 mM *N*-acetylneuraminate, or 250 μl of 100 mM *N*-acetylmannosamine, and 250 μl of 100 mM pyruvate), 250 μl of 200 mM phosphate buffer (pH 7.7), and 250 μl of the enzyme solution (0.1 unit). After incubation at 37°C, aliquots were taken at the indicated times. The reaction products were assayed as described in Fig. 1.

TABLE V. Michaelis constant and maximum velocity of *N*-acetylneuraminate lyase from *E. coli*. Increasing concentrations of each substrate were incubated with 0.01 unit of *N*-acetylneuraminate lyase at 37°C for 10 min in a 200 μl reaction mixture. The reaction products were determined by the method described in the text.

Substrate	K_m (mM)	V_{max} (μmol/mg enzyme/min)
N-Acetylneuraminic acid	3.6	154.5
N-Glycolylneuraminic acid	4.3	40.0

hydroxypyruvate, β-fluoropyruvate, 3-bromopyruvate, glyoxylate, and oxamate (Table VI). When methyl glyoxal was used as the substrate, a small amount of *N*-acetylmannosamine was consumed, and some thiobarbituric acid-positive product seemed to be formed as shown in Table VI. On thin layer chromatograms, however, no new spot could be detected.

Equilibrium Studies—Reaction mixtures containing an excess of enzyme were incubated either with *N*-acetylneuraminic acid or with *N*-acetylmannosamine plus pyruvate, and the reactions were followed until equilibrium was attained. A typical result is presented in Fig. 7. The two reactions, one in the direction of cleavage and the other in the direction of condensation, reached the same equilibrium point. The equilibrium constant, $K=[N\text{-acetylmannosamine}]\times[\text{pyruvate}]/[N\text{-acetylneuraminic acid}]$ was calculated to be 7.9×10^{-2} M, if the reaction was started either with 25 mM *N*-acetylneuraminic acid or 25 mM *N*-acetylmannosamine plus 25 mM pyruvate. The position of equilibrium, however, was dependent on the initial substrate concentration as has been pointed out by Brunetti *et al.* (*6*); the cleavage reaction was favored at lower substrate concentration, whereas the condensation reaction was favored at higher substrate concentration.

Molecular Weight—The molecular weight of the native *N*-acetylneuraminate lyase from *E. coli* was estimated to be 98,000 by gel filtration on Sephacryl S-300 as shown in Fig. 8. The same

TABLE VI. Substrate specificity studies with *N*-acetylneuraminate lyase from *E. coli*. The reaction mixture consisted of 250 μl of 200 mM aminosugar solution (*N*-acetylhexosamine or hexosamine), 250 μl of 200 mM pyruvate or its related compounds, 250 μl of 200 mM phosphate buffer (pH 7.7), and 250 μl of the enzyme solution (0.1 unit). After incubation at 37°C for 20 h, the reaction products were assayed by a thiobarbituric acid procedure (*19*). The remaining *N*-acetylhexosamine was determined with the Morgan-Elson color reaction modified by Brunetti *et al.* (*6*), and hexosamine was measured by an amino acid analyzer.

Substrate [a]	Amino sugar disappeared (μmol/ml)	TBA [b] (A_{550})
ManNAc+pyruvate	13.0	60.4
GlcNAc+pyruvate	0.1	0.2
GalNAc+pyruvate	0.1	0.1
ManN+pyruvate	0.2	0.7
GlcN+pyruvate	0.1	0.0
GalN+pyruvate	0.0	0.1
ManNAc+methyl glyoxal	0.9	0.8
ManNAc+phosphoenolpyruvate	0.0	0.2
ManNAc+lactate	0.2	0.2
ManNAc+α-ketobutyrate	0.0	0.1
ManNAc+acetoacetate	0.0	0.0
ManNAc+acetopyruvate	0.2	0.0
ManNAc+β-hydroxypyruvate	0.1	0.0
ManNAc+β-fluoropyruvate	0.0	0.0
ManNAc+3-bromopyruvate	0.0	0.0
ManNAc+glyoxylate	0.0	0.0
ManNAc+oxamate	0.1	0.0

[a] Abbreviations: ManNAc, *N*-acetyl-D-mannosamine; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; ManN, D-mannosamine; GlcN, D-glucosamine; GalN, D-galactosamine. [b] Absorbancy determined by thiobarbituric acid procedure.

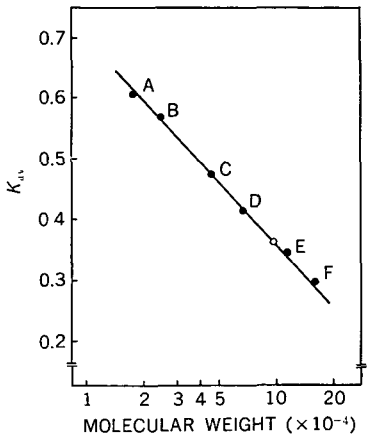

Fig. 8. Estimation of molecular weight of *N*-acetylneuraminate lyase by gel filtration on Sephacryl S-300. The standards used were (A) myoglobin (17,800), (B) chymotrypsinogen A (25,000), (C) ovalbumin (45,000), (D) bovine albumin (67,000), (E) lactate dehydrogenase (pig heart, 115,000), and (F) aldolase (rabbit muscle, 158,000). The open circle (○) indicates the position of *N*-acetylneuraminate lyase. Experimental details are given in "MATERIALS AND METHODS."

molecular weight was also given by gel filtration on Ultrogel AcA 44 or on Sephadex G-200.

On SDS-polyacrylamide gel electrophoresis, the purified enzyme gave a single band of subunit as shown in Fig. 2. A molecular weight of 33,000 for the subunit was estimated as shown in Fig. 9A.

In order to confirm the subunit molecular weight, the enzyme was also applied to a column of Sephacryl S-300 after reduction and alkylation of disulfide bonds, and was subjected to gel filtration in the presence of 6 M guanidine-HCl. The subunit molecular weight of the enzyme was also estimated to be 33,000 as shown in Fig. 9B. These results indicate that the *N*-acetylneuraminate lyase from *E. coli* probably consists of a molecule of 3 identical subunits.

Enzymatic Determination of N-Acetylneuraminic Acid—For the enzymatic determination of sialic acid (*N*-acetylneuraminic acid), a method was described to assay pyruvate by coupling *N*-acetylneuraminate lyase from *C. perfringens* with NADH and lactate dehydrogenase (*32*). Experiments were undertaken to examine whether *N*-acetylneuraminate lyase from *E. coli* can be substituted for that from *C. perfringens* in this assay system.

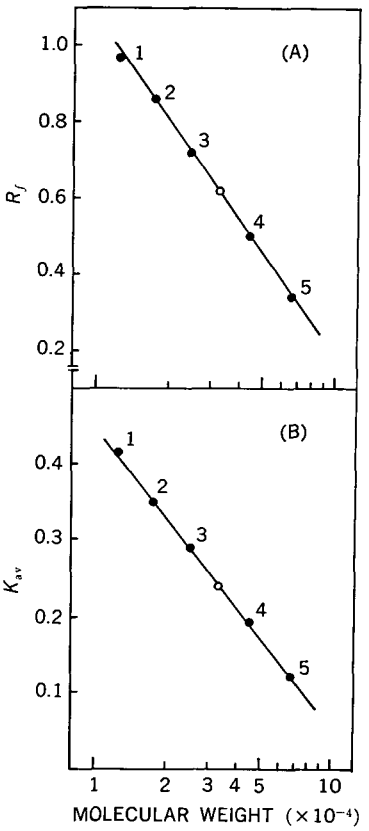

Fig. 9. Estimation of molecular weight of *N*-acetylneuraminate lyase by SDS-polyacrylamide gel electrophoresis (A) and by gel filtration on Sephacryl S-300 in 6 M guanidine-HCl (B). The standards used for the estimation were: 1, cytochrome *c*; 2, myoglobin; 3, chymotrypsinogen A; 4, ovalbumin; 5, albumin. The open circle (○) indicates the position of *N*-acetylneuraminate lyase. Experimental details are given in "MATERIALS AND METHODS."

The following components were added to a 3 ml cuvette with a 10 mm light path: 0.5 ml of sample solution, 1.0 ml of 30 mM phosphate buffer (pH 7.7), 0.2 ml of 4 mM NADH solution, and 5.0 units of lactate dehydrogenase. The volume was adjusted with water to 2.98 ml. After determination of the optical density of the solution at 340 nm, 1.0 unit of *N*-acetylneuraminate lyase was added to each cuvette to a final volume of 3.0 ml. The reference cell contained all components except for *N*-acetylneuraminic acid. The reaction was allowed to proceed at 37°C, and the changes in optical density at 340 nm were measured until the

reaction reached the end point. Under the reaction conditions shown in Fig. 10A, the reaction reached the end point in 40 min. If the amount of *N*-acetylneuraminic acid is plotted as a function of the change in optical density at 340 nm, a linear relationship can be obtained at each incubation time as shown in Fig. 10B. These results demonstrate that *N*-acetylneuraminate lyase from *E. coli*

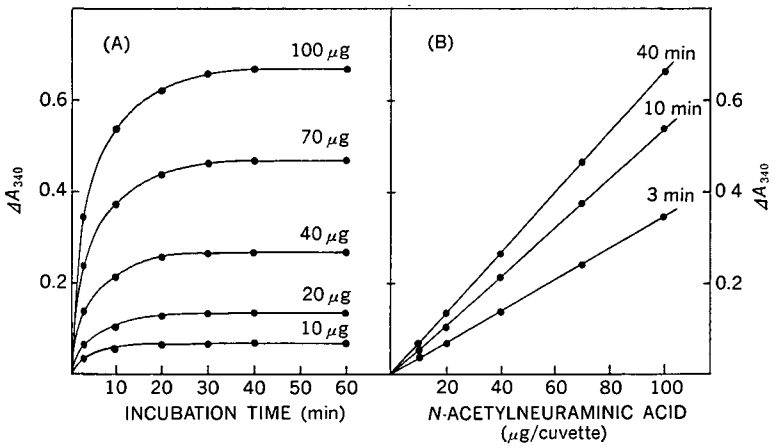

Fig. 10. Response of coupled *N*-acetylneuraminate lyase reaction to variation of *N*-acetylneuraminic acid concentration. Various amounts of *N*-acetylneuraminic acid (0–100 μg) were incubated at 37°C with the following components per 3.0 ml: 30 μmol of phosphate buffer (pH 7.7), 0.8 μmol of NADH, 5 units of lactate dehydrogenase, and 1 unit of *N*-acetylneuraminate lyase. The experimental details are given in the text. (A) Time course of the enzyme reaction. (B) Relationship between *N*-acetylneuraminic acid concentrations and changes in absorbance at 340 nm at the indicated incubation time.

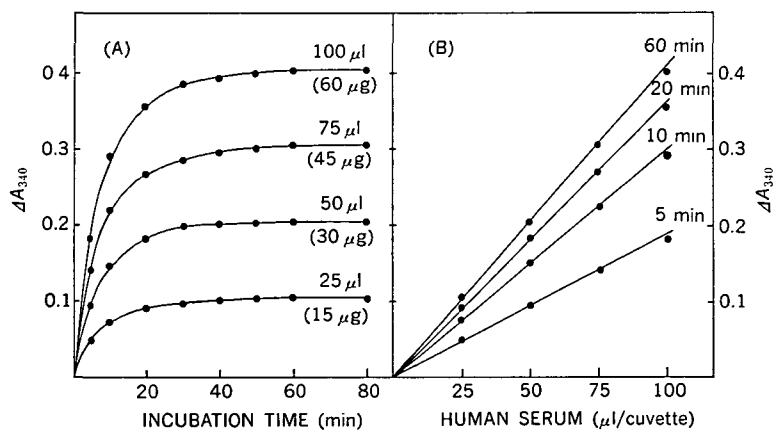

Fig. 11. Response of coupled *N*-acetylneuraminate lyase reaction to variation of bound *N*-acetylneuraminic acid concentration. Various amounts of bound *N*-acetylneuraminic acid (human serum, 25–100 μl) were incubated at 37°C with the following components per 3.0 ml: 30 μmol of phosphate buffer (pH 7.3), 0.8 μmol of NADH, 0.1 unit of neuraminidase, 5 units of lactate dehydrogenase, and 1 unit of *N*-acetylneuraminate lyase. The *N*-acetylneuraminic acid contents determined by Resorcinol method (*56*) are shown in the parentheses. (A) Time course of the coupled enzyme reaction. (B) Relationship between bound *N*-acetylneuraminic acid concentrations and changes in absorbance at 340 nm at the indicated incubation time.

can be used for the enzymatic determination of *N*-acetylneuraminic acid.

If the bound form of *N*-acetylneuraminic acid is to be determined, enzymatic hydrolysis of *N*-acetylneuraminic acid is necessary prior to the assay reaction (two-step assay system). If the coupled reaction consisting of the hydrolysis and the assay can be carried out simultaneously, the bound *N*-acetylneuraminic acid is easily determined in a single cuvette. Since the pH activity profile of *Arthrobacter ureafaciens* neuraminidase shows a broad optimum range from 4.5 to 8.0 with human serum (*33*) as the substrate, the coupled reaction was allowed to proceed in a single buffer system of pH 7.3. Neuraminidase from *A. ureafaciens* (0.1 unit) was added to the assay system of pH 7.3, and the *N*-acetylneuraminic acid content in the human serum was determined. The reaction reached the end point in 60 min under the conditions shown in Fig. 11A. In this case, a linear relationship can also be obtained at each incubation time as shown in Fig. 11B. The *N*-acetylneuraminic acid contents calculated here agreed with those determined by the two-step assay system. This means that the bound form of *N*-acetylneuraminic acid can be determined easily under these coupled reaction conditions. The times necessary to reach the end point become shorter, if more enzyme is used: the use of 0.25 units of neuraminidase, 3.0 units of *N*-acetylneuraminate lyase, and 5.0 units of lactate dehydrogenase brought the reaction to the end point within 25 min.

DISCUSSION

The existence of *E. coli* *N*-acetylneuraminate lyase was first reported in *E. coli* K 235, which produces colominic acid, a homopolymer of *N*-acetylneuraminic acid (*5*), but thereafter the enzyme was neither purified nor characterized. In this paper, *N*-acetylneuraminate lyase was purified from *E. coli* AKU 0007, which has no ability to produce colominic acid (*17*). This is the first report that deals with the purification of *E. coli* *N*-acetylneuraminate lyase to a homogeneous state and its detailed characterization. As *N*-acetylneuraminate lyase from *C. perfringens* has been well characterized (*4*, *5*, *8–16*), the enzymatic properties of *E. coli* *N*-acetylneuraminate lyase were compared with those of *C. perfringens* enzyme.

In regard to the specific activities, *N*-acetylneuraminate lyase purified from *E. coli* is more than 5 times as active as that from *C. perfringens*; the purest enzyme preparation so far obtained from *C. perfringens* has a specific activity of 167 nkat/mg enzyme (10.02 μmol/mg enzyme/min) (*16*) (Table I).

N-Acetylneuraminate lyase from *E. coli* has a pH optimum around 7.7 (Fig. 3), while the enzyme from *C. perfringens* has a pH optimum around 7.2 (*5*, *16*). Thus there is a slight difference in optimal pH. The enzyme from *C. perfringens* is shown to be markedly inhibited by Hg^{2+}, Ag^{+}, and Cu^{2+} (*14*, *16*), the behavior of *E. coli* enzyme toward metal ions seems to be similar to that of *C. perfringens* enzyme (Table III). It is reported that the thermostability of *N*-acetylneuraminate lyase from *C. perfringens* greatly increases in the presence of pyruvate (*34*). This is also the case with *E. coli* enzyme (Fig. 6). *E. coli* *N*-acetylneuraminate lyase seems to be a little more thermostable than *C. perfringens* enzyme. The K_m value for *N*-acetylneuraminic acid is reported to be 3.9 mM (*5*) or 3.3 mM (*12*) for the partially purified *N*-acetylneuraminate lyase from *C. perfringens* and 2.8 mM (*16*) for the pure enzyme. There was no marked difference between the K_m values of *E. coli* enzyme and *C. perfringens* enzyme.

It has been reported that the condensation of mannosamine and pyruvate was not catalized either by *C. perfringens* *N*-acetylneuraminate lyase (*5*) or by animal *N*-acetylneuraminate lyase (*6*). We confirmed that the commercial *N*-acetylneuraminate lyase preparation from *C. perfringens* (Sigma Chemical Company, A 5884) could not catalize this condensation reaction. A new spot, however, was detected on the thin layer chromatogram when *E. coli* *N*-acetylneuraminate lyase was incubated with mannosamine and pyruvate (Table VI). The *N*-acyl-free neuraminic acid is thought to be synthesized from mannosamine and pyruvate, but this compound is so unstable that it immediately cyclizes to give a product of an intramolecular Schiff base, 4-hydroxy-5-[1,2,3,4-tetrahydroxybutyl]-Δ'-pyrroline-2-carboxylic acid (*35*, *36*). Since the accumulation of the reaction product was small amount, it could not be isolated in this experiment. Further work is necessary to

identify the product.

Two pathways are known for the synthesis of *N*-acetylneuraminic acid in bacteria: *N*-acetylneuraminate synthetase, which condenses *N*-acetylmannosamine and phosphoenolpyruvate, and *N*-acetylneuraminate lyase, which condenses *N*-acetylmannosamine and pyruvate. *N*-Acetylneuraminate synthetase is found to be distributed in *Neisseria meningitidis* (*37*). Since the enzyme purified from *E. coli* can synthesize *N*-acetylneuraminic acid not from phosphoenolpyruvate but from pyruvate (Table VI), it can be identified as *N*-acetylneuraminate lyase.

N-Acetylneuraminate lyase from *C. perfringens* has been reported to have low substrate specificity with regard to the *N*-substituents of neuraminic acid (*9*, *11*); *N*-glycolyl-, *N*-monochloroacetyl-, *N*-formyl-, *N*-succinyl-, and *N*-benzyloxycarbonylneuraminic acid have little influence on the enzyme activity when compared with *N*-acetylneuraminic acid. As shown in Table V, *N*-acetylneuraminate lyase from *E. coli* was active against *N*-glycolylneuraminic acid, the enzyme from *E. coli* also seems to have low substrate specificity with regard to the *N*-substituents of neuraminic acid.

A molecular weight of 99,200 (*16*) or 92,000 (*14*) was determined for the native *N*-acetylneuraminate lyase from *C. perfringens*. After denaturation in SDS or urea, a molecular weight of 50,000 was demonstrated for the enzyme, indicating the existence of two subunits (*16*). The molecular weight of native *N*-acetylneuraminate lyase from *E. coli* was similar to that of *C. perfringens*, but the subunit molecular weight of the *E. coli* enzyme was smaller than for the *C. perfringens* enzyme (Figs. 8 and 9).

For the enzymatic determination of the bound form of sialic acid, a coupled reaction consisting of neuraminidase, *N*-acetylneuraminate lyase, and lactate dehydrogenase was proposed. The optimal pHs of bacterial neuraminidases are generally in the acidic range of pH 4.5 to 6.5 (*38*). *Arthrobacter ureafaciens* neuraminidase also has its maximum activity around pH 4.5 to 5.5 (*39*), but it shows a broad optimum range with glycoprotein as the substrate (*33*, *39*). Therefore, the coupled reaction could be conducted satisfactorily at pH 7.3 (Fig. 11). If the coupled reaction is conducted at an acidic pH, spontaneous degradation of NADH takes place, which makes accurate determination difficult.

Since the purified *N*-acetylneuraminate lyase from *E. coli* was shown to be free from NADH oxidase and NADH dehydrogenase (Table II), the experimental errors resulting from the degradation of NADH can be omitted. This is particularly important when a small amount of sialic acid has to be determined, especially when by the fluorometric procedure (*32*).

As significant changes in sialic acid levels in body fluids have been observed in many pathological states, the determination of sialic acid in serum or urine is of clinical significance (*33*, *40–50*). Automated methods for determining sialic acid have been described for this purpose (*51–53*). These methods, however, are based on color reactions such as either the thiobarbituric acid method or resorcinol method, so they are not strictly specific for sialic acid and are not suitable for the exact determination of sialic acid in serum, which usually contains only low levels of sialic acid and large amounts of substances which may interfere with the color reaction. A sensitive automated chromatographic method has been described for a highly specific assay of sialic acid (*54*), but it is relatively time-consuming.

Enzymatic methods have recently been developed for the determination of sialic acid (*33*, *44*, *55*). Although the enzymatic method is most specific, reliable, and easy to practice, the limited supply and the expense of the enzyme have prevented it from being adopted widely. The method of preparing *N*-acetylneuraminate lyase developed by us was shown to be suitable for the large-scale production of the enzyme with high activities. This will contribute to the simplified diagnostic assay of sialic acid in serum, urine, body fluids or tissues, which is expected to have clinical import.

The authors wish to thank to Mr. M. Sogawa for technical assistance.

REFERENCES

1. Heimer, R. & Meyer, K. (1956) *Proc. Natl. Acad. Sci. U.S.* **42**, 728–734
2. Brug, J., Esser, R.J.E., & Paerels, G.B. (1959) *Biochim. Biophys. Acta* **33**, 241–242
3. Popenoe, E.A. & Drew, R.M. (1957) *J. Biol. Chem.* **228**, 673–683
4. Comb, D.G. & Roseman, S. (1958) *J. Am. Chem. Soc.* **80**, 497–499

5. Comb, D.G. & Roseman, S. (1960) *J. Biol. Chem.* **235**, 2529–2537
6. Brunetti, P., Jourdian, G.W., & Roseman, S. (1962) *J. Biol. Chem.* **237**, 2447–2453
7. Sirbasku, D.A. & Binkley, S.B. (1970) *Biochim. Biophys. Acta* **206**, 479–482
8. Gantt, R., Millner, S., & Binkley, S.B. (1964) *Biochemistry* **3**, 1952–1960
9. Faillard, H., Ferreira do Amaral, C., & Blohm, M. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 798–802
10. Schauer, R. & Wember, M. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 1517–1523
11. Schauer, R., Wember, M., Wirtz-Peitz, F., & Ferreira do Amaral, C. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 1073–1080
12. Barnett, J.E.G., Corina, D.L., & Rasool, G. (1971) *Biochem. J.* **125**, 275–285
13. Suttajit, M., Urban, C., & McLean, R.L. (1971) *J. Biol. Chem.* **246**, 810–814
14. DeVries, G.H. & Binkley, S.B. (1972) *Arch. Biochem. Biophys.* **151**, 234–242
15. DeVries, G.H. & Binkley, S.B. (1972) *Arch. Biochem. Biophys.* **151**, 243–250
16. Nees, S., Schauer, R., Mayer, F., & Ehrlich, K. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 839–853
17. Uchida, Y., Tsukada, Y., & Sugimori, T. (1980) *Abstracts of Papers, Annual Meeting of the Agricultural Chemical Society of Japan* (in Japanese) p. 455
18. Reissig, J.L., Strominger, J.L., & Leloir, L.F. (1955) *J. Biol. Chem.* **217**, 959–966
19. Uchida, Y., Tsukada, Y., & Sugimori, T. (1977) *J. Biochem.* **82**, 1425–1433
20. Mackler, B. (1967) in *Methods in Enzymology* (Estabrook, R.W. & Pullman, M.E., eds.) Vol. 10, pp. 261–263, Academic Press, Inc., New York
21. King, T.E. & Howard, R.L. (1967) in *Methods in Enzymology* (Estabrook, R.W. & Pullman, M.E., eds.) Vol. 10, pp. 275–294, Academic Press, Inc., New York
22. Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265–275
23. Ornstein, L. (1964) *Ann. N.Y. Acad. Sci.* **121**, 321–349
24. Davis, B. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
25. Weber, K., Pringle, J.R., & Osborn, M. (1972) in *Methods in Enzymology* (Hirs, C.H.W. & Timasheff, S.N., eds.) Vol. 26, pp. 3–27, Academic Press, Inc., New York
26. Diezel, W., Kopperschläger, G., & Hoffmann, E. (1972) *Anal. Biochem.* **48**, 617–620
27. Andrews, P. (1964) *Biochem. J.* **91**, 222–233
28. Laurent, T.C. & Killander, J. (1964) *J. Chromatogr.* **14**, 472–474
29. Mann, K.G. & Fish, W.W. (1972) in *Methods in Enzymology* (Hirs, C.H.W. & Timasheff, S.N., eds.) Vol. 26, pp. 28–42, Academic Press, Inc., New York
30. Uchida, Y., Tsukada, Y., & Sugimori, T. (1973) *Agric. Biol. Chem.* **37**, 2105–2110
31. Kikutani, M. (1969) *Kagaku to Seibutsu* (in Japanese) **7**, 620–627
32. Brunetti, P., Swanson, A., & Roseman, S. (1963) in *Methods in Enzymology* (Colowick, S.P. & Kaplan, N.O., eds.) Vol. 6, pp. 465–473, Academic Press, Inc., New York
33. Taniuchi, K., Chifu, K., Hayashi, N., Nakamachi, Y., Yamaguchi, N., Miyamoto, Y., Doi, K., Baba, S., Uchida, Y., Tsukada, Y., & Sugimori, T. (1981) *Kobe J. Med. Sci.* **27**, 91–102
34. Kolisis, F.N., Sotiroudis, T.G., & Evangelopoulos, A.E. (1980) *FEBS Lett.* **121**, 280–282
35. Gielen, W. (1965) *Hoppe-Seyler's Z. Physiol. Chem.* **342**, 170–171
36. Gielen, W. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 329–333
37. Blacklow, R.S. & Warren, L. (1962) *J. Biol. Chem.* **237**, 3520–3526
38. Drzeniek, R. (1972) *Curr. Top. Microbiol. Immunol.* **59**, 35–74
39. Uchida, Y., Tsukada, Y., & Sugimori, T. (1979) *J. Biochem.* **86**, 1573–1585
40. Spiro, R.G. (1970) *Annu. Rev. Biochem.* **39**, 599–638
41. Tsurumi, K. (1974) *Rinsho Byori* (in Japanese) extra issue No. 20, pp. 103–110, Rinsho Byori Kankokai, Tokyo
42. Morishita, Y., Nakane, K., & Kosaka, A. (1976) *Rinsho Byori* (in Japanese) **24**, 411–414
43. Rosenberg, A. & Schengrund, C. (1976) in *Biological Roles of Sialic Acid* (Rosenberg, A. & Schengrund, C., eds.) pp. 275–294, Plenum Press, New York
44. Taniuchi, K., Miyamoto, Y., Uchida, Y., Chifu, K., Mukai, M., Yamaguchi, N., Tsukada, Y., Sugimori, T., Doi, K., & Baba, S. (1979) *Jpn. J. Clin. Chem.* **7**, 403–410
45. Murata, K. (1982) *Nihon Rinsho* (in Japanese) **40** (special autumnal issue), 224–226
46. Tanaka, A., Yamaoka, S., Issiki, G., & Tamada, A. (1982) *Nihon Rinsho* (in Japanese) **40** (special autumnal issue), 252–253
47. Corfield, A.P. & Schauer, R. (1982) in *Cell Biology Monographs* (Schauer, R., ed.) Vol. 10, pp. 5–50, Springer-Verlag, New York
48. Reutter, W., Kottgen, E., Bauer, C., & Gerok, W. (1982) in *Cell Biology Monographs* (Schauer, R., ed.) Vol. 10, pp. 263–305, Springer-Verlag, New York

49. Cantz, M. (1982) in *Cell Biology Monographs* (Schauer, R., ed.) Vol. 10, pp. 307–320, Springer-Verlag, New York
50. Taoka, Y. (1983) *Rinsho Byori* (in Japanese) extra issue No. 54, pp. 2–25, Rinsho Byori Kankokai, Tokyo
51. Gerbaut, L., Rey, E., & Lombart, C. (1973) *Clin. Chem.* **19**, 1285–1287
52. Rey, E., Gerbaut, L., & Lombart, C. (1975) *Clin. Chem.* **21**, 412–414
53. Morishita, Y., Nakane, K., & Kosaka, A. (1976) *Rinsho Byori* (in Japanese) **24**, 1008–1012
54. Krantz, M.J. & Lee, Y.C. (1975) *Anal. Biochem.* **63**, 464–469
55. Sugahara, K., Sugimoto, K., Nomura, O., & Usui, T. (1980) *Clin. Chim. Acta* **108**, 493–498
56. Cassidy, J.T., Jourian, G.W., & Roseman, S. (1965) *J. Biol. Chem.* **240**, 3501–3506