N-Acetyl-D-neuraminic acid synthesis in *Escherichia coli* K1 occurs through condensation of *N*-acetyl-D-mannosamine and pyruvate

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Two enzymes have been found to be involved in bacterial *N*-acetyl-D-neuraminic acid (NeuAc) synthesis: NeuAc synthase, which condenses *N*-acetyl-L,D-mannosamine and phosphoenolpyruvate, and NeuAc lyase or NeuAc aldolase, which condenses *N*-acetyl-D-mannosamine and pyruvate. When we used *Escherichia coli* K1 crude extracts, we observed the generation of NeuAc in the presence of *N*-acetylmannosamine and both phosphoenolpyruvate (NeuAc synthase activity) or pyruvate (NeuAc lyase activity). However, when crude extracts were fractionated by Sephacryl S-200 chromatography, NeuAc synthase activity disappeared. A chromatographic peak of NeuAc synthase activity was detected when column fractions were re-tested in the presence of the active NeuAc lyase peak. Furthermore, crude extracts converted phosphoenolpyruvate into pyruvate. Pyruvate depletion, due to the addition of pyruvate decarboxylase to the NeuAc synthase reaction mixture, blocked NeuAc formation. Moreover, after NeuAc lyase immunoprecipitation no NeuAc synthase was detected. These findings suggest that NeuAc synthase is not present in *E. coli* K1 and therefore that NeuAc lyase is the only enzyme responsible for NeuAc synthesis in this bacterium.

INTRODUCTION

N-Acetyl-D-neuraminic acid (sialic acid, NeuAc) is an acidic sugar frequently found as a component of eukaryotic carbohydrate structures (glycoproteins and glycolipids) [1–7]. In prokaryotic cells, sialic acid has also been found as a capsular polysaccharide constituent of a few genera of pathogenic bacteria [7–12]. Thus, *Escherichia coli* K1 serotypes and *Neisseria meningitidis* groups B and C produce a capsular homopolymer containing NeuAc residues α(2-8)- or α(2-9)-ketosidically linked that have been identified as pathogenic determinants. These polysialic acids protect against host defences [13], causing many neonatal meningitis and urinary tract infections [14–16].

Capsular polysialic acid biosynthesis starts with NeuAc formation by an enzymic system different from that of eukaryotic cells described (for a review see [17,18]). In bacteria, two enzymes have been implicated in NeuAc synthesis: (a) NeuAc synthase (EC 4.1.3.19), which condenses *N*-acetyl-D-mannosamine (ManNAc) and phosphoenolpyruvate (Pep) by the following reaction:

$$\text{ManNAc} + \text{Pep} \rightarrow \text{NeuAc} + P_i$$

and (b) NeuAc lyase or aldolase (EC 4.1.3.3), which condenses ManNAc and pyruvate (Pyr) by the following reaction:

$$\text{ManNAc} + \text{Pyr} \rightarrow \text{NeuAc}$$

NeuAc synthase has been found in *N. meningitidis* [19,20] but only in undialysed *E. coli* crude extracts [21] and never in animal tissues [18]. NeuAc lyase has been found in different bacterial [22–26] and eukaryotic [18,27,28] sources. Since the equilibrium of NeuAc lyase lies in the catabolic direction [18] and no activity has been found in animal tissues secreting sialic acid-containing polymers [18,25], NeuAc synthase has been proposed as being responsible for '*in vivo*' bacterial NeuAc synthesis, and NeuAc lyase could regulate the pool of intracellular NeuAc by a catabolic reaction [18,21,29]. In our previous studies we have detected the presence of both activities in crude extracts of *E. coli* K235 [30,31], a K1 serotype that produces a capsular homopolymer of sialic acid α(2-8)-linked, namely colominic acid [32]. Although the biosynthesis of this capsular polymer has been extensively studied [7,18,21,30,32–42], little is known about the enzyme(s), regulation and metabolic effectors involved in NeuAc formation.

In this paper we report that *E. coli* K1 crude extracts synthesize NeuAc using both Pep and Pyr as substrates. However, we also demonstrate that crude extracts degrade Pep to Pyr before the NeuAc synthesis, concluding that NeuAc lyase is the only enzyme responsible for NeuAc synthesis in *E. coli*.

MATERIALS AND METHODS

Chemicals

NeuAc, ManNAc, pyruvate, phosphoenolpyruvate, 3-bromopyruvic acid, *N*-bromosuccinimide, D-xylose, resorcinol, BSA, L-proline, 2-thiobarbituric acid, periodic acid and sodium arsenite were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *N*-Acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid (300 Ci/mol) was from Amersham International (Amersham, Bucks., U.K.). ManN[1-^{14}C]Ac (18 Ci/mol) was from ICN Biomedicals (ICN, Bucks., U.K.). Sephadex G-25 (PD-10), Protein A–Sepharose 4B and Sephacryl S-200 were purchased from Pharmacia Fine Chemicals (Sweden). Other reagents used were of analytical quality.

Culture media and growth conditions

Escherichia coli K1 (A.T.C.C. 13027) was obtained from the American Type Culture Collection. The strain was mantained on

Abbreviations used: NeuAc or sialic acid, *N*-acetyl-D-neuraminic acid; ManNAc, *N*-acetyl-D-mannosamine; Pep, phosphoenolpyruvate; Pyr, pyruvate; Glc, glucose; TLCK, Nα-*p*-tosyl-L-lysine chloromethane; DTT, dithiothreitol.

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Trypticase Soy Agar (Difco) and slants grown for 8 h at 37 °C were used for seeding liquid media as previously described [39]. Incubations were carried out in a rotary shaker (250 rev./min) at 37 °C for 14 h ($A_{540} = 2.0$) using a chemically defined medium (xylose/proline) ideal for colominic acid production [39]. When necessary, carbon or nitrogen sources were replaced by others, the C- or N-concentration being maintained constant.

Cell-free extracts and enzymic assays

Cell-free extracts (crude extracts) of *E. coli* K1 were obtained as previously described [30]. NeuAc synthase and NeuAc lyase activities were assayed by a modification of the method of Warren [43,44]. Incubation mixtures were contained in a final volume of 50 μl: 0.125 M Tris/HCl, pH 8.0, 10 mM ManNAc, 8.75 mM Pep (NeuAc synthase activity) or Pyr (NeuAc lyase activity) and 15 μl of protein extract. Incubations were carried out in a water bath at 37 °C for 30 min (or the time required). After incubations, the reactions were stopped by adding 137 μl of periodic acid solution (2.5 mg/ml in 57 mM H_2SO_4) and then incubated for an additional 15 min at the same temperature. After this time, 50 μl of sodium arsenite solution (25 mg/ml in 0.5 M HCl) was added and the tubes were shaken rigorously to ensure complete elimination of the yellow–brown colour. 2-Thiobarbituric acid solution (100 μl; 71 mg/ml adjusted to pH 9.0 with NaOH) was added and the tubes were heated to 100 °C for exactly 7.5 min. The pink–red colour generated was extracted by vigorous shaking in 1 ml of organic solution (butanol–acid) and the phases were then correctly separated in a microcentrifuge. The absorbance of the organic phase was measured at 549 nm with a Shimadzu UV-120-02 spectrophotometer.

The presence of Pyr or Pep in the reaction mixtures was determined spectrophotometrically using lactate dehydrogenase- or pyruvate kinase and lactate dehydrogenase-coupled assays respectively [45,46]. When the effect of pyruvate decarboxylase (from yeast, Sigma Chemical, Co., St. Louis, MO, U.S.A.) was assayed on synthase and lyase activities, incubations were carried out in 0.2 M citrate buffer, pH 7.0. After incubation (1 h) at 37 °C, the amounts of Pep and Pyr were determined spectrophotometrically.

To check the true degree of NeuAc generation and when NeuAc was used as effector, incubations were carried out with the radioactive substrate (ManN[1-^{14}C]Ac). In this case, 20 μl of the reaction mixture was adjusted for paper (Whatman 3 MM) chromatography and developed as described previously [39]. Under these conditions, the R_F value of the products generated was the same as that of standard NeuAc (*N*-acetyl[4,5,6,7,8,9-^{14}C]NeuAc) ($R_F = 0.6$). The R_F for ManN[1-^{14}C]Ac was 0.8.

Cleavage of NeuAc by NeuAc lyase activity was determined spectrophotometrically [30,45]. When the effect of Pyr was analysed, radioactive NeuAc was used as substrate.

Protein was measured by the method of Bradford [47] using BSA as standard.

One unit of enzyme was defined as the amount of enzyme that synthesizes or cleaves 1 nmol of NeuAc/min at 37 °C under the assay conditions. Specific activity was expressed as units/mg of protein.

Chromatographic analysis

Crude extracts of *E. coli* K1 were concentrated by ammonium sulphate precipitation (80% saturation), resuspended in a minimal volume (usually 2–3 ml) of 50 mM Tris/HCl, pH 8.0, containing 25 mM $MgCl_2$ and 1 mM dithiothreitol (DTT) and applied to a column (2.5 cm × 76 cm) of Sephacryl S-200 equilibrated and eluted with the same buffer. Fractions (2 ml) were collected and assayed for NeuAc synthase or NeuAc lyase activities.

When DEAE-chromatography was used, the resuspension was desalted by passing through a Sephadex G-25 PD-10 column equilibrated with this buffer. The desalted extract was injected into a fast-performance liquid chromatography (FPLC) system (Waters Millipore) equipped with a variable-wavelength visible/UV detector, a computer Spectra-Physics integrator (SP 4290), and an anion-exchange column (Nihon Waters DEAE SPW: 0.8 cm × 7.5 cm). The column was equilibrated and washed with the same buffer and proteins were then eluted with a KCl gradient (0–0.1 M). Fractions (2 ml) were collected and assayed for NeuAc synthase or NeuAc lyase activities.

Immunoprecipitation analysis

Rabbit hyperimmune sera to NeuAc lyase enzyme was obtained by immunizing animals with pure enzyme as previously described [48]. The immunoprecipitation was carried out as follows: crude extracts of *E. coli* K1 were treated with serum obtained against this enzyme in a final volume of 20 μl in the presence of protease inhibitors [1 mM PMSF and 0.5 mM *Nα-p*-tosyl-L-lysine chloromethane (TLCK)] for 1 h at room temperature [49,50].

Following the addition of Protein A–Sepharose 4B, the suspension was incubated for 1 h at 4 °C with shaking. After centrifugation at 5000 *g* for 3 min in a microcentrifuge, supernatants and pellets were assayed for NeuAc synthase or NeuAc lyase activities.

RESULTS AND DISCUSSION

Crude extracts synthesize NeuAc using both Pyr or Pep as substrates

NeuAc lyase and NeuAc synthase activities were analysed using cell extracts of *E. coli* K1 grown in a chemically defined liquid medium that has been reported as ideal for colominic acid production [39]. Cell extracts obtained by growth in this medium, which contained D-xylose and L-proline as the only carbon and nitrogen sources, showed the presence of both activities (48 and 10 units/mg of protein, respectively). Since Vimr and Troy [29,51] reported that NeuAc lyase from *E. coli* K12 and K12–K1 hybrid derivatives strains is an NeuAc-inducible and Glc-repressive enzyme, we assayed these activities in *E. coli* K1 extracts obtained by growth with different carbon sources. A higher NeuAc lyase activity (89 units/mg of protein) was detected when the carbon source was NeuAc. However, no significant differences were observed when the activity was assayed in extracts from bacteria grown in the presence of Pyr and Glc (42 and 40 units/mg of protein, respectively). These results suggest that NeuAc lyase is a NeuAc-inducible but not a Glc-repressive enzyme in *E. coli* K1. With regard to NeuAc synthase, a lower degree of activity was detected in Pyr-, Glc- and NeuAc-grown cell extracts (5, 4 and 6 units/mg of protein, respectively). This effect, similar in all cases, could be a consequence of the different metabolic conditions produced by these media [39], but not due to a repressive action of Glc.

NeuAc synthase activity is not saturated by Pep as substrate and disappears following liquid chromatography

Variations in substrate concentrations afforded a hyperbolic type of behaviour in the rate of the NeuAc lyase and NeuAc synthase

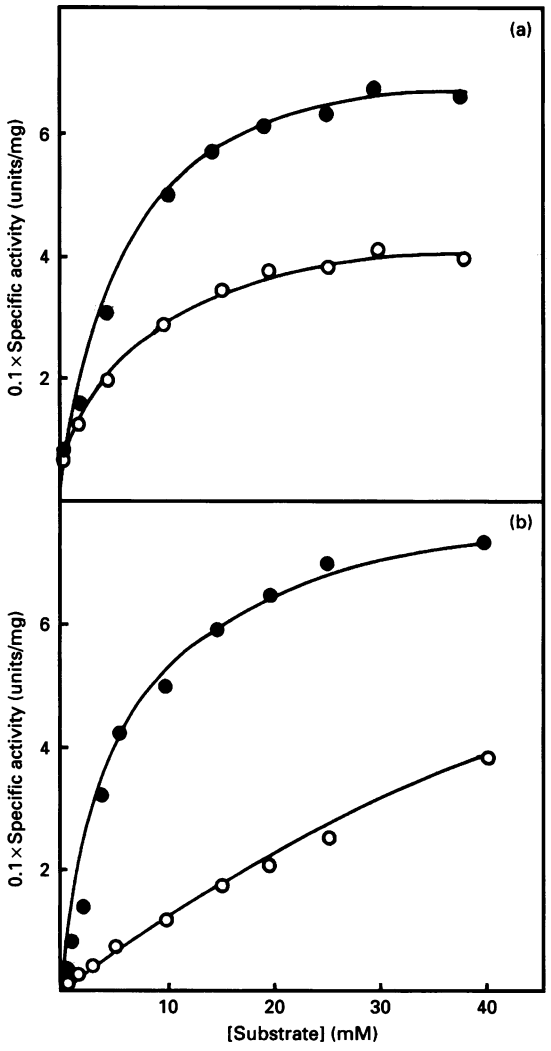

Figure 1 Effect of substrate concentration on NeuAc lyase (●) and NeuAc synthase (○) activities from *E. coli* K1 crude extracts

(**a**) ManNAc. (**b**) Pyr (NeuAc lyase) or Pep (NeuAc synthase).

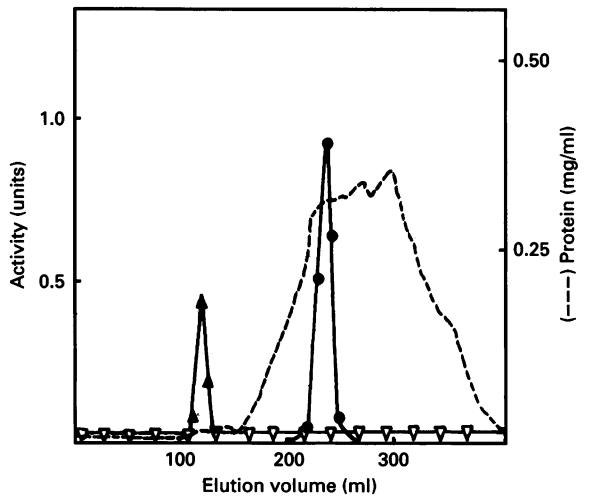

Figure 2 Profile of elution from Sephacryl S-200 column of *E. coli* K1 crude extracts

NeuAc lyase activity (●) and NeuAc synthase activity when coupled (▲) or not (▽) with the fraction containing maximal NeuAc lyase activity (230 ml).

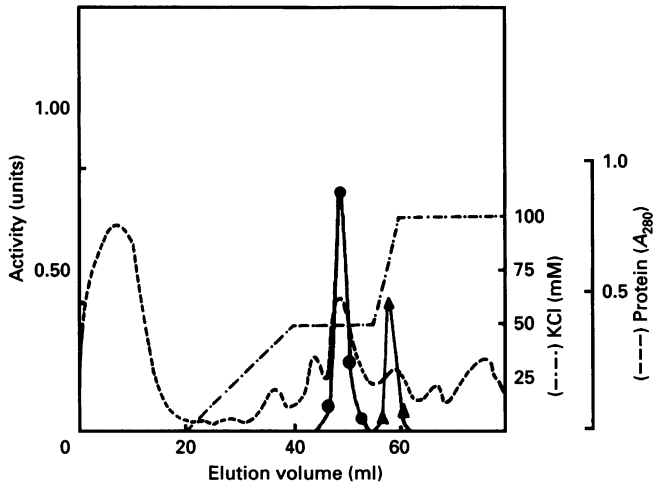

Figure 3 Profile of elution from FPLC-DEAE column of *E. coli* K1 crude extracts

NeuAc lyase activity (●) and NeuAc synthase activity when coupled (▲) with the fraction containing maximal NeuAc lyase activity (48 ml).

reactions (Figure 1). However, while both activities showed a similar behaviour against different ManNAc concentrations (Figure 1a), NeuAc synthase exhibited a lower affinity for Pep than NeuAc lyase for Pyr. Moreover, NeuAc synthase activity was not saturated by Pep, even when this substrate was used at a concentration of 40 mM (Figure 1b). These results suggest at least two different hypotheses: (1) NeuAc synthase is activated in the presence of high Pep concentrations, and (2) crude extracts used in these experiments modify Pep prior to NeuAc formation.

When crude extracts were chromatographed using gel filtration on Sephacryl S-200, NeuAc synthase activity disappeared (Figure 2). However, synthase activity was detected when column fractions were re-tested in the presence of the active NeuAc lyase peak (Figure 2). This synthase-coupled activity was also observed when commercial NeuAc lyase from *Clostridium perfringens* and from *E. coli* (Sigma Chemical Co., MO, U.S.A.) were added to the different column fractions (data not shown). Similar results were obtained when FPLC–DEAE-chromatography was used (Figure 3). The presence of a putative phosphatase activity in the crude extracts and in the column fractions with NeuAc synthase-coupled activity (Figures 2 and 3), which converts Pep into Pyr prior to NeuAc synthesis, could explain these results and would confirm the second hypothesis.

Crude extracts convert Pep into Pyr and NeuAc synthase activity is inhibited by Pyr decarboxylase

To confirm the above possibility, NeuAc synthase reactions without ManNAc and containing Pep and *E. coli* K1 crude extracts were incubated at 37 °C. The reactions were stopped at different times by protein elimination using a Centricon-10 ultrafiltration system (Amicon, MA, U.S.A.). Then, the amount of Pep and Pyr present was measured spectrophotometrically

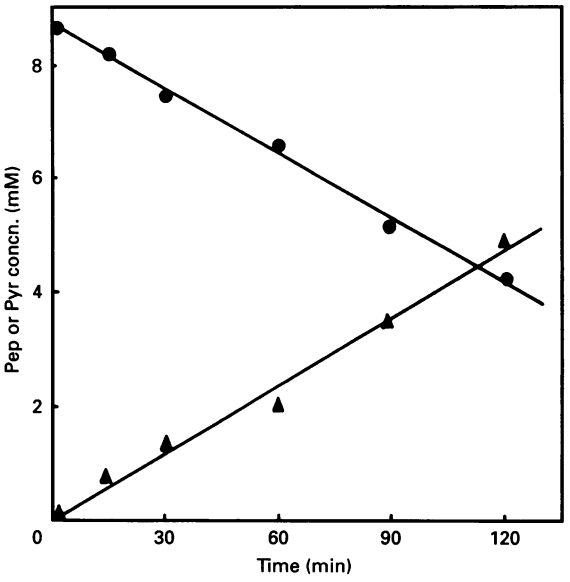

Figure 4 Time course of Pep disappearance (●) and Pyr appearance (▲) by NeuAc synthase activity from *E. coli* K1 crude extracts

In these experiments ManNAc was not present in the reaction mixtures.

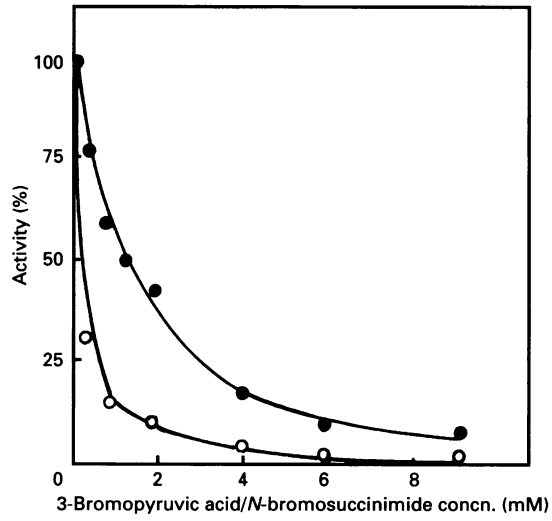

Figure 5 Effect of 3-bromopyruvic acid (●) and *N*-bromosuccinimide (○) concentrations on NeuAc lyase from *E. coli* K1

(see the Materials and methods section). Under these conditions, Pep disappeared and Pyr appeared rapidly, following a stoichiometric pattern (Figure 4). The results obtained suggest that the NeuAc generated in the NeuAc synthase reaction is also a consequence of NeuAc lyase activity. To prevent lyase activity, we added 2 units of Pyr decarboxylase (from yeast, Sigma Chemical Co., MO, U.S.A.) to the NeuAc synthase reaction mixture. Since Pyr decarboxylase rapidly converts the Pyr generated by crude extracts (but not the Pep) into acetaldehyde, then in the presence of this enzyme the NeuAc synthase reaction must synthesize NeuAc only through the NeuAc synthase action. When Pyr decarboxylase was present, NeuAc synthase activity was not detected (data not shown). These results demonstrate

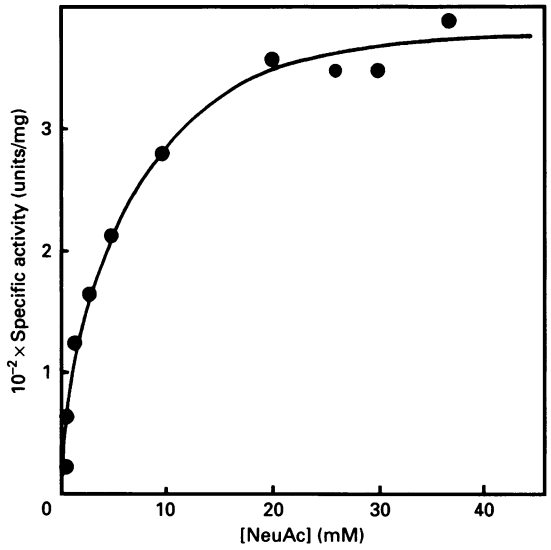

Figure 6 Effect of substrate concentration on NeuAc lyase activity (cleavage reaction)

that *E. coli* K1 crude extracts synthesize NeuAc by condensation of ManNAc with Pyr (NeuAc lyase activity) but not with Pep (NeuAc synthase activity). This conclusion was also confirmed by immunoprecipitation of crude extracts with antibodies against NeuAc lyase from *E. coli* K1. In the presence of these antibodies, obtained by rabbit immunization with purified enzyme (M. A. Ferrero, L. B. Rodríguez-Aparicio, C. González-Clemente and A. Reglero, unpublished work), NeuAc lyase activity was precipitated and NeuAc synthase activity was not detected either in the supernatant or in the precipitated fractions (data not shown).

The non-repressive NeuAc lyase described here, showed a similar behaviour to another enzyme reported in the literature [18,21,25,26,29,51]. In this sense, our enzyme was strongly inhibited by 3-bromopyruvic acid and *N*-bromosuccinimide (Figure 5), two known lyase inhibitors [18,26]. Moreover, the enzyme was also able to cleave NeuAc (Figure 6) and, in this case, the hydrolysis reaction was inhibited by Pyr (a reaction product). A concentration of 8.75 mM caused 80% inhibition. On the other hand, NeuAc inhibited the biosynthetic reaction. A concentration of 5 mM caused 30% inhibition. All these results suggest that NeuAc lyase from *E. coli* K1 can function to regulate the intracellular concentration of free sialic acid and to modulate the capsular polysialic acid synthesized by this bacterium. Equilibrium studies by analysis of the two reactions, cleavage and condensation directions, revealed an equilibrium constant, $K = [\text{NeuAc}]/([\text{ManNAc}] \times [\text{Pyr}])$, to be 12×10^{-5} M (Figure 7). Under these conditions the cleavage reaction was favoured at lower substrate concentrations, whereas the condensation reaction was favoured at higher substrate concentrations (Figure 7). Since *E. coli* K1 activates NeuAc to CMP-NeuAc for capsular biosynthesis [7,18,30,40], a low level of free intracellular sialic acid would be expected. In this state, the condensation reaction will be favoured.

A better understanding of NeuAc lyase from *E. coli* K1 by purification, characterization and gene analysis will make it possible to confirm its key function in bacterial sialic and polysialic acid metabolism. Moreover, this information should open the possibility of elaborating therapeutic approaches to

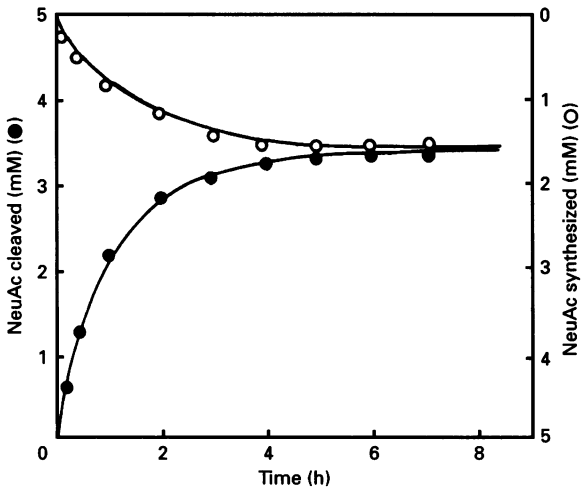

Figure 7 Equilibrium of NeuAc lyase reaction

Reaction mixtures (1 ml) containing an excess of enzyme were incubated either with 5 mM NeuAc (cleavage reaction) or with 5 mM ManNAc and 5 mM Pyr (condensation reaction) on 0.125 M Tris/HCl, pH 8.0. After incubation at 37 °C, aliquots were taken at the indicated times and the reaction products were quantified as described in the Materials and methods section.

controlling the synthesis of this pathogenic determinant. Further research on this topic is currently in progress.

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