N-Acetyl-D-neuraminic acid lyase generates the sialic acid for colominic acid biosynthesis in *Escherichia coli* K1

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Colominic acid is a capsular homopolymer from *Escherichia coli* K1 composed of α(2-8)-linked *N*-acetyl-D-neuraminic acid (NeuAc) residues. Recently, we have described that NeuAc synthesis in this bacterium occurs through the action of NeuAc lyase (EC 4.1.3.3) [Rodríguez-Aparicio, Ferrero and Reglero (1995) Biochem. J. **308**, 501–505]. In the present work we analysed and characterized this enzyme. *E. coli* K1 NeuAc lyase is detected from the early logarithmic phase of growth, is induced by NeuAc and is not repressed by glucose. The enzyme was purified to apparent homogeneity (312-fold) using two types of hydrophobic chromatographies (butyl-agarose and phenyl-Sepharose CL-4B), gel filtration on Sephacryl S-200, and anion-exchange chromatography on DEAE-FPLC. The pure enzyme, whose amino acid composition and N-terminal amino acid sequence are also established, has a native molecular mass, estimated by gel filtration, of 135 ± 3 kDa, whereas its molecular mass in SDS/PAGE was 33 ± 1 kDa. The enzyme was able to synthesize and cleave NeuAc in a reversible reaction. The maximal rate of catalysis was achieved in 125 mM Tris/HCl buffer, pH 7.8, at 37 °C. Under these conditions, the K_m values calculated for *N*-acetyl-D-mannosamine and pyruvate (condensation direction), and NeuAc (hydrolysis direction) were 7.7, 8.3 and 4.8 mM respectively. NeuAc synthesis by the pure enzyme was activated by Ca^{2+} and inhibited by Mn^{2+} and NeuAc, whereas the enzyme cleavage direction was inhibited by Ca^{2+}, Mn^{2+} and pyruvate. The reaction products, NeuAc and pyruvate, and Ca^{2+} are able to regulate the direction of this enzyme (synthesis or cleavage of sialic acid) and, accordingly, to modulate colominic acid biosynthesis.

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 constituent capsular polysaccharide of a few genera of pathogenic bacteria [7–14]. Thus, *Escherichia coli* K1 serotypes and *Neisseria meningitidis* groups B and C produce a capsular homopolymer composed of α(2-8)- or α(2-9)-ketosidically linked NeuAc residues that have been identified as pathogenic determinants. These polysialic acids protect against host defences [15–17] causing many neonatal meningitis and urinary tract infections [18–22].

Although the biosynthesis and structure of these bacterial polysaccharides have received considerable attention [1,8,9,11,23–29], little is known about the regulation and specific metabolic effectors involved in their biosynthesis, which starts with NeuAc formation by mechanisms different from those in animals. In eukaryotic cells, NeuAc synthesis implies the sequential action of three different enzymes with the formation of *N*-acetyl-D-mannosamine 6-phosphate (ManNAc-6-P) and NeuAc-9-P as intermediates [1,30–32]. However, in bacteria, sialic acid formation takes place by direct condensation of *N*-acetyl-D-mannosamine (ManNAc) with pyruvate (Pyr) (NeuAc lyase activity) or phosphoenolpyruvate (PEP) (NeuAc synthase activity) [33] as follows:

$$\text{ManNAc} + \text{Pyr (PEP)} \rightarrow \text{NeuAc}(+\text{P}_i)$$

Such bacterial enzymic activities also seem to be species-specific; thus NeuAc synthase, but not NeuAc lyase, has been found in *N. meningitidis* [34], and NeuAc lyase, and apparently NeuAc synthase [35], are also present in *E. coli* [33,36–38]. The fact that NeuAc formation for capsular polysialic acid synthesis takes place by different mechanisms to those in animal tissues opens the possibility of elaborate therapeutic approaches that should allow specific control of the synthesis of this pathogenic determinant. In this sense we have analysed the presence of this enzyme in *E. coli* K1, a bacterium that produces an α(2-8)-linked capsular polysialic acid: namely, colominic acid [8].

We have previously reported that NeuAc lyase is the only enzyme from *E. coli* K1 involved in the biosynthesis of NeuAc [33]. In the present paper, we now describe the purification methodology and the biochemical characterization of the NeuAc lyase from *E. coli* K1. Its physiological role in the regulation of the intracellular pool of free sialic acid and the modulation of capsular polysialic acid biosynthesis are also discussed.

MATERIALS AND METHODS

Chemicals

NeuAc, ManNAc, resorcinol, Pyr, PEP, D-xylose, BSA, NADH, lactate dehydrogenase, L-proline, 2-thiobarbituric acid, periodic acid, streptomycin sulphate, sugars, amino acids, nucleotides 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), butyl-agarose, phenyl-Sepharose CL-4B, Sephacryl S-200, Polybuffer changer gel (PBE-94), Polybuffer 74 and 96, and molecular-mass markers were purchased from Pharmacia Fine Chemicals (Sweden). Other reagents used were of analytical quality.

Temperature experiment media and growth conditions

E. coli K1 (A.T.C.C. 13027) was obtained from the American Type Culture Collection. The strain was maintained on Tryp-

Abbreviations used: NeuAc or sialic acid, *N*-acetyl-D-neuraminic acid; NeuGc, *N*-glycolylneuraminic acid; ManNAc, *N*-acetyl-D-mannosamine; PEP, phosphoenolpyruvate; Pyr, pyruvate; DTT, dithiothreitol.

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ticase 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 the required temperatures and times using a chemically defined medium (xylose/proline) ideal for colominic acid production [39]. When necessary, carbon or nitrogen sources were replaced by others, C- or N-concentrations being maintained constant.

E. coli K92 (A.T.C.C. 35860) and *Pasteurella haemolytica* A2 (A.T.C.C. 29694) were grown in Trypticase Soy Broth (Difco) as described previously [39].

Colominic acid determinations

The sialyl polymer produced by *E. coli* K1 and K92, and *P. haemolytica* A2 was analysed by the Svennerholm [40] methodology described elsewhere [39].

Enzymic assays

NeuAc lyase condensation activity was assayed by a modification [33] of the method of Warren [41,42]. Incubation mixtures contained final volumes of 50 μl: 0.125 M Tris/HCl pH 7.8, 10 mM ManNAc, 8.75 mM Pyr and 15 μl of protein extract. Incubations were carried out in a water bath at 37 °C for 30 min (or the required time).

Cleavage activity was determined spectrophotometrically, using a lactate dehydrogenase-coupled assay [26,43]. Incubation mixtures contained, in a final volume of 1 ml, 0.125 M Tris/HCl, pH 7.8, 5 mM NeuAc, 0.25 mM NADH, 4 units of lactate dehydrogenase and 50 μl of protein extract.

In some cases, when molecules that interfere with the thiobarbituric or spectrophotometric methods were used, NeuAc lyase activity (generation or cleavage of NeuAc) was followed by a radiometric assay. A 15-μl aliquot from the above reaction mixture containing radioactive ManN[1-^{14}C]Ac (6.5 nmol) (synthesis direction) or *N*-acetyl-[4,5,6,7,8,9-^{14}C]neuraminic acid (0.3 nmol) (cleavage direction) was subjected to paper chromatography (Whatman 3 MM) and developed as reported [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]neuraminic acid) ($R_F = 0.6$) and ManNAc (ManN[1-^{14}C]Ac) ($R_F = 0.8$).

Protein was measured by the method of Bradford [44], 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.

Purification of NeuAc lyase

Unless otherwise indicated, all procedures were carried out at 4 °C.

Step 1: preparation of the homogenized extract

E. coli K1 was grown in the chemically defined medium (xylose/proline) for 14 h (or the required time) [39]. Bacteria were collected by centrifugation (10000 ***g***, 10 min), washed twice with 25 mM Tris/HCl, pH 8, and resuspended in 50 mM Tris/HCl, pH 8, containing 25 mM $MgCl_2$ and 1 mM PMSF (TMP buffer). Cells (15 g wet weight/130 ml of buffer) were disrupted with 100 g of glass beads (Ballotini, 0.17–0.18 mm diameter) using a Braun MSK mechanical disintegrator. Cell debris was eliminated by centrifugation at 19000 ***g*** for 15 min.

Step 2: ultracentrifugation

The pellet from step 1 was discarded and the supernatant was ultracentrifuged at 250000 ***g*** for 60 min.

Step 3: streptomycin sulphate precipitation

Nucleic acids present in the supernatant from step 2 were precipitated by the addition of 0.75 % (w/v) streptomycin sulphate and removed by centrifugation at 35000 ***g*** for 20 min.

Step 4: ammonium sulphate fractionation

The supernatant obtained from step 3 was precipitated with ammonium sulphate. The fraction precipitating between 60 and 80 % (containing all the NeuAc lyase activity) was collected by centrifugation at 35000 ***g*** for 20 min.

Step 5: hydrophobic chromatography on butyl-agarose

The precipitate obtained from the above centrifugation was resuspended in TMP buffer and was passed through a Sephadex G-25 (PD-10) column to eliminate excess ammonium sulphate. Ammonium sulphate was added to the desalted extract to 25 % satn. and applied to a column (10 cm × 2 cm) of butyl-agarose equilibrated with the same buffer containing ammonium sulphate (25 % satn.). The column was washed with the same buffer and 2 ml fractions of this percolate were collected and enzyme activity measured.

Step 6: hydrophobic chromatography on phenyl-Sepharose CL-4B

Positive fractions of NeuAc lyase activity from step 5 were applied to a column (10 cm × 2 cm) of phenyl-Sepharose CL-4B. The column was exhaustively washed with 120 ml of TMP buffer containing ammonium sulphate (20 % satn.) and the enzyme was eluted with 120 ml of a 20–6.5 % gradient of ammonium sulphate in the same buffer. Fractions (1 ml) were collected at a flow rate of 6 ml/h.

Step 7: gel filtration on Sephacryl S-200

Fractions containing NeuAc lyase activity from the above step were mixed and precipitated with ammonium sulphate (80 % satn.). The precipitate was dissolved in 2 ml of TMP buffer and applied to a Sephacryl S-200 column (2.5 cm × 76 cm) equilibrated and eluted with the same buffer. Fractions (2 ml) were collected and assayed for NeuAc lyase activity.

Step 8: anion-exchange on DEAE-FPLC

Fractions containing NeuAc lyase activity from the above step were injected into an 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 5PW: 0.8 cm × 7.5 cm). The column was equilibrated and washed with the TMP buffer and proteins were then eluted with a 0 to 0.1 M KCl gradient. Fractions (2 ml) were collected and assayed for NeuAc lyase activity.

Using this procedure, a 312-fold purification of *E. coli* K1 NeuAc lyase was achieved (see Table 1).

Electrophoretic analysis

SDS/PAGE [45] was performed in 12 % slab gels under reducing conditions. Phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin

(43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14 kDa) were used as molecular-mass standards.

Amino acid analysis

The homogeneity-pure NeuAc lyase was hydrolysed in twice-distilled HCl (6 M) at 112 °C for 24 h in evacuated sealed tubes. Hydrolysates were analysed by HPLC as reported [46].

Amino acid sequencing

The purified enzyme was desalted by HPLC in a Vydac reverse-phase 5-μm C-4 column (type 214TP54, 0.46 cm × 5 cm) using a 30 min linear gradient from 20 to 95% acetonitrile (0.1% trifluoroacetic acid present). The protein, eluted between 58 and 61% acetonitrile, was freeze-dried, resuspended in 30 μl of 0.15% trifluoroacetic acid, and loaded on to the sequencer. Amino acid sequencing was carried out as described elsewhere [47].

Isoelectric point analysis

The isoelectric point of NeuAc lyase was analysed by chromatofocusing. A fraction eluted from DEAE-FPLC anion-exchange chromatography was loaded on to a chromatofocusing column (0.7 cm × 10 cm) packed with Polybuffer changer gel (PBE-94) previously equilibrated with 25 mM Tris/acetate buffer, pH 8.3. The column was washed and equilibrated with five volumes of the same buffer. Enzyme activity was eluted in a pH gradient created by the passage of a solution of Polybuffer 74 and 96 (7:3) diluted 1:10 with distilled water (final pH adjusted to 5.0 with acetic acid). Under these conditions the NeuAc lyase eluted at a pH of approx. 7.6.

Immunochemical methods

Rabbit hyperimmune sera to NeuAc lyase enzyme from *E. coli* K1 was obtained by immunizing the animals with pure enzyme (140 μg/dose) as described previously [33,48]. The serum thus obtained was then partially purified by passage through a Protein A–Sepharose column according to a previously described method [49]. Immunodetection of NeuAc lyase was carried out by immunoblot analysis on nitrocellulose sheets as previously described [50], using commercial or crude bacterial extracts as the protein source.

RESULTS AND DISCUSSION

Time course of the appearance of NeuAc lyase during culture

NeuAc lyase from *E. coli* K1 began to be synthesized from the early logarithmic phase of growth (5–10 h) when this bacterium was grown at 37 °C in xylose/proline, an ideal medium for colominic acid production [39]. The level of the enzyme increased linearly during the early logarithmic phase (Figure 1a), reaching a maximum after 18 h incubation, and from this time until 40 h it decreased continuously. This kind of kinetic behaviour, which parallels that of bacterial growth, is very similar to that observed for CMP-synthetase [28] and sialyltransferase [27], the other enzymes directly involved in the biosynthesis of colominic acid by *E. coli* K1. Additionally, the *in vivo* half-life of NeuAc lyase (determined by halting protein synthesis at 16 h of growth by adding 5 μg/ml chloramphenicol) was 54 h (Figure 1a), suggesting that the enzyme has a slow turnover rate.

Different authors have indicated that NeuAc lyase is a typical inducible enzyme in micro-organisms. It is produced only in the

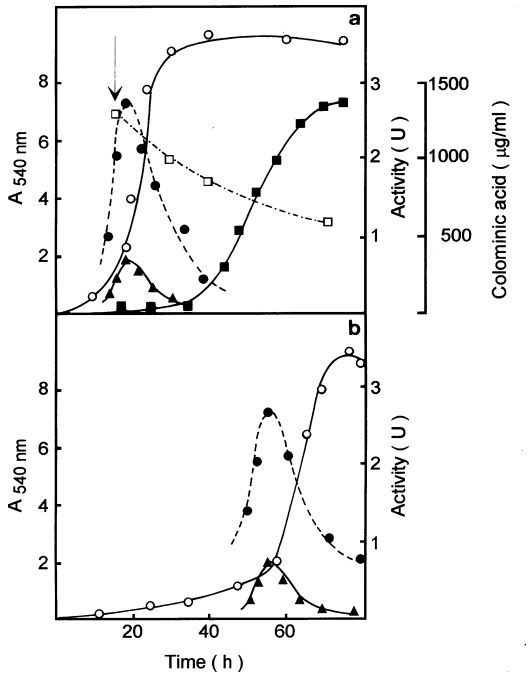

Figure 1 Time course of formation of NeuAc lyase from *E. coli* K1 grown at 37 °C (a) and 15 °C (b) in xylose/proline medium

NeuAc condensation (▲) and NeuAc cleavage (●) activities, bacterial growth (○) and colominic acid production (■) are shown. NeuAc cleavage activity when chloramphenicol (5 μg/ml) was present (□) is also shown. Arrow indicates time at which this antibiotic was added for determination of enzyme half-life.

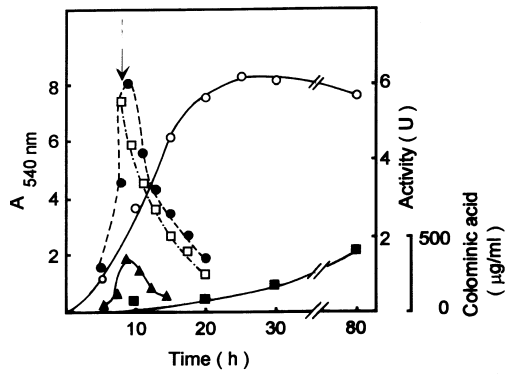

Figure 2 Time course of formation of NeuAc lyase from *E. coli* K1 grown at 37 °C in the presence of NeuAc (8 g/l) as carbon source

NeuAc condensation (▲) and NeuAc cleavage (●) activities, bacterial growth (○) and colominic acid production (■) are shown. □ shows NeuAc cleavage activity when glucose (0.2%, w/v) was present. Arrow indicates time at which this sugar was added.

presence of the substrate (NeuAc) in the growth medium or by biosynthetically derived sialic acid in bacterial strains that express a capsular polysialic acid [51,52]. Moreover, the degree of lyase induction appeared to be strain dependent [51]. When *E. coli* K1 was grown in NeuAc as the only carbon source, NeuAc lyase activity showed similar kinetic behaviour to that observed in xylose/proline medium and, although a decrease in colominic acid production was detected (65%), the level of the enzyme increased 2.5-fold (Figure 2). Moreover, the quantity of NeuAc

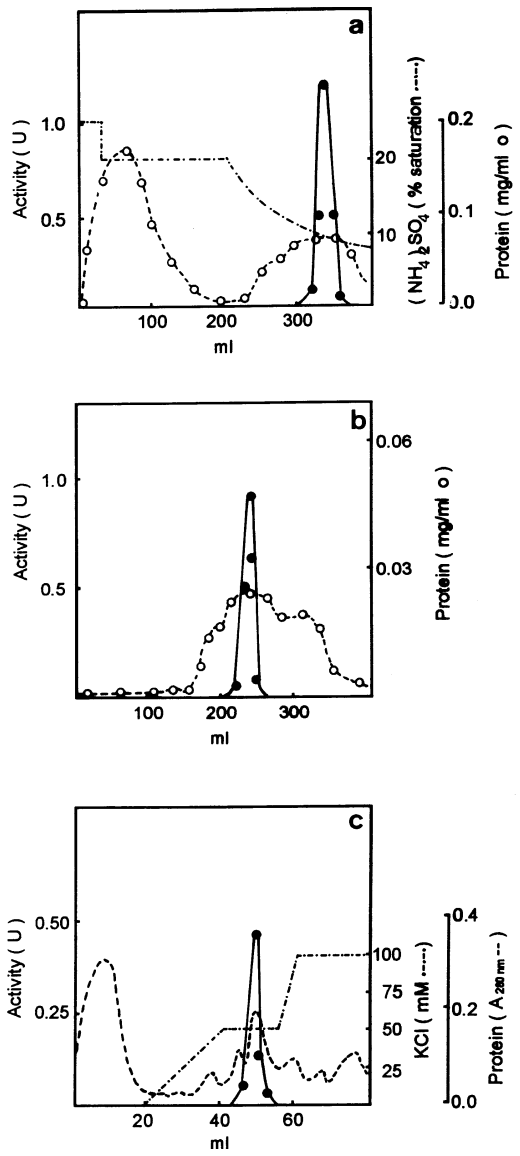

Figure 3 Profile of elution (●) of NeuAc lyase from *E. coli* K1 from a phenyl-Sepharose CL-4B column (a), Sephacryl S-200 (b), and DEAE-FPLC (c)

●, NeuAc condensation activity.

lyase present was not affected by the addition of glucose (0.2 %) to the NeuAc medium (Figure 2). These results suggest that the NeuAc lyase from *E. coli* K1 is NeuAc-inducible, but not a Glc-repressive enzyme as described previously [33].

On the other hand, previous studies have shown that when *E. coli* K1 is grown at a temperature below 20 °C, the quantity of colominic acid produced is negligible due to the absence of CMP-NeuAc synthetase at these temperatures [28,39], whereas synthesis increases markedly after the temperature is raised [28,39,53]. When NeuAc lyase synthesis was analysed at growth temperatures known to be restrictive (below 20 °C), this enzyme was also found to be present even when *E. coli* K1 was grown in the absence of NeuAc (Figure 1b). These results indicate that NeuAc lyase, the enzyme that synthesizes NeuAc in *E. coli* K1 [33], is also present in the absence of biosynthetically derived NeuAc (as, for instance, colominic acid).

Enzyme purification

A pure and active NeuAc lyase preparation was obtained from *E. coli* K1 extracts. The purification steps included butyl-agarose and phenyl-Sepharose chromatographies, followed by gel-filtration chromatography on Sephacryl S-200 and anion exchange on DEAE-FPLC (Figure 3). The purification scheme is summarized in Table 1. The most effective steps were the phenyl-Sepharose (Figure 3a) and DEAE-FPLC (Figure 3c) chromatographies. Exhaustive washings in these purification steps are very important for obtaining good results. Under these conditions (Table 1), the final preparation had a 312-fold enrichment in enzyme activity, with an overall recovery of 26 %. Other lyases from bacteria that do not produce polysialic acid have been purified with similar enrichments and activity yields: 358–740-fold and 21–24 % of activity yield for the lyases from *Clostridium perfringens* [54,55], and 72–90 and 42–12 % of activity yield for the lyases from *E. coli* C-600 and from *E. coli* AKU 0007, respectively [37,38]. The purification methods described in these sources [37,38,54,55] could not be successfully implemented for optimal lyase purification of *E. coli* K1. Moreover, the use of other ionic and hydrophobic chromatographic techniques gave no further increases in the specific activity yield of this enzyme.

Molecular-mass determination

The molecular mass of native NeuAc lyase from *E. coli* K1 was determined by gel-filtration chromatography in Sephacryl S-200 (calibrated with a kit of known molecular-mass protein stan-

Table 1 Purification of NeuAc lyase from *E. coli* K1

Treatment	Volume (ml)	Protein (mg)	Enzyme activity (units)*	Specific activity† (units/mg)	Yield of recovery (%)	Purification (fold)
Crude extract	125	96	4.62	0.048	100	1
Ultracentrifugation (250 000 ***g***, 60 min)	123	62	4.42	0.071	96	1.5
Streptomycin sulphate (0.75 %)	124	58	4.28	0.073	93	1.5
Ammonium sulphate precipitation (60–80 %)	24	33	4.12	0.124	89	2.5
Butyl-agarose	26	18	3.8	0.211	82	4.4
Phenyl-Sepharose CL-4B	25	4.2	2.9	1.44	63	30.0
Sephacryl S-200	23	1.2	2.3	1.91	50	39.8
DEAE-FPLC	5	0.08	1.2	15.0	26	312.5

* One unit is the amount of enzyme which synthesizes 1 nmol of NeuAc per min at 37 °C.
† Synthesis direction.

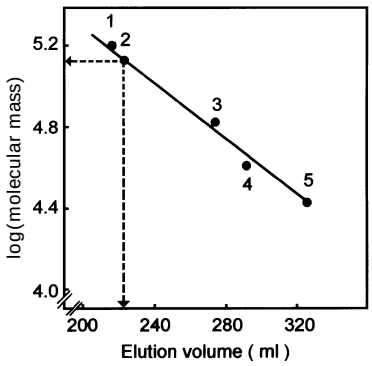

Figure 4 Determination of molecular mass of NeuAc lyase from *E. coli* K1

Molecular mass was calculated using a calibrated Sephacryl S-200 column with known proteins: 1, aldolase (158 kDa); 3, BSA (67 kDa); 4, ovalbumin (43 kDa); 5, carbonic anhydrase (29 kDa). 2, NeuAc lyase.

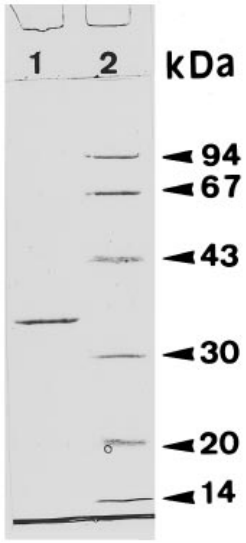

Figure 5 Electrophoretic mobility of purified NeuAc lyase from *E. coli* K1

A purified sample of enzyme was run on SDS/12%-PAGE (lane 1) and several molecular-mass standard proteins (phosphorylase *b*, 94 kDa; BSA, 67 kDa; ovoalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa) (lane 2). Proteins were stained with Coomassie Blue R-250.

dards) to be 135 ± 2 kDa (Figure 4). When running in SDS/12 %-PAGE, the denatured enzyme preparation gave a single and sharp protein band (Figure 5) whose apparent molecular mass was 33 ± 1 kDa. This finding suggests that the NeuAc lyase from *E. coli* K1 is a tetrameric protein with four similar chains (α_4-oligomeric structure). This molecular mass and the enzyme's quaternary structure differ from those of other lyases. Thus, *Clostridium perfringens* lyase has a dimer structure with a molecular mass of between 92 [55] and 99 kDa [54]. Moreover, different authors have reported a molecular mass for the *E. coli* lyases between 98 [38] and 105 kDa and a trimer structure, which is a very rare quaternary structure. However, our results for *E. coli* K1 lyase (135 ± 2 kDa and an α_4-oligomeric structure) are in agreement with Izard et al. [56], who have reported that the crystalline enzyme from another source (*E. coli* strain that does not produce sialylated polymer) has a tetrameric structure.

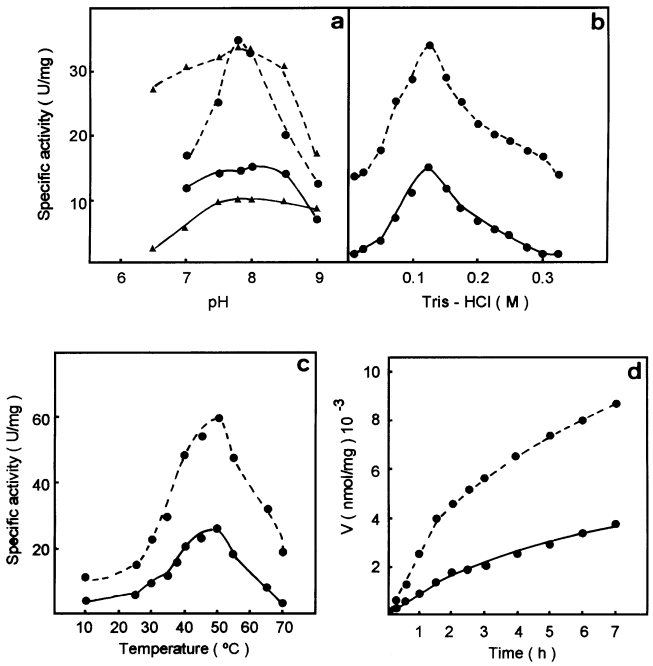

Figure 6 Effects of pH [Tris/HCl (●) and phosphate (▲) buffers] (a), ionic strength (b), temperature (c), and time (d) on NeuAc lyase activity from *E. coli* K1

Continuous and broken lines indicate the condensation and hydrolysis activity directions, respectively.

Optimization and pH

The effects of pH on the rate of synthesis or cleavage of NeuAc (Figure 6a) by NeuAc lyase were assayed. The highest activity in both directions was observed between 7.8 and 8.0 when Tris/HCl was used as buffer. In order to avoid the possible effect of variation in ionic strength, buffer molarity was modified between 10 and 325 mM. The variations in activity rates clearly indicate that the best conditions are achieved at 125 mM of Tris/HCl, pH 7.8 (Figure 6b). When the enzyme was incubated at temperatures ranging between 5 and 80 °C (Figure 6c), the highest activity was observed at 50 °C (in both directions). These results are similar to those previously described for NeuAc lyases (assayed only in the hydrolysis direction) from different bacterial sources [37,38,54,55].

Kinetic properties

The products generated by NeuAc lyase (in both directions) were linear over 90 min (Figure 6d) when incubations were carried out at 37 °C under the above conditions (pH 7.8 and 125 mM Tris/HCl).

The enzyme activity shows a hyperbolic type of behaviour for ManNAc and Pyr (synthesis of NeuAc) and NeuAc (cleavage direction) (Figure 7), the K_m values calculated for each being 8.3, 7.7 and 4.8 mM respectively. The K_m value for NeuAc was in the range of those observed for different NeuAc lyases from other sources [30,37,38,43,54,55]. However, since NeuAc lyase activity has usually been assayed in the cleavage direction (degradation of NeuAc) [54,55], no data about the K_m of ManNAc or Pyr are available. The similar substrate affinities shown for all the substrates employed (Figure 7) suggest that this enzyme plays an important role in the regulation of the intracellular free sialic

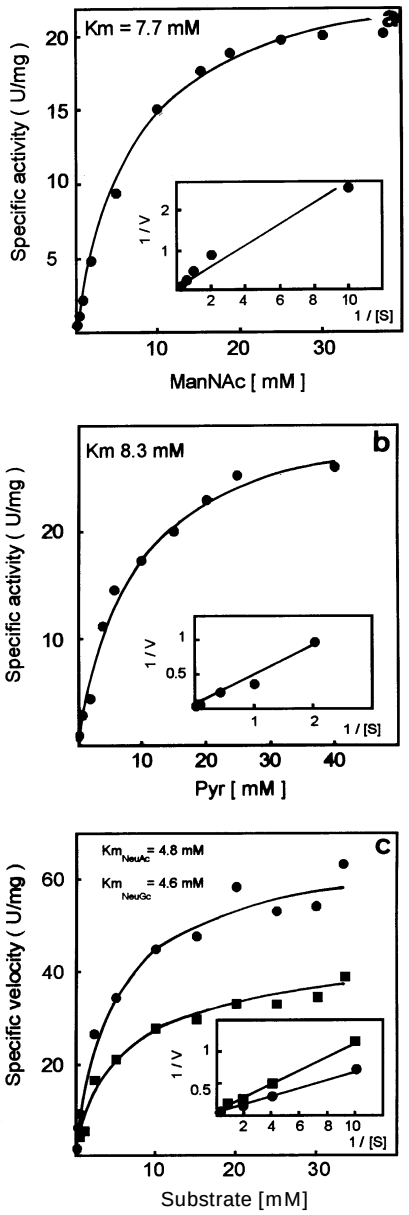

Figure 7 NeuAc lyase substrate analysis

Determination of K_m values for ManNAc (**a**) and Pyr (**b**) in the condensation direction, and for NeuAc (●) and NeuGc (■) in the hydrolysis direction (**c**). Each substrate was assayed under saturating conditions for the others, as described in the Materials and methods section. Kinetic parameters were calculated using the non-linear 'Enzfitter' (Elsevier) regression data analysis program.

acid concentration. Our previous analysis of the two reactions (synthesis and hydrolysis) have revealed an equilibrium constant ($K =$ [NeuAc]/([ManNAc] × [Pyr]), of 12×10^{-5} M [33]. Under these conditions the condensation direction is favoured at lower product (NeuAc) concentrations. Since *E. coli* K1 activates NeuAc to CMP-NeuAc for polysialic acid synthesis [7,26,28,57], a low level of free sialic acid would be expected and, in this situation, the NeuAc synthesis direction would be favoured.

Effects of temperature: enzyme stability

Purified NeuAc lyase from *E. coli* K1 is quite stable under prolonged storage at −20 °C in the same buffer used in the purification steps (TPM buffer, see the Materials and methods section) containing glycerol (20 %, v/v) as a cryoprotective agent. When the thermal stability of NeuAc lyase was examined, we observed that the enzyme was stable at 50 °C for at least 40 min, and 40 % inactivation was achieved at 80 °C for 10 min (Figure 8). Since at 50 °C a significant amount of protein was denatured and precipitated (36 %), temperature treatment can be used as a rapid method for partial enzyme purification. Additionally, the enzyme is stable at 37 °C for at least 24 h in the purification buffer (TMP). Accordingly, in ensuing experiments an incubation temperature of 37 °C, which is also the physiological temperature for *E. coli* K1 and optimal colominic acid production [39], was routinely used in both the synthesis and cleavage directions.

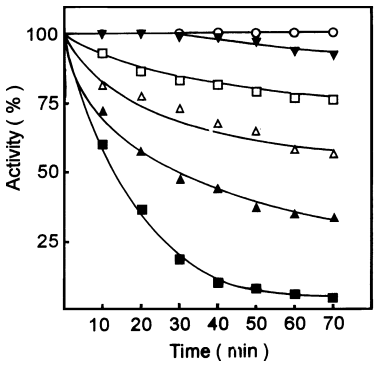

Figure 8 Effect of temperature on NeuAc lyase deactivation time

Incubations were carried out for different times at 37 °C (○), 50 °C (▼), 65 °C (□), 70 °C (△), 75 °C (▲) and 80 °C (■).

When the effect of substrates (ManNAc, Pyr or NeuAc) was tested against thermal deactivation, no modification in the temperature stability effect was detected even when high temperatures were used (results not shown). These results contrast with those previously reported where different lyases were protected in the cleavage direction when the reactions took place in the presence of both substrates (Pyr or ManNAc) [37,38].

Effect of Mn^{2+}, Ca^{2+} and other cations

The effect of several cations (10 mM, chloride form) on NeuAc lyase activity from *E. coli* K1 was studied. Whereas the addition of univalent cations (K^+, Na^+ and Li^+) to the reaction mixtures had no effect, the presence of certain bivalent cations such as Hg^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} strongly inhibited enzyme activity in both directions (98, 80, 96 and 78 % for condensation, and 96, 86, 99 and 80 % for the hydrolysis reaction, respectively). While similar effects have been reported when heavy metal ions have been analysed in the cleavage direction using other NeuAc lyase sources [37,38,55], the inhibition caused by Mn^{2+}, even at low concentrations (Figure 9), seems to be a specific characteristic of the *E. coli* K1 enzyme. Moreover, this inhibitory effect could account for the strong decrease (80 %) in colominic acid production detected when *E. coli* K1 was grown in xylose/proline medium with 10 mM Mn^{2+}. Ca^{2+} also showed specific behaviour against NeuAc lyase from this bacterium. However, when this cation was added to the reaction mixtures, we observed that NeuAc synthesis (condensation direction) was increased and NeuAc hydrolysis (cleavage direction) was strongly inhibited (Figure 9). A possible complexing of the NeuAc by this ion

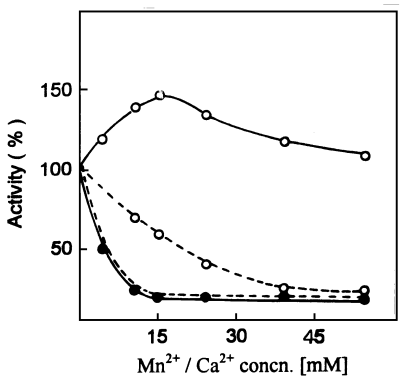

Figure 9 Effect of Mn^{2+} (●) and Ca^{2+} (○) chloride concentrations on NeuAc lyase activity

Continuous and broken lines indicate the condensation and hydrolysis activity directions, respectively.

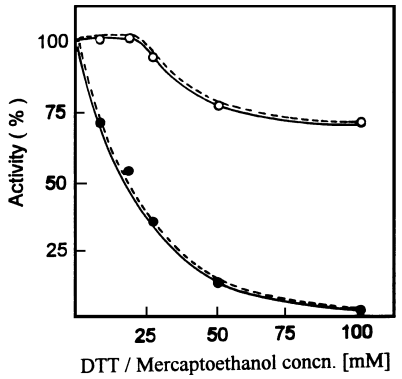

Figure 10 Effect of thiol-containing reagents on NeuAc lyase activity

DTT (●) and mercaptoethanol (○). Continuous and broken lines indicate the condensation and hydrolysis activity directions, respectively.

would explain this behaviour. Thus, in the presence of Ca^{2+} the condensation reaction of the enzyme from *E. coli* K1 would be favoured and the colominic acid biosynthesis would be increased. In this sense, when the bacterium was grown in xylose/proline medium with 20 mM Ca^{2+}, we observed a rise (120 %) in polysialic acid production, possibly related to this enzymic effect.

The specific regulation by Mn^{2+} and Ca^{2+} of NeuAc lyase and colominic acid production seems to confirm the important key function of this enzyme in the sialic acid metabolism of *E. coli* K1. The effect of Mn^{2+} and Ca^{2+} cations on NeuAc synthesis has also been described by Merker and Troy [35] when they assayed NeuAc synthase (the enzyme that condenses PEP and ManNAc, see the Introduction section) using crude extracts from different mutants of *E. coli*. Since under these conditions crude extracts convert PEP into Pyr prior to NeuAc synthesis [33], these similar effects support our previous results suggesting that NeuAc lyase is the only enzyme responsible for NeuAc generation in *E. coli* K1 [33].

Effect of ammonium sulphate and different molecules

NeuAc acid lyase from *E. coli* K1 was inhibited by ammonium sulphate, the salt used during the purification enzyme (see above).

Table 2 Amino acid composition of NeuAc lyase given as residues/mol of protein based on a molecular mass of 33 kDa

Abbreviation: ND, not determined.

Amino acid	No. of residues
Asn	16
Glx	28
Ser	15
Gly	40
His	8
Thr	10
Ala	23
Arg	11
Pro	14
Tyr	7
Val	21
Met	5
Cys	ND
Ile	12
Leu	59
Lys	14
Trp	ND

A concentration of 1.0 M (25 % satn.) caused a 30 % inhibition of both NeuAc cleavage and NeuAc condensation. This effect, although reversible by enzyme desalting, requires careful elimination of the ammonium sulphate present in the samples to achieve a good quantification of lyase activity.

Thiol-modifying and thiol-containing reagents also affected NeuAc lyase activity. In the presence of *p*-chloromercuribenzoate and *N*-ethylmaleimide (1 mM), the activity was strongly inhibited in both directions (93–98 and 75–73 % respectively). This effect is quite similar to that shown by other lyases [38,55] and, together with the inhibition caused by heavy metal ions (see above), suggests that the presence of thiol groups are essential for catalytic activity. When thiol-containing reagents [dithiothreitol (DTT) and mercaptoethanol] were added to the reaction mixtures (condensation and hydrolysis directions), we observed an inhibitory effect. As shown in Figure 10, the presence of DTT from 5 mM and mercaptoethanol from 20 mM decreases activity. These results contrast with those obtained by DeVries and Binkley [55] for the NeuAc lyase from *C. perfringens*, where an increase in hydrolysis activity (125 %) was observed in the presence of mercaptoethanol (10 mM). This different effect suggests the existence of certain differences among lyases obtained from other micro-organism sources, and involves the necessary maintenance of the specific disulphide groups (spatial structure) in the enzymic activity of NeuAc lyase from *E. coli* K1.

The analysis of other sugars, amino acids and nucleotides did not point to modifications in lyase activity (cleavage and condensation directions) even when high concentrations (20 mM) of these molecules were tested.

On the other hand, NeuAc lyase from *E. coli* K1 showed a slightly decreased activity in the presence of ampholytes contained in the buffers required for chromatofocusing, and under the conditions reported in the Materials and methods section the isoelectric point calculated for this enzyme was 7.6 ± 0.2. This isoelectric point is another differential characteristic of NeuAc lyases from other sources, such as *C. perfringens* (pI 4.7) and *E. coli* C600-SF8 [37,54] (pI 4.5), two micro-organisms that do not produce polysialic acid. Moreover, although the amino acid composition analysis (Table 2) showed a higher similarity to

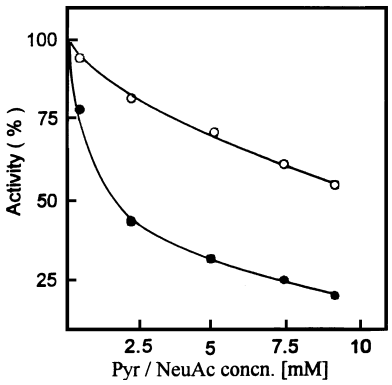

Figure 11 Effect of Pyr (●) and NeuAc (○) on the cleavage and condensation of NeuAc, respectively, by NeuAc lyase from *E. coli* K1

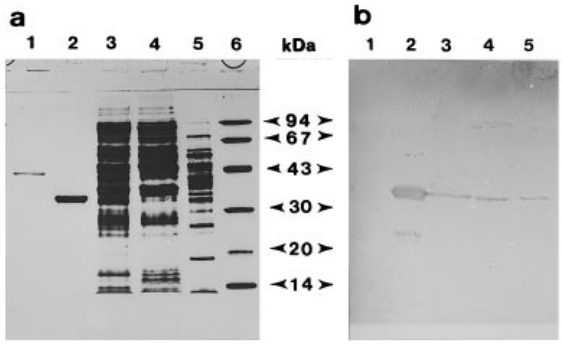

Figure 12 SDS/PAGE immunoblot analysis of NeuAc lyase from different bacterial sources

(**a**) SDS/PAGE stained with Coomassie Blue R-250. (**b**) Immunoblot analysed with anti-(NeuAc lyase from *E. coli* K1) as described in the Materials and methods section. Samples were: commercial NeuAc lyase from *C. perfringens* (from Sigma) and *E. coli* (from Boehringer) (lanes 1 and 2, respectively), and protein crude extracts from *E. coli* K1, *E. coli* K92 and *P. haemolytica* (lanes 3, 4 and 5, respectively). Lane 6 refers to molecular-mass standard proteins.

5 10 15
$^{+}H_3N$-Ala-Thr-Asn-Leu-Arg-Gly-Val-Met-Ala-Ala-Leu-Leu-(Leu)-Pro-Phe-Asp-Gln-(Cys)-Gln

Figure 13 N-terminal amino acid sequence of the purified NeuAc lyase from *E. coli* K1

Parentheses indicate a tentative assignment at that position.

other known lyases [56,58–60], this observation suggests the existence of some differences in the spatial structure of the enzyme from *E. coli* K1.

Substrate specificity

In an attempt to characterize the specificity of the NeuAc lyase from *E. coli* K1, different molecules were tested as substrates for the synthesis and hydrolysis of NeuAc. This enzyme showed high specificity for all the common substrates. Neither analogues of ManNAc (mannosamine, *N*-acetylgalactosamine, glucosamine and *N*-acetylglucosamine) nor analogues of Pyr (PEP, lactate, α-oxobutyrate, acetoacetate, and glyoxylate) were able to replace the original substrates in NeuAc lyase activity (synthesis direction). Moreover, when these compounds were added in the presence of the original substrates (ManNAc and Pyr), at a final concentration of 20 mM, no appreciable decrease in NeuAc synthesis was detected. These results indicate that other analogues can not be used as substrates or inhibitors of NeuAc synthesis and suggest that the ManNAc- and Pyr-binding sites of this enzyme are very specific.

In the cleavage direction, the NeuAc lyase from *E. coli* K1 was also able to use *N*-glycolylneuraminic acid (NeuGc) (Figure 7c). Although in this case a minor V_{max} was detected (70 %), the apparent affinity shown (K_m 4.6 mM) was similar to that obtained for NeuAc and to those described elsewhere for other lyases [37,38]. These results indicate that the modification in the NeuAc acetyl group due to hydroxylation is not very important for the cleavage of NeuAc lyase activity and suggest that, in this direction, the enzyme can probably use other modified sialic acids. Other sialyl-derivatives, such as sialyl oligomers (obtained by the hydrolysis of colominic acid, as described previously [61]) and colominic acid cannot be used as substrates and had no effect as inhibitors. These results indicate that the NeuAc lyase from *E. coli* K1 is not able to degrade NeuAc when this sugar appears as a polymer. Moreover, the absence of sialidase activity in this bacterium [62] suggests that NeuAc lyase is the enzyme responsible for the NeuAc formation of colominic acid biosynthesis.

Analysis of the reaction products as putative inhibitors showed that Pyr and NeuAc affected lyase activity in the cleavage and synthesis directions, respectively (Figure 11). The inhibitory effect elicited by the reaction products could regulate the direction of lyase activity in a similar fashion to the mechanism exerted by Ca^{2+} (see above). Thus, in the presence of high Ca^{2+} concentrations or low NeuAc levels, NeuAc synthesis would be favoured.

Immunoblot detection

In order to analyse the presence of NeuAc lyase from different sources, we obtained polyclonal antibodies against the enzyme from *E. coli* K1 (see the Materials and methods section). As shown in Figure 12, immunoblot analysis permitted us to detect the presence of this protein in strains of *E. coli* that produce (*E. coli* K1 and *E. coli* K92) or do not produce (commercial *E. coli* from Boehringer) polysialic acid and in *P. haemolytica*, another bacterium that has been reported to be a colominic acid producer strain [63]. However, the NeuAc lyase from *C. perfringens* (from Sigma) was not immunodetected (Figure 12b). These results suggest that the enzymes from *E. coli* and *P. haemolytica* are very different from that present in *C. perfringens*, whose enzyme even showed a different SDS/PAGE profile (Figure 12a).

On the other hand, the N-terminal amino acid sequence of the NeuAc lyase from *E. coli* K1 was analysed (Figure 13) and was found to be quite similar to the sequences established for other lyases from *E. coli* strains that do not produce colominic acid [37,56–59]. This finding, together with the similar results obtained with regard to amino acid composition analysis (Table 2) and certain kinetic parameters (see above), suggests that NeuAc lyase is very similar in all *E. coli* strains [36–38,56,60]. However, the significantly different pI (three units higher) and the specific effects of Mn^{2+} and Ca^{2+} (see above) shown by the NeuAc lyase from *E. coli* K1 indicate that the non-Glc-repressive enzyme from this bacterium has certain differences that may be fundamental for the synthesis of capsular polymer, suggesting the existence of evolutionary or adaptive changes in this enzyme. A punctual protein modification could account for the regulatory

effect that these cations have on the lyase activity, and consequently polysialic acid biosynthesis, of this bacterium.

The molecular information about the substrates and effector binding sites and the gene encoding this enzyme (currently in progress) could confirm the key role of NeuAc lyase in colominic acid metabolism and explain the differences observed among known lyases from different sources.

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REFERENCES

1 Corfield, A. P. and Schauer, R. (1982) in Sialic Acids. Chemistry, Metabolism and Function (Schauer, R., ed.), pp. 5–50, Springer-Verlag, New York
2 Van Rinsum, J., Van Dijk, W., Hoogwinkel, J. M. and Ferwerda, W. (1983) Biochem. J. **210**, 21–28
3 Rodríguez-Aparicio, L. B., Luengo, J. M., González-Clemente, C. and Reglero, A. (1992) J. Biol. Chem. **267**, 9257–9263
4 Montreuil, J. (1980) Adv. Carbohydr. Chem. Biochem. **37**, 157–223
5 Schachter, H., Jabbal, L., Hudgin, R. L., Pinteric, I., McGuire, E. J. and Roseman, D. (1970) J. Biol. Chem. **245**, 1090–1100
6 Troy, F. A. (1992) Glycobiology **2**, 5–23
7 Reglero, A., Rodríguez-Aparicio, L. B. and Luengo, J. M. (1993) Int. J. Biochem. **25**, 1517–1527
8 Barry, G. T. and Goebel, W. F. (1957) Nature (London) **179**, 206
9 Barry, G. T. (1959) Nature (London) **183**, 117–118
10 Liu, T.-Y., Gotschlich, E. C., Dunne, F. T. and Jonssen, E. K. (1971) J. Biol. Chem. **246**, 4703–4712
11 Bhattacharjee, A. K., Jennings, H. J., Kennedy, C. P., Martín, A. and Smith, I. C. P. (1975) J. Biol. Chem. **250**, 1926–1932
12 Bhattacharjee, A. K., Jennings, H. J., Kennedy, C. P., Martín, A. and Smith, I. C. P. (1976) Can. J. Biochem. **54**, 1–8
13 Silver, R. P. and Vimr, E. R. (1990) in The Bacteria. A Treatise on Structure and Function, XI, Molecular Basis of Bacterial Pathogenesis (Iglewski, B. H. and Clark, V. L., eds.), pp. 39–60, Academic Press, San Diego
14 Silver, R. P., Finn, C. W., Vann, W. F., Aaronson, W., Schneerson, R., Kretchmer, P. J. and Garon, C. F. (1981) Nature (London) **289**, 696–698
15 Schauer, R. (1981) Adv. Carbohydr. Chem. Biochem. **40**, 131–234
16 Finne, J. (1982) J. Biol. Chem. **257**, 11966–11970
17 Finne, J., Bitter-Suermann, D., Gorodis, C. and Finne, U. (1987) J. Immunol. **138**, 4402–4407
18 McCraken, G. H., Globe, M. P., Sarff, L. D., Mize, S. G., Schiffer, M. S., Robbins, J. B., Gotschlich, E. C., Orskov, I. and Orskov, F. (1974) Lancet **ii**, 246–250
19 Sarff, L. D., McCraken, G. H., Schiffer, M. S., Glode, M. P., Robbins, J. B., Orskov, I. and Orskov, F. (1975) Lancet **i**, 1090–1104
20 Kaijser, B., Hanson, L. A., Jodal, V., Linden-Johnson, G. and Robbins, J. B. (1977) Lancet **i**, 664–666
21 Mulder, C. J. J. and Zanen, H. C. (1984) Arch. Dis. Child **59**, 439–443
22 Pangburn, M. K. and Muller-Eberhard, H. J. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 2416–2420
23 Troy, F. A. (1979) Annu. Rev. Microbiol. **33**, 519–560
24 Kundig, F. D., Aminoff, D. and Roseman, S. (1971) J. Biol. Chem. **246**, 2543–2550
25 Yamasaki, R. and Bacon, B. (1991) Biochemistry **30**, 851–857
26 Rodríguez-Aparicio, L. B., Reglero, A., Ortiz, A. I. and Luengo, J. M. (1988) Biochem. J. **251**, 589–596
27 Ortiz, A. I., Reglero, A., Rodríguez-Aparicio, L. B. and Luengo, J. M. (1989) Eur. J. Biochem. **178**, 741–749
28 González-Clemente, C., Luengo, J. M., Rodríguez-Aparicio, L. B. and Reglero, A. (1989) FEBS Lett. **250**, 429–432
29 González-Clemente, C., Luengo, J. M., Rodríguez-Aparicio, L. B., Ferrero, M. A. and Reglero, A. (1990) Biol. Chem. Hoppe-Seyler **371**, 1101–1106
30 Comb, D. G. and Roseman, S. (1960) J. Biol. Chem. **235**, 2529–2537
31 Blacklow, R. S. and Warren, L. (1962) J. Biol. Chem. **237**, 3520–3526
32 Warren, L. and Felsenfeld, H. (1961) J. Biol. Chem. **237**, 1421–1431
33 Rodríguez-Aparicio, L. B., Ferrero, M. A. and Reglero, A. (1995) Biochem. J. **308**, 501–505
34 Masson, L. and Holbein, B. E. (1983) J. Bacteriol. **154**, 728–736
35 Merker, R. I. and Troy, F. A. (1990) Glycobiology **1**, 93–100
36 Ohta, Y., Shimosaka, M., Murata, K., Tsukada, Y. and Kimura, A. (1986) Appl. Microbiol. Biotechnol. **24**, 386–391
37 Aisaka, K., Igarashi, A., Yamaguchi, K. and Uwajima, T. (1991) Biochem. J. **276**, 541–546
38 Uchida, Y., Tsukada, Y. and Sugimori, T. (1984) J. Biochem. (Tokyo) **96**, 507–522
39 Rodríguez-Aparicio, L. B., Reglero, A., Ortiz, A. I. and Luengo, J. M. (1988) Appl. Microbiol. Biotechnol. **27**, 474–483
40 Svennerholm, L. (1958) Acta Chem. Scand. **12**, 547–554
41 Warren, L. (1959) J. Biol. Chem. **234**, 1971–1975
42 Warren, L. (1963) Methods Enzymol. **6**, 463–465
43 Comb, D. G. and Roseman, S. (1962) Methods Enzymol. **5**, 391–394
44 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
45 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
46 Bidlinmeyer, B. A., Cohen, S. A. and Tarvin, T. L. (1984) J. Chromatogr. **336**, 93–104
47 Hewick, R. M., Hunkapiller, M. W., Hood, L. E. and Dreyer, W. J. (1981) J. Biol. Chem **256**, 7990–7997
48 Rodríguez-Aparicio, L. B., Luengo, J. M., Ferrero, M. A. and Reglero, A. (1993) Int. J. Biochem. **25**, 427–432
49 Ey, P. L., Prowse, S. J. and Jenkin, C. R. (1978) Biochemistry **15**, 429–436
50 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
51 Vimr, E. R. and Troy, F. A. (1985) J. Bacteriol. **164**, 845–853
52 Vimr, E. R. and Troy, F. A. (1985) J. Bacteriol. **164**, 854–860
53 Troy, F. A. and McCloskey, M. A. (1979) J. Biol. Chem. **254**, 7377–7387
54 Nees, S., Schauer, R. and Mayer, F. (1976) Hoppe-Seyler's Z. Physiol. Chem. **357**, 839–853
55 DeVries, G. H. and Binkley, S. B. (1972) Arch. Biochem. Biophys. **151**, 234–242
56 Izard, T., Lawrence, M. C., Malby, R. L., Lilley, G. G. and Colman, P. M. (1994) Structure **2**, 361–369
57 Corfield, A. P. and Schauer, R. (1982) in Sialic Acids. Chemistry, Metabolism and Function (Schauer, R., ed.), pp. 195–261, Springer-Verlag, New York
58 Lin, C. H., Sugai, T., Halcomb, R. L., Ichikawa, Y. and Wong, C. H. (1992) J. Am. Chem. Soc. **114**, 10138–10140
59 Kong, P. C. M. and Itzstein, M. (1995) Tetrahedron Lett. **36**, 957–960
60 Otha, Y., Watenabe, K. and Kimura, A. (1985) Nucleic Acids Res. **13**, 8843–8852
61 Ferrero, M. A., Luengo, J. M. and Reglero, A. (1991) Biochem. J. **280**, 575–579
62 Vimr, E. R., Lawrisuk, I., Galen, J. and Kaper, J. B. (1988) J. Bacteriol. **170**, 1495–1504
63 Adlam, C., Knights, J. M., Mugridge, A., Williams, J. M. and Lindon, J. C. (1987) FEMS Microbiol. Lett. **42**, 23–25

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