The Preparation of CMP-Sialic Acids by using CMP-Acylneuraminate Synthase from Frog Liver Immobilized on Sepharose 4B

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A preparation of frog liver CMP-acylneuraminate synthase (2–10-fold enriched over the homogenate) obtained from DEAE-Sephadex A-50 chromatography of a 105000g liver supernatant was bound to Sepharose 4B by the CNBr method. The enzyme retained 80–100% activity on binding and showed similar properties to the purified soluble enzyme from the same source with respect to K_m , pH optimum and inhibition. The bound enzyme was stable to temperatures above 40°C, in contrast with the soluble enzyme, and could be stored for 4 months at 2°C with loss of 20% activity. The bound enzyme was used preparatively for the synthesis of radioactive and non-radioactive CMP-*N*-acetylneuraminic acid and CMP-*N*-glycolylneuraminic acid. With suitable substrate concentrations and ratios, yields of 80% and over can be achieved.

The current general interest in the biological roles of glycoconjugate-bound sialic acids in cell membranes and the intra- and extra-cellular environments has focused in part on the sialyltransferase systems involved in glycoconjugate biosynthesis. Such studies required the CMP-glycosides of sialic acids, either radioactively labelled in the sialic acid moiety or unlabelled. Current methods for the enzymic synthesis of these compounds involve the use of tissue extracts from submandibular glands (Schauer *et al.*, 1972; Schauer & Wember, 1973; Kean & Roseman, 1966) or frog liver (Lapina *et al.*, 1975; Ng & Dain, 1977) which, however, lead to problems in separation of the protein and nucleotide sugar fractions.

We report here a new synthesis of CMP-sialic acids using a partially purified frog liver CMP-acylneuraminate synthase (CTP-*N*-acylneuraminate cytidyltransferase; EC 2.7.7.43) coupled to Sepharose 4B. In addition, alterations in the behaviour of the bound enzyme relative to the soluble form are described.

Materials

Frog liver was obtained from exsanguinated animals (*Rana esculenta*) and stored at –15°C if not used immediately. AcNeu, GcNeu and AcylOAcNeu were isolated from bovine and pig submandibular glands (Schauer, 1978). [14 C]AcNeu (0.76 mCi/mmol)

Abbreviations used: AcNeu, *N*-acetylneuraminic acid; GcNeu, *N*-glycolylneuraminic acid; AcylOAcNeu, *O*-acetyl-*N*-acylneuraminic acids; AcylNeu, acylneuraminic (sialic) acids.

and [14 C]GcNeu (0.33 mCi/mmol) were prepared as described by Buscher & Schauer (1974).

CTP was generously given by Boehringer, Mannheim, Germany, and CMP and CDP were obtained from the same firm. CNBr and sodium pyrophosphate were obtained from E. Merck, Darmstadt, Germany, and Sepharose 4B and DEAE-Sephadex A-50 were from Pharmacia G.m.b.H., Freiburg, Germany. Other chemicals were obtained from E. Merck and were of the purest grade available. 2-Deoxy-2,3-dehydroAcNeu was synthesized by Dr. J. Haverkamp (University of Utrecht, Utrecht, The Netherlands). Aqua Luma scintillation fluid was purchased from Lumac Systems A.G., Basel, Switzerland.

Methods

Preparation of CMP-acylneuraminate synthase from frog liver

About 50g of liver from exsanguinated frogs was homogenized in an equal volume of 50mM-Tris/HCl buffer, pH 7.0, containing 1mM-2-mercaptoethanol. Homogenization was effected in an Ultra-Turrax homogenizer (Jahnke and Kunkel K.G., Staufen, Germany) at maximum setting for 90s, with cooling in an ice bath. This step and all subsequent operations were carried out at 2–4°C. The homogenate was centrifuged for 15min at 9000g and the supernatant collected and centrifuged at 105000g for 90min. The clear reddish supernatant (40–50ml, containing about 1g of protein) was applied to a column (50cm × 3cm) of DEAE-Sephadex A-50 previously equilibrated with 50mM-Tris/HCl buffer, pH 7.0, containing

1 mM-2-mercaptoethanol. After application of the sample, the column was washed with this buffer until all haemoglobin was removed. A linear gradient of 0–1 M-NaCl in the same buffer (2 × 500 ml) was applied, and fractions containing CMP-acylneuraminate synthase activity were pooled. Enzyme activity was eluted at 0.5 M-NaCl.

The enzyme pool (100–150 ml) was ultrafiltered under N₂ through an Amicon ultrafilter cell fitted with a PM 10 filter, at a N₂ pressure of 0.5 MPa. The total volume was decreased to about 30 ml. Enzyme activity ranged between 0.16 and 0.5 unit/ml (1 unit of activity forms 1 μmol of CMP-AcNeu/min), and the protein concentration was 10–20 mg/ml. This preparation was used for further studies.

Binding of the enzyme to Sepharose 4B

Sepharose 4B (1 vol., usually 20–30 ml of packed gel) was cooled in an ice bath, 2 M-Na₂CO₃ (1 vol.) was added and the suspension stirred for 90 s. After this time 1 vol. of CNBr in dioxan (150 mg of CNBr/ml of Sepharose) was added and stirring continued vigorously for 90 s. The suspension was then washed rapidly on a Buchner funnel with 100 vol. of ice-cold water and 50 vol. of ice-cold 100 mM-NaHCO₃, pH 8.5.

The wet slurry was immediately mixed with a sample of the enzyme extract diluted 1:1 (v/v) with 200 mM-NaHCO₃, pH 8.5, except for the protein-concentration curve, where various dilutions of protein with buffer were utilized to give a final concentration of 100 mM-NaHCO₃.

The suspension was left for 6 h or overnight at 4°C with occasional gentle shaking. The gel was then filtered on a Buchner funnel or, for gel volumes of less than 5 ml, on a Millipore filter system using normal filter paper (Schleicher and Schüll, no. 595), and the non-bound protein solution collected together with 2 vol. of a water wash for enzymic analysis. The gel was subsequently washed with 100 vol. of water. After suspension in 2 vol. of 2 M-NaCl the gel was filtered under gravity and a final volume of 2 M-NaCl sucked through; these washings were collected for assay of enzyme activity. The gel was further washed with 100 vol. of 2 M-NaCl and finally with 100 vol. of water.

The washed gel was suspended in 1 vol. of 100 mM-Tris/HCl buffer, pH 9.0, to block remaining activated groups, and left for 12 h at 4°C with occasional gentle shaking. The gel was washed again in the same manner as described before, taking samples for enzyme assay and washing sequentially with a total of 100 vol. in each case for water, 2 M-NaCl and water. The gel was stored at 4°C in 50 mM-Tris/HCl buffer, pH 7.0, containing 1 mM-2-mercaptoethanol and 1 mM-thymol as bactericide. The gel was washed as described above before use and resuspended in

50 mM-Tris/HCl buffer, pH 9.0, containing 1 mM-2-mercaptoethanol.

Assay of CMP-acylneuraminate synthase activities

Soluble enzyme. Activity was assayed by the method of Kean & Roseman (1966) modified as follows. The incubations contained enzyme extract (20 μl, 5.0–8.3 munits), 0.5 μmol of CTP, 0.5 μmol of AcNeu, 20 μmol of Tris, 2 μmol of MgCl₂ and 0.03 μmol of 2-mercaptoethanol in a total volume of 50 μl at pH 9.0. Incubation was for 30 min at 37°C unless noted otherwise.

After incubation the samples were cooled rapidly and 10 μl of 5.4 M-NaBH₄ was added. The samples were shaken for 15 min and 10 μl of acetone was added, followed by a further 15 min shaking. The samples were subsequently analysed for sialic acid content by the method of Warren (1959).

Bound enzyme. The assay described above for the soluble enzyme was used with 20 μl of a suspension of the enzyme-gel in 50 mM-Tris/HCl buffer, pH 9.0, containing 1 mM-2-mercaptoethanol. Larger incubations contained 100 μl of the gel suspension, 2.5 μmol of CTP, 2.5 μmol of AcNeu, 100 μmol of Tris, 10 μmol of MgCl₂, and 0.15 μmol of 2-mercaptoethanol in a total volume of 250 μl. In both of these incubations the gel was sedimented by centrifugation and a measured sample of the supernatant taken for borohydride reduction with 10 or 50 μl of 5.4 M-NaBH₄ as described, followed by acetone treatment and quantification with the thiobarbituric acid assay. Incubations were routinely for 30 min at 37°C. Gel volumes were determined for each individual incubation as described by Parker *et al.* (1977).

Alterations of substrate, Tris, MgCl₂, enzyme-gel, mercaptoethanol and further additions of inhibitors or activators were carried out within the above assay system, maintaining the other components as described without changing the total volume of the incubation. Time, pH and temperature were also varied within the assay system, whereby the degradation of CMP-AcylNeu at higher temperatures was measured at pH 9.0 for 30 min duration, and the results were corrected accordingly.

In experiments to determine the stability of the bound enzyme to repeated incubation, samples of the enzyme-gel suspension were increased to 500 μl and the total volume and substrate additions adjusted accordingly. After each incubation the reaction mixture was separated and the gel washed with 100 vol. of buffer by using a Millipore filter system as described before. The gel was submitted to further incubations with fresh buffer and substrates. Each reaction mixture was analysed to assess enzymic activity. In the case of only one substrate (AcNeu or CTP) being present in the incubation, samples of the washed gel were taken for assay in the standard procedure.

Assay of other enzyme activities

Acid and alkaline phosphatase and 5'-nucleotidase activities were assayed as described by Bergmeyer (1974). ($\text{Na}^+ + \text{K}^+$)-dependent adenosine triphosphatase activity was measured by the method of Hoelzl-Wallach & Ullrey (1964). Proteinase activity was assayed essentially as described by Laskowski (1955), except that azocasein was used as substrate and the absorbance measured at 330nm. CMP-AcylNeu hydrolase was assayed by the method of Van Dijk *et al.* (1976). Acylneuraminate pyruvate-lyase activity was tested as described by Corfield *et al.* (1976).

Synthesis of CMP-AcylNeu

Preparation of CMP-AcylNeu was carried out in incubation mixtures containing 8–10ml of immobilized enzyme-gel (approx. 800munits), 16–40 μmol of AcylNeu, 18–80 μmol of CTP, 8.25mmol of Tris, 800 μmol of MgCl_2 and 16.5 μmol of 2-mercaptoethanol in a total volume of 12–16ml at pH9.0. Radioactive syntheses were carried out in a mixture containing 4–5ml of immobilized enzyme-gel (300–500munits), 1.4–16 μmol of unlabelled AcylNeu, 1.6 μmol of [^{14}C]AcNeu or [^{14}C]GcNeu, 18–45 μmol of CTP, 3.3mmol of Tris, 300 μmol of MgCl_2 and 6 μmol of 2-mercaptoethanol in a total volume of 6ml at pH9.0. Incubation at 37°C was for 1.5 or 2h and CTP was added in two equal portions, one at zero time and the second at 45 or 60min. The samples were shaken gently during the incubation and after completion of the reaction cooled in an ice bath. The gel was filtered off and washed with 5ml portions of water until no more radioactivity was eluted. The washings were diluted to 100ml and applied to 5 or 10ml columns of Dowex 1 (X8; bicarbonate form; 200–400 mesh); the flow rate was 0.35ml/min at a temperature of 2–4°C. The column was previously washed with aq. 1mM- NH_3 . A linear gradient of 0–1M-triethylamine/ NaHCO_3 buffer, pH8.4 (2 $\times$ 50ml or 2 $\times$ 75ml), was applied to the column, and sialic acid- and nucleotide-containing fractions were determined. The identity of the different eluted fractions was evaluated by t.l.c. on cellulose (see below) and CMP-AcylNeu-containing fractions were pooled and freeze-dried. CMP-AcylNeu was separated from contaminating CMP by chromatography on a column (2cm $\times$ 80cm) of Sephadex G-25, eluted with aq. 1mM- NH_3 at a flow rate of 0.16ml/min at 2–4°C. CMP-AcylNeu was freeze-dried and stored at -15°C.

Chemical analyses

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Sialic acids were assayed by a micro-adaptation of the

thiobarbituric acid method (Warren, 1959). CMP-AcylNeu was assayed as described by Kean & Roseman (1966). Cytosine nucleotides including CMP-AcylNeu were quantified by using their respective molar absorption coefficients, and the purity of these compounds was checked by their absorbance ratios at 250, 260 and 280nm from published tables (P.L. Laboratories, 1973) after separation on Dowex 1 (X8; formate form; 200–400 mesh) as described by Hurlbert *et al.* (1954). Phosphate was measured by the method of Chen *et al.* (1956) as modified by Ames (1966).

Sialic acids were separated by t.l.c. on cellulose thin-layer plates prepared by Merck, prewashed in 0.1M-HCl and developed with butan-1-ol/propan-1-ol/0.1M-HCl (1:2:1, by vol.) (Schauer, 1978); the sialic acids were detected with the spray reagent of Böhm *et al.* (1954). CTP, CDP, CMP and CMP-AcylNeu were separated on cellulose plates with ethanol (95%)/1M-ammonium acetate (pH7.3) (7:3, v/v) as solvent; the plates were developed three or four times for optimal separation of the components (Comb *et al.*, 1966).

Radioactivity was measured on thin-layer plates by using a Berthold LB 2723 radio-thin-layer scanner and otherwise in a Packard Tri-Carb scintillation counter in 5ml of Aqua Luma scintillation fluid.

Results

Preparation and immobilization of the enzyme

The chromatography of the 105000g supernatant (obtained after homogenization of frog liver) on DEAE-Sephadex A-50 resulted in a 2–10-fold purification and 80–100% yield of CMP-AcylNeu synthase activity relative to the homogenate. This enzyme preparation could be bound to Sepharose by standard methods of CNBr activation, with yields of bound activity of up to 100%. Total recoveries were, however, usually of up to 150% when wash (non-bound) and gel (bound) activities were combined.

The binding reaction of the enzyme preparation with CNBr-activated Sepharose 4B was complete after 4h and no increase or loss of bound protein occurred for up to 20h. Under no conditions investigated was it possible to bind the total activity present, even when low protein concentrations were present. However, optimal binding conditions were observed when the ratio of protein concentration to gel volume was in the range 12–20mg of protein/ml of Sepharose 4B. Under these conditions 60–80% of total enzyme activity and 60–70% of total protein was bound. Poor binding and recovery of bound enzyme activity were observed if haemoglobin contaminated the preparation from DEAE-Sephadex A-50.

The enzyme preparation to be bound to Sepharose 4B showed low or negligible activities of enzymes that may have destroyed the substrates and products,

Table 1. *Bound and soluble enzyme activities determined in CMP-acylneuraminase synthase preparations from frog liver*

Results are representative of one enzyme preparation; similar results were obtained with other enzyme preparations. Values for CMP-acylneuraminase synthase given in parentheses represent the range measured in all experiments (14). Proteinase values were calculated relative to μg of trypsin under identical incubation conditions.

Enzyme	Activity before binding (munits/mg of protein)	Activity after binding (munits/ml of gel)
CMP-acylneuraminase synthase	11.3 (1.7–50)	63.3 (16.7–100)
CMP-acylneuraminase hydrolase	0.0	0.0
Acid phosphatase	2.33	0.72
Alkaline phosphatase	2.20	0.35
5'-Nucleotidase	0.38	0.26
Adenosine triphosphatase	3.26	0.0
Acylneuraminase pyruvate-lyase	0.013	0.56
Proteinase	2.1	0.31

especially CTP and CMP-AcNeu. The activities of the various enzymes tested are shown in Table 1. Incubation of CMP-AcNeu or CTP in 50mM-Tris/HCl buffer at 37°C and pH9.0 for up to 3h showed that CMP-AcNeu decomposition was less than 1% and CTP loss by conversion into CDP or CMP was also in this range. Formation of CMP from CTP under assay conditions in the presence of bound enzyme during prolonged incubations up to 3h was higher when the second substrate AcNeu was absent. Under normal incubation conditions with both substrates, CMP formation was less than 1% of the CTP concentration, and in the absence of AcNeu this value was 5–7%.

Stability

The maximal activity of the soluble and bound enzyme was found to depend on the presence of 2-mercaptoethanol or other thiol compounds. 2-Mercaptoethanol was the best thiol compound tested and showed a discrete activation maximum at 1mM. Higher and lower concentrations resulted in lower activity. The stability of the enzyme in the bound or soluble form could be improved by using 1mM-2-mercaptoethanol (Fig. 1). On average, the activity of batches of bound enzyme decreased linearly by 20% over 4 months when stored under the standard conditions noted in the Methods section.

Thymol (1mM) was included in the gel suspension as a bactericide; it had no influence on the CMP-

Fig. 1. *Effects of storage and 2-mercaptoethanol on soluble and bound CMP-acylneuraminase synthase activity* Soluble-enzyme activity with (■) and without (□) 1mM-2-mercaptoethanol in 400mM-Tris/HCl/40mM-MgCl₂ buffer, pH9.0, at 2–4°C, was measured as described in the text. Bound-enzyme activity with (●) and without (○) 1mM-2-mercaptoethanol in 400mM-Tris/HCl/40mM-MgCl₂ buffer, pH9.0, containing 1mM-thymol was measured at 2–4°C. Samples (50 μl) were taken from the gel, washed as described in the text and resuspended in fresh assay buffer before incubation. The activities in all cases are expressed as a percentage of the original activity at the start of the experiment.

acylneuraminase synthase activity. This was in contrast with NaN₃, which led to 70% inhibition of enzyme activity at 5mM.

The stability of the bound-enzyme preparation to repeated incubation is shown in Fig. 2. The decline in recoverable activity appeared to be due to CTP or a degradation product of CTP formed during the incubation.

Properties of the bound enzyme

The K_m values of 0.46 μM and 0.36 μM for CTP and AcNeu respectively and the pH optimum at pH9.0 found for the bound enzyme reflect those found for the soluble enzyme (Schauer *et al.*, 1977). Inhibition by CMP, CDP and pyrophosphate at 1mM was 43, 49 and 46% respectively of the non-inhibited reaction under standard incubation conditions, and inhibition by 2-deoxy-2,3-dehydro-AcNeu was 33% at 4.6mM.

Stimulation of bound-enzyme activity was observed for MgCl₂ (a maximum of 30% at 40mM) and 2-mercaptoethanol (210% at 1mM) relative to incubations without these compounds. The activation by 2-mercaptoethanol was optimal at 1mM, concentrations of 0.1mM and 10mM yielding 136% and 107% respectively.

The influence of temperature on the bound-enzyme activity showed a maximum of 40°C maintained to 60°C, whereas the soluble-enzyme activity decreased sharply at temperatures above 40°C.

Fig. 2. *Stability of the enzyme to repeated incubation* Samples of 500 μ l of gel were incubated at 37°C for 30 min with: 400 mM-Tris/HCl/40 mM-MgCl₂/1 mM-2-mercaptoethanol buffer, pH 9.0 (○); buffer containing 10 mM-AcNeu (□); buffer containing 10 mM-CTP (Δ); buffer containing 10 mM-AcNeu and 10 mM-CTP (●). After incubation the gel was removed by filtration and washed with 100 vol. of the Tris/HCl buffer; samples of this washed gel were taken and assayed for activity as described in the text. The remaining gel was subjected to up to five further incubation cycles. The activity was referred to that of a sample of the gel before the first incubation cycle and expressed as a percentage of this value.

Table 2. *Effect of substrate concentration and ratio on the yield of CMP-AcNeu on a preparative scale obtained by using immobilized CMP-acetylneuraminase synthase* Incubations were as described for radioactive syntheses in the Methods section, only the concentrations of CTP and AcNeu were varied. Between 200 and 300 munits of bound enzyme were used in these syntheses.

CTP/AcNeu ratio		Yield (%)
Initial	Total	
0.35:0.32 (1.1:1)	0.70:0.32 (2.2:1)	9
0.54:0.32 (1.7:1)	1.08:0.32 (3.4:1)	21
3.0:2.7 (1.1:1)	6.0:2.7 (2.2:1)	47
6.6:2.7 (2.4:1)	13.2:2.7 (4.8:1)	79

Discussion

The preparation of a suitably active enzyme fraction catalysing the formation of CMP-AcylNeu from CTP and AcylNeu has been achieved with frog liver. The isolation and properties of this enzyme have been investigated in this laboratory (Schauer *et al.*, 1977) and a preparation from the early stages of this purification was tested with regard to recovery of enzymic activity after binding to Sepharose 4B by the CNBr method.

Frog liver is chosen because of its ready availability and enzyme activity in a range compatible with larger-scale preparations of CMP-AcylNeu. The specific activity of the synthase in frog liver (0.7–5.0 munits/mg of protein) is in the range of the specific activities observed in bovine, pig and horse submandibular glands (Schauer *et al.*, 1972; Schauer & Wember, 1973). The absence of mucin from the liver system eliminates many problems encountered in submandibular-gland preparations, especially with regard to enzyme isolation, CMP-AcylNeu purification and enzyme assay. The active fraction used for binding to Sepharose 4B is rapidly obtained, and, when bound, shows low activities of enzymes likely to cause degradation of the substrates and CMP-AcylNeu produced under the synthase assay conditions. CMP-acetylneuraminase hydrolase activity is completely absent from the bound-enzyme fraction and, except for alkaline phosphatase, the remaining enzyme activities have pH optima lower than the synthase assay conditions of pH 9.0. Degradation of CTP to CMP by the bound enzyme under incubation conditions is observed at higher rates in the absence of AcylNeu, again underlining the high

Synthesis of CMP-AcylNeu

The immobilized-enzyme preparation was used successfully to prepare CMP-sialic acid glycosides. Optimal conditions for the synthesis were those where the concentration of CTP and AcylNeu were above enzyme saturation (≥ 1 mM and 2 mM for CTP and AcylNeu respectively) and the ratio of substrate concentrations CTP/AcylNeu was above 2.5:1 at the start of the incubation (see Table 2). Yields of 80% were obtained under these conditions. Samples analysed after ion-exchange purification on Dowex 1 (X8) contained CMP, necessitating Sephadex G-25 chromatography. After these procedures the glycoside preparations contained no CMP, CDP or CTP by t.l.c. criteria and had molar proportions of cytosine/phosphate/AcNeu of 1:1.15:1.05. Radioactive samples thus prepared and analysed showed specific radioactivities of CMP-AcylNeu corresponding to the dilution of [¹⁴C]AcylNeu in the incubation with unlabelled AcylNeu. GcNeu could be utilized to obtain similar yields to AcNeu, but *O*-acylated sialic acids proved to be poor substrates for the enzyme from frog liver and could not be utilized for synthesis of the corresponding CMP-glycosides.

activity of the CMP-acylneuraminase synthase bound to Sepharose in comparison with that of enzymes degrading substrates and products of the synthase reaction. Degradation of CMP-AcNeu to 2-deoxy-2,3-dehydroAcNeu at 37°C and pH 9.0 has been observed in this laboratory (J.-M. Beau, J. Haverkamp & R. Schauer, unpublished observations); however, the rate is not sufficient to decrease the CMP-AcylNeu concentration appreciably or to interfere significantly with the assays or syntheses reported here, considering the relatively high concentrations of 2-deoxy-2,3-dehydroAcNeu required to cause inhibition. The impure nature of the synthase preparation used for binding to Sepharose is probably responsible for the total recoveries of bound and non-bound activity over 100% of the starting material. Problems of dilution and high salt concentrations in the washing procedure may contribute to this observation. No investigation of this phenomenon was carried out because of the many possible causes and because a constant reproducible bound-enzyme activity is obtained using the method reported.

The properties of the bound enzyme reflect little change compared with the soluble enzyme with respect to substrate specificity [AcNeu and GcNeu are substrates and their *O*-acetylated derivatives are not, in contrast with the submandibular-gland enzymes from cow, pig and horse (Schauer *et al.*, 1972; Schauer & Wember, 1973)], K_m values, pH optima, activators and inhibitors. The activation by 2-mercaptoethanol supports the finding that the purified enzyme is a thiol-requiring enzyme (Schauer *et al.*, 1977) and that full activity is expressed in crude extracts only in the presence of optimal concentrations of this thiol compound. The stability of the enzyme preparation to temperatures above 40°C is due to a combination of factors. The physical immobilization prevents the rapid denaturation of the enzyme observed in the soluble state and may influence the maximum velocity and the K_m values at these higher temperatures. The apparent temperature-independence of reaction rate between 40 and 60°C under the assay conditions cannot be explained at present; the effect of assay time on the temperature-dependence has not been studied. Although corrections for substrate and product destruction and pH changes with temperature are made, the heterogeneity of the enzyme preparation and absence of detailed kinetic data for the pure enzyme preclude a valid kinetic explanation of the reaction rates above 40°C for the bound enzyme.

The stability of the bound synthase on storage relative to the soluble activity is not as marked as has been reported for immobilization of purified enzyme (Martinek *et al.*, 1977; Parker *et al.*, 1977). This is assumed to be a function of the heterogeneous protein preparation used in the binding step and the

low proteinase activity observed. In addition, the stability is markedly influenced by the presence or absence of 2-mercaptoethanol (Fig. 1), and this compound is important to maintain bound-enzyme activity on storage over periods of several months.

The stability of the bound enzyme during incubation is influenced by the presence of CTP. The repeated incubation of bound enzyme is shown in Fig. 2 and indicates that the presence of CTP or a metabolite formed from CTP produces a loss of enzyme activity not recoverable on washing. This limits the repeated use of the bound enzyme to two, or in highly active preparations, three cycles. The use of low-activity preparations for synthesis of CMP-AcylNeu may require larger volumes of enzyme-gel, in which case the observations on substrate concentrations and ratios must be followed as discussed below.

The immobilized enzyme can be used efficiently to synthesize CMP-AcylNeu (AcNeu and GcNeu, labelled or unlabelled) on a preparative scale. To ensure yields of 80% and over it is necessary to use substrate concentrations above saturation and an initial substrate ratio of CTP/AcNeu of above 2.5:1. The second addition of CTP is designed to ensure maximal conversion of AcylNeu into CMP-AcylNeu by providing a substrate excess for the second half of the incubation period. Temperatures above 37°C are not used owing to the degradation of CMP-AcylNeu at pH 9.0 (J.-M. Beau, J. Haverkamp & R. Schauer, unpublished work).

The separation of the reaction products on Dowex 1 (X8) yields CMP-AcNeu fractions free from CDP and CTP, but containing low concentrations of CMP. This contaminant is removed efficiently on Sephadex G-25 and the resulting CMP-AcylNeu is homogeneous on t.l.c. and shows equimolar ratios of cytosine, phosphate and AcNeu.

The advantages of such an immobilized CMP-acylneuraminase synthase preparation are evident in the rapidity and ease of removal of the enzyme activity from the reactants, eliminating the problems of contamination of the CMP-glycosides with low- and high-molecular-weight substances other than the reactants, as has been observed with the use of soluble-enzyme preparations, e.g. tissue extracts. The immobilized enzyme is prepared by simple and rapid procedures from readily available material and represents a valuable improvement in the preparation of these important compounds required in studies on glycoconjugate biosynthesis.

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