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Note

High-performance ion-exchange chromatographic separation of cytidine monophosphate-N-acetylneuraminic acid and cytidine nucleotides

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Cytidine monophosphate-N-acetylneuraminic acid (CMP-NeuAc) synthetase (E.C. 2.7.7.43) catalyzes the following reaction:

$$\text{Cytidine triphosphate (CTP)} + \text{NeuAc} \leftrightarrow \text{CMP-NeuAc} + \text{pyrophosphate}$$

The enzymatic activity has been measured by: (a) colorimetry, using resorcinol[1] and periodic acid–thiobarbituric acid[2–5]; (b) fluorometry[6]; and (c) NeuAc aldolase–nicotinamide–adenine dinucleotide (reduced) coupling[7–10]. One of the problems with these methods is that they are indirect. Following the enzymatic reaction, free sialic acid must first be destroyed prior to its hydrolysis from CMP-NeuAc and subsequent analysis.

We now report the development of a new procedure that separates enzymatically synthesized CMP-NeuAc from the enzyme substrates directly by high-performance liquid chromatography (HPLC). This procedure, which can detect approximately 160 fmoles of CMP-NeuAc, is approximately three orders of magnitude more sensitive than the other above-mentioned methods for CMP-NeuAc detection. Also, it is three orders of magnitude more sensitive than the approach recently described to measure CMP-NeuAc by HPLC[11]. In addition, this procedure can also be used preparatively for the purification of either non-radiolabeled or radiolabeled enzymatically synthesized CMP-NeuAc, because the separation of reaction substrates and products is done in a volatile buffer.

EXPERIMENTAL

Materials

CTP, NeuAc and CMP-NeuAc, were purchased from Sigma. Triethylamine (reagent grade) and glacial acetic acid (reagent grade) were purchased from Fisher Scientific. Cytidine 5′-monophosphate-[4-^{14}C]-N-acetylneuraminic acid (1.8 mCi/-

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mmol) was purchased from New England Nuclear. [9-^{3}H]-N-acetylneuraminic acid was synthesized enzymatically from N-acetyl-D-[6-^{3}H]mannosamine (22.8 Ci/mmol; New England Nuclear) as previously described by Warren and Glick[12].

High-performance liquid chromatography

For HPLC, a single M-45 pump (Waters) was used. All eluents were filtered through a 0.45-μm Millipore membrane filter under reduced pressure. The eluent gradient was generated with a low pressure, programmable gradient maker (Chromat-A-Trol Model II). Samples were injected using a syringe injector (Rheodyne Model 7125) with a 2-ml loop. A SynChropak AX-100 column (Synchrom, 25 cm $\times$ 4.1 mm I.D.) was used in all separations. The column was equilibrated with 10 m*M* triethylamine acetate (TEAA) buffer, pH 7.0. The eluent flow-rate was 2 ml/min at 2000 p.s.i., except when 1 *M* TEAA buffer, pH 7.0, was used in which case the rate was 1 ml/min. After injection of the sample, the column was eluted for 5 min with 10 m*M* TEAA buffer, pH 7.0. Thereafter the column was eluted for 10 min with a linear gradient of 10–75 m*M* TEAA buffer, pH 7.0. This was followed by 5 min of elution with a linear gradient of 75 m*M*–1 *M* TEAA buffer, pH 7.0. After further elution for 20 min with 1 *M* TEAA buffer, pH 7.0, the column was re-equilibrated for 20 min with 10 m*M* TEAA buffer, pH 7.0. The column was then ready for a new sample injection. Nucleotides were detected by monitoring the column effluent at 271 nm with an absorbance detector (Kratos, Spectroflow Model 757). Samples of 2 ml of the effluent were collected.Radioactivity of each fraction was determined following addition of 18 ml of Aquasol-2 (New England Nuclear). A Searle Analytic 91 liquid scintillation spectrometer was used to measure the radioactivity of the effluent fractions. Chromatographs were recorded on a multi-speed Servochart Recorder (Heath Kit, Model 1R-18M).

Enzymatic assay for CMP-NeuAc synthetase

The incubation mixture to measure CMP-NeuAc synthetase activity contained in a final volume of 0.25 ml: Tris · HCl, pH 8.5 (9 m*M* final concentration), magnesium chloride (10 m*M* final concentration), dithiothreitol (1 m*M* final concentration), CTP (2.5 m*M* final concentration), [9-^{3}H-]-N-acetylneuraminic acid (22.8 Ci mmol; 0.4 μ*M* final concentration) and different amounts of partially purified CMP-NeuAc synthetase from rat liver[13] (0.4 mg protein/ml; approximately 40 U/ml). The quantity of protein used and length of incubation are indicated in each experiment. Incubations were done at 37°C. The reaction was stopped by addition of cold water (1.75 ml). The reaction mixture was then immediately centrifuged in a Centricon filter (Amicon; 30 000 cutoff) for 30 min at 5000 *g*. The total deproteinated sample (2 ml) was then directly injected into the HPLC.

RESULTS

CMP-NeuAc synthetase was assayed as described under Experimental. The HPLC elution profile of the reaction substrates and products are shown in Fig. 1. The retention times for cytosine, NeuAc, CMP-NeuAc, CDP, and CTP were 1, 3.5, 12, 25, and 35 min, respectively. It was determined from separate experiments using the above HPLC separation that the enzymatic reaction was linear with time at leat

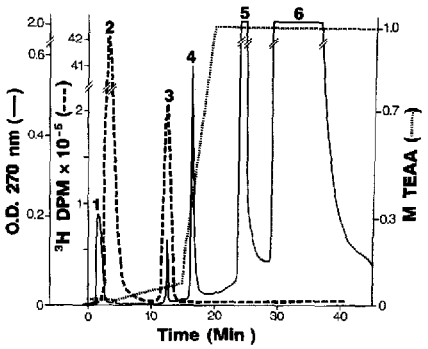
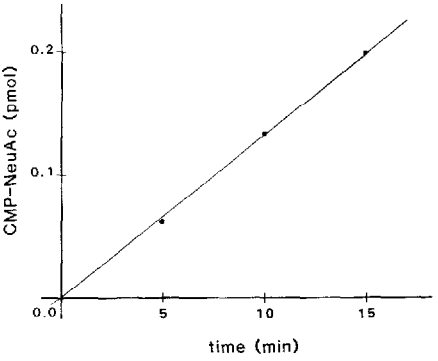


Fig. 1. HPLC radiochromatographic separation of CMP-NeuAc from substrates. Enzyme activity was measured as described under Experimental. The reaction mixture contained 70 μg of protein and was incubated for 10 min at 37°C. Following deproteination of the sample (see Experimental), standard CMP-NeuAc (30 μl; 1 m*M*) was added prior to injection into the HPLC column. The column was eluted with a gradient of TEAA buffer, pH 7.0, as described under Experimental. The abscissa indicates the time of elution following injection of the sample into the HPLC column. Peaks: 1 = cytosine; 2 = NeuAc; 3 = CMP-NeuAc; 4 = CMP; 5 = CDP; 6 = CTP.

up to 15 min (Fig. 2) and with protein at least up to 160 micrograms (Fig. 3).

Additional evidence for CMP-NeuAc identification and for its recovery from the HPLC column was obtained. In one experiment, standard CMP-[^{14}C]NeuAc was added to a deproteinated sample following an enzymatic assay as described under Experimental. Following injection of this mixture to the HPLC column, it was determined that all the ^{14}C radioactivity coeluted with a tritium-containing peak in a position where standard CMP-NeuAc eluted (not shown). Upon reinjection of an aliquot of this material into the HPLC column, it was determined that no degradation of CMP-NeuAc had occurred (not shown). In a second experiment, NeuAc was used at equimolar concentrations with CTP during the enzymatic assay (2.5 m*M*). The



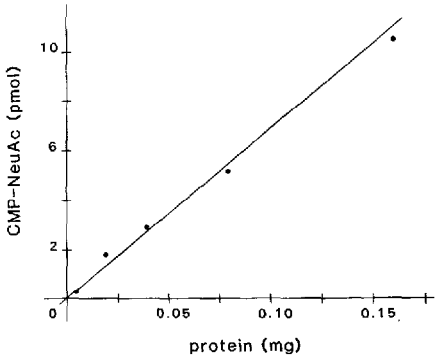


Fig. 2. Enzymatic synthesis of CMP-NeuAc *versus* time. Enzymatic assay conditions are described under Experimental. A 2-μg amount of protein was used. Each result shown is the average of three independent determinations.

Fig. 3. Enzymatic synthesis of CMP-NeuAc *versus* protein. Enzymatic assay conditions are described under Experimental. The incubation mixture contains different amounts of proteins; incubation was for 10 min at 37°C.

same elution profile from the HPLC column was obtained as described in Fig. 1 (not shown).

To determine the sensitivity of the assay, a mixture containing substrates as described under Experimental was incubated for 10 min at 37°C with 2 μg of protein. It was determined that 2730 cpm coeluted with standard CMP-NeuAc. From a separate incubation with radioactive NeuAc, but without enzyme, it was determined that the "background" radioactivity eluting in the same region as CMP-NeuAc was 450 cpm. Thus, in the previous incubation with enzyme, 2280 cpm were due to authentic CMP-NeuAc.

Using as internal standard [^{3}H]toluene, it was determined that the efficiency of tritium counting was 28%. The total net dpm comigrating with CMP-NeuAc were, therefore, 8140. From the specific activity of the [9-^{3}H]NeuAc, it was determined that this corresponded to 0.16 pmoles. From a separate experiment in which four times the same aliquot of a sample was injected into the HPLC column, it was determined that the coefficient of variation in the determination of the reaction products was 5.2%.

DISCUSSION

The need to develop a highly sensitive assay for the detection of CMP-NeuAc was motivated by our desire to recover CMP-NeuAc synthetase activity following its electrophoresis in and elution from a native gel. We found that the procedure described here can be applied both to enzyme still in a gel and to enzyme that has been electro-eluted from the gel. In the former case, a slice of native gel containing the enzyme can be used as source of enzyme in the assay described in Methods.

The principal advantages of the procedure described are: (1) its extremely high sensitivity, making it approximately 1000-fold more sensitive than any other published procedure so far; (2) its reproducibility; (3) its relative lack of interference by other compounds (because the assay measures directly the enzymatically synthesized CMP-NeuAc); (4) its relative simplicity (following the reaction, the sample needs very little manipulation before it can be injected into the chromatograph); and (5) its feasibility to be used preparatively because a volatile buffer is used.

The principal disadvantage of the described assay is the relatively long time (approximately 60 min) it takes to measure the activity of each sample. It is, therefore, not suitable when enzymatic activity of a large number of samples needs to be determined. For such cases, the published colorimetric approaches would appear to be more suitable.

Because of the different times of elution of free NeuAc, CMP, and cytidine CMP-NeuAc in the described procedure, this chromatographic procedure can also be used for the preparation of the sugar nucleotide radiolabeled in the NeuAc or cytidine moiety. It can also be used for detailed radiochemical analyses of the CMP-NeuAc components themselves, following acid hydrolysis. We are currently using the above-described assay for the final stages of purification of rat liver CMP-NeuAc synthetase.

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REFERENCES

1 L. Svennerholm, *Biochim. Biophys. Acta*, 24 (1957) 604–611.
2 L. Warren, *J. Biol. Chem.*, 234 (1959) 1971–1975.
3 L. Warren, *Methods Enzymol.*, 6 (1963) 463–465.
4 D. Aminoff, *Virology*, 7 (1959) 355–357.
5 D. Aminoff, *Biochem. J.*, 81 (1961) 384–392.
6 H. H. Hess and E. Rolde, *J. Biol. Chem.*, 239 (1964) 3215–3220.
7 P. Brunetti, G. W. Jourdian and S. Roseman, *J. Biol. Chem.*, 237 (1962) 2447–2453.
8 R. Gantt, S. Milliver and S. B. Brinkley, *Biochemistry*, 3 (1964) 1952–1960.
9 R. Schauer, M. Wember, F. Wirtz-Peitz and C. Ferreira Doamaral, *Hoppe-Seyler's Z. Physiol. Chem.*, 350 (1971) 111–115.
10 W. Ferwerda, C. M. Blok and J. Heijlman, *J. Neurochem.*, 36 (1981) 1492–1499.
11 C. R. Petrie, III and W. Korytnyk, *Anal. Biochem.*, 131 (1983) 153–159.
12 L. Warren and M. C. Glick, *Methods Enzymol.*, 8 (1966) 131–133.
13 M. Perez, A. Vannier and C. B. Hirschberg, *Fed. Proc., Fed. Am. Soc., Exp. Biol.*, 41 (1982) 662.