Nuclear Cytidine 5'-Monophosphosialic Acid Synthetase*

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EDWARD L. KEAN†

From the Departments of Ophthalmology and Biochemistry, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106

SUMMARY

Evidence is presented documenting the nuclear location of the enzyme, cytidine 5'-monophosphosialic acid (CMP-sialic acid) synthetase. This enzyme catalyzes the reaction:

\[
\text{CTP} + \text{sialic acid} \xrightleftharpoons{\text{Mg}^{2+}} \text{CMP-sialic acid} + \text{PP}_i
\]

From previous studies it had been inferred that this enzyme was cytoplasmic. Studies are reported here that describe the following. (a) The purity of the nuclei that were used in these investigations. Nuclei were isolated from hypertonic sucrose solutions from the retina of the hog and from liver, kidney, spleen, and brain of the rat. Nuclear purity (i.e. absence of cytoplasmic contamination) was established on the basis of enzymatic, chemical, and morphological criteria. (b) The activity of CMP-sialic acid synthetase in these nuclei. The yields of CMP-sialic acid synthetase in the nuclei recovered from the different tissues varied from 18 to 53% of the activities present in the initial homogenates. When corrected for the yield of nuclei, based on DNA content, the yields of enzyme ranged from 54 to 90%. This was accompanied by increases in specific activities of 3- to 13-fold. CMP-sialic acid synthetase by nuclei from the retina of the hog was identified by chemical, enzymatic, chromatographic, and electrophoretic procedures. (c) Some of the properties of nuclear CMP-sialic acid synthetase are described. The following apparent K_m values were observed for the enzyme from nuclei of rat liver: N-acetylneuraminic acid, 0.72 mM; N-glycolylneuraminic acid, 1.4 mM; CTP, 0.48 mM; Mg^{2+}, 6.75 mM. The optimal pH for the reaction is between 8.5 and 8.6 in Tris buffer. The effect of other metals and nucleotide triphosphates on the activity of the enzyme was measured.

Cytidine 5'-monophosphosialic acid synthetase catalyzes the following reaction:

\[
\text{CTP} + \text{sialic acid} \xrightarrow{\text{Mg}^{2+}} \text{CMP-sialic acid} + \text{PP}_i
\]

(2-4). Studies from several laboratories over the past few years have shown that the incorporation of sialic acid into the sialic acid-containing polymers, such as the glycoproteins and gangliosides, requires CMP-sialic acid as the sialic acid donor (5-8). Knowledge of the intracellular location of the sialic acid "activating" enzyme may aid in understanding more fully the cellular processes involved in the biosynthesis of these heteropolymers. Previous studies dealing with the isolation and properties of this enzyme have used extraction procedures that resulted in its solubilization (2-4, 8-10). Although the subcellular distribution of this enzyme had not been reported, it might have been inferred from these studies that CMP-sialic acid synthetase was cytoplasmic. Recently (11), however, high enzymatic activity was observed in the "nuclear fraction" from hog retina. In order to establish whether or not the nucleus, an organelle discarded in previous isolation procedures, was an intracellular site for the localization of this enzyme, the present report describes further studies on CMP-sialic acid synthetase in highly purified nuclei isolated from the retina of the hog and from liver, kidney cortex, brain, and spleen of the rat. The degree to which this activity might have been due to cytoplasmic contamination was evaluated by determining the purity of the nuclei. This was established on the basis of morphological, enzymatic, or chemical criteria (or all three). The activities and yields of CMP-N-AN synthetase in nuclei from these tissues are described and a product of the reaction catalyzed by the nuclear enzyme from hog retina, CMP-N-AN, was identified. Some of the properties of the enzyme obtained from rat liver are described.

MATERIALS AND METHODS

N-Acetylneuraminic acid and nucleotide triphosphates were obtained from Calbiochem. Cytochrome c (type VI) NADP⁺, NADH, NADPH, crystalline lactate dehydrogenase (rabbit muscle, type II), RNA (type XI from yeast), rotenone, alkaline phosphatase (E. coli), Tris, and glucose 6-phosphate (barium salt) were purchased from Sigma. DNA (calf thymus) and phenazine methosulfate were obtained from General Biochemicals. N-Glycolylnearaminic acid was prepared by the procedure of Brunetti, Swanson, and Roseman (12). The author is grateful to Dr. Saul Roseman for a sample of N-AN-aldolase purified from Clostridium perfringens (12).

Analytical Procedures—Enzymatic activities were calculated from initial rates measured under conditions of linearity with

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† To whom requests for reprints should be addressed at The Department of Ophthalmology, Laboratory for Ophthalmology Research, Case Western Reserve University, Cleveland, Ohio 44106.
‡ The abbreviations used are: N-AN, N-acetylneuraminic acid; N-GN, N-glycolylnearaminic acid; N-AN-aldolase, the specific aldolase that cleaves N-AN and N-GN to pyruvate and N-acetylmannosamine; TBA, thiobarbituric acid.
Table I

RNA, DNA, and protein relationships in homogenates and nuclei from several tissues

| Tissue            | Whole homogenate | Nuclei               | Yield of DNA in recovered nuclei |
|-------------------|------------------|----------------------|---------------------------------|
|                   | RNA/DNA (%)      | Protein/DNA (%)      | DNA/DNA (%)                     |                                  |
| Hog retina        | 0.60             | 0.14                 | 0.14                            | 20                              |
| Rat tissues       | 3.0              | 2.22                 | 2.22                            | 52                              |
| Liver             | 1.2              | 0.11                 | 0.11                            | 61                              |
| Kidney cortex     | 1.7              | 0.16                 | 0.16                            | 80                              |
| Spleen            | 0.42             | 0.084                | 0.084                           | 76                              |

N-AN was measured by the TBA assay of Warren (29), by the resorcinol method (27), and fluorometrically after reaction with N-AN-aldolase and lactate dehydrogenase (12). CMP-N-AN was measured by the TBA-NaBH₄ procedure (4, 10).

Paper chromatography was performed with the following solvent systems: A, 95% ethanol-1.0 M Tris-HCl, pH 7.5, 65:35; B, 1-butanol-pyridine-water, 6:4:3; C, 95% ethanol-1.0 M NH₄ acetate, pH 7.5, 7:3. Paper electrophoresis was performed on a Gilson high voltage electrophorator with the following systems: I, 1% sodium tetraborate, pH 9.4 at 55 volts per cm; II, 0.05 M potassium phosphate, pH 7.1 at 44 volts per cm. Paper chromatography was performed with Whatman No. 1 paper and Schleicher and Schuell 589 blue paper. Paper electrophoresis was carried out on Whatman No. 3MM paper.

Silicic acid was located on dried paper chromatograms and electrophoreograms after spraying with TBA reagent according to the procedure of Warren (28). CMP-N-AN was located on paper initially by ultraviolet quenching, after which the entire papergrams were cut into areas 0.5 × 1 inch and minced and the TBA-NaBH₄ assay was carried out on the individual zones (3).

CMP-N-AN was isolated from incubation mixtures by ion exchange chromatography. After incubation, the reaction was stopped by the addition of EDTA, pH 8.5, to a final concentration of 0.17 M. An aliquot of the incubation mixture was placed over a column of Dowex 1-bicarbonate (200 to 400 mesh) and elution was carried out as indicated previously (4). Peak tubes containing borohydride-stable TBA-reactive material were pooled and evaporated to dryness several times on a rotary evaporator at 30°C. The material was further purified by paper chromatography with Solvent System C by descending technique on Schleicher and Schuell 589 blue ribbon filter paper. After chromatography the paper was dried at room temperature and the ultraviolet quenching, TBA-NaBH₄-positive area, whose migration corresponded to that of authentic CMP-N-AN was cut out. The material was eluted from the paper at 4°C with water previously adjusted to pH 8.0 with NH₄OH.

Electron microscopy was performed on nuclei previously fixed at 4°C in 4% glutaraldehyde in 0.125 M sodium phosphate buffer, pH 7.3, followed by fixation with 2% OsO₄ in acetate-barbital buffer for 1 hour. After dehydration through graded alcohols the pellets were embedded in Maraglas. Thin sections were cut with an LKB ultramicrotome, stained with uranyl acetate and lead hydroxide and examined with an RCA 3G electron microscope.

Preparation of Nuclei—Retinas were dissected from hog eyes obtained from a local slaughterhouse. Nuclei from retinas were prepared from both fresh tissue and retinas that had been stored at −20°C for several days and then thawed at 4°C. Nuclei were also prepared from tissues obtained from 200- to 300-g male rats fasted overnight and then killed by decapitation. All of the following operations were conducted between 0°C and 4°C unless otherwise indicated. After mincing, the tissues were homogenized and nuclei were isolated according to the procedure of Chaveau, Moule, and Rouiller (29) as described by Busch (30). The tissues were homogenized in a solution of 2.4 M sucrose-3.3 mM CaCl₂ (10 ml of buffer per g, wet weight, of tissue), with a Teflon Potter-Elvehjem homogenizer having a 0.01-inch clearance. After homogenization, the sucrose concentration was 60 to 61% by weight as determined with an Abbé refractometer. The homogenate was centrifuged at 40,000 × g for 60 min. The pellet was washed by resuspension in a solution of 1.0 M sucrose-

Sodium azide (10 mM) was used to inhibit cytochrome c oxidase instead of sodium cyanide (personal communication from Dr. C. Hoppel).

Samples were previously incubated for 10 min at room temperature in the presence of 1% potassium cholate, pH 7.0 (personal communication from Dr. C. Hoppel).

time and protein content. The following enzymatic activities and substances were assayed by the indicated methods: CMP-N-AN synthetase by the thiobarbituric acid assay after reduction with sodium borohydride (TBA-NaBH₄) (4, 10); NADH-cytochrome c reductase (NADH : cytochrome c oxidoreductase, EC 1.6.2.1) by the procedure described by Hoppel and Cooper (13); succinic dehydrogenase (succinate:phenazine methosulphate oxidoreductase, EC 1.3.99.1) plus 6-phosphogluconate dehydrogenase (6-phospho-D-glucuronate:NADP+ oxidoreductase EC 1.1.1.44) by following the rate of reduction of NADPH with glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) by the method of Harper (14), and the liberated phosphate by the procedure of Ames (15); the combined activities of glucose 6-phosphate dehydrogenase (d-glucose 6-phosphate:NADP+ oxidoreductase, EC 1.1.1.48) plus 6-phosphogluconate dehydrogenase (6-phospho-D-glucuronate:NADP+ oxidoreductase EC 1.1.4.44) by following the rate of reduction of NADPH with glucose 6-phosphate as substrate according to the procedure of Lörh and Waler (16); N-AN-aldolase by the procedure of Comb and Roseman (17); RNA by the procedure of Ceriotti (18) or by ultraviolet absorption according to the procedure described by Munro and Fleck (19). DNA was measured by the diphenylamine reaction according to the procedure of Munro and Fleck (19). DNA was measured by the method of Burton (20) or by following the rate of reduction of NADP with glucose 6-phosphate (EC 1.1.1.49) plus 6-phosphogluconate dehydrogenase (6-phospho-D-glucuronate:NADP+ oxidoreductase EC 1.1.1.48) by following the rate of reduction of NADPH with glucose 6-phosphate as substrate according to the procedure of Lörh and Waler (16); N-AN-aldolase by the procedure of Comb and Roseman (17); RNA by the procedure of Ceriotti (18) or by ultraviolet absorption according to the procedure described by Munro and Fleck (19).
The reduction of cytochrome c at 550 nm. The activity of succinic dehydrogenase was measured by following the rate of reduction of NADP+ as determined with glucose 6-phosphate as substrate represents a summation of glucose 6-P and 6-P-gluconate dehydrogenase activities. The activity of glucose 6-phosphatase was determined by measuring the rate of release of Pi from glucose G-phosphate. NADH-cytochrome c reductase was measured spectrophotometrically by following the reduction of 2,6-dichloroindophenol at 600 nm, with phenazine methosulfate as an electron acceptor. N.D. indicates not detected.

Rat liver Glucose 6-phosphate and 6-phosphogluconate dehydrogenases
Glucose 6-phosphatase
NADH-cytochrome c reductase
Succinic dehydrogenase

Hog retina Glucose 6-phosphate and 6-phosphogluconate dehydrogenases
NADH-cytochrome c reductase
Succinic dehydrogenase

Tissue Enzyme assayed Enzyme activities Activity of homogenate in nuclei

| Tissue     | Enzyme assayed          | Homogenate | Nuclei | Homogenate | Nuclei | %   |
|------------|-------------------------|------------|--------|------------|--------|-----|
| Rat liver  | Glucose 6-phosphate and 6-phosphogluconate dehydrogenases | 2.24       | N.D.   | 0.0150     | N.D.   | 0.00 |
|            | Glucose 6-phosphatase   | 11.8       | 0.024  | 0.0639     | 0.011  | 0.20 |
|            | NADH-cytochrome c reductase | 0.26%     | 0.20   | 0.00%      | 0.25   | 0.25 |
|            | Succinic dehydrogenase  | 7.47       | N.D.   | 0.0530     | N.D.   | 0.00 |
| Hog retina | Glucose 6-phosphate and 6-phosphogluconate dehydrogenases | 1.15       | N.D.   | 0.0234     | N.D.   | 0.00 |
|            | NADH-cytochrome c reductase | 0.26%     | 0.20   | 0.00%      | 0.25   | 0.25 |
|            | Succinic dehydrogenase  | 2.36       | 0.0080 | 0.048      | 0.0041 | 0.34 |

a Less than 1%.
b Less than 2%.

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|            | Succinic dehydrogenase  | 7.47       | N.D.   | 0.0530     | N.D.   | 0.00 |
| Hog retina | Glucose 6-phosphate and 6-phosphogluconate dehydrogenases | 1.15       | N.D.   | 0.0234     | N.D.   | 0.00 |
|            | NADH-cytochrome c reductase | 0.26%     | 0.20   | 0.00%      | 0.25   | 0.25 |
|            | Succinic dehydrogenase  | 2.36       | 0.0080 | 0.048      | 0.0041 | 0.34 |

a Less than 1%.
b Less than 2%.

1.0 mM CaCl2 (1 ml per g of fresh tissue) with a Dounce homogenizer. The suspension was centrifuged at 8,000 × g for 5 min and the nuclear pellet was resuspended in a solution of 0.25 M sucrose.3.3 mM CaCl2 (1 ml per g of fresh tissue). Nuclei were also prepared from rat liver by the procedure of Blobel and Potter (31) where indicated.

Sucrose interferes in the TBA assay used for the determination of the activity of CMP-sialic acid synthetase with the conditions determined in this and in previous studies (4, 10). The incubation mixtures contained 1.25 μmoles; CTP, 1.25 μmoles; Tris-HCl buffer, pH 8.5, 45 μmoles; MgCl2, 5 μmoles; and enzyme. All particulate preparations were resuspended by homogenization before sampling. One unit of enzyme is defined as that quantity producing 1 μmole of CMP-sialic acid per hour when incubated at 37°C.

RESULTS

Purity of Nuclei

Chemical Analyses—Comparison of the ratios of RNA to DNA and protein to DNA (Table I) of the whole homogenates to that of the nuclei indicates that considerable enrichment of DNA relative to protein and RNA was achieved in nuclei from each of these tissues. Variation has been reported in these relationships among different tissues (32) and in nuclei purified by several techniques (33). The present values are in accord with the compiled data in the literature (30, 32, 33). Recovery of DNA in nuclei from rat liver in the present studies (51%) is similar to that reported previously (29, 34). The relatively low recoveries of RNA in the nuclei (per cent of the whole homogenate: retina, 5; liver, 4; kidney, 6; spleen, 15; brain, 3) reflects the decrease in contamination of the nuclei with cytoplasmic RNA. In addition, since some RNA is indigenous to the nucleus, its presence cannot be regarded exclusively as a sign of cytoplasmic contamination (33).

Cytoplasmic Enzyme Markers

The extent of cytoplasmic contamination of nuclei from hog retina and rat liver was evaluated also by analyzing for the presence of several marker enzymes. When compared to the enzymatic activities present in the initial homogenates, little or no activities of supernatant, microsomal, or mitochondrial enzymes were detected in the nuclear preparations from these tissues (Table II). Sonic oscillation of the nuclear fractions or the initial homogenates did not result in increases in these enzymatic activities. In all cases in which no activity was detected in nuclear preparations, full activity was observed when aliquots of the initial homogenate were added to the nuclei.

Nuclei prepared from rat liver had the same low order of cytoplasmic contamination as determined by the activity of NADH-cytochrome c reductase (0.26% of the activity found in the initial homogenate) as nuclei isolated from hog retina. In the presence of rotenone (3 μM) no depression was observed in the activity of NADH-cytochrome c reductase of nuclei from hog retina, while about 10% reduction in this activity was detected in nuclei from rat liver. Although the rotenone-insensitive activity of NADH-cytochrome c reductase may indicate the presence of membranes or outer mitochondrial membranes (35), Kastning and Kasper (36) suggest that this enzymatic activity is an integral component of the nuclear membrane. These studies were not performed on the nuclei from the other tissues used in this investigation.

TABLE II

Extent of cytoplasmic contamination of nuclei as assessed by enzyme markers

| Tissue     | Enzyme assayed          | Homogenate | Nuclei | Homogenate | Nuclei | %   |
|------------|-------------------------|------------|--------|------------|--------|-----|
| Rat liver  | Glucose 6-phosphate and 6-phosphogluconate dehydrogenases | 2.24       | N.D.   | 0.0150     | N.D.   | 0.00 |
|            | Glucose 6-phosphatase   | 11.8       | 0.024  | 0.0639     | 0.011  | 0.20 |
|            | NADH-cytochrome c reductase | 0.26%     | 0.20   | 0.00%      | 0.25   | 0.25 |
|            | Succinic dehydrogenase  | 7.47       | N.D.   | 0.0530     | N.D.   | 0.00 |
| Hog retina | Glucose 6-phosphate and 6-phosphogluconate dehydrogenases | 1.15       | N.D.   | 0.0234     | N.D.   | 0.00 |
|            | NADH-cytochrome c reductase | 0.26%     | 0.20   | 0.00%      | 0.25   | 0.25 |
|            | Succinic dehydrogenase  | 2.36       | 0.0080 | 0.048      | 0.0041 | 0.34 |

a Less than 1%.
b Less than 2%.

*Purchased from Kontes Glass Company, Vineland, New Jersey.*
Morphology

When examined by light microscopy, the nuclear preparations appeared homogeneous. A preparation from hog retina contained less than 2% nonnuclear particulates as determined by counting 2000 particles of a suspension of nuclei in a hemocytometer.

Nuclei obtained from hog retina and rat liver were examined by electron microscopy, although a quantitative evaluation of nuclear purity by this technique was not carried out. The general appearance and morphological integrity of the nuclei from rat liver were similar to previous reports describing nuclei isolated by procedures similar to that used here (29, 30, 34, 37). The majority of the nuclei in the rat liver preparations were hepatic cell nuclei. Variation in size and shape of nuclei was observed in preparations from hog retina and probably reflected the cellular heterogeneity of this tissue (38). While relatively few of the nuclei were damaged in the liver preparation, some damaged and disrupted nuclei were observed in the preparations from hog retina.

CMP-N-AN Synthetase

Initial Extraction—Except for the report by the author (11) previous studies dealing with the preparation of CMP-N-AN synthetase from mammalian tissues (2, 4, 8–10) used procedures that removed nuclei. The procedure of Kean and Roseman (4, 10), for example, involved soaking or homogenizing the tissues in 0.1 M potassium phosphate buffer, pH 7.5, followed by centrifugation at 36,000 × g for 1 hour. When this procedure was applied to hog retina the yield of enzyme in the supernatant solution was 3.1 units per g, wet weight, of tissue. However, when the retinas were homogenized with 2.4 M sucrose-3.3 mM CaCl₂, 0.25 M sucrose-1.8 mM CaCl₂, or 0.25 M sucrose, from 14 to 28 units per g, wet weight, of retina were obtained in the whole homogenate, i.e. up to 9-fold greater yield of enzyme. Similarly, the yield of enzyme in the supernatant solution of the 0.1 M potassium phosphate extract of rat liver was about one-half that obtained after homogenization by the procedure of Chaveau et al. (29).

After homogenization in 2.4 M sucrose-3.3 mM CaCl₂ and centrifugation, three major fractions are obtained: the nuclei, an intermediate liquid phase, and a plug of tissue floating on the surface composed of cell debris, some whole cells, nuclei, and cytoplasmic constituents. Recovery studies with a preparation from hog retina showed the following distribution of CMP-N-AN synthetase activity in terms of the percentage of the initial homogenate: nuclei, 28%; floating plug, 64%. Very little activity was detected in the intermediate liquid fraction.

Nuclear Enzyme

Effect of Enzyme Concentration, Incubation Time, Yield, and Purification—The dialysis procedure (to remove sucrose) completely disrupted the nuclei. When sampled after resuspension by hand homogenization the rate of the reaction of the dialyzed particulate preparation from hog retina was proportional to enzyme concentration and incubation time (Fig. 1, A and B). The preparations from the other tissues behaved in a similar manner except that the rate of the reaction of preparations from rat liver was linear with incubation time only for about 10 min. The yields of CMP-N-AN synthetase in nuclei recovered from the several tissues that were investigated varied from 18 to 53% of the activities present in the initial homogenates (Table III). When corrected for the yield of nuclei (as measured by the yield of DNA (Table I)), from 54 to 90% of the total activity could be ascribed to the nuclei from these different tissues. The yield of enzyme in nuclei recovered from rat liver in seven preparations varied from 28 to 38% of the activity present in the whole homogenate. Nuclei prepared from rat liver by the procedure of Blobel and Potter (31) contained 27% of the CMP-N-AN synthetase activity of the whole homogenate. Nuclei prepared from fresh and frozen retinas had similar CMP-N-AN synthetase activities, and the yield of enzyme in nuclei recovered from the retinas from four different batches of hog eyes varied from 17 to 28%. Increases in the specific activities of nuclear CMP-N-AN synthetase compared to that in

TABLE III
Activities and yields of CMP-N-AN synthetase in nuclei from several tissues

Nuclei were isolated from fresh tissue according to the procedure of Chaveau et al. (29). The whole homogenates and nuclei refer to these fractions after being dialyzed against 0.01 M Tris buffer, pH 7.5, containing 1% mercaptoethanol (see text). CMP-N-AN synthetase activity was determined at 37° under conditions in which product formation was proportional to protein concentration and time of incubation, and under conditions of optimal substrate and magnesium concentrations and pH as determined in this and previous studies (4, 10); N-AN and CTP, 5 mM; MgCl₂, 20 mM; Tris, pH 8.6. CMP-N-AN formation was measured by the TBA-NaBH₄ procedure. These activities were obtained from the same fractions whose nucleic acid and protein relationships were described in Table I. The yield of enzyme in "recovered nuclei" refers to the per cent of the activity in the whole homogenate, while the corrected enzyme yield is this value corrected for the recovery of DNA which appears in Table I.

| Tissue     | Enzyme activity | Yield of enzyme in recovered nuclei | Corrected enzyme yield |
|------------|-----------------|-------------------------------------|------------------------|
|            | Homogenate      | Nuclear                            | %                      |
|            | enzyme units/g, wet wt, tissue | enzyme units/ mg protein | %                      |
| Hog retina | 18 3.2          | 0.29 1.8                           | 18 90                  |
| Rat tissues|                 |                                    |                        |
| Liver      | 95 26           | 0.73 9.7                           | 28 54                  |
| Kidney cortex | 26 9.4          | 0.49 4.5                           | 36 59                  |
| Brain      | 19 3.5          | 0.38 3.0                           | 18 60                  |
| Spleen     | 30 16           | 0.42 1.3                           | 53 70                  |
TABLE IV

Analysis of CMP-N-AN synthesized by nuclei from hog retina

After a large scale incubation with nuclei from hog retina, CMP-N-AN was isolated from the incubation mixture by ion exchange and paper chromatography, as indicated in the text. N-AN was determined by the TBA method with and without sodium borohydride treatment. Cytidine was assayed by ultraviolet light absorption at 280 nm at pH 2.0 (ε = 13 × 10³). Total phosphate was measured after acid hydrolysis.

| Constituent                        | Molar ratio |
|------------------------------------|-------------|
| N-AN (+NaBH₄)                      | 1.00        |
| N-AN                               | 1.06        |
| Phosphate (total)                  | 1.05        |
| Cytidine                           | 0.95        |
| Phosphate (inorganic)              | 0.00        |

Fig. 2. Effect of the concentration of N-AN on the reaction rate. In addition to N-AN, the standard reaction mixture contained 1.25 μmoles of CTP, 40 μmoles of Tris-Cl buffer (pH 8.0), 5 μmoles of MgCl₂, and enzyme from rat liver nuclei (0.068 mg of protein) in a total volume of 0.25 ml. Incubations were carried out for 10 min at 37°C, and CMP-N-AN was measured by the TBA-NaBH₄ method. ●, S/v; ○, CMP N-AN formed.

Fig. 3. Effect of the concentration of N-GN on the reaction rate. Nuclei were prepared from rat liver by the procedure of Blobel and Potter (31). Incubations were carried out for 5 min with the standard incubation mixture (see text and legend to Fig. 2) and enzyme from these nuclei (0.063 mg of protein). The concentration of N-GN was varied as indicated. ●, S/v; ○, CMP N-AN formed.

Fig. 4. Effect of the concentration of CTP on the reaction rate. The conditions were the same as described in the legend to Fig. 2, except that each reaction mixture contained 1.25 μmoles of N-AN and the concentration of CTP was varied as indicated. ●, S/v; ○, CMP N-AN formed.

The whole homogenates of the different tissues varied from 3- to 13-fold (Table III).

Identification of Product

CMP-N-AN (1.2 μmoles) produced enzymatically by nuclei from hog retina was isolated by ion exchange and paper chromatography as indicated under “Methods.” Chemical analyses for N-AN, borohydride-stable N-AN, cytidine, and total phosphate showed that these components were present in approximately equimolar proportions (Table IV), the relationship expected for CMP-N-AN.

When the product (0.02 μmole) was exposed to 0.5 M sodium acetate buffer, pH 4.0 at 37°C, for 1 hour approximately 85% of the N-AN was made susceptible to borohydride reduction. This acid lability is similar to that observed by Warren and Blacklow for CMP-N-AN (3).

When incubated with 4 units of *E. coli* alkaline phosphatase for 1 hour at 37°C, pH 8.1, no phosphate was liberated from the product (0.02 μmole). However, phosphate was liberated under these conditions when the product (CMP-N-AN) had previously been exposed to 0.1 N HCl for 15 min at room temperature.

CMP-N-AN (0.0025 to 0.006 μmole) synthesized by retinal nuclei was resistant to cleavage by N-AN-aldolase unless previously hydrolyzed (0.1 N HCl for 15 min at room temperature). These incubations were carried out in the presence of N-AN-aldolase (0.6 unit) and lactic dehydrogenase; the oxidation of NADH was measured fluorometrically (12). Under conditions when the reaction proceeded to about 90% of completion with acid-hydrolyzed CMP-N-AN, the nonhydrolyzed product was about 7% susceptible to the combined action of N-AN-aldolase and lactic dehydrogenase. N-AN (0.005 μmole) added to the tubes containing nonhydrolyzed CMP-N-AN was completely cleaved.

Paper Chromatography and Electrophoresis

CMP-N-AN synthesized by retinal nuclei and by hog submaxillary gland extracts had similar paper chromatographic and electrophoretic characteristics. The *Rf* values of CMP-N-AN synthesized by retinal nuclei and by extracts of the hog submaxillary gland were, respectively: Solvent A, 0.66 and 0.61; Solvent B, 0.016 and 0.016; Solvent C, 0.68 and 0.64. After electrophoresis the following anodic mobilities were observed for CMP-N-AN from these two sources: Solvent I, 16.5 cm and 17.0 cm; Solvent II, 14.5 cm and 14.9 cm. All of these systems except B can readily distinguish between CMP-N-AN, N-AN, and CMP.
Fig. 5. Effect of varying the concentration of MgCl₂ on the reaction rate. The conditions were the same as described in Fig. 2, except that each reaction mixture contained 1.25 μmoles of N-AN and the concentration of MgCl₂ was varied as indicated.

![Graph showing the effect of MgCl₂ concentration on the reaction rate.](image)

**Some Properties of Nuclear CMP-Sialic Acid Synthetase**

Differences in some of the properties of the nuclear and soluble enzymes (previously studied (3, 4, 9)) might exist if these two forms were distinct enzymes. In order to characterize the nuclear system further, some of the properties of nuclear CMP-sialic acid synthetase were examined.

**Effect of Substrate Concentration on Reaction Rate**—The effect on the rate of the reaction of nuclear CMP-sialic acid synthetase of variation in the concentrations of N-AN and N-GN is shown in Figs. 2 and 3. The apparent \( K_m \) value for N-AN is 0.72 mM, and for N-GN, 1.40 mM. The apparent \( K_m \) for CTP, obtained from similar studies (Fig. 4), is 0.48 mM. These values were calculated from Lineweaver-Burk plots which were obtained after analyses of the data by the method of least squares.

**Nucleotide Specificity and Effect of Magnesium and Other Metals on Reaction Rate**—The effect of varying the concentration of magnesium chloride on the rate of the reaction is shown in Fig. 5. The apparent \( K_m \) for Mg²⁺ is 0.75 mM. At 20 mM, a concentration at which magnesium showed maximal stimulation, the following metal chlorides exhibited the indicated activities as compared to that of magnesium: Mn²⁺, 30%; Ca²⁺, 15%; Cd²⁺, 3%; Cu²⁺, Co²⁺, and Zn²⁺, inactive.

When substituted for CTP in the standard incubation procedure, ATP, ITP, and TTP were inactive, while GTP, dCTP, and dUTP were about 4, 5, and 7% as active as CTP, respectively. No activity was observed in the absence of either N-AN or CTP.

**Effect of pH**—The effect of variation in pH on the reaction rate is seen in Fig. 6. In Tris buffers the optimal rate of the reaction was obtained between pH 8.3 and 8.6.

These properties are very similar to those of the soluble CMP-sialic acid synthetase as described previously.

### Stability and Storage

The enzyme is more stable when prepared and stored in the presence of exogenous sulfhydryl compounds, such as mercaptoethanol or glutathione. Essentially full activity of CMP-sialic acid synthetase of the dialyzed nuclear preparation was retained for about 2 weeks when stored at 0°. The enzyme was inactivated by freezing and thawing or lyophilizing, losing from 50 to 80% of enzymatic activity with each freeze and thaw cycle. When these latter operations were carried out in the presence of glutathione, however, this lability was not encountered. Thus, after being lyophilized in the presence of 0.15 M GSH, pH 7.0, and subsequent storage at −20° for 1 month, full activity was observed when reconstituted with water. The reconstituted preparation retained over 90% of the initial enzymatic activity when stored for an additional month at −20°. When kept at 0°, the same stability was observed as previously in the absence of GSH, when stored for the same length of time.

| Source of nuclei | RNA/DNA | Yield of CMP-sialic acid synthetase in recovered nuclei | Activity % |
|------------------|---------|-------------------------------------------------------|----------|
| Original Preparation | 0.16    | 27                                                   | 18       |
| After Wash 1      | 0.19    | 10                                                   | 10       |
| After Wash 2      | 0.15    | 18                                                   | 17       |

### Phosphate-extracted Nuclei

Nuclei isolated from rat liver by the procedure of Chaveau et al. (29) were homogenized with a solution of 0.1 M potassium phosphate, pH 7.5, containing 1% mercaptoethanol and centrifuged for 30 min at 40,000 × g. The residue was washed once with the same buffer. After dialysis against 0.01 M Tris, pH 7.5, containing 1% mercaptoethanol, 90% of the recovered enzymatic activity (70%) was present in the potassium phosphate extract. About a 90-fold increase in specific activity was obtained in the potassium phosphate extract of the nuclei (30 units per mg of protein) as compared to the activity present in the whole homogenate (0.66 unit per mg of protein).
Washing of Nuclei

Nuclei prepared from rat liver by the procedure of Blobel and Potter (31) were washed by resuspension in 0.25 M sucrose-TKM\(^+\) buffer with a Dounce homogenizer, followed by centrifugation for 10 min at 1000 × g. The supernatant solution was discarded and the pellet was resuspended in the same medium for analysis. The pellet from a second aliquot of nuclei was washed again by resuspension with a stirring rod, followed by centrifugation as above. The ratio of enzyme activity to DNA content was constant throughout these steps (Table V), indicating that the observed decrease in yield of CMP-sialic acid synthetase (from 27% to 18%) was probably due to a disruption of nuclei during the procedures and not merely a washing out of enzyme from the nucleus.

Mixing Experiments

Experiments were performed in an attempt to determine whether purified nuclei could adsorb CMP-sialic acid synthetase. These studies involved mixing nuclei (obtained from rat liver by the procedure of Chaveau et al. (29)) with the soluble form of the enzyme (obtained after extracting the tissue with 0.1 M potassium phosphate buffer, pH 7.5). The soluble enzyme was dialyzed against 0.01 M Tris buffer, pH 7.5, containing 1% mercaptoethanol, and the extract was adjusted to 0.25 M sucrose. The soluble preparation and the nuclei were mixed in the cold over a period of 20 min with a Dounce homogenizer. After the mixing procedure, nuclei were resolated by a modification of the procedure of Blobel and Potter (31). Adsorption was indicated by an increase in total activity of nuclear enzyme as compared to that of control nuclei. The results from these studies were not conclusive. No increase in enzymatic activity of nuclei was detected in experiments in which soluble enzyme from 1.7 g of rat liver was mixed with nuclei equivalent to that obtained from 2.2 g of liver. However, because of the high endogenous activity of the nuclei, the sensitivity of these studies was such that an adsorption by the nuclei of over 30% of the activity to which they had been exposed would have been required in order to be detected.

Difficulty was also encountered in interpreting results from experiments in which a concentrated source of soluble enzyme was used (obtained by ammonium sulfate fractionation of the soluble enzyme). A search is in progress to obtain viable nuclei that have little or no endogenous enzyme for these studies.

DISCUSSION

It is difficult, if not impossible, by currently available techniques to eliminate completely the contamination of nuclei by cytoplasmic elements. The extent of this contamination can be estimated by measuring the activities of certain indicator enzymes and by chemical analyses. These evaluations, in addition to morphological appearance, provide criteria for defining nuclear purity. As a result of analyses of this type, the nuclei from both hog retina and rat liver used in the present studies can be described as being relatively “pure”; i.e. they contained little, if any, cytoplasmic contamination. The chemical data for the nuclei from rat kidney, brain, and spleen are also in accord with this description. These considerations together with the activities of CMP-sialic acid synthetase in these nuclei indicate that at least one form of the sialic acid activating enzyme exists as a nuclear enzyme and in relatively high concentration. Many of the properties of the nuclear and soluble forms of the enzyme (described previously (3, 4, 9, 10)) are similar: K\(_m\) values, nucleotide specificity, pH optimum, metal requirement. It is not possible from these studies to determine whether the activities of the soluble enzyme represent a different species of sialic acid-activating enzyme or reflect solubilization of the nuclear enzyme during the preparative procedures. However, the soluble enzyme extracted from tissues with phosphate buffer (2, 4, 10, 11) must also contain the nuclear enzyme.

Relatively few enzymes are nuclear in location. Evidence presented in this report supports the observation that a key enzyme involved in the biosynthetic pathways of sialic acid-containing polymers, CMP-sialic acid synthetase, is located in the nucleus of the cell. The demonstration that this is a nuclear enzyme, even if this is not its exclusive site, may be of importance in understanding the metabolism of the sialic acid-containing compounds. Until now these processes have been observed, in the main, as extranuclear events. Although the role of this synthetase in the nucleus is not presently clear, as pointed out by Allfrey (39) “...where the absence of contamination has been established by independent tests, the presence of any enzyme activity in isolated nuclei must be regarded as significant to the function of the nucleus. . . .” Several sialyl transferases have been described that utilize CMP-sialic acid as a substrate for the biosynthesis of sialic acid-containing heteropolymers (5-8). In the preparation of most of these enzymes, procedures were used that involved the removal of nuclei. (Sialyl-transferase activity was reported to be present, however, in the “nuclei and cell debris,” in addition to the synaptosomes, in the preparations from embryonic chick brain that are involved in the biosynthesis of gangliosides (7).) In addition to the extranuclear locale of most of the sialyl transferases that have been described, several studies have also reported the low concentration or virtual absence of sialic acid from the nucleus (36, 40-42). There thus appears to be a geographic separation within the cell between the site of the activation of sialic acid and its utilization for the biosynthesis of glycoproteins and gangliosides. Since sialic acid residues occupy end positions on the carbohydrate chains of most of these polymers, its attachment may thus terminate further extension of these segments. Control over this process may occur at the level of the transferase reactions, but also may be exerted by mechanisms that regulate the availability of “active sialic acid” for use as a substrate by the extranuclear sialyl transferases. The nuclear location of CMP-sialic acid synthetase may play a role in this control process.

The nature of the attachment of CMP-sialic acid synthetase to the nucleus is not known. The presence of this enzyme in purified nuclei in high concentration as shown in this report strongly suggests that this is the actual site of this enzyme in situ. Studies are in process with immunohistochemical techniques in order to substantiate further the cellular location of CMP-sialic acid synthetase.

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1 The TKM buffer used by Blobel and Potter (31) is composed of 0.05 M Tris-HCl (pH 7.5), 0.025 M KCl, and 0.005 M MgCl\(_2\).
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