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SUBCELLULAR AND REGIONAL DISTRIBUTION OF CMP-*N*-ACETYLNEURAMINIC ACID SYNTHETASE IN THE CALF KIDNEY

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SUMMARY

The subcellular localization of the enzyme CMP-*N*-acetylneuraminic acid (CMP-NANA) synthetase was studied in the different regions of the calf kidney. The enzyme appeared to be localized in the nuclear fractions. The possibility that CMP-NANA synthetase was adsorbed to the nuclear membranes during homogenization and subsequent isolation of the nuclei was excluded by removal of the membranes from purified nuclei by solubilization with Triton X-100. This treatment did not remove the CMP-NANA synthetase activity from the nuclei.

About 11% of the CMP-NANA synthetase activity was recovered in the soluble fractions of the different regions of the calf kidney. To investigate whether this enzyme activity could be of nuclear origin too, some properties of the nuclear-bound and the soluble enzyme of the cortex of the kidney were compared. Both enzyme preparations exhibited identical pH and temperature optima. The apparent K_m values for NANA, CTP and Mg^{2+} for both enzyme preparations were also almost identical.

Because (a) no differences were detectable between the two enzyme preparations and (b) it was observed that nuclei appear to be sensitive to homogenization, it is concluded that CMP-NANA synthetase recovered in the soluble fraction may be released from maltreated nuclei during homogenization.

INTRODUCTION

In sialoglycoprotein biosynthesis, sialyltransferase is responsible for the incorporation of *N*-acetylneuraminic acid (NANA). It has been shown[1–3] that for this incorporation CMP-NANA is required as a sialic acid donor. This sugar nucleotide is synthetized by CMP-NANA synthetase, an enzyme discovered in bacteria (*Neisseria meningitidis*) and in animal tissue (submaxillary gland) by Warren and Blacklow[4] and Roseman[5], respectively. The existence of the enzyme in a variety of animal tis-

Abbreviation: NANA, N-acetylneuraminic acid.

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sues was shown by others[6–14]. In 1969 and in 1970, Kean[9,10] reported a nuclear localization of CMP-NANA synthetase in rat liver and some other rat tissues. His observations were supported by the reports of Gielen *et al.*[11,12] for leucocytes and rat brain and Van den Eijnden and co-workers[13,15] for calf brain.

Kean[9,10] and Gielen *et al.*[11,12] could not completely exclude the possibility that the nuclear localization of CMP-NANA synthetase is artificial, because of a possible specific adsorption to the nuclear membranes during homogenization of the tissue. Moreover they always found a small amount of enzyme activity in the soluble fraction resulting after high-speed centrifugation of the homogenate.

In investigating the enzymes involved in the biosynthesis of sialic acid containing glycoproteins in the different regions of the kidney, we tried to obtain definite proof about the subcellular localization of CMP-NANA synthetase in these regions of the kidney. In this report we describe the regional distribution, the subcellular localization and some properties of CMP-NANA synthetase in the calf kidney.

Part of this work has been presented previously in a preliminary form[16,17].

MATERIALS AND METHODS

Materials

Kidneys from 3-month-old calves were obtained from a local slaughterhouse. The organs were removed from the animals as soon as possible after death and were transported on ice to the laboratory.

All chemicals were analytical grade and were obtained from commercial sources.

Tissue homogenization and fractionation

Part of the calf kidney was sliced with a scalpel and the different regions were dissected. After being minced with rotating razor blades, a 12.5% (w/v) homogenate was prepared from 2–3 g of the desired region of the kidney in 0.45 M sucrose containing 0.68 mM EDTA, and 0.01 M Tris–HCl buffer (pH 7.4). Homogenization was performed with a Teflon–glass Potter–Elvehjem homogenizer at 1400 rev./min, by 1 up-and-down stroke with a loose-fitting pestle (0.2-mm clearance) followed by 6 up-and-down strokes of a tightly-fitting pestle (0.03-mm clearance). The 12.5% (w/v) homogenate was filtered over a 25-μm nylon sieve to get rid of undisrupted tissue. The residue on the sieve was washed with the 0.45 M sucrose medium until the filtrate was 7% (w/v).

To study the subcellular localization of CMP-NANA synthetase in the different regions of the calf kidney, the 7% (w/v) homogenates were separated into three particulate fractions and one soluble fraction by centrifuging the homogenates, successively, for 10 min at 230 $\times$ g_{av} (unbroken cells and nuclei), 20 min at 16 500 $\times$ g_{av} (mitochondria and lysosomes) and 90 min at 105 000 $\times$ g_{av} (microsomes and soluble fraction). The particulate fractions were suspended in the 0.45 M sucrose medium. From the different subcellular fractions samples were taken for enzyme assays and for the assay of protein, DNA and RNA. Routinely, the samples were frozen at -40 °C, except those for the assay of lactate dehydrogenase and CMP-NANA synthetase. All operations were carried out at 0–4 °C.

Preparation of purified nuclei

The procedure used to purify the nuclear fraction from cytoplasmatic contaminants is a modification of the method of Chauveau *et al.*[18].

In order to maintain the integrity of the nuclei during isolation, the tissue was homogenized in 0.45 M sucrose containing 1 mM $MgCl_2$ and 0.01 M Tris–maleate buffer (pH 6.4) (ref. 19). Homogenization was performed as in the preceeding section. The crude nuclear pellet obtained by centrifugation at 230 × g_{av} for 10 min, was resuspended in the same 0.45 M sucrose medium with a tightly-fitting (0.03-mm clearance) Potter–Elvehjem homogenizer. The suspension was layered on a 1.75 M sucrose cushion (d_0 °C = 1.230) containing 1 mM $MgCl_2$ and 0.01 M Tris–maleate buffer (pH 6.4). The sucrose gradient was centrifuged at 30 000 × g_{av} for 2 h. The pellet containing the purified nuclei was resuspended in 0.25 M sucrose containing 1 mM $MgCl_2$ and 0.01 M Tris–maleate buffer (pH 6.4), unless otherwise indicated.

Treatment of nuclei with Triton X-100

In order to remove the nuclear membranes, the purified nuclei were suspended in 1% Triton X-100 solution in 0.25 M sucrose containing 1 mM $MgCl_2$ and 0.01 M Tris–maleate buffer (pH 6.4). The suspension was kept at 0–4 °C for 30 min under occasional shaking and was centrifuged subsequently at 230 × g_{av} for 10 min. The detergent solubilizes the nuclear membranes, including adsorbed cytoplasmatic materials, whereas the nucleoplasma containing the chromatin and nucleoli is not affected[15,18–22]. The pellet was resuspended in 0.25 M sucrose containing 1 mM $MgCl_2$ and 0.01 M Tris–maleate buffer (pH 6.4).

Extraction of soluble nucleoplasmatic materials from nuclei

The purified nuclei were suspended in 0.25 M sucrose containing 0.1 M KCl and 0.04 M potassium acetate buffer (pH 6.0). After standing at 0–4 °C for 30 s, the suspension was centrifuged at 230 × g_{av} for 10 min. The extraction procedure was repeated and the supernatants were combined. At the end the pellet was resuspended in 0.25 M sucrose containing 1 mM $MgCl_2$ and 0.01 M Tris–maleate buffer (pH 6.4). Under the conditions applied only soluble nuclear constituents like mRNA, tRNA and proteins are extracted[19,23], whereas the nuclei remain structurally intact[15].

Assay of CMP-NANA synthetase

The assay system for the measurement of CMP-NANA synthetase activity contained the following components: 1 μmole NANA, 1 μmole CTP, 8 μmoles $MgCl_2$, 32 μmoles Tris–HCl buffer (pH 9.0) and enzyme in a total volume of 0.2 ml. After incubation at 37 °C for 30 min, the reaction was stopped on ice and the CMP-NANA formed was assayed by the thiobarbituric acid method[24], after reduction of the non-reacted NANA with $NaBH_4$, according to the method of Warren and Blacklow[4]. All samples were run in triplicate. Because of the interference of sucrose with the thiobarbituric acid assay, prior to the assay of CMP-NANA synthetase activity the samples were dialyzed against 300 vol. of 0.01 M Tris–HCl buffer (pH 9.0), containing 0.1% β-mercaptoethanol and 1 mM $MgCl_2$. Dialysis was carried out overnight at 0–4 °C.

Assay of cytoplasmatic enzyme markers

Succinate–2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium reductase

activity was assayed according to the method of Pennington[25], except that the formazan produced was extracted with isoamylalcohol.

β-Galactosidase was assayed by a procedure adapted from Sellinger *et al.*[26]. The incubation mixture contained 1.25 mM *p*-nitrophenyl-β-D-galactopyranoside as substrate in 0.05 M citrate–HCl buffer (pH 5.0) and 0.01% Triton X-100, in a total volume of 1.0 ml. After incubation at 37 °C for 30 min the reaction was terminated by the addition of 3 ml 0.25 M glycine–Na_2CO_3 buffer (pH 10.7) and the amount of the *p*-nitrophenol liberated was determined spectrophotometrically.

(Na^+–K^+)-activated ouabain-sensitive ATPase was assayed according to the method of Bonting *et al.*[27]. Liberated phosphate was measured by the method of Taussky and Shorr[28].

Glucose 6-phosphatase was assayed according to the method of De Duve *et al.*[29] with the assay of liberated phosphate as above.

Lactate dehydrogenase was determined by direct spectrophotometric measurement of the oxidation of NADH[30].

Assay of DNA and RNA

The samples to be analyzed were heated at 100 °C for 10 min to inactivate the nucleases[14]. The nucleic acids were extracted with 0.9 M $HClO_4$ (70 °C for 20 min)[31] from the resulting pellets, after the extraction of acid-soluble constituents and of lipids[14]. In aliquot parts of the extracts, DNA was assayed by the diphenylamine method of Burton[32] and RNA was assayed by the orcinol method according to Munro and Fleck[33], with calf thymus DNA and yeast RNA as the respective standards. To correct for the interference of DNA in the orcinol assay, standard DNA samples were co-assayed.

Determination of protein

Protein was determined according to the method of Lowry *et al.*[34] with horse serum as a standard. Prior to assay the protein was precipitated with 1 vol. 10% trichloroacetic acid, because of the interference of sucrose with the assay of protein; the precipitate was dissolved in 1.0 M NaOH.

RESULTS

Regional distribution of CMP-NANA synthetase

In Table I the distribution of CMP-NANA synthetase over the different regions of the calf kidney is given. The enzyme activity per gram wet weight and per mg DNA is approximately the same for the three regions. However, the activity of CMP-NANA synthetase expressed per mg protein is twice as much for medulla and papilla as for the cortex.

Subcellular distribution of CMP-NANA synthetase

By means of differential centrifugation of the 7% (w/v) homogenates of the different regions of the calf kidney, four subcellular fractions were obtained as is shown for the cortex in Table II.

From the DNA recovery it appears that about 75% of the nuclei are recovered in the nuclear fraction. Because in this fraction only the relative specific activity of

TABLE I

REGIONAL DISTRIBUTION OF CMP-NANA SYNTHETASE IN CALF KIDNEY

The enzyme activity is given per g wet wt, per mg protein and per mg DNA for cortex, medulla and papilla of calf kidney. Means ± S.E. are given; the number of experiments performed are given in parentheses. 1 unit of enzyme activity is defined as the amount of enzyme producing 1 μmole of CMP-NANA per h at 37 °C.

Region of calf kidney	*CMP-NANA synthetase activity*		
	Per g wet wt	*Per mg protein*	*Per mg DNA*
Cortex	16.2 ± 1.4 (9)	0.185 ± 0.024 (9)	5.1 ± 1.1 (8)
Medulla	16.6 ± 2.5 (4)	0.377 ± 0.044 (4)	6.9 ± 1.3 (3)
Papilla	15.5 ± 1.0 (3)	0.331 ± 0.140 (4)	6.5 ± 2.0 (3)

the plasma membrane marker ($(Na^+–K^+)$-ATPase) is higher than in the homogenate, it can be concluded that plasma membranes form the second main component of the nuclear fraction. Only slight contamination of soluble cell components was detectable in this fraction as is shown by the low relative specific activity of lactate dehydrogenase.

The remaining 25% of the nuclei are recovered in the mitochondrial–lysosomal fraction. This latter subcellular fraction also contains heavy microsomes as follows from the relative specific activities of $(Na^+–K^+)$-ATPase and glucose 6-phosphatase.

From the values obtained for the mitochondrial and lysosomal enzyme markers in the soluble and microsomal fractions, it can be concluded that parts of the mitochondria and lysosomes are disrupted as a result of the necessarily crude homogenization procedure.

When the subcellular distribution of CMP-NANA synthetase for the cortex of the calf kidney (Table II) is compared with the distribution of DNA, it appears that 77% of the enzyme activity in the homogenate is recovered in the nuclear fraction, a value close to that for the recovery of DNA in this fraction, *i.e.* 73%. The remaining enzyme activity was recovered mainly in the mitochondrial and lysosomal fractions and in the soluble fraction; only about 1% was recovered in the microsomal fraction. From the relative specific activities in the various subcellular fractions it appears that only the nuclear fraction is enriched in CMP-NANA synthetase activity (6.2-fold), whereas the other subcellular fractions are impoverished (0.1–0.5-fold).

The results thus far obtained are in agreement with the findings of Kean[9,10,35], Gielen *et al.*[11,12] and Van den Eijnden and co-workers[13,15] concerning the nuclear localization of CMP-NANA synthetase. To make sure that this nuclear localization is not artificial, we decided to purify the nuclei and to subject them subsequently to a Triton X-100 treatment, to solubilize the nuclear membranes.

Purity of the nuclei

When the purified nuclei from cortex, medulla and papilla were examined by light microscopy, these preparations appeared to be homogeneous: contamination by non-nuclear particles was less than 1%.

The recovery of the nuclei after purification was calculated from the yield of DNA in the recovered nuclei. These values were for cortex, medulla and papilla, 27, 53 and 48%, respectively. In Table III the degree of purification of the nuclei

TABLE II

SUBCELLULAR DISTRIBUTION OF CMP-NANA SYNTHETASE AND OF VARIOUS ENZYMATIC MARKERS, DNA AND PROTEIN IN THE CORTEX OF THE CALF KIDNEY

DNA, succinate–2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium (succinate–INT) reductase, β-galactosidase, (Na^+–K^+)-activated ouabain-sensitive ATPase ((Na^+–K^+)-ATPase), glucose 6-phosphatase and lactate dehydrogenase were measured as markers for nuclei, mitochondria, lysosomes, plasma membranes and cytosol, respectively. The ratios between the percent of enzyme activity and percent of protein in the different subcellular fractions (relative specific activities) are given for the marker enzymes. The amounts of protein (mg), DNA (mg) and enzyme activity (units) are given for the homogenate per g wet wt of tissue in parentheses. 1 unit of enzyme activity is defined as the amount of enzyme producing 1 μmole of product per h. Means of 2–4 experiments are given.

Fraction	*Protein (%)*	*DNA (%)*	*CMP-NANA synthetase*			*Relative spec. act.*				
			Units/g wet wt of tissue	*%*	*Relative spec. act.*	*Succinate–INT reductase*	*β-Galactosidase*	*(Na^+-K^+)-ATPase*	*Glucose 6-phosphatase*	*Lactate dehydrogenase*
Homogenate (7%, w/v)	100 (108)	100 (3.6)	18.61 ± 5.75	100	1.0	1.0 (98)	1.0 (31)	1.0 (96)	1.0 (940)	1.0 (6700)
Nuclear fraction (230 × g_{av}, 10 min)	12.5	73	14.34 ± 4.34	77.0	6.2	0.9	0.8	2.6	1.0	0.3
Mitochondrial–lysosomal fraction (16 500 × g_{av}, 10 min)	35.6	26	3.32 ± 1.57	17.8	0.5	2.1	1.4	1.6	1.3	0.3
Microsomal fraction (105 000 × g_{av}, 90 min, pellet)	11.3	0	0.21 ± 0.08	1.1	0.1	0.7	0.6	1.5	3.9	0.2
Soluble fraction (105 000 × g_{av}, 90 min, supernatant)	40.7	0	2.10 ± 1.31	11.3	0.3	0.1	0.7	0.2	0.7	2.1

TABLE III

PROTEIN/DNA AND RNA/DNA RATIOS IN HOMOGENATES AND PURIFIED NUCLEI FROM CORTEX, MEDULLA AND PAPILLA OF CALF KIDNEY

Region of calf kidney	*Protein/DNA ratio (mg/mg)*		*RNA/DNA ratio (mg/mg)*	
	Homogenate	*Purified nuclei*	*Homogenate*	*Purified nuclei*
Cortex	38.5	1.85	1.86	0.31
Medulla	14.1	1.03	1.62	0.28
Papilla	25.0	1.08	1.75	0.32

towards protein and RNA is given: a 14–23-fold enrichment towards protein and a 5–6-fold enrichment towards RNA was obtained for DNA in the purified nuclear fractions, relative to the homogenates of the three regions.

The protein/DNA and the RNA/DNA ratios for the purified nuclei from cortex, medulla and papilla are comparable with the ratios found for rat liver by Busch[36] (4 and 0.25, respectively) and for rat kidney by Kean[10] (1.5 and 0.11, respectively).

The degree of contamination of the purified nuclei by cytoplasmatic constituents was checked by assay of appropriate marker enzymes. The data given in Table IV indicate that cytoplasmatic contamination was minimal in the purified nuclei.

Activity of CMP-NANA synthetase in the purified nuclei

As a result of the purification of the nuclei the specific CMP-NANA synthetase activity is increased 2–4 times in comparison to the crude nuclear fractions, and when compared to the homogenates, as much as 12–25 times. The CMP-NANA synthetase/DNA ratio in the purified nuclei is only slightly lower than in the crude nuclear fraction. This is in sharp contrast with the 6–10-fold lowering of the marker enzyme/DNA ratios as a result of purification of the nuclei.

Treatment with Triton X-100 and extraction with 0.10 M KCl–0.04 M potassium acetate buffer (pH 6.0) of purified nuclei

It appears that after Triton X-100 treatment of purified nuclei from the cortex of the calf kidney, the bulk of CMP-NANA synthetase activity (72%) is recovered in the nuclear pellet, together with the bulk of DNA (85%) and RNA (83%) (Table V). Because purified nuclei are sensitive to homogenization (refs 10 and 37; unpublished results), the somewhat lower recovery of CMP-NANA synthetase, when compared to DNA in the Triton X-100-treated nuclei, can be explained by leakage of enzyme activity from somewhat disrupted nuclei. The same results were obtained for the other two regions.

The effect of extraction of purified nuclei of cortex with 0.10 M KCl–0.04 M potassium acetate buffer (pH 6.0) in isotonic solution is also given in Table V. As can be seen, 90% of DNA and 88% of RNA and only 35% of the CMP-NANA synthetase activity were recovered in the pellet containing the salt-extracted nuclei. Comparable results were obtained after salt extraction of purified nuclei of medulla and papilla.

Comparison of the properties of the nuclear-bound CMP-NANA synthetase with the 105 000 × g_{av} supernatant enzyme fraction (soluble enzyme)

It was shown in Table II that 11.3% of the total activity of CMP-NANA syn-

TABLE IV

ACTIVITY/mg DNA OF CMP-NANA SYNTHETASE AND CYTOPLASMATIC ENZYME MARKERS IN NUCLEI BEFORE AND AFTER PURIFICATION FOR CORTEX, MEDULLA AND PAPILLA OF CALF KIDNEY

Means of 2–3 experiments are given. INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium. Values are expressed as units/mg DNA unless otherwise indicated. See Table II for more details.

Region of calf kidney	*Protein (%)*	*DNA (%)*	*CMP-NANA synthetase*	*Succinate–INT reductase*	*$(Na^+$-$K^+)$-ATPase*	*β-Galactosidase*	*Lactate dehydrogenase*
Cortex							
nuclear fraction	13.7	73	4.3	3.82	8.90	1.54	33.21
purified nuclei	2.4	27	3.8	0.17	1.27	0.36	2.76
Medulla							
nuclear fraction	16.3	82	5.0	3.43	—	1.22	45.
purified nuclei	2.6	53	4.3	0.51	—	0.21	5.91
Papilla							
nuclear fraction	12.3	83	5.1	1.81	—	1.03	20.5
purified nuclei	3.2	48	4.9	0.29	—	0.11	2.17

TABLE V

EFFECT OF TRITON X-100 TREATMENT AND SALT EXTRACTION ON THE CMP-NANA SYNTHETASE CONTENT OF PURIFIED NUCLEI OF CALF KIDNEY CORTEX

Purified nuclei obtained from cortex were suspended in 0.1 M KCl–0.04 M potassium acetate (pH 6.0)–0.25 M sucrose or in 1% Triton X-100 in 0.25 M sucrose–1 mM $MgCl_2$–0.01 M Tris–maleate buffer (pH 6.4) and were treated further as indicated in Materials and Methods. After centrifugation at 230 × g_{av} for 10 min the pellets and supernatants were assayed for CMP-NANA synthetase activity and for protein, DNA and RNA content. The results are expressed as percentages of the values obtained for untreated nuclei. Means of 3 experiments are given. N.D., not determined.

	Recovery (%) after Triton X-100 treatment		*Recovery (%) after salt extraction*	
	Pellet	*Supernatant*	*Pellet*	*Supernatant*
CMP-NANA synthetase activity	72	36	35	57
Protein	67	25	73	26
DNA	85	N.D.	90	N.D.
RNA	83	N.D.	88	N.D.

thetase of the homogenate of the cortex was recovered in the soluble fraction. To investigate whether this enzyme is different from the nuclear-bound CMP-NANA synthetase, we measured some properties of both enzyme preparations. For the nuclear-bound enzyme we used the enzyme preparation obtained after extraction of the purified nuclei with 0.1 M KCl–0.04 M potassium acetate buffer (pH 6.0). This latter preparation we shall call the "KCl-extracted enzyme". The activities of both enzyme preparations were measured under conditions where product formation was linear with enzyme amounts.

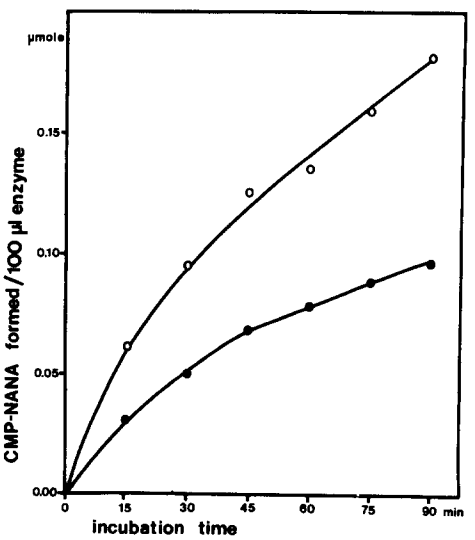

Fig. 1. Relationship between incubation time and product formation of the KCl-extracted and the soluble CMP-NANA synthetase of the calf kidney cortex. The assay conditions were the same as described in the text except for the variation in incubation time as indicated in the figure. ○—○, KCl-extracted enzyme; ●—●, soluble enzyme.

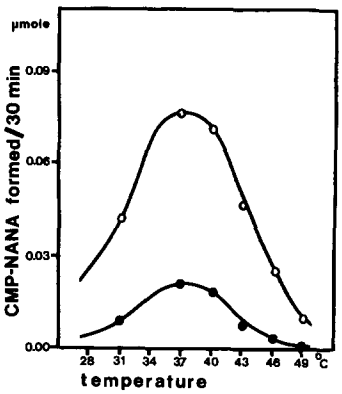

Fig. 2. Effect of temperature on product formation of the KCl-extracted and the soluble CMP-NANA synthetase of calf kidney cortex. The incubation mixtures were the same as described in the text. Incubations were carried out at the temperatures indicated for 30 min. ○—○, KCl-extracted enzyme; ●—●, soluble enzyme.

Effect of incubation time. In Fig. 1 the relationship between product formation and incubation time is given for the soluble enzyme and the KCl-extracted enzyme Both preparations show a non-linear behaviour with incubation times of less than 30–45 min. When incubation is carried out for longer periods, a more linear behaviour is shown.

Effect of temperature and pH. For the effect of incubation temperature on product formation, two identical curves were obtained for the soluble and the KCl-extracted enzyme. Both curves show an optimal enzyme activity at 37 °C (Fig. 2).

The dependence of product formation on pH variation is given in Fig. 3. The KCl-extracted as well as the soluble enzyme has a pH optimum from pH 8.5 to 9.0.

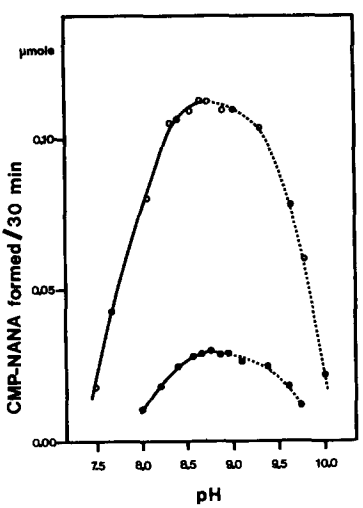

Fig. 3. Effect of pH variation on the product formation of the KCl-extracted and the soluble CMP-NANA synthetase of calf kidney cortex. The pH values were measured at room temperature before the addition of enzyme. Incubation time, 30 min. ———, 0.160 M Tris–HCl buffer; - - -, 0.160 M glycine–NaOH buffer; ○, KCl-extracted enzyme; ●, soluble enzyme.

TABLE VI

APPARENT K_m VALUES OF CMP-NANA SYNTHETASE FOR NANA, CTP AND Mg^{2+}

For the determination of the apparent K_m values for NANA, CTP and Mg^{2+}, the assay conditions were the same as described in the text, apart from a variation in the concentration of NANA (0–10 mM), CTP (0–10 mM) and Mg^{2+} (0–40 mM), respectively. The apparent K_m values were calculated from Lineweaver–Burk plots by the method of the least squares.

Region of calf kidney	*Enzyme preparation*	*Apparent K_m (mM)*		
		NANA	*CTP*	*Mg^{2+}*
Cortex	KCl-extracted	1.6	1.6	3.0
Cortex	Soluble	1.5	1.9	3.0
Medulla	KCl-extracted	1.4	2.4	2.1
Papilla	KCl-extracted	1.9	2.1	3.6

Apparent K_m values for NANA, CTP and Mg^{2+}. In Table VI the apparent K_m values for NANA, CTP and Mg^{2+} are given for the KCl-extracted and the soluble enzyme of cortex, together with the apparent K_m values for the KCl-extracted enzymes of medulla and papilla. The apparent K_m values for the soluble and KCl-extracted CMP-NANA synthetase of cortex are almost equal.

The K_m values for the KCl-extracted enzymes of medulla and papilla are in the same range as the K_m values for the cortical CMP-NANA synthetase.

Stability and storage. When the soluble as well as the KCl-extracted enzyme was dialyzed overnight in the absence of β-mercaptoethanol at 0–4 °C, both preparations lost about 75% of enzyme activity. No loss of enzyme activity was observed when the enzymes were dialyzed in the presence of β-mercaptoethanol and were stored subsequently under the same conditions at 0–4 °C for about a week. The enzymes were inactivated by freezing and thawing (both in the presence and in the absence of β-mercaptoethanol), loosing 20% of enzyme activity with each freeze and thaw cycle. It did not make any difference whether the freezing temperatures were −180 or −40 °C. However, storage of whole tissue at −40 or −180 °C had no influence on the CMP-NANA synthetase activity for at least 6 months.

After ultrasonic treatment (MSE sonifier) of the soluble and the KCl-extracted enzyme preparations for 15 min, no loss of activity was observed. When the treatment was carried out for longer periods, a gradual decrease in enzyme activity was noticed for both preparations.

DISCUSSION

The distribution of CMP-NANA synthetase and DNA over the various subcellular fractions of the different regions of the calf kidney shows that a close parallel exists between the distribution of CMP-NANA synthetase and DNA, except for the soluble fractions (*cf.* Table II). This parallel between DNA and CMP-NANA synthetase is maintained after purification of the nuclei, as is shown clearly from the data of Table IV. The observations presented point to a nuclear localization of CMP-NANA synthetase in the calf kidney, which is in agreement with the findings of several authors for other mammalian tissues[9–13,15,35]. However, Kean[10] and Gielen *et al.*[12]

suggested that this nuclear localization of CMP-NANA synthetase could be articifial because of a possible specific adsorption of the enzyme to the nuclear membranes during homogenization. In order to investigate this suggestion, the nuclei were fractionated by (a) Triton X-100 treatment and (b) osmotic shock. After Triton X-100 treatment, which is known to solubilize the nuclear membranes[15,18–22], the bulk of the CMP-NANA synthetase activity remains in the nuclear pellet. After osmotic shock, which was performed by subjecting a purified nuclear preparation to dialysis against a hypotonic medium, all CMP-NANA synthetase activity was recovered in the high-speed supernatant of the retained fraction (unpublished results). In this experiment too, CMP-NANA synthetase is separated easily from the nuclear membranes as these membranes are recovered in the high-speed pellet. With these two experiments a possible adsorption of the enzyme to the nuclear membranes is excluded and we can conclude that CMP-NANA synthetase is localized indeed in the nucleus, a conclusion which holds for all three regions of the calf kidney.

By selective salt extraction it is possible to determine the subnuclear localization of nuclear constituents[15,19,23]. The bulk of the CMP-NANA synthetase was easily extractable under the conditions applied (Table V)[19,23], which means that CMP-NANA synthetase is localized in the nuclear sap. We have no explanation for the fact that our results concerning the subnuclear localization (as demonstrated by lysis and salt extraction of the nuclei) of CMP-NANA synthetase, are in disagreement with the results of Gielen *et al.*[12] for rat brain CMP-NANA synthetase. They found that after lysis of brain cell nuclei, all enzyme activity was recovered in the nuclear sediment, *i.e.* CMP-NANA synthetase in rat brain is localized in the nuclear membranes or in some other sedimentable nuclear constituent[12].

In a series of experiments we investigated some properties of the nuclear CMP-NANA synthetase and of the enzyme occurring in the soluble fraction (see Table II), to determine whether there are two types or only one type of NANA-activating enzyme. All properties appeared to be the same for both enzyme preparations (Figs 1–3, Table VI).

In his study about CMP-NANA synthetase in hog eyes, Kean[10] compared the properties of the nuclear enzyme with literature values obtained for soluble enzyme preparations prepared by extraction from whole tissue with phosphate solutions[4,6,8,9]. Because these latter preparations also contain the nuclear enzyme[10], Kean could not decide whether the soluble enzyme was different from the nuclear enzyme or not. However, in our experiments the soluble enzyme fraction was not prepared by extraction of the tissue with a salt solution and therefore could not contain nuclear CMP-NANA synthetase solubilized by salt extraction. The similarity in characteristics between the nuclear-bound enzyme and the enzyme occurring in the soluble fraction suggests that only one type of CMP-NANA synthetase exists. The CMP-NANA synthetase activity in the particulate fractions (Table II) can be readily explained by the presence of well-preserved nuclei and/or damaged nuclei. The occurrence of enzyme activity in the soluble fraction cannot be explained in this way. However, because (a) Blobel and Potter[37] reported that about 50% of the purified nuclei, obtained by their method, were more or less damaged, (b) we observed that no differences exist between the nuclear-bound enzyme and the enzyme occurring in the soluble fraction, and (c) we observed that after lysis of the nuclei all CMP-NANA synthetase activity was recovered in the high-speed supernatant, it may be possible,

that the existence of CMP-NANA synthetase activity in the soluble fraction can be explained by leakage of enzyme activity from damaged nuclei.

The conclusions about the nuclear localization of CMP-NANA synthetase are valid for cortex, medulla and papilla of the calf kidney. From this fact and from the approximately equal CMP-NANA synthetase/DNA ratios (Table I) and apparent K_m values for NANA, CTP and Mg^{2+} (Table VI) for the three regions of the calf kidney, we may conclude that nuclei of every cell type of the calf kidney contain about the same CMP-NANA synthetase activity.

It can be expected that enzymes which have a nuclear localization are related to the function of the nucleus[23]. Up to now no functional relationship with the nucleus has been found for CMP-NANA synthetase. However, it is possible that the latter is localized in the nucleus to separate the CMP-NANA production from cytoplasmatic processes such as the synthesis of NANA[38] and the incorporation of NANA into glycoproteins by sialyltransferase[39,40]. It is known that CMP-NANA can exert a regulatory influence on its own synthesis by feed-back inhibition of UDP-*N*-acetylglucosamine-2-epimerase[38,41], an enzyme which is responsible for the conversion of UDP-*N*-acetylglucosamine into *N*-acetylmannosamine, one of the precursors of NANA[38]. Because NANA mostly occupies end positions in the carbohydrate chains of glycoproteins, it is imaginable that intervening in the supply of CMP-NANA for sialyltransferase can have repercussions on sialoglycoprotein biosynthesis. Feed-back inhibition by CMP-NANA as mentioned above, might be one way of regulating glycoprotein biosynthesis. The nuclear localization of CMP-NANA synthetase could open a second indirect regulatory way of sialoglycoprotein biosynthesis as a consequence of the separation of the site of synthesis of CMP-NANA from the sites of CMP-NANA utilization by the nuclear membrane.

Shoyab and Bachhawat[7,42] reported the existence of a CMP-NANA degrading enzyme in a variety of rat tissues. It could be possible that this enzyme is also involved in the regulation of the CMP-NANA concentration in the cell. Whether this latter enzyme plays an active role in this system is under investigation in our laboratory.

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