On the presence of two non-specific nucleotide-sugar-hydrolysing enzymes in rat liver

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Evidence is presented for the occurrence of two different non-specific nucleotide-sugar hydrolases in rat liver and other rat tissues. These two enzymes (I and II) were separated by chromatography on a 5'-AMP-aminohexyl-Sepharose column. Enzyme I is most probably identical with phosphodiesterase I (EC 3.1.4.1). Enzyme II appeared to be identical with an enzyme described in literature as 'CMP-sialic acid hydrolase' [Kean & Bighouse (1974) *J. Biol. Chem.* **249**, 7813–7823], since almost all activity with CMP-*N*-acetylneuraminate as substrate was recovered in this enzyme fraction. CMP-*N*-acetylneuraminate was a poor substrate for Enzyme I, whereas deoxythymidine-5'-*p*-nitrophenyl phosphate and all nucleoside-diphosphosugars tested were good substrates for both Enzyme I and II. Therefore it is suggested that CMP-*N*-acetylneuraminate is used as an additional substrate to discriminate between the activities of Enzyme I and II in homogenates or membrane preparations. The various substrates appeared to be competitive inhibitors of each other, suggesting that, in each enzyme preparation, only one enzyme is responsible for the hydrolysis of the various substrates. The dissimilar properties of the two enzymes are substantiated by studying the subunit molecular masses (Enzyme I, 125 kDa; Enzyme II, 50–55 kDa), the sensitivity towards Triton X-100, Sarkosyl and sodium dodecyl sulphate and towards trypsin treatment. It is discussed whether the α -*N*-acetylglucosamine phosphodiesterase described by Varki & Kornfeld [(1981) *J. Biol. Chem.* **256**, 9937–9943] is identical with one of the nucleotide-sugar hydrolases described here.

The presence in animal tissue of enzymes hydrolysing various nucleotide-sugars has been demonstrated by several authors (Shoyab & Bachhawat, 1969; Skidmore & Trams, 1970; Bachorik & Dietrich, 1972; Decker & Bischoff, 1972; Kean & Bighouse, 1974; Van Dijk *et al.*, 1976; Abney *et al.*, 1976; Haugen & Skrede, 1977; Nelson *et al.*, 1977; Spik *et al.*, 1979; Fukui *et al.*, 1981). The physiological function of the enzyme(s) is unknown. In estimating glycosyltransferase activity complications arise because of the depletion of radio-labelled nucleotide-sugar substrates by the action of nucleotide-sugar hydrolases which are present in the assay mixture (Faltynek *et al.*, 1981). It is not known if the depletion of substrates for glycosyltransferases demonstrated under conditions *in vitro*

has any significance *in vivo*. Studies are therefore required to establish the actual functions of the enzymes and their physiological significance. Furthermore, insight is needed regarding the specificity of the enzymes. Nucleotide-sugars can be hydrolysed enzymically by the non-specific enzyme phosphodiesterase I/nucleotide pyrophosphatase (Decker & Bischoff, 1972; Evans *et al.*, 1973; Haugen & Skrede, 1977) as well as by specific enzymes such as CMP-*N*-acetylneuraminate hydrolase (Shoyab & Bachhawat, 1969; Kean & Bighouse, 1974; Van Dijk *et al.*, 1976, 1977, 1983) and UDP-glucose hydrolase (Skidmore & Trams, 1970; Bachorik & Dietrich, 1972; Spik *et al.*, 1979). The properties of the various enzymes, however, with respect to pH, ion requirements, subcellular localization etc. are very much alike. Moreover, it has been reported that nucleotide-sugars like UDP-glucose and UDP-*N*-acetylglu-

Abbreviations used: SDS, sodium dodecyl sulphate; AH-Sepharose, aminohexyl-Sepharose; IgG, immunoglobulin G.

cosamine could inhibit the activity towards CMP-*N*-acetylneuraminate competitively (Kean & Bighouse, 1974). Thus the question has been raised as to whether a single enzyme is responsible for the hydrolysing activity towards all nucleotide-sugars. There are also indications that more than one nucleotide-sugar hydrolase exists, namely the uneven distribution of CMP-*N*-acetylneuraminate hydrolase activity and the phosphodiesterase I activity over the hepatocyte surface (Van Dijk *et al.*, 1977) and the tissue distribution of the sulphohydrolytic enzyme activity towards 3'-phosphoadenosine 5'-phosphosulphate and adenosine 5'-phosphosulphate (Fukui *et al.*, 1981).

In this paper, evidence is provided that two non-specific nucleotide-sugar hydrolases are present in various rat tissues. Parts of this study have been published in a preliminary form (Van Dijk *et al.*, 1979; Muilerman *et al.*, 1981).

Experimental

Materials

Sarkosyl NL-35 was purchased from Ciba-Geigy. Nucleotide-sugars were obtained from Sigma, except CMP-*N*-acetylneuraminate, which was synthesized enzymically in our own laboratory (Van Dijk *et al.*, 1981). Nitrocellulose was purchased from Schleicher and Schüll (0.45 μ m Selekttron, BA 85). 2'-Deoxythymidine-5'-*p*-nitrophenyl phosphate, trypsin and trypsin inhibitor (from hen's-egg white) were obtained from Boehringer, and Triton X-100 was obtained from Merck. Concanavalin A-Sepharose, Sephacryl S-400, AH-Sepharose 4B, Blue Sepharose CL 6B and DEAE-Sepharose CL 6B were purchased from Pharmacia (Sweden).

Preparation of tissue homogenates and rat liver membranes

Unless otherwise stated, male Wistar rats (200–300 g) were used, and were deprived of food for 24 h before being killed by decapitation. All experiments were carried out at 4°C. Homogenates of tissues (10%, w/v) were prepared in standard buffer [50 mM-Tris/HCl buffer, (pH 8.9)/5 mM-MgCl₂, containing 0.1% (v/v) Triton X-100] and were sonicated before use (MSE Sonifier; twice for 1 min; amplitude 5 μ m). For the preparation of membranes, rat livers were perfused with 0.01 M-phosphate buffer (pH 7.2)/0.14 M-NaCl. The livers were then cut into small pieces, washed with 1 mM-NaHCO₃ and homogenized in 1 mM-NaHCO₃ (1:9, v/v) with nine up-and-down strokes (1400 rev./min) of an all-glass Potter-Elvehjem-type homogenizer. Cell debris was removed by centrifugation at 100 g for 5 min and the particulate fraction of rat liver was obtained finally by centri-

fugation of the supernatant at 21000 g for 30 min (10 $\times$ 100 ml rotor, MSE 21). The pellet obtained is henceforward referred to as 'rat liver membranes'.

Partial purification of nucleotide-sugar hydrolases I and II from rat liver membranes

Rat liver membranes from 35–40 g of rat liver were extracted with 5% (v/v) Triton X-100 in standard buffer (5.9 ml/g of wet liver tissue) for 1 h. After centrifugation at 100000 g for 75 min (8 $\times$ 50 ml rotor; MSE PEGASUS) the supernatants were collected and applied to a column of concanavalin A-Sepharose (4 cm $\times$ 5 cm) at a flow rate of 100 ml/h. The eluate was reapplied three times to ensure maximal protein binding. After washing with 100 ml of 1% Triton X-100/1 M-NaCl in standard buffer, elution of the enzyme activities was performed with 300 ml of 0.5 M- α -methyl mannopyranoside/1% Triton X-100/1 M-NaCl in standard buffer at a flow rate of 40 ml/h. After desalting over Sephadex G-25 (100 cm $\times$ 2.5 cm column) the concanavalin A-Sepharose eluate was applied to a 5'-AMP-AH-Sepharose affinity column (Bischoff *et al.*, 1975) (10 cm $\times$ 1.5 cm) and eluted with a shallow linear NaCl gradient (0–0.8 M) in standard buffer containing 1% Triton X-100. Two main fractions were collected by pooling the fractions eluted by 0.14–0.20 M-NaCl (Enzyme I) and by 0.21–0.37 M-NaCl (Enzyme II) from the column.

After dialysis against standard buffer containing 1% Triton X-100, both enzyme fractions were concentrated over a short column of DEAE-Sepharose CL-6B (column size 1 cm $\times$ 1 cm for Enzyme I and 3 cm $\times$ 1.5 cm for Enzyme II). The elution was performed with 5–10 ml of 2 M-NaCl/1% Triton X-100 in standard buffer at 10 ml/h per cm². Enzyme I and II were further purified by Sephacryl S-400 chromatography (100 cm $\times$ 2.6 cm column) in standard buffer containing 1% Triton X-100.

Preparation of antibodies against nucleotide-sugar-hydrolysing Enzymes I and II

Enzyme I and II obtained after Sephacryl S-400 chromatography were used to raise antibodies in rabbits. The procedure used for the immunization and bleeding of the animals and the preparation of the IgG fractions was described previously (Muilerman *et al.*, 1982). The IgG fractions obtained were stored at –40°C.

SDS/polyacrylamide-gel electrophoresis and electric transfer (blotting) of the protein bands on nitrocellulose

The immunological detection of inactive enzyme protein on nitrocellulose was done as reported by us previously (Muilerman *et al.*, 1982), with some slight modifications. Instead of detect-

ing of the enzyme protein with a precipitating chromogenic substrate, the nitrocellulose strips were cut into 0.5 cm pieces and each piece was incubated in a reaction mixture suitable for the assay of the nucleotide-sugar-hydrolysing activity to be tested.

Assays of enzymic activities and protein

The following enzyme activities were assayed according to the literature or to methods described previously: nucleotide-sugar hydrolase (Van Dijk *et al.*, 1981); phosphodiesterase I (Bischoff *et al.*, 1975); alkaline phosphatase (Bessey *et al.*, 1946); 5'-CMP nucleotidase was assayed at pH 7.4 in 50 mM-Tris/acetate buffer/5 mM-MgCl₂ and liberated phosphate was determined as described by Van Dijk *et al.* (1981). Protein was determined with Coomassie Brilliant Blue (Bradford, 1976) by using the Bio-Rad protein assay kit.

Results

Enzymic hydrolysis of nucleotide-sugars in various rat tissues

In Table 1 the rates of enzymic hydrolysis of nucleotide-sugars in various rat tissues are compared with the activity of phosphodiesterase I (assayed with deoxythymidine-5'-*p*-nitrophenyl phosphate), since this enzyme is reported to be a non-specific phosphodiesterase/pyrophosphatase (Bischoff *et al.*, 1975; Abney *et al.*, 1976). It appeared that the three nucleoside-diphosphosugars tested were hydrolysed to the same extent in all tissues tested, all three showing about the same ratio of activity with respect to phosphodiesterase I

activity, except for brain and stomach. When CMP-*N*-acetylneuraminate was used as a substrate, no direct correlation with the hydrolysis of the nucleoside-diphosphosugars or the activity of phosphodiesterase I was apparent.

Segregation of two nucleotide-sugar hydrolase activities from rat liver

(a) *Sucrose-density-gradient centrifugation of detergent extracts of rat liver membranes (not shown).* Depending on the detergent used during extraction of rat liver membranes, different results were obtained for the solubilization of nucleotide-sugar-hydrolysing activities. One peak of enzyme activity was obtained in linear 10–40%-(w/v)-sucrose density gradients (100 000 g, 63 h, 3 × 25 ml rotor, MSE 65) when Sarkosyl NL-35 was used as solubilizing agent, and the activity pattern was independent of the nucleotide-sugar used as a substrate. When Triton X-100 was used, the enzyme activities towards UDP-galactose and CMP-*N*-acetylneuraminate were enriched at 23.3 and 25.8% sucrose respectively. The activity pattern with deoxythymidine-5'-*p*-nitrophenyl phosphate as substrate coincided with the pattern obtained with UDP-galactose.

(b) *Column chromatography on 5'-AMP-AH-Sepharose 4B.* As described in the Experimental section, partial purification of two nucleotide-sugar hydrolase activities was obtained by starting with a Triton X-100 extract of rat liver membranes. CMP-*N*-acetylneuraminate, UDP-galactose or deoxythymidine-5'-*p*-nitrophenyl phosphate was used to discriminate between the two enzyme activities.

Table 1. *Enzymic hydrolysis of nucleotide-sugars in various rat tissues relative to the activity of phosphodiesterase I* The activity of phosphodiesterase I was assayed with, as substrate, deoxythymidine 5'-*p*-nitrophenyl phosphate (dTMP-pNP). Results are means for three experiments. For endometrium and ovary, 12-month-old female rats were used and for the other tissues 5-months-old male rats. Other abbreviations used: n.d., not determined; NeuAc, *N*-acetylneuraminate; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine.

Tissue	Substrate ...	Enzyme activity (nmol/min per mg of protein)				
		dTMP-pNP	Ratio relative to dTMP-pNP			
			CMP-NeuAc (× 100)	UDP-Gal (× 10)	UDP-Glc (× 10)	UDP-GlcNAc (× 10)
Intestine (mucosa)		472 ± 120	0.4	4.0	4.5	4.3
Kidney		333 ± 44	1.0	4.8	5.0	5.6
Liver		135 ± 32	5.3	4.2	5.6	5.9
Pancreas		118 ± 27	0.6	7.7	8.3	5.9
Stomach (mucosa)		41 ± 9	2.1	1.5	1.6	1.5
Brain		8 ± 4	3.3	0.4	0.4	0.4
Submandibular gland		388 ± 86	1.1	4.6	5.3	5.0
Epididymis		357 ± 86	7.1	4.8	5.3	5.3
Ovary		326 ± 87	1.4	3.3	n.d.	n.d.
Endometrium (mucosa)		372 ± 126	2.0	7.1	n.d.	n.d.

The first step in the partial purification was performed by affinity chromatography on concanavalin A-Sepharose. From the affinity columns tested (concanavalin A-, Red A-, lentil- and Blue Sepharose), concanavalin A-Sepharose gave the best results.

Apparently part of the hydrolase activity, and especially the activity detectable with CMP-*N*-acetylneuraminate, was bound irreversibly to the concanavalin A-Sepharose beads, since it could not be eluted by EDTA, increasing amounts of α -methyl mannopyranoside or NaCl, and remained detectable in large amounts on the beads. Segregation of the two nucleotide-sugar hydrolase activities was obtained on chromatography of the post-concanavalin A-Sepharose fraction on a 5'-AMP-AH-Sepharose column as described by Bischoff (1975) by using, however, a very shallow NaCl gradient. Enzyme I (0.14–0.20M-NaCl) showed high activity with UDP-sugars and deoxythymidine-5'-*p*-nitrophenyl phosphate and a low activity with CMP-*N*-acetylneuraminate, whereas Enzyme II (0.21–0.37M-NaCl) showed a relatively low activity with UDP-sugars and deoxythymidine-5'-*p*-nitrophenyl phosphate and relatively high activity with CMP-*N*-acetylneuraminate (Fig. 1). On rechromatography, Enzyme I and Enzyme II respectively were eluted from the 5'-AMP-AH-Sepharose column at the same NaCl concentrations as in the first column passage (not shown).

After submitting both enzyme preparations to Sephacryl S-400 column chromatography the nucleotide-sugar hydrolases were eluted in the region of the void volume. Calibration with protein standards (ferritin, catalase, aldolase and albumin) indicated a molecular mass of approx. 550 kDa for both Enzymes I and II.

From the ratios of the activities with different substrates (Table 2) it appears that, for Enzyme II, the ratio CMP-*N*-acetylneuraminate/UDP-galactose is 15.6 times higher than the same ratio for Enzyme I, whereas the activity ratio UDP-galactose/deoxythymidine-5'-*p*-nitrophenyl phosphate is about the same for both enzyme preparations. So both nucleotide-sugar hydrolase I and II can hydrolyse non-specifically various nucleotide-sugars, with the exception of CMP-*N*-acetylneuraminate, which is a good substrate only for nucleotide-sugar hydrolase II.

When the enzyme preparations obtained by Sephacryl S-300 chromatography were subjected to SDS/polyacrylamide-gel electrophoresis, several protein bands were still visible (Figs. 2b, 2d and 2g). Further purification could be achieved by preparative electrophoresis in the presence of Sarkosyl (Elovson, 1980), but the recovery of the enzyme activities, and especially of Enzyme II, was very poor as a result of inactivation due to Sarkosyl. The properties of the enzymes as described below, therefore, were determined for the partially puri-

Fig. 1. 5'-AMP-AH-Sepharose 4B chromatography of the post-concanavalin A-Sepharose enzyme fraction. The nucleotide-sugar-hydrolysing enzyme activities were assayed with UDP-galactose (□), UDP-glucose (○), UDP-*N*-acetylglucosamine (Δ), deoxythymidine-5'-*p*-nitrophenyl phosphate (♦) and CMP-*N*-acetylneuraminate (●—●) in $\mu\text{mol/min per ml}$. Elution was performed with 0–0.8M-NaCl in standard buffer containing 1% Triton X-100 (●----●) at 40 ml/h. Fractions of volume 7 ml were collected.

Table 2. Segregation of nucleotide-sugar hydrolases I and II

The enzymes were partially purified from rat liver up to, and including, chromatography on Sephacryl S-300 as described in the Experimental section. The mean results for three preparations are given. In parentheses are given the activities per mg of protein relative to the liver homogenate. See Table 1 for abbreviations used.

Enzyme fraction	Substrate ...	Nucleotide-sugar hydrolase activity ($\mu\text{mol}/\text{min}$)			Ratio			Protein (mg)
		CMP-NeuAc	UDP-Gal	dTMP-pNP	100 $\times$ A/B	100 $\times$ A/C	100 $\times$ B/C	
		(A)	(B)	(C)				
Liver homogenate		320 (1)	705 (1)	1800 (1)	45.0	17.8	39.2	4800
Post-concanavalin A-Sepharose		57 (3.8)	428 (15)	1392 (17)	13.3	4.1	30.7	225
Enzyme I		2.8 (32)	178 (623)	476 (650)	1.6	0.6	37.3	2.0
Enzyme II		20.5 (78)	82 (141)	191 (129)	25.0	10.7	42.9	4.0

fied enzyme preparations I and II obtained by Sephacryl S-400 chromatography. 5'-CMP nucleotidase activity was not detectable in Enzyme I and showed a specific activity relative to the homogenate of 2.2 in Enzyme II. The specific activity of alkaline phosphatase relative to the homogenate in Enzyme I was 0.5, and in Enzyme II it was below the detectable level (cf. Table 1). So, in rat liver, non-specific nucleotide-sugar pyrophosphatase/phosphodiesterases are not identical with alkaline phosphatase, as was suggested by Hawley *et al.* (1983) for the enzyme activities present in human serum.

Further substantiation of the differences between nucleotide-sugar hydrolases I and II

(a) *Subunit molecular mass obtained by SDS/polyacrylamide-gel electrophoresis* (Fig. 2). Subunit molecular masses of Enzyme I and II were determined by SDS/polyacrylamide-gel electrophoresis, followed by blotting on nitrocellulose and detection of the inactive enzyme proteins with the enzyme immunoassay described in the Experimental section.

Most of the enzyme protein of Enzyme I was recovered at a molecular mass of approx. 125 kDa (Fig. 2a), whereas Enzyme II was recovered in the bottom of the 5.3% gel (Fig. 2c). Electrophoresis of Enzyme II on 13% gels indicated a subunit molecular mass of 50–55 kDa for the enzyme protein (Figs. 2e–2g). In the studies with Enzyme II the UDP-galactose hydrolase activity (Fig. 2e) appeared to migrate with the CMP-*N*-acetylneuraminase hydrolase activity (Fig. 2f). Similar results were obtained for Enzyme I and II when deoxythymidine-5'-*p*-nitrophenyl phosphate was used as a substrate in the enzyme immunoassays (not shown).

(b) *Sensitivity to trypsin treatment*. Treatment with trypsin [0.1 mg of trypsin/mg of protein; 0.05 M-Tris/HCl (pH 7.4)/5 mM-MgCl₂/0.1% (v/v) Triton X-100] did not affect the activity of Enzyme I (measured with UDP-galactose during an incu-

bation period of 4 h at 37°C. The activity of Enzyme II (measured with CMP-*N*-acetylneuraminase), however, was affected strongly by the action of trypsin within the first ½ h of incubation. Similar results were obtained for Enzymes I and II when deoxythymidine-5'-*p*-nitrophenyl phosphate was used as a substrate in the assays.

(c) *Sensitivity to SDS* (Fig. 3). The activity of Enzyme I decreased slowly with increasing SDS concentrations when CMP-*N*-acetylneuraminase (Fig. 3b, open symbols) or UDP-galactose (Fig. 3a, open symbols) were used as substrate; with deoxythymidine-5'-*p*-nitrophenyl phosphate as substrate, a more rapid decrease of Enzyme I activity was noticed (Fig. 3c, open symbols). In contrast, the activity of Enzyme II decreased very rapidly as SDS concentrations as low as 0.2–0.8 mg/ml, independent of the substrate used (Fig. 3, closed symbols).

(d) *Competition studies*. In Fig. 4, Dixon (1953) plots are given of the inhibition by CMP-*N*-acetylneuraminase and by UDP-galactose of the enzyme activities of nucleotide-sugar hydrolases I and II measured with deoxythymidine-5'-*p*-nitrophenyl phosphate. The type of inhibition shown by both nucleotide-sugars is best described as competitive, since the family of Dixon plots intersect above the horizontal axis, and replots of the slopes of the Dixon plots (Fig. 4, inset) fitted a straight line through the origin. Inhibition constants of about 0.2 mM were obtained for both compounds for Enzyme II, and, respectively, of 0.3 and 2 mM for UDP-galactose and CMP-*N*-acetylneuraminase for Enzyme I. The K_i values are close to the K_m values obtained by us for UDP-galactose and CMP-*N*-acetylneuraminase for Enzyme I (0.3 as against 1 mM) and Enzyme II (0.1 as against 0.2 mM). From these results it is strongly suggested that in both enzyme preparations only one enzyme is responsible for the hydrolysis of the various substrates. This was also apparent from studies with Enzyme II, in which UDP-galactose and CMP-*N*-acetylneuraminase were used as competing sub-

Fig. 2. Subunit molecular masses of the nucleotide-sugar hydrolase I and II obtained by SDS/polyacrylamide-gel electrophoresis

Samples of Enzyme I and II were submitted to electrophoresis in the presence of SDS and β -mercaptoethanol (Laemmli, 1970), followed by blotting of the protein patterns on nitrocellulose and detection of the inactive enzyme protein subunits by the enzyme immunoassay described in the Experimental section. 5.3%- (a-d) or 13%- (e-g) polyacrylamide gels were used. (a and f), activity patterns of Enzymes I (a) and II (f) obtained by enzyme immunoassay of the nitrocellulose blots with UDP-galactose as substrate. (c and e), activity patterns of Enzyme II obtained by enzyme immunoassay of the nitrocellulose blots with CMP-N-acetylneuramine as substrate from 5.3%- (c) and 13%- (e) polyacrylamide gels. (b, d and g), Coomassie Blue pattern of Enzyme I on 5.3%-polyacrylamide gels (b) and of Enzyme II on 5.3%- (d) and 13%- (g) polyacrylamide gels.

Fig. 3. Effect of various amounts of SDS on the activities of nucleotide-sugar hydrolases I and II with UDP-galactose (a), CMP-N-acetylneuramine (b) and deoxythymidine-5'-p-nitrophenyl phosphate (c) as substrate. Enzyme activities were measured after preincubation in the various SDS concentrations indicated in the Figure for 15 min at 37°C. ○, Nucleotide-sugar hydrolase I; ●, nucleotide-sugar hydrolase II.

Fig. 4. Dixon (1953) plots of the inhibition of the activities of Enzymes I and II with deoxythymidine-5'-*p*-nitrophenyl phosphate as substrate by UDP-galactose (a, b) and CMP-N-acetylneuraminic acid (c, d).

The incubation mixtures contained the indicated concentrations (mM) of deoxythymidine-5'-*p*-nitrophenyl phosphate (dTMP-pNP) alone or in the presence of various concentrations of UDP-galactose (UDP-Gal) or CMP-N-acetylneuraminic acid (CMP-NeuAc) indicated on the abscissa. Assays were performed by determination of v_0 as described for the assay of phosphodiesterase I (Bischoff *et al.*, 1975) in the presence of exogenously added alkaline phosphatase (0.125 unit; Boehringer-Mannheim, Grade I) to prevent inhibition by the phosphorylated products formed (Van Dijk *et al.*, 1981; Kean & Bighouse, 1974). The $1/v$ values on the ordinate are expressed as $1/(\mu\text{mol of } p\text{-nitrophenol/min per ml of enzyme preparation I or II})$. The lines were drawn by least-squares linear-regression analysis. The insets show replots of the slope of the lines obtained versus the reciprocal of the corresponding concentrations of deoxythymidine-5'-*p*-nitrophenyl phosphate.

strates (H. G. Muilerman, P. L. Koppen, G. J. M. Hooghwinkel & W. Van Dijk, unpublished work). With CMP-*N*-acetylneuraminate as inhibitor, a non-linear behaviour was found at higher concentrations both for Enzyme I and Enzyme II. This non-linearity is indicative of a mixed-type inhibition of this compound at higher concentrations (Dixon, 1953). Kean & Bighouse (1974) reported the same phenomenon for the inhibition by UDP-galactose of the enzymic hydrolysis of CMP-*N*-acetylneuraminate in rat liver plasma membranes.

Discussion

Evidence is presented here for the occurrence of two enzymes capable of hydrolysing nucleotide-sugars. From the pattern on 5'-AMP-AH-Sepharose-column chromatography (Fig. 1), it was concluded that both hydrolases (Enzyme I and Enzyme II) are characterized by a certain specificity towards the nucleotide-sugars tested. Nucleotide-sugar-hydrolysing Enzyme I is most probably identical with the non-specific nucleotide-sugar pyrophosphatase/phosphodiesterase (phosphodiesterase I) previously described by Evans *et al.* (1973), Bischoff *et al.* (1972, 1975) and others (Haugen & Skrede, 1977), since most of the activity with deoxythymidine-5'-*p*-nitrophenyl phosphate and with UDP-galactose as substrate was recovered in this fraction (Table 2 and Fig. 1). Nucleotide-sugar hydrolase II appears to be identical with CMP-sialic acid hydrolase described by ourselves (Van Dijk *et al.*, 1976, 1977, 1983) and others (Shoyab & Bachhawat, 1969; Kean & Bighouse, 1974), since almost all activity with CMP-*N*-acetylneuraminate as substrate was recovered in Enzyme II. So our results strongly suggest that a nucleotide-sugar-hydrolysing enzyme specific only for CMP-sialic acid (CMP-sialic acid hydrolase) is not distinctly separable, and that the CMP-sialic acid hydrolase activity described in the literature appears to belong to the so-far-undescribed non-specific nucleotide-sugar pyrophosphatase/phosphodiesterase, Enzyme II. However, it is also concluded from these results that CMP-*N*-acetylneuraminate can still be used to discriminate between the two non-specific enzymes, as Enzyme I exhibited a very low activity towards CMP-*N*-acetylneuraminate (the ratio of the activities obtained with CMP-*N*-acetylneuraminate and UDP-galactose as substrate is 15.6 times higher for Enzyme II compared with Enzyme I (Table 2). Further proof of the dissimilarity between these enzymes was obtained by (i) studying the tissue distribution of the enzymes in rat (Table 1), (ii) the subunit molecular masses (Fig. 2), (iii) the changes in activity as a function of the time-dependent treatment with trypsin, (iv) the changes in activity

brought about by the addition of SDS to the enzyme preparations (Fig. 3), and (v) the differences in K_i values for both enzymes obtained for UDP-galactose and CMP-*N*-acetylneuraminate (Fig. 4). The native enzyme preparations showed apparent molecular masses of 550 kDa (see the Results section). The subunit molecular mass, however, was different for both enzymes. Most enzyme protein of nucleotide-sugar hydrolase I was recovered at about 125 kDa and of nucleotide-sugar hydrolase II at 50–55 kDa on SDS/polyacrylamide gels (Fig. 2). The existence of lower-molecular-mass forms of phosphodiesterase I in SDS-containing gels was also mentioned by Tran-Thi *et al.* (1981) and by Schneider *et al.* (1982).

Besides variations in the activities of nucleotide-sugar hydrolases I and II in various rat tissues (Table 1), physiological differences in their activities were observed in normal and in regenerating rat liver (Van Dijk *et al.*, 1977, 1983). In those reports, Enzyme I was referred to as 'phosphodiesterase I' and Enzyme II as 'CMP-*N*-acetylneuraminate hydrolase'. Moreover, we had shown that, in rat liver, the activities of Enzyme I and II were distributed unevenly over the hepatocyte surface (Van Dijk *et al.*, 1977). In those studies, deoxythymidine-5'-*p*-nitrophenyl phosphate was used as substrate for the detection of phosphodiesterase I activity. Since this compound is also a good substrate for nucleotide-sugar hydrolase II, the values reported for the phosphodiesterase I activity measured in tissue homogenates are too high. In future studies, correction should be made for the contribution of the activity of nucleotide-sugar hydrolase II present in the same homogenate by using CMP-*N*-acetylneuraminate as substrate, since more than 90% of the activity with this substrate appears to reside in Enzyme II (Table 2 and Fig. 1).

Although most activity of the enzymes appears to be present in the plasma membrane of the liver cells (Kean & Bighouse, 1974; Van Dijk *et al.*, 1976; Bischoff *et al.*, 1975), other reports have appeared showing the presence of Enzymes I or II in the endoplasmic reticulum (Bischoff *et al.*, 1975; Tran-Thi *et al.*, 1981) or in Golgi membranes (Munro *et al.*, 1975). Varki & Kornfeld (1981) have isolated an enzyme from a smooth-membrane fraction of rat liver that is capable of cleaving the outer phosphodiesterase-linked α -*N*-acetylglucosamine residues present in the high-mannose oligosaccharides on newly synthesized lysosomal enzyme. The purification scheme used by Varki & Kornfeld (1981) corresponds remarkably to the procedure used by us for the preparation of the nucleotide-sugar hydrolases from total rat liver membranes described both here and in a preliminary form (Muilerman *et al.*, 1981), resulting also in an

enzyme preparation with a high molecular mass (500 kDa) in Triton X-100. In addition, the enzyme preparation has been shown to be a nucleotide-sugar hydrolase too, since, at pH 9.0, UDP-*N*-acetylglucosamine appeared to be a substrate (Varki & Kornfeld, 1981). Identity of α -*N*-acetylglucosamine phosphodiesterase with one of the nucleotide-sugar hydrolases studied by us is strongly suggested by these results. Fairly recently Varki *et al.* (1983) have reported that the enzyme cleaves the C–O bond rather than the O–P bond, and therefore acts by a glycosidase-type mechanism. However, the characterization was carried out at pH 5 and not at pH 9, which appears to be the optimum for the hydrolysis of UDP-*N*-acetylglucosamine by this enzyme (Varki & Kornfeld, 1981; Waheed *et al.*, 1981) and also for the nucleotide-sugar hydrolases studied by us (Evans *et al.*, 1973; Kean & Bighouse, 1974; Van Dijk *et al.*, 1976). At pH 9 a pyrophosphatase/phosphodiesterase-type mechanism for nucleotide-sugar hydrolases I and II was obtained by us when the products of the enzymic hydrolysis of UDP-*N*-acetylglucosamine, UDP-galactose and CMP-*N*-acetylneuraminate at this pH were analysed by high-pressure liquid chromatography (H. G. Muilerman, P. L. Koppen, G. J. M. Hooghwinkel & W. Van Dijk, unpublished work). Identity of nucleotide-sugar hydrolases I and II with the enzyme studied by (Varki & Kornfeld, 1981; Varki *et al.*, 1983) therefore supposes a pH-dependent variation in the enzymic reaction mechanisms. This possibility awaits further study.

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