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CMP-*N*-ACETYLNEURAMINIC ACID HYDROLASE, AN ECTOENZYME DISTRIBUTED UNEVENLY OVER THE HEPATOCYTE SURFACE

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Summary

The regional localization of CMP-*N*-acetylneuramic acid hydrolase at the hepatocyte surface was studied by using plasma membranes and hepatocytes isolated from rat liver.

1. By homogenization of the rat liver plasma membrane preparations and subsequent discontinuous sucrose gradient centrifugation, one light and two heavy membrane fractions were obtained. The origin of these three subfractions is discussed based on the specific activities in the three fractions of 5′-nucleotidase, alkaline phosphatase and Mg^{2+}-ATPase and on electron microscopic examination of the fractions. Evidence is given suggesting that the light fraction is derived from the bile canalicular surface of the plasma membrane, and that the heavy fractions are derived predominantly from the sinusoidal and lateral surfaces of the liver cell membrane. CMP-AcNeu hydrolase was present at highest specific activity in one of the heavy subfractions. Therefore it is concluded that CMP-AcNeu hydrolase is located preferentially in the sinusoidal and/or lateral plasma membrane parts of the liver cell.

2. Experiments with intact and disintegrated hepatocytes isolated from rat liver indicated that CMP-AcNeu hydrolase is located at the surface of the cell membrane, with its functional group directed to the outside.

Introduction

Hydrolases of nucleotide sugars, the activated substrates for the glycosylation reactions of glycoproteins and glycolipids, are reported to be localized predominantly in cellular plasma membranes [1—5]. Little is known about the function of these enzymes in cellular metabolism, although, seen in the light of

Abbreviations: AcNeu, *N*-acetylneuraminic acid; INT, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride.

the accumulating evidence on the occurrence of glycosyltransferases at the cellular surface [6], one can speculate about an involvement of the hydrolases in the regulation of ectocellular glycoconjugate metabolism. To gain insight in the role these hydrolases can play in cell-surface phenomena, it is of importance to know in which differentiated area and at what side of the plasma membrane the hydrolases exert their function. Thus far, for one hydrolase, UDPgalactose pyrophosphatase, a localization at the external side of the plasma membrane is reported [4].

For CMP-AcNeu hydrolase, a phosphodiesterase, a plasma membrane localization was established by Kean and Bighouse [1] for rat liver and by our group [5] for the cortex of calf kidney. We now report on the regional plasma membrane localization of rat liver CMP-AcNeu hydrolase.

We have investigated this by (a) comparing the distribution of CMP-AcNeu hydrolase in "light" and "heavy" subfractions of isolated rat liver plasma membrane, with the distribution of established plasma membrane enzymes, and (b) comparing the CMP-AcNeu hydrolase activity of disintegrated and intact hepatocytes isolated from rat liver, assuming that CMP-AcNeu can not pass the intact cell membrane [7,8].

Materials and Methods

Materials

CMP-[*Ac*-^{14}C]AcNeu and non-radioactive CMP-AcNeu were prepared as described previously [9]. All other chemicals were obtained from commercial sources and were of analytical grade.

Preparation of rat liver plasma membrane and its subfractions

Rat livers were obtained from male Wistar rats, weighing about 200 g, which which were starved for 24 h and than killed by decapitation. Plasma membrane enriched fractions of rat livers were prepared by the method of Emmelot et al. [10], using 5 livers for each preparation. For the determination of enzyme activities the final membrane preparation was suspended in 1 mM Tris · HCl buffer (pH 8) and was stored at −20°C until use.

For the preparation of plasma membrane subfractions the final membrane preparation obtained by the method of Emmelot et al. was suspended in 2 ml 8.6% (w/v) sucrose/5 mM Tris · HCl buffer (pH 7.4). In accordance with the method described by Toda et al. [11], the plasma membrane suspension was homogenized in a glass-Teflon homogenizer by 10 up-and-down strokes of the tightly fitting Teflon pestle, rotating at 1400 rev./min. To the homogenized plasma membranes was added 21 ml 45% (w/v) sucrose/5 mM Tris · HCl buffer (pH 7.4). This suspension was divided over three 23-ml centrifuge tubes and overlayered successively with 8 ml 38% (w/v) sucrose/5 mM Tris · HCl buffer (pH 7.4). The tubes were centrifuges for 90 min at 77 500 × g_{av} in a MSE 3 × 23 rotor. This resulted in a narrow band (B_1) at the upper interface, a diffuse band (B_2) at the lower interface and a pellet (P). Fractions B_1, B_2 and P were washed twice by means of dispersion and centrifugation at 100 000 × *g* for 60 min in 1 mM Tris · HCl buffer (pH 8), and were stored frozen in the same buffer at −20°C until use.

Isolation of rat liver hepatocytes and incubation conditions for the assay of CMP-AcNeu hydrolase activity

The isolation of rat liver hepatocytes was performed by a modification of the method of Berry and Friend [12], according to Johnson et al. [13] using a technique described by Williamson et al. [14]. At zero time 95% and after 60 min incubation 85% of the hepatocytes appeared to be viable as determined by Trypan Blue exclusion and lactate dehydrogenase activity leakage by the isolated hepatocytes. The hepatocytes obtained from one liver, were suspended in 0.5 mM Ca^{2+}-Krebs solution (pH 7.4). Two experiments were performed using different hepatocytes preparations.

Immediately after isolation of the hepatocytes, the suspension was divided in two equal parts. Part A was incubated as intact cells and Part B was homogenized prior to incubation by means of ultrasonic treatment for three 30-s periods. Varying amounts of cell were incubated in 5 mM Ca^{2+}-Krebs solution (pH 7.4), containing 1.5 mM CMP-[^{14}C]AcNeu (0.68 Ci/mol) in a total volume of 1 ml in conical incubation tubes. Incubation was performed for 30 or 60 min (see legend to Fig. 2) at 37°C under continuous shaking in a atmosphere of O_2 : CO_2 (95 : 5, v/v). The reaction was stopped by the addition of 0.2 ml 1 M EDTA. After the addition of 1.2 ml 96% ethanol (—20°C) the tubes were centrifuged at 1000 × *g* for 15 min. The pellets were resuspended in 2 ml 50% ethanol (—20°C) and centrifuged as above; this washing step was repeated one more time. In aliquots of the combined supernatants the radioactive amounts of [^{14}C]AcNeu and CMP-[^{14}C]AcNeu were estimated after chromatographic separation of the compounds. For Expt. 1 chromatography was carried out using a Dowex Ag 1-X8 column (0.8 × 2 cm) as described previously for Method B, for the assay of CMP-AcNeu hydrolase activity [5], except that the column was washed first with 5 ml water to eluate the high molecular weight bound [^{14}C]AcNeu, that might be incorporated by sialyltransferase(s) in endogenous acceptors. Not more than 0.001 of the total radioactivity, corresponding to 1.5 nmol AcNeu, appeared to be high molecular weight bound. In Expt. II [^{14}C]AcNeu and CMP-[^{14}C]AcNeu were separated by paper chromatography on Whatman 3 MM paper. Elution with 95% ethanol 0.6 M NH_4OH (7 : 3, v/v) as a solvent mixture and the estimation of the radioactivities were performed as described previously for Method C for the assay of CMP-AcNeu hydrolase activity [5]. The values obtained were corrected for the temperature-dependent hydrolysis of CMP-[^{14}C]AcNeu, which was determined by running control samples containing no hepatocytes under the same conditions.

Other methods

In other than hepatocytes preparations CMP-AcNeu hydrolase activity was assayed using the $NaBH_4$-thiobarbituric acid assay as described previously (Method A) [5], except that each sample was incubated for 5, 10 and 15 min. The assay of other enzyme activities and the determination of protein was performed as described previously [5].

For the electron microscopic examination of the plasma membrane preparations the samples were fixed in Karnovsky's solution [15] and post-fixed in 1% OsO_4. After dehydration in a series of alcohols through propylene oxide, the samples were embedded in Epon-Araldite. Utrathin sections, stained with

TABLE I

ACTIVITIES OF CMP-AcNeu HYDROLASE AND MARKER ENZYMES IN PLASMA MEMBRANE FRACTIONS OF RAT LIVER

The plasma membrane fractions were prepared as described in Materials and Methods. Unless otherwise indicated the values are the means ±S.D. of the results of three independent experiments. Phosphodiesterase I was assayed using p-nitrophenyl thymidine 5′-phosphate as a substrate [5].

Enzyme	Enzyme activity (μmol/h/g liver)				
	Homogenate	Plasma membrane *	Plasma membrane subtractions *		
			B_1	B_2	P
(1)	(2)	(3)	(4)	(5)	(6)
CMP-AcNeu hydrolase	19.1 ± 3.0	1.10 ± 0.18	0.14 ± 0.02	0.34 ± 0.04	0.71 ± 0.09
Phosphodiesterase I	2851 ± 217	269 ± 59	84.9 ± 7.4	136.2 ± 6.2	136.2 ± 13.8
Alkaline phosphatase	46.9 ± 1.7	—	0.65 ± 0.18	0.30 ± 0.09	0.66 ± 0.11
Mg^{2+}-ATPase	494 ± 20	16.1 ± 4.1	12.3 ± 1.0	5.6 ± 1.2	14.6 ± 1.0
5′-Nucleotidase	748 ± 47	24.2 ± 5.2	13.5 **	7.4 **	9.8 **
Succinate-INT dehydrogenase	61 **	0.07 ± 0.03	—	—	—
β-Galactosidase	19.6 ***	0.03 ***	—	—	—
α_1-Acid glycoprotein sialyl-transferase	3.05 **	0.002 **	—	—	—
Glucose-6-phosphatase	368 **	0.26 ± 0.03	—	—	—
Protein †	110.6 ± 7.4	0.115 ± 0.026	0.023 ± 0.003	0.042 ± 0.007	0.156 ± 0.02

* For the preparation of the subfractions and for the chemical and enzymatic assays in the intact plasma membrane fraction, different plasma membrane preparations were used. Since the former plasma membranes preparations showed to have a higher protein content than the latter, direct comparison between specific enzyme activities in the subfractions and in the whole plasma membrane preparation is not allowed.

** Mean of two experiments.

*** One single experiment.

† Expressed as mg/g liver.

uranyl acetate and lead citrate, were examined with a Philips 301 electron microscope operating at 60 kV.

Results and Discussion

Activity of CMP-AcNeu hydrolase in rat liver plasma membranes

Plasma membranes of rat liver were prepared by the method of Emmelot et al. [10], starting from the nuclear fraction. The purity of the preparations was checked by measuring the activities of several marker enzymes. The results are shown in Table I, columns 3 and 8. The established plasma membrane enzymes were enriched highly in specific activity relative to the respective values in the rat liver homogenate: phosphodiesterase I, 90 times; Mg^{2+}-ATPase, 30 times; and 5′-nucleotidase, 27 times. The recoveries from the homogenate of these enzymes in the membrane preparations were between 3 and 9%. The specific activities in the plasma membrane fraction of enzymes representative for mitochondria, lysosomes, Golgi complex, and endoplasmatic reticulum (succinate-INT reductase, β-galactosidase, α_1-acid glycoprotein sialyltransferase and

Specific enzyme activity (μmol/h/mg protein)						
Homogenate	Plasma membrane *	Plasma membrane subfractions *				
		B_1	B_2	P	Ratio B_2/B_1	Ratio P/B_1
(7)	(8)	(9)	(10)	(11)	(12)	(13)
0.17 ± 0.03	9.7 ± 0.6	6.3 ± 0.2	8.2 ± 2.8	4.6 ± 0.1	1.30	0.73
25.78 ± 0.75	2342 ± 109	3756 ± 276	1400 ± 119	873 ± 36	0.37	0.23
0.42 ± 0.02	—	28.8 ± 10.1	7.2 ± 1.5	4.3 ± 0.2	0.25	0.15
4.5 ± 0.2	138 ± 15	546 ± 35	133 ± 12	94 ± 8	0.24	0.17
7.6 ± 1.0	211 ± 16	597 **	174 **	63 **	0.29	0.11
0.68 **	0.63 ± 0.26	—	—	—	—	—
0.17 ***	0.15 ***	—	—	—	—	—
0.027 **	0.017 **	—	—	—	—	—
4.1 **	2.3 ± 0.3	—	—	—	—	—
—	—	—	—	—	—	—

glucose-6-phosphatase, respectively) were decreased 0.5—0.9 times and the recoveries of the enzyme activities were very low, 0.05—0.15%. These results indicate that the plasma membrane preparations obtained were only slightly contaminated with other subcellular material *. Electron microscopic observation of the plasma membranes confirmed this conclusion. As is shown in Fig. 1A, the plasma membrane preparations were composed of sheets of membranes frequently connected by desmosomes and tight junctions.

In the plasma membrane preparations the enrichment in specific activity (57 times) and the recovery from the homogenate of CMP-AcNeu hydrolase activity (5.7%) are of the same order as those obtained for the plasma membrane marker enzymes phosphodiesterase I, Mg^{2+}-ATPase and 5'-nucleotidase.

* It is reported by Emmelot et al. [10] that by using their method for the isolation of plasma membranes, these membranes are obtained at low yield but at high purity, showing a preparation representative for the liver plasma membrane, save perhaps some of the blood front linings. The low yield of the method is due to incomplete homogenization of the tissue (in order not to fragment the membrane sheets to much), entrapment of membrane fragments in the nuclear pellets and loss of membrane fragments in the supernatants.

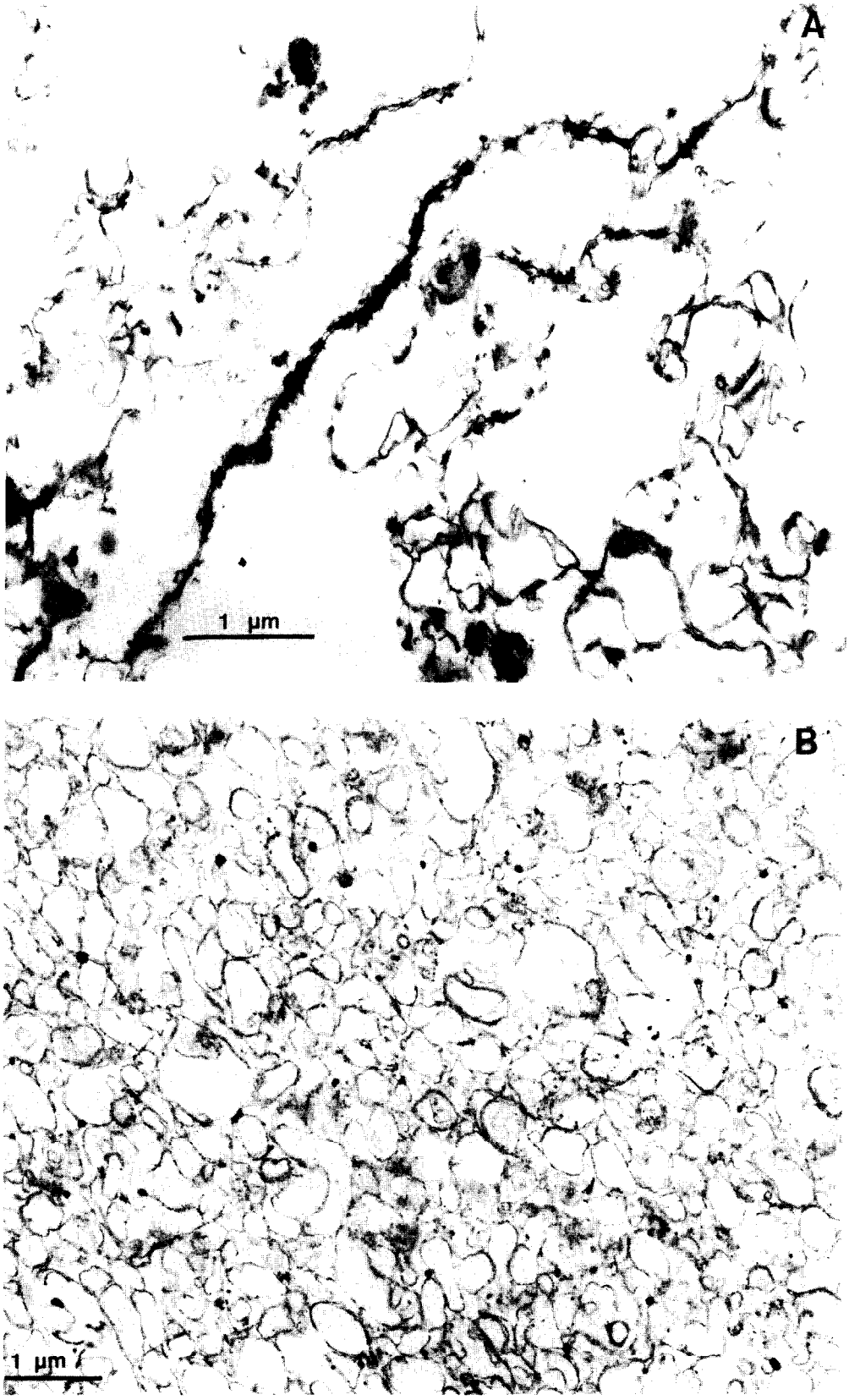

Fig. 1. For legend, see opposite page.

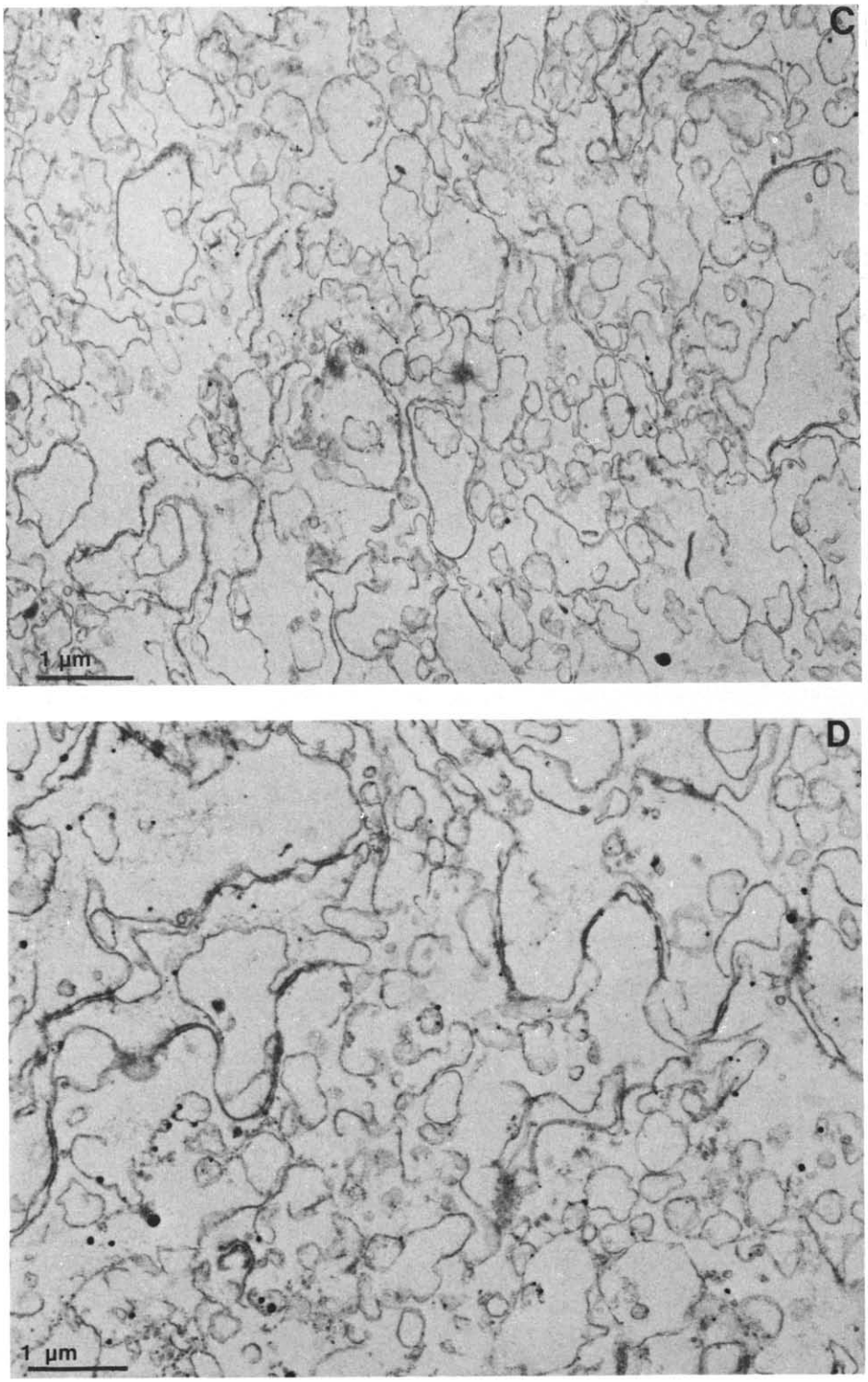

Fig. 1. Electron micrographs of a plasma membrane preparation of rat liver and its subfractions B_1, B_2 and P. (A) Plasma membranes obtained by the method of Emmelot er al. [10], magnification 19 000×. (B) Subfraction B_1, showing membrane vesicles of various sizes, magnification 14 300×. (C) Subfraction B_2, composed of sheets of membranes, occasionally interconneted and membrane vesicles, magnification 14 300×. (D) Subfraction P, composed predominantly of large membrane fragments, often interconnected, and some membrane vesicles, magnification 14 300×.

From this it is concluded that CMP-AcNeu hydrolase is localized in the plasma membranes of the rat liver cells. Our findings confirm the results of Kean and Bighouse [1] concerning a plasma membrane localization of CMP-AcNeu hydrolase in rat liver.

Distribution of CMP-AcNeu hydrolase activity over the subfractions of the rat liver plasma membrane

The liver cell possesses a cell membrane that can be divided into three distinct areas, the blood sinusoidal surface, the bile canalicular surface and the lateral surface contiguous to the neighbouring cell, containing the junctional complexes. It is possible to fractionate the isolated liver cell membrane into two or three subfractions, differing in density, morphology and enzymatic composition [11,16,17]. It was shown by Evans and co-workers [16,17] and by Toda et al. [11], that the lighter fraction, which is vesicular in nature, is derived most probably from the bile canalicular surface of the hepatocyte, whereas the heavier fraction(s) is derived from the sinusoidal and lateral surfaces of the cell.

In order to obtain information about the sub-plasma membrane localization of CMP-AcNeu hydrolase, we have sub-fractionated our plasma membrane prepation using the method of Toda et al. [11] (see Materials and Methods). In agreement with their results we obtained two membrane fractions, B_1 and B_2, which banded, respectively, at the upper and lower interfaces of the discontinuous sucrose gradient. In addition, however, we obtained a pellet. This discrepancy from the results of Toda et al. can be explained by the fact that different methods were used for the preparation of total plasma membranes. This is supported by the results of Wisher and Evans [17], who, starting also from a plasma membrane preparation isolated according to Emmelot et al., on fractionation obtained one light and two heavy plasma membrane subfractions. Fig. 1B shows that Fraction B_1 is composed of membrane vesicles of various sizes whereas fractions B_2 and P (Figs. 1C and 1D) are composed of sheets of membranes, occasionally interconnected, and membrane vesicles.

Analysis of our subfractions for the activity of the plasma membrane enzymes, showed that in the light fraction, B_1, the specific activities of phosphodiesterase I, alkaline phosphatase, Mg^{2+}-ATPase and 5′-nucleotidase were several times higher than in the two heavy plasma membrane subfractions, B_2 and P (see Table I). This is clearly shown by the ratios of the specific enzyme activities of the various fractions, as given in the last two columns of Table I. Histochemical studies have indicated that alkaline phosphatase, Mg^{2+}-ATPase and 5′-nucleotidase stain preferentially the bile canalicular surface of the hepatocyte [18,19]. Since the light subfraction, B_1, possesses the highest specific activities of these enzymes, it seems reasonable to conclude that this fraction is enriched in membranes originating from the bile front of the liver cell membrane. This is in agreement, with conclusions drawn by Evans [16] and Toda et al. [11]. They reported 3—7 times elevated specific activities of alkaline phosphatase, Mg^{2+}-ATPase and 5′-nucleotidase in the light membrane subfraction (comparable with our Fraction B_1) over the heavy subfraction(s) (our Fractions B_2 and P), whereas the distribution of protein over the various subfractions also is compatible with our results. Based on the higher insulin-bind-

ing activity [17] and the higher specific adenylate cyclase activity [11] in their subfraction(s), Evans and Toda concluded that these fractions were enriched in membrane fragments derived from the sinusoidal and lateral surfaces of the cell. Although we were not able to measure these two activities, the similarities in enzymatic distribution over and in electron microscopic appearance of the fractions with the results of Evans and co-workers and Toda et al., do suggest that our heavy membrane Fractions B_2 and P are derived also predominantly from the sinusoidal and lateral surfaces of the liver cell.

The distribution of CMP-AcNeu hydrolase activity over the various plasma membrane subfractions, differs markedly from the distribution of the other enzyme activities. It is shown in Table I that, contrary to the results obtained with the four plasma membrane enzymes, the highest specific CMP-AcNeu hydrolase activity is measured in Fraction B_2. It does not seem likely that specific inactivation of CMP-AcNeu hydrolase during subfractionation is responsible for this different distribution, since (1) both CMP-AcNeu hydrolase and phosphodiesterase I are recovered to the same extent in the subfractions with respect to the whole plasma membrane fraction, and no reports are available in literature about inactivation of phosphodiesterase I during subfractionation, and (2) no inactivation of CMP-AcNeu hydrolase activity was detectable in total plasma membrane preparations and its subfractions after storage at various sucrose concentrations at 0—4°C for several days (unpublished results). The different distribution of CMP-AcNeu hydrolase with respect to the other plasma membrane enzymes suggest that CMP-AcNeu hydrolase is localized preferentially at the sinusoidal and/or lateral parts of the cell membrane, and most probably not at the bile canalicular part of the cell membrane of the hepatocytes. This result is in good agreement with our observation regarding the plasma membrane localization of CMP-AcNeu hydrolase in the cortex of calf kidney [5]. In these cells CMP-AcNeu hydrolase appeared to be localized preferentially at the brush border side of the tubular cell membrane, which faces the lumen of the tubule.

A second important observation is that CMP-AcNeu hydrolase and phosphodiesterase I appear to be distributed in a different way over the liver cell membrane fractions (Table I). Being a phosphodiesterase too, the question has been discussed [1,5] whether CMP-AcNeu hydrolase is a specific phosphodiesterase or not. The apparent different localization of CMP-AcNeu hydrolase and phosphodiesterase I in the rat liver plasma membrane, seems to be a further argument in favour of the specificity of CMP-AcNeu hydrolase.

Activity of CMP-AcNeu hydrolase in intact and disintegrated hepatocytes isolated from rat liver

To answer the question whether CMP-AcNeu hydrolase exerts its action at the internal or at the external side of the plasma membrane, intact and disintegrated hepatocytes, isolated from rat liver, were incubated in the presence of CMP-[^{14}C] AcNeu as described in Materials and Methods. In Fig. 2 the results of two experiments are presented using different hepatocytes preparations for each experiment and two incubation times, namely 30 min for Expt. II (Curves a and b) and 60 min for Expt. I (Curves a and d). Curves b and c (full lines) represent the CMP-AcNeu hydrolase activity of the intact hepatocytes prepara-

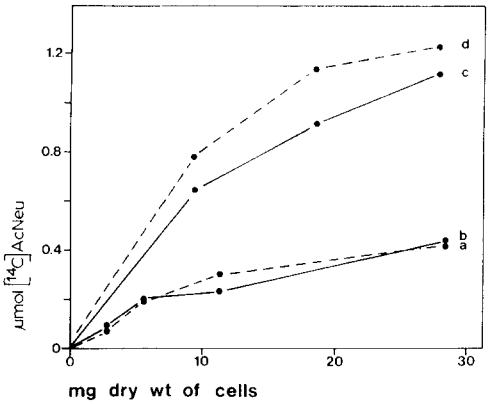

Fig. 2. CMP-AcNeu hydrolase activity in intact and in desintegrated isolated hepatocytes. Isolated hepatocytes were prepared from rat liver as described in Materials and Methods. The hepatocytes suspension was divided in two equal parts. One part was incubated as intact cells and the other part after disintegration by ultrasonic treatment. The results of two experiments are given using different hepatocytes preparations and different incubation times. in Expt. II (curves a and b) the cells were incubated for 30 min; in Expt. I (curves c and d) the cells were incubated for 60 min (see Materials and Methods for further details). The CMP-AcNeu hydrolase activity of the intact cells is given by the full lines (b and c) and the hydrolase activity of the disintegrated cells is given by the broken lines (a and d).

tions and Curves a and d (broken lines) the enzyme activity of the disintegrated cells. From the increasing production of [^{14}C]AcNeu when higher amounts of cells are incubated and when the incubation time is doubled, it appears that we are dealing with an enzymatic hydrolysis of CMP-AcNeu. With disintegrated hepatocytes the enzyme activity is the same (Curve a) or somewhat higher (Curve d) than with intact hepatocytes (Curves b and c, respectively). There is evidence that nucleotide sugars can not pass the intact cell membrane [7,8]. Consequently, the enzymatic hydrolysis of CMP-AcNeu obtained with the intact cells, must be the result of the action of CMP-AcNeu hydrolase present at the surface of the cell membrane. The small differences in CMP-AcNeu hydrolase activity between the intact and the disintegrated hepatocytes might be due to some activity present in the endoplasmic reticulum of the cell or simply by activation of the enzyme as a result of ultrasonic treatment of the cells [5]. So it appears that in rat liver plasma membranes CMP-AcNeu hydrolase is an ectoenzyme, present at the outside of the cellular membrane.

General conclusions

The result presented in this report strongly suggests that CMP-AcNeu hydrolase in rat liver is localized at the sinusoidal and/or lateral surfaces of the hepatocytes, with its active site directed to the outside. An external localization was reported also for another nucleotide sugar hydrolase, namely UDPgalactose pyrophosphatase [4]. The same author has reported that this enzyme is a glycoprotein showing dual specificity towards nucleotide pyrophosphate and phosphodiester bonds [3]. Our results concerning the different distributions of phosphodiesterase I and CMP-AcNeu hydrolase activities over the plasma membrane subfractions, do suggest that CMP-AcNeu is not hydrolized by the non-specific phosphodiesterase/nucleotide pyrophosphatase complex. Definite

proof, however, must await further investigations. The function of CMP-AcNeu hydrolase in cellular metabolism is not known, although a number of possible functions have been discussed by us [5] and by Kean and Bighouse [1]. However, the fact that CMP-AcNeu hydrolase of rat liver appears to be an ecto-enzyme that is distributed not evenly over the plasma membrane, as was shown also for the hydrolase of calf kidney cortex [5], might add a new dimension of future investigations towards the role of CMP-AcNeu hydrolase in cellular metabolism.

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