Analytical Study of Microsomes and Isolated Subcellular Membranes from Rat Liver. IX. Nicotinamide Adenine Dinucleotide Glycohydrolase: A Plasma Membrane Enzyme Prominently Found in Kupffer Cells

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ABSTRACT The distribution of nicotinamide adenine dinucleotide (NAD) glycohydrolase in rat liver was investigated by subcellular fractionation and by isolation of hepatocytes and sinusoidal cells. The behavior of NAD glycohydrolase in subcellular fractionation was peculiar because, although the enzyme was mainly microsomal, plasma membrane preparations contained distinctly more NAD glycohydrolase than could be accounted for by their content in elements derived from the endoplasmic reticulum or the Golgi complex identified by glucose-6-phosphatase and galactosyltransferase, respectively. When microsomal and plasmalemmal preparations were brought to equilibrium in a linear-density gradient, NAD glycohydrolase differed from these enzymes and behaved like 5'-nucleotidase and alkaline phosphodiesterase I. NAD glycohydrolase was markedly displaced towards higher densities after treatment with digitonin. This behavior in density-gradient centrifugation strongly suggests that NAD glycohydrolase is an exclusive enzyme of the plasma membrane. NAD glycohydrolase differed clearly from other plasmalemmal enzymes when the liver was fractionated into hepatocytes and sinusoidal cells; its specific activity was considerably greater in sinusoidal cell than in hepatocyte preparations. Further subfractionation of sinusoidal cell preparations into endothelial and Kupffer cells by counterflow elutriation showed that NAD glycohydrolase is more active in Kupffer cells. We estimate that the specific activity of NAD glycohydrolase activity is at least 65-fold higher at the periphery of Kupffer cells than at the periphery of hepatocytes. As the enzyme shows no structure-linked latency and is an exclusive constituent of the plasma membranes, we conclude that it is an ectoenzyme that cannot lead to a rapid turnover of the cytosolic pyridine nucleotides.

The nicotinamide adenine dinucleotide (NAD)[1] glycohydrolase reaction appears barely consistent with the metabolic role of the pyridine nucleotides, particularly in the liver. Indeed, the high activity of NAD glycohydrolase in hepatocytes should result in a rapid turnover of the pyridine coenzymes through the cycle proposed by Gholson (26). The resynthesis of NAD from nicotinamide requires three ATP molecules and expends

[1] *Abbreviations used in this paper*: NAD, nicotinamide adenine dinucleotide; NPC_1 and NPC_2, nonparenchymal cells; PC, parenchymal cells.

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five high-energy phosphate bonds. So far there has been no identification of any cellular function that could justify the large expenditure of energy due to the rapid turnover of an NAD pool.

Early studies by Alivisatos and Denstedt (1) showed that NAD glycohydrolase is located on the surface of blood cells. In order to explain the rapid destruction of endogenous pyridine nucleotides after grinding cells, the authors suggested that NAD glycohydrolase is an ectoenzyme in all tissues. The observation that NAD glycohydrolase is fully active, or sensitive to nonpenetrating inhibitors in undisrupted Ehrlich ascite cells (14, 17), peritoneal macrophages (7), and splenic cells (7, 31, 32) is in agreement with this surmise.

However, at present it is thought that the subcellular distribution of the enzyme in liver cells is more complex. It was first found that NAD glycohydrolase is concentrated in the microsome fraction of the rat, mouse, hamster, rabbit, and pigeon liver (27). This distribution was confirmed by Nakazawa et al. (35), who showed that the nuclear polyADPribose synthesis contributes only weakly to the breakdown of NAD in rat liver homogenates, and concluded that the authentic NAD glycohydrolase is localized essentially in the endoplasmic reticulum membrane. Subsequently, Bock et al. (19) found that various membrane preparations, including rough and smooth microsomes and heavy and light plasma membrane fractions, demonstrate a distinctly higher specific activity of NAD glycohydrolase than do whole liver homogenates; they came to the conclusion that NAD glycohydrolase is present in the endoplasmic reticulum and the plasmalemmal membrane of the hepatocytes.

This dual localization of NAD glycohydrolase in liver is puzzling in several respects. The presence of NAD glycohydrolase in the endoplasmic reticulum implies a rapid turnover of the cytosolic pyridine coenzymes, as recalled above, if no compartmental hindrance to their breakdown is present. In addition, except for minor differences in carbohydrate composition, comparative studies on the plasmalemmal and microsomal NAD glycohydrolase from rat liver did not reveal noticeable differences in their biochemical (24) and immunological (18) properties, and in their half-lives (19). Finally, there is at present no clear-cut example of an enzyme being a constituent of the endoplasmic reticulum and of plasma membranes in liver cells (11, 47).

In this work, we have examined the distribution of NAD glycohydrolase in the rat liver at the cellular and subcellular levels. Our results show that the enzyme is present only in the plasma membranes, although its behavior in differential centrifugation is very similar to that of authentic enzymes of the endoplasmic reticulum. In fact, the enzyme is largely associated with sinusoidal cells, mainly Kupffer cells. Some of these results have been presented in abstract form (3, 10, 37).

MATERIALS AND METHODS

Products were obtained from the following sources: [*carbonyl*-^{14}C]nicotinamide adenine dinucleotide (NAD), ammonium salt (sp act 59 Ci/mol), The Radiochemical Centre Ltd. (Amersham, England); collagenase type I, Triton X-100, and chromatographic alumina, neutral type WN-3, grade I, Sigma Chemical Co. (St. Louis, Mo); pronase, B grade, Calbiochem-Behring Corp. (San Diego, CA); Metrizamide, Nyegaard and Co., A/S (Oslo, Norway); Biofluor, New England Nuclear (Boston, MA).

Subcellular Fractionation of Rat Liver: Livers from fasted female rats of the Wistar strain were perfused through the portal vein (2) and homogenized (4). Differential centrifugation of the homogenate gave the nuclear fraction and the cytoplasmic extract, which was further fractionated into large granules, microsomes, and final supernate (4). Microsomes were subfractionated by isopycnic centrifugation in the E-40 rotor (9) through the sucrose gradient without previous treatment (13), or after treatment with digitonin (5). Plasma membranes were prepared by flotation of low-speed sediments through sucrose layers (38) and were analyzed by density equilibration in a gradient of sucrose directly, or after treatment with digitonin (47).

Isolation of Hepatocytes and Sinusoidal Cells: Three cell preparations, designated parenchymal, nonparenchymal$_1$, and nonparenchymal$_2$ (PC, NPC$_1$, and NPC$_2$), were obtained as described by van Berkel (42) using male Wistar rats. This method (Fig. 1) combines the procedures developed by Berry and Friend (15), and by Mills and Zucker-Franklin (30), to isolate hepatocytes and sinusoidal cells, respectively. In short, the liver was perfused at 37°C for 8 min with Hanks' balanced salt solution. A small lobe (≈70 mg) was tied off and removed to determine the specific activities of enzymes in the whole tissue. Perfusion was then continued for 10 min, using the same solution supplemented with 0.05% collagenase. The liver was excised,

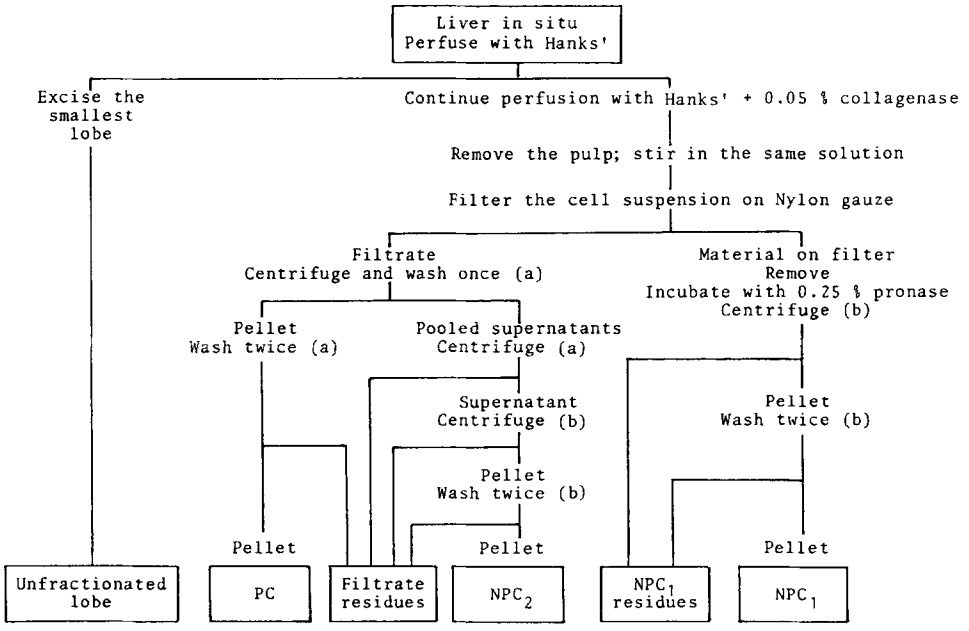

FIGURE 1 Flow diagram for the isolation of PC, NPC$_1$, and NPC$_2$ cell preparations. Adapted from van Berkel (42). Centrifugations were carried out for 30 s at 50 *g* (*a*) or for 5 min at 400 *g* (*b*).

cut into pieces, and incubated for 15 min at 37°C in Hanks' buffer with 0.05% collagenase. The cell suspension was filtered through nylon gauze. The filtrate was fractionated by differential pelleting giving the PC preparation enriched in hepatocytes (30 s at 50 *g*), the NPC_2 preparation enriched in sinusoidal cells (5 min at 400 *g*), and residues which were saved (filtrate residues) to establish the enzyme distributions quantitatively. The material on the filter was removed and incubated for 1 h at 37°C in Hanks' balanced salt solution which contained 0.25% pronase. Sinusoidal cells (NPC_1 preparation) were recovered by centrifugation (5 min at 400 *g*) and washing; the supernates were saved for analysis (NPC_1 residues).

In other experiments, sinusoidal cells were isolated and subfractionated by counterflow elutriation (Fig. 2), as described in detail by Nagelkerke et al. (34). To understand our results it is worth recalling that: (*a*) Kupffer and endothelial cells are isolated without incubation with pronase; (*b*) the yield in Kupffer cells in the filtrate is improved by extensive washing of the material retained on the filter; (*c*) cross-contamination by hepatocytes and large vesicles derived from hepatocytes (blebs) is minimized by counterflow elutriation and flotation in a Metrizamide solution. The second centrifugal elutriation step was carried out at 2,500 rpm, yielding three preparations (designated I, II, and III) at the flow speeds indicated in the legend of Fig. 2.

Rat hepatocytes were also isolated and cultured in plastic Petri dishes coated with collagen, by a technique that combines the procedure of Seglen (39, 40) and of Wanson et al. (46), as described by Limet et al. (28).

Before the biochemical assays, the various cell preparations were disrupted with a Dounce-type homogenizer (20 strokes with the tight-fitting pestle) in a medium consisting of 0.25 M sucrose, 3 mM imidazole-HCl, pH 7.4, and of the reagents introduced with the supernatants (filtrate residues and NPC_1 residues).

Biochemical Assays: NAD glycohydrolase was assayed in a final volume of 0.25 ml in the presence of 1.5 mM [*carbonyl*-^{14}C]nicotinamide adenine dinucleotide (45,000–60,000 cpm), 1 mM EDTA, and 50 mM 4-morpholinoethanesulfonic acid-KOH buffer, pH 6.5. After incubation for 30 min at 37°C, the reaction was stopped by refrigeration in melting ice and addition of 0.5 ml of a cold solution made of 0.1 M $NaHCO_3$-Na_2CO_3 buffer at pH 10 and 2 mM nicotinamide. The mixtures were quickly passed over 2-cm columns of alumina packed in Pasteur pipettes. Test tubes and columns were immediately rinsed with 2.5 ml of the cold bicarbonate-carbonate-nicotinamide solution. Eluates were recovered in scintillation vials and added with 20 ml of Biofluor for counting. Blanks were run in the same conditions except that the substrate was added after the alkaline reagent.

Protein was assayed according to Lowry et al. (29), or when high sensitivity was needed according to Böhlen et al. (20). In both cases, bovine serum albumin was used as a standard. Glucose-6-phosphatase, galactosyltransferase, alkaline phosphodiesterase I, cytochrome *c* oxidase, and 5′-nucleotidase were assayed manually under the conditions described earlier (12). When specified the activity of glucose-6-phosphatase was corrected for that of other phosphatases still active after incubation for 15 min at pH 5 and 37°C (22).

Light Microscopy of the Isolated Cell Fractions: Cytochemical staining for peroxidase was done according to Wisse et al. (48), by incubation at 37°C for 20 min in the presence of diaminobenzedine (0.05%) and H_2O_2 (0.02%).

Cells were also fixed in suspension with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2, recovered as a pellicle by centrifugation (5 min, 10,000 *g*) on a cushion of human plasma high-speed supernate, postfixed with osmium tetroxide (2%), stained with uranylacetate, and embedded in Epon. Thin sections were stained with toluidine blue for light microscopy. Stereological analysis of the photomicrographs was made with the multipurpose test system of Weibel et al. (45) to determine the volumetric fraction corresponding to various types of liver cells. Equidistance was 0.94 cm in the hexagonal lattice which consisted in 120 lines arranged in 15 rows.

RESULTS

Enzyme Kinetics

Factors that influence the NAD glycohydrolase reaction were examined in detail by using cytoplasmic extracts and microsomes. The enzyme was fully active in the presence of EDTA. This chelating agent was included in the reaction medium to inhibit the synthesis of polyADPribose, which also produces nicotinamide but is Mg^{2+}-dependent (36). The reaction followed Michaelian kinetics. Almost identical K_m

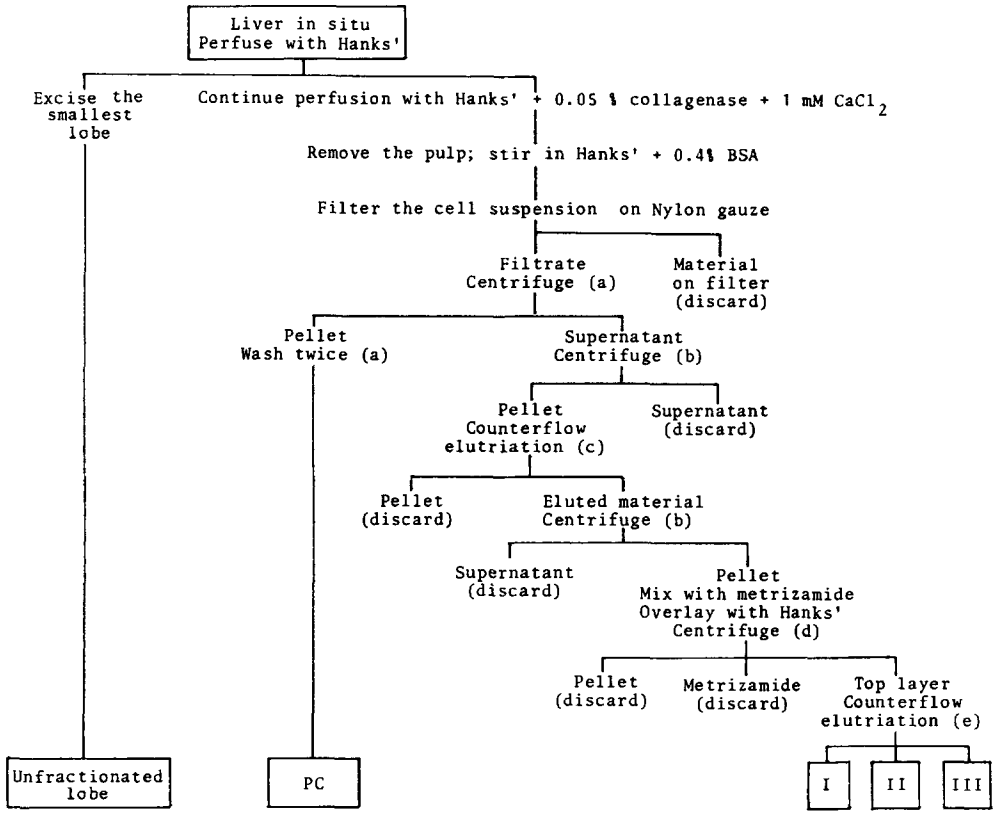

FIGURE 2 Flow diagram for the isolation of sinusoidal cell preparations by counterflow elutriation. Adapted from Nagelkerke et al. (34). Centrifugations were twice for 30 s at 50 *g* (*a*), 10 min at 400 *g* (*b*), counterflow elutriation at 1,500 rpm and 18 ml/min flow speed (*c*), 15 min at 1,400 *g* (*d*), and counterflow elutriation at 2,500 rpm (*e*) at flow speeds of 13.5 ml/min (material discarded), 20 ml/min (I), 25 ml/min (II), and 30 ml/min (III).

TABLE I

*Distribution of NAD Glycohydrolase and Various Constituents after Differential Centrifugation of Rat Liver Homogenates**

Constituent	Experiments	Nuclear fraction	Large granules	Microsomes	Final supernate	Recovery
	n			%		
NAD glycohydrolase	5	15.9 ± 4.7	3.3 ± 2.0	69.0 ± 11.4	2.7 ± 2.0	90.9 ± 13.6
Protein	5	18.1 ± 3.4	24.7 ± 1.3	20.7 ± 2.0	36.5 ± 3.8	100.0 ± 5.2
Glucose-6-phosphate	5	9.2 ± 5.2	12.6 ± 2.9	77.4 ± 7.4	2.9 ± 1.4	102.1 ± 8.9
Galactosyltransferase	1	3.2	1.5	79.3	7.3	91
Alkaline phosphodiesterase I	5	42.9 ± 7.7	7.3 ± 4.0	52.1 ± 7.9	1.3 ± 0.4	103.6 ± 7.4
5′-Nucleotidase	5	39.6 ± 7.5	18.0 ± 3.1	54.0 ± 6.8	6.2 ± 1.5	117.8 ± 14.2
Alkaline phosphatase	4	24.8 ± 5.1	11.0 ± 1.1	32.0 ± 1.8	30.1 ± 4.5	97.9 ± 5.0

* Results are given as means ± SD, in percent of the sum of the absolute values found in the whole liver (cytoplasmic extract + nuclear fraction). Enzyme activities and protein content per gram of liver were similar to those reported earlier (4). The specific activity of NAD glycohydrolase was 14.8 ± 1.5 (23 experiments) and 11.1 ± 0.7 (five experiments) in liver homogenates from female and male animals, respectively.

values (≃50 μM) were measured with microsomes and cytoplasmic extracts obtained from the same liver homogenate, indicating that endogenous NAD did not interfere with measurements although enzyme activities were assayed under nearly maximal velocity conditions (1.5 mM labeled NAD). The pH-activity curve had a broad maximum around 6.5. The enzyme showed no clear-cut evidence of structure-linked latency when the influence of Triton X-100 and of hypotonic conditions was examined. The increase in activity did not reach 10%.

Distribution of NAD Glycohydrolase after Differential Centrifugation

The centrifugation behavior of NAD glycohydrolase was compared with that of various reference enzymes, in particular 5′-nucleotidase, alkaline phosphodiesterase I and alkaline phosphatase for plasma membranes, galactosyltransferase for Golgi complex elements, and glucose-6-phosphatase for the endoplasmic reticulum. Reference enzymes for mitochondria, lysosomes, and peroxisomes were also assayed in some cases, but are generally not considered here because they dissociated obviously from NAD glycohydrolase.

Fractionation of liver homogenates by differential centrifugation into nuclear fraction, large granules, microsomes, and final supernate gave the distributions shown in Table I. The mean sedimentation profiles derived from the data for NAD glycohydrolase, alkaline phosphodiesterase I, and glucose-6-phosphatase are presented graphically in Fig. 3. Glucose-6-phosphatase and galactosyltransferase were recovered mainly in the microsomes, whereas alkaline phosphodiesterase I, 5′-nucleotidase, and alkaline phosphatase exhibited the nucleo–microsomal distribution characteristic of various plasma membrane-associated enzymes (4, 5). NAD glycohydrolase was found mainly in the microsomes, with a yield of 76% of the total recovered activity, identical to that of glucose-6-phosphatase. A slight excess of NAD glycohydrolase relative to glucose-6-phosphatase is noted in the nuclear fraction.

NAD Glycohydrolase in Plasma Membrane Preparations

The results shown in Table I and Fig. 3 apparently question the occurrence of NAD glycohydrolase at the periphery of liver cells. Consequently, plasma membranes were prepared and assayed for NAD glycohydrolase and reference enzymes. As seen in Table II, the preparations contain a significant amount of cytochrome *c* oxidase and glucose-6-phosphatase,

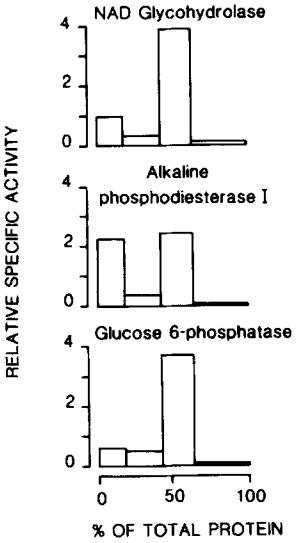

FIGURE 3 Average distribution pattern of NAD glycohydrolase, alkaline phosphodiesterase I, and glucose-6-phosphatase after fractionation of rat liver homogenates by differential centrifugation. Nuclear fraction, large granules, microsomes, and final supernate are shown by blocks, from left to right, respectively. The ordinate scale gives the relative specific activity of the enzyme (percentage of total activity/percentage of total protein). The abscissa scale gives cumulatively the percentage of protein.

TABLE II

*Yield and Relative Specific Activity of NAD Glycohydrolase Compared with the Values of Reference Enzymes in Plasma Membrane-rich Fractions Obtained from Rat Liver**

Constituent	Yield‡	Relative specific activities§
	%	
NAD glycohydrolase	19.6 ± 5.3	5.7 ± 0.9
Protein	3.6 ± 1.4	1
Glucose-6-phosphatase	4.0 ± 1.4	1.1 ± 0.2[a]
Galactosyltransferase	3.8 ± 1.9	1.0 ± 0.2[a]
Cytochrome c oxidase	9.1 ± 2.5	2.7 ± 0.5[a]
Alkaline phosphodiesterase I	41.5 ± 10.6	12.2 ± 2.5[a]
5′-Nucleotidase	31.5 ± 10.0	9.1 ± 1.7[b]
Alkaline phosphatase	22.4 ± 7.6	6.5 ± 2.0

* Values are given as means ± SD in five experiments, except for galactosyltransferase which was determined in three experiments.

‡ Yield is the percent of the sum of the activities, or of the amounts in the case of protein, recovered in all fractions. These sums comprised between 89 and 102% of values in the homogenate.

§ Relative specific activity is defined in the legend of Fig. 3. Where indicated enzymes differ from NAD glycohydrolase at $P < 0.001$ (a), or $P < 0.01$ (*b*).

indicating that inner mitochondrial membranes and endoplasmic reticulum-derived membranes together account for half the protein. This preparation was preferred to the alternative with its high purification and low yield, in view of the heterogeneity expected to occur among plasma membrane domains. The specific activity of the reference enzymes for

these membranes reaches 10 or more when the values are corrected for the fraction of enzyme activity in the final supernate (see Table I). NAD glycohydrolase was present with a distinctly greater yield and a correspondingly greater relative specific activity than glucose-6-phosphatase and galactosyltransferase, but in both respects it remained significantly below the values arrived at for the particle-bound activity of the plasma membrane reference enzymes. These results suggest that at least a part of the NAD glycohydrolase activity in liver is associated with plasma membranes.

To investigate this tentative conclusion plasma membrane preparations were centrifuged in a linear sucrose gradient, either directly, or after treatment with digitonin. As seen in Fig. 4, NAD glycohydrolase was shifted to higher equilibrium density after reaction with digitonin. The increase in median density was similar to that of other plasma membrane enzymes (Table III). The behavior of protein reflects the contamination of plasma membranes by membranes that are not significantly shifted, or that are shifted less, such as the Golgi elements identified by galactosyltransferase. These results, which agree completely with those reported in more detail (2 and 47), strengthen the conclusion that NAD glycohydrolase is present in plasma membranes.

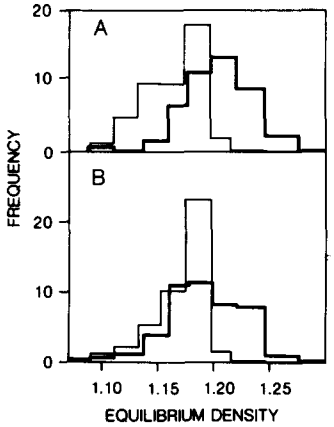

FIGURE 4 Density distribution of NAD glycohydrolase (*A*) and protein (*B*) in control and digitonin-treated plasma membrane-rich fractions. Yields of NAD glycohydrolase, expressed in percent of the liver content, were 20.3 and 16.3 in the two plasma membrane preparations used; the relative specific activities were 5.6 and 6.0, respectively. The first preparation (thin line) was centrifuged in the E-40 rotor, through a sucrose-H_2O gradient extending linearly from 1.10 to 1.25, for 140 min at 40,000 rpm. Digitonin (0.25 mg/mg protein) was added to the second preparation (thick line) before subfractionation as above. Recoveries of NAD glycohydrolase after density gradient centrifugation were 110 and 100%, respectively.

TABLE III

*Median Densities of Constituents in Untreated and Digitonin-treated Plasma Membrane Preparations Obtained from Rat Liver**

Constituent	Untreated	Digitonin-treated	Difference
NAD glycohydrolase	1.170	1.205	0.035
5′-Nucleotidase	1.163	1.197	0.034
Alkaline phosphatase	1.181	1.214	0.033
Alkaline phosphodiesterase I	1.173	1.205	0.032
Galactosyltransferase	1.146	1.167	0.021
Protein	1.178	1.191	0.013

* Values derived from the density distributions of protein and enzymes in the experiments described in the legend of Fig. 4.

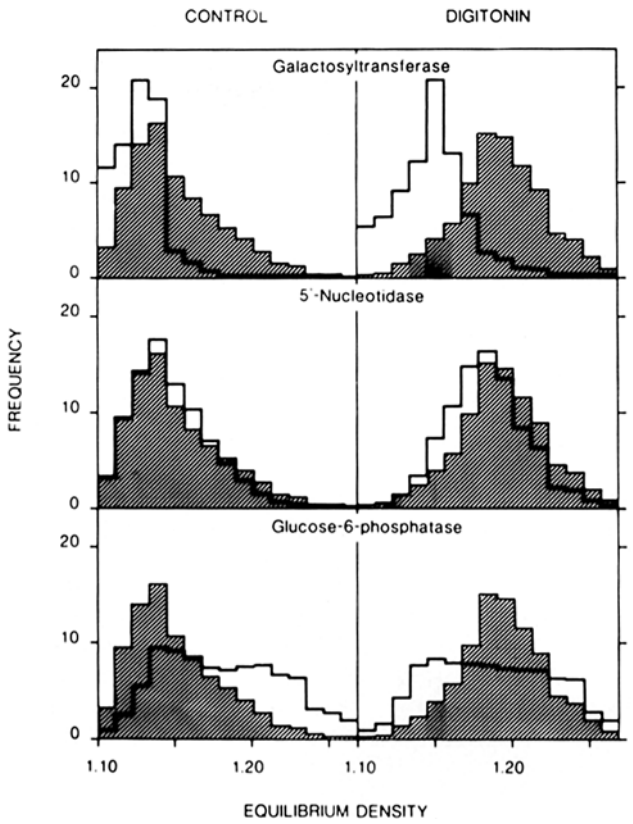

FIGURE 5 Density distribution of NAD glycohydrolase, compared with that of glucose-6-phosphatase, galactosyltransferase, and 5′-nucleotidase after isopycnic centrifugation of microsomes in a sucrose gradient. The distributions shown on the left side are the average results of three experiments in which microsomes have been brought to density equilibrium in a linear gradient of sucrose, without previous treatment, under the conditions given in the legend of Fig. 4. The distributions shown on the right side are the average results of three other experiments in which the microsomes were subjected to the treatment with digitonin before subfractionation in the density gradient. The distributions obtained were normalized for averaging as described elsewhere (9). The mean yields of NAD glycohydrolase in the microsome fractions used were 77% of the total activity in liver, and recoveries after density gradient centrifugation comprised between 71 and 115%. The distribution of NAD glycohydrolase is shown by the shaded histograms superimposed on the distribution profiles of the other enzymes.

Density Distribution of the Microsomal NAD Glycohydrolase

Taken together, the results described in the preceeding sections were still consistent with a dual localization of NAD glycohydrolase in both the plasma membranes and the endoplasmic reticulum. On the other hand, NAD glycohydrolase could belong exclusively to a particular type of plasma membranes that are broken down into small-sized fragments. This second explanation is strongly supported by the density distribution of the microsomal NAD glycohydrolase shown in Fig. 5. When microsomes were subfractionated by isopycnic centrifugation in a linear sucrose gradient, NAD glycohydrolase followed closely 5′-nucleotidase. Its activity was low compared with that of glucose-6-phosphatase in subfractions of density >1.2, making its occurrence in rough vesicles unlikely. In experiments performed on digitonin-treated mi-

crosomes, the density shift of NAD glycohydrolase was slightly greater than that of 5′-nucleotidase. This shift led to a marked dissociation from galactosyltransferase and glucose-6-phosphatase in the low-density subfractions. The average median density of NAD glycohydrolase increased by more than 0.04 density units after digitonin treatment, in contrast to glucose-6-phosphatase, which was not perceptibly influenced. Therefore, the microsomal NAD glycohydrolase cannot be assigned to membranes of the endoplasmic reticulum or of the Golgi apparatus. The possible presence of NAD glycohydrolase in the Golgi complex was also ruled out by the finding that the yield of this enzyme in Golgi-rich fractions was no greater than that of other plasma membrane enzymes (results not shown).

NAD Glycohydrolase Activity in Isolated Liver Cells

Inasmuch as the liver parenchyma consists of hepatocytes and various types of sinusoidal cells, a possible explanation for the difference between NAD glycohydrolase and other plasma membrane enzymes in differential centrifugation was that NAD glycohydrolase belongs mainly to cells yielding small plasma membrane fragments upon homogenization. This possibility was investigated by isolating hepatocytes and sinusoidal cells according to van Berkel (42). A PC preparation containing mainly hepatocytes (44) and two different NPC preparations enriched in sinusoidal cells (43) are obtained. NPC_1 is cleaner than NPC_2 because the pronase treatment reduces the contamination by hepatocytes. However enzymes of the cell periphery were partially inactivated by this treatment. The loss of activity under the conditions used to isolate NPC_1 was established in preliminary experiments and the values measured were corrected accordingly (see Table IV). Protein and enzymes were also assayed in the residue fractions to establish the distributions quantitatively.

The distribution of enzymes and protein between the various cell preparations is shown in Table IV. NAD glycohydrolase differed from the other constituents in that its yield in PC was noticeably low and the bulk of its activity (≃80%) was retained on the filter (NPC_1 and NPC_1-residues). This peculiar behavior is reflected in the specific activities given in Table V. NAD glycohydrolase is the only enzyme that has a much-reduced activity in PC compared with that in the unfractionated lobe (difference significant at $P < 0.001$). It also shows the highest increase in NPC_1. Consequently, the specific activity in NPC_1 is ≃70 times that in PC. This ratio is only ≃5 for alkaline phosphodiesterase I and ≃7 for 5′-nucleotidase. The differences are less sharp when the specific activities in NPC_2 are compared with those in the PC preparation. NAD glycohydrolase still shows the highest ratio, but mainly as a consequence of its low activity in PC. Glucose-6-phosphatase (Table V) and the fructose-1,6-diphosphate-activated pyruvate kinase (not shown) indicate that NPC_2 is significantly contaminated by material of hepatocytic origin. Light and electron microscopy revealed that although intact hepatocytes were not observed in NPC_2, sinusoidal cells were contaminated by membrane-bounded vesicles of hepatocytic origin, described earlier (33). These vesicles are of similar size to sinusoidal cells and contain membrane-associated enzymes at a higher concentration than do hepatocytes (33). Such vesicles may thus account for a significant fraction of the 5′-nucleotidase and alkaline phosphodiesterase I in the NPC preparations.

TABLE IV

*Distribution of Enzymes and Protein after Fractionation of the Liver into PC, NPC_1, and NPC_2 Preparations**

Constituent	Cell preparation				
	PC	NPC_2	Filtrate residues	NPC_1‡	NPC_1 residues‡
			%		
NAD glycohydrolase	3.2	2.0	14.1	7.4	73.3
Alkaline phosphodiesterase I	22.2	10.7	31.7	7.0	28.4
5′-Nucleotidase	16.7	5.0	30.7	5.1	42.5
Glucose-6-phosphatase§	35.1	0.7	64.2	ND‖	ND‖
Protein	21.2	1.0	52.8	0.8	24.2

* Average results of two experiments in which PC, NPC_1, and NPC_2 cell preparations were obtained as described in Materials and Methods. Figures give the percent of enzyme activity and protein, relative to the total amount in the cell preparations plus the residues.

‡ Values were corrected for the loss of activity after pronase treatment: NAD glycohydrolase, 54%; alkaline phosphodiesterase I, 67%; 5′-nucleotidase, 17%.

§ Activity labile at 37°C and pH 5.

‖ ND, not detectable after incubation with pronase.

Table V also shows that the specific activity of each enzyme is similar in the reconstituted tissue material (preparations plus residues) and in the unfractionated lobe, except for a tendency of NAD glycohydrolase to be more active in the former. The correction made for the loss of NAD glycohydrolase on incubation with pronase might thus be somewhat excessive. This would not seriously weaken the singularity of this enzyme because the average NPC_1/PC ratio is 32 without correction, a value still far above those of 5′-nucleotidase and alkaline phosphodiesterase I after correction. Alternatively, the unfractionated lobe may not be an exact reference for the bulk of this organ. Specific activities of NAD glycohydrolase in whole liver homogenates (see Table I) were closer to that found in the reconstituted tissue material.

When hepatocytes isolated in a PC fraction were cultured, the activity of NAD glycohydrolase vanished within 24 h whereas glucose-6-phosphatase and alkaline phosphodiesterase I activities remained at 87 and 134% of the initial values, respectively.

NAD Glycohydrolase Activity in Sinusoidal Cells Obtained by Counterflow Elutriation

Another procedure for isolating sinusoidal cells avoids the use of pronase. Although the many steps involved preclude quantitative work, the procedure results in cell preparations free from hepatocyte-derived structures (34). As can be seen in Table VI, this method yields sinusoidal cells (preparations I–III) similar to NPC_1 with respect to the activity of NAD glycohydrolase, but with a much lower level of alkaline phosphodiesterase I and half the activity of 5′-nucleotidase. The low activity of alkaline phosphodiesterase I in these cell preparations may indicate a low degree of contamination by hepatocyte-derived vesicles if it is assumed that this enzyme is prominent in plasma membranes of hepatocytes. The average ratios of the specific activities of NAD glycohydrolase in preparations II and III to those in the PC preparations were 74 and 81, respectively.

TABLE V

*Specific Activity of Enzymes in Various Cell Preparations**

Preparation	NAD glycohydrolase	Alkaline phosphodiesterase I	5′-Nucleotidase	Glucose-6-phosphatase§
		mU/mg protein		
Unfractionated lobe	9.73 ± 2.50 (6)	112.5 ± 30.3 (7)	71.8 ± 8.6 (4)	47.4 ± 10.8 (4)
Preparations + residues‡	13.8 ± 4.6 (4)	144 ± 27 (3)	76.5 (2)	50.3 ± 6.1 (3)
PC	2.49 ± 1.05 (7)	137 ± 31.4 (7)	64.7 ± 17.4 (4)	79.5 ± 10.2 (4)
NPC_2	32.2 ± 2.5 (4)	908 ± 396 (3)	376 ± 123 (3)	41 ± 12.2 (3)
NPC_1	136 ± 7.6 (4)	621 ± 398 (3)	430 ± 253 (3)	ND‖
		Specific activity relative to that in PC		
NPC_2	16.7 ± 6.4 (4)	6.9 ± 1.4 (3)	6.2 ± 1.4 (3)	0.51 ± 0.08 (3)
NPC_1	69.4 ± 24 (4)	4.9 ± 2.6 (3)	6.8 ± 2.3 (3)	

* Cell preparations were obtained from perfused livers as described in Materials and Methods. Values give means ± standard deviations, with the number of experiments in parentheses. Activities in the unfractionated lobes and in the PC preparations derived from the experiments shown in Table VI were included in the statistics.

‡ Values estimated from the total activities and total protein recovered in the various cell preparations and in the residues.

§ Activity labile at pH 5 and 37°C.

‖ ND, not detectable after incubation with pronase.

TABLE VI

*Morphology and Enzyme Properties of Cell Preparations Obtained by Counterflow Elutriation**

	Unfractionated lobe	Cell preparations I	II	III
		Test points lying in cells (% of total)‡		
Kupffer cells		22	81	76
Endothelial cells		55	2	1
Other cells and unidentified cells		23	17	20
Total number of points lying in cells counted		167	155	108
		mU/mg protein§		
NAD glycohydrolase	8.9; 12.5	115; 157	185; 231	174; 266
Alkaline phosphodiesterase I	107; 106	28; 70	27; 53	29; 64
5′-Nucleotidase	73	254	217	186
		Relative specific activity‖		
NAD glycohydrolase		12.9; 12.5	20.8; 18.5	19.6; 21.3
Alkaline phosphodiesterase I		0.26; 0.66	0.25; 0.50	0.27; 0.60
5′-Nucleotidase		3.48	2.97	2.55

* Sinusoidal cells and PC preparations were isolated from collagenase-perfused livers by a method including differential centrifugation, elutriation at 1,800 rpm and 18 ml/min flow rate, and flotation through 17.5% Metrizamide, exactly as described by Nagelkerke et al. (34). The cells were then injected in the elutriation rotor spinning at 2,500 rpm and different fractions were collected as described under Materials and Methods.

‡ The cell fractions obtained in one experiment were examined by light microscopy as described in the Materials and Methods section. Photomicrographs at ×810 magnification were analyzed with the multipurpose test system (45) for volumetric estimation of Kupffer cells and endothelial cells.

§ Values are the results of two experiments; in each set the first value corresponds to the preparation examined by light microscopy.

‖ Ratio of the specific activity in the cell preparation to that in the unfractionated lobe.

The cell composition of preparations I, II, and III was established by stereological analysis of light photomicrographs obtained from thin sections stained with toluidine blue. Identification of Kupffer cells was confirmed by 3,3′-diaminobenzedine peroxidase staining (48). In agreement with Nagelkerke et al. (34), endothelial cells were washed out at a lower flow speed (preparation I) than Kupffer cells (preparations II and III) in the final elutriation step. The morphological values given in Table VI are related to the volumetric fraction of each cell species, which is comparable with the specific activities of enzymes. NAD glycohydrolase activity correlates with the content of the preparations in Kupffer cells, but the values suggest that the enzyme may occur in endothelial cells with a specific activity about three times lower.

DISCUSSION

NAD glycohydrolase is largely recovered in microsomes after differential centrifugation of liver homogenates, and its activity in plasma membrane preparations exceeds the value obtained for enzymes associated with contaminating endoplasmic reticulum and Golgi complex elements. Similar results reported by others have been interpreted as indicating either attachment of the enzyme to the endoplasmic reticulum membrane (35) or a dual location in the endoplasmic reticulum and plasma membrane of hepatocytes (19).

Although compatible with the data presented earlier, this conclusion must be reconsidered because the microsomal NAD glycohydrolase differs strikingly from authentic enzymes of the endoplasmic reticulum in density gradient analysis (Fig. 5). Like 5′-nucleotidase, but unlike glucose-6-phosphatase, NAD glycohydrolase is recovered essentially in low-density subfractions (<1.2 density units) when microsomes are brought to equilibrium in a sucrose gradient, and it is shifted to subfractions of higher density after treatment of the microsomes with digitonin. It is clear that the amount of NAD glycohydrolase associated with the endoplasmic reticu-

lum, if any, must be small in comparison with the total amount present in the microsomes, and cannot explain the high yield of this enzyme in the microsomal fraction. Our earlier studies (5, 41, 47) have brought biochemical and morphological evidence that the digitonin shift is typical of the plasma membranes, including possibly endocytotic or other cytoplasmic vesicles closely related with the cell periphery. In spite of its microsomal character, NAD glycohydrolase is thus specifically associated with that kind of subcellular entity, which we have designated a2 (13). Vesicles derived from the Golgi complex and bearing several glycosyltransferases are also shifted by digitonin (5, 47), but the magnitude of the shift is smaller, and the dissociation between galactosyltransferase and NAD glycohydrolase after treatment with digitonin (Fig. 5) is sharp enough to exclude this alternative localization. Other studies on the rat liver enzyme failed to demonstrate a clear-cut difference between the NAD glycohydrolase of microsomes and that of plasma membrane preparations. Both enzymes have nearly the same half-life (19), give a single continuous precipitation line in Ouchterlony double-diffusion immunochemical tests (18), and show similar biochemical characteristics (24). Such experimental data are consistent with NAD glycohydrolase being a constituent of a single membrane entity in the liver.

The question thus became how to explain that NAD glycohydrolase is recovered in microsomes in great excess over 5′-nucleotidase. A similar question has been raised earlier by the distribution of cholesterol (4) and protein-bound sialic acid (2), which were both partly assigned to the endoplasmic reticulum (8, 25). The explanation had to be sought in the differentiation of the hepatocyte periphery into biochemically distinct domains, or in the existence of several varieties of cells within the liver parenchyma. Plasma membrane preparations obtained from low-speed sediments are derived from the peribiliary border, junction complexes, and adjoining portions (reviewed in reference 23); the extent to which the sinusoidal border of hepatocytes and the plasma membranes of sinusoidal cells contribute to these preparations is not known exactly.

The answer is mainly that NAD glycohydrolase is prominently present in Kupffer cells, less so in endothelial cells, and low in hepatocytes. This is shown by the relative yields in NAD glycohydrolase and protein in PC preparations (Tables IV and V). In some experiments the specific activity fell to 15% of that in the whole liver (Table V). The presence of a minor part of NAD glycohydrolase in hepatocytes might explain the difference between the density profiles in microsome and plasma membrane preparations (compare Figs. 4 and 5). Further evidence for the association of the bulk of NAD glycohydrolase with Kupffer cells is the recovery of up to 80% of the enzyme activity in the material retained on the nylon filter. This property of adherence has been utilized to separate macrophages from other cells of the spleen (6). Such selective retention allowed the specific activity of NAD glycohydrolase in NPC_1 to be raised by a factor of 10–13 over its value in the whole liver and of ≃70 over PC. Finally, the assignment of the enzyme to Kupffer cells is supported by the counterflow elutriation results (Table VI). The enrichment factor in NAD glycohydrolase activity was ≃21 in preparations II and III. It is a minimum value for Kupffer cells, as they make up no more than 80% of the cell volume in these preparations. Comparison of the cell composition and enzyme properties of preparation I suggests an enrichment factor of 7 in endothelial cells.

An approximation of the distribution of NAD glycohydrolase between various liver cells is obtained when our results are combined with the stereological data reported by Blouin et al. (16). Hepatocytes, endothelial cells, and Kupffer cells make up 78.8, 2.8, and 2.1% of the parenchymal volume, respectively, and their respective contributions to the plasma membrane surface area are 73.5, 15.2, and 4.3%. Assuming that the percent volume is equivalent to the percent protein, and that the relative specific activities derived for NAD glycohydrolase in the various cell types are valid for the whole liver despite the low yields in the sinusoidal cell preparations, the percentage of the enzyme activity that may be assigned to hepatocytes, endothelial cells, and Kupffer cells is 14, 24, and 53, respectively. In reference to the surface areas of the respective plasma membranes, these estimates indicate that the activity of NAD glycohydrolase per unit surface area of membrane in Kupffer cells may be 8 times higher than in endothelial cells, and 65 times higher than in hepatocytes. In this sense, NAD glycohydrolase may be taken as a reference enzyme for the plasma membrane of Kupffer cells. The true differences between these various cells may be greater than estimated, as the PC preparations may contain some ghosts of sinusoidal cells. It is perhaps significant that the NAD glycohydrolase activity fell on cultivation of hepatocytes. Interestingly, the specific activity reaches 510 mU/mg of protein in resident peritoneal macrophages of the mouse (21), a value 30 times that of mouse liver homogenates (unpublished results of C. Darte). In these macrophages, also, NAD glycohydrolase is associated with subcellular elements characteristically shifted by digitonin (21). The microsomal distribution of NAD glycohydrolase shows that the plasma membranes of Kupffer cells are largely reduced to microsomal elements when the liver is homogenized, and explains how authentic constituents of plasma membranes, e.g., cholesterol and protein-bound sialic acid, occur in microsomes in excess over 5′-nucleotidase and other enzymes characteristic of plasma membranes.

The activity of NAD glycohydrolase within the cytosol would lead to a rapid turnover of the pyridine coenzymes and to a large expenditure of cellular energy (26). In agreement with others (18), we have found no evidence for a structure-linked latency of NAD glycohydrolase in rat liver. This enzyme would therefore act freely on the pyridine nucleotides of the cytosol if it were present in the endoplasmic reticulum, but because it is inserted in the plasma membrane, it is probably shielded from these coenzymes. Recently, Muller et al. (32) have shown convincingly that NAD glycohydrolase is, indeed, an ectoenzyme in beef splenocytes. The exclusive localization of the liver NAD glycohydrolase in elements derived from the plasma membrane and its prominent association with sinusoidal cells rule out a major role for this enzyme in the breakdown of cytosolic pyridine nucleotides in liver parenchymal cells.

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