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HETEROGENEOUS DISTRIBUTION OF THE SODIUM-DEPENDENT ALANINE TRANSPORT ACTIVITY IN THE RAT HEPATOCYTE PLASMA MEMBRANE

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Summary

The localization of the sodium-dependent alanine uptake activity in rat liver cells was studied. Fractions representative of the canalicular, the contiguous (lateral) and the blood-sinusoidal surface of the hepatocyte were isolated by means of centrifugal fractionation and density gradient centrifugation. The distribution of various marker-enzyme activities in conjunction with the occurrence of alanine transport activity was studied both in fractions obtained after zonal density gradient centrifugation, and in the subcellular fractions mentioned above.

It is concluded that the sodium-dependent alanine transport activity is primarily located in the blood-sinusoidal plasma membrane of the hepatocyte.

Introduction

In view of its obvious role in the maintenance of the intracellular amino acid pools and thus in liver metabolism, many studies have been devoted during the past decade to the uptake of glucogenic amino acids in the liver. Experimental techniques in the study of amino acid transport have evolved from liver perfusion towards the use of isolated hepatocytes [1—4]. The resulting parameters are often related to the rate of metabolic conversions.

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

In the isolated hepatocyte the various parts of the plasma membrane, viz. sinusoidal (blood-side), contiguous (lateral) and canalicular (bile-side) membrane, are exposed to the same medium. A complicating factor in such studies is that the amino acid transport activity may not be equally distributed with equal activity along the plasma membrane of the hepatocyte. Furthermore, plasma membrane regions that are not directly accessible for the substrate in the intact liver may influence the observed uptake in the isolated cell. In this respect, it should be noted that substantial differences in transport properties exist in the brush-border and the basolateral membranes from transporting epithelia like intestine [5—7] and kidney [8—10].

We recently described the isolation of a plasma membrane vesicle preparation from rat liver which retained transport activity for amino acids [11]. This preparation enabled us to characterize the L-alanine transport system in this tissue [12]. In order to increase our knowledge about the role of amino acid transport in the physiology of the hepatocyte, and knowing the uneven distribution of various plasma membrane marker enzymes in this cell [13], we decided to study the subcellular localization of the sodium-dependent alanine uptake activity.

Materials and Methods

Methods

Tissue preparation. Male Wistar rats, weighing 0.2—0.3 kg and fed ad libitum, were killed by decapitation. The liver was immediately removed, minced with scissors and homogenized in 5 vols. of ice-cold 0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM Hepes-KOH (pH 7.5). Homogenization was performed in a loose-fitting Dounce homogenizer (0.14 mm diameter clearance) with 21 strokes and subsequently in a tight-fitting Dounce homogenizer (0.06 mm diameter clearance) with one stroke. All operations were performed at 0—4° C.

Centrifugal fractionation. The homogenate was filtered through a nylon net (pore size 0.4 mm), diluted with 4 more vols. of homogenization buffer and then made 1 mM EDTA by the addition of 100 mM EDTA-KOH (pH 7.5). Fractionation by centrifugation resulted in a debris (10 min, $270 \times g$), in nuclear (10 min, $1100 \times g$), heavy microsomal (30 min, $20\,000 \times g$) and light microsomal (60 min, $100\,000 \times g$) pellets and in a remaining supernatant.

Density gradient centrifugation. The nuclear pellet was further fractionated by resuspending it in homogenization buffer (plus 1 mM EDTA) and layering it on top of a discontinuous gradient prepared from 8 ml of 49% (w/v) sucrose, 4.5 ml of 41% (w/v) sucrose, 4.5 ml of 39.5% (w/v) sucrose and 8 ml of 20% (w/v) sucrose, all in 10 mM Hepes-KOH (pH 7.5). Analogously, the light microsomal pellet was layered on top of a discontinuous gradient prepared from 8 ml of 46% (w/v) sucrose, 9 ml of 33% (w/v) sucrose and 8 ml of 20% sucrose, also in 10 mM Hepes-KOH (pH 7.5). After centrifugation for 2.5 h at $50\,000 \times g_{av}$ in the SW 25.1 rotor of the Beckman Spinco L_{50} ultracentrifuge, subfractions banding at the 49/41% (CC) interface or the 39.5/20% (C) interface (nuclear fraction), or at the 46/33% (S) interface or the 33/20% (LS) interface (light microsomal fraction) were collected, diluted 1 : 1 with homogenization buffer and centrifuged for 60 min at $100\,000 \times g$. The remaining

pellets were resuspended in homogenization buffer by pipetting through a needle of 1.6 mm internal diameter and stored in 0.3-ml aliquots in liquid nitrogen. For reasons to be explained in Results, the various fractions are named contiguous-canalicular (CC), canalicular (C), sinusoidal (S) and light-sinusoidal (LS), respectively.

In some experiments the composition of the nuclear 41/49% interface subfraction was studied further. This was performed by carefully layering this subfraction in the middle of a linear gradient of 35—52% (w/v) sucrose in 10 mM Hepes-KOH (pH 7.5). After placing 6 ml of 10 mM Hepes-KOH (pH 7.5) on top, the gradient was centrifuged 17—18 h at 50 000 × g_{av} in the SW 25.1 rotor of the Beckman Spinco L_{50} ultracentrifuge. Approx. 23 fractions were collected with a density-gradient-removing apparatus (Auto-Densi-flow II_c, Buchler Instruments, Searle Analytic Inc., Fort Lee, NJ, U.S.A.) connected with a fraction collector (LKB, Bromma, Sweden). Sucrose concentrations were measured with an Abbe refractometer at 20° C.

Density gradient centrifugation in a zonal rotor. This was performed by loading the M.S.E. B XIV zonal rotor with a linear gradient of 20—55% (w/v) sucrose (500 ml total volume) and a cushion of 70 ml of 66% (w/v) sucrose, both in 10 mM Hepes-KOH (pH 7.5). After the application of 60-ml sample and 20-ml homogenization buffer as an overlay on top, the gradient was centrifuged for 2 h at 41 000 rev./min (83 000 × g_{av}). After deceleration of the rotor the gradient was pumped out by the introduction of a solution of 66% (w/v) sucrose and divided into approx. 28 fractions by means of a fraction collector. For the determination of alanine transport activity, 4.5-ml aliquots were diluted 1 : 1 with homogenization buffer and centrifuged for 30 min at 100 000 × g. The remaining pellets were resuspended in 0.5 ml homogenization buffer and stored in liquid nitrogen until use. The remainder of the gradient fractions was used for the determination of various marker-enzyme activities and stored at −18° C.

Assay of enzyme activities, RNA and protein. The activities of $(Na^+ + K^+)$-ATPase (EC 3.6.1.3), cytochrome *c* oxidase (EC 1.9.3.1.), 5′-nucleotidase (EC 3.1.3.5), glucose-6-phosphatase (EC 3.1.3.9) and alkaline phosphatase (EC 3.1.3.1), as well as the concentrations of protein and RNA, were determined as described previously [11,14]. The Mg^{2+}-ATPase activity (EC 3.6.1.3) was determined parallel to that of $(Na^+ + K^+)$-ATPase [11,15], but in the absence of KCl and in the presence of 2.1 mM ouabain. Leucine aminopeptidase activity (EC 3.4.11.1) was determined by the method of Nagel et al. [16], and acid phosphatase activity (EC 3.1.3.2) at pH 4.5 by the method of Lisman et al. [17]. Hormone-sensitive adenylate cyclase activity (EC 4.6.1.1) was determined as described by Wisher and Evans [13], but in the presence of 2 mM theophyllin and 0.5 mM EGTA, and with or without 10 μM glucagon. The amount of cyclic AMP formed was measured by competition with added cyclic [^{3}H]AMP for binding to a cyclic AMP-binding protein [18]. In gradient fractions the presence of the protein was estimated by measuring the 280 nm absorbance ($A_{280\ nm}$) as previously described [19].

Transport assay. The sodium-dependent alanine transport capacity of the various fractions was determined by measuring the difference in the alanine taken up by the fractions after 20 s at pH 7.5 and 25° C in the presence of

0.2 mM L-[2,3-^{3}H]alanine and 100 mM NaSCN or KSCN as described previously [11].

Materials

Cytochrome *c* (horse heart), Na_2ATP, Na_2AMP, glucose-6-phosphatase and the cyclic AMP assay kit were obtained from Boehringer; glucagon from Serva; L-[2,3-^{3}H]alanine from The Radiochemical Centre, Amersham; Hepes, theophyllin and bovine serum albumin (fraction V) were purchased from Sigma. All other reagents are of analytical grade.

Results

In our previous work [11,12] we characterized the sodium-dependent alanine transport system from rat liver by means of a plasma-membrane vesicle preparation resulting from the heavy microsomal (30 min, 20 000 × *g*) fraction. This fraction had been purified by means of centrifugation in a discontinuous gradient of sucrose. The material at the 20/39.5% (w/v) sucrose interface was used in our transport studies. However, in view of the contamination of this plasma-membrane vesicle preparation with other intracellular organelles [11] and in order to obtain insight into the heterogeneity of the plasma-membrane vesicles themselves, we decided to subject the heavy microsomal fraction to centrifugation in a continuous sucrose gradient (20—55%, w/v) in the M.S.E. B XIV zonal rotor. This rotor type was chosen because of the large amount of sample (derived from 34 g rat liver, wet weight) that could be applied to the gradient. This enabled us to study the distribution of sodium-dependent alanine transport activity parallel to the distribution of various marker-enzyme activities. After centrifugation (2 h, 83 000 × g_{av}) the sodium-dependent alanine transport activity is mainly located between 33 and 48% (w/v) sucrose and correlates very well with the distribution of the plasma-membrane marker enzyme ($Na^+ + K^+$)-ATPase, but less with that of the other marker enzyme activities (Fig. 1). The 5′-nucleotidase activity has a much wider distribution, whereas the Mg^{2+}-ATPase activity partly coincides with the peak activity of the mitochondrial marker enzyme, cytochrome *c* oxidase. The sharp peak of cytochrome *c* oxidase activity at 48—53% (w/v) sucrose, together with the absence of this activity at lower sucrose concentrations, may indicate the absence of fragmented mitochondria in this fraction [19], which illustrates the smoothness of our homogenization procedure. The distribution of leucine aminopeptidase activity partially coincides with that of the lysosomal marker enzyme acid phosphatase. Although leucine aminopeptidase is thought to be associated with the globular knobs which coat the liver plasma membranes lining the bile spaces [20], it has been demonstrated also in lysosomes [21]. The endoplasmic reticulum marker enzyme, glucose-6-phosphatase, has a broad distribution throughout the gradient, partially running parallel to the distribution of RNA. The latter may indicate the presence of rough endoplasmic reticulum. The observed distribution of sodium-dependent alanine transport activity shows that the procedure which was developed for the isolation of plasma-membrane vesicles with alanine transport activity [11], leads to partial loss of material from the heavy microsomal (30 min, 20 000 ×

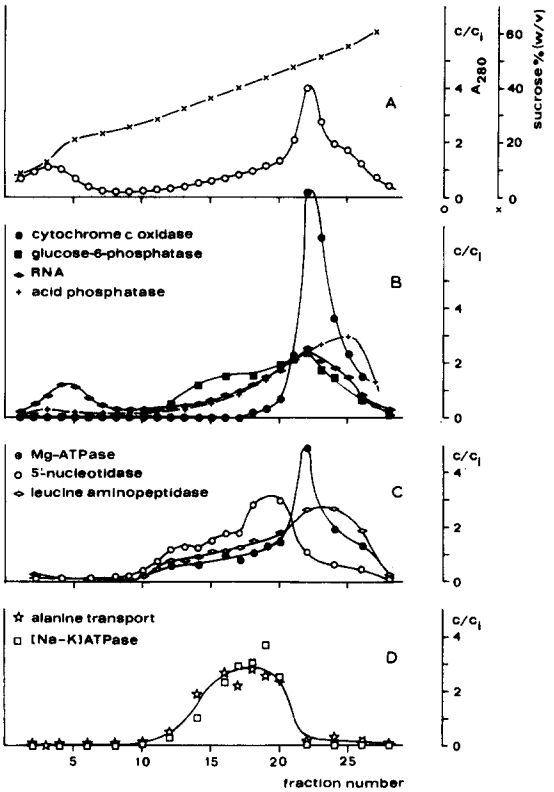

Fig. 1. Enzyme-distribution patterns after density-gradient centrifugation (2 h, 83 000 × g_{av}) of a heavy microsomal fraction (30 min, 20 000 × *g*) of rat liver in the M.S.E. B XIV zonal rotor (single experiment). Results are expressed as the quotient (c/c_i) of the fraction of the total activity, divided by the fraction of the total volume, present in each of the samples (except for sucrose). A value of c/c_i greater than 1 indicates an enrichment of a substance in the fraction. (A) X———X, distribution of sucrose; ○———○, 280 nm absorbance. (B) ●———●, cytochrome *c* oxidase activity; ■———■, glucose-6-phosphatase activity; ◆———◆, distribution of RNA; +———+, acid phosphatase acitivity; (C) ⊕———⊕, Mg^{2+}-ATPase activity; ○———○, 5'-nucleotidase activity; ◇———◇, leucine aminopeptidase activity; (D) ☆———☆, sodium-dependent alanine transport activity; □———□, ($Na^+ + K^+$)-ATPase activity.

g) fraction, since in this procedure the 20—39.5% (w/v) sucrose interface fraction was used. However, this procedure is the best compromise between recovery of alanine transport activity and minimal contamination with intracellular organelles.

Although the zonal gradient experiment indicated that there is a positive correlation between ($Na^+ + K^+$)-ATPase and Na^+-dependent alanine transport, we felt that further evidence was needed before we could decide whether the two activities are located in the same region of the plasma membrane. On the basis of reports on the isolation and separation of various parts of the plasma membrane of the hepatocyte [13,22], we developed a simplified and quick procedure for the isolation of subcellular fractions enriched in distinct parts of the plasma membrane (see Materials and Methods). It should be noted that this procedure was not aimed at complete recovery of the various activities. The sucrose concentrations used in the discontinuous sucrose gradients

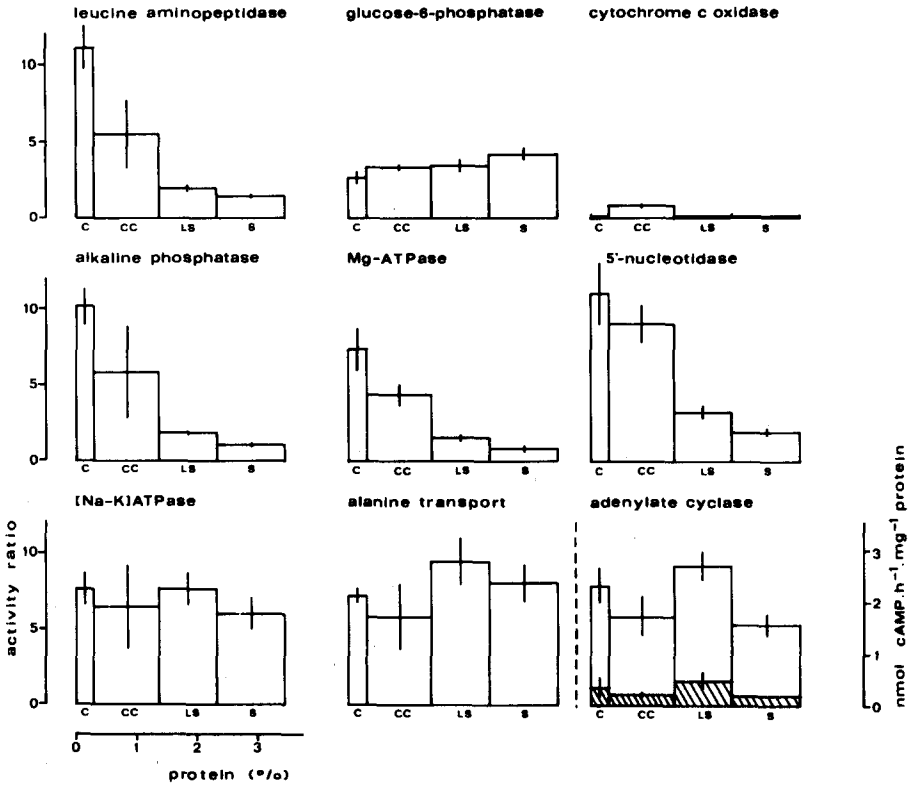

Fig. 2. Activity of various marker enzymes and sodium-dependent alanine transport in the canalicular (C), contiguous-canalicular (CC), light-sinusoidal (LS) and sinusoidal (S) fractions of rat liver. The means of the ratios of the specific activities (enzymes) or transport activity in each fraction to the homogenate value is plotted with standard errors against the protein content of each fraction (as percentage of the homogenate protein content). The adenylate cyclase activities are expressed in absolute values in nmol cyclic AMP formed/h per mg protein in the presence (total bars) or in the absence (shaded bars) of 10 μg glucagon. The number of experiments as well as the homogenate values are similar to those shown in Table I.

were chosen on the basis of pilot experiments in which the enzyme-distribution pattern was studied after centrifugation in continuous sucrose gradients (not shown). In analogy to Evans [23], the nuclear fraction (10 min, $1100 \times g$) separated upon centrifugation in a discontinuous gradient in a low-density bile-canalicular plasma-membrane fraction (C) and a high-density plasma-membrane fraction containing the canalicular-contiguous complexes (CC). The light microsomal fraction (60 min, $100\,000 \times g$) from the same homogenate was separated into a light-sinusoidal (LS) and sinusoidal (S) plasma-membrane fraction.

In these subcellular fractions we have studied the activities of various marker enzymes and of sodium-dependent alanine transport (Fig. 2). We have plotted the enrichment in the specific activities of the marker enzymes over the homogenate values (which are given in Table I) against the protein recovery in each fraction. An exception has been made for the glucagon-stimulated adenylate cyclase activities which are plotted as nmol cyclic AMP formed/h per mg pro-

TABLE I

SPECIFIC ACTIVITIES OF ENZYMES AND SODIUM-DEPENDENT ALANINE TRANSPORT IN RAT LIVER HOMOGENATE

Means with standard errors are presented with the number of experiments in parentheses.

Enzyme	Specific activity (μmol/h per mg protein)
Leucine aminopeptidase	0.52 ± 0.03 (3)
Glucose-6-phosphatase	4.31 ± 0.25 (3)
Cytochrome *c* oxidase	19.2 ± 2.7 (3)
Alkaline phosphatase	0.31 ± 0.06 (3)
Mg^{2+}-ATPase	6.79 ± 0.63 (5)
5′-Nucleotidase	3.66 ± 0.30 (5)
$(Na^+ + K^+)$-ATPase	0.41 ± 0.02 (3)
	nmol cyclic AMP/h per mg protein
Adenylate cyclase + glucagon	3.21 ± 0.20 (3)
— glucagon	0.12 ± 0.03 (3)
	pmol alanine/20 s per mg protein
Na^+-dependent alanine transport	22 ± 2 (3)

tein. This was done because of the unusually high specific activities of adenylate cyclase found in the homogenate (Table I) in comparison with the findings of others [13]. Compared to the one used in literature [13], small changes have been introduced in our adenylate cyclase assay with respect to the glucagon concentration and by the introduction of theophyllin, an inhibitor of phosphodiesterase activity. However, we verified that this cannot explain the observed discrepancy. Homogenization in 1 mM $NaHCO_3$, which was used by Wisher and Evans [13], instead of our homogenization medium (Materials and Methods) resulted in similar values. A possible explanation may lie in the presence of an activating cytoplasmic protein factor [24] or GTP [25]. These substances will be present only in the homogenate but not in the washed and purified plasma-membrane fractions. For this reason we have plotted absolute specific activities of adenylate cyclase in Fig. 2. From the results shown therein, two main groups of plasma-membrane marker enzymes can be distinguished. The first group, consisting of leucine aminopeptidase, alkaline phosphatase, Mg^{2+}-ATPase and 5′-nucleotidase, is highly enriched in the canalicular fraction and to a somewhat lesser extent in the canalicular-contiguous fraction. Much lower enrichments are observed in the light-sinusoidal and sinusoidal fractions. The presence of members of this group of enzymes has been demonstrated in mammalian bile [26]. In addition, alkaline phosphatase was cytochemically localized at the canalicular plasma-membrane surface [27] and a similar location was postulated for 5′-nucleotidase on the basis of ligand-binding properties [28]. The second group, consisting of $(Na^+ + K^+)$-ATPase and glucagon-stimulated adenylate cyclase, has a quite different distribution and correlates very well with the distribution of sodium-dependent alanine transport. Although relatively high activities are present in the canalicular and the canalicular-contiguous fractions, similar activities are present in the light-sinusoidal and the sinusoidal fractions in contrast to the activities of enzymes from the first group. Both $(Na^+ + K^+)$-ATPase and glucagon-stimulated ade-

nylate cyclase are claimed to be mainly located in the sinusoidal plasma membrane on the basis of cytochemical findings [27] and of the glucagon-binding properties of this plasma membrane type [28]. Finally, the distribution of cytochrome *c* oxidase activity shows that only in the canalicular-contiguous fraction can an appreciable activity be found. The glucose-6-phosphatase activity gradually increases towards the (microsomal) sinusoidal (LS and S) fractions. It therefore appears that our canalicular and canalicular-contiguous fractions are indeed enriched in bile-canalicular plasma membranes. However, also sinusoidal plasma membranes are present in view of the relatively high activity of $(Na^+ + K^+)$-ATPase and glucagon-stimulated adenylate cyclase. In contrast to this, the light-sinusoidal and sinusoidal fractions appear to be relatively free from canalicular plasma membranes.

In various experiments, a large variation in marker-enzyme distribution over the canalicular and the canalicular-contiguous fractions was observed. In all cases, however, a strong correlation exists between sodium-dependent alanine uptake activity, and the activities of both $(Na^+ + K^+)$-ATPase and glucagon-stimulated adenylate cyclase, thus confirming our previous results. The large variation in our findings may be caused by the fragility of the canalicular-contiguous complex which affects the reproducibility of the homogenization procedure. This is illustrated in Fig. 3. When the contiguous-canalicular fraction, which had been obtained at the 41/49% (w/v) sucrose interface in the normal isolation procedure, was redistributed by centrifugation in a continuous gradient of sucrose, two peaks of plasma-membrane marker-enzyme activity were observed (open symbols). Next to a large peak of $(Na^+ + K^+)$-ATPase, 5′-nucleotidase and leucine aminopeptidase activities in the middle of the gradient, a smaller activity peak was observed at lower density (25—33%, w/v). In this low-density peak a small enrichment was observed in 5′-nucleotidase and leucine aminopeptidase activities compared to $(Na^+ + K^+)$-ATPase activity. Pre-treatment of the canalicular-contiguous fraction by dilution, centrifugation for 60 min at $100\,000 \times g$, and resuspension by forcing the suspension through a wide needle before application to the continuous sucrose gradient, resulted in a completely different enzyme-distribution pattern (closed symbols). The majority of the 5′-nucleotidase and leucine aminopeptidase activities (and to a somewhat lesser extent the $(Na^+ + K^+)$-ATPase activity) was shifted towards the top of the gradient with little activity remaining in the middle of the gradient. Similar results were obtained by Evans [29] who could fractionate a plasma-membrane fraction of density 1.17 from mouse liver into a light (density 1.12) and a heavy (density 1.18) subfraction by means of rehomogenization in a tight-fitting Dounce homogenizer. Our results confirm that the canalicular-contiguous fraction is indeed rather fragile and can easily be fractionated into a light subfraction, relatively enriched in leucine aminopeptidase and 5′-nucleotidase activities, and a heavy subfraction relatively enriched in $(Na^+ + K^+)$-ATPase activity. In view of the apparent location of the various plasma-membrane marker enzymes mentioned above, the light subfraction may represent bile-canalicular plasma membranes of vesicular aspect [29], whereas the remaining heavy subfraction represents the contiguous plasma-membrane complex with, perhaps, some adhering residual sinusoidal plasma-membrane pieces. The canalicular-contiguous plasma-membrane frac-

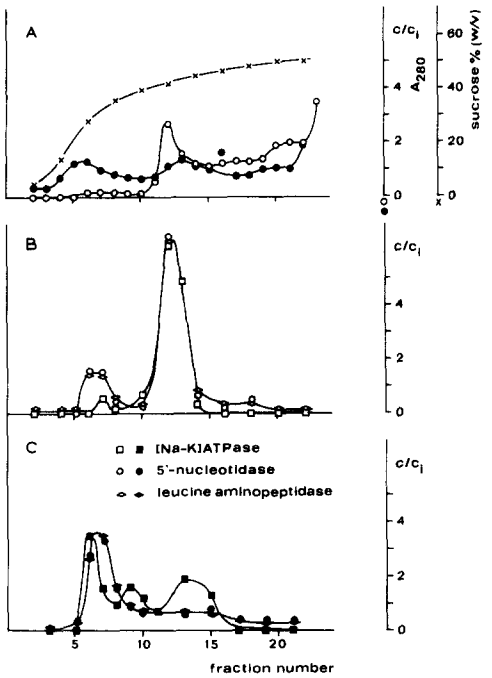

Fig. 3. Enzyme-distribution patterns after density-gradient centrifugation (17—18 h, 50 000 × g_{av}) of a nuclear (10 min, 1100 × g) fraction from rat liver. The nuclear fraction is purified by centrifugation in a discontinuous gradient of sucrose as described in Materials and Methods. The 41/49% (w/v) sucrose interface fraction is applied either directly in the middle of the gradient (control, open symbols), or after dilution with 2.4 vols. Hepes-KOH (pH 7.5), centrifugation for 60 min at 100 000 × g and resuspension in homogenization buffer by means of a syringe fitted with a needle of 1.6 mm internal diameter (closed symbols). The c/c_i value is defined in the legend of Fig. 1. (A) X———X, distribution of sucrose; ○———○ and ●———●, 280 nm absorbance; (B,C) □———□ and ■———■, ($Na^+ + K^+$)-ATPase activity; ○———○ and ●———●, 5'-nucleotidase activity; ◇———◇ and ◆———◆, leucine aminopeptidase activity.

tion obtained in our procedure, therefore, appears to be of mixed canalicular and contiguous origin and is transformed in bile-canalicular plasma-membrane vesicles and contiguous plasma-membrane complexes during resuspension (see Materials and Methods).

Discussion

The purpose of our study was to determine whether the sodium-dependent alanine uptake activity is homogeneously distributed along the plasma membrane of the hepatocyte, or concentrated in a distinct part of this membrane. In our previous studies [11,12] we used a crude liver plasma-membrane fraction to study the characteristics of L-alanine transport without discriminating between the plasma-membrane vesicles as to their subcellular origin.

The distribution patterns observed after density-gradient centrifugation of the heavy microsomal fraction (Fig. 1) show that a strong correlation exists between sodium-dependent alanine transport and ($Na^+ + K^+$)-ATPase, but not with other marker enzymes. This experiment not only confirms our previous

conclusion that the sodium-dependent alanine transport activity is located in the liver plasma membrane [11], but also suggests that it is located in a specific part of this membrane. Further support is obtained from the results shown in Fig. 2. An identical distribution is observed for the activities of sodium-dependent alanine transport, $(Na^+ + K^+)$-ATPase and glucagon-stimulated adenylate cyclase, whereas another group of enzymes located in the plasma membrane shows a completely different distribution. The first two plasma-membrane marker enzymes are thought to be associated with the sinusoidal plasma membrane, whereas the second group is postulated to represent the bile-canalicular plasma membrane [26,28]. A complicating factor might be the presence of the contiguous plasma membrane in the canalicular-contiguous fraction. Whereas the sinusoidal and the canalicular part of the plasma membrane easily vesiculate upon prolonged homogenization (Refs. 13 and 29; Fig. 3), the rigidity of the contiguous membrane prevents vesiculation [29], thus making alanine transport studies impossible. In any case, the presumed low activity of the plasma-membrane marker enzyme in the contiguous plasma membrane [13,23] precludes strong conclusions with regard to the properties of this part of the membrane. Furthermore, the experiments on the rehomogenization of the canalicular-contiguous fraction (Fig. 3) illustrate the fragility of this fraction. Its repercussions on the variability of the values obtained for the canalicular and canalicular-contiguous fractions are evident in the data shown in Fig. 2. From our results, in combination with results of Blitzer and Boyer [27] and Carey and Evans [28], we conclude that the sodium-dependent alanine transport activity is mainly, if not exclusively, located in the blood-sinusoidal plasma membrane.

Recently, it has been suggested that there are differences between parenchymal cells in the central and in the peripheral portions of the liver lobule [30]. However, it is not clear whether such differences would have consequences for the function and/or structure of the cell membrane of the various parenchymal cells.

Rat hepatocytes, isolated by collagenase perfusion, have been frequently used in the study of amino acid transport [1—4]. The question arises as to whether the heterogeneous localization of sodium-dependent alanine transport, as demonstrated in this paper, is still preserved in isolated liver cells. This question becomes even more important in the study of the uptake of bile acids in isolated hepatocytes [31,32] where an uptake at the sinusoidal surface may be accompanied by a concomitant release at the bile surface. Further studies to elucidate this point are in progress in our laboratory.

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