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PURIFICATION OF PLASMA MEMBRANES OF RAT LIVER. APPLICATION OF ZONAL CENTRIFUGATION TO ISOLATION OF CELL MEMBRANES

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SUMMARY

1. Subcellular components of rat liver were separated with the zonal centrifuge. The distribution of mitochondria, lysosomes, and endoplasmic reticulum was monitored by analysis of fractions for succinate dehydrogenase (EC 1.3.99.1), acid phosphatase (EC 3.1.3.2), and glucose-6-phosphatase (EC 3.1.3.9), respectively. 5'-Nucleotidase (EC 3.1.3.5) was used to measure the distribution of plasma membrane among the subfractions.

2. Fractionation in a density gradient of sucrose resulted in separation of 5'-nucleotidase with two general classes of particles, one of relatively low sedimentation rate, and the other of high sedimentation rate. Whereas more rapidly sedimenting 5'-nucleotidase-rich membranes were free of glucose-6-phosphatase, the slowly sedimenting particles were coincident with glucose-6-phosphatase activity.

3. Plasma membranes were isolated by a combination of rate-zonal and isopycnic centrifugation.

INTRODUCTION

Fractions of plasma membrane prepared from the nuclear sediment of rat liver have been the subject of several studies[1-4]. Isolation of these fractions requires several differential-sedimentation steps to remove most of the other cell structures. The very low yield of plasma membranes in these fractions, as well as indications of cross-contamination by other organelles[5] make it necessary to pursue additional avenues for isolation and purification of plasma membranes. The utility of a pure preparation of membranes for the study of antigen content and distribution, as well as for other studies such as membrane structure and transport of biologically active substances is obvious.

The zonal centrifuge has been found to be a useful instrument for separating subcellular particles of liver[6]. Because of the large volume which zonal rotors can accommodate, it is possible to use a large amount of starting sample and thereby increase the quantity of end-product. This use of a large volume of fluid should also

Abbreviation: INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride.

reduce the need for repeated washing of a fraction to free it of particles having similar sedimentation velocities.

The identification of plasma membranes in subcellular fractions is contingent on a marker, either biochemical, enzymatic, antigenic, or ultrastructural. The presence of desmosomes in membranes isolated from the crude nuclear fraction of rat liver provides proof that such fractions contain plasma membranes[1,3,4]. It has been shown histochemically that 5'-nucleotidase is located primarily at the plasma membrane in rat-liver cells[7].

The present study was undertaken in an effort to establish the localization of plasma membranes in subfractions of rat-liver homogenates and to develop more effective methods of isolating plasma membranes. Fragments of plasma membrane of rat-liver cells were followed quantitatively during subcellular fractionation, utilizing 5'-nucleotidase (EC 3.1.3.5) as a putative marker. The presence of mitochondria, lysosomes, or endoplasmic reticulum in various fractions was detected by assaying for succinate dehydrogenase (EC 1.3.99.1), acid phosphatase (EC 3.1.3.1), and glucose-6-phosphatase (EC 3.1.3.9), respectively.

MATERIALS AND METHODS

Initial fractionation of liver

The livers of adult male and female albino rats (Sprague–Dawley strain, 250–300 g body wt.) were obtained after fasting for 12 h. Livers were perfused *in situ* through the portal vein with 5 mM Tris buffer (pH 7.6) containing 0.08 M sucrose at 4°. All subsequent handling of liver during homogenization and fractionation was at 4°.

The liver was cut into several pieces and pressed through a net of stainless-steel wire. The fibrovascular skeleton which was retained on the wire was discarded. The pulp was collected, weighed, and then homogenized by hand in a glass homogenizer with a teflon-coated pestle, until more than 90 % of the cells were broken, using 9 ml of perfusion medium per g wet wt. of liver. The homogenate was centrifuged at 1000 × *g* for 10 min, and subsequently at 20000 × *g* and 105000 × *g* as indicated in the text. The resulting pellets were suspended in 1 mM Tris (pH 7.6) for protein and enzyme analysis.

Isolation of subcellular fractions by zonal centrifugation

A suitable continuous density gradient was established in a BXV rotor (1660 ml, prototype: Oak Ridge National Laboratories) using a gradient pump (Beckman Instruments). In most experiments the gradient was formed from solutions of 20 and 55% (w/w) sucrose containing 5 mM Tris (pH 7.6). The structure of the density gradient was changed from this for a few experiments, either by reshaping the pump cam or by using different stock solutions of sucrose. 40 ml of the sample were applied to the gradient, followed by 5 mM Tris (pH 7.6) to give a sample *plus* buffer volume of 120 ml. An integrator for measuring total centrifugal force ($\omega^2 t$) was used (supplied by the Oak Ridge National Laboratory). The speed and period of centrifugation were varied to observe differences in separation patterns. 40-ml fractions were collected after centrifugation by displacing the gradient with 55% sucrose. The effluent was monitored at 260 and 280 nm using a Beckman DU spectrophotometer. Density measurements were made on each fraction with a refractometer (20°). The fractions were

concentrated by dilution with equal volumes of deionized water followed by centrifugation at 20000 × *g* for 20 min in a Servall SS-34 rotor. The pellets were each brought to a volume of 2 ml in Tris–sucrose medium for enzyme and protein analysis.

Equilibrium density-gradient centrifugation

In some of the experiments a part of the material from selected fractions was placed on a continuous density gradient of sucrose prepared in 5- or 30-ml tubes and centrifuged at 63000 × *g* or 130000 × *g*, for 2 h using Spinco rotors SW-25.1 or SW-39, respectively. Sampling of resultant bands for enzyme and protein analysis was made using a j-shaped pipette or by collecting sequential fractions resulting from displacement of the gradient with 55 % sucrose.

Enzyme assays

All assays were modified to the use of small sample volumes in order to extend the number of tests possible on each fraction. Enzyme velocities were determined on the basis of 15 min of incubation with the appropriate substrate at 37°. Where activities were low, the time of incubation was extended to 40 min. Reactions were terminated by the addition of cold 10 % trichloroacetic acid in a volume equal to that of the reaction mixture. The precipitate in trichloroacetic acid-treated reaction mixtures was removed by centrifugation at 1000 × *g* for 5 min. Three of the assays, acid phosphatase, 5'-nucleotidase, and glucose-6-phosphatase, involved the release of inorganic phosphate from substrate. Activities for these enzymes were expressed as μmoles of inorganic phosphate released per 40 ml of zonal fraction per min, or as μmoles of inorganic phosphate released per mg protein per min. Corrections were made for the presence of inorganic phosphate formed in the absence of substrate, and phosphate released due to non-enzymic hydrolysis of substrate. An appropriate dilution of tissue fractions was made before enzyme analysis, based on the range of activity which was expected in each fraction.

5'-Nucleotidase was determined with disodium AMP as substrate. Reaction mixtures contained 5 mM AMP, 0.01 M Mg^{2+}, 0.1 M glycine buffer (pH 8.5) and tissue fraction[8]. For the assay of glucose-6-phosphatase the disodium salt of glucose 6-phosphate was used at 0.01 M in the reaction mixture. The reaction was carried out at pH 6.0 in 0.1 M succinate–acetate buffer. Control mixtures were prepared by combining an aliquot of each mixture with an equal volume of 10 % trichloroacetic acid immediately after addition of enzyme to the buffered substrate. Acid phosphatase was determined at pH 5.0 (0.05 M acetate) using 0.05 M β-glycerophosphate as substrate. Succinate dehydrogenase was assayed by measuring the formazan formed from reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT)[9]. The reaction mixtures contained 0.05 M succinate, 0.01 % INT, 0.1 M Tris–HCl (pH 8.0) and tissue fraction. The formazan of INT was measured at 490 nm after extraction into ethyl acetate.

Chemical analyses

Inorganic phosphate was assayed by a micro version of the method of FISKE AND SUBBAROW[10]. A Coleman spectrophotometer with a microcell having a 1-cm light path was used at 650 nm. Protein was measured by the ninhydrin method[11], with albumin standards.

RESULTS

Initial fractionation

By examining liver samples with a phase-contrast microscope during the course of homogenization it was possible to decide when most of the cells were broken, and large membrane fragments could be seen.

A hypotonic medium (0.08 M sucrose, pH 7.6) was chosen for preparing the liver homogenate to exploit the osmotic damage to cells and get maximum cell breakage with a minimum of mechanical trauma. Use of this medium rather than 1 mM bicarbonate buffer appeared to increase the yield of membrane, and most of the nuclei remained intact.

Scattered throughout the homogenate were occasional slender elongated structures which resembled the 'y', 'v', and 'L' configurations described by NEVILLE[1]. The majority of such membrane structures, interpreted as plasma membranes by phase contrast, sedimented after 10 min at 1000 × *g*. Occasional membranes were found in the supernatant of the 1000 × *g* pellet.

As is indicated in Table I, 8–15 % of the total amount of succinate dehydrogenase, glucose-6-phophatase, and acid phosphatase was recovered in the initial pellet after 10 min centrifugation at 1000 × *g*. This fraction was enriched with 5'-nucleotidase, the putative marker for plasma membrane, as shown by its content of 23 % of the activity of whole homogenate.

Examination of the pellet obtained after 30 min centrifugation of the 1000 × *g* supernatant material at 20000 × *g* revealed that one half of the total 5'-nucleotidase activity was associated with this fraction. However, a similar percentage of the total succinate dehydrogenase and 74 % of the total glucose-6-phosphatase activity were found in the 20000 × *g* pellet. The supernatant material from the 20000 × *g* sedimentation step was then brought to 105000 × *g* for 1 h. The resultant pellet and supernatant fluid each contained less than 10 % of the total activity of each of the four enzymes, with the exception that 29 % of the total acid phosphatase was located in the soluble fraction (supernatant of 105000 × *g*). The low ionic strength of the homogenizing medium was sufficient to cause moderate disruption of lysosomes.

It was concluded from the results of analysis of the initial fractions that further separation procedures using the 1000 × *g* sediment might be productive, since there

TABLE I

FRACTIONATION OF RAT LIVER

Values listed are the mean of activities obtained for each fraction in 4 separate experiments.

Fraction	*5'-Nucleotidase*		*Succinate dehydrogenase*		*Glucose-6-phosphatase*		*Acid phosphatase*	
	μmole/min per mg	*Total* (%)	*μmole/min per mg*	*Total* (%)	*μmole/min per mg*	*Total* (%)	*μmole/min per mg*	*Total* (%)
Whole homogenate	0 08	100.0	0.23	100.0	0 07	100.0	0.13	100.0
1000 × *g* sediment	0.20	23.0	0 15	8.9	0.08	14 4	0 10	11.2
20000 × *g* sediment	0 27	50.1	0.55	55.7	0 24	74.0	0.31	52.1
105000 × *g* sediment	0 13	5.7	0.04	0.9	0.09	6.4	0.13	5.6
105000 × *g* supernatant	0.02	8.2	0.03	7.0	0.01	6 9	0.08	29.1

was enrichment of this fraction with 5'-nucleotidase and membranes, while mitochondria, lysosomes, and endoplasmic reticulum were not concentrated in this fraction.

Zonal fractionation

At first a continuous density gradient was established in the BXV rotor by pumping from stock solutions of 20 and 55% (w/w) sucrose. The resulting gradient was one of gradually increasing density from 1.08 to 1.25 g/ml.

The sample applied to the density gradient was the 1000 × *g* sediment prepared as described above from 20 g of rat liver. After centrifugation to $7.8 \cdot 10^8\ \omega^2 t$ (4200 rev. per min, 1 h) the contents of the zonal rotor were removed by displacement with 55% sucrose, and fractions of 40 ml each were collected. Nuclei and any other structures which had come to rest against the rotor wall were collected on disassembly of the rotor.

On the basis of established sedimentation properties of mitochondria, lysosomes, and endoplasmic reticulum it was expected that under the above conditions there would be migration of mitochondria into the gradient, but little migration of lysosomes and endoplasmic reticulum. From the preliminary studies described above on the content of fractions obtained at different sedimentation velocities it was expected that plasma membranes would move into the density gradient more rapidly than mitochondria.

The results of analysis of the zonal fractions recovered are given in Figs. 1a and 1b. By phase-contrast examination plasma membranes were found predominantly in Tubes 8–16. However, these tubes also contained a large quantity of mitochondria, detected by phase-contrast study as well as by determination of succinate-dehydrogenase activity. There was a biphasic distribution of 5'-nucleotidase in fractions having a density below 1.18 g/ml. That peak of activity in fractions at a density around 1.16 g/ml was associated with large membrane casts interpreted as plasma membranes.

That these membranes had approached isopycnic density was determined in other experiments by ultracentrifugation of membrane fractions in a continuous density gradient of sucrose (SW-25 rotor, Beckman Spinco).

As shown in Fig. 1a the greater part of the 5-'nucleotidase in zonal fractions was associated with structures having a lower sedimentation velocity. No conclusion

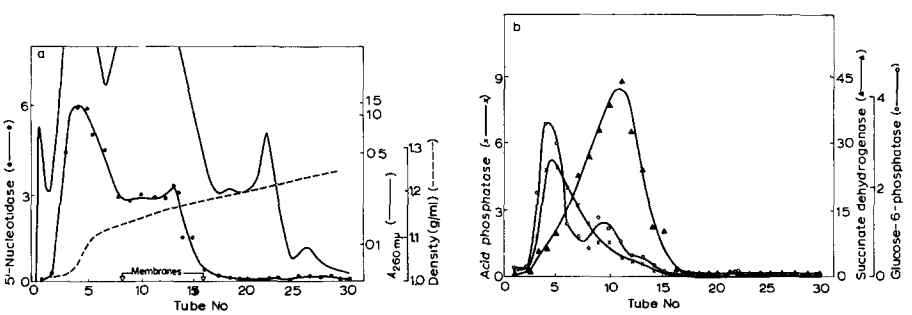

Fig. 1. Zonal fractionation of nuclear sediment from 20 g of liver. Centrifugation was to $\omega^2 t = 7.8 \cdot 10^8$ (4200 rev./min, 1 h, BXV rotor). The sucrose gradient had a density spread of 1.08–1.25 g/ml. Enzyme units are given as μmoles P_i or formazan per 40 ml per min. a. Absorbance, density, and 5'-nucleotidase profiles of zonal fractions. b. Profiles of acid phosphatase, succinate dehydrogenase, and glucose-6-phosphatase in zonal fractions.

could be made from the initial zonal fractionation as to whether the 5'-nucleotidase segregating with the more slowly sedimenting structures was due to small pieces of plasma membrane or whether it might be due to other organelles, such as endoplasmic reticulum or lysosomes.

5'-Nucleotidase was also found in zonal fractions containing predominantly nuclei. Addition of total 5'-nucleotidase values for all of the zonal fractions *plus* nuclei and accompanying material from the rotor wall gave a total recovery value of 54.6 μmoles inorganic phosphate released per min. Of this total 5.4 μmoles were attributable to structures having a density greater than 1.22 g/ml. Membrane structures were visable in the high-density fractions by phase-contrast examination, but no attempt was made to separate these from nuclei, since sedimentation velocities and density properties of such membranes and nuclei were similar.

Although enzyme analysis indicated a difference in distribution of various organelles after zonal fractionation under the conditions described above, there was considerable overlap of endoplasmic reticulum, lysosomes, and mitochondria (Fig. 1b). The peak concentration of mitochondria in effluent from the rotor was reached before the peak of phase-contrast-visible membranes, indicating a higher rate of sedimentation for membranes.

One major objective of these experiments was to use the zonal centrifuge as a preparative device for the isolation of plasma membranes in large quantity. Since at this point it seemed that a single passage through the zonal centrifuge might not adequately free plasma membranes from other subcellular constituents, it became important to know if further separation of particles might be accomplished by a second passage of zonal fractions through additional density gradients. It was also of interest to know if freezing and thawing would have a significant effect on partly separated fractions. Purification procedures would be simplified if processing the tissue derivatives could be done in large quantities and in stages where a long delay period might make temporary storage in the frozen state desirable.

Consequently, that part of selected fractions from the initial zonal separation which was not used for analysis was subjected to a second zonal fractionation. The material from the original zonal fractionation was collected by dilution with deionized water, and the pellet from centrifugation for 30 min at 20000 × g was resuspended and frozen until it was convenient to perform the second zonal run.

The fractions of the initial zonal separation which were of immediate interest were those containing plasma membranes visible under phase contrast. A sample obtained by pooling material from Tubes 11–16 (Fig. 1) was diluted to 40 ml and applied over a gradient of identical composition to that for the initial zonal run (1.08–1.25 g/ml, $7.8 \cdot 10^8$ $\omega^2 t$).

A profile of 5'-nucleotidase and succinate-dehydrogenase activities in these fractions is shown in Fig. 2. There was a clear separation of 5'-nucleotidase activity into two peaks. The more rapidly sedimenting membranes located at a density of 1.16 g/ml, identical to the position which these structures attained in the initial zonal run. The more slowly sedimenting structures giving the second peak of 5'-nucleotidase were barely visible by phase contrast. It is presumed that these structures are small fragments of plasma membrane that derived from larger fragments by freezing and thawing, and the additional handling necessitated by a second zonal fractionation.

Mitochondria were found in fractions of the second run which were intermediate

between fractions giving the two peaks of 5′-nucleotidase activity. There was some contamination of fractions of each of the 5′-nucleotidase peaks by mitochondria. Glucose-6-phosphatase and acid-phosphatase activities were reduced in fractions of the second zonal run relative to 5′-nucleotidase and succinate dehydrogenase. Glucose-6-phosphatase was detectable in small quantity in fractions which also contained 5′-nucleotidase. Acid phosphatase was present but barely detectable in fractions which contained succinate dehydrogenase as well as 5′-nucleotidase.

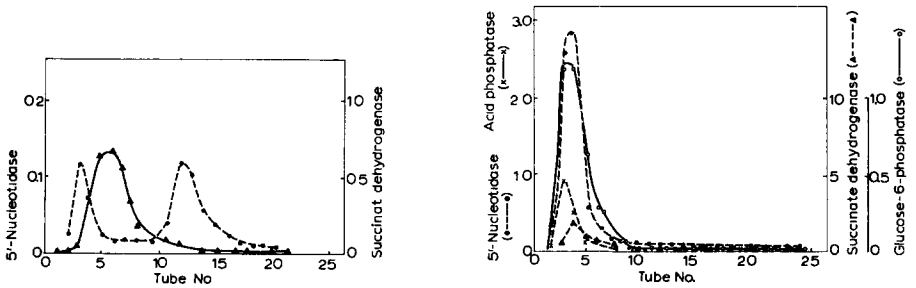

Fig. 2. Fractionation of partially purified liver plasma membranes by recycling pooled membrane-rich zonal fractions on a second zonal density gradient. Equal parts of material from Tubes 11–16 (Fig. 1) of the first zonal separation procedure were pooled and applied to a second density gradient (1.08–1.25 g/ml, BXV rotor). Centrifugation was to $\omega^2 t = 7.7 \cdot 10^8$ (4200 rev./min, 1 h). Enzyme activities (μmoles P_i or formazan per 40 ml per min) were low because of the limited amount of sample used for fractionation.

Fig. 3. Fractionation of partially purified liver by recycling pooled fractions having slowly sedimenting particles with high 5′-nucleotidase activity. Equal parts of material from Tubes 3–7 (Fig. 1) of the first zonal separation procedure were pooled and applied to a second density gradient (1.08–1.25 g/ml, BXV rotor). Centrifugation was to $\omega^2 t = 7\ 8 \cdot 10^8$ (4200 rev./min, 1 h). Enzyme activities are expressed as μmoles P_i or formazan per 40 ml per min.

A second zonal fractionation was performed on previously frozen fractions which contained the more slowly sedimenting structures that were associated with 5′-nucleotidase in the initial zonal fractionation (Tubes 3–7, Fig. 1). The density gradient and total centrifugal force used for this experiment were identical to that for the two zonal fractionations described above. Results of analysis of the fractions obtained are shown in Fig. 3.

The amount of 5′-nucleotidase relative to glucose-6-phosphatase was similar in the first few fractions of the second zonal run as in the first few fractions of the initial zonal run. Notably absent was any indication of a biphasic distribution of 5′-nucleotidase, in contrast to the two major peaks of 5′-nucleotidase which were found in the initial zonal run. None of the rapidly sedimenting plasma membranes were seen in the sample as applied to the gradient, nor were any such structures seen in subfractions. Whereas acid phosphatase approached a similar level of activity to 5′-nucleotidase in the first few fractions of the initial zonal run, the relative levels of acid phosphatase in the first few fractions of the secondary run were much lower. This finding indicated that 5′-nucleotidase activity was independent of acid phosphatase. The reduction in acid phosphatase most likely was a result of loss due to disruption of lysosomes on freezing and thawing. As was expected, succinate dehydrogenase in the fractions of the second zonal run was minimal, since relatively few mitochondria were in the starting sample. No zone of particles was found deeper in the gradient.

The absence of such a zone of rapidly sedimenting particles was taken as evidence that no extensive aggregation of particles had occurred due to freezing of the sample prior to the second fractionation.

In these experiments it was shown that 5'-nucleotidase was associated with structures having widely different sedimentation velocities, as well as densities. 5'-Nucleotidase was demonstrated to be independent of succinate dehydrogenase and acid phosphatase, markers for mitochondria and lysosomes. Its independence of glucose-6-phosphatase was not certain. 5'-Nucleotidase was found to be associated with a class of rapidly sedimenting membrane structures, interpreted as plasma membranes. These located at a density of 1.16–1.18 g/ml in a density gradient of sucrose and were easily detected by their appearance on examination with a phase-contrast microscope.

Thereafter, attention was given primarily to isolation of the membranes having a density of 1.16–1.18 g/ml in sucrose wihch were detectable by a phase-contrast examination.

Because of the broad overlap of mitochondria and membranes which occurred in the initial zonal run at $7.8 \cdot 10^8$ $\omega^2 t$, it appeared that reduction of centrifugal force might effect better separation of membranes from mitochondria by preventing mitochondria from moving as far into sucrose gradient. To evaluate this a similar density gradient was prepared, but the total centrifugal force was reduced to $2.0 \cdot 10^8$ $\omega^2 t$. The sample used for fractionation in this case was nuclear sediment from 10 g of liver.

Results of analysis of fractions obtained at $2.0 \cdot 10^8$ $\omega^2 t$ are given in Fig. 4. In this experiment plasma membranes sedimented slightly ahead of mitochondria. As in the experiment performed at $7.8 \cdot 10^8$ $\omega^2 t$, membranes reached a density near 1.16 g/ml. Although the zone covered by mitochondria was not as broad at $2.0 \cdot 10^8$ $\omega^2 t$ as at $7.8 \cdot 10^8$ $\omega^2 t$, there was still a large quantity of mitochondria in membrane fractions.

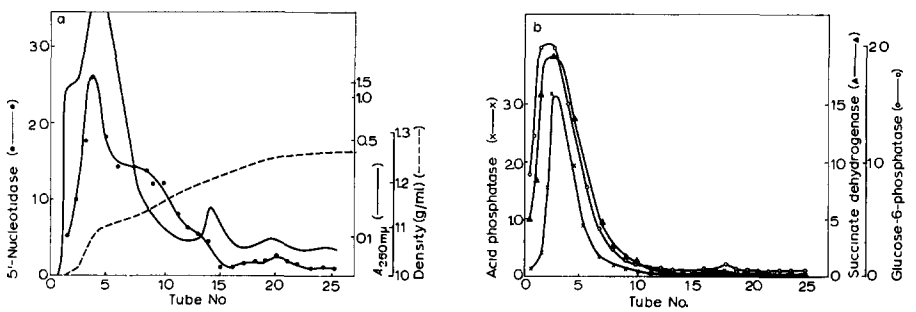

Fig 4 Zonal fractionation of nuclear sediment from 10 g of liver. A continuous density gradient of sucrose ranging from 1.08 to 1.25 g/ml was used in the BXV rotor Centrifugation was to $\omega^2 t = 2.0 \cdot 10^8$. Enzyme units are μmoles P_i or formazan per 40 ml per min.

In Tubes 7–13 where membranes were abundant, the amount of 5'-nucleotidase recovered per mg protein was much greater than in other fractions in this run. The specific activity was greatest in Tube 10, being 1.6 μmoles inorganic phosphate per min per mg. Occasional nuclei and damaged cells could be found in membrane fractions. Erythrocytes which had not been completely removed by perfusion of the liver

located at a density around 1.20 g/ml in Tubes 14–15. Many nuclei were recovered in Fractions 18–25. It was concluded that the use of a gradient with a spread of 1.08–1.25 g/ml was not optimal at $2.0 \cdot 10^8$ $\omega^2 t$, since there was contamination of membrane fractions at each side of the membrane zone by other structures.

In an attempt to isolate the plasma membranes free of other organelles a density gradient was prepared which would let membranes move as far as possible before approaching isopycnic density, thus allowing the differences in sedimentation velocities of mitochondria and membranes to have maximum effect. This gradient was established to give a density range between 1.08 and 1.15 g/ml, with 55 % sucrose at the periphery of the rotor. A total centrifugal force of $2.0 \cdot 10^8$ $\omega^2 t$ was chosen in order to limit the movement of mitochondria into the density gradient.

In Fig. 5 are given the results of zonal fractionation of the nuclear sediment of homogenate from 10 g of rat liver under the newly devised conditions. After 25 min, at 3000 rev./min, plasma membranes were identifiable in fractions over a wide range of the gradient (Tubes 8–23). Most of the membranes had not achieved their isopycnic density position. Although most of the total 5′-nucleotidase activity was accounted for in the first seven zonal fractions, the enzyme was detected in most fractions. Calculation of specific activity (Fig. 5) showed the greatest activity per unit protein in fractions with membranes. A determination of specific activity was not obtained for Tubes 14–27 because of the limited quantity of material recovered from these fractions. Plasma membranes by phase contrast were in greatest abundance in Tubes 8–13.

Many nuclei had penetrated the 55 % sucrose cushion at the periphery of the rotor, coming to rest against the rotor wall. Some nuclei remained in Fractions 28–32, where the density increased greatly over the preceding fractions. Erythrocytes which had not been removed during perfusion of the liver obtained a density position slightly less than nuclei and located in a narrow zone (Tubes 28–29). None of the four enzymes showed an increased activity in fractions which contained erythrocytes.

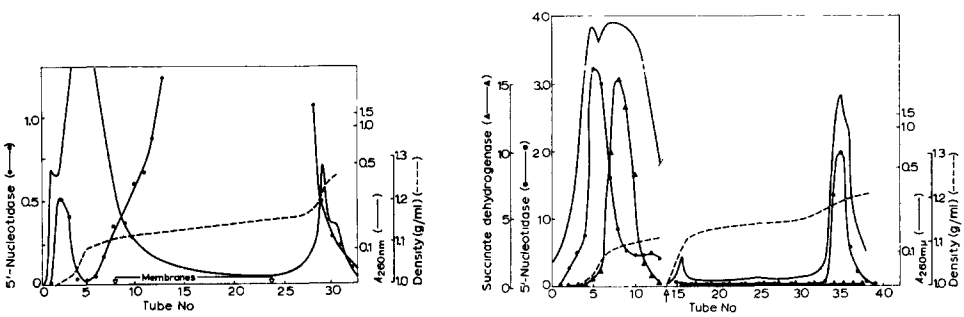

Fig. 5. Zonal fractionation of nuclear sediment from 10 g of liver, using a gradient of lowered density (1.08–1.15 g/ml). Centrifugation was to $2.0 \cdot 10^8$ $\omega^2 t$ (BXV rotor). The specific activity for 5′-nucleotidase (μmoles P_i per mg per min) obtained for each tube is plotted, except for Tubes 14–27 where the quantity of material recovered was so small that protein values were not determined.

Fig. 6. Isolation of plasma membranes by a combination of rate and isopycnic zonal centrifugation. The nuclear sediment from 10 g of liver was fractionated using a gradient of sucrose having a range of 1.08–1.15 g/ml. 40-ml fractions were collected at two separate stages (↑ indicates point of interruption for first unloading of rotor). Fractions 1–13 were obtained after $2.0 \cdot 10^8$ $\omega^2 t$ while Fractions 14–39 were obtained after centrifugation of the unremoved material to $\omega^2 t = 1.6 \cdot 10^{10}$. Enzyme units are μmoles of formazan per 40 ml per min.

In the experiments described above it appeared that separation of mitochondria from membranes on the basis of sedimentation differences alone would not be feasible using the zonal centrifuge. Therefore, separation based on a combination of sedimentation rate and isopycnic density differences was attempted (Fig. 6). To accomplish this a two-stage centrifugation was performed (N. G. ANDERSON, personal communication). The first stage consisted of partial separation of components of the 1000 $\times$ g sediment at low speed (3000 rev./min until $\omega^2 t = 2.0 \cdot 10^8$), as described above using a sucrose gradient with a density spread of 1.08–1.15 g/ml. At $\omega^2 t = 2.0 \cdot 10^8$ a volume of 520 ml was removed from the rotor by displacement with 55 % sucrose (Tubes 1–14). The displaced fluid included most of the mitochondria, lysosomes, and endoplasmic reticulum contained in the sample introduced into the zonal centrifuge. However, some mitochondria were retained in the rotor, because further displacement of fluid resulted in considerable loss of plasma membranes. After displacement of the more slowly sedimenting particles, the speed was increased to 20000 rev./min until $\omega^2 t = 1.6 \cdot 10^{10}$ (1 h). The entire contents of the rotor were then unloaded.

The membranes were collected as a sharp peak at 1.18–1.19 g/ml. Erythrocytes were found at 1.20 g/ml. A few mitochondria which were not completely removed in the first unloading step were detected microscopically in Fractions 34–35, which contained the greatest quantity of membranes. These mitochondria could be effectively removed by dilution and collection of membranes by sedimentation at 750 $\times$ g for 10 min.

The method of isolation of membranes based on differential sedimentation and isopycnic centrifugation was adopted as that most suitable for obtaining membranes in quantity. The yield of membranes by this procedure was 2.3 mg as protein from 10 g of fresh liver. The plasma membranes thus obtained were free of nuclei, endoplasmic reticulum, lysosomes, and mitochondria. There was a 10–20-fold increase in specific activity of 5′-nucleotidase in these membranes as compared to the activity of this enzyme in whole homogenate of liver.

DISCUSSION

Isolation of a single class of organelles from the cell is complicated by the fragmentation of such organelles during processing. Isolation of plasma membranes from hepatic cells is even more difficult than with some other types of cells because of the greater quantity of cytoplasmic structures present in hepatic cells. The isolation of a plasma membrane fraction from hepatic cells by NEVILLE[1] stimulated subsequent investigators to study membranes prepared by similar methods.

EMMELOT *et al.*[3] found several enzymes which were concentrated in membranes prepared by a modification of the method of NEVILLE. These enzymes, *i.e.*, 5′-mononucleotidase, NAD^+ pyrophosphatase (EC 3.6.1.9), Mg^{2+}-ATPase (EC 3.6.1.4), and (Na^+-K^+-Mg^{2+})-ATPase are also shared by the microsomal fraction, which is composed mostly of endoplasmic reticulum. The question which must eventually be answered deals with the extent to which such sharing of enzymes by various subcellular fractions is a result of contamination of a particular fraction by organelles which are found predominantly in another fraction.

Histochemical studies by light and electron microscopy have indicated that 5′-nucleotidase is localized to the plasma membrane, specifically where the membrane

folds to form the microvilli of bile canaliculi and to a lesser extent where microvilli are exposed to the blood sinusoids[7]. Other data obtained from the study of subcellular fractions of rat liver prepared by differential centrifugation have shown that 5'-nucleotidase activity is located in the nuclear and microsomal fractions[12,13]. The 5'-nucleotidase in the nuclear fraction appears to be due to contamination by relatively large structural components of the cell which probably have their origin from that part of parenchymal cells in contact with bile canaliculi[1]. Very little or no 5'-nucleotidase is present in rat-liver nuclei isolated free from other structures[14].

The segregation of 5'-nucleotidase with subcellular particles which separate by rate-zonal centrifugation into at least two zones using a density gradient of sucrose confirms the results of EL-AASER *et al.*[15]. One of the zones of activity obtained as reported herein was associated with fractions which contained an abundance of rough endoplasmic reticulum as seen by electron microscopy. The second zone contained plasma membranes identifiable by phase-contrast and electron microscopy. The presence of desmosomes in plasma membrane fractions confirmed the identity of these membranes with surface membranes of hepatic epithelial cells.

With freezing and thawing of membranes there appears to be partial breakage into smaller fragments having reduced sedimentation rate (Fig. 2). That plasma membranes may undergo further breakage after homogenization is complete, was suggested in NEVILLE's observation[1] of the disappearance of 'y', 'v', and 'L' configurations during the final stages of membrane isolation.

With the methods described here fractions have been obtained which have at least a 15-fold increase in specific activity of 5'-nucleotidase when compared to whole homogenate of liver. In density gradients of sucrose some of these membranes have an equilibrium density of 1.16–1.19 g/ml. A significant quantity of membranes having 5'-nucleotidase activity obtain a density in sucrose greater than 1.19 g/ml, while some membranes obtain a density less than 1.16 g/ml.

If computed in terms of 5'-nucleotidase activity about one fourth of the total plasma membrane in whole homogenate of rat liver is recoverable in the unwashed nuclear sediment. Of this at least one half consists of slowly sedimenting structures which in these experiments were not separated from glucose-6-phosphatase activity. About 20–25 % of that 5'-nucleotidase in the 1000 $\times$ *g* sediment is recoverable as plasma membrane which resembles that isolated according to the method of NEVILLE. The yield of slightly above 2 mg membrane as protein by zonal fractionation is similar to yields reported by others using modifications of the method of NEVILLE[14]. This represents only 5 % of the total 5'-nucleotidase in whole homogenate. The remaining 20 % of 5'-nucleotidase from the 1000 $\times$ *g* sediment assumes a density in sucrose greater than 1.25 g/ml under the conditions for zonal fractionation used here. It is likely that at least a part of the 5'-nucleotidase that was not separated from nuclei in these experiments was due to entrapment of plasma membrane by ruptured nuclear particles and residual connective tissue fibers[14].

By zonal centrifugation a relatively large quantity of tissue homogenate can be processed for purification of plasma membrane. A convenient method combining differential centrifugation and separation based on equilibrium density has been adopted for preparation of membranes. These membranes can be obtained free of apparant contamination by other subcellular organelles.

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