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COMPARATIVE STUDIES ON ENZYME MARKERS OF LIVER PLASMA MEMBRANES

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SUMMARY

1. We found that the standard isolation procedures for the plasma membrane fraction, as established for rat liver, cannot necessarily be used for other species without modifications.

2. Liver cell fractions from the rat, guinea pig, rabbit and cat were prepared by sucrose density gradient centrifugations. The activities of 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5), nucleotide pyrophosphatase (dinucleotide nucleotidohydrolase, EC 3.6.1.9), *p*-nitrophenylphosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) and leucyl-β-naphthylamidase (L-leucyl-peptide hydrolase, EC 3.4.1.1) were assessed as "characteristic" membrane markers.

3. There was a wide species variation in the specific activities of the different enzymes. Furthermore, the activity ratios of these enzymes could not be used to define a subcellular fraction as "characteristically plasma membrane".

4. We have concluded that plasma membranes could be isolated from the guinea pig liver with no modification to the methods usually employed for rat liver. Purified membrane fractions could not be isolated from rabbit or cat liver under the same conditions.

INTRODUCTION

"Isolated plasma membrane" preparations from mammalian tissues usually are characterized by their morphology and by biochemical analyses. Lipid composition is somewhat characteristic, but the specific activity of "marker enzymes" is reported most commonly in the characterization of plasma membrane preparations. Substantial variations have been reported, however, for these enzymic activities, not only from one species to another and from one organ to another, but also from one laboratory to another. Therefore, we have addressed part of our studies to the following questions: (1) Are certain enzymes characteristic of plasma membranes to the exclusion of all other subcellular elements? (2) Since many cells exhibit a polarity with respect to function and morphology, what are the elements (as biochemical information) which are common to all the surfaces of the cell? (3) Are some of the surfaces isolated preferentially by any particular preparative procedure? (4) Can any isolation procedure, proven successful in one species, be utilized in

other species? (5) Granting substantial species variation in enzymic activities, could the activity ratios for various marker enzymes be considered characteristic? (6) Can preparative procedures be devised for membrane isolation in which the "marker enzymes" are solely used as guides?

In order to reduce the variables, we have maintained as many factors as possible constant. We have restricted ourselves here to the use of the liver as a membrane source. We have used the same enzymatic methods throughout, and we have used widely accepted procedures for isolation of plasma membranes without adaptations.

MATERIALS AND METHODS

Source of materials

AMP, glucose 6-phosphate, and L-leucyl-β-naphthylamide: Sigma Chemical Co.; NADH: P. and L. Biochemicals, Inc.; *p*-nitrophenylphosphate (disodium salt): Nutritional Biochemicals Corp., converted to di-Tris salt by ion-exchange column chromatography; 2-amino-2-methyl-1-propanol (aminopropanol buffer): Eastman Organic Chemicals, pH adjusted with HCl; sucrose, ribonuclease-free, density-gradient grade: Mann Research Laboratory.

Preparation of cell fractions

Male Sprague–Dawley rats, 125–150 g; male NIH strain (non-inbred) guinea pigs, 375–800 g; male New Zealand white rabbits, 3 kg; and a female domestic cat, 2.5 kg, were fasted overnight prior to sacrifice. The livers were excised, and after the gall bladders were removed where applicable, placed on ice and minced. All succeeding steps were carried out at 0–4°C. The procedures of Neville[1] or Ray[2] for the isolation of plasma membranes were followed. All homogenization procedures were performed with the same Dounce homogenizer (clearance 0.10–0.15 mm). For sucrose gradients all percentages are expressed as w/w and all densities (d) as d_{20}^{20}.

As described by Neville[1], the minced liver was homogenized in 1 mM $NaHCO_3$ medium with 8 strokes. The filtered homogenate was adjusted in volume and is referred to as Fraction 1 (See Table I for flow-sheet of procedure). The low-speed supernate is referred to as Fraction 2. The low-speed pellet after conversion into 44.0 % (d = 1.204) sucrose solution is Fraction 3. From the discontinuous sucrose gradient, after centrifugation, three fractions were separated: a compact float on the 42.3 % (d = 1.199) sucrose (Fraction 4), a particulate layer at the 42.3–44.0 % sucrose interphase (Fraction 5), and a pellet (Fraction 6). The float (Fraction 4) was subjected to continuous sucrose gradient centrifugation. Depending upon species, 3 or 4 distinct bands resulted. A turbid band about 5 mm wide (about in the 5.5 (d = 1.024) to 11 % (d = 1.04) sucrose range) always occurred and was marked as Fraction 7. The membrane layer, after washing, was marked Fraction 8.

In the experiments carried out by the Ray[2] procedure, livers were homogenized in 1 mM $NaHCO_3$ *plus* 0.5 mM $CaCl_2$ medium with 25 strokes of the loose-fitting Dounce homogenizer. After filtration and final volume adjustment, the homogenate was centrifuged at low-speed. Ray's pellet III, after final adjustment to 48 % in sucrose, is marked Fraction III. The discontinuous sucrose gradient after centrifugation yielded discrete, narrow particulate bands at the 37.0–41.0 % (d = 1.163–1.184),

the 41.0–45.0 % (d = 1.184–1.205), the 45.0–48.0 % (d = 1.205–1.221) sucrose interphases, and a pellet. These are referred to as Fractions D, E, F and P, respectively. All layers were washed and resuspended in the same manner as described, and the pellet was resuspended in the medium.

Enzymic assays

The 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity was measured by incubation of samples for 20 min at 37° C in a final volume of 0.2 ml 75 mM aminopropanol buffer, pH 9.0, containing 5.0 mM AMP and 10 mM Mg^{2+}. The incubation mixtures contained approx. 20 μg of membrane protein for the rat or amounts yielding comparable enzyme activity for other species or fractions. The reaction was terminated by addition of 0.8 ml 0.2 M $HClO_4$. After centrifugation at high speed, aliquots of the supernate were analyzed for liberated inorganic phosphate by the method of Chen *et al.*[3].

Nucleotide pyrophosphatase (dinucleotide nucleotidohydrolase, EC 3.6.1.9) activity was measured by incubating samples for 30 min at 37° C in a final volume of 0.2 ml 75 mM aminopropanol buffer, pH 9.0, containing 0.5 mM NADH, 10 mM K^+, and 0.05 mM Co^{2+}. Usually a 4–5 times greater amount of enzyme protein was used than in the nucleotidase assay. The reaction was terminated by addition of 0.3 ml 0.5 M $HClO_4$, and the liberated inorganic phosphate analyzed in the supernate as described above.

Nonspecific phosphomonoesterase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) (p-nitrophenylphosphatase) activity was measured by incubating samples for 20 min at 37° C in a final volume of 0.9 ml 67 mM aminopropanol buffer, pH 9.0, containing 0.55 mM Tris–p–nitrophenylphosphate, 55 mM K^+ and 11 mM Mg^{2+}. The amount of protein used was comparable to that in the nucleotidase assay. The reaction was terminated by addition of 0.1 ml 1.8 M NaOH. Method blanks were prepared by incubating the complete mixture omitting the enzyme. After centrifugation at high speed, the supernate was analyzed spectrophotometrically at 420 nm for p-nitrophenol.

Leucyl-β-naphthylamidase (L-leucyl-peptide hydrolase, EC 3.4.1.1) activity was measured according to the method described by Mellors[4] with the following modifications: in a final volume of 0.5 ml of 0.1 M phosphate buffer, pH 7.4, the concentration of the substrate L-leucyl-β-naphthylamide was 0.1 mM and usually 5 μg membrane protein, or amounts yielding equivalent activity from other fractions, was used as enzyme source. The reaction mixture was incubated for 15 min at 37° C and then transferred to an ice-bath. 2 ml of 0.1 M borate buffer, pH 10.7, was added to stop the reaction. The liberated β-naphthylamine was determined with an Aminco–Bowman spectrophotofluorometer.

Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) was assayed essentially as described previously[5] but in order to inhibit alkaline and acid phosphatases 4 mM EDTA and 1 mM KF were added.

Protein and lipids

Protein was determined by the method of Lowry *et al.*[6], after first solubilizing the sample with 2 vol. of 5 % sodium deoxycholate solution, using human plasma albumin as standard. Total lipids were estimated, after extraction with chloroform–methanol (2:1, v/v) by the dichromate oxidation method of Bragdon[7].

TABLE I

DISTRIBUTION OF 5′-NUCLEOTIDASE ACTIVITY IN LIVER FRACTIONS DURING THE MEMBRANE ISOLATION PROCEDURE OF NEVILLE[1]

$\frac{\text{ooo (oo\%)}}{\text{ooo (oo\%)}}$: $\frac{\mu\text{moles (\% of total) } P_i \text{ formed from AMP per h per g wet wt liver}}{\text{mg (\\% of total) protein in fraction per g wet wt liver}}$

Flow-sheet of membrane preparation

(1) Homogenate —2700 rev./min, 10 min→ (3) Pellet —25000 rev./min, 120 min→ (4) Float $d<1.19$ —2000 rev./min, 60 min→ (8) Membranes $1.16<d<1.23$

(2) Supernate

(5) Interphase layer $1.19<d<1.20$

(7) Float $d<1.02$

(6) Pellet $d>1.20$

Rat liver

(1) $\frac{608\ (100\%)}{200\ (100\%)}$	(3) $\frac{110\ \ (18\%)}{9.3\ \ (4.7\%)}$	(4) $\frac{47.5\ \ (7.8\%)}{1.18\ (0.6\%)}$		(8) $\frac{7.21\ (1.2\%)}{0.24\ (0.12\%)}$
	(2) $\frac{390\ (64\%)}{194\ (97\%)}$	(5) $\frac{1.0\ \ (0.16\%)}{0.11\ (0.06\%)}$		(7) $\frac{6.59\ (1.1\%)}{0.16\ (0.08\%)}$
		(6) $\frac{28.6\ \ (4.7\%)}{4.19\ (2.1\%)}$		

Guinea pig liver

(1) 91.1 (100%) / 189 (100%)

(2) 65.3 (72%) / 176 (93%)

(3) 24.1 (26%) / 10.5 (5.5%)

(4) 18.1 (20%) / 1.78 (0.94%)

(5) 0.29 (0.31%) / 0.12 (0.06%)

(6) 4.26 (4.6%) / 3.37 (1.8%)

(7) 1.00 (1.1%) / 0.24 (0.13%)

(8) 2.88 (3.2%) / 0.30 (0.16%)

Rabbit liver

(1) 135 (100%) / 218 (100%)

(2) 97.7 (72%) / 170 (78%)

(3) 35.3 (26%) / 44.7 (21%)

(4) 14.3 (10.6%) / 15.1 (6.9%)

(5) 1.07 (0.8%) / 1.10 (0.5%)

(6) Not collected

(7) 3.83 (2.8%) / 2.61 (1.2%)

(8) 6.14 (4.5%) / 4.70 (2.1%)

Cat liver

(1) 124 (100%) / 182 (100%)

(2) 101 (81%) / 178 (98%)

(3) 17.4 (14%) / 12.6 (6.9%)

(4) 2.99 (2.4%) / 2.42 (1.3%)

(5) 4.30 (3.5%) / 1.01 (0.55%)

(6) 3.07 (2.5%) / 2.38 (1.3%)

(7) 0.60 (0.48%) / 0.44 (0.24%)

(8) 0.18 (0.14%) / 0.23 (0.13%)

RESULTS

Variations were observed in the appearance of cell fractions when livers of different species were used. In the low speed centrifugation, rabbit liver homogenate produced about 4 times more pellet than that obtained from the rat. This difference was apparent through the first sucrose gradient of the Neville procedure, where for the rabbit and the cat the "float" above the 42.3 % sucrose (Fraction 4) was a wide, non-compact band containing a proportionately larger amount of protein (Table I). The "float" derived from the guinea pig and the rat was compact.

There also appeared to be considerable species variation when the second sucrose gradient fractions of the Neville procedure were obtained. The plasma membranes of the rat always were located in a discrete particulate layer at the 37–50 % (d = 1.163–1.232) sucrose interphase. For the guinea pig, the equivalent layer existed in a diffuse but particulate band in the 27–37 % (d = 1.114–1.163) sucrose range. Since its morphology, as observed by phase contrast microscopy (magnifications from 500 to 1250 ×), was similar to the membrane fraction of the rat, it was also designated as Fraction 8. Since the guinea pig plasma membranes were consistently of slightly lighter density than those of the rat, we suggest that the second sucrose gradient be modified slightly: a 20-ml linear sucrose gradient from 5.5 to 27.0 % is placed on top of 6 ml of 37.0 % and 3 ml of 42.3 % sucrose layers. The plasma membranes concentrate as a particulate band within the 37 % sucrose layer. With the rabbit and the cat, particulate bands did layer on the 50 % sucrose cushion, but these were not purified membranes, as discussed later. Sometimes an opaque band extended downward from Fraction 7 into the higher density range of about 11–16 % sucrose (d = 1.044–1.065). This was especially noticeable in the cat liver preparation, but this fraction was not assayed.

The relation of the distribution of 5'-nucleotidase activity to cellular protein in rat, guinea pig and rabbit liver fractions obtained during the Neville procedure is shown in Table I. The numbering of fractions is arbitrary, but by reading through the top line from left to right in each box in Table I one can conveniently follow the main steps leading to the purified plasma membrane preparation, *i.e.* Fraction 8. The absolute and relative yield of plasma membranes can also be obtained from Table I. Although the overall recovery of both enzyme activity and tissue protein approached 100 %, as much as 95–98 % of the "characteristic enzyme marker" was discarded before Fraction 8 was obtained. The pattern of distribution of the 5'-nucleotidase activity was similar in all species studied, though significant enrichment in the membranes was obtained only with rat and guinea pig livers (Table II). In the cat, the enzyme activities were enriched in Fraction 5, rather than Fraction 4, a fact which can account for the lack of increase in the specific activity of "marker enzymes" in the subsequent fractions.

Plasma membrane preparations obtained by the Ray procedure confirm these species differences. We have employed this procedure also in an attempt to isolate plasma membranes from the livers of 18–20-day-old rat fetuses. No particulate fraction was observed at any of the sucrose interphases, and the lipid and enzymic analyses of the heavy pellet revealed no characteristics of plasma membranes.

The specific activity of other "plasma membrane marker enzymes" follow the same pattern as that of 5'-nucleotidase (Table II). The differences between rat or

TABLE II

ACTIVITY OF "PLASMA MEMBRANE MARKER ENZYMES" IN LIVER FRACTIONS AND LIVER PLASMA MEMBRANE PREPARATIONS

Data are given in μmoles substrate metabolized per mg protein per h. The values represent averages. The number of preparations used is indicated in parentheses. Purification = Fraction 8/liver homogenate or Fraction D/liver homogenate.

Fraction	*Rat*	*Guinea pig*	*Rabbit*	*Cat*
	5'-Nucleotidase			
Neville procedure:				
Liver homogenate	2.29 (4)	0.39 (2)	0.80 (2)	0.68 (1)
Fraction 4	45.0 (4)	7.05 (2)	0.80 (3)	1.23 (1)
Fraction 5	8.35 (2)	1.80 (2)	0.93 (3)	4.26 (1)
Fraction 7	42.5 (4)	2.95 (2)	1.25 (3)	1.40 (1)
Fraction 8	37.2 (4)	7.29 (2)	0.89 (3)	0.81 (1)
Purification	16.0	19.0	1.1	1.2
Ray procedure:				
Liver homogenate	3.20 (2)	—	0.97 (1)	—
Fraction D	44.1 (2)	—	0.87 (1)	—
Purification	14.0	—	0.90	—
	Nucleotide pyrophosphatase			
Neville procedure:				
Liver homogenate	0.62 (2)	0.044 (2)	—	0.31 (1)
Fraction 4	3.59 (2)	1.14 (2)	0.29 (1)	0.34 (1)
Fraction 5	0.97 (2)	0.30 (2)	—	0.57 (1)
Fraction 7	4.87 (2)	0.40 (2)	0.34 (1)	0.85 (1)
Fraction 8	3.62 (2)	0.96 (2)	0.27 (4)	0.32 (1)
Purification	5.8	22.0	—	1.0
Ray procedure:				
Liver homogenate	1.03 (1)	—	—	—
Fraction D	4.80 (1)	—	0.39 (1)	—
Purification	4.7	—	—	—
	p-Nitrophenylphosphatase			
Neville procedure:				
Liver homogenate	0.20 (2)	0.11 (2)	0.83 (4)	0.09 (1)
Fraction 4	4.38 (2)	0.16 (2)	1.01 (4)	0.24 (1)
Fraction 5	0.42 (2)	0.17 (2)	1.58 (3)	1.04 (1)
Fraction 7	3.09 (2)	0.14 (1)	1.91 (4)	0.21 (1)
Fraction 8	6.03 (2)	0.13 (2)	1.14 (4)	0.19 (1)
Purification	30.0	1.2	1.4	2.1
Ray procedure:				
Liver homogenate	0.25 (1)	—	1.19 (1)	—
Fraction D	3.99 (1)	—	1.16 (1)	—
Purification	16.0	—	0.98	—
	Leucyl-β-naphthylamidase			
Neville procedure:				
Liver homogenate	0.32 (4)	0.38 (3)	—	—
Fraction 4	4.41 (2)	3.14 (2)	—	—
Fraction 7	5.43 (2)	2.30 (1)	—	—
Fraction 8	4.60 (4)	3.96 (2)	—	—
Purification	14.0	10.0	—	—

TABLE III

CORRELATION OF PROTEIN CONTENT AND ENZYME ACTIVITIES OF TWO FRACTIONS OF THE NEVILLE PROCEDURE FOR LIVER MEMBRANE PREPARATION

The values represent averages. The number of preparations used is indicated in parentheses after each species name. Only one determination was made of the nucleotide pyrophosphatase activity of the rabbit.

	Rat (2)	*Guinea pig* (2)	*Rabbit* (3)	*Cat* (1)
Protein content (mg/g liver protein)				
Fraction 4	5.77	12.0	44.7	12.6
Fraction 8	1.48	2.24	16.1	1.2
Percent recovery of Fraction 4 in Fraction 8				
Protein	25.8	18.7	36.7	9.5
Enzyme activity:				
5′-nucleotidase	21.5	21.1	42.4	6.2
nucleotide pyrophosphatase	25.5	11.2	38.0	9.1
p-nitrophenylphosphatase	35.8	15.0	41.5	7.4

guinea pig liver fractions and those from rabbit or cat are evident. In the guinea pig liver, however, the specific activity of alkaline *p*-nitrophenylphosphatase in any of the fractions did not increase to levels significantly higher than that of the homogenate. Species differences are readily apparent also with regard to the content of these enzymes in whole liver (Table II).

There appears to be no further biochemical purification attained by the second density gradient centrifugation of the Neville procedure, *i.e.* the specific enzyme activities in Fraction 8 are not significantly different from those in Fraction 4 (Table II). Accordingly, the percentages of protein content and of enzyme activities of Fraction 4 recovered in Fraction 8 appear to be correlated (Table III).

The total lipid content of some of the liver fractions is shown in Table IV. The

TABLE IV

LIPID CONTENT OF LIVER FRACTIONS AND LIVER PLASMA MEMBRANE PREPARATIONS

The values represent averages. The number of preparations used is indicated in parentheses.

Fraction	*mg total lipids/mg protein*			
	Rat	*Guinea pig*	*Rabbit*	*Cat*
Neville procedure				
Fraction 4	0.50 (2)	0.64 (2)	0.28 (3)	0.63 (1)
Fraction 5	0.27 (2)	0.29 (2)	0.38 (2)	0.25 (1)
Fraction 7	0.64 (2)	1.43 (3)	0.53 (2)	0.51 (1)
Fraction 8	0.41 (2)	0.49 (3)	0.29 (3)	0.36 (1)
Ray procedure				
Fraction III	0.51 (1)		0.31 (1)	
Fraction D	0.50 (1)		0.32 (1)	
Fraction E	0.40 (1)		0.32 (1)	
Fraction F			0.25 (1)	

lighter fractions, *i.e.* Fractions 4 or 7 of the Neville procedure, have somewhat higher lipid:protein ratios than the heavier ones, *i.e.* Fraction 5 or 8. These variations of lipid content do not seem to correlate with those of the enzyme activities. The step from Fraction 4 to Fraction 8 represents loss of somewhat more lipids than of proteins.

DISCUSSION

Neville[1,8] has worked out a procedure to isolate plasma membranes from rat liver. In its original[8] or modified[1,9] applications, it appears to yield morphologically pure hepatocyte membrane preparations. It was noted, however, that in order to obtain optimal yield or purity the procedure had to be modified slightly when applied to livers of rats on different diets or to hepatomas[9]. The present results suggest that Neville's method does not necessarily yield acceptable plasma membrane preparations, as characterized by marker enzymes, when other species are used. Unlike most isolation procedures for other subcellular fractions, the method for the isolation of plasma membrane fragments may have to be worked out individually for different species, tissues and/or physiological and pathological conditions.

The Neville method has been used successfully for the isolation of plasma membranes from guinea pig[10] and mouse[11] livers. Our experience with the guinea pig liver confirms this, although we would recommend our slightly modified second sucrose gradient since a less disperse membrane fraction was obtained. The liver plasma membranes in these species are probably similar in composition. It was reported, however, that there are significant differences in "marker enzyme" specific activities; *e.g.* 5'-nucleotidase activity is lower in mouse[11] and guinea pig[10] than in rat. Leucyl-β-naphthylamidase activity was similar in guinea pig and in rat liver. It is important to emphasize in this respect that corresponding data from different laboratories vary significantly despite nearly identical assay procedures (Table V). Therefore, conclusions with regard to species or tissue differences should be drawn only from data obtained under standardized conditions.

The enzyme activities measured in this study were considered to be characteristic of liver plasma membranes in general. According to our observations, alkaline *p*-nitrophenylphosphatase, however, is not a characteristic marker for guinea pig membranes. The "marker enzyme" designation appears to be questionable also with regard to different tissues of the same species. 5'-Nucleotidase was not found in rat adipose cell plasma membranes[12]. Alkaline glycerophosphatase does not characterize liver plasma membrane preparations in the rat[9] or in guinea pig[10] while kidney membranes and intestinal mucosa preparations have high specific activities[10]. In certain pathologic conditions, however, this enzyme may be elevated in liver membranes[9]. Plasma membranes from essential fatty acid deficient rats have a reduced 5'-nucleotidase activity in contrast to (Na^+-K^+)-ATPase activity which had remained unchanged[13]. Certain enzymes of rat- and mouse-hepatoma plasma membranes were found to differ from liver membranes in their specific activity and stability[14,15]. It was concluded, however, that "no enzymatic change common to all the hepatoma membranes has as yet been observed." All these observations indicate that our concepts of the relationship of plasma membrane "marker enzymes" to the quality of membrane preparations need substantial refinement.

We have estimated the theoretical yield of membranes using the data of Weibel

TABLE V

COMPARATIVE DATA ON PLASMA MEMBRANE "MARKER ENZYMES"

Enzyme	*Species*	*Tissue*	*Specific activity (μmoles substrate metabolized/mg protein per h)*			*Assay conditions*				*Reference*
			Plasma membrane	*Light fraction*	*Heavy fraction*	*pH*	Mg^{2+} (*mM*)	K^{+} (*mM*)	*Other*	
5'-Nucleotidase	Rat	liver	82.0			7.5	10	—	—	2
				32.6	11.8	7.5	10	—	—	18
			32.2			7.2	5	100	—	9
				167.0	29.4	7.4	10	100	—	20
			13.5–44.5			7.2	5	100	—	21
			51.0			7.2	5	100	—	11
			7.7			7.2	5	100	—	22
			97–128			9.1	10	—	—	28
			58			7.4	—	—	—	30
		fat cell	0.0			7.4	3.5	5	Na: 118	12
	Mouse	liver	26.5	75.1	15.2	7.4	10	100	—	20
			13.2			7.2	5	100	—	11
	Guinea pig	liver	1.2–3.3			7.2	5	100	—	10
		kidney	3.5			7.2	5	100	—	10
		intestinal mucosa	11.5–13.8			7.2	5	100	—	10
		brain	0.01–0.24			7.2	5	100	—	10
		erythrocyte	0.0			7.2	5	100	—	10
	Bovine	liver	48.6			7.4	10	100	—	23
	Pig	liver		26.8	14.8	7.4	10	100	—	24
		lymphocyte	10.1			7.4	10	100	—	25
		thymus	3.8			7.4	10	100	—	25

p-Nitrophenylphosphatase	Rat	liver	8.0			9.0	2.5	—	—		2
				0.15	0.46	10.0	5	—	—		18
			0.92			8.9	—	—	—		9
			1.5			8.9	5	—	—		11
			13.1			10.25	10	—	—		22
			4.2			8.9	5	—	—		30
		intestine	4.9			9.2	4.2	—	Zn:0.8		26
		kidney	108			9.5	—	—	KF		31
	Mouse	liver	1.5			8.9	5	—	—		11
Nucleotide pyrophosphatase (NAD)	Rat	liver	5.7			7.4	5	—	—		9
			20–22			9.0	—	—			28
			84–138			9.6	10	—	—		29
	Pig	liver		4.59	5.47	7.4	5	—	—		24
Leucyl-β-naphthylamidase	Rat	liver	3.6–4.1			7.2	—	—	—		27
			9.8–10.3			7.2	—	—	—		21
			3.7			7.2	—	—	—		11
			11.1			7.0					22
	Mouse	liver	3.4			7.2	—	—	—		20
			3.4			7.2	—	—	—		27
			3.5			7.2	—	—	—		11
	Guinea pig	liver	12.0–14.2			7.0	—	—	—		10
		kidney	81.5–91.5			7.0	—	—	—		10
		intestinal mucosa	35.5–42.3			7.0	—	—	—		10
		brain	0.0–0.03			7.0	—	—	—		10
		erythrocyte	0.0			7.0	—	—	—		10

et al.[16] and assuming a membrane thickness of 100 Å. The calculations indicated that the theoretical yield from 1.0 cm^3 of liver (1.06 g) should be 2.85 mm^3 of membrane volume, or 3.3 mg membrane material (d = 1.16 g/ml). Since liver plasma membranes contain about 60 % protein and 40 % lipids by dry weight, a yield of 1.98 mg membrane protein per gram of wet weight liver should be expected (= 1.1 % of total liver protein). Thus, for enzymes which are exclusively associated with the plasma membrane a 100-fold purification would constitute an acceptable value.

D. M. Neville, Jr (personal communication) estimated that his procedure gave a yield of about 15 % and our data show a recovery of 0.28±0.05 mg protein per gram wet weight rat liver in Fraction 8 (about 14 % of the presumed theoretical value). At the same time only about 1.2 % of the total liver 5'-nucleotidase was recovered in this fraction.

The problem is that these so called marker enzymes are often present in subcellular elements other than plasma membranes. In particular, in the case of 5'-nucleotidases and *p*-nitrophenylphosphatases, we have separated a series of isoenzymes which made the determination of the purification factor difficult (unpublished data). For similar reasons, enzyme activity ratios can hardly define the presumed purification process nor can they characterize plasma membranes in general.

In our experiments the specific activities of "marker enzymes" did not change substantially during the second gradient centrifugation of the Neville procedure, *i.e.* from Fraction 4 to Fraction 8, which is also in agreement with the findings of Pohl *et al.*[17] on adenyl cyclase activity. We have tabulated the average change of specific activity of a series of enzymes during the purification process (Table VI) and expressed the specific activity of Fraction 8 as percent of that in Fraction 4. In the case of the guinea pig it appears as if contamination by microsomes was substantially reduced, taking glucose-6-phosphatase as an index. By and large, however, the specific activities of the other enzymes declined somewhat as well. Therefore, we assume that Fraction 4 is mostly plasma membrane material. In the rat, this fraction had a protein yield of approximately 1.1 mg per g of wet weight liver, representing an estimated total of about 1.85 mg of membranes, or near the theoretical yield. House and Weidemann[18] reported a recovery of 3.0 mg membrane protein per g liver in their light *plus* heavy plasma membrane fractions.

If the purification process had been accompanied by a drastic change in the protein:lipid ratio, it might have represented ablation of some membrane proteins

TABLE VI

CHANGES IN SPECIFIC ACTIVITY OF ENZYMES IN THE PLASMA MEMBRANE FRACTIONS DURING THE PURIFICATION PROCESS

Figures represent specific activity in Fraction 8 as percent of Fraction 4. The values represent averages. The number of experiments is indicated in parentheses.

Enzyme	*Guinea pig*	*Rat*
Glucose-6-phosphatase	49.5 (2)	78.9 (2)
5'-Nucleotidase	88.4 (4)	82.7 (4)
Leucyl-β-naphthylamidase	107 (4)	90.2 (4)
Nucleotide pyrophosphatase	84.2 (2)	100 (2)
p-Nitrophenylphosphatase	81.2 (2)	137 (2)

(or enzymes) by these procedures. This was not the case (Table IV), and consequently, Step 4→ Step 8 separates lighter membranes from the heavier ones.

The present finding that the second density gradient centrifugation of the Neville procedure did not yield substantial biochemical purification supports the validity of a simplified method such as described by Ray[2] or Chandrasekhara and Narayan[19]. Furthermore, the high specific activities of Fraction 7 of the second centrifugation are likely to represent "lighter", probably more vesiculated and smaller membrane fragments corresponding to the "light" as opposed to the "heavy" plasma membrane fractions obtained in some other studies (Table V). Differences in absolute and relative specific activities of the "marker enzymes" in such membrane subfractions may mean that they contain specific portions of the limiting membrane[11]. On the other hand, such differences might reflect variations in the degree of disruption and fragmentation of the membrane with associated possible changes in the physicochemical status of the enzyme proteins.

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