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A procedure for the rapid isolation from rat liver of plasma membrane vesicles exhibiting Ca^{2+}-transport and Ca^{2+}-ATPase activities

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A technique is described for the isolation of a plasma membrane-enriched preparation from a rat liver post-mitochondrial fraction by using discontinuous Percoll density-gradient centrifugation. The procedure is simple, of high reproducibility and yield and requires a total isolation time of only 90min. The preparation consists almost exclusively of membrane vesicles and is enriched approx. 26-fold in plasma membrane-localized enzymes with minor contamination (less than 10%) with membranes derived mainly from the endoplasmic reticulum and Golgi apparatus. Approx. 20% of the fraction comprises tightly-sealed vesicles in the inverted orientation which are capable of accumulating calcium ions and exhibiting vanadate-insensitive Ca^{2+}-ATPase activity. The properties of these activities, including insensitivity to vanadate, oxalate, and to *p*-chloromercuribenzoate as well as a lack of requirement for added Mg^{2+}, contrast markedly with the reported properties of Ca^{2+} transport by the endoplasmic reticulum isolated from rat liver. The technique may have wide application in the study of plasma membrane-associated activities in rat liver, particularly in relation to sinusoidal membrane surface-related events.

Many procedures used for the preparation of plasma membranes from rat liver employ variations of the technique first described by Neville (1960) as modified by Emmelot *et al.* (1964). These yield a morphologically heterogeneous fraction consisting of vesicles and membrane sheets joined by tight junctions (Wallach & Lin, 1973). Those membranes which sediment at relatively low *g*-forces are believed to represent primarily the bile-front hepatocyte surface, and vesicles obtained from this material generally are considered to be bile-canalicular in origin (Neville, 1960; Scharschmidt & Keeffe, 1981).

Despite the wide-spread use of these procedures to prepare plasma membranes from rat liver, several difficulties are associated with their use. These include the apparent heterogeneity of the preparation in terms of cell type and cell surface, the low yield obtained and the long preparation times that are required (Fleischer & Kervina, 1974).

In contrast, plasma-membrane vesicles of low density may be obtained from liver post-mitochondrial fractions. These membranes derive largely from the blood-front hepatocyte surface following the rupture of the sinusoidal microvilli (reviewed in Evans, 1977). Presumably it is with this latter membrane surface that many effectors of liver metabolism such as polypeptide hormones and catecholamines interact *in situ*.

In the present study we describe a simple procedure for the rapid isolation of a homogeneous, vesicular plasma-membrane fraction from a liver post-mitochondrial fraction by using discontinuous density gradients of Percoll. High yields, a short preparation time and the use of iso-osmotic media are noteworthy features of the procedure. Moreover, Ca^{2+} transport and Ca^{2+}-ATPase have been studied with this preparation.

An account of this work was presented at a meeting of the Australian Biochemical Society in May 1983 (Epping & Bygrave, 1983).

Experimental

Plasma membrane isolation

Male Wistar-strain rats of approx. 350g body wt. were obtained from the Department of Bio-

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chemistry Animal House at the Australian National University and were fed *ad libitum* prior to use. The animal was anaesthetized by intraperitoneal administration of sodium pentabarbitone (100mg/kg body wt.). The liver was exposed and blanched to remove non-parenchymal material by severing the inferior vena cava and infusing Krebs–Henseleit (1932) buffer via the portal vein. The bicarbonate buffer at 37°C contained 120mM-NaCl, 28mM-$NaHCO_3$, 1.2mM-KH_2PO_4, 4.8mM-KCl, 1.2mM-$MgSO_4$, 1.25mM-$CaCl_2$ and was equilibrated with O_2/CO_2 (19:1) immediately prior to use. Perfusions were performed by hand using a 50ml syringe plus 16-gauge needle.

Excised lobes were rinsed with 0.25M-sucrose/5mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH (pH7.4)/0.5mM-EGTA at 4°C, weighed and homogenized by hand in the same medium (50%, w/w) using 15 passes of a loose-fitting Teflon pestle (clearance 0.053cm) in a glass Elvehjem-type homogenizer (Thomas, size C). The crude suspension was filtered through nylon gauze (0.5mm mesh) and the filtrate was designated as the homogenate fraction. All subsequent operations were performed at 4°C.

The liver homogenate was centrifuged at 7710$g_{av.}$ for 10min in a Sorvall RC5-B centrifuge and the supernatant, together with any floating membranes, was removed carefully from the pellet and congealed lipid with a Pasteur pipette. This was diluted with 1.5vol. of 75% (v/v) Percoll prepared as follows. Commercially available Percoll (ρ 1.130g/l) was slowly adjusted to pH7.4 with HCl at 4°C, the refractive index was standardized at 1.3530 with water (designated 100% Percoll stock) and solutions of 10–75% (v/v) Percoll were prepared in 0.25M-sucrose/5mM-Hepes/KOH (pH7.4). Gradients were prepared with ice-cold buffers and used within 30min. Then, four 6ml portions were transferred carefully with a 10ml syringe to the bottom of pre-poured, discontinuous Percoll/sucrose density gradients prepared in 15ml Corex tubes as outlined in Fig. 1. Loaded gradients were centrifuged at 48000$g_{av.}$ for 2min, excluding acceleration time, in an SS-34 rotor fitted with rubber inserts. Deceleration took place with the brake applied. Milk-coloured membrane aggregates at the buffer/10% Percoll interface (fraction A1, Fig. 1) were collected by pipette and pooled. The total time required for isolation following liver excision was 35min.

Of fraction A1, 5ml were incubated at 37°C for 10min, 2ml of 75% (v/v) Percoll were added and the membranes were re-centrifuged on a second, modified, discontinuous Percoll gradient at 4°C in which 2.5ml of 18% (v/v) Percoll was substituted for the 18–30% layers. Fraction A2 was collected, incubated and re-centrifuged on a third gradient, identical with the second, to yield fraction A3 which was utilized without delay.

For high reproducibility of membrane purity and yield, close attention to detail, in particular temperature, is essential (see, e.g., de Pierre & Karnovsky, 1973).

Ca^{2+} transport and Ca^{2+}-dependent ATPase

Uptake of $^{45}Ca^{2+}$ by freshly-isolated membrane vesicles was determined by a membrane filtration technique. Incubations were performed at 37°C in 500μl containing 150mM-KCl, 5mM-Hepes/KOH (pH7.4) and 50μM-EDTA. Membrane protein (0.5mg/ml) was pre-incubated for 120s prior to the addition of 1mM-ATP (pH7.4) and the required concentration of radiolabelled Ca^{2+} (200μCi/mmol of Ca^{2+}), e.g. 10μM-free Ca^{2+} = 132μM total $CaCl_2$ (ATP present) or 60μM total $CaCl_2$ (ATP absent).

Samples of the medium were taken after 10–120s and filtered under vacuum through pre-soaked, Sartorius membrane filters (0.2μm pore size) to remove non-sequestered Ca^{2+}. Filters were washed immediately with 2.0ml of 150mM-KCl/5mM-Hepes/KOH (pH7.4)/0.5mM-EGTA at 4°C, dried and dissolved in toluene/2-methoxyethanol (2:1, v/v) containing 6g of 5-(biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD)/l and 1% (v/v) Triton X-100. Samples were counted for radioactivity with a Beckman LS-330 scintillation counter. Specific radioactivities were determined by counting unfiltered aliquots. Initial rates of ATP-dependent $^{45}Ca^{2+}$ uptake were determined by drawing a tangent to the curve at zero time.

Ca^{2+}-activated ATPase was determined during a 2–10min incubation period by inorganic phosphate (P_i) liberation using identical assay conditions as described for Ca^{2+} transport. The ATPase rate was linear over this period. Assays were terminated by the addition of 1ml of 20% (w/v) trichloroacetic acid at 4°C and control incubations were terminated concurrently with ATP addition.

In the absence of exogenously-added $MgCl_2$, free Mg^{2+} was reduced to nM concentrations by the chelatory action of EDTA, which has a high affinity for both Ca^{2+} and Mg^{2+}.

Free Ca^{2+} ion concentrations were computed at selected total Ca^{2+} concentrations by the iterative algorithm COMICS (Concentration Of Metal Ions and Complexed Species) originally developed by Perrin & Sayce (1967) and modified to run on a Univac 1100 system. The following apparent equilibrium ($-\log_{10}$) constants from Sillen & Martell (1964, 1971) were used: $EDTA^{4-} + H^+$ (9.91); $HEDTA^{3-} + H^+$ (5.98); $H_2EDTA + H^+$ (2.86); $EDTA^{4-} + Ca^{2+}$ (10.11); $HEDTA^{3-} + Ca^{2+}$ (3.51); $EDTA^{4-} + Mg^{2+}$ (8.73); $HEDTA^{3-}$

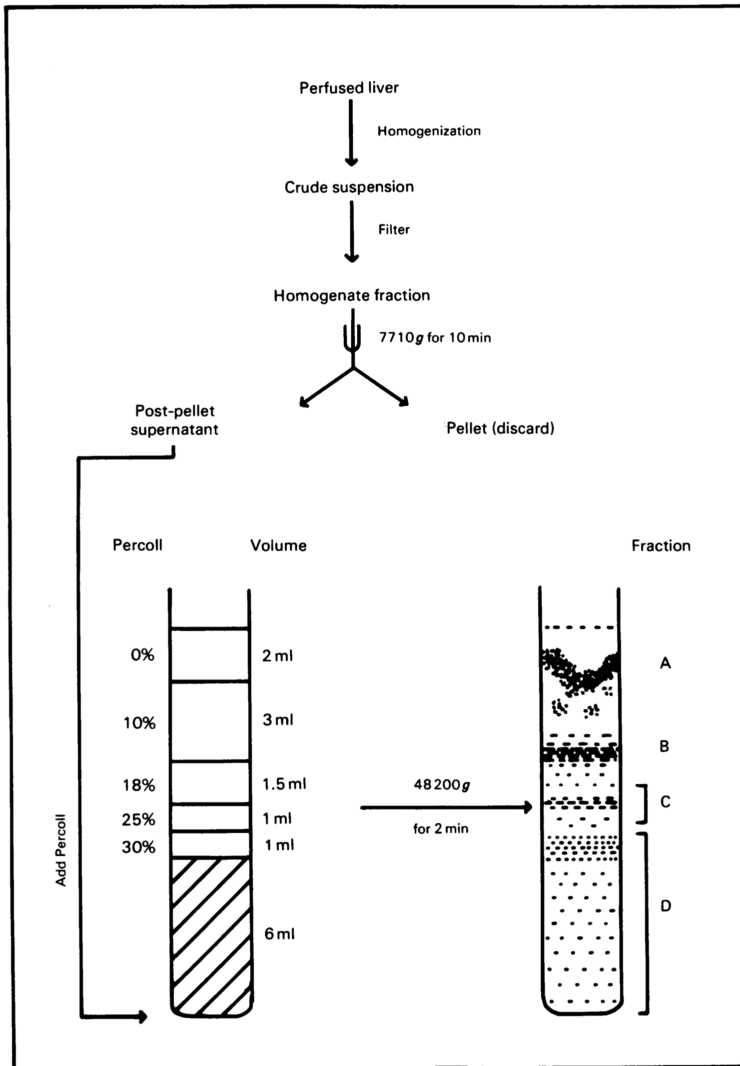

Fig. 1. *Isolation of liver fractions on Percoll gradients*

$+Mg^{2+}$ (2.28); $ATP^{4-}+H^+$ (6.52); $HATP^{3-}+H^+$ (3.87); $ATP^{4-}+Ca^{2+}$ (3.94); $HATP^{3-}+Ca^{2+}$ (2.13); $ATP+Mg^{2+}$ (4.28); and $HATP^{3-}+Mg^{2+}$ (2.29). These values are in close agreement with those determined by Durham (1983) at 37°C and *I*0.16.

Electron microscopy

Membranes were fixed in equal volumes of 6% glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.4) for 3h, washed (3 × 20min) in 0.1 M-cacodylate buffer and post-fixed with OsO_4 in 0.1 M-cacodylate buffer for 90min. Samples were again washed three times, stained *en bloc* in 2% aqueous uranyl acetate (60min), washed and series-dehydrated in alcohols prior to embedding in Spurr resin. Thin sections stained in Reynolds' lead citrate stain were examined with a Philips 301 transmission electron microscope. Many different fields were examined and random sampling was performed by cutting the pellet at various levels.

Analytical procedures

The following marker enzymes were assayed as described: cytochrome oxidase, EC 1.3.99. 1 (a mitochondrial marker), Reinhart & Bygrave (1981); NADPH–cytochrome *c* reductase, EC 1.6.99.2 (an endoplasmic reticulum marker), Dallner *et al.* (1966); catalase, EC 1.11.1.6 (a peroxisomal marker), Luck (1965); UDP-galactose:

N-acetylglucosamine galactosyltransferase, EC 2.4.1.38 (a marker for Golgi apparatus), Bretz & Staubli (1977); alkaline phosphatase, EC 3.1.3.1 (a plasma membrane marker), Pekarthy *et al.* (1972); acid phosphatase, EC 3.1.3.2 (a lysosomal marker), Baudhuin (1974); 5′-nucleotidase, EC 3.1.3.5, and glucose 6-phosphatase, EC 3.1.3.9 (markers for plasma membranes and endoplasmic reticulum, respectively), Reinhart & Bygrave (1981); ouabain-sensitive ($Na^{+}+K^{+}$)-ATPase, EC 3.6.1.3 (a plasma membrane marker), Seiler & Fleischer (1982). [^{3}H]Ouabain binding was determined as described by Mansier *et al.* (1983).

Materials

Millipore membrane filters came from Millipore Corp., Bedford, MA, U.S.A., and Corex tubes were obtained from Dupont Instruments, Freehold, NJ, U.S.A. Percoll was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. UDP-[^{14}C]galactose, [G-^{3}H]ouabain and $^{45}CaCl_2$ were obtained from Amersham International, and sodium orthovanadate came from BDH. $Na_3VO_4.14\,H_2O$ was used to calculate the concentration of vanadate solutions which were prepared in 150mM-KCl/30mM-Hepes/KOH (pH7.4). Cytochrome *c*, *p*-nitrophenylphosphate and Na_2ATP (vanadium-free) came from Boehringer Mannheim, and AMP came from Merck, Darmstadt. Calmodulin, Dowex, nucleotides and all other chemicals were from Sigma or were of equivalent analytical grade.

Results

Electron microscopy

Fraction A1 obtained from Percoll gradients consists predominantly of a homogeneous population of smooth membranous vesicles (Fig. 2). Fragmented membranes constitute only a minor proportion of total membranes with occasional lateral plasma membranes clearly apparent by virtue of adherent filamentous material. Electron-dense profiles e.g. lysosomes and peroxisomes, and flattened cisternal structures originating from the Golgi apparatus also are present although in substantially lesser proportions. Vesicles derived from the rough endoplasmic reticulum and which are readily identified in microsomal preparations obtained by using buffers identical with those adopted in this study (Reinhart & Bygrave, 1981) seldom are encountered in fraction A1 (Fig. 2, arrow). Likewise, intact nuclei mitochondria and collagen fibres essentially are absent from the preparation (see, e.g., Fleischer & Kervina, 1974, for a comparison).

Morphometric studies performed using five separate preparations of plasma membrane-enriched fraction A3 consistently demonstrated smooth membranous vesicles with a mean diameter equal to $0.18\pm0.04\,\mu$m ($n=50$ profiles) and a quantitative reduction in electron-dense material.

Marker enzyme analysis

Plasma membrane-enriched fraction A3 was shown to be highly enriched in 5′-nucleotidase activity as depicted by a 26-fold increase in homogenate specific activity (Table 1). The alternative plasma membrane marker alkaline phosphatase also was enriched 18-fold in this preparation, whereas only a 5-fold increase in UDP-galactosyltransferase was observed. The preparation was substantially depleted of mitochondrial, peroxisomal and lysosomal activities (the latter being enriched in fraction C1). Endoplasmic reticulum marker enzyme specific activities in fraction A3 decline to 23–43% of homogenate values.

Enzyme deactivation in the course of Percoll plasma membrane isolation appears unlikely since close to 100% recovery could be demonstrated for all marker enzyme activities studied. An exception was acid and alkaline phosphatases, where minor latency was indicated (Table 2). Despite the loss of 58% of homogenate 5′-nucleotidase activity in the low speed pellet fraction, 28% of remaining microsomal activity was recovered in fraction A3. This followed three successive Percoll gradient centrifugations and represents a recovery of 11% of initial homogenate enzyme activity and only 0.43% of homogenate protein. The pellet contained 82% of alkaline phosphatase activity, although some error possibly due to enzyme latency is associated with this value (initial recovery = $138\pm34\%$).

By contrast, markers for the endoplasmic reticulum generally remain at positions of comparatively high bouyant density ($>1.09\,g/cm^3$) in Percoll gradients with a net recovery of only 0.1–0.2% of total homogenate activity in plasma membrane-enriched fraction A3.

Some 2.3% of homogenate UDP-galactosyltransferase activity was retrieved in fraction A3. Membranes from more dense organelles such as mitochondria were almost completely removed either in the initial centrifugation or following failure to redistribute in Percoll gradients. Hence these membranes were collected in fraction D1. Fraction A3 thus contained less than 0.1% of homogenate cytochrome oxidase, catalase and acid phosphatase activities.

A comparison was made between fraction A3 membranes and two liver plasma-membrane preparations isolated by the procedure described by Emmelot *et al.* (1964). The relative specific enrichment of 5-nucleotidase, alkaline phospha-

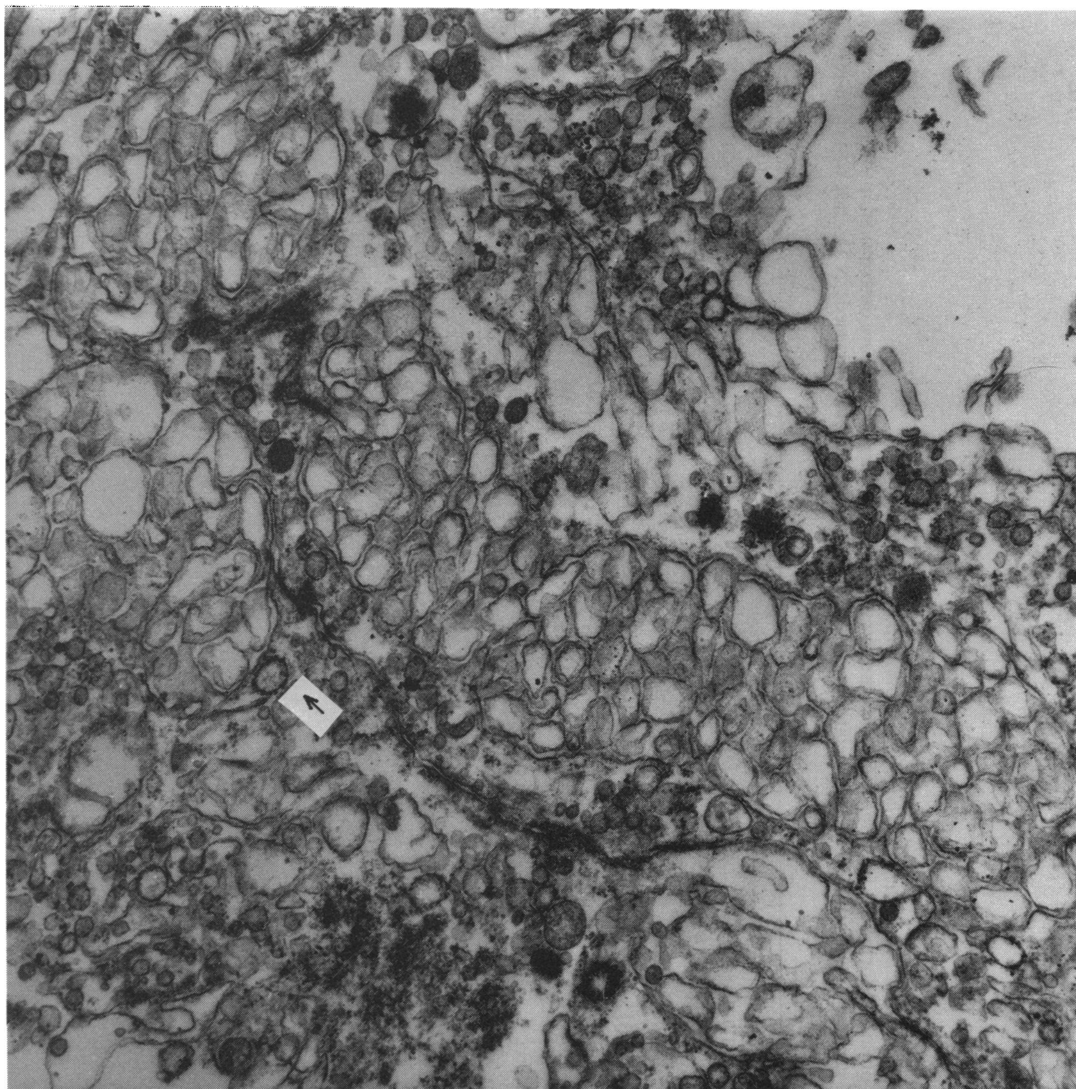

Fig. 2. *Electron micrographs of membranes from Percoll density gradients*
Liver fraction A1 is shown; magnification 28000×.

tase, NADPH–cytochrome *c* reductase and cytochrome oxidase activities in the latter membranes was found to approximate 27-, 81-, 0.24-, and 0.39-fold, respectively. The contrast between alkaline phosphatase and cytochrome oxidase activities of those membranes in our hands compared with plasma membranes from Percoll gradients (Table 1) clearly relates to the dissimilarity in membrane type and corresponding techniques employed in the isolation of the two preparations (see the Discussion).

Orientation of plasma membrane vesicles

The extent of re-sealing associated with plasma membrane vesicle preparation A3 was assessed by examining the accessibility of a number of substrates to their active sites on enzymes which have been shown to be localized on one side only of the plasma membrane. Those sites which face the intravesicular space of tightly-sealed vesicles are inaccessible to added substrate unless the membranes are rendered permeable by incubation with detergents, or freeze-thawing (Steck *et al.*, 1970; Ber *et al.*, 1980; Akyempon & Roufogalis, 1982; Seiler & Fleischer, 1982; Sips *et al.*, 1982; Mansier *et al.*, 1983). The results were regarded as estimates only, however, since some important assumptions are inherent when using this approach. These include even distribution of enzyme activities on all plasma-membrane surfaces, resolution of total

Table 1. *Specific enzyme activities in liver fractions*

Marker enzyme activities in liver subcellular fractions obtained during the isolation of plasma membrane-enriched fraction A3 were determined in six fractionation studies. Fraction A3 enrichment refers to the ratio of enzyme activity in fraction A3 relative to the homogenate. Abbreviation used: N.D., not determined.

		Activity in fraction													
Organelle or constituent	Marker (unit)	Homo-genate	Pellet	Super-natant	A1	B1	C1	D1	A2	B2	C2	D2	A3	Enrichment (fold)	n
Plasma membrane	5′-Nucleotidase (μmol/h per mg)	5.08 ±0.13	4.39 ±0.38	5.23 ±0.46	45.6 ±4.4	35.9 ±4.1	13.6 ±1.8	1.65 ±0.21	102 ±11	29.9 ±3.3	11.9 ±1.3	9.4 ±0.7	130.5 ±6.8	26	4
	Alkaline phosphatase *nmol/h per mg	0.033 ±0.011	0.045 ±0.010	0.049 ±0.015	0.26 ±0.06	0.25 ±0.04	0.13 ±0.01	0.03 ±0.01	0.62 ±0.15	0.25 ±0.07	0.13 ±0.05	0.13 ±0.02	0.58 ±0.09	18	3
Endoplasmic reticulum	NADPH–cytochrome *c* reductase (μmol/h per mg)	0.79 ±0.01	0.62 ±0.07	0.87 ±0.01	0.84 ±0.13	0.91 ±0.10	0.84 ±0.12	0.86 ±0.16	0.43 ±0.08	0.54 ±0.02	1.01 ±0.08	1.03 ±0.09	0.18 ±0.05	0.23	4
	Glucose 6-phosphatase (μmol/h per mg)	3.5 ±0.2	3.7 ±0.4	3.7 ±0.4	4.3 ±0.4	4.5 ±0.3	5.0 ±0.3	3.2 ±0.2	2.4 ±0.1	1.8 ±0.3	4.9 ±0.7	3.3 ±0.7	1.5 ±0.1	0.43	4
Golgi apparatus	UDP-galactosyl-transferase (μmol/h per mg)	11.0 ±1.0	3.0 ±2.0	21.0 ±2.0	259 ±51	100 ±26	25.0 ±6.0	2.5 ±0.5	142 ±29	N.D	N.D.	N.D.	59.0 ±11.0	5.3	3
Mitochondria	Cytochrome oxidase (μmol/h per mg)	2.74 ±0.06	4.56 ±0.13	0.31 ±0.02	0.76 ±0.54	N.D.	N.D.	N.D.	0.28 ±0.17	N.D.	N.D.	N.D.	0.28 ±0.11	0.10	3
Peroxisomes	Catalase (arbitrary units)	1.6 ±0.2	1.3 ±0.4	1.8 ±0.2	1.1 ±0.4	1.4 ±0.3	1.5 ±0.2	1.2 ±0.5	0.3 ±0.1	0.9 ±0.1	N.D.	N.D.	0.3 ±0.2	0.18	2
Lysosomes	Acid phosphatase (μmol/min per mg)	33.0 ±0.7	47.2 ±4.8	28.1 ±3.3	44 ±21	63 ±11	166 ±40	40 ±6	5.6 ±0.8	N.D.	N.D.	N.D.	3.4 ±1.3	0.10	3
Protein	(mg/10g of liver)	1096	655	420	18.6	11.3	7.1	404	5.8	1.9	3.4	8.2	4.7	–	6

Table 2. *Balance sheet for marker enzyme recovery*

The recovery of total enzyme activity was calculated for (i) the initial centrifugation, and (ii), (iii) and (iv) Percoll density centrifugations as described in the Experimental section. All values presented are percentages of total homogenate activity. Abbreviation used: N.D., not determined.

		Recovery (%)														
			(i)		(ii)				(iii)				(iv)			
Organelle	Marker	Homogenate	Pellet	Supernatant	A1	B1	C1	D1	A2	B2	C2	D2	A3	Total	Mean	*n*
Plasma membrane	5′-Nucleotidase	100	58 ±5	39.4 ±3.5	15.2 ±1.5	7.3 ±0.8	1.7 ±0.2	12.0 ±1.0	10.6 ±1.2	1.0 ±0.1	0.7 ±0.1	1.4 ±0.1	11.0 ±0.6	(i) 97 (ii) 92 (iii) 90	93±2	4
	Alkaline phosphatase	100	82 ±17	56.4 ±17.4	13.5 ±3.0	7.8 ±1.1	2.5 ±0.2	35.5 ±3.4	9.9 ±2.4	1.3 ±0.3	1.2 ±0.2	3.0 ±0.5	7.6 ±1.2	(i) 138 (ii) 105 (iii) 114	119±10	3
Endoplasmic reticulum	NADPH–cytochrome *c* reductase	100	47 ±5	42.4 ±0.6	1.8 ±0.3	1.2 ±0.1	0.7 ±0.1	40.3 ±7.6	0.29 ±0.05	0.12 ±0.02	0.40 ±0.03	0.98 ±0.09	0.10 ±0.03	(i) 89 (ii) 104 (iii) 99	97±3	4
	Glucose 6-phosphatase	100	64 ±7	41 ±5	2.1 ±0.2	1.4 ±0.1	0.95 ±0.05	34.4 ±2.1	0.37 ±0.02	0.09 ±0.01	0.44 ±0.06	0.71 ±0.15	0.19 ±0.01	(i) 105 (ii) 95 (iii) 77	92±8	4
Golgi apparatus	UDP-galactosyl-transferase	100	16 ±7	73 ±7	40 ±8	9.4 ±2.4	1.5 ±0.4	10.1 ±1.7	6.8 ±1.4	N.D.	N.D.	N.D.	2.3 ±0.4	(i) 89 (ii) 84	87±3	3
Mitochondria	Cytochrome oxidase	100	99 ±3	4.0 ±0.3	0.5 ±0.3	N.D.	N.D.	N.D.	0.05 ±0.03	N.D.	N.D.	N.D.	0.04 ±0.02	(i) 103	103±3	3
Peroxisomes	Catalase	100	60 ±7	31 ±10	1.9 ±0.2	0.9 ±0.2	0.6 ±0.1	28.0 ±1.2	0.1 ±0.3	0.1 ±0.1	N.D.	N.D.	0.08 ±0.05	(i) 91 (ii) 101	96±5	2
Lysosomes	Acid phosphatase	100	86 ±9	33 ±4	2.3 ±1.1	2.0 ±0.3	3.3 ±0.8	45 ±7	0.09 ±0.01	N.D.	N.D.	N.D.	0.04 ±0.02	(i) 120 (ii) 159	140±14	3
	Protein	100	59.8 ±1.2	38.3 ±1.2	1.70 ±0.34	1.03 ±0.13	0.65 ±0.10	36.9 ±1.1	0.53 ±0.14	0.17 ±0.02	0.31 ±0.04	0.75 ±0.17	0.43 ±0.07	(i) 98 (ii) 105 (iii) 104	102±2	6

enzyme activity is optimal using detergents and activities are not activated directly by detergents.

The accessibility of 5′-AMP and ouabain to their extracellular-facing binding sites on the ectoenzymes $(Na^{+}+K^{+})$ATPase (Sips *et al.*, 1982) and 5′-mononucleotidase (Mansier *et al.*, 1983) was determined in freshly-isolated membranes by P_i liberation and [^{3}H]ouabain binding, respectively. Results for the latter activity are presented in Table 3.

A latent activity representing the percentage of sealed inside-out vesicles, and which corresponds to approx. 20% of total enzyme activity, was observed for both activities following multiple freeze/thaw cycles or incubation of freshly-isolated membranes with the detergents Triton X-100, sodium deoxycholate or sodium dodecyl sulphate. A value of 22% sealed inside-out vesicles was obtained from four [^{3}H]ouabain binding experiments and optimum detergent concentrations showed close agreement with reported values in other tissues (Seiler & Fleischer, 1982; Grinstein & Cohen, 1983). The levels of specific ouabain binding in fresh membranes (2.79 ± 0.15 pmol/mg of protein) and in frozen/thawed membranes incubated at 25°C for 30 min with 0.8 mg of sodium deoxycholate/mg of protein (3.78 ± 0.43 pmol/mg of protein), and the low value of non-specific (i.e. total minus specific) [^{3}H]ouabain binding also corresponded well with published data (Mansier *et al.*, 1983).

Ouabain and ATP bind to opposite sides of the membrane-spanning enzyme, $(Na^{+}+K^{+})$-ATPase. Consequently, latent ouabain-sensitive $(Na^{+}+K^{+})$-ATPase activity frequently has been used to assess the total proportion of sealed vesicles in plasma membrane preparations, since this activity is not expressed in vesicles which are impermeable to either ouabain, i.e. sealed in the inside-out orientation, or ATP, i.e. sealed in the right-side-out orientation (Bers *et al.*, 1980).

Less than 25% of fraction A3 plasma membrane vesicles were tightly sealed as could be determined from the expression of latent $(Na^{+}+K^{+})$-ATPase activity using optimum concentrations of sodium dodecyl sulphate to enhance membrane permeability (Fig. 3). Sodium dodecyl sulphate was used in preference to Triton X-100 since the latter detergent was found to inhibit directly both $(Na^{+}+K^{+})$-ATPase activity and [^{3}H]ouabain binding to the enzyme (results not shown).

The data obtained in these experiments suggest that plasma membrane fraction A3 comprises 25% of tightly-sealed substrate-impermeable vesicles of which the majority retain an inverted orientation.

Ca^{2+}-transport and Ca^{2+}-ATPase

When ambient Mg^{2+} is buffered to nM concentrations in the presence of EDTA, a stable, Ca^{2+}-activated ATPase activity could be demonstrated in fraction A3. This activity was activated half-maximally by 1.6 μM free Ca^{2+} and was not attributable to $(Na^{+}+K^{+})$-ATPase activity in the preparation, since minimal activity was observed at low concentrations of free Ca^{2+} (Fig. 4*a*).

Under the conditions of these experiments, ADPase and AMPase activities in the preparation contribute less than 10% of the P_i liberated, and the

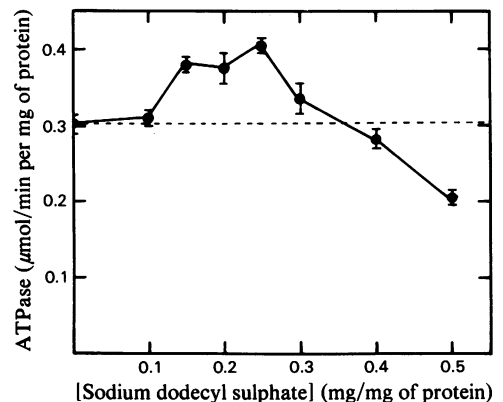

Fig. 3. *Determination of vesicle orientation*
Latent ouabain-sensitive $(Na^{+}+K^{+})$-ATPase in fraction A3 was determined in three experiments following a 30 min preincubation at 20°C of membranes (1 mg/ml) in buffer containing 0–0.5 mg of sodium dodecyl sulphate/mg of protein.

Table 3. *5′-Nucleotidase latency in freshly-prepared fraction A3*

Incubation	Activity (μmol of P_i/min per mg of protein)
Control	1.45 ± 0.09 ($n = 7$)
+0.075% Triton X-100	1.76 ± 0.10 ($n = 7$)
+0.3 mg of sodium dodecyl sulphate/mg of protein*	1.78 ± 0.08 ($n = 7$)
∴ Sealed inside-out vesicles ≃ 18%	

* Membranes (1 mg/ml) were preincubated for 30 min at 25°C with sodium dodecyl sulphate.

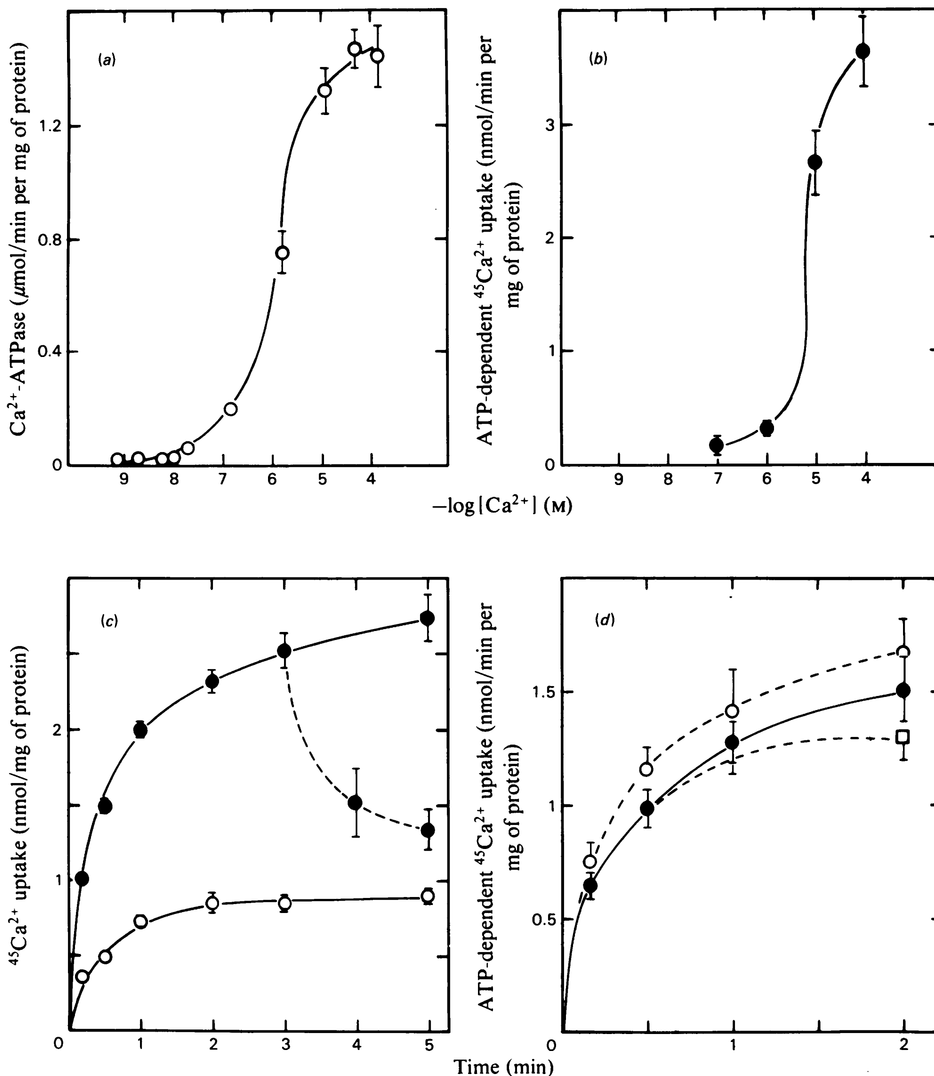

Fig. 4. *Ca^{2+}-transport and Ca^{2+}-ATPase activity in plasma membrane-enriched fraction A3*
(*a*) Ca^{2+}-ATPase (○) was examined using four preparations of fraction A3. The buffer contained 150mM-KCl, 5mM-Hepes/KOH (pH7.4), 5mM-ATP, 1mM-EDTA and 0–3.5mM-total $CaCl_2$. (*b*) ATP-supported Ca^{2+} uptake (●) also was determined as a function of free Ca^{2+} concentration in two preparations of fraction A3 as described in (*a*). (*c*) Uptake of $^{45}Ca^{2+}$ was measured using 10μM free ambient Ca^{2+} in the absence (○) and in the presence (●) of 1mM-ATP as described in the Experimental section; 1μM-A23187 was added after 3min. Mean results for four separate experiments are shown. (*d*) ATP-supported $^{45}Ca^{2+}$-uptake was determined as the difference between the curves shown in (*c*). (●) denotes control incubations (no additions), ±50μM-*p*-chloromercuribenzoate, ±3μM-Ruthenium Red, or ±1–10μg of calmodulin/ml. (○) and (□) denote the presence of 10mM-oxalate and 10μM-free Mg^{2+}, respectively.

higher basal activity which was observed when EGTA was used to buffer Ca^{2+} could be correlated with Mg^{2+}-ATPase activity in the preparation (results not shown).

Membrane vesicles in fraction A3 rapidly sequester $^{45}Ca^{2+}$ in a Ruthenium Red-insensitive, ATP-dependent manner when identical experimental conditions to those used in the Ca^{2+}-ATPase studies are adopted (see Figs. 4*b*, 4*c* and 4*d*). The initial rate of ATP-supported Ca^{2+} transport is approx. 3.5nmol of Ca^{2+}/min per mg, which compares with 1.3μmol of ATP/min per mg for Ca^{2+}-ATPase activity in the same assay, i.e. with no added Mg^{2+}, and 10μM-free Ca^{2+} present.

The Ca^{2+}-transport activity was activated half-maximally by 6 µM free Ca^{2+} and apparently was not due to contaminating microsomal membranes in the preparation, since the activity was insensitive to vanadate, *p*-chloromercuribenzoate, Mg^{2+} (0.1–1 mM total $MgCl_2$), oxalate and calmodulin (Fig. 4*d*). All of these agents have been shown to modulate the Ca^{2+}-transport system which is present in liver microsomes (Moore *et al.*, 1975; Bygrave, 1978; Dawson & Fulton, 1983; Moore & Kraus-Friedmann, 1983; Epping & Bygrave, 1984). Moreover, the pH optimum of fraction A3 Ca^{2+}-transport activity, i.e. pH 7.4, also differs from that of the microsomal system, i.e. pH 6.8 (Dawson, 1982). Vanadate, Mg^{2+} and oxalate also were without effect on the Ca^{2+}-ATPase in this study (results not shown).

Discussion

The two important features of this paper are the description of a new procedure for the isolation of plasma membranes from rat liver and the demonstration in this fraction of a significant Ca^{2+}-transport and Ca^{2+}-ATPase activity.

The use of Percoll (Pertoft *et al.*, 1978) to isolate plasma membranes complements our previous study on the successful rapid isolation of mitochondria from a rat liver homogenate (Reinhart *et al.*, 1982*a*) and discloses some significant innovations over existing isolation techniques. Most notable was the very short isolation time: membranes enriched 26-fold in 5′-nucleotidase activity were isolated within 90 min and a 35 min total isolation time was all that was required to obtain membranes enriched 9-fold in 5′-nucleotidase activity. This compares with an isolation time of approx. 16–18 h for similar low-density plasma membranes from liver post-mitochondrial fractions by using alternative techniques (Wisher & Evans, 1975).

The procedure is highly reproducible and simple, since ultracentrifuges and other complex equipment are not required. Membrane resuspension also was not required at any stage, and yields and purity of isolates were considerably better than those reported for procedures currently available for rat liver. A striking feature of the preparation was the low density (1.02–1.04 g/cm^3) of the membranes. This may be attributed to the use of iso-osmotic media throughout the isolation procedure and the high proportion of vesicles in the preparation.

Morphological markers have been used to determine the membrane purity of most earlier plasma-membrane preparations (Touster *et al.*, 1970). However, the true composition of those preparations usually is uncertain as a result of incomplete or insufficient analyses of marker enzymes (see de Duve, 1971; de Pierre & Karnovsky, 1973; Wallach & Lin, 1973).

Estimates of approx. 90% minimal plasma membrane composition in membrane preparations have been calculated on the basis of 20–23-fold enrichment of 5′-nucleotidase activity (Yunghans & Morré, 1973; Jelsema & Morré, 1978). Such calculations, however, are not always strictly accurate. For example, the preparation may be enriched in small membrane fragments which contain a particular marker in abundance, and the possibility of Golgi and endoplasmic reticulum markers comprising intrinsic plasma membrane constituents cannot be eliminated. These arguments ultimately prohibit anything but approximate calculations to be made. Subject to the validity of such approaches, fraction A3 arguably could be described as being of at least comparable to, if not higher, in purity than the above preparations.

Glucose 6-phosphatase and NADPH–cytochrome *c* reductase are localized on smooth and rough endoplasmic reticulum vesicles (Leskes *et al.*, 1971*a*,*b*; Amar-Costesec & Baufay, 1981) and in Golgi membranes (Howell *et al.*, 1978). Endoplasmic reticulum membranes comprise 19% of total hepatocyte protein (de Pierre & Karnovsky, 1973) and 90% pure endoplasmic reticulum fractions have been estimated with only a 3.7-fold increase in homogenate glucose 6-phosphatase activity (Jelsema & Morré, 1978). From this, an approximate endoplasmic reticulum contamination in fraction A3 of the order of 10% may be calculated, subject to similar criticisms as described above. Compatible with the 90% pure plasma membrane estimate, the glucose 6-phosphatase relative specific activity in this study (0.43 ± 0.03) compares well with that of alternative Neville-type membranes (0.2–1.5) and in particular with other liver plasma membrane isolates which derive from post-mitochondrial fractions [1.1 ± 0.1, Tousler *et al.* (1970); 0.12 ± 0.04, House *et al.* (1972); 1.0 ± 0.5, Hubbard *et al.* (1983); 1.7, Lin *et al.* (1983); 2.0 ± 0.6, Taylor *et al.* (1983)].

Persistent Golgi contamination is a common feature of low-density liver plasma membrane preparations and has not been examined in studies prior to 1975. The similar density of membranes containing Golgi and endoplasmic reticulum markers (Amar-Costesec *et al.*, 1974) has prohibited effective marker separation on sucrose gradients (Wisher & Evans, 1975) or by using aqueous polymer two-phase systems (Hino *et al.*, 1978). This resulted in the isolation of low density 'plasma membrane' preparations enriched as much as 33-fold (Brown *et al.*, 1976) and 65-fold (Wisher & Evans, 1975) in UDP-galactosyltransferase activity. Rat liver Golgi preparations typi-

cally are enriched more than 70-fold in this activity (Bergeron *et al.*, 1973; Fleischer, 1974; Hino *et al.*, 1978; Morré *et al.*, 1970) and demonstrate low 5′-nucleotidase activity. A 110-fold increase in enzyme activity has been correlated with an 84% pure Golgi preparation (Jelsema & Morré, 1978). Consequently, an upper limit on the contribution of the Golgi apparatus towards fraction A3 membranes in this study (relative specific enrichment = 5.3; Table 1) may be approximated at 4%.

Evaluation of endoplasmic reticulum and Golgi contamination is likely to represent an overestimate, however, since multiple pathways for membrane recycling could lead to the insertion of Golgi elements containing both Golgi and endoplasmic reticulum-derived glycoproteins, etc., into the cell membrane, thus becoming intrinsic plasma membrane activities (reviewed in Evans, 1980; Farquhar, 1983). Moreover, the unique specificity of microsomal markers for the endoplasmic reticulum is not clearly established. Only a very minor contamination by other intracellular membranes in fraction A3 is indicated by the low recovery (<0.1% of homogenate activity) of markers for these organelles (Tables 1 and 2) and by electron microscopy.

Whilst fraction A3 is likely to comprise a mixture of sinusoidal and canalicular membranes, two lines of evidence argue in favour of a putative sinusoidal-surface origin of many of the membranes in fraction A3. Firstly, blood sinusoids distribute with microsomes in centrifugation profiles as demonstrated by the binding of ^{125}I-labelled wheat germ agglutinin (Chang *et al.*, 1975; Taylor *et al.*, 1983). Secondly, the sinusoidal microvilli generate vesicles of approx. 0.2μm diameter in place of large fragments during homogenation (Evans, 1980). Indeed, the density of sinusoidal plasma membranes (approx. 1.03g/cm^3) is believed to be considerably lower than for other plasma-membrane surfaces (Evans, 1980) and is similar to that observed in the present study (1.02–1.04g/cm^3). A third feature is that specific [^{3}H]adrenaline binding to an extent of 100fmol/mg of protein was achieved with fraction A3 (R. J. Epping & F. L. Bygrave, unpublished work).

'Sinusoidal' plasma-membrane isolates recently have been reported by Hubbard *et al.* (1983) and Taylor *et al.* (1983). The former preparation (4–5h isolation time) was isolated from low-speed pellets, was of variable purity, e.g. 15–41-fold enrichment of 5′-nucleotidase, and was heavily contaminated with endoplasmic reticulum. Taylor *et al.* (1983) claim good separation of Golgi, endoplasmic reticulum and plasma membranes by using Ficoll gradients (24h centrifugation); however, Golgi and endoplasmic reticulum enrichment of 26-fold and 1.4-fold, respectively, persisted in that preparation. Other reports which presume to provide an alternative to existing plasma-membrane isolation techniques suffer either from low (4–15-fold) enrichment of 5′-nucleotidase activity over homogenate values (Sips *et al.*, 1982; Chan & Junger, 1983; Wallace *et al.*, 1983) or exhibit relative specific enrichment of glucose 6-phosphatase activities which are as high as 0.9–1.7 (Wallace *et al.*, 1983; Hubbard *et al.*, 1983; Taylor *et al.*, 1983; Lin *et al.*, 1983) or which cannot be accurately assessed (Famulski & Carafoli, 1982).

Because the endoplasmic reticulum of rat liver contains a Ca^{2+}-transport system with apparently greater activity *in vitro* than that of the plasma membrane, it becomes very important to consider the extent to which those plasma membrane fractions which are reported to carry out Ca^{2+} transport are contaminated with endoplasmic reticulum. For example, the preparation of Chan & Junger (1983) shows vanadate-sensitive, Mg^{2+}-dependent Ca^{2+}-transport activity which may be endoplasmic reticulum-localized.

Data from experiments described in the present paper add to the growing body of evidence that plasma membrane Ca^{2+} transport and Ca^{2+}-ATPase (see also Lotersztajn *et al.*, 1981; Iwasa *et al.*, 1982) have properties quite different from those of microsomal Ca^{2+} transport and Ca^{2+}-ATPase. For instance, plasma membrane Ca^{2+} transport does not require added Mg^{2+} (the present work), is unaffected by the presence of vanadate, *p*-chloromercuribenzoate, oxalate and calmodulin (Fig. 4*c*; see also Kraus-Friedman *et al.*, 1982), has a pH optimum of approx. 7.4 and reaches saturation by approx. 3min. These properties differ sharply from those reported for microsomal Ca^{2+} transport (cf., e.g., Moore *et al.*, 1975; Bygrave, 1978; Dawson, 1982; Kraus-Friedmann *et al.*, 1982; Epping & Bygrave, 1984).

In this regard, it is of interest that Ca^{2+}-transport activity by the rat liver plasma membrane fraction of Prpić *et al.* (1984), which also was prepared by Percoll density gradient centrifugation, was found to be dependent on added Mg^{2+}, was stimulated by oxalate, exhibited considerably lower rates of Ca^{2+} transport and was not saturated even after 30min of transport. Other features of their preparation that differ from those described in the present work were its primary source (the pellet was obtained from a 1464g × 10min centrifugation of the liver homogenate) and the heterogeneous appearance of the fraction as revealed by electron microscopy. Finally, we have not been able to detect any significant reproducible stable alteration to either Ca^{2+} transport or Ca^{2+}-ATPase activity in plasma membrane fraction A3 following the challenge of a

perfused rat liver (Reinhart *et al.*, 1982*b*) with the α-adrenergic agonist, phenylephrine for 3 min (cf. Lin *et al.*, 1983; Prpić *et al.*, 1984).

In conclusion, the novel features including ease and rapidity of isolation, high reproducibility and yield, the vesicular nature of the membranes and the high enrichment of Ca^{2+}-ATPase activity make the Percoll technique a distinctively innovative alternative to the procedures currently available for the isolation of rat liver plasma membranes. They also render the preparation as the material of choice with which to study a wide variety of liver (sinusoidal?) plasma membrane-related events which previously could not be approached by using available preparations.

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