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PREPARATION AND PROPERTIES OF PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM FRAGMENTS FROM ISOLATED RAT FAT CELLS

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SUMMARY

1. Plasma membrane and endoplasmic reticulum of free fat cells were isolated in high yield as a mixed microsomal fraction by sucrose gradient centrifugation.

2. The semipermeable behavior of these particles was explored by observing shifts in isopycnic density in continuous polymer (Dextran) gradients over a range of pH and ionic conditions.

3. Using a discontinuous Dextran gradient, a plasma membrane fraction was partially purified. The plasma membrane fraction showed a 12–16-fold enrichment of 5'-nucleotidase over the homogenate. Na^+–K^+-stimulated ATPase was recovered in highest specific activity in this fraction. Mitochondrial contamination was 3 % where endoplasmic reticulum contamination was 27 %.

4. A sensitive radioassay for 5'-nucleotidase is described.

INTRODUCTION

Preparation of free, intact fat cells[1] has proven an extremely useful tool in the study of the cellular response to hormones. Certain major steps in this response involve the plasma membrane, namely: "specific" hormone binding[2,3] and, subsequently, hormone-induced alteration in plasma membrane-bound functions such as glucose transport[4,5] and adenyl cyclase activity.[6,7]. In order to study the organizational and molecular basis of these functions of the plasma membrane, a suitable preparation from a hormone-responsive tissue is required. Herein we report on a fractionation of free fat cells designed to yield membrane vesicles exhibiting semipermeable behavior. After cell disruption, such a fraction was isolated by isopycnic sucrose gradient centrifugation. We have characterized the semipermeable properties of the plasma membrane and endoplasmic reticulum vesicles comprising this microsomal fraction, using polyglucose (Dextran) continuous density gradients. We have applied this information to effect the partial purification of plasma membrane on a discontinuous Dextran gradient.

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MATERIALS AND METHODS

Crude collagenase, lots CLS9FM and CLS9KA, was obtained from Worthington Biochemicals. Dextran 110 is a product of Pharmacia, Inc. It was unnecessary to dialyze Dextran solutions prior to use, as the conductivity of the directly dissolved material was always less than $1 \cdot 10^{-4}$ Ω^{-1}/cm. All water used in membrane fractionation was doubly glass-distilled and deionized. Bovine serum albumin (Cohn Fraction V), AMP, ATP, oxidized cytochrome *c*, NADH and Triton X–100 were obtained from Sigma Chemical Co. [^{3}H]AMP and $H_3{}^{32}PO_4$ were obtained from New England Nuclear. 2,5-Diphenyloxazole (PPO) and 2,5-bis-2-(5-*tert*-butylbenzoxazolyl)-thiophene (BBOT) were obtained from the Packard Instrument Co., Inc.

Cell isolation

Male Sprague-Dawley strain rats, 130–150 g, were supplied by Gofmoor Farms, Westboro, Mass. The animals were allowed free access to food and sacrificed within 1 week. Isolated fat cells were prepared by the method of RODBELL[1]. Pooled distal epididymal fat pads were incubated for 30–60 min in glucose-free Krebs–Ringer bicarbonate buffer containing 4 % bovine serum albumin and 2 mg/ml collagenase. Undigested fragments were removed by filtering through several layers of fine mesh gauze. The cells were washed 3 times in 0.25 M sucrose, 5 mM Tris–HCl, pH 7.4, at room temperature and centrifuged in an International clinical centrifuge at 250 × *g* for 1 min.

Cell disruption

The fat cell can be disrupted by gentle liquid shear as follows: The packed cells were resuspended in approx. 20 ml of 0.25 M sucrose, 5 mM Tris–HCl (pH 7.4) and aspirated through a 16 cannula into a 30-ml plastic syringe fitted with a Swinny filter holder (Millipore, Cat. No. XX30-01200) containing a stainless steel photo-etched support screen, without a filter. The support screen, with apertures of approx. 200 μm, acted as a sieve and provided the necessary shearing force. The cell diameter ranged from 50–100 μm. The cell suspension was rapidly aspirated and ejected 5–6 times within 90 sec. No intact cells could be seen by phase microscopy thereafter. This homogenate was centrifuged at 20000 × *g* for 3 min in a Sorvall centrifuge (model RC 20) at 20°. The resulting pellet and cloudy infranate were aspirated from below the fat cake. This suspension was immediately placed in an ice bath and made 1 mM in EDTA. It was redispersed by 5–6 cycles of aspiration and ejection through the Swinny filter holder–sieve apparatus. By phase microscopy the homogenate now consisted of unaggregated vesicles of various sizes, intact nuclei and occasional fat droplets.

Microsomal isolation

All subsequent centrifugations were done in a Beckman L2-50 preparative ultracentrifuge. 10 ml of the homogenate was layered onto a 27-ml linear sucrose gradient, 27.6–54.1 % (w/v) in sucrose (approx. $\rho_{4^\circ} = 1.1$–1.2) containing 5 mM Tris–HCl (pH 7.4), 1 mM EDTA. Gradients were centrifuged in a SW 27 rotor at 25000 rev./min at 4° for 90 min, yielding the banding pattern diagrammed in Fig. 1. The layer above the gradient was discarded. The upper "microsomal" zone, down to a ρ_{4° of approx. 1.15, was aspirated, diluted with 3 vol. of water and centrifuged at 40000 rev./min in

a 40 rotor for 30 min. The supernatant was discarded; the resuspended unwashed pellet constituted the "microsomal" fraction used for enzymatic analyses. The two bands at $\rho_4°$ 1.180 and 1.185 were aspirated, diluted with 5 vol. of water and centrifuged 30 min at 40000 rev./min. The yellow pellets were resuspended in 0.25 m sucrose, 10 mM Tris–HCl (pH 7.4) and constituted the mitochondrial fraction. The remaining gradient was aspirated from the pellet and the pellet resuspended in the same buffered sucrose. This was the nuclear fraction. To free the microsomal pellet of adsorbed and trapped soluble proteins, it was resuspended in 10 mM Tris–HCl (pH 8.6) by homogenization through a 27-gauge needle. The microsomes were centrifuged in a 40 rotor at 40000 rev./min for 30 min and similarly resuspended in 1 mM Tris–HCl (pH 8.6). After recentrifugation, this washed osmotically shocked microsomal pellet was resuspended in 1 mM Tris buffer.

Continuous Dextran gradients

A series of 10 ml Dextran-110 gradients ($\rho_4°$ 1.01–1.10) were prepared with 1 mM Tris of varying pH and varying $MgSO_4$ concentration. For those gradients not containing Mg^{2+}, the washed, lysed microsomes were resuspended in 2 ml of 1 mM Tris buffer and layered directly onto the matching Dextran gradients containing 1 mM Tris buffer at the same pH. Above pH 7.0, Cl^- was the counter ion; below pH 7.0, acetate.

In gradients containing Mg^{2+}, the washed microsomes were resuspended in 1 mM Tris–HCl (pH 8.6) containing the desired cation concentration and dialyzed against 200 vol. of buffer for 2 h with one buffer change. The microsomes were then layered onto the Dextran gradient of corresponding buffer and cation concentration. The gradients were centrifuged to equilibrium at 4° in an SW 41 rotor at 40000 rev./min (approx. 200000 $\times$ g (average)) for 12–15 h. The tubes were punctured at the bottom and 0.5-ml fractions were collected by slow displacement from above with air, using a micro-buret syringe. The fractions were stored at −86°.

Discontinuous Dextran gradients

Two ml of a suspension of washed, lysed and dialyzed microsomal membranes were layered onto 3 ml of Dextran solutions of varying composition and density. These discontinuous gradients were centrifuged in an SW 50 rotor at 40000 rev./min. Essentially identical distributions were obtained after 3 or 12 h centrifugation. The contents were divided into two fractions: (1) the material above and at the interface between the Dextran and buffer solutions and (2) the material in the pellet and Dextran.

Plasma membrane separation

The washed, lysed microsomes were dialyzed against 200 vol. of buffer containing 1 mM Tris–HCl (pH 8.6) 0.5 mM $MgSO_4$ for 2 h with one buffer change. The suspension was layered onto approx. 2 vol. of Dextran solution, $\rho_4°$ 1.0400–1.0425, containing 1 mM Tris–HCl (pH 8.6) 0.5 mM $MgSO_4$. This discontinuous gradient was centrifuged in an SW 50 rotor 3 or more h at 40000 rev./min or 12–15 h at 25000 rev./min. The contents were collected in two fractions by aspiration from the top with a micropipette: (1) The material above and at the interface (plasma membrane fraction); (2) the material in the Dextran and pellet (endoplasmic reticulum fraction). These

fractions were stored frozen at −86° either directly or after centrifugation at 40 000 rev./min for 30 min and resuspension in 0.25 M sucrose 10 mM Tris–HCl, pH 8.6.

Analytical methods

Nuclear counts were performed in a hemocytometer after staining with methylene blue. Densities were measured by pycnometry in a converted 200 μl "self-adjusting" micropipette, calibrated with water.

Protein was determined by two methods. The procedure of LOWRY[8] was used, with crystalline bovine serum albumin as a standard. In addition, membrane protein was estimated by intrinsic protein fluorescence[9]. Aliquots of membrane fractions were suspended in 2 ml of 0.05 % sodium dodecyl sulfate and excited at 286 nm in a Hitachi Perkin-Elmer spectrofluorometer MPF 2A. Fluorescence emission was measured at 338 nm. Tryptophan was used as a standard. The conversion of mg tryptophan to mg adipocyte protein was obtained by comparison with protein values obtained on the same sample by the Lowry technique. The conversion factor (mg tryptophan per mg adipocyte protein) was 40.0. There was no significant interference by buffers or gradient media even at submicrogram protein concentrations. This method was rapid, reproducible and more sensitive than the Lowry method (1.0 μg protein yielding easily measureable readings). It was especially useful at low protein concentrations in the presence of substances known to interfere with the Lowry procedure.

5'-Nucleotidase (EC 3.1.3.5) was assayed in a 1-ml volume containing 50 μmoles Tris–HCl pH 8.0 or 9.0, 0.18 μmoles Mg^{2+}, 0.02 μmoles AMP as sodium salt, tracer [^{3}H]AMP and membrane suspension. Incubation at 37° was continued 30 or 60 min. Blank samples contained no enzyme. The reaction was stopped by the addition of 0.2 ml of 0.25 M $ZnSO_4$. Protein and unhydrolyzed AMP were precipitated by addition of 0.2 ml 0.25 M $Ba(OH)_2$, while adenosine remained in the supernatant. The tubes were centrifuged at full speed for 10 min in an International centrifuge, model H (table top). An 0.7-ml aliquot of the supernatant was aspirated and counted in 10 ml of a toluene-triton X-100 (2:1, v/v) scintillation mixture containing 2,5 diphenyloxazole (5 g/l) and BBOT (1.0 g/l). Under these conditions the rate of reaction was linear until over 80 % of the substrate has been hydrolyzed.

ATPase (EC 3.6.1.3) was assayed in a 1.5-ml volume containing membrane suspension, 1.0 μmole ATP, 1.08 μmole Mg^{2+}, 0.09 μmole EDTA 20 μmole Tris–HCl (pH 7.4). Either 180 μmoles of choline chloride or 150 μmoles NaCl *plus* 30 μmoles KCl were present. γ-[^{32}P]ATP was synthesized by the method of WEISS *et al.*[10] and was included in tracer quantities. Samples were incubated 30–60 min at 37° and all assays were performed in triplicate. The reaction was stopped by the addition of 3 ml of a suspension containing 4 % Norit, 0.1 M HCl, 0.2 mg/ml bovine serum albumin, 1 mM phosphate and 1 mM pyrophosphate. 30 min incubation at 4° was allowed for adsorption of unhydrolyzed ATP, after which the suspension was passed through a millipore filter directly into a scintillation vial, and the residue washed twice with 3 ml of a solution containing 0.01 M HCl and 1 mM phosphate. Cerenkov radiation of $^{32}P_i$ counted directly in aqueous solution. Na^+–K^+ stimulation was calculated as the difference in specific activity in the presence and absence of these cations.

Succinate cytochrome *c* reductase (EC 1.3.99.1) was measured in a 1-ml volume containing 0.1 μmoles cytochrome *c*, 10 μmoles KCN, 8.0 μmoles phosphate buffer (pH 7.4), 66 μmoles sodium succinate and membrane suspension. The reduction of

cytochrome *c* was followed by monitoring the absorbance at 550 nm in a Cary 14 recording spectrophotometer. The reference cuvette contained the above components *minus* sodium succinate. This activity has been consistently associated with the inner membrane system of the mitochondrion[11,12].

NADH oxidase (EC 1.6.99.3) was measured in a 1-ml vol. containing 10 μmoles NADH, 16 μmoles Tris–HCl (pH 7.4) 0.66 μmoles $K_3Fe(CN)_6$, and enzyme suspension. The oxidation of NADH was followed by monitoring the absorbance at 340 nm in a Cary 14 recording spectrophotometer. The reference cuvette contained the above components *minus* enzyme suspension. This activity has been found in the endoplasmic reticulum and the outer membrane of the mitochondrion[11,13].

RESULTS

Preliminary fractionation

The disruption and fractionation of the fat cells was monitored by phase microscopy and recovery of homogenate 5′-nucleotidase. Vigorous mechanical homogenization in a Potter–Elvehjem homogenizer at 4° led to a congealed lipid cake from which cellular elements could not be extracted reproducibly. Less vigorous homogenization in the cold gave inadequate cellular breakage, while homogenization at 20°, where the fat is liquid, led to inordinate heating of the homogenate. Nitrogen cavitation[14] and hypotonic lysis proved unsatisfactory because of nuclear rupture. The sieving method obviated these problems.

In early studies, the homogenate prepared in the absence of chelating agents contained many aggregated clumps of nuclei and membrane fragments which could not be dispersed by further homogenization. This aggregation could be visibly overcome by the addition of 1 mM EDTA immediately after homogenization (Table I). Differential centrifugation of the resuspended lipid-free homogenate led to partial distribution of 5′-nucleotidase and NADH oxidase into the nuclear (1000 × *g* for 10 min) and mitochondrial (20000 × *g* for 10 min) pellets. This pattern was not altered by further homogenization or chelating agents. In sucrose gradients without chelating agents a similar distribution of 5′-nucleotidase was seen. In the presence of 1 mM EDTA, however, 76% of the recovered 5′-nucleotidase and 100% of the Na^+–K^+-stimulated ATPase was in a region of density $\rho < 1.10$–1.15 (Table II). It appears that during homogenization the plasma membrane is broken into fragments of widely varying size, compromising separation by differential centrifugation. The attainment of equilibrium density in sucrose gradients is, however, largely independent of vesicle size. In addition, co-aggregation between cellular elements occurs, leading to losses

TABLE I

EFFECT OF CHELATING AGENTS ON THE DISTRIBUTION OF PLASMA MEMBRANE IN A SUCROSE GRADIENT EXPRESSED AS PERCENT OF RECOVERED 5′-NUCLEOTIDASE

The fractions of a sucrose gradient are as shown in Fig. 1. A lipid-free adipocyte homogenate was resuspended and centrifuged in the presence and absence of 1 mM EDTA.

	No EDTA	*1 mM EDTA*
Microsomal	34.0	80.0
Mitochondrial	34.2	16.9
Nuclear	31.8	3.1

TABLE II

PRELIMINARY FRACTIONATION OF FAT CELL HOMOGENATE ON SUCROSE GRADIENTS

Distribution of enzyme activities in the fractions of the sucrose gradient as described in MATERIALS AND METHODS. Specific activity expressed as μmoles·mg^{-1}·h^{-1} at 37° for phosphohydrolases; μmoles·mg^{-1}·min^{-1} at 25° for NADH oxidase and succinic cytochrome *c* reductase. Percent values are percent of recovered activity. Results are given as ± S.E. — indicates no activity detectable, Numbers in parentheses indicate number of experiments.

	Protein (5)	*5'-Nucleotidase* (5)		*(Na⁺–K⁺)-ATPase* (2)		*NADH oxidase* (4)		*Succinate cytochrome c reductase* (4)	
	Mg^{2+}	*Spec. act.*	*Percent*	*Spec. act.*	*Percent*	*Spec. act.*	*Percent*	*Spec. act.*	*Percent*
Homogenate	27.65 ± 4.87	0.60 ± 0.10		0.83 1.01		1.14 ± 0.10		0.113 ± 0.014	
Microsomal	3.96 ± 0.54	2.73 ± 0.59	76.0 ± 2.8	4.91 3.38	100	2.26 ± 0.29	66.3 ± 3.2	0.012 ± 0.0005	3.3 ± 1.2
Mitochondrial	2.47 ± 0.40	0.83 ± 0.07	16.4 ± 1.1	—	0.0	1.75 ± 0.08	30.6 ± 2.9	0.592 ± 0.06	92.0 ± 4.2
Nuclear	0.56 ± 0.05	2.02 ± 0.45	7.6 ± 3.0	—	0.0	0.74 ± 0.06	3.1 ± 0.6	0.273 ± 0.06	4.7 ± 3.2

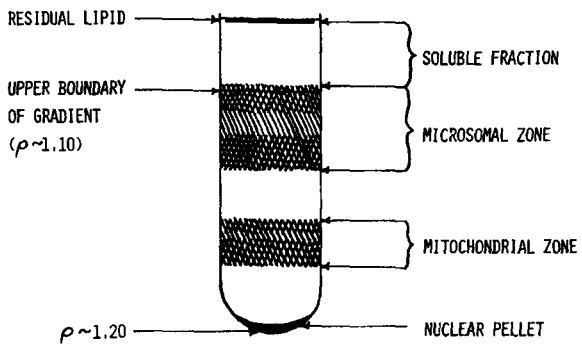
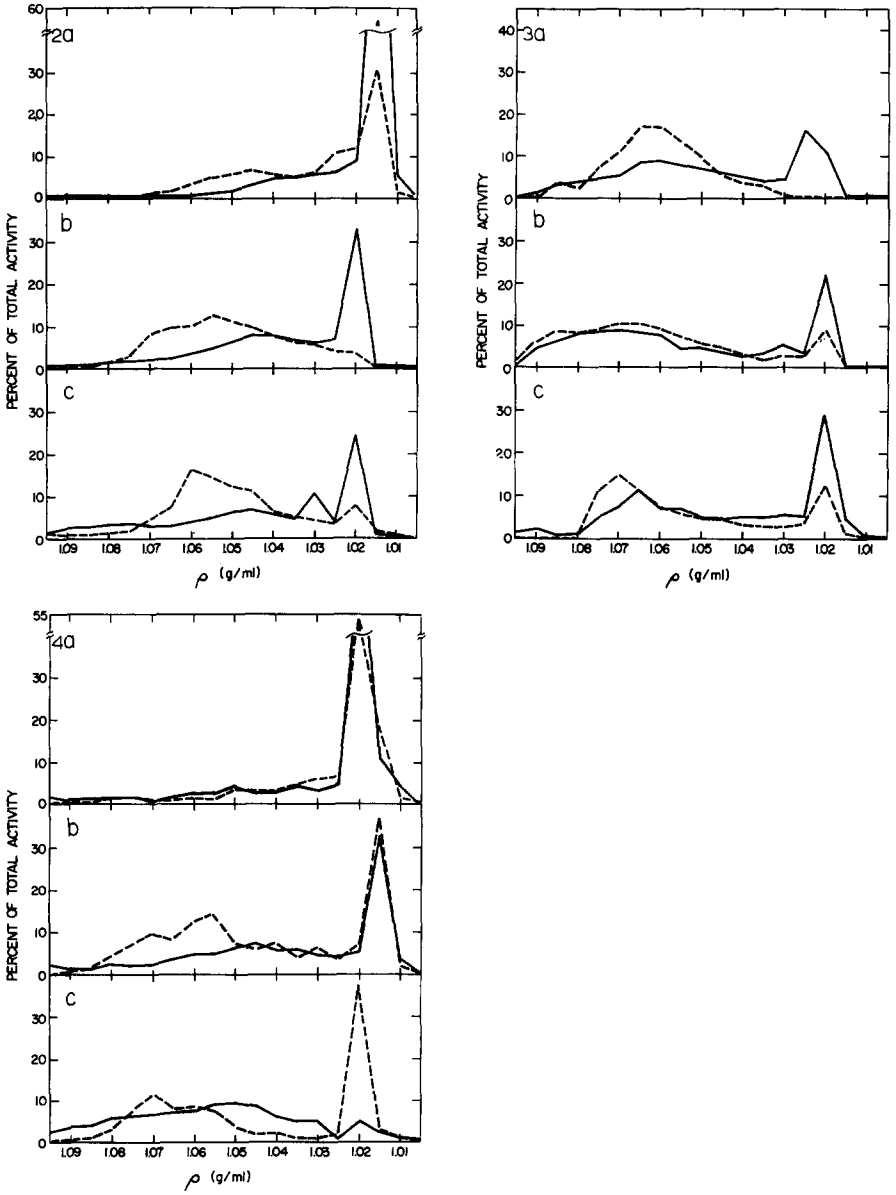


Fig. 1. Diagramatic representation of sucrose gradient (27.6–54.1%, w/v, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4) containing a "lipid-free" adipocyte homogenate, after 90 min centrifugation.

of plasma membrane into all fractions of the sucrose gradient. This co-aggregation is effectively reversed by EDTA (Table I).

The composition of the fractions of the sucrose gradient can be seen in Fig. 1 and



Figs. 2, 3 and 4. Isopycnic distribution of microsomal enzymes in continuous Dextran gradients. ———, 5′-nucleotidase; – – – –, NADH oxidase. Ordinate indicates percent of total recovered activity. Abscissa is $\rho_4°$.

Gradient:	*2a*	*2b*	*2c*	*3a*	*3b*	*3c*	*4a*	*4b*	*4c*
$[Mg^{2+}]$(mM)	0.0	0.1	0.5	1.0	1.5	2.0	0.0	0.0	0.0
pH	8.6	8.6	8.6	8.6	8.6	8.6	7.0	6.5	6.0

Table II. Of the total activities recovered, the upper microsomal region contains 76 % of the 5'-nucleotidase, 100 % of the (Na^+–K^+)-ATPase, and 66 % of the NADH oxidase, with only 3.3 % mitochondrial contamination. The mitochondrial zone contains 16.4 % of the 5'-nucleotidase. On rehomogenization of the mitochondrial zone and recycling through an identical sucrose gradient, approx. 50 % of its nucleotidase content was released into a density region corresponding with that of the microsomal zone. The resuspended nuclear pellet contained intact nuclei with little other membranous material.

Gradient experiments

The behavior of washed, lysed microsomal membranes in continuous Dextran gradients of varying pH and Mg^{2+} concentration can be seen in Figs. 2–5. At pH 8.6 in the absence of divalent cations, both 5'-nucleotidase (plasma membrane) and NADH oxidase (endoplasmic reticulum) show a sharp peak at ρ 1.01–1.02, with a trail down to ρ 1.05. With the addition of $1 \cdot 10^{-4}$ M Mg^{2+}, the modal density of the endoplasmic reticulum is shifted to ρ 1.05, with considerable spread. The sharp peak of endoplasmic reticulum at ρ 1.01–1.02 is virtually abolished. The plasma membrane, however, appears bimodal. A portion of the activity shifts to a modal density of 1.04, but a ρ 1.01–1.02 peak, somwehat diminished, persists. At $5 \cdot 10^{-4}$ M Mg^{2+} the endoplasmic reticulum is shifted to a slightly higher density, but the plasma membrane distribution, while similar to that at $1 \cdot 10^{-4}$ M is more complex, with several peaks. At $1 \cdot 10^{-3}$ M Mg^{2+}, the plasma membrane is still bimodal, the denser component showing complete overlap with the endoplasmic reticulum. Aggregation becomes apparent at this Mg^{2+} concentration in the regions of highest density. As the Mg^{2+} concentration is raised to to $2 \cdot 10^{-3}$ M, the aggregation becomes more extensive, but the density distribution of the endoplasmic reticulum and plasma membrane remains essentially as at $1 \cdot 10^{-3}$ M.

Progressive lowering of the pH in the absence of added divalent cations, effect little change in density distribution until pH 6.5, where a portion of both endoplasmic reticulum and plasma membrane shift to a higher density. In contrast to the effects of

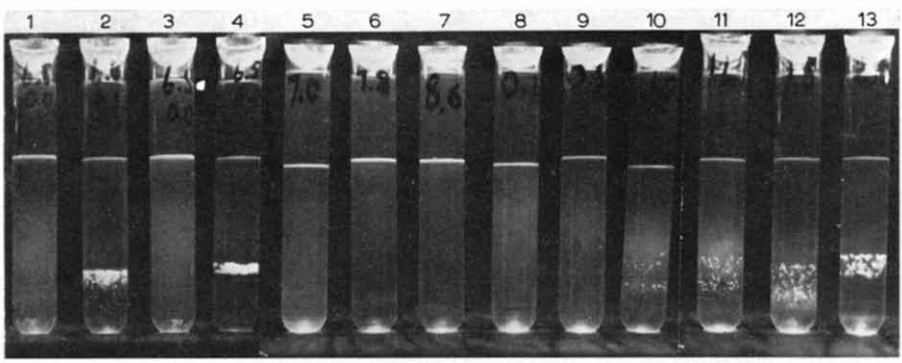


Fig. 5. Isopycnic distribution of microsomes in continuous Dextran gradients. Composition of gradients as follows:

Gradient No.:	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
[Mg^{2+}](mM)	0.	0.1	0.0	0.1	0.0	0.0	0.0	0.1	0.5	1.0	1.25	1.5	2.0
pH	6.0	6.0	6.5	6.5	7.0	7.8	8.6	8.6	8.6	8.6	8.6	8.6	8.6

TABLE III

COMPARISON OF PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM DISTRIBUTION IN CONTINUOUS *versus* DISCONTINUOUS DEXTRAN GRADIENTS

Plasma membrane and endoplasmic reticulum distributions calculated from percent of recovered 5'-nucleotidase and NADH oxidase, respectively, in a given region. Gradients were prepared as described in MATERIALS AND METHODS and centrifuged 12–15 h at 40000 rev./min.

Gradient conditions			*Species*	*Percent of species remaining at or above the density barrier*		*Percent of species calculated to remain at or above the density barrier which actually moves past the barrier*
pH	*Mg^{2+} (mM)*	*Barrier density*		*Calculated from continuous gradient*	*Observed in discontinuous gradient*	
8.6	0.1	1.030	Plasma membrane	46.5	36.5	21.5
			Endoplasmic reticulum	17.6	5.8	67.0
8.6	0.1	1.040	Plasma membrane	65.5	43.1	34.2
			Endoplasmic reticulum	32.9	7.9	75.5
8.6	0.5	1.042	Plasma membrane	55.9	44.7	20.2
			Endoplasmic reticulum	34.5	8.1	76.5
8.6	1.0	1.067	Plasma membrane	82.5	69.2	16.1
			Endoplasmic reticulum	78.8	52.3	33.7

Mg^{2+} addition, addition of H^+ to pH 6.0 shifts the plasma membrane uniformly to higher density, but causes a bimodal distribution of endoplasmic reticulum. The addition of $1 \cdot 10^{-4}$ M Mg^{2+} at pH 6.5 and 6.0 led to a total aggregation of particles, in excess of that seen with 20-fold greater Mg^{2+} concentration at pH 8.6.

In order to achieve separation of plasma membrane from endoplasmic reticulum, a discontinuous rather than continuous Dextran gradient system was used. This was based on the observations outlined in Table III. Under all conditions tested, a greater percentage of the microsomal protein passed through a barrier of a given density than was calculated from the results of the corresponding continuous Dextran gradient. The feature that results in enhanced separation of plasma membrane from endoplasmic reticulum is that in all cases, of the material which was calculated to be retained at the barrier, a 2- to 3-fold greater percentage of endoplasmic reticulum than plasma membrane actually passed on through the barrier. Optimal separation was achieved in discontinuous gradients containing $5 \cdot 10^{-4}$ M Mg^{2+}, $1 \cdot 10^{-3}$ M Tris–HCl (pH 8.6), with a density barrier at 1.040–1.042 (Table IV). Under these conditions well over 90 % of the NADH oxidase activity passes through the interface, while approx. 45 % of the applied 5'-nucleotidase is retained at and above the interface. The results of a typical fractionation can be seen in Table V.

DISCUSSION

Properties and recovery of enzyme markers

5'-Nucleotidase has proven a useful plasma membrane marker in several tissues[15–17], and this appears to hold true for adipose tissue. All the homogenate activity measurable at pH 8.0 could be sedimented at 40000 rev./min for 30 min. At pH 9.0 an additional soluble phosphatase was detectable. The particulate enzyme had

TABLE IV

PLASMA MEMBRANE PURIFICATION ON DISCONTINUOUS DEXTRAN GRADIENTS

Distribution of protein and enzyme activities recovered in the fractions of the discontinuous dextran gradient. Procedure described in MATERIALS AND METHODS under *Plasma membrane separation*. Summary of three experiments. Specific activities as in Table I. Results are given as ± S.E.

	Protein (%) *Total*	*5′-Nucleotidase* (%)		*(Na^+-K^+)-ATPase* (%)		*NADH oxidase* (%)	
		Spec. act.	*Total*	*Spec. act.*	*Total*	*Spec. act.*	*Total*
Pellet and dextran (endoplasmic reticulum fraction)	78.3 ± 3.2	2.85 ± 0.61	55.3 ± 4.4	0.82 ± 0.31	55.2 ± 5.4	3.61 ± 0.28	91.9 ± 2.8
Interface and buffer (plasma membrane fraction)	21.7 ± 2.9	8.65 ± 2.32	44.7 ± 4.3	2.89 ± 1.15	44.8 ± 5.4	1.18 ± 0.20	8.1 ± 1.3

TABLE V

RESULTS OF A SINGLE REPRESENTATIVE ADIPOCYTE FRACTIONATION

Distribution of protein and enzyme activities within a representative adipocyte fractionation. Total column represents all the activity recovered in that fraction. Units are μmoles/h for phosphohydrolases; otherwise μmoles/min. — indicates no activity detectable. PM, plasma membrane. E.R., endoplasmic reticulum.

	Protein mg	*5′-Nucleotidase*		*Mg^{2+}-ATPase*		*(Na^+-K^+)-ATPase*		*NADH oxidase*		*succinate cytochrome c reductase*	
		Total	*Spec. act.*	*Total*	*Spec. act.*	*Total*	*Spec. act.*	*Total*	*Spec. act.*	*Total*	*Spec. act.*
Homogenate	25.51	20.40	0.80	195.0	7.65	21.15	0.83	29.83	1.17	2.38	0.0931
Mitochondrial	2.60	2.68	1.03	85.5	22.50	—	—	3.90	1.50	1.69	0.651
Nuclear	0.57	0.63	1.10	6.59	11.57	—	—	0.32	0.56	0.03	0.054
Microsomal	4.08	13.37	3.28	71.0	17.38	20.06	4.92	12.28	3.01	0.07	0.0176
PM fraction	0.40	5.17	12.92	8.01	20.00	1.74	4.34	0.54	1.35	—	—
ER fraction	1.43	5.49	3.84	19.47	13.61	1.72	1.20	4.98	3.48	—	—

a broad pH optimum between 7.0 and 9.5. The plasma membrane fraction showed a 12- to 16-fold greater specific activity over the corresponding homogenate. Estimation of the total recovery of homogenate activity in the final plasma membrane fraction is complicated because approx. 20 % of original activity decays over the course of the fractionation, and sucrose and Dextran gradients yield only 85 % recovery of the applied activity. Ignoring these considerations leads to an estimate of 24.5 % recovery of homogenate plasma membrane; correcting for them gives an estimate of 34 0 % recovery.

The inability of McKEEL AND JARETT[18] to demonstrate 5'-nucleotidase activity in their preparation of plasma membrane is possibly due to the relative insensitivity of their assay conditions. A plasma membrane fraction prepared by their method was found to contain significant 5'-nucleotidase activity when measured by the radioassay described above (J. AVRUCH, unpublished data).

Na^+–K^+-stimulated ATPase, another generally plasma membrane[13,19] marker, was fractionated in parallel fashion to 5'-nucleotidase, but proved unsatisfactory as a routine plasma membrane marker. 72–100 % of the homogenate activity was recovered in the microsomal fraction with essentially none present in the mitochondrial or nuclear fraction. However, the Na^+–K^+ stimulation decayed (50–70 %/24 h) more rapidly than the Mg^{2+}-stimulated ATPase and generally represented only a 20–25 % (occasionally up to 50 %) increase over the Mg^{2+}-activated ATPase. This latter activity was found in every fraction, with little evidence of enrichment. 52–70 % of the homogenate Mg^{2+}-stimulated ATPase was recovered in particulate fractions. The activity associated with the "mitochondrial" and nuclear fractions showed a consistent 2–10 % inhibition in the presence of 100 mM Na^+, 15 mM K^+.

NADH oxidase is presently the only enzymatic endoplasmic reticulum available for adipose tissue. If it is not intrinsically present in the plasma membrane, then the final plasma membrane fraction is contaminated with endoplasmic reticulum to the extent of 27 %. This is virtually identical to that found by McKEEL AND JARETT[18] in their preparations of fat cell plasma membrane. Smooth endoplasmic reticulum has generally proved the component most difficult to separate from plasma membrane. The endoplasmic reticulum fragments tend to parallel those of the plasma membrane in size and density under a variety of conditions. For example, in various preparations of liver plasma membrane[20–22], endoplasmic reticulum contamination ranged from 10–20 %. Preparations of Ehrlich ascites cell plasma membrane contained from 12–32 % endoplasmic reticulum. However, some of the markers commonly used for endoplasmic reticulum, such as NADH oxidase[23] and RNA[24], may be represented in plasma membrane as well. This uncertainty allows only a maximal estimate of endoplasmic reticulum contamination in the present case.

Cell disruption and preliminary separation

The plasma membrane and endoplasmic reticulum differ from other subcellular particulate components (lysosomes, mitochondria, nuclei) in that the former (especially the plasma membrane) can assume a wide variety of sizes and densities upon cell disruption. This has contributed to the difficulty of separation of plasma membrane with good yield and purity by the simple centrifugation techniques that suffice for more discrete, homogeneous subcellular particulates. The size and density of plasma membrane fragments depend on the method of cellular disruption, the resistance of

various areas of the membrane to the disrupting force, the presence of surface specializations and the tendency to spontaneous vesiculation. Many cell membranes tend to bud into small closed vesicles; this tendency may be influenced by the ionic-osmotic environment[25,26]. In general, it is least marked in whole cells, but may occur[27]. Cellular disruption appears to greatly enhance this phenomenon. These vesicles vary in size and show semipermeable behavior; thus their equilibrium density will be a function of their environment[28].

Little is known concerning the resistance of membranes to mechanical fragmentation. In rat kidney[29] for example, the endoplasmic reticulum undergoes extensive fragmentation with mechanical homogenization, while at least a fraction of the plasma membrane is quite resistant. In general, areas of surface specialization (*e.g.* desmosomes, the complex outer coats around the sarcolemma, brush border) resist both mechanical fragmentation and spontaneous vesiculation. On cell breakage, the particles tend to be of large size and of open sheetlike structure, whose density is much less dependent on environment than is the case with vesicles[30,31]. This resistance can be overcome with greater homogenizing force, resulting in the disintegration of these structures into vesicles [32,33]. WARREN *et al.*[34] have attempted to increase the resistance of the plasma membrane to homogenization by "toughening" the surface of L cells with various substances. It appeared more reasonable to tailor the techniques of cell disruption and preliminary fractionation to the characteristics of the membrane to be isolated. The surface of the isolated fat cell appears unspecialized, tends to vesiculate spontaneously and is very sensitive to mechanical homogenization. An attempt was made to exploit these properties by extensively fragmenting the cells, harvesting the membranes as vesicles and subfractionating this mixed vesicle population. With the sieving technique the cells can be totally and reproducibly disrupted and separated from the released lipid in 5 min, without appreciable heating. The preservation of nuclei is in excess of 95 %.

The microsomal region of the sucrose gradient contains 76–100 % of the recovered plasma membrane and 3 % of the mitochondria. It contains no nuclei by phase microscopy. The distribution of the endoplasmic reticulum is more difficult to evaluate, as the NADH oxidase activity is present in the mitochondrial outer membrane as well. A consistent proportion of the total recovered NADH oxidase (66.3 %) appeared in the microsomal fraction.

Dextran gradient studies

The subfractionation of a mixed microsomal vesicle population was based on principles evolved from the study of Ehrlich ascites cell microsomes[29,35,36]. The surfaces of membranous vesicles contain fixed charges. The charges on the inner surface of the membranes create an asymmetrical distribution of the permeant ionic species of the bathing medium, according to the Gibbs–Donnan equilibrium. In media of high ionic strength (*e.g.* NaBr) or high osmotic activity (*e.g.* sucrose), this intravesicular Donnan osmotic effect is rendered trivial. At low ionic strengths and osmotic activities, however, this effective intravesicular osmotic pressure becomes significant and the vesicles swell until ionic–osmotic equilibrium with the bathing medium is reached. In such a swollen state, the isopycnic density is far below the density of the membrane matrix, due to the large contribution of the internal aqueous compartment. This latter situation appears to obtain in polymer (Dextran) gradients at low ionic strength (1 mM

Tris–HCl, pH 8.6). Neutralization of a portion of the internal fixed charge should diminish the expansive forces within these vesicles. This has been accomplished by manipulation of pH and/or divalent cation concentration. In Figs. 2 and 3 the effect of progressive increase in Mg^{2+} concentration from $1 \cdot 10^{-4}$ to $2 \cdot 10^{-3}$ M is illustrated. The vesicles progressively increase in density over the range $1 \cdot 10^{-4}$ to $1 \cdot 10^{-3}$ M. Of special note is that the titration of endoplasmic reticulum and plasma membrane proceed differently. H^+ titration can also effect density shifts, which appear qualitatively different. The density behavior of adipocyte vesicles, while qualitatively similar to that seen with Ehrlich ascites cell microsomes[28], is quantitatively different. The increase in density on cation titration is greater for Ehrlich ascites cell vesicles, especially endoplasmic reticulum.

Distinct from the phenomena discussed above is that of particle aggregation. In these studies, as in others[37], aggregation was a function of ionic and H^+ concentrations. With increasing cation concentration, visible aggregates first appear at $1 \cdot 10^{-3}$ M Mg^{2+}. As the cation concentration is increased to $2 \cdot 10^{-3}$ M, the extent of aggregation steadily increases without any significant density changes. A similar situation is seen as pH is lowered. Although large density shifts have occurred by pH 6.0, the aggregation is minor. At pH 6.0, however, the sensitivity to Mg^{2+}-induced aggregation is increased markedly. A partial explanation for this behavior is that aggregation is promoted as fixed charged groups on the outer surface of the vesicles are shielded or neutralized. This diminishes electrostatic repulsion between particles and promotes inelastic collisions. Finally, it would appear that even under conditions of density overlap between endoplasmic reticulum and plasma membrane, aggregates are found at higher density than non-aggregated particles.

The titrations of both inner and outer surface charges are pertinent to the separation of plasma membrane from endoplasmic reticulum on a discontinuous rather than continuous Dextran gradient. In such a discontinuous gradient, vesicles are rapidly sedimented to the interface, resulting in a high particle concentration. Aggregation is thereby fostered and is most likely between particles of low electrostatic repulsion. If these particles are those with a higher density as well (*i.e.* endoplasmic reticulum), these factors are reinforcing and the efficiency of the separation is enhanced over that seen in a continuous gradient. The assumptions underlying these arguments have been extensively detailed[36,38]. Two, however, deserve mention here: (1) The bulk of the vesicles membranes retain their original right-side-out orientation; (2) the differences in surface charge densities between endoplasmic reticulum and plasma membrane are greater than the variation within either species.

This work reports the efficient reduction and separation of fat cell plasma membrane into a microsomal fraction. This mixed microsomal population has been subfractionated into plasma membrane and endoplasmic reticulum on the basis of surface charge-properties.

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