PREPARATION AND CHARACTERIZATION OF A PLASMA MEMBRANE FRACTION FROM ISOLATED FAT CELLS

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ABSTRACT

A rapid method of preparing plasma membranes from isolated fat cells is described. After homogenization of the cells, various fractions were isolated by differential centrifugation and linear gradients. Ficoll gradients were preferred because total preparation time was under 3 hr. The density of the plasma membranes was 1.14 in sucrose. The plasma membrane fraction was virtually uncontaminated by nuclei but contained 10% of the mitochondrial succinic dehydrogenase activity and 25–30% of the RNA and reduced nicotinamide adenine dinucleotide cytochrome *c* reductase activity of the microsomal fraction. Part of the RNA and NADH-cytochrome *c* reductase activity was believed to be native to the plasma membrane or to the attached endoplasmic reticulum membranes demonstrated by electron microscopy. The adenyl cyclase activity of the plasma membrane fraction was five times that of Rodbell's "ghost" preparation and retained sensitivity to epinephrine. The plasma membrane ATPase activity was five times that of the homogenate and microsomal fractions. Electron microscopic evidence suggested contamination of the plasma membrane fraction by other subcellular components to be less than the biochemical data indicated.

INTRODUCTION

The plasma membrane of fat cells has been suggested as the site of initial action for many hormones (1). A membrane-bound enzyme, adenyl cyclase, seems to be involved in the primary response to several hormones such as catecholamines, ACTH[1], glucagon, and TSH, which are known to stimulate lipolysis in fat tissue (2). Furthermore, catecholamines seem to have a different receptor site for stimulating adenyl cyclase than do ACTH and glucagon (3, 4). Stimulation of the adenyl cyclase activity increases the level of 3′,5′-cyclic AMP, which plays a key role in regulating lipolysis as a secondary messenger (2).

It has been suggested that the antilipolytic activity of insulin results from its ability to lower 3′,5′-cyclic AMP levels by acting on adenyl cyclase (5, 6). However, insulin affects adenyl cyclase activity only in intact cellular systems and

[1] The following abbreviations are used in this paper: ACTH, adrenocorticotrophic hormone; TSH, thyroid stimulating hormone; 3′,5′-cyclic AMP, cyclic adenosine-3′,5-monophosphate; ATP, adenosine triphosphate; AMP, adenosine monophosphate; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate; Tris, Tris(hydroxymethyl)aminomethane; SDH, succinic dehydrogenase; ATPase, adenosine triphosphatase; Pi, inorganic phosphate; NADH, reduced nicotinamide adenine dinucleotide; ER, endoplasmic reticulum.

not when added to broken cell systems. Other actions of insulin, such as stimulation of glucose uptake and amino acid incorporation into protein, as well as the inhibition of the antilipolytic actions of other hormones, can be imitated by phospholipase C (7, 8). These findings suggest that the plasma membrane is the common site of action of these two agents.

In order to study adequately the interaction of these various hormones with the plasma membrane of fat cells and with the enzyme adenyl cyclase, a preparation of intact plasma membranes still containing the adenyl cyclase activity is desirable. In the present study a procedure is described for preparing such a fraction, and it is characterized both morphologically and biochemically. Part of this work has been previously reported in brief (9).

MATERIALS AND METHODS

Animals

Groups of 12–16 fed, male rats of the Wistar strain (National Laboratory Animal Co., Creve Coeur, Mo.) weighing 150–180 g were used for each fractionation experiment. This weight range provided the maximum yield of plasma membranes. The rats were fed *ad libitum* on Purina lab chow (Ralston Purina Co., St. Louis, Mo.) and were allowed free accesss to tap water.

Reagents

Collagenase was purchased from Worthington Biochemical Corp. (Freehold, N.J.). Uniformly ^{14}C-labeled ATP, and cyclic AMP-^{3}H were obtained from New England Nuclear Corp. (Boston, Mass.) and Schwartz Bioresearch, Inc. (Orangeburg, N.Y.). ATP, horse heart cytochrome *c*, type VI, calf thymus DNA, and yeast RNA were obtained from Sigma Chemical Co. (St. Louis, Mo.). 2,6-Dichlorophenolindophenol was obtained from Fisher Scientific Co. (St. Louis, Mo.). Sodium succinate was supplied by C. F. Boehringer and Soehne GMBH (Mannheim, Germany). Fatty acid-poor BSA Fraction V was puchased from Nutritional Biochemicals Corporation (Cleveland, Ohio). Ficoll is a product of Pharmacia (Uppsala, Sweden). All other chemicals were standard commercial products of reagent grade quality.

Fractionation Procedures

Isolated Fat Cells

The distal halves of epididymal fat pads from 12–16 decapitated rats were pooled, and isolated fat cells were prepared as described by Rodbell (10), except that 1% BSA was used. The incubation was stopped after 25–30 min when only two to four small lumps of undigested tissue were visible after gentle hand swirling of each incubation flask. The isolated fat cells were gently filtered through a fine mesh Japanese silk screen and washed with 2 volumes of the modified Krebs buffer used for incubation. The cell suspension was centrifuged at 1000 *g* for 30 sec and the infranatant was removed. The fat cells were diluted with 4 volumes of a slightly chilled solution (medium I) that contained 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4 and were recentrifuged at 1000 *g* for 30 sec. All subsequent steps in the fractionation procedure were carried out at 4°C.

Fig. 1 is a flow diagram that illustrates the over-all fractionation scheme. The "*g*" values refer to the average centrifugal force at the midportion of the centrifuge tube. The fractionation procedure was monitored by phase microscopy in initial experiments.

The Homogenate Fraction

The washed, isolated cells were gently mixed with 8 ml of medium I and decanted into a prechilled glass homogenizer fitted with a Teflon pestle (clearance 0.006–0.009 in.). Additional medium I was used to bring the final homogenization volume to 35 ml. Homogenization consisted of 10 up and down strokes at 1800 rpm. The crude homogenate was transferred quickly to prevent congealing of the fat and was divided into 27 ml (*A*) and 8 ml (*B*) portions (see below). Several milliliters of portion *A* were aspirated from the bottom of the tube to avoid contamination with fat; this constituted the "homogenate" fraction to be used for assay purposes.

Potter-Elvehjehm glass homogenizers fitted with Teflon pestles (clearance 0.004–0.006 in.) were used to resuspend all subsequent pellets. Unless otherwise noted, six strokes at 1500 rpm were used in the resuspension steps.

The Nuclear Fraction

Portion *A* of the homogenate was centrifuged at 16,000 *g* for 15 min. This resulted in the separation of a tightly packed fat cake floating on top of a turbid intermediate layer (*S1*, Fig. 1) and a white pellet (*P1*, Fig. 1). The intermediate layer was used to prepare a microsomal fraction (see below). The pellet was resuspended in 8 ml of medium I by using six strokes at 1000 rpm and was centrifuged at 1000 *g* for 10 min. The supernatant (*S2*, Fig. 1) was aspirated and used to prepare plasma membrane and gradient mitochondrial fractions (see below). The pellet (*P2*, Fig. 1) was resuspended in 1–2 ml of a solution (medium II) that contained 0.25 M sucrose and 10 mM Tris-HCl, pH 7.4, and comprised the nuclear fraction.

PREPARATION OF FAT CELL FRACTIONS

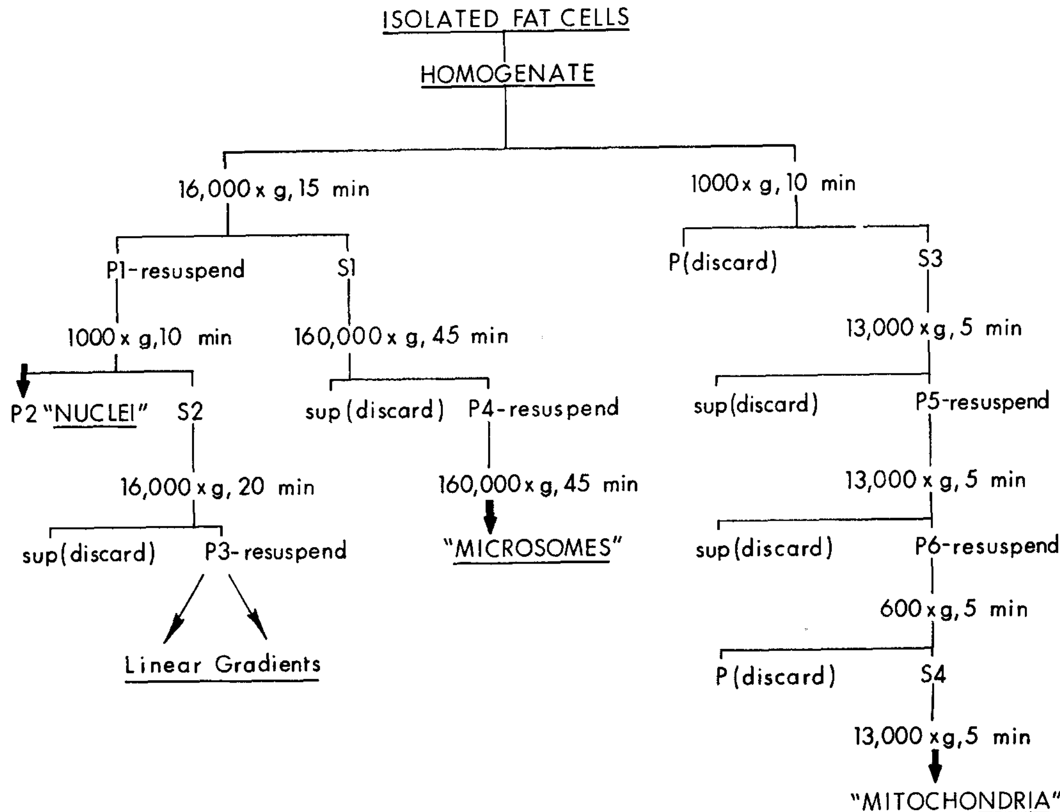


FIGURE 1 Schematic representation of the procedure for fractionation of isolated fat cells.

THE MICROSOMAL FRACTION

Fraction *S1* (Fig. 1) was centrifuged at 160,000 *g* for 45 min. The pellets (*P4*, Fig. 1) were resuspended in medium I and recentrifuged at 160,000 *g* for 45 min. The pellet, resuspended to 1–2 ml in medium II, formed the "microsomal" fraction.

THE PLASMA MEMBRANE AND GRADIENT MITOCHONDRIAL FRACTIONS

The postnuclear supernatant (*S2*, Fig. 1) was centrifuged at 16,000 *g* for 20 min. The pellet (*P3*, Fig. 1) was resuspended in 8 ml of medium I. 4-ml portions were layered carefully on 26-ml linear gradients of two types (see below). All gradients were prepared on the day of use and allowed to equilibrate in the cold room for several hours.

LINEAR SUCROSE GRADIENTS: The gradients formed were 1.05 density at the top and 1.25 density at the bottom, and contained 1 mM EDTA and 0.005 M Tris-HCl, pH 7.4. Centrifugation at 24,000 rpm for 90 min in a Beckman L-4 ultracentrifuge SW 25.1 rotor (Beckman Instruments, Palo Alto, Calif.) resulted in the separation of two finely particulate bands at densities of 1.14 and 1.18 (Fig. 2). The bands were removed separately and the 1.14 d band was diluted 4:1 (v:v) with medium I. After being centrifuged at 16,000 *g* of 15 min, the pellet was resuspended in 1–2 ml of medium II; this was the plasma membrane fraction (sucrose).

Similarly, the 1.18 d band was removed, diluted 6:1 (v:v) with medium I and centrifuged at 16,000 rpm for 10 min. The resulting pellet, resuspended in 1–2 ml of medium II, constituted the gradient mitochondrial fraction (sucrose).

LINEAR FICOLL-SUCROSE (FICOLL) GRADIENTS: Solutions containing 5 and 25% Ficoll (w/v) in 0.25 M sucrose were filtered through 0.8 μ Millipore filters (Millipore Corp., Bedford, Mass.) before use. The Ficoll had previously been dialyzed against deionized distilled water for 18–24 hr to remove salts, and had been lyophilized. Centrifugation

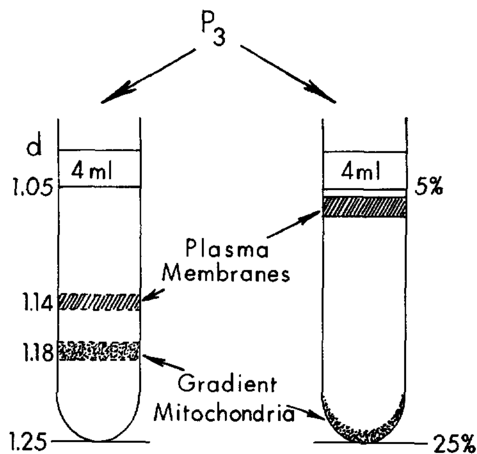


FIGURE 2 Schematic representation of linear gradients after centrifugation of fraction *P3* from Fig. 1.

for 30 min at 24,000 rpm (SW. 25.1 rotor) resulted in the formation of a sharp band just below the sample interface and a layer at the bottom of the tube (Fig. 2).

The band was removed, diluted 4:1 (v:v) with medium I and centrifuged for 15 min at 16,000 *g*. The resulting pellet was resuspended to 1–2 ml in medium II to form the plasma membrane fraction (Ficoll). The layer at the bottom of the gradient was resuspended to 10 ml with medium I and centrifuged for 15 min at 16,000 *g*. The pellet was resuspended in 1–2 ml of medium II to form the gradient mitochondrial fraction (Ficoll).

The Mitochondrial Fraction

Portion *B* (8 ml) of the crude homogenate was used to prepare a mitochondrial pellet as outlined in Fig. 1. The pellet, resuspended to 1–2 ml in medium II, formed the mitochondrial fraction.

Electron Microscopy

Minced epididymal fat pads and isolated cells were fixed in 3% glutaraldehyde followed by 1 or 2% osmium tetroxide in 0.1 M sodium cacodylate-HCl buffer, pH 7.4, dehydrated in ethanol, and embedded in Epon 812. Aliquots of fractions prepared as described under "fractionation procedures" were pelleted and processed similarly, except that after osmication the pellets were rinsed twice in 0.9% saline, immersed for 1 hr in 1% uranyl acetate in 0.3 M sodium acetate, pH 7.0, and rerinsed twice in saline. Ultrathin sections were mounted on uncoated grids, stained with alcoholic uranyl acetate and Reynold's lead citrate (11), and lightly carbon coated. Sections were photographed with a Philips 200 electron microscope operating at 60 kv.

Biochemical Studies

General

Fractions were prepared as described under *Fractionation Procedures* and were diluted to contain 0.5–2.0 mg of protein/ml of medium II.

Protein

The colorimetric procedure described by Lowry et al. (12) was used. Daily standards were prepared from stock solutions of BSA fraction V.

RNA

Aliquots of isolated fractions were extracted and assayed by a modification of the Schmidt-Thannhauser procedure described by Fleck and Munro (13). Purified yeast RNA was used as a standard, and aliquots were carried through the extraction procedure to check for recovery.

DNA

The diphenylamine colorimetric reaction was used with the modifications described by Burton (14). Calf thymus DNA was used as a standard, and aliquots were carried through the extraction procedure to check for recovery.

Adenyl Cyclase

The double isotope assay used has been described in detail by Cryer et al. (15). Corrections for double isotope were performed by a program developed for the Olivetti Programma 101 calculator (Olivetti Underwood Corp., New York).

Succinic Dehydrogenase

The succinate-dependent reduction of indophenol to leucoindophenol was followed spectrophotometrically by the method of Bachmann et al. (16).

Phosphatases

ATPase: The assay system in a final volume of 1.0 ml contained either 20 mM Tris-HCl, pH 7.4 (Tris-ATPase) or 20 mM $NaHCO_3$ that was equilibrated with 95% O_2–5% CO_2 and adjusted to pH 7.4 with 0.1 N NaOH (HCO_3-ATPase), 118 mM NaCl, 5 mM KCl, 3.5 mM $MgSO_4$, 3.5 mM ATP, distilled water, and 0.03–0.2 mg of fraction protein. Incubations were carried out in triplicate for 10 min at 37°C in a Dubnoff shaker water bath (Precision Scientific Co., Chicago, Ill.). "Zero" time controls, in which the fraction aliquots were added to the complete reaction mixture at 4°C after the addition of perchloric acid, were used routinely. The reactions were stopped by adding 0.3 ml of cold 3.0 M perchloric acid, allowed to stand at 4°C for 10 min, centrifuged, and 1.0 ml aliquots were neutralized with 0.35 ml of 2 N potassium bicarbonate. The precipitate was sedimented and aliquots of the supernatant were assayed in duplicate for inorganic phosphate by the method of Fiske and Subbarow (17).

5′-NUCLEOTIDASE: The assay conditions were identical to those for Tris-ATPase, except that adenosine-5′-monophosphate was used as substrate.

NADH-cytochrome *c* Reductase

The reduction of cytochrome *c* was followed spectrophotometrically as described by Dallner et al. (18).

RESULTS

General Remarks

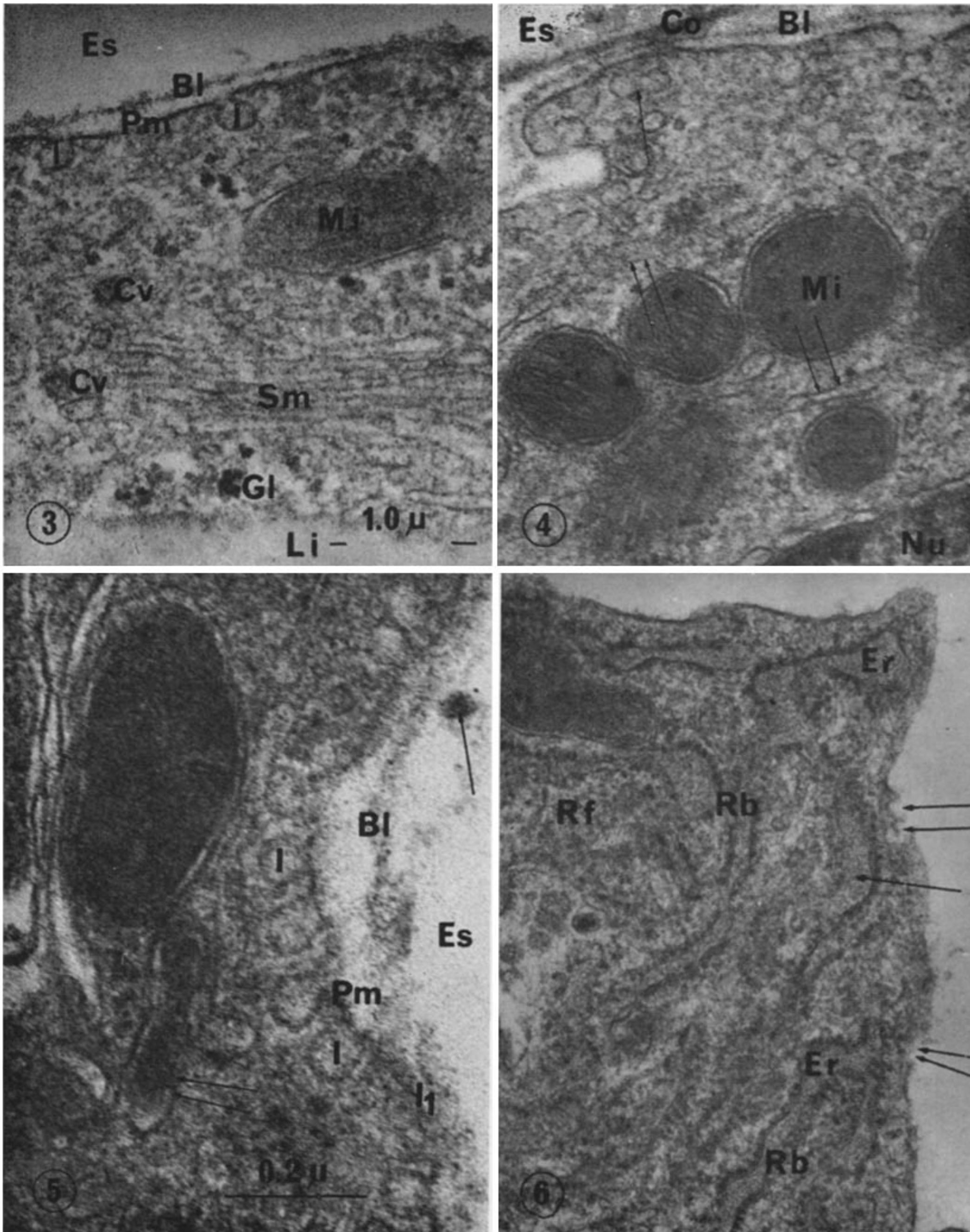
Initial attempts to purify plasma membranes from hypotonic lysates of isolated fat cells prepared according to Rodbell's method (19) were unsuccessful. A postnuclear 16,000 *g* pellet of the lysate was fractionated on a sucrose gradient and vesicles with invaginations similar to those on the plasma membrane of intact fat cells were found near 1.14 d. However, electron microscopy revealed that the vesicles were highly contaminated with cytoplasmic contents including severely damaged mitochondria, ER and nuclear fragments. Hypotonic lysing was abandoned for these reasons, and subsequent experiments utilized isotonic sucrose homogenates exclusively.

Homogenization conditions were of critical importance to the success of the present method. Homogenization in an isotonic sucrose medium was found to minimize damage to cellular organelles, especially mitochondria and nuclei. This was particularly desirable for studies in which the distribution and properties of ATPase in the fat cell were investigated. Increasing sucrose concentration beyond 0.25 M did not increase mitochondrial preservation during fractionation. EDTA was empirically found to be necessary in the homogenization medium to achieve reproducible separation of plasma membrane vesicles from other components in the fat cell.

The final yield of plasma membranes was critically dependent on the conditions used to resuspend *P1*. Phase and electron microscopy of *P1* prior to resuspension showed vesicles varying in diameter from 10 μ to several hundred angstroms, plus mitochondria and, rarely, rough ER. The conditions of resuspension was chosen to selectively rupture the larger vesicles and release entrapped organelles and cytoplasmic contents, while leaving the less contaminated smaller vesicles and free organelles relatively intact. Too vigorous grinding at this step resulted in the formation of vesicles that would not resediment at 16,000 *g* and greatly reduced the final yield of plasma membranes. With both types of gradients, the final yield of plasma membrane fraction protein from 16 rats was 1.0–1.5 mg/20–25 mg of homogenate protein.

Electron Microscopy

Intact adipose tissue and isolated cells were examined to establish the morphologic features characteristic of the major subcellular components. The basal lamina that surrounded fat cells *in situ* (Figs. 3–5) was removed by collagenase and was not seen around isolated fat cells (Fig. 6) as has been mentioned by Rodbell (1). The mitochondria in both types of preparations had extremely dense matrices (Figs. 3–6). Profiles of smooth membranes



FIGS. 3–5 Portions of intact white adipose cells from adult rat epididymal fat pads are shown.

FIGURE 3 Lamellar arrays of smooth membranes, (*Sm*); plasma membrane, (*Pm*) and its invaginations, (*I*); coated vesicles, (*Cv*); a mitochondrion, (*Mi*); probable glycogen, (*Gl*) aggregates; extracellular space, (*Es*); basal lamina, (*Bl*); part of a lipid inclusion, (*Li*). × 20,000.

FIGURE 4 A portion of cytoplasm near the nucleus (*Nu*) containing mitochondria (*Mi*) with dense matrices and dense granules. The remaining cytoplasm is filled with fused (arrow) and unfused vesicles and tubular smooth membrane profiles (double arrows). Extracellular space, (*Es*); collagen, (*Co*); basal lamina, (*Bl*). × 20,000.

FIGURE 5 Details of the plasma membrane (*Pm*) and its invaginations (*I*). The invaginations consist of a rounded portion, a neck, and a diaphragm. Note that dense material is seen outside the basal lamina (*Bl*) in the extracellular space (*Es*), (arrow), within plasma membrane invaginations (I_1), and in cytoplasmic vesicles (double arrows). × 100,000.

FIGURE 6 A concentration of rough endoplasmic reticulum (*Er*) is present in an isolated fat cell. The cisternae are filled with amorphous dense material (arrow). Numerous bound ribosomes (*Rb*) and free ribosomes (*Rf*) are present. The basal lamina is absent, and breaks (double arrows) in the plasma membrane are seen. × 20,000.

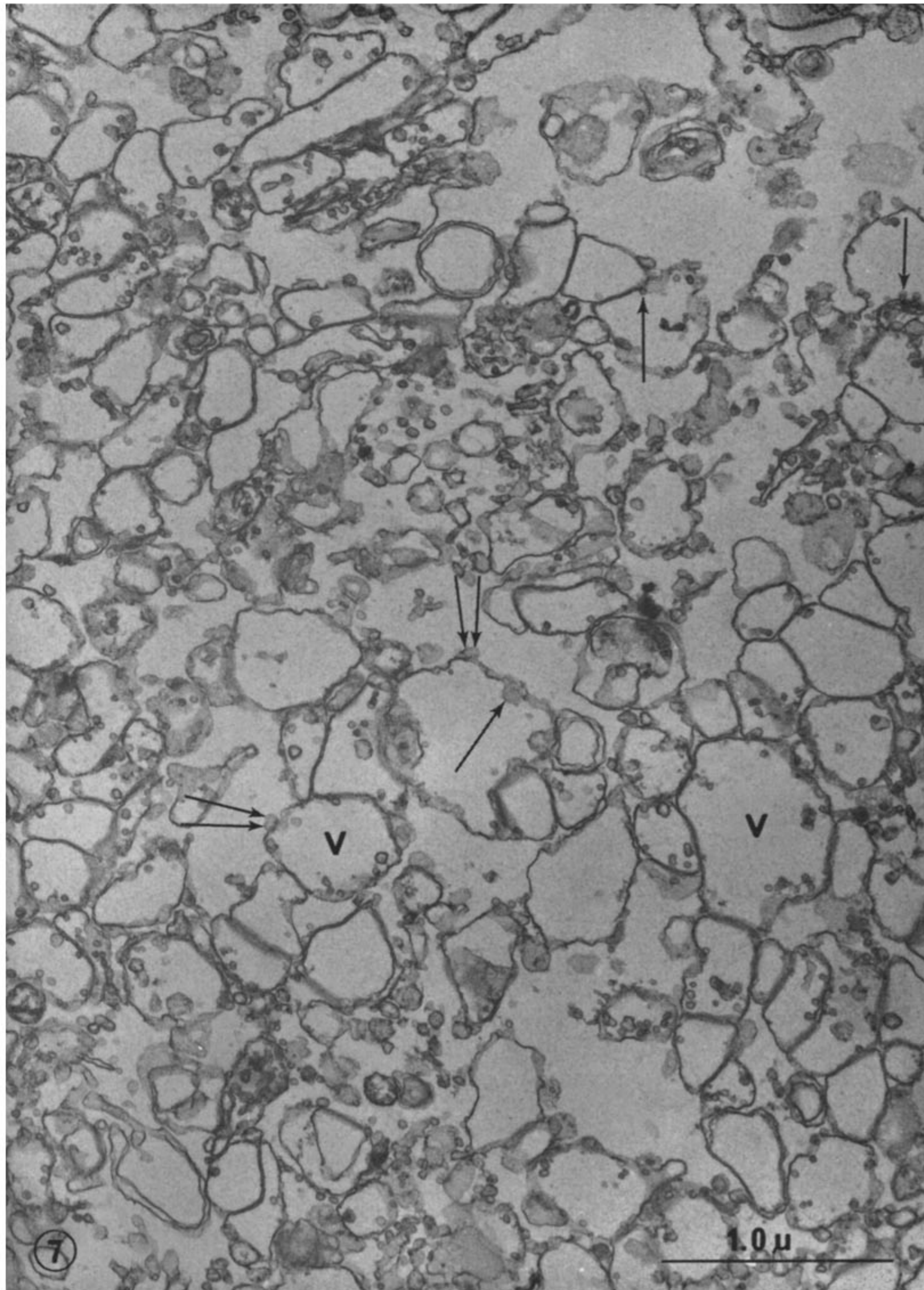


FIGURE 7 Plasma membrane fraction prepared on a Ficoll gradient. Most of the larger vesicles (V) resemble *in situ* plasma membranes in having numerous flask-shaped invaginations (arrows). Outpouchings of the vesicle membranes are also seen (double arrows). No structures resembling mitochondria are seen in this field. × 35,000.

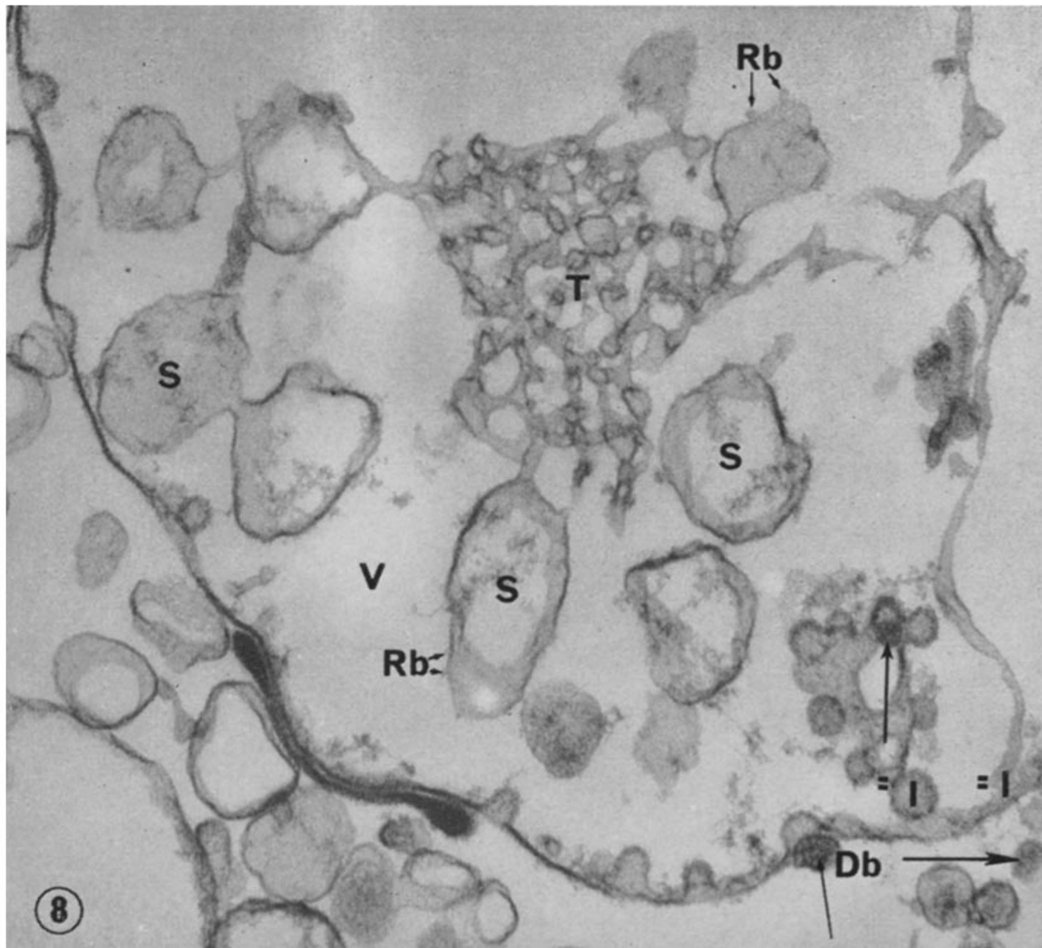


FIGURE 8 A plasma membrane vesicle (*V*) from a Ficoll gradient preparation contains a reticulum formed of predominately smooth sacs (*S*) and a central tubular meshwork (*T*). A few ribosomes (*Rb*) are attached to some of the sacs. The amorphous material in many of the sacs resembles the contents of endoplasmic reticulum cisternae *in situ* (cf. Fig. 6). Several points of communication with the plasma membrane invaginations (*I*) are seen (=). Dense bodies (*Db*) similar to those seen *in situ* are seen within a plasma membrane invagination (small arrow), within fused vesicles (medium arrow), and in the extracellular space (large arrow). × 80,000.

appeared as circles, lamellar stacks, or flattened sacs throughout all portions of the cytoplasm (Figs. 3 and 4). Scattered aggregates of glycogen were present in some cells (Fig. 3). Small clusters of rough ER were occasionally seen. As shown in Fig. 6, ribosomes were tightly packed, and the paired ER membranes, which were randomly arranged with occasional branching, contained abundant amorphous material in the intramembranous space. It should be emphasized that smooth ER membranes were abundant in all portions of most cells, whereas rough ER was found with difficulty in only a few cells. Since intact fat cells were often up to 100 μ in diameter, the chance of missing a cellular component, such as rough ER that was concentrated at particular portions of the cytoplasm, was great. Conversely, the observation of smooth ER in every portion of the cell in random sections indicated the ubiquity of this component in fat cells.

Characteristic invaginations of the fat cell plasma membrane previously described by Williamson (20) proved to be the most useful morphologic marker for this component during isolation. In some portions of intact and isolated cells the invaginations were closely spaced (Fig. 5), but in other portions they were widely separated (Figs. 3 and 4) or were not seen (Fig. 6). The invaginations were flask-shaped or rounded, with the neck bridged by a single membrane 25 A thick. Vesicles similar in size to the rounded portions of the invaginations were often seen throughout the cytoplasm, but differed slightly in that some contained dense material (Fig. 6), or were "coated" with filamentous material (Fig. 3). Fusion of adjacent vesicles was frequently noted (Fig. 4).

The fractions that were isolated and studied biochemically as in Materials and Methods had the following ultrastructural appearances:

PLASMA MEMBRANE FRACTIONS: The plasma membrane fractions from Ficoll and sucrose gradients were similar. Fig. 7 illustrates a typical plasma membrane preparation from a Ficoll gradient. Most of the profiles consisted of membranous sacs roughly 0.5–2.0 μ in diameter that had numerous invaginations and microvesicles similar to the ones seen in intact fat cells. Occasional altered mitochondria were present, but there was a striking discrepancy between the number observed by electron microscopy and the number that should have been observed on the basis of the biochemical studies. Only rarely were components with double membranes or cristae observed, indicating an extremely low contamination by mitochondria. The major contaminating components of this fraction other than mitochondria were portions of smooth and rough ER. Identification of ER in fractions (Fig. 8) was based on the tendency to form swollen, interconnected sacs by a process presumably similar to the one first described by Palade and Siekevitz (21), on the presence of intact or altered ribosomes attached to the outer surface of the sacs, and on the similarity of the material contained in the sacs to the contents observed in the ER of intact and isolated fat cells.

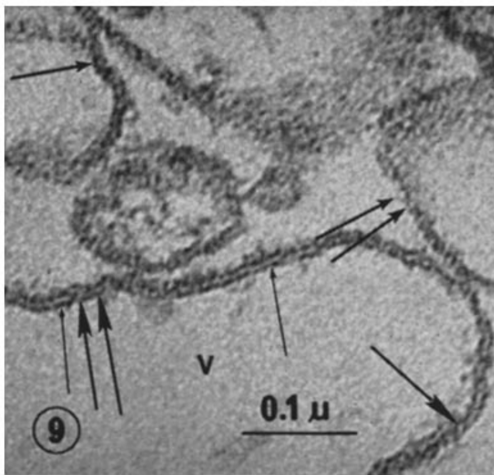


FIGURE 9 Adjacent portions of several plasma membrane vesicles (*V*) from Ficoll gradients display "trilaminar" (small arrow), "globular" (medium arrow), and "twisted" (large arrow) substructural patterns. × 150,000.

Interconnections between the outer surface membrane of the isolated vesicles, i.e., plasma membrane, and the inner sacs were common (Fig. 8). Also both the sacs that seemed to equate with the ER in intact cells and the plasma membrane invaginations contained a homogeneous matrix of identical electron opacity, possibly indicating the transitional nature of the invaginations as sites of exchange between the external environment and the ER. These observations might explain the presence of large but constant amounts of ER marker enzymes associated with the plasma membrane fraction. Another observation that reinforced the transitional role of the invaginations is that they frequently fused to form large, irregular sacs (Fig. 8) similar in matrix density, size, and shape to the presumed ER sacs. Another feature occurring at the plasma membrane, which suggested a dynamic process akin to pinocytosis, was the presence of dense particles lying outside but adjacent to invaginations (Fig. 8) or seemingly being incorporated into invaginations (Fig. 8) or contained with small vesicles internal to the plasma membrane (Fig. 8). Similar observations were made in intact cells (Fig. 6), but the nature of the dense particles is unknown.

A portion of several plasma membrane derived vesicles is shown at high magnification in Fig. 9. Several substructural configurations of the isolated plasma membranes were observed, including a trilaminar "unit membrane" pattern, a globular pattern, and a twisted pattern.

MITOCHONDRIA: The mitochondria prepared by differential centrifugation only (Fig. 10) were similar to the gradient mitochondrial frac-

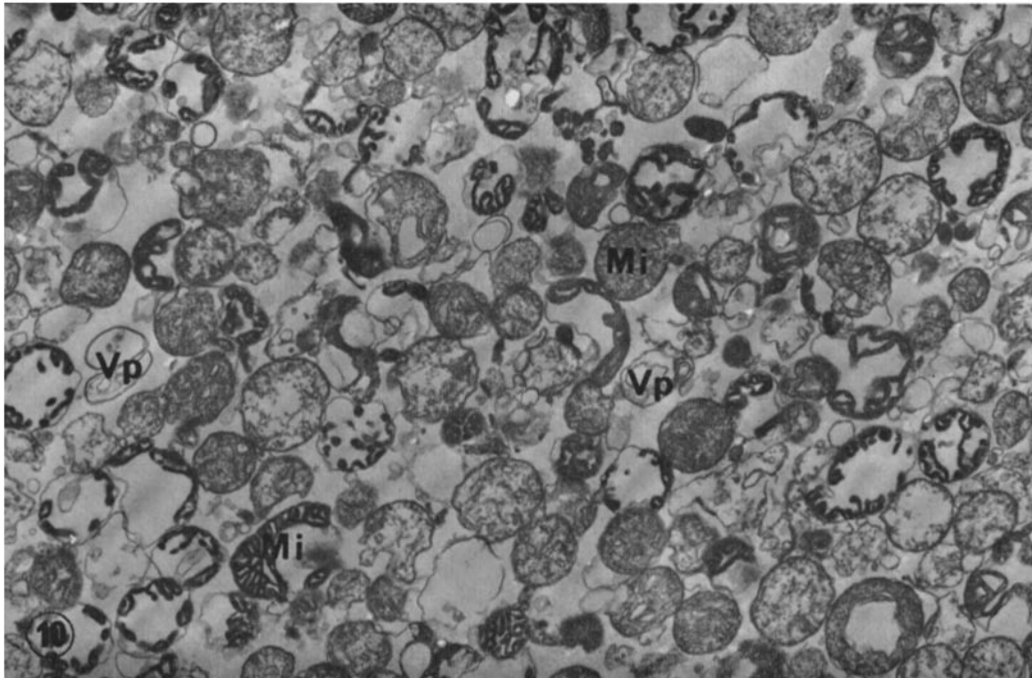


FIGURE 10 Mitochondrial fraction prepared by differential centrifugation and repeated washings. Vesicular contamination has not been eliminated (*Vp*). Mitochondria, (*Mi*). × 20,000.

tions. In all preparations the mitochondria were swollen, and occasional loss of the outer membrane was seen. Since estimation of the biochemical purity of the plasma membrane fraction depended on the purity of the mitochondrial fraction, it should be emphasized that all mitochondrial fractions were found to contain vesicles that could often be identified as originating from the plasma membrane by numerous microvesicular invaginations.

MICROSOMAL FRACTION: A typical microsomal fraction is illustrated in Fig. 11. Most of the fraction seemed to consist of both open and closed fragments of membranes and altered ribosomal material. Some of the smallest vesicles were similar in size to microvesicular invaginations of the plasma membrane, but the large amount of smooth ER present in intact cells was considered a more likely source of the small vesicular component.

Biochemical Studies

The biochemical purification of a cellular component fraction depends on increasing the specific activity of known marker enzymes of the component, while at the same time establishing maximum contamination by other cellular organelles by using marker enzymes of the components characteristic of these organelles. The major cellular structures of fat cells that should sediment in the 16,000 *g* pellet besides plasma membranes are nuclei, mitochondria, ribosomes, and ER membranes. The major biochemical markers for the plasma membrane of the fat cell are adenyl cyclase (1) and possibly ATPase (22).

The following data attempt to establish the purity of the plasma membrane fraction of fat cells by using the above criteria.

Nuclear Contamination

DNA measurement has been used to equate with nuclear content. This assumption was verified by the finding that 85% of the homogenate DNA was found in the nuclear fraction (Table I). The plasma membrane preparation contained only 0.58% of the total DNA content of the homogenate and had only 0.1% of the DNA specific activity of the nuclear fraction (Table I).

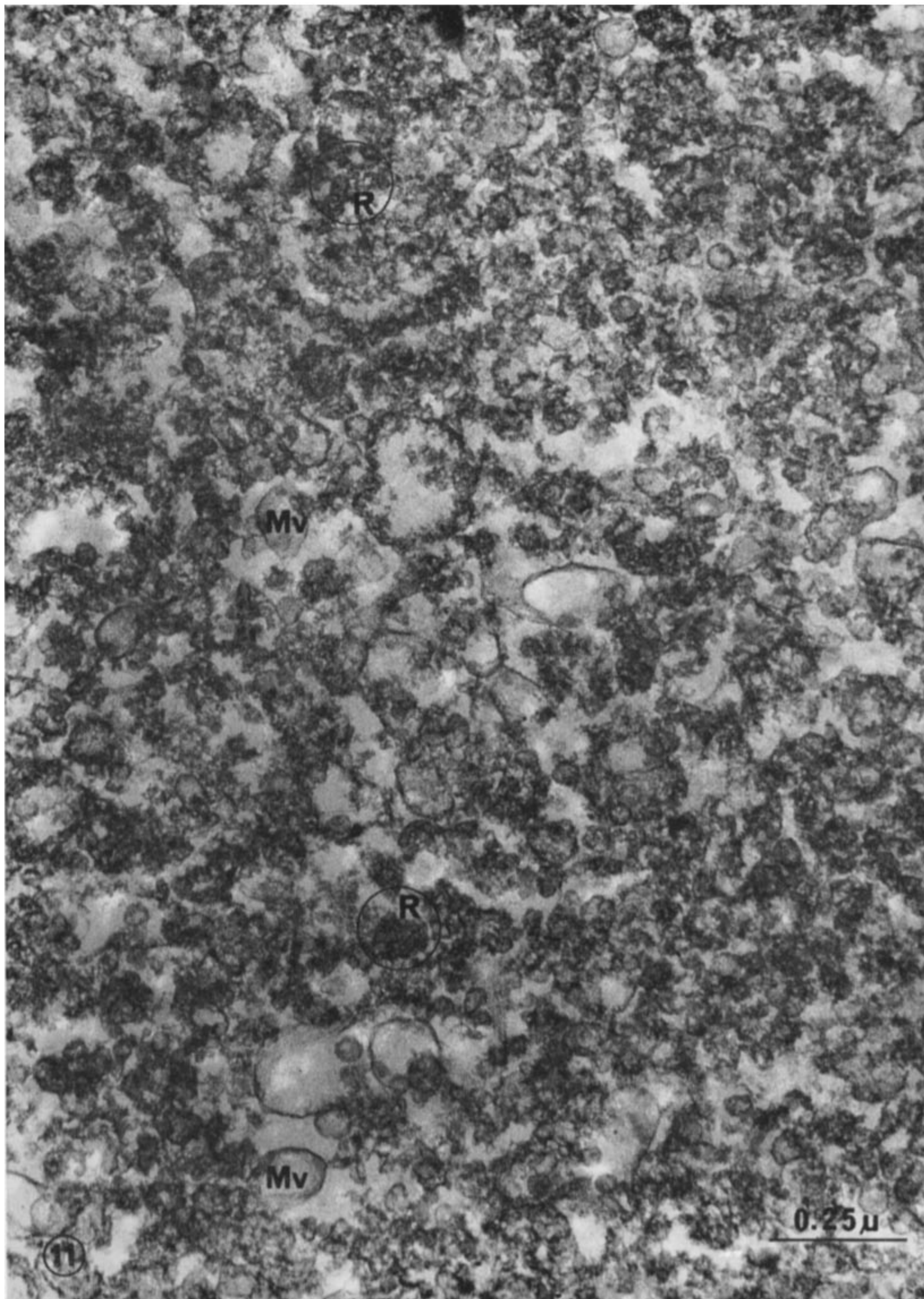


FIGURE 11 Microsomal fraction. Membrane vesicles (*Mv*) and altered ribosomal material (*R*) are the major morphologic components of this fraction. × 80,000.

Mitochondrial Contamination

SDH was chosen as an enzyme marker for mitochondria, since it is exclusively found in this organelle where it is tightly bound to the inner membrane (16). The data in Table II support this choice of an enzyme marker for fat cell mitochondria. Of the original homogenate SDH activity, only 2% was found in the plasma membrane fraction.

When the plasma membrane SDH specific activity was compared to the SDH valves of the mitochondria from the same experiment, then the linear sucrose preparation of plasma membranes was least contaminated with mitochondria (Table III). However, if the SDH specific activity of plasma membranes from linear Ficoll gradients was compared with SDH values of mitochondria from linear sucrose gradients, the contamination of plasma membranes from Ficoll and sucrose gradients was quite similar.

The value presented for the mitochondrial contamination of the plasma membranes was the maximum value. The true or lowest contamination by mitochondria could be calculated only if a pure preparation of mitochondria were available. The adenyl cyclase data presented below showed the mitochondria to be contaminated about 25% by plasma membranes, assuming the adenyl cyclase activity to be specific for plasma membrane. Electron micrographs confirmed the impurity of the mitochondrial fractions. Thus, it appeared that the plasma membrane preparation was contaminated with between 5 and 10% mitochondria.

The microsomal fraction contained a small amount of SDH activity, which indicated about 2% mitochondrial contamination.

Microsomal Contamination

Microsomal contamination was estimated by two different measurements. RNA content was used to represent primarily ribosomal contamination; NADH-cytochrome *c* reductase was used as an enzyme marker to represent the ER membranes of the microsomal fraction.

TABLE I

DNA Distribution

Fraction	% Recovery	μat DNA-P/mg protein	Contamination
			%
Homogenate	100.00	0.127×10^{-3}	
Nuclear fraction	85.00	0.129	
Plasma membrane fraction (Ficoll)	0.58	0.118×10^{-3}	0.092

TABLE II

SDH Distribution in Fat Cell Fractions

Fraction	mμmoles succinate oxidized/mg protein per min	% Recovery
Homogenate	9.0	100.0
P1	22.0	81.0
P2 (nuclei)	26.0	24.0
Mitochondria	49.0	61.0
Microsomes	2.1	1.7
Plasma membranes (Ficoll)	7.9	2.0

RNA Measurements

Data in Table IV show, that the plasma membrane fraction from Ficoll gradients contained about 29% as much RNA as the microsomal fraction. This represented only 2% of the original homogenate RNA or 2.4 mg RNA/100 mg protein.

NADH-cytochrome c Reductase Measurements

The highest specific activity of this enzyme was found in the microsomal fraction, and the lowest activity was found in the mitochondria (Table IV). The plasma membrane fraction contained about 27% of the specific activity found in the microsomal fraction. This percentage value was extremely consistent from experiment to experiment, although the absolute activities of the two fractions varied from experiment to experiment.

TABLE III

SDH Activity in Mitochondrial, Plasma Membrane, and Microsomal Fractions of Fat Cells

	Linear sucrose gradient		Linear Ficoll gradient		Differential centrifugation	
	Plasma membranes (8)	Gradient mitochondria (8)	Plasma membranes (29)	Gradient mitochondria (14)	Mitochondria (15)	Microsomes (12)
Specific activity	3.53 ± 0.83	61.0 ± 5.14	6.59 ± 0.56	46.8 ± 2.7	49.0 ± 3.43	1.30 ± 0.14

Specific activity expressed as mμmoles succinate oxidized/mg protein per 10 min. Values represent mean ± standard error of the mean. Numbers in parenthesis represent the number of experiments. Mitochondria were obtained from gradients or by differential centrifugation.

TABLE IV

RNA and NADH-cytochrome c Reductase Activity of Microsomal, Plasma Membrane, and Mitochondrial Fractions of Fat Cells

Fractions	NADH-cytochrome *c* reductase	RNA
Microsomes	337.7 ± 46.0 (11)	82.7 ± 6.6 (3)
Plasma membranes	111.6 ± 10.1 (11)	23.9 ± 1.5 (3)
Mitochondria	65.6 ± 8.8 (11)	

NADH-cytochrome *c* reductase activity is expressed as mμmoles NADH oxidized/mg protein per min. RNA content is expressed as μg RNA/mg protein. Values represent means ± standard error of the means. Number in parenthesis represents the number of experiments.

Phosphatase Activity

The ATPase activities of plasma membranes prepared from linear Ficoll and sucrose gradients were quite similar. The use of Tris or bicarbonate buffers made little difference (Table V). This data illustrated that the plasma membrane and mitochondria had very similar ATPase activity, and that, depending on the mode of preparation, the mitochondrial activity could be increased while the plasma membrane activity remained constant. The ATPase activity of the plasma membrane fraction was five times that of the homogenate.

The microsomal fraction had a very low ATPase activity compared to the other two fractions (Table V). Furthermore, the buffer used made no difference in activity. This low activity in the microsomes suggested the lack of cross-contamination by plasma membranes in this fraction.

In none of the fractions could 5′-nucleotidase be detected. Even the plasma membrane fraction had no measurable activity of this enzyme, which is usually associated with plasma membranes.

Adenyl Cyclase Activity

Data in Table VI demonstrated that basal adenyl cyclase activity in the plasma membrane preparation was about three times that of "ghost" preparations, while the sodium fluoride values were 6-10 times the value of the ghosts. Thus the fluoride stimulated the adenyl cyclase activity in the plasma membrane preparation some four to eight times the basal rate, while the ghost preparation was stimulated only two to three times the basal rate. Epinephrine stimulated the cyclase activity to about 30% of the fluoride value. Detailed studies of hormonal effects on the adenyl cyclase activity of this membrane preparation will be reported separately.

The microsomal fraction was found to have virtually no adenyl cyclase activity, which was consistent with the ATPase data, suggesting very little plasma membrane content in the microsomal fraction.

The mitochondrial fraction had no basal adenyl cyclase activity, while the sodium fluoride value was about 30% of the plasma membrane value. This marked stimulation by fluoride could

TABLE V

ATPase Activity of Plasma Membrane, Mitochondrial, and Microsomal Fractions of Fat Cells

Fraction	Source	Tris buffer	Bicarbonate buffer
		μmoles Pi/mg protein per 10 min	
Homogenate	Isolated fat cells		1.86 ± 0.13 (3)
Plasma membrane	Linear sucrose gradient	8.0 ± 1.07 (4)	7.0 ± 0.84 (7)
	Linear Ficoll gradient	8.0 ± 1.13 (3)	9.3 ± 0.63 (8)
Mitochondria	Linear sucrose gradient	8.5 ± 2.26 (3)	11.4 ± 1.38 (7)
	Linear Ficoll gradient		10.1 ± 0.52 (3)
	Differential centrifugation	6.5 ± 0.40 (3)	8.3 ± 0.53 (5)
Microsomes	Differential centrifugation	1.7 ± 0.20 (7)	1.6 ± 0.13 (11)

Values represent mean ± standard error of the mean. Numbers in parenthesis represent the number of experiments.

be explained by its inhibition of the high ATPase activity of the mitochondria, which competed much too strongly for the substrate under basal conditions. From Table V it can be observed that the ATPase activity of the mitochondria was some 10^3 times that of the plasma membrane adenyl cyclase activity. With the fluoride present, the adenyl cyclase activity and the plasma membrane contamination of the mitochondrial fraction became apparent and were consistent with the electron microscopic observations.

TABLE VI

Adenyl Cyclase Activity of Plasma Membrane, Microsomal, and Mitochondrial Fractions of Fat Cells

	Basal	NaF
	picomoles/mg protein per 10 min ± S.D.	
Ghosts	651 ± 32	1669 ± 46
Experiment I		
Plasma membranes	2090 ± 249	9218 ± 220
Microsomes	80 ± 43	697 ± 17
Experiment II		
Plasma membranes	1816 ± 194	16338 ± 495
Mitochondria	0	4736 ± 222

The data from ghost preparations of fat cells are taken from a report by Cryer et al. (15) from this laboratory. The other data are from triplicate determinations in single experiments.

DISCUSSION

The present report describes an easy and rapid preparation of fat cell plasma membranes. Alternative gradient systems, sucrose and Ficoll, have been tested, both giving comparable preparations. The location of the plasma membrane fraction at sucrose density of 1.14 is in agreement with the density of plasma membranes from HeLa cells (23) and liver cells (24).

The ease of handling the Ficoll gradient and the short centrifugation time required made it the gradient of choice. With the Ficoll system, membrane preparations were available for study within 2½–3 hr after sacrificing the rats.

This speed is of importance, since adenyl cyclase activity in fat cell ghosts has been reported to be labile and to decrease rapidly with time. The stability of this enzyme with the current membrane preparation is now being studied.

The plasma membrane fraction seemed to be a relatively clean preparation. Nuclear contamination was virtually absent. Mitochondrial contamination as judged by SDH measurements was reduced to at least the 10% level, if not more so, as indicated by electron microscopy. This represented less than 2% of the original mitochondrial content and was in contrast to the contamination in the Rodbell ghost preparation (19), which contained 25% of the original mitochondria and was contaminated close to 50% when the data were converted into specific activity.

Microsomal contamination of the plasma membrane fraction was the most difficult to quantitate. The RNA and NADH-cytochrome c reductase activities were similar to, or only slightly higher than, those found in isolated plasma membranes of HeLa cells (23) and liver cells (24). Boone et al. have recently reported the RNA content of a plasma membrane preparation of HeLa cells to be less than 3 mg/100 mg protein (23), similar to the present value of 2.4 mg/100 mg protein. Other membrane preparations (24, 25) have been reported to contain from 10 to 20 μg RNA/mg protein. If it is assumed that most of the RNA is membrane RNA, then ribosomal contamination could be reduced to a rather small percentage as indicated by electron microscopy.

Emmelot and Benedetti (24) have concluded that isolated liver plasma membranes contained at least some intrinsic NADH-cytochrome c reductase activity, amounting to about 15–20% that of the microsomes. If this be the case for fat cells, then the plasma membrane fraction would be contaminated with true microsomal activity by only 7–12% instead of 27%. Some of the NADH-cytochrome c reductase activity of the present plasma membrane fraction might be derived from contaminating outer mitochondrial membranes that are known to contain this enzymatic activity (26). On the basis of the available RNA and enzymatic data, it is likely that the true microsomal contamination of the present plasma membrane preparation was about 10%, which would also seem realistic from the electron microscopic observations.

The purity of the plasma membrane preparation was substantiated further by the fact that adenyl cyclase activity of this preparation was increased over that of ghost preparations. This latter preparation had lost 86% of the starting protein, which meant that the adenyl cyclase could have been purified up to sevenfold. Thus, the plasma membrane fraction described here has not only a four- to fivefold increase of specific activity over the ghost preparation but could have about a 30-fold increase of adenyl cyclase specific activity over the starting material. Furthermore, the total lack of this enzyme in the microsomes demonstrates that the plasma preparation could not be heavily contaminated, otherwise this increase in specific activity could not have been obtained.

Studies have been reported claiming that the majority of ATPase activity of fat cells is located on the plasma membrane (22). The present data demonstrate that the plasma membrane fraction contained a high activity of ATPase, comparable to the mitochondria. In contrast the microsomes had a very low ATPase activity. Mitochondria from sucrose gradients seemed to be activated to a small degree compared to mitochondria prepared from Ficoll gradients or differential centrifugation. Within the same experiment, values obtained in Tris buffer were always lower than values from bicarbonate buffer. The ATPase activity was tested in bicarbonate buffer as well as Tris, because of the use of the former buffer for physiological studies. Detailed studies on the characterization and distribution of ATPase activity in the fat cell and its fractions will be presented in a separate manuscript.

It has been shown previously (20), as well as demonstrated in this study, that many small vesicles are attached to or are adjacent to the plasma membrane of intact fat cells. This has proven to be a useful structural marker, for these same small vesicles are seen attached to or inside the larger vesicles of our preparation which was believed to be derived from plasma membranes. The role of these small vesicles in fat cells remains to be elucidated. The electron microscopic evidence reported here, that dense material was found in the extracellular space, in invaginations of the plasma membrane, and in small vesicles probably derived from the plasma membrane invaginations in both intact fat cells and plasma membrane fractions, suggested a dynamic process similar to pinocytosis. Recent studies by Cushman and Rizak (27) have demonstrated energy-dependent uptake of colloidal-gold particles into plasma membrane invaginations of isolated fat cells.

Morphologically it was possible to recognize the various structures that might be contaminating the plasma membrane fraction. From electron micrographs, the mitochondrial contamination was certainly not greater than 10% and was probably less. The larger pieces of ER in the microsomal fraction were recognized by the heavy amorphous material present inside these vesicular structures. This was consistent with the criteria for ER morphology described by Palade and Siekevitz (21) and with observations on intact fat cells. By these criteria the microsomal contamination seemed to be no greater than 10–15% on morphologic grounds. Both the mitochondrial and microsomal contaminations were primarily

trapped inside the larger plasma membrane vesicles and were not loose contaminations interspersed with the plasma membranes. In fact, electron microscopy indicated some continuity between the plasma membrane and ER membranes. Further washing of the preparation would not reduce this type of contamination.

A rapid procedure has been described for preparing a relatively clean preparation of plasma membranes from isolated fat cells. The adenyl cyclase and ATPase activity have been greatly enriched. This preparation should prove useful in future studies on the interactions of hormones with structural and biochemical properties of the plasma membrane.

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