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AN IMPROVED TECHNIQUE FOR PREPARATION OF SKELETAL MUSCLE CELL PLASMA MEMBRANE

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SUMMARY

1. An improved technique of plasma membrane isolation from rat skeletal muscle is presented. The procedure is based on the cautious disruption of the muscle fibers, elimination of actomyosin by salt solutions and separation of sarcolemma from organelles by differential centrifugation. This is followed by a centrifugation on a discontinuous sucrose density gradient. The plasma membranes are yielded in the form of vesicles. They are characterized and their purity is assessed by electron microscopy, chemical and biochemical tests.

2. The fraction of pure plasma membrane is found to have a high specific activity in plasma membrane markers: 5′-nucleotidase, (Na^+,K^+)-stimulated Mg^{2+}-dependent ouabain-sensitive ATPase and a high molar ratio of cholesterol to phospholipids; these markers have lower or negligible specific activities in the other fractions. The enzymatic tests performed to determine the cross-contamination by cell organelles are: succinate dehydrogenase and NADH: cytochrome *c*-reductase rotenone insensitive, for mitochondria and outer-mitochondrial membrane; NADPH: cytochrome *c*-reductase for sarcoplasmic reticulum; IDP phosphohydrolase for Golgi apparatus; acid phosphatase for lysosomes; and tentatively lactate-dehydrogenase for cytoplasm. These activities are poorly represented or absent in the plasma membrane fraction.

INTRODUCTION

Several methods were described for the purification of sarcolemma from skeletal muscle. The procedures used for the separation of the sarcolemma from the contractible proteins were: washing of the disrupted cells with strong salt solutions [1–3] or the use of one or several of the following procedures: "aging" of the cell segments, washing in moderate salt solutions, or incubation at 37 °C within a limited

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Abbreviation: EGTA, ethyleneglycolbis(β-aminoethylene)-*N*,*N*′-tetraacetic acid.

pH range [4–7]. The purification of the sarcolemma tubules or sheets was obtained by differential centrifugation.

More recently two techniques were described to obtain, instead of sarcolemma, plasma membranes in the form of vesicles. The plasma membranes were separated from actomyosin by strong salt solutions [8] or by filtration [9]. The final purification was obtained in both cases by centrifugation on a continuous sucrose density gradient.

Phase-contrast microscopy and electron microscopy were the criteria used to characterize the sarcolemma tubules [2, 4, 5, 10–12] or the plasma membrane vesicles [8, 9]. Attempts were made to characterize the sarcolemma and plasma membranes [2, 3, 8–18] and to appreciate their contamination with organelles by biochemical assays [8, 9, 10, 17].

The aim of this study is to obtain pure plasma membranes starting from the sarcolemma preparation obtained according to Kono and Colowick [2] and Sulakhe et al. [3].

The plasma membranes are separated from the other layers composing the sarcolemma and from the eventually remaining organelles by rehomogenization in a high density sucrose solution and centrifugation on a sucrose discontinuous gradient.

The plasma membranes are characterized and their purity checked by electron microscopy and biochemical assays.

METHODS

Preparation of plasma membrane vesicles

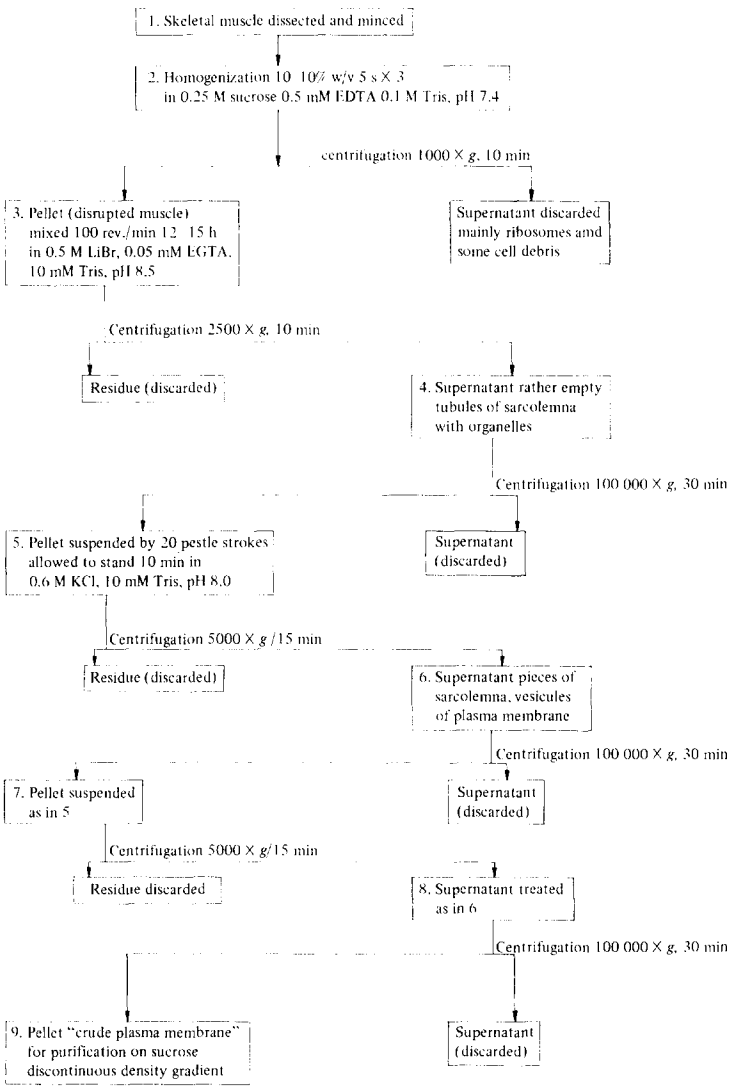
A schematic diagram of the procedure is shown in Scheme 1 and Fig. 1. Male albino rats weighing 200–300 g were killed and the skeletal muscles from the hind legs were dissected out quickly. All subsequent steps of the membrane preparations were carried out at 4 °C.

(a) *Disruption.* The muscles were minced, suspended in 10 vol. (w/v) 0.25 M sucrose, 0.5 mM EDTA and 0.1 M Tris (pH 7.4) and homogenized three times for 5 s (usually in batches of 5 g of muscle/50 ml of buffer) with a Polytron Pt 10 homogenizer (Professor P. Willems, Pat. Kinematica GmbH, Luzern, Switzerland) at a set of 5 (about 17 000 rev./min).

The homogenate was centrifuged at $1000 \times g$ for 10 min.

(b) *Solubilization of actomyosin and differential centrifugation.* The sediment was washed and extracted (12 h, 4 °C with mixing at 100 rev./min) with LiBr solution (0.5 M LiBr, 0.05 mM ethyleneglycolbis(β-aminoethylene)-*N*,*N*′-tetraacetic acid (EGTA), 16 mM Tris–HCl (pH 8.5), using 50 ml per 5 g of homogenized muscle. The jelly-like suspension was spun at $2500 \times g$ for 10 min. The supernatant was centrifuged at $100\,000 \times g$ for 30 min. The pellet was suspended in 0.6 M KCl, 10 mM Tris–HCl, pH 8.0, by 20 strokes with a plastic pestle in a small volume of solution, then adjusted to the final volume: 30 ml per 10 g of homogenized muscle. This suspension was allowed to rest for 10 min and was centrifuged at $5000 \times g$ for 15 min. The supernatant was further centrifuged at $100\,000 \times g$ for 30 min, and the resulting pellet submitted to another KCl washing. A pellet of "crude plasma membrane" was obtained.

(c) *Discontinuous sucrose density gradient* (*Figs 1A and 1B*). The crude plasma membrane pellet was submitted twice to a 5-s homogenization (Polytron Pt 10 about



Scheme 1. Preparation of crude plasma membrane vesicles (all steps carried out at 4 °C).

5000 rev./min) in 50% sucrose solution in 0.05 M Tris–HCl buffer at pH 7.5. The protein concentration was estimated and adjusted by adding 50% sucrose at a concentration of 1 mg per ml. 6 ml of this solution were transferred to each centrifuge tube. 8 ml of 45%, 10 ml of 41 p. 100 and 6 ml of 36 p. 100 sucrose solutions in 0.05 M Tris–HCl buffer, pH 7.5, were then successively layered. Centrifugation was operated at 100 000 × *g* for 90 min (rotor SW 25).

The resulting bands were removed and diluted (1:4, v/v) using 0.05 M Tris–HCl buffer, pH 7.5, and centrifuged at 100 000 × *g* for 15 min.

The final pellets were suspended in ion-free distilled water and the protein concentration was estimated before analysis.

Characterization

(1) *Morphological controls.* All the steps of the purification of crude plasma membranes and sucrose density gradient centrifugation were controlled by phase-contrast and electron micrography (not shown).

(2) *Chemical determinations.* Protein: protein determination was made by the procedure of Lowry et al. [19]. Cholesterol: total cholesterol was determined by the Liebermann–Bürchard reaction using the method of Chiamori and Henry [20]. Phospholipids: lipids were extracted using the Folch et al. [21] procedure. Lipid phosphorus was determined according to Bartlett [22]. Total phospholipid content was calculated assuming that 770 is the average molecular weight of the phospholipids. Deoxyribonucleic acid: determinations using the diphenylamine method of Croft and Lubran [23] were carried out.

(3) *Enzyme assays.* The enzyme assays of: 5′-nucleotidase (EC 3.1.3.5) [24]; (Na^+,K^+)-stimulated, Mg^{2+}-dependent ouabain-sensitive ATPase (EC 3.6.1.3) [3]; succinate dehydrogenase (EC 1.3.99.1) [25]; NADH: cytochrome *c* reductase rotenone insensitive (EC 1.6.99.3) [26]; NADPH: cytochrome *c* reductase (EC 1.6.2.3) [27]; acid phosphatase (EC 3.1.3.2) [28]; lactate dehydrogenase (EC 1.1.1.27) [29]; IDP phosphohydrolase (EC 3.6.1.9) [30]; NADH diaphorase (EC 1.6.99.3) [31]; and active Ca^{2+} uptake [32] were performed.

The P_i released in the 5′-nucleotidase, ATPase, acid phosphatase, and IDP phosphohydrolase assays was estimated by the method of Fiske and SubbaRow [33].

RESULTS

Preparation of crude plasma membranes

Our attempts to prepare sarcolemma from skeletal muscles resulted in preparations contaminated by organelles and in which the integrity of the sarcolemma was not preserved.

The following steps were found to have a high influence on yield, purity and integrity of the obtained sarcolemma: homogenization length and strength; time between decapitation of the animal and extraction of myofibrillar proteins in LiBr; duration and speed of mixing during LiBr extraction; time of resuspension in KCl buffer.

Sucrose density gradient centrifugation

After discontinuous gradient centrifugation, four fractions were recovered (Fig. 1B): (a) thin band at the upper half 41% sucrose, (b) a larger band at the 41/45 % sucrose layers interface, (c) a homogeneous one at the 45/50 p. 100 interface, (d) a pellet of varying size.

When starting from 10 g of muscle, 8.06 ± 1.66 mg of crude plasma membrane proteins were obtained; after sucrose density gradient centrifugation 6.72 ± 0.76 mg of proteins were recovered.

Most of the proteins were recovered in Fraction a ($1.59 \pm 0.32 = 24\%$ of recover edproteins) and in Fraction b ($4.25 \text{ mg} \pm 0.77 = 64\%$ of recovered proteins).

Smooth vesicles associated with plasma membrane enzymes were found in the fractions a and b. We could not succeed in separating the plasma membrane vesicles of Fraction b by rewashing with KCl and recentrifugation on a second sucrose gradient.

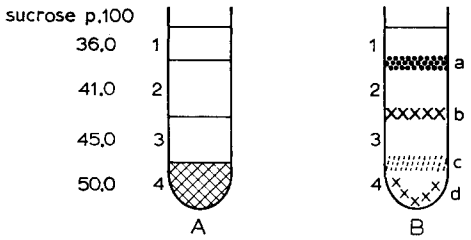


Fig. 1. Density gradient centrifugation. Centrifugation was performed on SW 25 rotor at 100 000 × *g* for 90 min. a, purified plasma membrane; b, impure plasma membrane, sarcoplasmic reticulum cytoplasma, mitochondria; c, mitochondria, cytoplasma, lysosomes, nuclei; d, lysosomes, nuclei, sarcoplasmic reticulum.

Plasma membrane enzymes (*Table I*):

41% of the 5'-nucleotidase total activity and 26% of the ATPase total activity recovered after sucrose gradient centrifugation are in Fraction a. High quantities of these plasma membrane enzymes were also recovered in Fraction b.

The highest specific activities of 5'-nucleotidase and (Na^+,K^+)-stimulated Mg^{2+}-dependent ouabain-sensitive ATPase (total ATPase) were measured in Fraction a (1.65 μM P_i/mg protein per 60 min, and 15 μM P_i/mg protein per 60 min). These specific activities were 1 μM P_i/mg protein per 60 min for 5'-nucleotidase and 9 μM P_i/mg protein per 60 min for total ATPase in Fraction b; very low specific activities were found in Fraction c and in Pellet d.

Cholesterol to phospholipids molar ratio (*Table I*)

The average value was 0.65 in Fraction a. This ratio was lower than 0.50 in all the other fractions.

DNA and organelle enzymes (*Table II*)

The succinate dehydrogenase, and NADH:cytochrome *c* reductase rotenone-insensitive enzymes were assayed for mitochondria and the outer membranes of mitochondria; NADPH:cytochrome *c* reductase and IDP phosphohydrolase were assayed for the sarcoplasmic reticulum and Golgi apparatus; DNA was tested for nuclei, acid phosphatase for lysosomes and lactate dehydrogenase for cytoplasma. These enzymes were low or absent in Fraction a; DNA was absent in this fraction.

Most of the total activities of these enzymes were recovered in Fraction b i.e. 78% of succinate dehydrogenase, 82% of NADH:cytochrome *c* reductase rotenone insensitive enzymes, and 96.5% of NADPH:cytochrome *c* reductase.

DISCUSSION

(I) Technique of preparation

Plasma membranes and sarcoplasmic reticulum behave differently from the other subcellular components. They can assume a wide variety of sizes and densities (buoyant densities on a sucrose gradient for instance) which are dependent on the resistance of these membranes and on their tendency to vesiculation. Both factors are influenced by the structure of the membrane and ionic osmotic environment.

TABLE I

SPECIFIC ACTIVITY OF PLASMA MEMBRANE ENZYMES AND MOLAR RATIO OF CHOLESTEROL TO PHOSPHOLIPIDS AFTER SUCROSE DENSITY GRADIENT CENTRIFUGATION

Results are given as average value of five experiments ± S.D. Units: ATPase, acid phosphatase, 5′-nucleotidase and IDP phosphohydrolase (determination of P_i by the method of Fiske and SubbaRow [33]: 1 U = amount of enzyme catalyzing the liberation of 1.0 μmole of orthophosphate/1 mg protein per 60 min. NADPH: cytochrome *c* reductase: 1 U = change of optical density of 1.0 min at 550 nm at 25 °C in a 1-cm light path/1 mg protein. Rotenone insensitive NADH:cytochrome *c* reductase: 1 U = change of optical density of 1.0/min at 550 nm at 38 °C in a 1-cm light path/1 mg protein. Lactate dehydrogenase: 1 U = change of optical density of 1.0/min at 340 nm at 25 °C in 1-cm light path/1 mg protein. Succinate dehydrogenase: 1 U = variation of optical density at 490 nm/1 μg of nitrogen.

Fraction	5′-Nucleotidase (U/mg protein per h)	ATPase (U/mg protein per h)			Cholesterol (μg/mg protein)	Phospholipids (μg/mg protein)	Cholesterol/ phospholipids molar ratio
		Total	(Na^+,K^+)-stimulated	Mg^{2+}			
a	1.65 ± 0.33	14.95 ± 3.71	11.05 ± 1.51	3.89 ± 3.25	133.02 ± 19.71	417.92 ± 114.18	0.65 ± 0.13
b	1.02 ± 0.28	9.17 ± 4.75	5.55 ± 3.26	3.24 ± 2.59	156.42 ± 48.72	654.02 ± 183.72	0.46 ± 0.03
c	0.49 ± 0.09	3.09 ± 0.41	1.11 ± 0.61	1.33 ± 1.25	51.04 ± 10.50	394.26 ± 131.94	0.25 ± 0.02
d	0.31 ± 0.14	3.14 ± 1.82	1.64 ± 1.39	1.50 ± 0.48	41.80 ± 9.12	294.14 ± 58.63	0.28 ± 0.06

TABLE II

SPECIFIC ACTIVITY OF ORGANELLE ENZYMES AND DNA AFTER SUCROSE DENSITY GRADIENT CENTRIFUGATION

Results are given as the average value of five experiments ± S.D. *U/mg protein per h (for units see Table I).

Fraction	Succinate dehydrogenase* ($\times 10^{-2}$)	NADH:cytochrome *c* reductase* rotenone insensitive ($\times 10^{-2}$)	NADHP:cytochrome *c* reductase* ($\times 10^{-2}$)	IDP phospho-hydrolase*	Acid phosphatase* ($\times 10^{-2}$)	Lactate dehydrogenase* ($\times 10^{-2}$)	DNA (μg/mg protein)
a	0.103±0.073	1.71 ± 1.71	1.02±1.39	0.57±0.18	7.52± 3.12	2.02±0.93	4.38± 4.27
b	8.432±3.237	19.26 ± 8.87	34.96±9.72	2.62±1.14	40.92±10.45	7.92±2.84	103.25± 17.81
c	20.624±9.104	26.960±15.920	5.69±3.76	0.78±0.57	41.25±12.48	7.97±5.27	357.30±175.09
d	4.574±2.461	20.06 ± 8.69	19.62±6.28	1.65±0.82	71.49± 11.13	1.33±1.15	752.48±105.63

The sarcolemma of skeletal muscle cell is a strong envelope which comprises several layers: the plasma membrane which is in contact with the cell cytoplasma, the cell coat, the basement membrane and an outermost layer of collagen fibers [34–36]. This structure if not altered offers a high resistance to disrupting forces (mechanical and chemical). The myofibrils are bound together by what was defined by Peters [37] and later Mc Collester and Semente [38, 39] as a "cytoskeleton" located within elements of the sarcoplasmic reticulum and of the Z membranes system which form complex interlocking networks surrounding the individual myofibrils. Elimination of the myofibrils consists of breaking this cytoskeleton by chemical and biochemical forces.

Mitochondria, nuclei, and sarcoplasmic reticulum as confirmed by direct morphological control by light or electron microscopy are not damaged during mechanical homogenization (in our conditions), because they are embedded in the myofibrils and protected by the sarcolemma.

During the chemical elimination of actomyosin by LiBr, the sarcoplasmic reticulum is vesiculated and also some of the organelles, but the sarcolemma structure is not damaged and separation can be performed by differential centrifugation. The conditions of resuspension in KCl are critical because then, breaking of the sarcolemma structure can occur with subsequent vesiculation of the plasma membranes if the homogenization is too long or too strong. At the end of KCl washing, the crude plasma membrane pellet is a mixture containing nearly 60% of sarcolemma and plasma membranes and 40% of organelles. Most of the sarcoplasmic reticulum has been eliminated.

A discontinuous sucrose density gradient was preferred to a continuous one, although the first can provide an artificial concentration of heterogeneous material. In our procedure, the plasma membranes were prepurified and the preparations which were submitted to gradient centrifugation contained about the same proportion of impurities to be eliminated. Our goal was to separate a fraction of pure plasma membranes from all remaining subcellular components of known density and a discontinuous gradient provided a compact and well-separated fraction of plasma membranes. The fact of collecting the plasma membrane vesicles in a rather wide scale of densities (between 1.16 and 1.14) might generate contamination by vesicles from other origins and required a cautious control of these possible contaminations.

(II) Evaluation of contamination and characterization of the plasma membrane vesicles

We had to rely mainly on data published for other tissues to choose the organelle marker enzymes: so far, only a few data are published for skeletal muscle [2, 8, 9, 17, 13].

Various enzymes and chemical estimations known to be specific for the plasma membranes in different tissues were tested in the skeletal muscle plasma membranes: 5′-nucleotidase [9]; (Na^+,K^+)-stimulated Mg^{2+}-dependent ouabain-sensitive ATPase [3, 8, 9, 13, 14, 16, 17], adenylate cyclase [17]; cholesterol and phospholipids content and molar ratio [8, 9, 15, 17].

Contamination. Succinate dehydrogenase which was used as a marker of mitochondria [40, 41] was assayed, as well as NADH:cytochrome *c* reductase rotenone insensitive for the outer membrane of mitochondria [42] the buoyant density of

which was found to be similar to the one we found for the plasma membrane.

We found a similar specific activity for NADH diaphorase, previously tested as sarcoplasmic reticulum specific in muscle plasma membranes [8], in all the fractions of the gradient. We also tested NADPH:cytochrome *c* reductase which was found to be associated with purified sarcoplasmic reticulum fractions and absent from the plasma membrane fraction of chick embryos fibroblasts [43]. The plasma membrane fraction of our preparations exhibited negligible activity.

This absence of sarcoplasmic reticulum contamination was confirmed by our attempts to determine the Ca^{2+} uptake by the different fractions of our preparations. The specific activity of the "crude" plasma membranes before density gradient centrifugation was 0.022 μmole Ca^{2+}/mg protein in 10 min at 20 °C. This specific activity was far lower than the one found for sarcoplasmic reticulum isolated from rabbit skeletal muscle in discontinuous sucrose gradient, after elimination of actomyosin by 0.6 M LiBr: 3 μmoles Ca^{2+}/mg proteins in 10 min at 20 °C (Chevallier, J., personal communication). No Ca^{2+} uptake could be determined in the (a) and (c) fractions and Pellet d. The specific activity of Fraction b was 0.019 μmole Ca^{2+}/mg proteins in 10 min at 20 °C.

Lactate dehydrogenase tested tentatively for cytoplasma was recovered everywhere on the gradient with a similar specific activity. Hultin and Westort [13], working on sarcolemma from chicken muscle, found that lactate dehydrogenase was associated with the sarcolemma without concluding whether this was the situation in vivo or an artefact of the preparation.

Plasma membrane characterization. Determinations of adenylate cyclase activity are being conducted at present.

(1) *5'-Nucleotidase.* In the case of skeletal-muscle plasma membrane preparations, no 5'-nucleotidase activity was found by Severson et al. [17], this absence was due to the solubility of this enzyme under the conditions of purification used. Kidwai et al. [9] detected a 5'-nucleotidase activity in his preparation. The specific activity found in the plasma membrane fraction was about three times higher than ours.

(2) *Cholesterol to phospholipids ratio.* According to Coleman and Finean [45], plasma membranes are characterized by a high molar ratio of cholesterol to phospholipids (0.50–1.30 according to the tissues). Boegman et al. [8] and Kidwai et al. [9] found, respectively, ratios varying from 0.10 to 0.20. Severson et al. [17] found a ratio of 0.60. In our preparations this ratio was 0.65 in the plasma membrane fraction, and 0.45 in Fraction b. The preparation of sarcolemma we obtained according to the technique of Sulakhe et al. [3] always gave higher ratios varying from 1.00 to 1.30 in proportion to the amount of impurities.

(3) *(Na^+,K^+)-stimulated Mg^{2+}-dependent ouabain-inhibited ATPase.* (Na^+,K^+)-stimulated Mg^{2+}-dependent ATPase (total ATPase) was recognized as a marker from plasma membranes. Ouabain-inhibited ATPase ((Na^+,K^+)-ATPase) was found to be specifically associated with plasma membranes [8, 9] or sarcolemma [13, 14, 16, 17] from skeletal muscle. It was absent in the sarcoplasmic reticulum or mitochondrial fraction of this tissue.

Our specific activity for total ATPase: 16 μM P_i/mg protein per 60 min, are low if compared to others [3, 8, 9, 14] which range from 10 to 70 μM P_i/mg protein per 60 min. But the percentage of ATPase inhibited by ouabain ((Na^+,K^+)-ATPase): 70–75 %, is high if compared to other results: 10–40 % [3, 9, 14, 16, 17] and

comparable on this point with the results of Boegman et al. [8] who isolated a fraction of ATPase with 87 % inhibition by ouabain. Specific activities for (Na^+, K^+)-ATPase in other studies: from 3 to 15 μM P_i/mg protein per 60 min (excepting the results of Boegman et al. [8]), are lower or comparable with ours: 12 μM P_i/mg protein per 60 min [3, 9, 14, 16, 17].

Our purification procedure yielded a fraction of smooth vesicles free of sarcoplasmic reticulum, mitochondria and other organelle enzymes. These vesicles exhibited enzyme activities and chemical evidence i.e. 5'-nucleotidase, (Na^+, K^+)-stimulated Mg^{2+}-dependent ouabain-inhibited ATPase and cholesterol to phospholipids molar ratios which are recognized as specific for plasma membranes.

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