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ISOLATION AND PROPERTIES OF SKELETAL MUSCLE PLASMA MEMBRANE

ABDUL M. KIDWAI, MARTHA A. RADCLIFFE, EDA Y. LEE and EDWIN E. DANIEL
Department of Pharmacology, University of Alberta, Edmonton, Alberta (Canada)
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SUMMARY

1. Subcellular fractions, namely plasma membrane, sarcoplasmic reticulum and mitochondria were isolated from rat skeletal muscle by the use of a continuous density gradient prepared by mixing 2 M and 0.25 M sucrose in an ISCO density gradient former.

2. The contractile proteins were insoluble under the conditions and were removed by filtration.

3. On examination with the electron microscope the subcellular fractions were fairly homogenous. The unit membrane structure of plasma membrane and sarcoplasmic reticulum were evident at high magnifications, the former being thicker.

4. $(Na^+ + K^+)$-ATPase and 5′-nucleotidase (EC 3.1.3.5) were found to be concentrated in plasma membrane fraction.

Cytochrome *c* oxidase (EC 1.9.3.1) was measured as a mitochondrial marker and was present in Fraction F_3 exclusively.

5. Cholesterol content was highest in plasma membrane fraction and a ratio of cholesterol:phospholipid was found to be 0.11 in plasma membrane, 0.025 in sarcoplasmic reticulum and 0.013 in mitochondria.

6. [^{3}H] Leucine incorporation was maximum in the sarcoplasmic reticulum during a period of 2–5 min.

7. This method yielded fairly pure subcellular fractions: namely, plasma membrane, sarcoplasmic reticulum and mitochondria during a short period of time (4 h) without extraction by salts or other drastic procedures.

INTRODUCTION

Isolation of plasma membrane from skeletal muscle was attempted by several groups[1–4] and various modifications are in use since then[5,6]. The methods so far reported require extraction of membrane fraction or homogenate with high salt concentrations or extraction at 37 °C in the presence of alkali. These treatments might change the structure and properties of the plasma membrane. It has been shown that some membrane proteins can be isolated by extraction with salts, while others require more drastic treatment, *e.g.* use of organic solvents[7].

Encouraged by the purity and simplicity of smooth[8] and heart[9] muscle preparations, we applied the same technique to skeletal muscle. By introducing minor

modifications we were able to isolate the plasma membrane, sarcoplasmic reticulum and mitochondria from rat skeletal muscle by density gradient centrifugation. We evaluated the purity of our preparation by electron microscopy, lipid composition, enzymatic markers and protein biosynthesis. This is the first method which allows the isolation of all the subcellular fractions on a single density gradient centrifugation without any extraction by salt or alkali. We have been able to use the subcellular fractions isolated by this method to study calcium uptake[10] and study the structural proteins of skeletal muscle plasma membrane (Kidwai, A. M., unpublished).

MATERIALS AND METHODS

Female Wistar rats weighing 200–240 g were killed by a blow to the head.

Chemicals

The following chemicals were obtained as indicated; ATP, AMP, Tris, ouabain and cholesterol from Sigma Chemical Co.; histidine base, sucrose, acetic acid, acetic anhydride and dinitrofluorobenzene from Fisher Co.; [^{3}H]leucine from Schwartz/Mann; wire cloth from Small Parts Inc., 6901 N.E. 3rd Ave, Miami, Fla. (U.S.A.).

Density measurements

Density of the sucrose gradient was determined on fractions collected by density gradient fractionator (ISCO Model D) using ISCO Golden Retriever Fraction Collector Model 327. Fractions of 10 drops were collected and percentage of sucrose measured in Abbe-3L Refractometer (Bausch and Lomb) at 20 °C.

Centrifugation

All centrifugations were performed between 0–4 °C in a Beckmann $L_2$65B or L using rotors SW-40 or SW 36 for density gradient centrifugation and rotors 40 or 65 angle head were used for differential centrifugation.

Density gradient

Sucrose density gradient was prepared according to Kidwai *et al.*[8] using 2 M and 0.25 M sucrose solutions (68.4 g of sucrose dissolved in distilled water to a final volume of 100 ml and 8.5 g dissolved in water to a final volume of 100 ml for 2.0 M and 0.25 M sucrose solutions respectively). Densities were determined as described above.

Scintillation counting, electron microscopy and homogenization

[^{3}H]Leucine was counted in Picker-Nuclear Spectrometer using Aquasol cocktail (New England Nuclear).

Electron microscopy as described earlier[9]. Willems Polytron homogenizer PT 20 was purchased from Brinkmann Instruments Inc. (Canada) Rexdale, Ontario.

Chemical determinations

Lipid phosphate was determined according to Chen *et al.*[11].

Cholesterol was extracted by the method of Folch *et al.*[12] and color development procedure was followed as described by Schoenheimer and Sperry[13]. Protein

was determined by the method of Lowry *et al.*[14] using bovine serum albumin as standard.

Enzymatic determinations

5′-Nucleotidase (EC 3.1.3.5) and cytochrome *c* oxidase (EC 1.9.3.1) were determined as mentioned earlier[8].

$(Na^+ + K^+)$-ATPase was determined in 40 mM histidine–HCl buffer, pH 7.4, 3 mM ATP (magnesium salt), 100 mM NaCl, 10 mM KCl and suitably diluted subcellular fractions in a total volume of 1 ml; incubated at 37 °C for 15 min; reaction stopped by adding 1 ml of 10% trichloroacetic acid. The tubes were kept cold and centrifuged to remove protein and the supernatant used for P_i determinations by the method of Fiske and SubbaRow[15]. Freshly prepared ouabain (10^{-3} M) was used to study the inhibition of $(Na^+ + K^+)$-ATPase.

Protein biosynthesis

Protein biosynthesis was followed essentially by the method of Florini[16] with few modifications as described below. A 10% homogenate of muscle was prepared in 0.25 M sucrose containing 0.01 M $MgCl_2$, 0.08 M KCl and 0.05 M Tris (pH 7.6) adjusted with HCl. Incubation mixture contained 10 ml of homogenate after filtration to remove contractile proteins and connective tissue, 10^{-3} M ATP (disodium salt), GTP 10^{-4} M, L-[4,5-$^3H_2$]leucine 0.9 pmole (5.0 μCi) was added to the homogenate. The homogenate (20 ml) was incubated at 37 °C for 15 min. At the end of this period the homogenate was divided into two equal volume aliquots; one served as control without any ATP or GTP added to it. The second received appropriate amounts of ATP and GTP followed by L-[4,5-$^3H_2$]leucine. The incubation continued for another 2 or 5 min. After the incubation period, the homogenate was chilled and centrifuged at $104000 \times g$ for 30 min at 0 °C. The sediment fractionated on density gradient and the subcellular fractions were precipitated by equal volumes of 10% trichloroacetic acid which contained 1 mg/ml of unlabelled leucine. Precipitates were washed twice with 5 ml cold trichloroacetic acid; extracted with 5 ml trichloroacetic acid in a boiling water bath for 15 min, washed with 5 ml ethanol–ether (1:1, v/v) and extracted overnight with chloroform–methanol (3:2, v/v) to remove possible amino acid lipid complexes. The precipitates were suspended in 0.1 M NaOH and protein determined. An aliquot was counted for radioactivity. To determine N-terminal labelling we used the same procedure as was used by Florini[16].

Filtration device

In order to remove contractile proteins which were insoluble under our conditions of homogenization, we constructed a filtration device described in detail in Methods in Enzymology (Biomembranes[17]) using an acrylic cylinder as a support. One O ring of smaller diameter was fused in the bottom of the cylinder. Several O rings of the same diameter were stacked inside the cylinder but not fused. Circular stainless steel wire cloth of different mesh sizes were sandwiched between the rings, mesh size 16 at the top and 80 at the bottom. This filter can be used on a Buchner funnel and a flask under slight vacuum.

RESULTS

Preparation of material

Muscles from hind legs of two rats were removed, dissected free of connective tissue and nerves. They were chopped into small pieces, weighed and suspended in 0.25 M sucrose (5 g muscle in 20 ml sucrose solution).

Homogenization

5-g batches were homogenized at a time in 0.25 M sucrose prepared in glass distilled water and kept cold at 0–5 °C. Polytron PT 20 equipped with a timer set at 15 s was turned on at near maximum speed. The chopped muscle in sucrose was held in an ice bath while homogenizing. Care was taken not to homogenize too long, otherwise mitochondrial damage occurred and the resultant fragments of mitochondria contaminated other subcellular fractions.

For smaller amounts of tissue to be processed, generator PT 10 can be used and for large amounts, generator PT 35 would be suitable.

Filtration

This was an essential step in the preparation of plasma membrane from skeletal muscle. This step helped to remove contractile proteins, connective tissue, cell debris *etc.* without any differential centrifugation to be performed which tended to lower the yield of the plasma membrane fraction. Use of several filters of mesh sizes helped to remove the various sizes of particles and allowed the filtration to proceed smoothly. Alternatively, the homogenate can be filtered through a series of filters set up separately, but this would be time consuming.

The filtrate was clear pinkish in color. Slight turbidity did not interfere with the density gradient centrifugation, since the contractile proteins settled down at the bottom of the gradient tube.

The filtrate was centrifuged at $104000 \times g$ for 30 min. The sediment was resuspended in a small volume of 0.25 M sucrose.

All the above steps were performed in cold 0–4 °C.

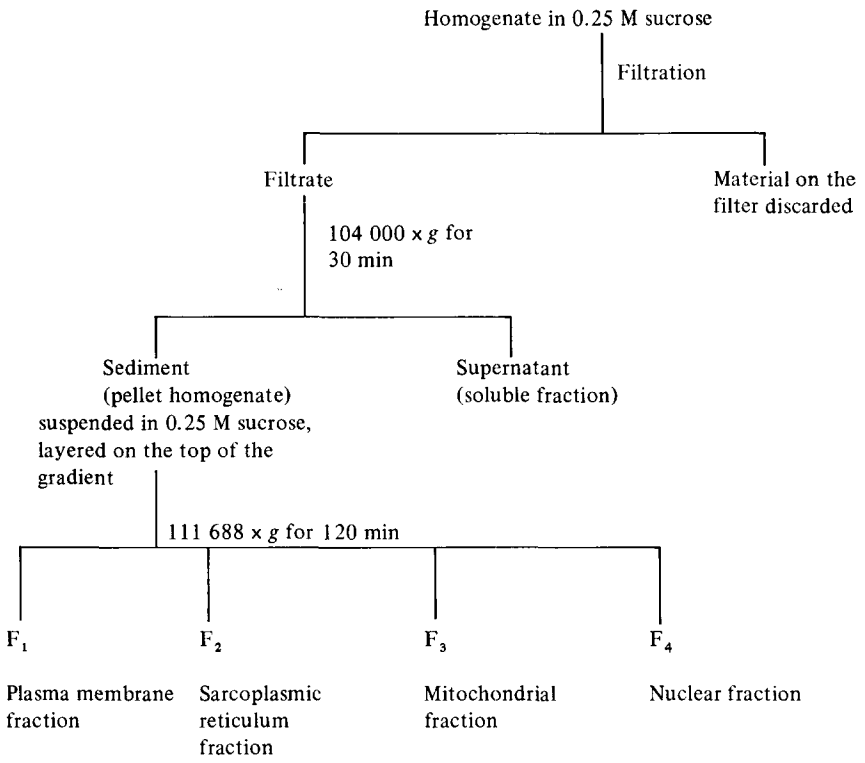
Density gradient centrifugation

Freshly prepared density gradient (0–4 °C) tubes were used to load the filtered centrifuged material on the top of the gradient and centrifuged at $111688 \times g$ (SW-40 rotor or SW-36 rotor) in a Spinco centrifuge (Model L or L_2) for 2 h (Scheme 1).

At the end of the run, the tubes had three distinct zones. The first was located at the interphase of the loading medium and density gradient. The second zone was more diffused, starting just below the first zone and continuing up to the middle of the gradient tube where a thick band of mitochondria can be seen. The lower part of the tube consisted of nuclei and contractile proteins which escaped the filtration (Fig. 1).

The fractions were either collected by Pasteur pipette or in an ISCO density gradient fractionator Model D.

Each zone was diluted with distilled water to approx. the sucrose concentration of 0.25 M and centrifuged at $104000 \times g$ for 30 min. The pellets were either fixed for electron microscopy or suspended in 0.25 M sucrose and left frozen until used.



Scheme 1. Procedure for isolation of subcellular fractions.

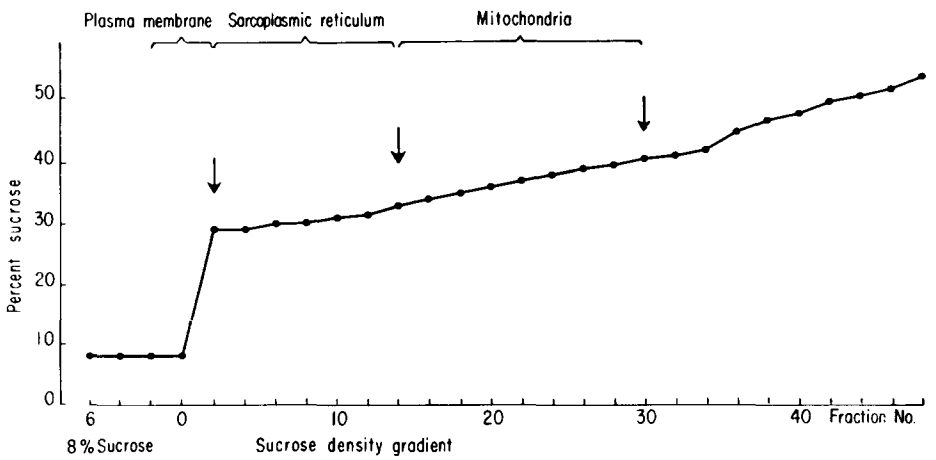


Fig. 1. Representative values of sucrose concentration and approximate position of each band after density gradient equilibration. 8% sucrose is the loading medium of the material. ●—●, percent sucrose as determined in the refractometer.

The first compact band at the interphase of the loading medium and the density gradient was designated as F_1 (plasma membrane), the second more diffused band was F_2 (sarcoplasmic reticulum) and the third F_3 (mitochondrial fraction). These

fractions corresponded to the following sucrose concentrations (Fig. 1); 29% for F_1, 29–33% for F_2 and 33–40% for F_3 as measured in refractometer.

Electron microscopy

Electron microscopy of the fractions revealed the vesicular nature of Fractions F_1 and F_2 while F_3 was typical mitochondria with a double membrane and cristae.

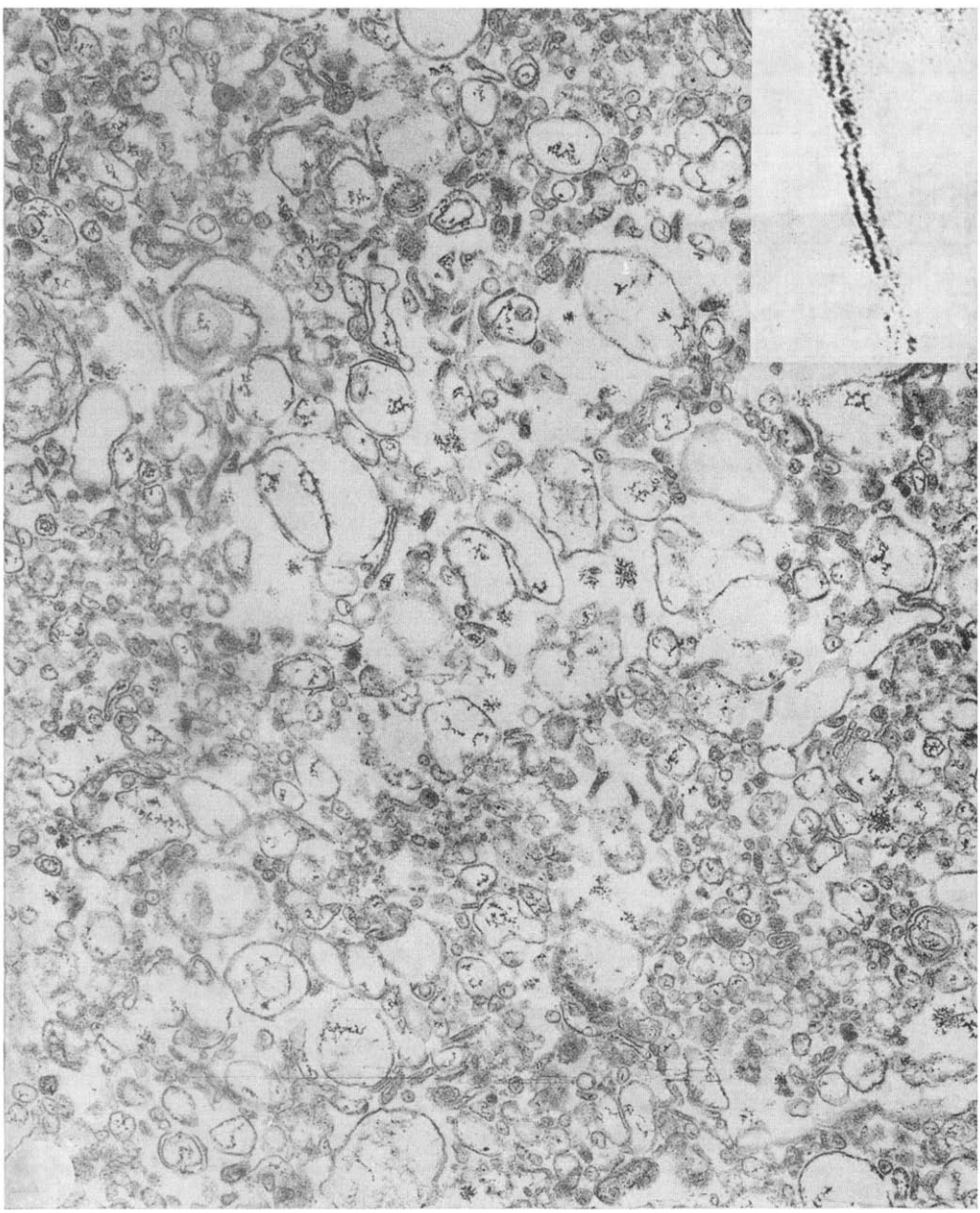


Fig. 2. Electron micrograph of plasma membrane fraction (F_1). Magnification $30 \cdot 10^3$. Vesicles of various sizes are present. Inset is triple-layered structure obtained at high magnification $250 \cdot 10^3$.

As shown in Fig. 2, vesicles of various sizes were present in Fraction F_1. At higher magnification, the membrane revealed a triple layered structure (inset) with an average thickness of 95 ± 3 Å (S.E.).

A typical electron micrograph of Fraction F_2 (Fig. 3) also revealed vesicles of various shapes and sizes and at higher magnification showed a triple layered

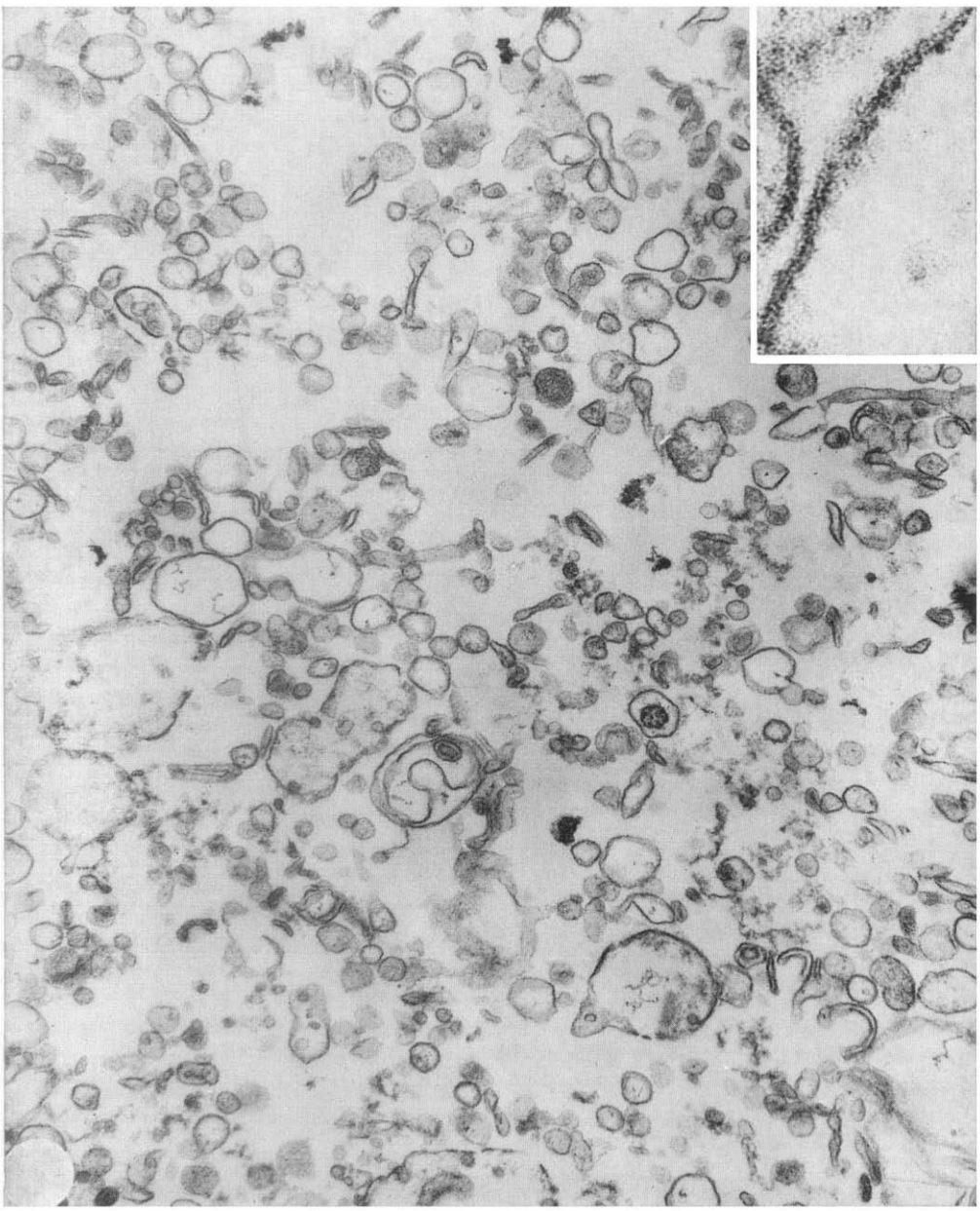


Fig. 3. Electron micrograph of sarcoplasmic reticulum (F_2). Magnification $30 \cdot 10^3$. Inset $250 \cdot 10^3$.

structure which had an average thickness of 56 ± 2 Å (S.E.) Fraction F_2 in spite of similar morphological characteristics as that of plasma membrane was definitely heavier than plasma membrane and differed as to its chemical and enzymatic characteristics.

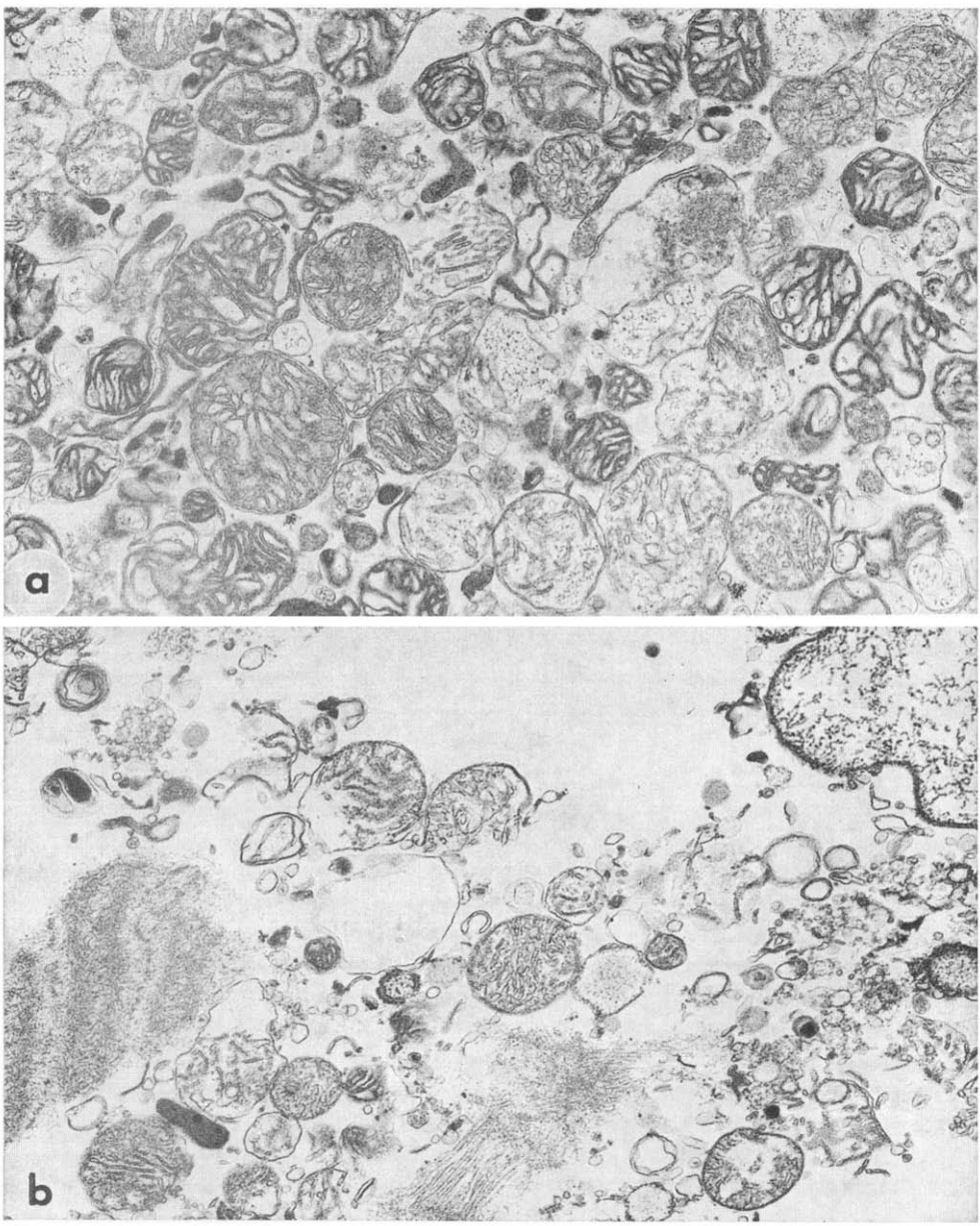


Fig. 4 (a) Electron micrograph of mitochondrial fraction (F_3). Magnification $20 \cdot 10^3$. (b) Electron micrograph of nuclear fraction (F_4). Magnification $20 \cdot 10^3$. Heterogenous fraction consisting of nucleus, mitochondria and contractile proteins.

Fraction F_3 (Fig. 4a) was a very uniform and homogenous preparation of mitochondria, identified by their characteristic double membrane and cristae.

The fraction collected from the bottom of the density gradient tube (Figs 4b and 5) showed the presence of nuclei, mitochondria, contractile proteins, collagen, *etc.* Due to the heterogenous character of this fraction it was not included in some studies.

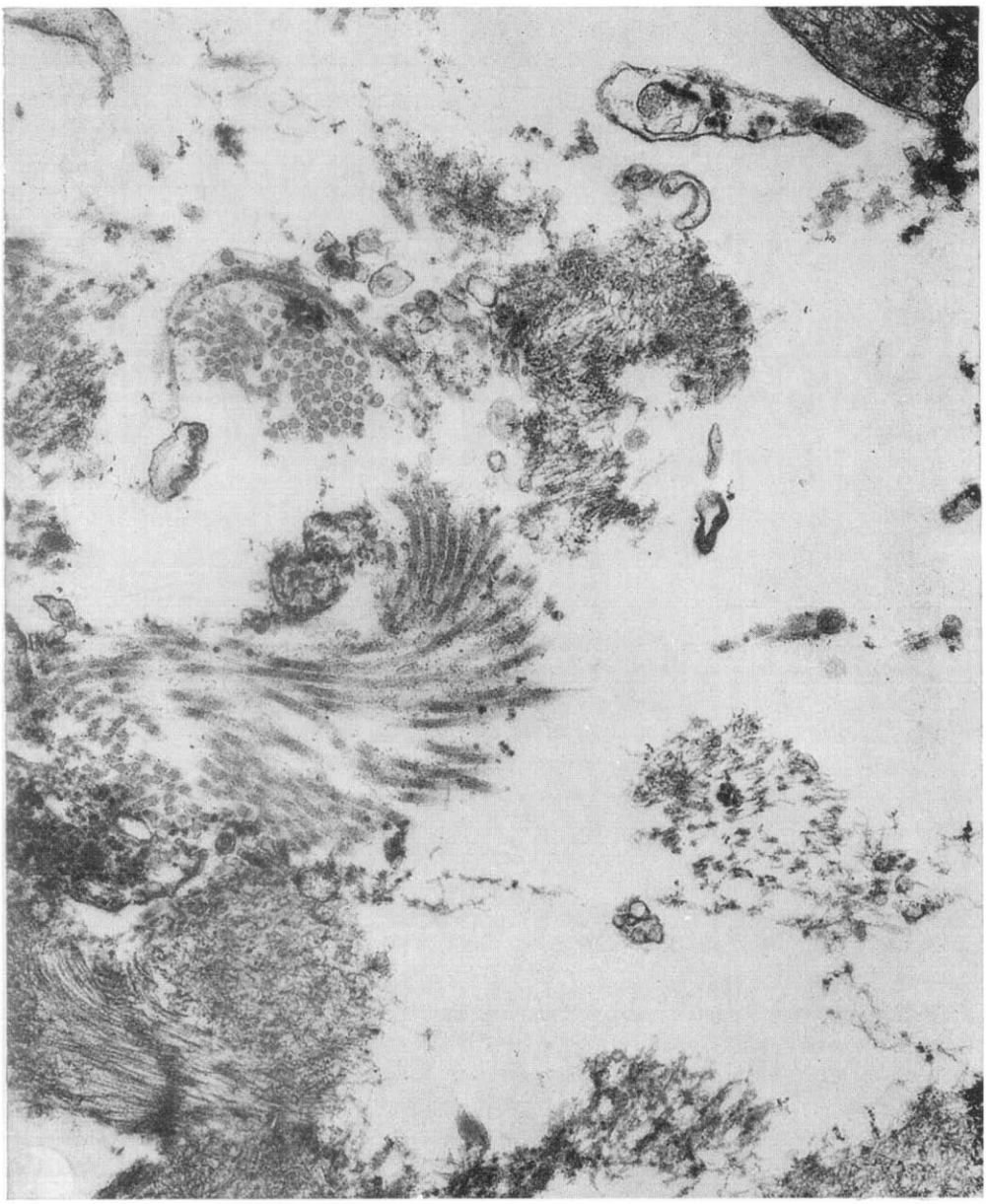


Fig. 5. Electron micrograph of nuclear fraction F_4. Magnification $30 \cdot 10^3$. A different area showing collagen and contractile proteins.

Distribution of protein and 5'-nucleotidase activity in particulate fractions

To determine the relative distribution of protein and 5'-nucleotidase and yield of plasma membrane we calculated the total protein content in each fraction. In Table I the protein content of plasma membrane (F_1), endoplasmic reticulum (F_2), mitochondria (F_3) and nuclear fraction (F_4) were 0.16, 0.23, 0.28 and 0.68 mg per g wet wt of the tissue, while the distribution of 5'-nucleotidase over the various fractions amounted to 44.4, 15.1, 33.6 and 6.9%.

5'-Nucleotidase was not determined in the homogenate because of the possible presence of soluble non-specific phosphatases and AMP deaminases. The former enzyme can hydrolyze any nucleotide and substrates like β-glycerophosphate, the latter enzyme can deplete the substrate by converting AMP into IMP. β-Glycerophosphate was hydrolyzed by homogenate but this activity was absent from the subcellular particles.

TABLE I

YIELD OF PLASMA MEMBRANE

Subcellular fraction	*Distribution of protein (mg/g of wet wt)*	*Distribution of 5'-nucleotidase activity (%)*
F_1	0.16 ± 0.05 (5)	44.4 ± 4 (5)
F_2	0.23 ± 0.02 (5)	15.1 ± 1 (5)
F_3	0.28 ± 0.1 (5)	33.6 ± 1.5 (5)
F_4	0.68 ± 0.5 (5)	6.9 ± 2 (5)

Enzymatic Markers

$(Na^+ + K^+)$-ATPase. Fraction F_1 which was equilibrated at the interphase of the loading medium and the density gradient possessed the maximum specific activity of $(Na^+ + K^+)$-ATPase. Other fractions had no significant activity of this enzyme. In Table II, the enzyme activity is given under different conditions. About 76 μmoles P_i released in the presence of $Na^+ + K^+ + Mg^{2+}$ while 60 μmoles was in the presence of Mg^{2+} alone in fraction F_1. Ouabain at a concentration of 3 mM

TABLE II

$(Na^+ + K^+)$-ATPase OF SUBCELLULAR FRACTION FROM SKELETAL MUSCLE

Values are expressed as μmoles P_i released/mg protein/h. The numbers in parentheses are the number of experiments performed.

Additions	*F_1 (plasma membrane)*	*F_2 (sarcoplasmic reticulum)*	*F_3 (mitochondria)*
$Mg^{2+} + Na^+ + K^+$	76 (3)	112 (2)	60 (2)
$Mg^{2+} + Na^+ + K^+$ + ouabain (10^{-3} M)	61 (3)	117 (2)	56 (2)

inhibited the $Na^+ + K^+$ stimulation almost completely. The specific activity of the ouabain-sensitive enzyme was 15 μmoles/mg protein/h. ATPase activities of F_2 and F_3 were not sensitive to ouabain and the activation by ($Na^+ + K^+$) could be achieved by either cation.

5'Nucleotidase. 5'-Nucleotidase activity was determined in all the subcellular fractions obtained by density gradient centrifugations and compared with that of the pellet homogenate. Fraction F_1 had this enzyme 17 times enriched when compared to the pellet homogenate. Other subcellular fractions; namely, sarcoplasmic reticulum (F_2) and mitochondria (F_3) had a smaller amount of this enzyme activity (Table III). The pellet homogenate was free of contractile proteins and hence had a higher specific activity of 5'-nucleotidase than in a complete homogenate.

TABLE III

SKELETAL MUSCLE 5'-NUCLEOTIDASE AND CYTOCHROME *c* OXIDASE ACTIVITIES

5'-Nucleotidase activity expressed as μmoles P_i released per mg protein per h. Cytochrome *c* oxidase activity as reduction in absorbance at 550 nm/min/mg proteins. Numbers in parentheses are the number of experiments performed on separate preparations.

Fraction	*5'-Nucleotidase*	*Cytochrome c oxidase*
F_1 (plasma membrane)	5.8 ±0.7 (4)	0.0±0.0 (2)
F_2 (sarcoplasmic reticulum)	2.7 ±0.55 (4)	0.0±0.0 (2)
F_3 (mitochondria)	1.7 ±0.5 (4)	8.0±2 (2)
F_4 (nuclear)	0.23±0.7 (4)	—
Pellet homogenate	0.34±0.4 (4)	—

Cytochrome c oxidase. Cytochrome *c* oxidase activity was measured as a mitochondrial marker (Table III). This enzyme was exclusively present in the mitochondrial fraction (F_3) identified by electron microscopy. Occasionally this enzyme was found in other fractions as well, but careful homogenization eliminated such activity.

Lipid composition

In order to differentiate various membranes, we determined phospholipid and cholesterol, assuming that plasma membrane should have higher cholesterol content as compared with that of intracellular membranes[18]. In Table IV total cholesterol

TABLE IV

LIPID COMPOSOTION OF SKELETAL MUSCLE SUBCELLULAR FRACTIONS

Values are expressed as μmoles/mg protein. Numbers in parentheses are the numbers of experiments performed on separate preparations.

Fractions	*Total cholesterol*	*Total phospholipid*	*Ratio cholesterol: phospholipid*
F_1 (plasma membrane)	0.18±0.024 (6)	1.6±0.2 (5)	0.11
F_2 (sarcoplasmic reticulum)	0.04 ±0.004 (6)	1.6±0.3 (5)	0.025
F_3 (mitochondria)	0.025±0.007 (3)	1.9±0.5 (6)	0.013

content in plasma membrane was found to be 0.18 μmole, while fractions F_2 and F_3 identified as sarcoplasmic reticulum and mitochondria possessed much less cholesterol per mg of protein. Phospholipid content was not much different (F_1, 1.6; F_2, 1.6; and F_3, 1.9 μmoles/mg protein). This observation was consistent with others, except the total cholesterol content seemed to be much lower than reported earlier[18,19]; however, the cholesterol:phospholipid ratio was found to be highest in the plasma membrane fraction.

Protein biosynthesis

Due to the absence of any accepted marker for sarcoplasmic reticulum in skeletal muscle, we decided to measure the protein biosynthetic activity of sarcoplasmic reticulum and other subcellular organelles to differentiate sarcoplasmic reticulum from plasma membrane. Earlier *in vivo* work by Ray *et al.*[20] has established that incorporation of [^{3}H[leucine into smooth reticulum of rat liver was maximum within a few minutes after injection of [^{3}H]leucine, while plasma membrane continued the incorporation much longer.

TABLE V

[^{3}H]LEUCINE INCORPORATION INTO VARIOUS FRACTIONS OF SKELETAL MUSCLE

The values below were calculated after subtracting the appropriate zero-time blank values. pmoles [^{3}H]leucine/mg protein. Values in parentheses are the number of experiments.

Fractions	*pmoles of [^{3}H]leucine*	
	2 min	*5 min*
F_1 (plasmic reticulum)	1.4 ± 1.1 (3)	2.2 ± 1.0 (5)
F_2 (sarcoplasmic reticulum)	19.0 ± 7.3 (3)	19.2 ± 5.4 (5)
F_3 (mitochondria)	0.0 (3)	0.0 (5)
Soluble fraction	0.0 (3)	0.0 (5)

In Table V the [^{3}H[leucine incorporation is given in various fractions of skeletal muscle. It was observed that incorporation of [^{3}H]leucine was highest in sarcoplasmic reticulum if the incubation time was 2 min. The incorporation of [^{3}H]leucine in fraction F_2 (sarcoplasmic reticulum) was 13 times higher than the plasma membrane. Significant labelling took place in plasma membrane in the absence of added ATP and GTP. This leucine was removed by treatment with dinitrofluorobenzene and washed according to Florini[16]. However, the incorporation of leucine in sarcoplasmic reticulum was not reduced to any significant extent by the same procedure. In 5 min the incorporation of leucine not removed by the above treatment was significant in plasma membrane and increased with time.

DISCUSSION

We succeeded in isolating plasma membrane and other subcellular fractions from smooth[8] and heart muscle[9] by density gradient centrifugation and were able to separate plasma membrane, sarcoplasmic reticulum and mitochondria on the basis of their density differences. In order to compare the properties of plasma

membrane of smooth and heart muscle to that of skeletal muscle, we used the same method of isolation to prepare the subcellular fractions from skeletal muscle as well.

However, the large amount of insoluble contractile proteins made it impossible to use density gradient centrifugation for the whole homogenate. The difficulty was overcome by using a special filter to remove the unbroken cells, connective tissue and contractile proteins in one filtration step. The filtrate was then concentrated by centrifugation and loaded on the gradient and successfully fractionated into different fractions.

Electron microscopy revealed the homogenous vesicular nature of the plasma membrane fraction as well as the sarcoplasmic reticulum fraction. Sarcoplasmic reticulum was mainly smooth type, but heavier than plasma membrane on sucrose density gradient. Due to the absence of Ca^{2+} or Mg^{2+} at these stages of isolation, it is conceivable that ribosomes detach from the sarcoplasmic reticulum and remain in the soluble fraction. Thus, none of our subcellular fractions looked like rough endoplasmic reticulum under electron microscopy. The plasma membrane and sarcoplasmic reticulum both revealed a triple layered structure at high magnification, but the thickness of sarcoplasmic reticulum unit membrane was much less than the plasma membrane.

Only the plasma membrane fraction possessed measurable activity of $(Na^+ + K^+)$-ATPase. Another enzyme which is often used as a marker of plasma membrane is 5′-nucleotidase[21,22]. In our preparations this enzyme had the maximum specific activity in fraction F_1 (plasma membrane). Other fractions (F_2 and F_3) also possessed this activity but this cannot be taken as conclusive evidence of the presence of plasma membrane in other fractions, because it is quite possible that this enzyme is present in sarcoplasmic reticulum and lysosomes as well[23,24]. Lysosomes, which reportedly contain this activity, could be isolated with mitochondria[25]. In addition, the possible continuity of sarcoplasmic reticulum with the T tube system[26] suggests that fragments containing both sarcoplasmic reticulum and plasma membrane may occur. If so, 5′-nucleotidase might be found with either the plasma membrane or the sarcoplasmic reticulum or both depending on the location of their combined fragments. Although we have not been able to localize T tubes in our gradient (sucrose density), we assume that isolated T tubules as extensions of plasma membrane would possess a density close to plasma membrane thus will equilibrate at the same density as does plasma membrane, while combined fragments might occur in either fraction.

Our yields of plasma membrane were found to be close to the published values[5,6]. We had an added advantage that we can recover all the subcellular fractions from a single gradient. The highest value was found to be for F_4 which contained most of the nuclei and some of the contractile proteins which escaped filtration.

Highest specific activity of 5′-nucleotidase was found in F_1 (plasma membrane) but 33% of the total activity was in the mitochondrial fraction which could be due to the plasma membrane contamination or the enzyme activity originating from lysosomes[24].

Cytochrome *c* oxidase was exclusively localized in the mitochondrial fraction (within the sensitivity of our method) but was occasionally found in other fractions if homogenization was not properly controlled.

It has been well established that there is a difference in lipid composition of various membranes[18,19,21]. We determined total cholesterol and total phospho-

lipid in our fractions in order to differentiate between plasma membrane and sarcoplasmic reticulum. We tried various methods of cholesterol determination by thin layer separation and color development by the method of Zlatkis *et al.*[27], and Schoenheimer and Sperry[13], but we found the cholesterol content much lower per mg protein as compared to that reported by others[18,19]. However, we consistently got higher values of cholesterol in plasma membrane as compared to those of sarcoplasmic reticulum and mitochondria. Phospholipid content was not much different from fraction to fraction, which is consistent with observations by others. The overall low values in our experiments could be due to the absence of any washing procedures which tend to remove certain membrane proteins. Saline is sometimes enough to remove some loosely bound proteins, while many methods so far reported for skeletal muscle plasma membrane preparations involve extraction with high salt solutions, which probably increases the lipid content per mg protein of the membranes.

In liver, glucose-6-phosphatase is an acceptable marker for endoplasmic reticulum but other tissues do not possess this enzyme to a significant extent. Ca^{2+} uptake property of sarcoplasmic reticulum may be a good marker but the possibility of plasma membrane possessing the same property cannot be excluded[28–31]. Recent studies on plasma membranes showed ATP-dependent Ca^{2+} binding when isolated from chick embryo fibroblasts[28], crab nerve[29], kidney cortex of rabbit[30] and human blood platelets[31]. Instead we used protein biosynthesis as a marker for endoplasmic reticulum. This should be localized in our gradient with sarcoplasmic reticulum.

Studies on protein biosynthesis of plasma membrane[20,32] have established that the incorporation of [^{3}H]leucine occurred in plasma membrane much later than endoplasmic reticulum. For this reason we selected a shorter time of incubation to enable us to use protein biosynthesis as a marker of sarcoplasmic reticulum.

We were able to demonstrate significant uptake of [^{3}H]leucine by sarcoplasmic reticulum in 2 min. Only after increasing the time of incubation was incorporation of this amino acid in plasma membrane fractions observed as well.

We were unable to detect mitochondrial contamination in the plasma membrane fraction using electron microscopy and cytochrome *c* oxidase enzyme as a marker. Fraction F_2 designated as sarcoplasmic reticulum had the highest protein incorporation activity and a possible contamination of plasma membrane by this fraction does not exceed 7% (Table V) assuming plasma membrane does not incorporate [^{3}H]leucine in 2 min. Collagen was not detected electronmicroscopically in any fraction except in Fraction F_4 which sedimented at the bottom of the tube.

In summary, we have used a combination of criteria for the assessment of the purity of membranes, morphology, specific activity of the enzymes, lipid composition and protein biosynthesis. Our plasma membrane and sarcoplasmic reticulum fractions differ in density, in thickness of membrane, in cholesterol:phospholipid ratios and in enzyme distributions including certain activities selectively localized in each fraction. We conclude that the present method has the advantage of being mild, fast and yields fairly pure subcellular fractions using a single density gradient centrifugation.

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