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ISOLATION OF PLASMA MEMBRANE VESICLES, DERIVED FROM TRANSVERSE TUBULES, BY SELECTIVE HOMOGENIZATION OF SUBCELLULAR FRACTIONS OF FROG SKELETAL MUSCLE IN ISOTONIC MEDIA

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Summary

A new technique for isolating fragmented plasma membranes from skeletal muscle has been developed that is based on gentle mechanical disruption of selected homogenate fractions. ($Na^+ + K^+$)-stimulated, Mg^{2+}-dependent ATPase was used as an enzymatic marker for the plasma membrane, Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase as a marker for sarcoplasmic reticulum, and succinate dehydrogenase for mitochondria. Cell segments in an amber low-speed (800 × *g*) pellet of a frog muscle homogenate were disrupted by repeated gentle shearing with a Polytron homogenizer. Sarcoplasmic reticulum was released into the low-speed supernatant, whereas most of the plasma membrane marker remained in a white, fluffy layer of the sediment, which contained sarcolemma and myofibrils. Additional gentle shearing of the white low-speed sediment extracted plasma membranes in a form that required centrifugation at 100 000 × *g* for pelleting. This pellet, the fragmented plasma membrane fraction, had a relatively high specific activity of ($Na^+ + K^+$)-stimulated ATPase compared with the other fractions, but it had essentially no Ca^{2+}-stimulated ATPase activity and only a small percentage of the succinate dehydrogenase activity of the homogenate.

Experimental evidence suggests that the fragmented plasma membrane fraction is derived from delicate transverse tubules rather than from the thicker, basement membrane-coated sarcolemmal sheath of muscle cells. Electron microscopy showed small vesicles lined by a single thin membrane. Hydroxy-

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Abbreviations: INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride; EGTA, ethyleneglycol-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

proline, a characteristic constituent of collagen and basement membrane, could not be detected in this fraction.

Introduction

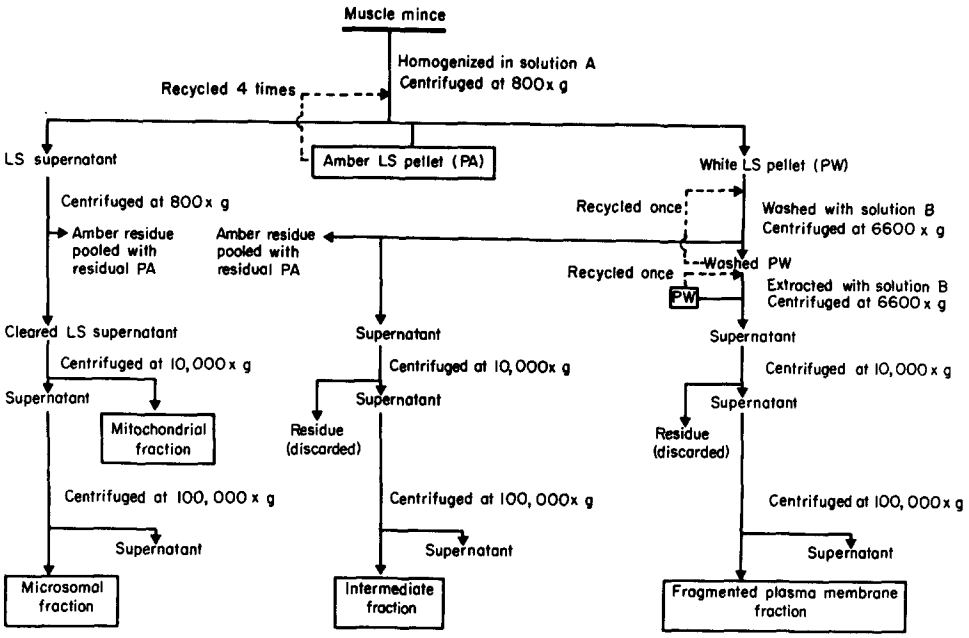
Studies of the biological properties of the skeletal muscle cell surface have been hampered by the difficulty of isolating plasma membranes from these cells in suitable form. Most such efforts have depended on separation of the sarcolemma from homogenates. Emptying of contractile proteins from cell segments has required extensive extraction with either hypotonic solutions [1,2] or hypertonic solutions such as 0.4 M LiBr and 0.6 M KCl [3—6]. In this approach the basement membrane and collagen fibers attached to the outer surface of the cell permit the sarcolemmal segments to be pelleted at low centrifugal speeds, at least in the earlier stages, and progress of the purification can be observed by phase-contrast microscopy. However, some membrane proteins may be lost during the extractions [7—10].

The plasma membrane of skeletal muscle cells is also found in delicate invaginations, the transverse tubules, which appear to lack a basement membrane (cf. Figs. 1 and 2 of Ref. 11). Lau et al. [10] have demonstrated the presence of transverse tubular vesicles in microsomal preparations of rabbit skeletal muscle, but it is difficult to separate plasma membrane cleanly from the more abundant sarcoplasmic reticulum of the microsomal fraction [10,12—14]. Observations described in this paper suggest that plasma membrane vesicles can be isolated from transverse tubules by gentle shearing of broken sarcolemmal segments from which most of the sarcoplasmic reticulum has been removed. This finding is the basis of a new fractionation procedure that permits excellent separation of plasma membrane from sarcoplasmic reticulum without recourse to hypotonic or hypertonic solutions.

Materials and Methods

Chemicals. Reagent-grade materials were purchased as follows: ouabain monohydrate, INT, and INT formazan from Sigma; EGTA from Eastman Kodak; crystallized bovine plasma albumin from Armour Laboratories; bovine achilles tendon collagen from Worthington; calf thymus DNA from Calbiochem; and Na_2ATP, pyruvate kinase and lactate dehydrogenase from Boehringer Mannheim. The pyruvate kinase suspension had approx. 2200 units/ml; 1 unit, as defined by the supplier, utilized 1 μmol phospho*enol*pyruvate/min per ml at 25° C. The lactate dehydrogenase suspension had approx. 2750 units/ml; each unit catalyzed the conversion of pyruvate to lactate at 1 μmol/min per ml at 25° C.

Homogenization of muscle and separation of amber and white low-speed pellets. Female *Rana pipiens*, 6—8 cm long, obtained from the Mogul-Ed Company of Oshkosh, Wisconsin, were maintained at 7° C in polyethylene pans containing 3 mM NaCl solution; they were used within 6 weeks of receipt. After pithing of the animals, the muscles were removed from the hindlimbs, trimmed of larger vessels, nerves and thicker pieces of connective tissue, and



Scheme 1. Procedure for isolation of subcellular fractions. LS, low-speed; PA, amber pellet; PW, white pellet.

collected in ice-cold frog-Ringer bicarbonate solution [15] equilibrated with a gas phase of 95% O_2/5% CO_2. For each fractionation 20—50 g of muscle were blotted and weighed, minced finely with a razor blade on an ice-cold glass plate, and suspended in 3 vols. of solution A (0.19 M sucrose, 0.1 mM $CaCl_2$ and 4 mM Tris-HCl, pH 8, at 0°C). Sucrose at this concentration is isotonic with respect to the extracellular fluid of frogs.

The fractionation procedure is outlined in Scheme 1. Throughout this procedure 1 vol. of solution will mean 1 ml per g of original muscle weight.

The suspension of minced muscle was homogenized in an ice bath with a Polytron PT-35 homogenizer (Brinkmann Instruments) for 5 min at low speed (approx. 800 rev./min). Clumps of connective tissue were removed from the blade at 1-min intervals and discarded. The resulting suspension was designated the crude homogenate fraction. An aliquot was set aside for analyses; to ensure uniform sampling for assays this aliquot was homogenized for an additional 15 min at low speed.

Subsequent steps were conducted at 0—5°C. The homogenate was centrifuged for 5 min at 800 × *g*. The turbid supernatant was removed and saved. The amber, clumpy pellet was resuspended in 3 vols. of solution A, homogenized for 5 min at approx. 600 rev./min and centrifuged for 5 min at 800 × *g*. The amber pellet was resuspended, homogenized for 10 min and centrifuged as before, three more times, for a total of five homogenizations.

As these homogenizations progressed, part of the amber pellet was converted into a more voluminous, fluffy white pellet that rested on top of the denser amber layer after centrifugation. These white layers were collected separately

and pooled to form the white low-speed pellet fraction, which was saved for additional washing and extraction. The amber pellet that remained after the fifth homogenization was combined with other amber pellets, obtained at steps that will be described later, to form a pooled residual amber, low-speed pellet fraction that was set aside for chemical and enzymatic analyses.

Preparation of mitochondrial and microsomal fractions. The supernatants obtained from the five initial homogenizations and centrifugations of muscle were combined, and turbidity was partially cleared by centrifugation for 15 min at 800 × *g*. The small, pale amber pellet was pooled with the amber pellet obtained earlier. The cleared supernatant was centrifuged for 15 min at 10 000 × g_{max} in the JA-10 rotor of a Beckman J-21 centrifuge to pellet a tan mitochondrial fraction. The supernatant was centrifuged for 30 min at 100 000 × g_{max} in a Spinco type 35 rotor to pellet a pale yellow microsomal fraction, which was suspended in 1—2 ml of solution B (0.19 M sucrose and 4 mM Tris-HCl, pH 8, at 0° C).

Derivation of an intermediate fraction from washing of the white low-speed pellet. To wash the pooled white low-speed pellets, they were stirred for 1 min at the lowest speed at which the Polytron PT-35 could be operated (approx. 400 rev./min), then diluted with 1 vol. of solution B and centrifuged for 15 min at 6600 × g_{max} in the JS-7.5 swinging bucket rotor of a Beckman J-21 centrifuge. The small amount of supernatant was removed, and the loosely packed white layer that constituted the main portion of the pellet was separated from a pale amber, more clumpy bottom layer. This amber material was pooled with the final amber pellet from the homogenate and set aside. The white layer was diluted with 2 vols. of solution B, stirred for 1 min at approx. 400 rev./min, then diluted with 2 more vols. of solution B and centrifuged for 15 min at 6600 × *g*.

The supernatants of these two wash steps were pooled and centrifuged for 15 min at 10 000 × *g*; the small pellet was discarded. Centrifugation of the supernatant for 60 min at 100 000 × *g* yielded a pellet, called the intermediate fraction, which was suspended in the same manner as the microsomal fraction.

Isolation of the fragmented plasma membrane fraction. The washed, white, low-speed pellet obtained in the preceding steps was suspended in 4 vols. of solution B and homogenized with the Polytron PT-35 at low speed (approx. 600 rev./min). The suspension was then diluted with 2 more vols. of solution B and centrifuged for 15 min at 6600 × *g*. The extraction was repeated by adding 3 vols. of solution B to the white pellet, homogenizing for 10 min at low speed, diluting with 2 more vols. of solution B, and centrifuging for 15 min at 6600 × *g*. The precipitate from these two extraction steps was called the residual white, low-speed pellet fraction.

The two extracts were pooled and centrifuged for 15 min at 10 000 × *g*; the small pellet was discarded. The supernatant was centrifuged for 60 min at 100 000 × *g* to pellet the fragmented plasma membrane fraction, which was resuspended in 1—2 ml of solution B. The subcellular fractions were stored at —20° C.

Sucrose density gradient centrifugation. Conical chambers (Buchler Instruments) were employed to prepare 14 ml of a shallow linear gradient, 1.5—0.75 M sucrose, in a cellulose nitrate tube of a Spinco SW 27.1 rotor. To

avoid a sharp transition of density from the tissue sample to the main gradient, it was topped with 2 ml of a shorter, steeper gradient, 0.75—0.19 M sucrose. 4 mM Tris-HCl, pH 8, was included in all solutions. The tissue sample, in 1 ml of solution B, was layered over the upper gradient. After centrifugation for 2 h at 100 000 × *g*, fractions were collected with an Auto-densiflow pump (Buchler Instruments). Sucrose concentrations were measured with an Abbe refractometer. Each fraction was diluted with 6 ml of H_2O and centrifuged in a Spinco type 50 rotor for 60 min at 100 000 × *g*. Pellets were resuspended in a small volume of 0.19 M sucrose and stored at −20° C.

Chemical analyses. Protein was determined by the method of Lowry et al. [16] using bovine plasma albumin as standard. Tissue samples were diluted at least 10-fold with 0.19 M sucrose, and standards were prepared in the same solution; under these conditions sucrose and Tris did not interfere significantly. DNA was measured fluorometrically by the method of Kissane and Robins [17] as modified by Hinegardner [18]. Phospholipids were extracted from subcellular fractions according to the procedure of Bligh and Dyer [19], and total phosphate was determined by the technique of Bartlett [20]. For assays of hydroxyproline the protein of tissue fractions was first purified by the procedure of Winnick [21]; then protein hydrolysates were analyzed in the manner described by MacKenzie and Tenaschuk [22].

Coupled spectrophotometric assay of ATPases. Two forms of ATPase (EC 3.6.1.3) were used as markers: $(Na^+ + K^+)$-activated, Mg^{2+}-dependent ATPase ($(Na^+ + K^+)$-ATPase) for plasma membrane, and Ca^{2+}-activated, Mg^{2+}-dependent ATPase (Ca^{2+}-ATPase) for sarcoplasmic reticulum.

The procedure of Schwartz et al. [23] for $(Na^+ + K^+)$-ATPase was modified to provide optimal conditions for measuring both this enzyme and Ca^{2+}-ATPase sequentially. The relative activities of the two enzymes were more helpful than absolute activities for observing how modifications in the fractionation procedure affected purification of subcellular fractions. Measurement of both enzyme activities in the same tissue sample helped to minimize variations of relative activity, which were otherwise troublesome, among aliquots of coarse suspensions such as those of crude homogenate and low-speed pellets.

The basal assay mixture contained 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 1.9 mM EGTA, 0.2 mM phospho*enol*pyruvate, 0.16 mM NADH, 2 mg of bovine plasma albumin per ml and 3 units of pyruvate kinase and 1 unit of lactate dehydrogenase per ml. To each cuvette was added an amount of tissue fraction that would give a change of 0.003—0.030 absorbance units per min after the reaction was started by adding 0.1 ml of ATP solution (1 mM final concentration). The stock solution of EGTA was adjusted to pH 7.4 with Tris, and additional Tris-HCl at pH 7.4 was added to give a final concentration of 40 mM Tris in the reaction system. The reaction mixture was maintained at 30° C and was stirred continually with a magnetically driven stirring sphere.

Baseline readings were recorded at 340 nm on a Beckman Acta II spectrophotometer between 5 and 10 min after the reaction was initiated. Then 0.05 ml ouabain solution (0.2 mM final concentration) was added to inhibit the $(Na^+ + K^+)$-ATPase [23], and activity was recorded for another 5 min. Next 0.1 ml $CaCl_2$ solution (2 mM total final concentration of Ca^{2+}; 0.1 mM excess over EGTA) was added to activate Ca^{2+}-ATPase for the final rate measurement,

bringing the reaction volume to 3 ml. Readings were corrected for the effects of successive dilutions on the absorbance of NADH. Reaction rates remained linear for at least 30 min, and the activity of each type of ATPase was proportional to the amount of tissue sample added. Enzyme activities were calculated as μmol of ATP hydrolyzed per h. Duplicate assays agreed within 20%. Subcellular fractions were tested as soon after preparation as possible, usually within 1 day.

Assay of succinate dehydrogenase. The reaction mixture for the succinate dehydrogenase (EC 1.3.99.1) assay contained in a 12 × 75-mm test tube: 10 mM Tris-HCl at pH 7.5, 100 mM sodium succinate, 0.8 mM INT, 1 mM NaN_3, and 0.2 ml of a subcellular sample in solution B. The reagents were warmed to 30° C for 5 min, and tissue sample was added to initiate the reaction. The final volume was 0.5 ml. After 10 min of additional incubation at 30° C, 1.5 ml of 95% ethanol was added to stop the reaction and to extract the purple, water-insoluble formazan produced by reduction of INT. The tube was immediately shaken for 5 s on a vortex mixer, then kept on ice for 20—30 min. Tissue particles and precipitated proteins were removed by centrifugation for 10 min at 800 × *g*. The absorbance of 1 ml of the clear supernatant fluid was read at room temperature at 458 nm against a reagent blank that had been incubated without tissue.

When succinate was omitted from the reaction mixture, negligible color was formed in the presence of tissue samples under the conditions employed. In standard tests on mitochondrial fractions, the amount of formazan produced was proportional to the amount of mitochondria present when the final absorbance was in the range of 0.030—0.400 units. Duplicate determinations on subcellular fractions agreed within 10%. The molar absorbance of chemically produced INT-formazan in an alcohol-water extract was $2.16 \cdot 10^4\ l \cdot mol^{-1} \cdot cm^{-1}$.

This method is a modification of procedures described by Defendi and Pearson [24] and Shelton and Rice [25], who used ethyl acetate, a more volatile solvent, for extraction. We found that alcohol was easier to pipette and extracted formazan from tissue samples more effectively than ethyl acetate. However, for crude homogenate suspensions sequential applications of alcohol and 2 ml of ethyl acetate were required; neither solvent alone extracted the dye completely. The factors that affect this assay system will be described in detail in a separate report.

Electron microscopy. A cell membrane suspension in solution B was centrifuged for 60 min at 100 000 × *g* in a Spinco SW 27.1 rotor to form a thin pellet, which was fixed with 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4, for 2 h at 4° C. After additional fixation with 1% OsO_4, it was embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate.

Results

Fractionation of muscle homogenates by differential centrifugation and density gradient centrifugation

Almost all of the DNA of muscle homogenates was recovered in pellets ob-

TABLE I

DISTRIBUTION OF PROTEIN, DNA AND PHOSPHOLIPID IN SUBCELLULAR FRACTIONS

Results of a representative fractionation performed in winter on 32 g of frog skeletal muscle are shown. Supernatants from all centrifugations at 100 000 × *g* were pooled to obtain the cytosol, which was analyzed only for protein. Each value is the mean of duplicate determinations. The percentage of homogenate material recovered in the subcellular fractions was 94% for protein, 90% for DNA, and 77% for phospholipid.

Fraction	Protein (mg/g muscle)	DNA		Phospholipid	
		μg/g muscle	μg/mg protein	μmol phosphorus/g muscle	μmol phosphorus/mg protein
Crude homogenate	117	580	5.0	6.5	0.055
Residual amber pellet *	45	460	10.2	2.9	0.064
Residual white pellet **	33	60	1.8	0.7	0.021
Mitochondrial	0.75	1.6	2.1	0.4	0.53
Microsomal	1.1	1.5	1.4	0.6	0.54
Intermediate	0.47	0.7	1.5	0.3	0.64
Fragmented plasma membrane	0.15	0.3	2.0	0.08	0.53
Cytosol	30				

* Residual amber, low-speed pellet obtained after five cycles of homogenization.
** Residual white, low-speed pellet after extraction of fragmented plasma membranes.

tained at 800 × *g* (Table I), but nuclei were only a minor constituent of these pellets. A much higher specific activity of DNA (approx. 200 μg/mg protein) has been reported for isolated nuclei of rat skeletal muscle [26]. The mitochondrial fraction, pelleted at 10 000 × *g*, was characterized by a high specific activity of succinate dehydrogenase (Table II). The microsomal fraction, pelleted from the postmitochondrial supernatant at 100 000 × *g*, was distinguished by a high specific activity of Ca^{2+}-ATPase.

The microsomal fraction also contained ($Na^+ + K^+$)-ATPase activity. Attempts were made to separate this activity from the Ca^{2+}-ATPase activity on sucrose density gradients of varying steepness, with and without the addition of $CaCl_2$ (2 mM), EGTA (4 mM) or sodium pyrophosphate (2 mM). Although individual peaks could be separated, Ca^{2+}-ATPase activity was diffuse and predominant in each gradient. In a shallow two-step gradient (Fig. 1), where separations were somewhat better than in steeper gradients, there was a major peak of Ca^{2+}-ATPase activity in the lower part of the centrifuge tube at a density of approx. 1.16 g/ml, but significant activity extended into regions of lower density. Meissner [27] found that heterogeneous sarcoplasmic reticulum fragments are dispersed over a broad zone in sucrose density gradients.

($Na^+ + K^+$)-ATPase activity was also spread out, with peaks at densities of approx. 1.09, 1.14 and 1.16 g/ml (Fig. 1). Similar multiple peaks of plasma membrane material have been observed in gradients of rabbit skeletal muscle homogenates by Caswell et al. [28]. Other investigators [12—14] have also obtained overlapping zones of activity for ($Na^+ + K^+$)-ATPase and Ca^{2+}-ATPase in gradients.

TABLE II

ACTIVITIES OF ENZYMES IN MUSCLE CELL FRACTIONS

The tissue fractions are from the same experiment as in Table I. Each value is the mean for duplicate assays. The activity recovered in subcellular fractions was 60% for $(Na^+ + K^+)$-ATPase, 82% for Ca^{2+}-ATPase, and 92% for succinate dehydrogenase.

Fraction	$(Na^+ + K^+)$-ATPase *		Ca^{2+}-ATPase *		Activity ratio: Ca^{2+}-ATPase/ $(Na^+ + K^+)$-ATPase	Succinate dehydrogenase **		Activity ratio: succinate dehydrogenase/ $(Na^+ + K^+)$-ATPase
	Activity	Specific activity	Activity	Specific activity		Activity	Specific activity	
Crude homogenate	166	1.4	460	3.9	2.8	18	0.15	0.11
Residual amber pellet	63	1.4	285	6.3	4.5	8.9	0.20	0.14
Residual white pellet	28	0.85	6.4	0.19	0.23	2.8	0.085	0.10
Mitochondrial	2.8	3.7	7.2	9.6	2.6	4.2	5.6	1.5
Microsomal	2.5	2.3	69	63	28	0.21	0.19	0.084
Intermediate	1.7	3.6	8.5	18	5.0	0.067	0.14	0.039
Fragmented plasma membrane	2.0	13	<0.02 ***	<0.13	<0.01	0.12	0.80	0.060

* μmol ATP converted to ADP/h per g muscle (activity) or per mg protein (specific activity).

** μmol formazan produced/h per g muscle (activity) or per mg protein (specific activity).

*** At the lower limit of detectability.

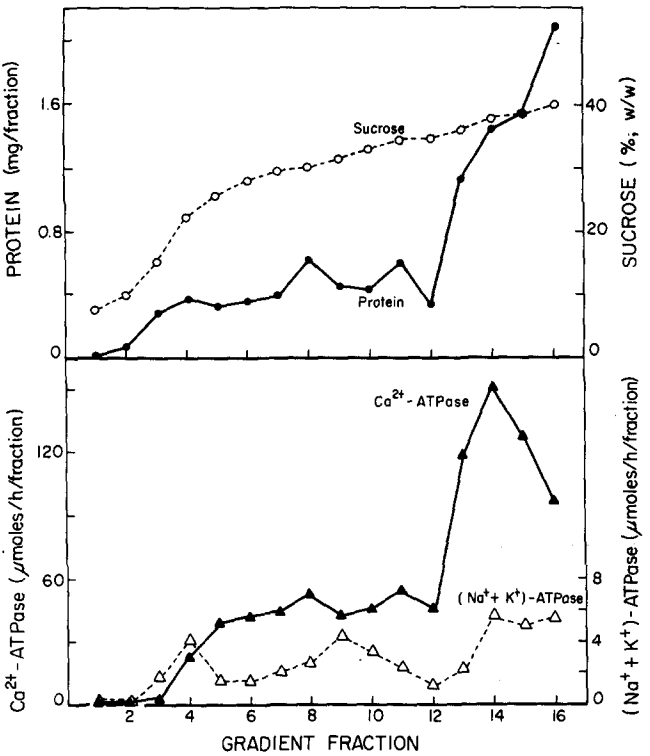


Fig. 1. Sucrose density gradient fractionation of a microsomal preparation. The gradient was loaded with 1 ml of a microsomal suspension; after centrifugation, 1.1-ml fractions were collected, starting at the top of the gradient.

Isolation of a fragmented plasma membrane fraction free of significant contamination by sarcoplasmic reticulum

Separation of plasma membrane from sarcoplasmic reticulum was achieved more readily by selective homogenization of low-speed pellets consisting largely of short cell segments with relatively intact contents. Repeated homogenization of the amber pellet material caused the myofibrils to swell so that they protruded from the ends of the cell segments (Fig. 2). Eventually the sarcolemmal walls broke down, releasing swollen fibrils and finely divided granular matter which, together with irregular pieces of sarcolemmal walls, constituted a voluminous, white, upper layer of the low-speed pellets. The presence of expanded myofibrillar elements in the white sediment was confirmed by its rapid shrinkage, to approximately one-fourth of its original volume, upon addition of ATP and $MgCl_2$ (each at 2 mM final concentration).

One biochemical distinction between the two layers was particularly advantageous for purifying plasma membranes: the ratio of Ca^{2+}-ATPase to $(Na^+ + K^+)$-ATPase activity was much lower in the white portion than in the amber (Table II). Apparently the disruption of cell segments released sarcoplasmic reticulum more readily than plasma membrane from low-speed pellets. A small additional amount of Ca^{2+}-ATPase activity was removed from the

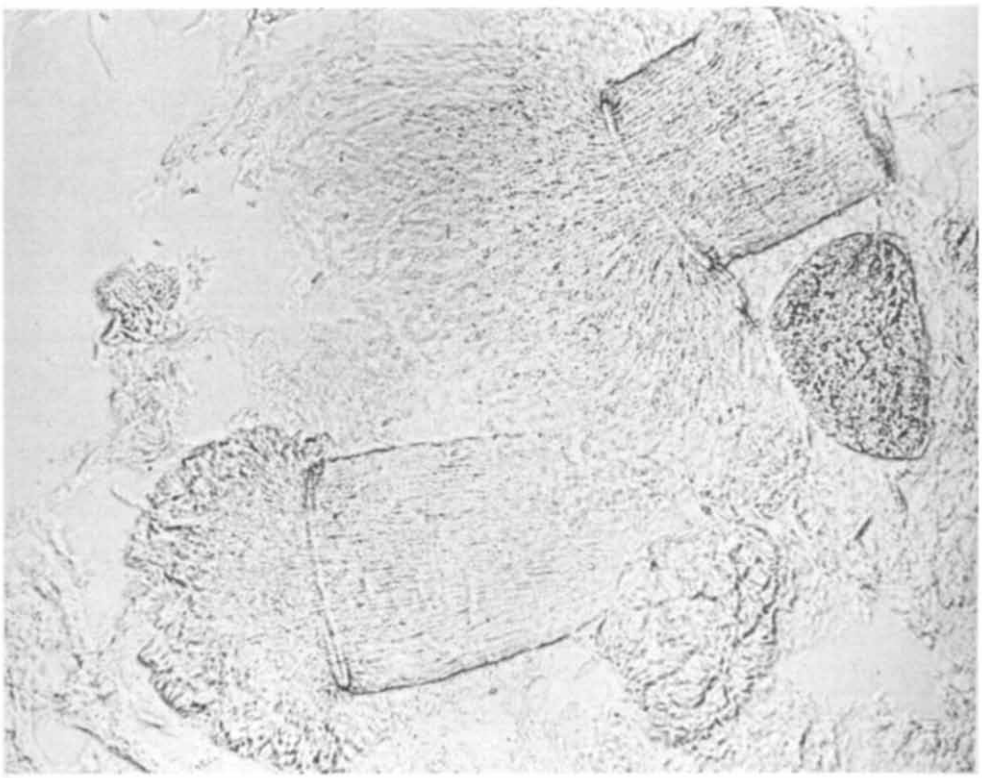


Fig. 2. Phase contrast micrograph of cell segments in an amber low-speed pellet. Note the swelling and extrusion of myofibrils after two homogenizations. Magnification 360X.

pooled white low-speed pellets by brief washing; this activity was recovered in the intermediate membrane fraction, which had less Ca^{2+}-ATPase activity than the microsomal fraction.

Gentle homogenization of the washed white sediment released material, enriched in $(Na^+ + K^+)$-ATPase activity, that was pelleted in the fragmented plasma membrane fraction. This fraction was essentially devoid of Ca^{2+}-ATPase activity. Thus one of the major difficulties we had encountered in the purification of plasma membranes from skeletal muscle was resolved. The virtual absence of Ca^{2+}-ATPase activity in the fragmented plasma membrane fraction was confirmed in 11 other preparations in which the white low-speed pellet was homogenized under various conditions of duration, speed and suspending medium. The yield of $(Na^+ + K^+)$-ATPase tended to be slightly higher when solution B was used for the final extraction, but activity was negligible in either case. Post and Sen [29] found $(Na^+ + K^+)$-ATPase to be less stable in the presence of Ca^{2+}. In our tests the specific activity of $(Na^+ + K^+)$-ATPase ranged from 8 to 21 μmol/h per mg protein, with most values falling in the middle of this range.

Comprehensive assay results for all the major fractions of a single fractionation are given in Tables I and II. Similar results were obtained in eight other

studies in which a more limited number of subcellular fractions were examined each time to observe the effects of modifications of the experimental conditions (duration of homogenization or centrifugation, volume of suspending medium). Despite moderate fluctuations of absolute enzyme activities, the basic pattern of relative activities among the tissue fractions was unchanged.

Other properties of isolated membrane fractions

The total $(Na^+ + K^+)$-ATPase activity recovered in the subcellular fractions (Table II) was 37 μmol/h per g of muscle. This does not include the residual amber pellet, where the enzyme activity appears to be associated mainly with whole cell segments. Only 17% of the subcellular activity was found in the fractions pelleted at 100 000 × *g*; a small additional amount was associated with the mitochondrial fraction. The remaining 76% was in the residual white pellet. Further homogenization of this pellet at higher speeds, up to 15 000 rev./min for 2 min, extracted only a few percent more of the activity. Thus $(Na^+ + K^+)$-ATPase activity appears to be associated with two distinct types of plasma membrane; one is readily fragmented at low shearing forces, while the other is firmly retained in the white low-speed pellet even after extensive homogenization.

Mitochondrial contamination of the fragmented plasma membrane fraction was low but difficult to eliminate. The mitochondrial fraction itself had 57% of the total subcellular succinate dehydrogenase activity, exclusive of the amber pellet. A significant portion (38%) was in the residual white pellet. Close association of one class of mitochondria with myofibrils [30] may help to explain why it is generally difficult to separate mitochondria from muscle homogenates. Repeated washing did not appreciably lower the mitochondrial content of our white pellet fractions. Isolated intact mitochondria were readily pelleted at 10 000 × *g* and migrated to the bottom of the sucrose density gradients. On the other hand, the microsomal and fragmented plasma membrane fractions contained small amounts of succinate dehydrogenase activity, possibly associated with submitochondrial particles, which remained in the upper portions of gradients and could not be separated from plasma membranes by centrifugation.

This type of contamination increased several-fold when the white pellet was homogenized at higher speeds. Therefore the Polytron was run at low speeds at all stages of the procedure described in this report. Only 1.6% of the succinate dehydrogenase activity of subcellular fractions (0.7% of homogenate activity) was in the fragmented plasma membrane fraction under these conditions (Table II).

The supernatants from the final (100 000 × *g*) centrifugations were too dilute for enzyme assays. After centrifugation of these fluids for 60 min at 250 000 × *g*, some $(Na^+ + K^+)$-ATPase but no Ca^{2+}-ATPase or succinate dehydrogenase activity could be detected in the pellets. The supernatant of the intermediate fraction was the best source of $(Na^+ + K^+)$-ATPase activity, but even here the specific activities were so low, 2—5 μmol/h per mg protein, that extra-high-speed centrifugation was not utilized regularly for isolation of plasma membranes.

The ratio of phospholipid phosphorus to protein was significantly higher in

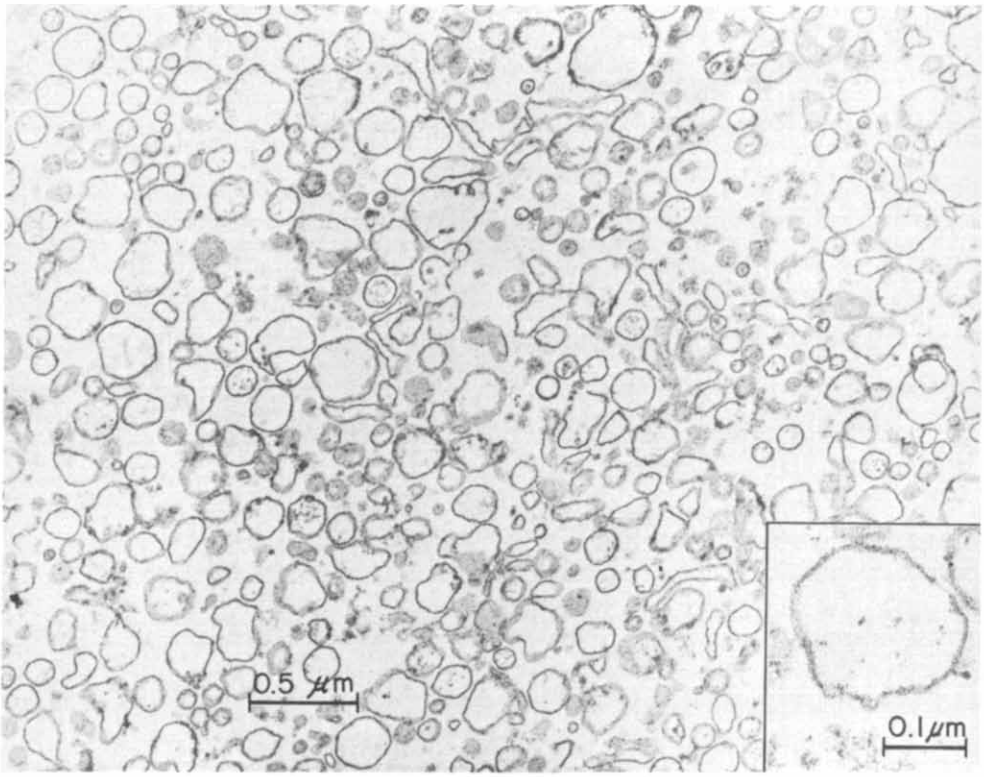


Fig. 3. Electron micrograph of a fragmented plasma membrane fraction. The field is typical of those observed in thin sections taken from various locations in a high-speed pellet. Numerous membranous vesicles are seen; magnification 45 000X. Insert: A trilaminar structure is evident in the membranes at higher magnification (171 000X).

the mitochondrial, microsomal, intermediate and fragmented plasma membrane fractions than in the crude homogenate (Table I). These findings, which are compatible with partial purification of membranes in these fractions, are similar to values reported for plasma membranes from rabbit skeletal muscle [5].

Electron microscopy showed that the fragmented plasma membrane fraction was composed largely of membranous vesicles (Fig. 3). At higher magnification a trilaminar structure could be discerned in the membranes. No basement membrane was associated with the vesicles, in contrast to descriptions of emptied sarcolemmal tubes by other investigators [3,31]. Kono and his colleagues [32] found appreciable amounts of hydroxyproline in extracts of their purified sarcolemmal fractions. We prepared sarcolemma from frog skeletal muscle by the method of Kono and Colowick [3] and found 0.070 μmol hydroxyproline per mg protein. In bovine achilles tendon collagen, we found 0.60 μmol/mg protein, which agrees well with the analyses of Kefalides and Winzler [33]. In contrast, three samples of our fragmented plasma membrane fraction had no detectable hydroxyproline (<0.002 μmol/mg protein).

Discussion

We postulate that the $(Na^+ + K^+)$-ATPase activity of the fragmented plasma membrane fraction is derived from transverse tubules. It is reasonable to expect that these delicate intracellular extensions of plasma membrane could be broken off from the sarcolemma by gentle shearing. Caswell et al. [28] have identified transverse tubular vesicles morphologically in microsomal preparations as components of characteristic triad junctions. The transverse tubules appeared to bind [^{3}H]ouabain and to possess $(Na^+ + K^+)$-ATPase activity. We have not found triad junctions in electron micrographs of our microsomal or fragmented plasma membrane fractions, but triads are fragile and may have been disrupted by our more prolonged homogenization.

Observations by other investigators [2,3,31], as well as results of the present studies, suggest that the $(Na^+ + K^+)$-ATPase remaining in the white low-speed pellet after prolonged homogenization resides mainly in the plasma membrane of the sarcolemma, which includes a basement membrane and an outer coat of collagen fibers. Although basement membrane stays associated with the plasma membrane of the sarcolemma during extensive washing with hypertonic salt solutions [3,32], no basement membrane was seen in electron micrographs of our fragmented plasma membrane fraction that had been isolated by gentle homogenization alone. Furthermore, hydroxyproline was not present in this preparation.

The validity of using $(Na^+ + K^+)$-ATPase as a marker for identifying plasma membranes in muscle homogenates has been well supported by recent studies with a variety of experimental techniques [6,10,14,34—36]. A high activity of Ca^{2+}-ATPase is characteristic of sarcoplasmic reticulum of skeletal muscle, but low activities have been reported in other subcellular fractions, including plasma membranes [34,37—40]. We found Ca^{2+}-ATPase predominantly in the microsomal fraction; it was essentially absent from the fragmented plasma membrane fraction. Succinate dehydrogenase, which is localized in the inner membrane of mitochondria [41], provides a means of distinguishing these organelles from other types of cellular membranes [42,43]. The tetrazolium test is a convenient means of demonstrating relative enzyme activities in tissue fractions.

Although enzyme activities are highly useful for identifying types of membranes, especially in the early stages of fractionation, they should be regarded as qualitative rather than quantitative markers. The ratio of $(Na^+ + K^+)$-ATPase activity to phospholipid phosphorus is twice as high in the residual white pellet, which is enriched in sarcolemma, as in the fragmented plasma membrane fraction, 40 vs. 20 μmol/h per μmol phosphorus. We do not yet know whether this difference reflects a variation of enzyme concentration in plasma membranes from anatomically distinct portions of the cell or is related to physical changes in the membranes at different stages of fractionation.

The specific activity of $(Na^+ + K^+)$-ATPase in the fragmented plasma membrane fraction, 8—21 μmol/h per mg protein, is within the range reported for muscle plasma membrane fractions that have been partially purified by treatment with hypertonic salt solutions or detergents [44,45] or by sucrose density gradient centrifugation [46]. In our experience treatment of muscle homo-

genates with 0.4 M LiBr or 0.6 M KCl produces membrane pellets that are gummy and difficult to suspend for washing and analysis. In contrast, the fragmented plasma membrane pellets described in this report are easily resuspended.

The yield of $(Na^+ + K^+)$-ATPase activity in the fragmented plasma membrane fraction is low, ranging from 1.3 to 3.9 μmol/h per g muscle in 12 preparations. One reason for the low yield is that much of the activity in the homogenate is in the sarcolemma, which remains in the residual white pellet. Our yield is much higher, however, than that for plasma membranes derived from the transverse tubules of rabbit skeletal muscle [10] where the specific activity was 6 μmol/h per mg protein, and the protein content was approx. 4 mg/80 g muscle, giving a yield of 0.3 μmol/h per g muscle. In most other procedures reported for isolating plasma membranes of skeletal muscle, whole homogenate was not assayed before the first centrifugation or filtration, and sufficient information is not available for calculating the yield of $(Na^+ + K^+)$-ATPase per g muscle.

From a practical standpoint, the yield and purity of our fragmented plasma membrane fraction are high enough to permit further studies of its chemical and biological properties.

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References

1 McCollester, D.L. (1962) Biochim. Biophys. Acta 57, 427—437
2 Westort, C. and Hultin, H.O. (1966) Anal. Biochem. 16, 314—319
3 Kono, T. and Colowick, S.P. (1961) Arch. Biochem. Biophys. 93, 520—533
4 Boegman, R.J., Manery, J.F. and Pinteric, L. (1970) Biochim. Biophys. Acta 203, 506—530
5 Severson, D.L., Drummond, G.I. and Sulakhe, P.V. (1972) J. Biol. Chem. 247, 2949—2958
6 Andrew, C.G. and Appel, S.H. (1973) J. Biol. Chem. 248, 5156—5163
7 Rosenberg, S.A. and Guidotti, G. (1969) J. Biol. Chem. 244, 5118—5124
8 Marchesi, S.L., Steers, E., Marchesi, V.T. and Tillack, T.W. (1970) Biochemistry 9, 50—57
9 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
10 Lau, Y.H., Caswell, A.H. and Brunschwig, J.-P. (1977) J. Biol. Chem. 252, 5565—5574
11 Franzini-Armstrong, C. (1970) J. Cell Biol. 47, 488—499
12 Katz, A.M., Repke, D.I., Upshaw, J.E. and Polascik, M.A. (1970) Biochim. Biophys. Acta 205, 473—490
13 Forgue, M.-E. and Freychet, P. (1975) Diabetes 24, 715—723
14 Barchi, R.L., Bonilla, E. and Wong, M. (1977) Proc. Natl. Acad. Sci. U.S. 74, 34—38
15 Holloszy, J.O. and Narahara, H.T. (1967) J. Gen. Physiol. 50, 551—562
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
17 Kissane, J.M. and Robins, E. (1958) J. Biol. Chem. 233, 184—188
18 Hinegardner, R.T. (1971) Anal. Biochem. 39, 197—201
19 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917
20 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
21 Winnick, T. (1950) Arch. Biochem. 27, 65—74

22 MacKenzie, S.L. and Tenaschuk, D. (1975) J. Chromatogr. 104, 176—177
23 Schwartz, A., Allen, J.C. and Harigaya, S. (1969) J. Pharmacol. Exp. Ther. 168, 31—41
24 Defendi, V. and Pearson, B. (1955) J. Histochem. Cytochem. 3, 61—69
25 Shelton, E. and Rice, M.E. (1957) J. Natl. Cancer Inst. 18, 117—125
26 Edelman, J.C., Edelman, P.M., Knigge, K.M. and Schwartz, I.L. (1965) J. Cell Biol. 27, 365—378
27 Meissner, G. (1975) Biochim. Biophys. Acta 389, 51—68
28 Caswell, A.H., Lau, Y.H. and Brunschwig, J.-P. (1976) Arch. Biochem. Biophys. 176, 417—430
29 Post, R.L. and Sen, A.K. (1967) in Methods in Enzymology (Estabrook, R.W. and Pullman, M.E., eds.), Vol. 10, pp. 762—768, Academic Press, New York
30 Palmer, J.W., Tandler, B. and Hoppel, C.L. (1977) J. Biol. Chem. 252, 8731—8739
31 Zacks, S.I., Vandenburgh, H. and Sheff, M.F. (1973) J. Histochem. Cytochem. 21, 895—901
32 Kono, T., Kakuma, F., Homma, M. and Fukuda, S. (1964) Biochim. Biophys. Acta 88, 155—176
33 Kefalides, N.A. and Winzler, R.J. (1966) Biochemistry 5, 702—713
34 Sulakhe, P.V., Drummond, G.I. and Ng, D.C. (1973) J. Biol. Chem. 248, 4150—4157
35 Tada, M., Kirchberger, M.A., Iorio, J.-A.M. and Katz, A.M. (1975) Circ. Res. 36, 8—17
36 Schimmel, S.D., Kent, C. and Vagelos, P.R. (1977) in Methods in Cell Biology (Prescott, D.M., ed.), Vol. 15, pp. 289—301, Academic Press, New York
37 Nayler, W.G. (1977) in Mammalian Cell Membranes (Jamieson, G.A. and Robinson, D.M., eds.), Vol. 3, pp. 147—171, Buttersworths, London
38 Quist, E.E. and Roufogalis, B.D. (1977) J. Supramol. Struct. 6, 375—381
39 Weidekamm, E. and Brdiczka, D. (1975) Biochim. Biophys. Acta 401, 51—58
40 Hui, C.-W., Drummond, M. and Drummond, G.I. (1976) Arch. Biochem. Biophys. 173, 415—427
41 Schnaitman, C. and Greenawalt, J.W. (1968) J. Cell Biol. 38, 158—175
42 Hogenboom, G.H., Schneider, W.C. and Pallade, G.E. (1948) J. Biol. Chem. 172, 619—635
43 Cleland, K.W. and Slater, E.C. (1953) Biochem. J. 53, 547—556
44 Rogus, E., Price, T. and Zierler, K.L. (1969) J. Gen. Physiol. 54, 188—202
45 Akera, T., Larsen, F.S. and Brody, T.M. (1969) J. Pharmacol. Exp. Ther. 170, 17—26
46 Kidwai, A.M., Radcliffe, M.A., Lee, E.Y. and Daniel, E.E. (1973) Biochim. Biophys. Acta 298, 593—607