Biochem. J. (1982) **202**, 289–301
Printed in Great Britain

Biochemical heterogeneity of skeletal-muscle microsomal membranes

Membrane origin, membrane specificity and fibre types

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(*Received 23 June 1981/Accepted 23 October 1981*)

1. Microsomes were isolated from rabbit fast-twitch and slow-twitch muscle and were separated into heavy and light fractions by centrifugation in a linear (0.3–2 M) sucrose density gradient. The membrane origin of microsomal vesicles was investigated by studying biochemical markers of the sarcoplasmic-reticulum membranes and of surface and T-tubular membranes, as well as their freeze-fracture properties. 2. Polyacrylamide-gel electrophoresis showed differences in the Ca^{2+}-dependent ATPase/calsequestrin ratio between heavy and light fractions, which were apparently consistent with their respective origin from cisternal and longitudinal sarcoplasmic reticulum, as well as unrelated differences, such as peptides specific to slow-muscle microsomes (mol.wts. 76000, 60000, 56000 and 45000). 3. Freeze-fracture electron microscopy of muscle microsomes demonstrated that vesicles truly derived from the sarcoplasmic reticulum, with an average density of 9 nm particles on the concave face of about $3000/\mu m^2$ for both fast and slow muscle, were admixed with vesicles with particle densities below $1000/\mu m^2$. 4. As determined in the light fractions, the sarcoplasmic-reticulum vesicles accounted for 84% and 57% of the total number of microsomal vesicles, for fast and slow muscle respectively. These values agreed closely with the percentage values of Ca^{2+}-dependent ATPase protein obtained by gel densitometry. 5. The T-tubular origin of vesicles with a smooth concave fracture face in slow-muscle microsomes is supported by their relative high content in total phospholipid and cholesterol, compared with the microsomes of fast muscle, and by other correlative data, such as the presence of $(Na^+ + K^+)$-dependent ATPase activity and of low amounts of Na^+-dependent membrane phosphorylation. 6. Among intrinsic sarcoplasmic-reticulum membrane proteins, a proteolipid of mol.wt. 12000 is shown to be identical in the microsomes of both fast and slow muscle and the Ca^{2+}-dependent ATPase to be antigenically and catalytically different, though electrophoretically homogeneous. 7. Basal Mg^{2+}-activated ATPase activity was found to be high in light microsomes from slow muscle, but its identification with an enzyme different from the Ca^{2+}-dependent ATPase is still not conclusive. 8. Enzyme proteins that are suggested to be specific to slow-muscle longitudinal sarcoplasmic reticulum are the flavoprotein NADH:cytochrome b_5 reductase (mol.wt. 32000), cytochrome b_5 (mol.wt. 17000) and the stearoyl-CoA desaturase, though essentially by criteria of plausibility.

It seems now well established that the protein and enzymic composition of skeletal-muscle microsomes, currently denoted as fragmented sarcoplasmic reticulum, is much more complex for slow-twitch muscles than for fast-twitch muscles (Margreth *et al.*, 1974*a*, 1975, 1977, 1980; Salviati *et al.*, 1979; Damiani *et al.*, 1981).

This apparent specificity in membrane composition in relation to fibre type encompasses a variety of biochemical aspects, namely the presence in slow-muscle microsomes of: (i) endogenous protein kinase(s) and phosphorylatable protein substrates (Kirchberger & Tada, 1976; Margreth *et al.*, 1977,

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0306-3283/82/020289-14$01.50/1

1980; Heilmann *et al.*, 1977), similarly to those in cardiac-muscle microsomes (Tada *et al.*, 1978); (ii) a cytochrome b_5-linked stearoyl-CoA desaturase system, with composition similar to, or identical with, that of the analogous system of liver microsomes (Salviati *et al.*, 1979, 1981); (iii) a higher binding of α-tocopherol to membrane phospholipids and a higher content of phospholipid arachidonate (Salviati *et al.*, 1980). Furthermore, a polymorphism of the sarcoplasmic-reticulum Ca^{2+}-dependent ATPase, according to the type of muscle, is supported by recent immunological evidence (DeFoor *et al.*, 1980; Damiani *et al.*, 1981) and kinetic data (Wang *et al.*, 1979).

On the other hand, the use of different methods for isolating and purifying muscle microsomes, and the variety of electrophoretic techniques employed for their analysis, have been themselves the origin of views (see, e.g., Campbell *et al.*, 1980) that are at variance with that according to which sarcoplasmic-reticulum membranes have a highly simplified protein composition, consisting of the Ca^{2+}-dependent ATPase, calsequestrin and high-affinity Ca^{2+}-binding protein and a proteolipid (Meissner & Fleischer, 1971; MacLennan, 1974).

The extensive studies of Meissner (1975), and of Caswell and his co-workers (Lau *et al.*, 1977, 1979*a*,*b*; Brandt *et al.*, 1980), have demonstrated a heterogeneity in protein composition between heavy and light vesicles, in relation to their origin from the cisternal and the longitudinal component of the intact sarcoplasmic reticulum respectively. Conversely, from other lines of investigation there is increasing evidence that in some muscles, such as chicken breast muscle (Scales & Sabbadini, 1979; Malouf & Meissner, 1979) and dog heart muscle (Jones *et al.*, 1978; Malouf & Meissner, 1980), the isolated microsome fraction has a mixed composition, i.e. of sarcoplasmic-reticulum vesicles and of T-tubule fragments and/or fragments derived from the sarcolemma.

In the light of these observations, we have re-investigated the problem of the biochemical heterogeneity of the microsomes of fast-twitch and slow-twitch muscle of the rabbit, in relation to the membrane origin of the constituent vesicles.

Materials and methods

Methods

All chemicals were analytical products and were obtained from Merck A.G. (Darmstadt, Germany). Substrates and alkaline phosphatase (type VII) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pyruvate kinase, lactate dehydrogenase, cholesterol oxidase, cholesteryl esterase and peroxidase were obtained from Boehringer (Mannheim, Germany). [G-^{3}H]Ouabain, [γ-^{32}P]ATP and [8-^{3}H]adenosine 3′:5′-cyclic monophosphate were from The Radiochemical Centre (Amersham, Bucks., U.K.). Ionophore A23187 was purchased from Calbiochem (Behring Co., La Jolla, CA, U.S.A.). Mitochondrial F1-ATPase was a gift from Professor A. Bruni, Institute of Pharmacology, University of Padova.

Preparative procedures

New Zealand male adult rabbits weighing about 2.5 kg were used. The animals were fed a stock laboratory diet. The combined soleus and semitendinosus were used as representative slow muscles and the adductor as a fast muscle. Muscle microsomes were prepared by techniques described previously for procedure A (Margreth *et al.*, 1977; Salviati *et al.*, 1979) or by the following procedure for procedure B. A crude microsome fraction was isolated from 10% homogenates in 0.3 M-sucrose containing 2 mg of phenylmethanesulphonyl fluoride/litre, by centrifuging the mitochondria-free supernatant at 80000 ***g*** for 60 min. The microsomal pellet was resuspended in 0.3 M-sucrose/1 mM-Hepes, pH 7.5, at a protein concentration of about 10 mg/ml, and kept overnight in the cold room. The suspension was diluted with an equal volume of 0.3 M-sucrose/1.2 M-KCl/1 mM-Hepes, pH 7.5, and immediately centrifuged at 100000 ***g*** for 60 min. The final sediment, after washing once with 0.25 M-sucrose, was resuspended in the same solution. About 8–10 mg of microsomal membrane protein was loaded on top of 11.5 ml of a linear (0.3–2 M) sucrose density gradient and centrifuged at 140000 ***g*** for 90 min in a Beckman SV 40 rotor. About 1 ml fractions from the gradient, or the pooled light fractions (0.7–0.9 M-sucrose) and heavy fractions (1.2–1.5 M-sucrose) were used for enzymic and protein analysis.

EDTA treatment of muscle microsomes obtained by procedure B was carried out by the method of Duggan & Martonosi (1970). Proteolipid was extracted from muscle microsomes obtained by procedure A, essentially as reported by MacLennan (1974). Calsequestrin was isolated and purified from rabbit hind-leg and back muscles by the procedure of MacLennan (1974).

Assays

ATPase activities were all measured by a spectrophotometric enzyme-coupled assay (Warren *et al.*, 1974) at 37°C, in a final volume of 3 ml in the presence of 0.15 mM-NADH, 0.5 mM-phosphoenolpyruvate, 5 units of pyruvate kinase and 5 units of lactate dehydrogenase.

Basal ATPase activity was assayed in a medium of the following composition: 20 mM-histidine, pH 7.2; 100 mM-KCl; 5 mM-$MgSO_4$; 2 mM-ATP; 0.2 mM-EGTA. The reaction was started by adding

10–60 μg of microsomal protein. The absorbance change at 340 nm was continuously monitored in a Unicam spectrophotometer. Extra (Ca^{2+}-dependent) ATPase activity was measured from the incremental rate, after addition of 0.2 mM-$CaCl_2$, in the absence and in the presence of Ca^{2+} ionophore A23187 (1 μg/ml).

($Na^+ + K^+$)-dependent ATPase activity was assayed in a medium of the following composition: 20 mM-histidine, pH 7.4; 100 mM-NaCl; 10 mM-KCl; 5 mM-$MgSO_4$; 1 mM-EGTA; 5 mM-disodium ATP, in the presence and in the absence of 0.1 mM-ouabain. The reaction was started by adding 25–100 μg of microsomal protein and was recorded as described above.

Ca^{2+}-loading rate was measured by the murexide method (Scarpa, 1979) at 25°C, in a medium containing, in a final volume of 3 ml, 20 mM-histidine, pH 7.2, 100 mM-KCl, 10 mM-$MgSO_4$, 5 mM-oxalate, 0.1 mM-murexide, 0.3 mM-$CaCl_2$ and 0.5–1 mg of microsomal protein. The reaction was started by adding 1 mM-ATP and the absorbance was monitored at 540–570 nm in a double-beam Perkin–Elmer spectrophotometer.

Potassium *p*-nitrophenyl phosphatase activity was assayed in the presence and in the absence of 2 mM-ouabain, by the method of Skou (1974).

Ca^{2+}-dependent formation of ^{32}P-labelled phosphoenzyme was carried out as described by Meissner *et al.* (1973). Na^+-dependent formation of ^{32}P-labelled phosphoenzyme was carried out as described above, except that the medium contained 10 mM-histidine, pH 7.4, 5 mM-$MgSO_4$, 0.4 mM-EGTA, 100 mM-NaCl and 0.1 mM-[γ-^{32}P]-ATP.

[^3H]Ouabain binding was assayed by the method of Lau *et al.* (1979*b*), in a medium containing, in a final volume of 0.3 ml, 40 mM-Tris/HCl, pH 7.4, 120 mM-NaCl, 10 mM-$MgSO_4$, 1 mM-EGTA, 10 mM-Tris/ATP, 0.05% deoxycholate, 40–80 μg of microsomal protein and [^3H]ouabain in the concentration range from 5 nM to 5 μM. Non-specific [^3H]ouabain binding was measured in the absence of ATP and in the presence of 1 mM-ouabain. Incubation was at 37°C and was terminated after 30 min by adding unlabelled 1 mM-ouabain. After diluting with 1 ml of cold medium (without ouabain), [^3H]ouabain bound was determined by a Millipore-filtration technique (0.45 μm Millipore filters). Specific binding was calculated from the difference between total and non-specific binding.

[^3H]Cyclic AMP binding was measured by the method of Gilman & Murad (1974) on about 100 μg of microsomal protein by a Millipore-filtration technique (0.45 μm Millipore filters).

Rotenone-insensitive NADH:cytochrome *c* reductase, NADH:$K_3Fe(CN)_6$ reductase, succinate:cytochrome *c* reductase and stearoyl-CoA desaturase activities and cytochrome b_5 concentration were measured as described previously (Margreth *et al.*, 1971; Salviati *et al.*, 1979).

Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of muscle microsomes was carried out by disc-gel electrophoresis by the method of Weber & Osborn (1969) in 10% polyacrylamide gels, or by slab-gel electrophoresis by the method of Laemmli (1970), either with 10% polyacrylamide gels or with linear (5–15%) polyacrylamide-gradient gels. Staining of the gels with Coomassie Blue and destaining were done as described by Volpe *et al.* (1981). Densitometric scanning of disc gels was done in a Gilford spectrophotometer and that of slab gels under conditions identical with those described by Volpe *et al.* (1981).

Immunological assays

Chicken antibodies against the sarcoplasmic-reticulum Ca^{2+}-dependent ATPase of rabbit fast muscle were tested with muscle microsomes by a two-step competitive enzyme-linked immunosorbent assay, as described previously (Damiani *et al.*, 1981). Pre-incubation was carried out in 0.1 M-sodium phosphate buffer, pH 7.2, containing 0.15 M-NaCl, 0.05% Tween 20 and 0.02% NaN_3 at a final concentration of anti-(rabbit fast-muscle, Ca^{2+}-dependent ATPase) immunoglobulin G of 10 μg/ml and at concentrations of microsomal protein varying between 0.05 μg and 50 μg of protein. A purified Ca^{2+}-dependent ATPase preparation from fast muscle was used for coating microtitre wells, at a coating concentration of 5 μg/ml.

Lipids

Lipids were extracted from unfractionated muscle microsomes obtained by procedure A, or from light microsomal fractions (procedure B), with chloroform/methanol as solvent, by the method of Folch *et al.* (1957), as modified by Rouser & Fleischer (1967). Phospholipids were purified by silicic acid column chromatography and were separated by t.l.c. (Rouser & Fleischer, 1967). Each phospholipid species was identified by co-chromatography with the appropriate standard, and was determined by digesting the material scraped from the plate with $HClO_4$, at 180°C, and by measuring the P_i released (Rouser *et al.*, 1970).

Cholesterol was measured in chloroform/methanol extracts by the method of Searcy *et al.* (1960) or directly on muscle microsomes by the enzymic method of Gamble *et al.* (1978).

Electron microscopy

Unfractionated and light microsomes from fast and slow muscle were partly unfixed and partly fixed with 1% glutaraldehyde added to the suspending medium. Fixation was performed at 4°C for 30min. Both fixed and unfixed specimens were gradually infiltrated with 20% glycerol and left in 20% glycerol overnight. Samples were frozen in liquid Freon 22, cooled and stored in liquid N_2, and fractured in a Balzer BAF 301 freeze-etching apparatus at −100°C and 2.66×10^{-4} Pa (2×10^{-6} Torr). The fracture faces were rotatory shadowed with platinum/carbon from angles between 30 to 35°, at 60rev./min. Replicas were examined in a Siemens Elmiskop 1A electron microscope.

Stereological measurements

Random pictures were taken at 30000× magnification and enlarged to a final print magnification of 150000×. To calculate the density distribution of the intramembrane particles, a test circle corresponding to an area of $5.024 \times 10^{-3} \mu m^2$ was chosen. The test area was centred on microsomal concave (P) and convex (E) fracture faces, taking care to ensure that all the circle was on the microsomal membrane. The number of intramembrane particles in the test area was counted. No statistically significant differences in particle-density distribution were found between fixed and unfixed specimens.

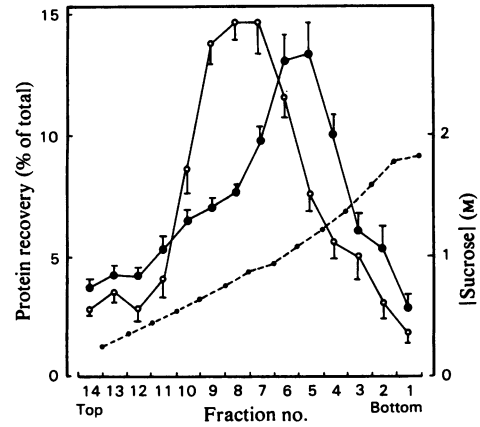


Fig. 1. *Distribution profile of microsomal membrane protein from fast and slow muscle after sucrose-density-gradient centrifugation*

Microsomes were isolated from fast and slow muscle of the rabbit by procedure B, and were fractionated by centrifuging at 140000*g* for 90min on a linear (0.3–2M) sucrose gradient (see the Materials and methods section). Sucrose concentrations (----) were determined by refractometry. Protein concentration in each fraction (about 1ml) from the gradient was determined by the method of Lowry *et al.* (1951). Values, expressed as the percentage of protein recovered in each fraction, are the means of 25 experiments for slow muscle (●) and of 18 experiments for fast muscle (○). Vertical bars represent ±S.E.M.

Results

Fig. 1 shows the protein profile of microsomes from fast muscle and slow muscle after centrifuging for 1.5h on a continuous sucrose density gradient. The profile of fast-muscle membranes is characterized by a broad band, with a peak at about 0.8M-sucrose. In the case of slow-muscle membranes, the main protein peak appears to be displaced at higher sucrose densities, though a prominent shoulder is observed in the region of the gradient corresponding to the major band of fast-muscle microsomes.

In spite of these differences, the pattern of distribution of Ca^{2+}-dependent ATPase activity among the several fractions appears to be similar for the microsomes of fast and slow muscle, with a peak in specific activity at about 0.8M-sucrose (Figs. 2*a* and 2*b*). Extra (Ca^{2+}-dependent) ATPase activity is found to be in a constant ratio to that of basal ATPase in all fractions from either kind of muscle, though this ratio is markedly lower for slow than for fast muscle.

The activity profile of rotenone-insensitive NADH:cytochrome *c* reductase activity (Fig. 2*c*), a characteristic marker of slow-muscle sarcoplasmic reticulum (Salviati *et al.*, 1979, 1981), corresponds closely to that of basal and Ca^{2+}-dependent ATPase activities, except for the presence of a small second peak in fractions near the bottom of the gradient, also shown to contain trace amounts of succinate:cytochrome *c* reductase activity from contaminating fragments of the mitochondrial inner membrane. On the other hand, most of the [^{3}H]-cyclic AMP-binding activity (Fig 2*d*), associated with slow-muscle microsomes (Margreth *et al.*, 1975), appears to be distributed among the heavy fractions from membranes.

Contrastingly, both ouabain-sensitive ($Na^+ + K^+$)-dependent ATPase and [^{3}H]ouabain-binding activity of slow-muscle microsomes show a dual distribution with peaks at about 0.75M- and 1.4M-sucrose densities respectively (Fig. 2).

Heterogeneity elements between 'heavy' and 'light' microsomes and according to muscle type

Separation methods by sucrose-density-gradient centrifugation have been mostly used for isolating sarcoplasmic-reticulum vesicles derived from the cisternal and longitudinal portions of the intact sarcoplasmic reticulum (Meissner, 1975; Lau *et al.*,

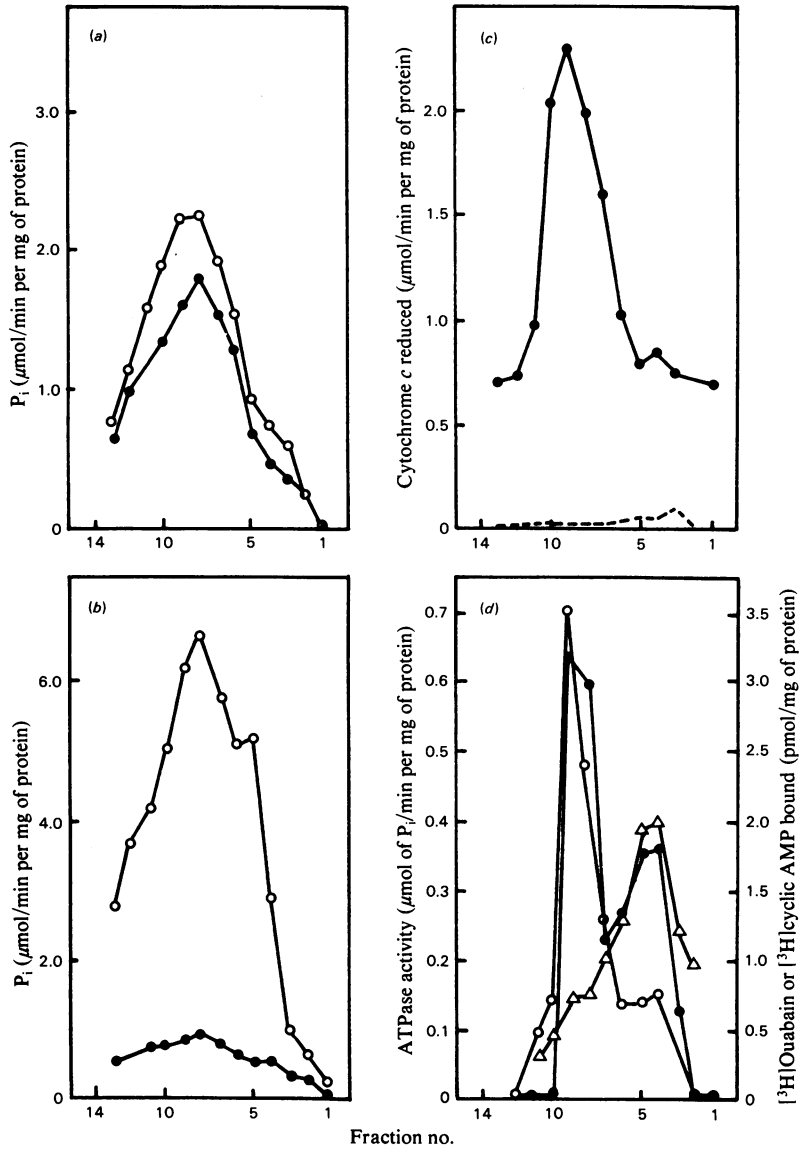


Fig. 2. *Distribution profile of microsome-associated enzymic activities and of [^{3}H]ouabain-binding and [^{3}H]cyclic AMP-binding activities after sucrose-density-gradient centrifugation*

All activities were measured, as described in the Materials and methods section, on microsomes isolated and fractionated by procedure B. Values from representative experiments are given. (*a*) Slow-muscle microsomes. ●, Basal ATPase activity; ○, Ca^{2+}-activated ATPase activity. (*b*) Fast-muscle microsomes. ●, Basal ATPase activity; ○, Ca^{2+}-activated ATPase activity. (*c*) Slow-muscle microsomes. ●, Rotenone-insensitive NADH:cytochrome *c* reductase activity; ----, succinate:cytochrome *c* reductase activity. (*d*) Slow-muscle microsomes. ●, (Na^+ + K^+)-dependent ATPase activity, ouabain-sensitive fraction; ○, [^{3}H]ouabain-binding activity; △, [^{3}H]cyclic AMP-binding activity.

1977; Michalak *et al.*, 1980), based on the different content of calsequestrin (see also Jorgensen *et al.*, 1979; Campbell *et al.*, 1980).

The results obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the pooled fractions from the gradient of fast-muscle microsomes, in the 1.2–1.5 M-sucrose (fraction nos. 4 and 5) and the 0.9–0.7 M-sucrose (fraction nos. 8

Table 1. *Fractional values of major protein constituents of muscle microsomes*

Muscle microsomes were prepared and fractionated by sucrose-density-gradient centrifugation, as reported in the Materials and methods section (procedure B). Light fractions are the pooled fractions (fraction nos. 8 and 9; see Fig. 1) from the gradient corresponding to sucrose densities between 0.7 and 0.9 M-sucrose. Heavy fractions: pooled fraction nos. 4 and 5, between 1.2 and 1.5 M-sucrose. The percentage values of total protein represented by the Ca^{2+}-dependent ATPase protein (mol.wt. 100000), M_{55} protein (mol.wt. 55000; Meissner & Fleischer, 1971) and calsequestrin [apparent mol.wt. 44000 in the Weber and Osborn (1969) system (W.O.); apparent mol.wt. 64000 in the Laemmli (1970) system (L); Michalak *et al.*, 1980) were determined by densitometry of sodium dodecyl sulphate/polyacrylamide electrophoretic gels, after staining with Coomassie Blue. Values, expressed as percentages of total stainable protein, are means ± S.E.M. for the numbers of determinations on different membrane preparations shown in parentheses.

		ATPase		M_{55}		Calsequestrin	
Microsomes		W.O.	L	W.O.	L	W.O.	L
Fast muscle	Unfractionated	71.0 ± 3.0 (9)	70.8 (2)	6.7 ± 0.6 (9)	7.6 (2)	7.7 ± 0.7 (9)	8.1 (2)
	Heavy fraction	60.0 ± 0.3 (3)	58.1 (2)	6.0 ± 0.1 (3)	6.5 (2)	20.0 ± 0.2 (3)	17.8 (2)
	Light fraction	82.5 ± 1.3 (3)	78.6 (2)	8.1 ± 0.2 (3)	9.4 (2)	2.9 ± 0.1 (3)	5.7 (2)
Slow muscle	Unfractionated	31.8 ± 0.4 (6)	24.9 ± 0.8 (4)	11.2 ± 0.7 (6)	12.1 ± 0.9 (4)	8.7 ± 0.4 (6)	7.9 ± 0.8 (4)
	Heavy fraction	23.5 (2)	20.5 (2)	13.0 (2)	15.3 (2)	21.0 (2)	20.5 (2)
	Light fraction	53.2 ± 3.3 (6)	41.0 (2)	11.4 ± 1.4 (6)	10.7 (2)	3.4 ± 0.4 (6)	8.7 (2)

and 9) density range (heretofore termed heavy and light microsomes respectively) are in agreement with these previous observations on the same type of preparations. Densitometric measurements show (Table 1) that the ATPase protein/M_{55} protein ratio remains fairly constant (about 10:1) through the fractions from the gradient, whereas the calsequestrin/M_{55} protein ratio decreases from a high value of about 4:1 in the heavy vesicles to about 0.4:1 in the light vesicles, due to the selective depletion of calsequestrin. By contrast, the ATPase protein/M_{55} protein ratio increases from heavy to light fractions of slow-muscle microsomes along with the decrease of calsequestrin.

The enrichment of ATPase protein in the light-microsomal fraction of slow muscle appears to be accompanied by a somewhat parallel increase of Ca^{2+}-dependent ATPase activity, steady-state level of Ca^{2+}-dependent phosphorylated protein and of Ca^{2+}-loading rate, as well as by an increased immunological reactivity with antibody to purified Ca^{2+}-dependent ATPase from fast muscle (Table 2). However, the absolute activity values remain very low compared with the values for the corresponding fractions from fast muscle, which has previously been explained by the existence of catalytic and antigenic differences between the sarcoplasmic-reticulum Ca^{2+}-dependent ATPases of fast and slow muscle (Damiani *et al.*, 1981). Conversely, basal ATPase activity of the light-microsomal fraction of slow muscle appears to be highest by any comparison (Table 2). In additional experiments it was found that this activity was not inhibited by either 1 μM-vanadate, 5 mM-azide or 1 μg of oligomycin/ml, and was only 15% inhibited by *N*-ethylmaleimide, tested by the method of Malouf & Meissner (1980).

In agreement with the observed pattern of distribution of rotenone-insensitive NADH:cytochrome *c* reductase activity in the sucrose density gradient (Fig. 2*c*), the results in Table 2 further show that the light-microsomal fraction of slow muscle is enriched in all components of the stearoyl desaturase system (i.e. the specific flavoprotein NADH:cytochrome b_5 reductase, measured from the rate of reduction of $K_3Fe(CN)_6$, cytochrome b_5 and the desaturase), compared with the original microsome fraction.

Freeze-fracture electron microscopy of muscle microsomes

Methods have been developed to identify the isolated microsomal vesicles from muscle homogenates in relation to the membrane of origin, on the basis of differences in density of freeze-fracture intramembrane particles (Scales *et al.*, 1977; Scales & Sabbadini, 1979; Scales, 1981). Sarcoplasmic-reticulum membranes are characterized by 9 nm particles, with characteristically asymmetrical distribution between the concave and convex faces (Packer *et al.*, 1974; Scales & Inesi, 1976). On the other hand, T-tubule membranes that are symmetrical and show a very low particle density on both the concave and convex fracture faces can be distinguished from both sarcoplasmic-reticulum membranes and plasma membranes (Scales & Sabbadini, 1979; Scales, 1981).

Freeze-fracturing of sarcoplasmic-reticulum microsomal preparations from fast and slow muscle (Plate 1) reveals rounded vesicles with particles associated mostly with the concave fracture face. Membrane sheets, most probably of sarcolemmal origin (Scales & Sabbadini, 1979), are occasionally

Table 2. *Comparison between light- and heavy-microsomal fractions of fast and slow muscle with respect to ATPase and Ca^{2+} transport activities, antigenic properties and activity of stearoyl-CoA desaturase system*

The isolation and fractionation of muscle microsomes were as in Table 1. Mg^{2+}-dependent (basal)ATPase activity was assayed spectrophotometrically as reported in the Materials and methods section. Extra ATPase is the increment of activity after adding 0.2 mM-$CaCl_2$. The steady-state level of Ca^{2+}- and Mg^{2+}-dependent phosphorylated protein and Ca^{2+} accumulation were determined as reported in the Materials and methods section. Immunological cross-reactivity was determined by competitive enzyme-linked immunosorbent assay; 50% inhibitory level is the concentration of microsomal protein required for 50% inhibition of binding of anti-(rabbit fast-muscle Ca^{2+}-dependent ATPase) antibody to antigen-coated wells. NADH–$K_3Fe(CN)_6$ reductase activity, stearoyl-CoA desaturase activity and cytochrome b_5 were measured spectrophotometrically as reported in the Materials and methods section. Values are given as means ± S.E.M. for the numbers of determinations reported in parentheses, when more than three determinations were carried out. NADH:$K_3Fe(CN)_6$ reductase and stearoyl-CoA desaturase activities and cytochrome b_5 content of fast-muscle microsomes were not determined, since they were already found to be very low (Salviati *et al.*, 1979; Margreth *et al.*, 1977).

	Fast-muscle microsomes			Slow-muscle microsomes		
	Unfractionated	Light fraction	Factor of purification	Unfractionated	Light fraction	Factor of purification
ATPase activity (μmol of P_i/min per mg of protein)						
Basal ATPase	0.71 ± 0.07 (9)	0.92 ± 0.12 (5)	1.3	1.20 ± 0.06 (8)	1.81 ± 0.03 (6)	1.5
Extra ATPase	4.97 ± 0.62 (9)	5.43 ± 0.74 (5)	1.1	0.45 ± 0.06 (8)	0.68 ± 0.03 (6)	1.5
Extra ATPase in the presence of ionophore A23187	9.06	17.73 (3)	2.0	1.05	1.82 (3)	1.7
Steady-state level (nmol of [^{32}P]P_i/mg of protein)	3.05 (3)	4.34 (3)	1.4	0.31 ± 0.04 (6)	0.54 (3)	1.7
Ca^{2+}-loading rate (μmol of Ca^{2+}/min per mg of protein)	1.18 ± 0.06 (4)	1.89 (2)	1.6	0.18 ± 0.04 (4)	0.45 (2)	2.5
Immunological cross-reactivity with antibody for fast-muscle Ca^{2+}-dependent ATPase (50% inhibitory level, μg of protein/ml)	1.6	1.4 (2)	1.1	30 (3)	8 (2)	3.8
NADH:$K_3Fe(CN)_6$ reductase (μmol of $K_3Fe(CN)_6$ reduced/min per mg of protein)	—	—	—	3.52 ± 0.58 (6)	5.71 ± 0.78 (6)	1.6
Cytochrome b_5 (nmol/mg of protein)				0.21 (2)	0.35 (2)	1.7
Stearoyl-CoA desaturase (nmol of oleate formed/min per mg of protein)	—	—	—	0.34 (2)	0.68 (2)	2.0

seen in the unfractionated microsomes (Plate 1*a*) but never in the light fraction. Determination of the particle-density distribution on the concave face of the light-microsomal vesicles showed two different classes of vesicles, one with a low number of particles (less than 1000/μm^2) and the other with a particle density greater than 2000/μm^2. The percentage of concave faces with very few particles accounted for 16% of total vesicles, in the case of the fast muscle, and for 43%, in the case of the slow muscle. On the other hand, about 90% of the convex faces examined had particle densities below 1000/μm^2, without any significant difference between the microsomes of fast and slow muscle.

Interestingly, the average particle density in the particle-rich vesicles was found to be virtually the same for fast and slow muscle, with values of 3383 ± 905/μm^2 and 3184 ± 995/μm^2 (means ± S.D.) respectively. These values are lower than the reported density of 5730/μm^2 for particles in the concave faces of the isolated sarcoplasmic reticulum from rabbit predominantly fast muscle (Scales & Inesi, 1976) but are in good agreement with the more recent data reported by Scales (1981).

Content of ($Na^+ + K^+$)-dependent ATPase in muscle microsomes

Conflicting results have appeared concerning the true content of the ($Na^+ + K^+$)-dependent ATPase enzyme in muscle microsomal preparations (Besch

et al., 1976; Lau *et al.*, 1977; Jones *et al.*, 1978; Scales & Sabbadini, 1979). A comparison with these previous results must take into account the influence of the sidedness of the microsomal vesicles, according to the particular parameter used (cf., e.g., Bers *et al.*, 1980).

Our data (Fig. 3) show that ATP-dependent [^{3}H]ouabain binding to light-microsomal fractions is greater for slow muscle than for fast muscle, due to differences in both affinity and total number of binding sites. However, these values, as well as the previously reported (Damiani *et al.*, 1981) values for K^+-dependent ouabain-sensitive *p*-nitrophenyl phosphatase activity, also measured in a light-microsomal fraction (11.7 and 1.9 nmol/min per mg of protein, on average, for slow and fast muscle respectively) appear to be very low, absolutely and relatively to the ($Na^+ + K^+$)-dependent ATPase activity values. Ouabain-sensitive ($Na^+ + K^+$)-dependent ATPase activity of slow-muscle microsomes, with an average value of 0.36 μmol of P_i/min per mg of protein, was found to be about 4-fold as high compared with the corresponding fraction from fast muscle, and in the upper range of values reported by Jones *et al.* (1980) for microsomal preparation from heart muscle after detergent treatment.

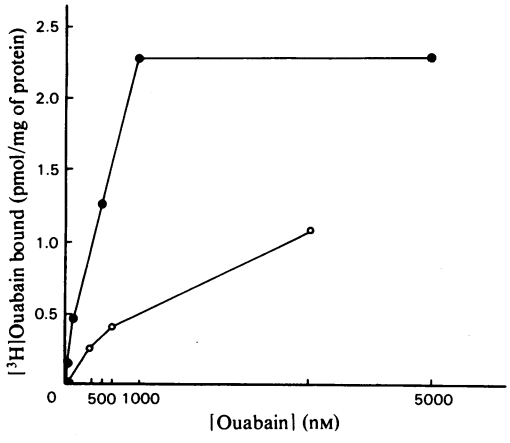


Fig. 3. *Saturation curve of muscle microsomes for [^{3}H]ouabain*

Light microsomes were isolated from fast and slow muscle, as described in the Materials and methods section and in the legends to Fig 1 and Table 1. [^{3}H]Ouabain was determined as reported in the Materials and methods section, in the concentration range between 5 nM and 5 μM. Results are representative values from paired determinations on muscle microsomes from fast and slow muscle of the same rabbit. ●, Slow-muscle microsomes; ○, fast-muscle microsomes.

A high ratio of ($Na^+ + K^+$)-dependent ATPase to K^+-dependent *p*-nitrophenyl) phosphatase and ATP-dependent ouabain-binding activities would imply that, in our microsomal preparations, the ($Na^+ + K^+$)-dependent ATPase is located in a population of inside-out vesicles (Bers *et al.*, 1980), as are predominantly those derived from T-tubules (Lau *et al.*, 1977; Brandt *et al.*, 1980). Under these conditions the average membrane content of ($Na^+ + K^+$)-dependent ATPase is best estimated from the steady-state values of Na^+-dependent membrane protein phosphorylation, since the Na^+-binding site of the ATPase will be exposed on the external surface of the vesicles. On the basis of an experimental value of 0.19 and 0.07 nmol of [^{32}P]P_i/mg of protein for light microsomes of slow and fast muscle and taking into account a reported value of about 6 nmol of [^{32}P]P_i/mg of protein for purified ($Na^+ + K^+$)-dependent ATPase (Jorgensen, 1974), the amount of ATPase protein bound to these fractions was calculated to be approx. 3% and 1% of the total membrane protein, in the stated order.

Lipid composition of muscle microsomes

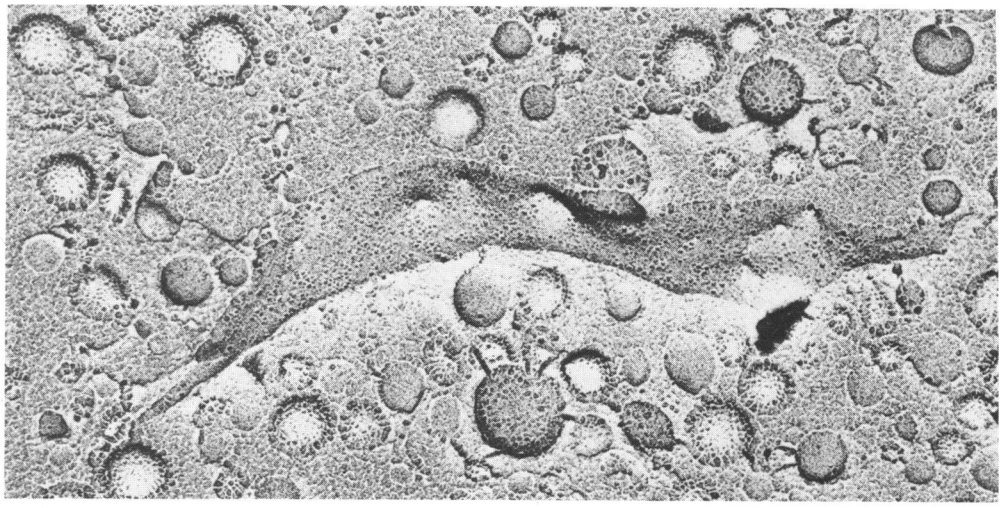
Skeletal-muscle plasma-membrane fragments (Fiehn *et al.*, 1971; Kidway *et al.*, 1973) and isolated T-tubules (Lau *et al.*, 1979*a*) were both shown to differ from sarcoplasmic-reticulum membranes by a higher cholesterol/phospholipid ratio, and relatively higher content of sphingomyelin and phosphatidylserine. Lipid-related parameters have

EXPLANATION OF PLATE 1

Freeze-fracture electron microscopy of microsomes from rabbit slow and fast muscle

(*a*) Unfractionated microsomes from rabbit slow muscle. The specimen was frozen in liquid Freon 22 without chemical fixation and without glycerol infiltration and rotatory-shadowed from 35°. A few large sheets, very likely derived from the sarcolemmal membrane, are present together with microsomal vesicles. Magnification 90000×. (*b*) Light microsomes from rabbit slow muscle. The sample was fixed in glutaraldehyde, infiltrated in 20% glycerol before freezing and rotatory-shadowed from 30°. The fraction appears to be heterogeneous with regard to both the size of the microsomal vesicles and number of intramembrane particles. Magnification 90000×. (*c*) Light microsomes from rabbit fast muscle. The specimen was treated as in (*b*). The microsomal population is more homogeneous than that shown in (*b*). Magnification 90000×.

(*a*)



(*b*)

(*c*)

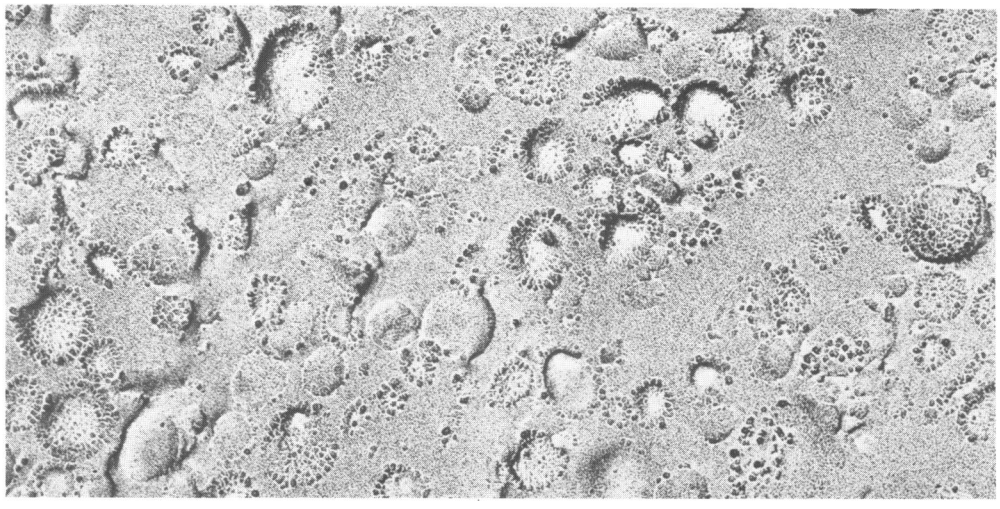


Table 3. *Lipid composition of microsomes from rabbit fast and slow muscle*

All analyses were carried out on microsomes isolated by procedure A (for experimental details see the Materials and methods section). Cholesterol (non-esterified cholesterol + cholesteryl esters) was determined on chloroform/methanol extracts by the method of Searcy *et al.* (1960), or by the method of Gamble *et al.* (1978) (marked with an asterisk). Total phospholipids were determined as reported in the Materials and methods section. Values are means ± S.E.M. for the numbers of determinations reported in parentheses. Percentage values of the several phospholipid species are the averages of the results obtained in two separate experiments, which varied by less than 10%.

	Fast muscle	Slow muscle
Total cholesterol (μmol/mg of protein)	0.035 ± 0.002 (3)	0.086 (2)
	0.039*	0.075*
Total phospholipids (μmol of P/mg of protein)	0.55 ± 0.02 (6)	0.96 ± 0.04 (6)
Phospholipid species (% of total phospholipid P)		
Phosphatidylcholine	74.2	69.8
Phosphatidylethanolamine	12.4	16.5
Phosphatidylinositol	8.3	12.2
Phosphatidylserine	1.9	1.1
Sphingomyelin	1.0	0.1
Cardiolipin	—	0.5
Not identified	2.4	—

therefore been used as a further criterion to characterize our microsomal preparations.

As determined in unfractionated microsomes (Table 3), the phospholipid/protein ratio appears to be about 2-fold higher for slow compared with fast muscle, but phospholipid composition appears to be similar for both muscles, and also similar to that reported for purified sarcoplasmic-reticulum fragments from rabbit fast muscle (Meissner & Fleischer, 1971). Thus phosphatidylcholine constitutes the major phospholipid species, and phosphatidylethanolamine and phosphatidylinositol the other two major lipids, whereas sphingomyelin and phosphatidylserine are present in very low amounts. The content of cholesterol in slow-muscle microsomes is higher than in the corresponding fraction from fast muscle, but the cholesterol/phospholipid molar ratio appears to be essentially the same.

By comparison with unfractionated microsomes, light microsomes were found to be enriched in phospholipids (cf. Lau *et al.*, 1979*a*), with values of 0.83 μmol of phospholipid phosphorus/mg of protein for fast muscle and 1.21 μmol of phospholipid phosphorus/mg of protein for slow muscle, on average. The respective values for total cholesterol concentration, measured by the method of Gamble *et al.* (1978), were 0.03 ± 0.01 ($n = 5$) and 0.12 ± 0.02 ($n = 3$) μmol/mg of protein (means ± S.D.), i.e. about 6% and 20% of the reported values for isolated T-tubules (Lau *et al.*, 1979*a*).

Patterns of protein composition of muscle microsomes according to the type of muscle

Slab-gel electrophoresis is shown to resolve the protein material in the range mol.wt.-65000–55000 into two distinct bands, for fast-muscle microsomes, and into five bands, for slow-muscle microsomes, both unfractionated (Fig. 4). By co-electrophoresis with molecular-weight markers, the common peptides were identified as calsequestrin and high-affinity

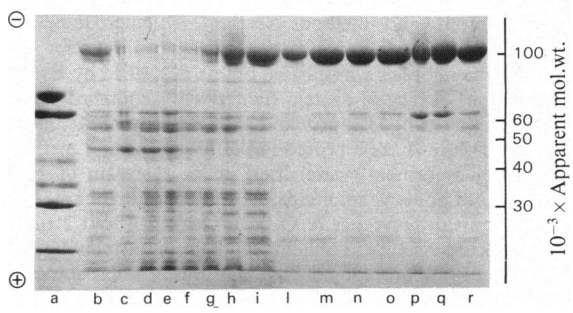


Fig. 4. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of microsomes from fast and slow muscle*

Muscle microsomes were isolated and fractionated as described in the Materials and methods section and in the legend to Fig. 1 and Table 1. (a) Molecular-weight markers in the following order: bovine serum albumin (mol.wt. 68000); pyruvate kinase (mol.wt. 57000); aldolase (mol.wt. 40000); lactate dehydrogenase (mol.wt. 36000); carbonic anhydrase (mol.wt. 29000); cytochrome b_5 (mol.wt. 17000); (b) unfractionated microsomes from slow muscle; (c–i) individual gradient fractions (4–9) of slow-muscle microsomes (cf. Fig. 1), in the stated order: (l) fraction no. 10 from gradient of microsomes from fast muscle; (m) fractions nos. 8 and 9; (n–q) individual gradient fractions (7–4) of fast-muscle microsomes, in the stated order; (r) unfractionated microsomes from fast muscles. Electrophoresis was carried out as described by Laemmli (1970), using a linear polyacrylamide-gel gradient (see the Materials and methods section). About 15 μg (b, h and i), 10 μg (c–g) or 8 μg of protein (l–r) was applied per gel.

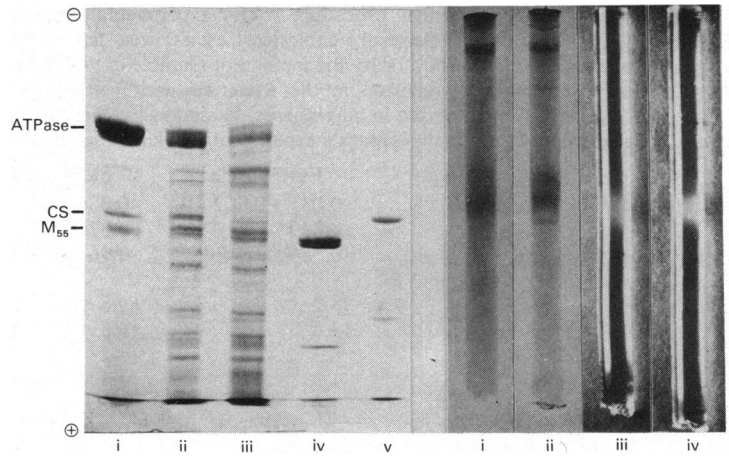


Fig. 5. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of muscle microsomes, calsequestrin, F1-ATPase and of proteolipid*

(*a*) Muscle microsomes (prepared by procedure B), calsequestrin and F1-ATPase were obtained as described in the Materials and methods section. (i) Microsomes from fast muscle; (ii) microsomes from slow muscle; (iii) EDTA-treated microsomes from slow muscle; (iv) purified F1-ATPase; (v) partially purified calsequestrin. Electrophoresis was carried out as described by Laemmli (1970) in 10% polyacrylamide gel. About 20 μg (i), 40 μg (ii and iii) or 5 μg of protein (iv and v) was applied per gel. Abbreviations used: ATPase, Ca^{2+}-dependent ATPase; CS, calsequestrin; M_{55}, protein band of mol.wt. 55000. (*b*) Proteolipid was extracted from muscle microsomes isolated by procedure A, as reported in the Materials and methods section. Portions of the chloroform/methanol extract (about 10 μg of protein) were dried under a stream of N_2 and dissolved in 0.1 M-sodium phosphate buffer (pH 7.0)/1% sodium dodecyl sulphate/ 1% β-mercaptoethanol. Electrophoresis was carried out on 12.5% polyacrylamide gels, as described by Weber & Osborn (1969). About 20 μg of protein was applied per gel. The fluorescent proteolipid was detected visually in oblique u.v. light in unstained gels (iii and iv), and was then stained with Coomassie Blue (i and ii). (i) and (iii), proteolipid from fast-muscle microsomes; (ii) and (iv), proteolipid from slow-muscle microsomes.

Ca^{2+}-binding protein (with apparent mol.wts. about 64000 and 54000, under the specified conditions). The peptides specific to slow-muscle microsomes have mol.wts. of approx. 60000, 56000 and 52000. Additional peptides that are virtually absent in the unfractionated microsomes from fast muscle correspond to mol.wts. of approx. 45000, 36000, 32000 (NADH:cytochrome b_5 reductase; cf. Salviati *et al.*, 1981), 29000 and 28000. Low-molecular-weight peptides, such as cytochrome b_5 (mol.wt. about 17000), also specific to slow-muscle microsomes (cf. Salviati *et al.*, 1981), can likewise be seen. By comparison, and in agreement with the densitometric data in Table 1, light microsomes appear to be depleted of calsequestrin (h, i and m of Fig. 4), and those from slow muscle also of the peptides of mol.wt. 56000 and 45000. Conversely, the latter fractions are enriched in peptides of mol.wt. 76000, 60000, 54000 and 28000, as well as in cytochrome b_5, as expected from the data in Table 2.

Fernandez *et al.* (1980) found peptides of mol.wt. about 50000 in sodium dodecyl sulphate/polyacrylamide gels of a standard microsomal preparation from rabbit fast muscle, and suggested that these peptides might be identical with the subunits of F1-ATPase of mol.wt. 54000 and 50000. Our results show (Fig. 5*a*) that a peptide doublet, in a similar molecular-weight range, is present in sodium dodecyl sulphate/polyacrylamide gels of slow-muscle microsomes, whether untreated or after EDTA treatment, which leads to a selective loss of calsequestrin. This peptide doublet, however, differs from that found with purified F1-ATPase, whose larger subunit only (mol.wt. 54000) appears to have an electrophoretic mobility similar to that of the high-affinity Ca^{2+}-binding protein. This can be explained most easily by chance identity of the respective molecular weights.

Proteolipid

The results in Fig. 5(*b*) show that extracts from slow-muscle microsomes (see the Materials and methods section) contain a major proteolipid that can be detected in sodium dodecyl sulphate/polyacrylamide gels by its fluorescence in u.v. light and that has an apparent mol.wt. of 12000, i.e. identical with that of the purified proteolipid from the microsomes of fast muscle.

Discussion

Recent freeze-fracture stereological studies of skeletal-muscle microsomal fractions have lent support to the view that these fractions, even when isolated under conditions yielding a purified sarcoplasmic-reticulum membrane fraction by biochemical criteria, are in fact mixtures of membranes derived both from these membranes and T-tubules (Scales *et al.*, 1977; Scales & Sabbadini, 1979; Scales, 1981). On the basis of the present observations this appears to apply to a greater extent to the microsomes of slow-twitch muscle, compared with fast-twitch muscle.

We assume that the separation of microsomes into two distinct classes of high and of low particle density on the concave face is a valid criterion for distinguishing between sarcoplasmic-reticulum and non-sarcoplasmic-reticulum vesicles (Scales, 1981). We further assume that each intramembrane 9nm particle represents an oligomer of Ca^{2+}-dependent ATPase polypeptides (Scales & Inesi, 1976). If so, one should be able to confirm a direct correlation between the fractional value of vesicles with high particle density and the percentage of total membrane protein accounted for by the Ca^{2+}-dependent ATPase monomer; and this is exactly what one observes by comparing these fractional values obtained for light-microsomal fractions (84% and 57% of the total number of vesicles for fast and slow muscle respectively) with the corresponding percentage values of ATPase protein monomer of mol.wt. 100000 (83% and 53% for fast and slow muscle respectively).

The following experimental evidence further supports the interpretation that the non-sarcoplasmic-reticulum vesicles present in our microsomal preparations are derived from the T-tubules and not from the plasma membrane. (i) Under the conditions used for homogenization of the tissue and isolation of the microsomes, plasma-membrane fragments, when present, appeared as membrane sheets rather than in a vesicular membrane-bound form. (ii) It is inferred from study of [^{3}H]ouabain-binding activity, K^+-dependent ouabain-sensitive *p*-nitrophenyl phosphatase and ($Na^+ + K^+$)-dependent ouabain-sensitive ATPase activities (see the Results section) that the non-sarcoplasmic-reticulum vesicles with membrane-bound ($Na^+ + K^+$)-dependent ATPase activity are mostly inside-out vesicles. It was reported that this orientation is predominant in vesicles derived from fragmentation of the T-tubules (Lau *et al.*, 1979*b*), at variance with sarcolemmal vesicles, which tend to be predominantly right-side out (Jones *et al.*, 1980; Bers *et al.*, 1980). (iii) We found the content of total phospholipids in slow-muscle microsomes to be higher than the content of the plasma membrane, with a reported value of 0.6 μmol of phospholipid phosphorus/mg of protein (Lau *et al.*, 1979*a*; Brandt *et al.*, 1980), and, with respect to the light fractions, to be somewhat intermediate between that of the corresponding fractions from fast muscle and of purified T-tubules, which have the highest phospholipid/protein ratio (Lau *et al.*, 1979*a*).

An interesting question to ask is why the yield of T-vesicles in the isolated microsomal fraction has to vary between fast and slow muscle? In this regard, and since the distribution profile of T-vesicles in sucrose density gradients appeared to be similar to that of vesicles derived from the longitudinal sarcoplasmic reticulum, their source of origin might have to be traced to free T-tubules rather than to T-tubules junctionally associated with sarcoplasmic-reticulum cisternae. The latter can be dissociated from sarcoplasmic-reticulum triads only by treatment in a French press (Lau *et al.*, 1977, 1979*a*; Brandt *et al.*, 1980).

Morphometric data on fast-twitch and slow-twitch fibres of the guinea-pig (Eisemberg & Kuda, 1976) show that the surface-area development of the sarcoplasmic-reticulum longitudinal tubules, at the A-band level in particular, is much greater for fast-twitch than for slow-twitch fibres, both absolutely and relative to the junctional sarcoplasmic reticulum. According to our own qualitative observations (Margreth *et al.*, 1974*b*), there are even more marked differences in the case of rabbit slow fibres, which are also characterized by very small triad structures. The higher yield of T-vesicles in the microsomes from rabbit slow muscle could therefore result from both the poor development of the longitudinal sarcoplasmic reticulum and a greater surface area of free versus junctional sarcoplasmic reticulum.

The relative contribution of T-tubular protein to the average protein composition of our light-microsomal fractions must take into account the higher phospholipid/protein ratio in T-vesicles, compared with sarcoplasmic-reticulum vesicles (Brandt *et al.*, 1980; Scales, 1981). From these values, as well as cholesterol values, T-tubular protein may be calculated to represent about 5% and 20% of total microsomal protein of fast and of slow muscle respectively. On the other hand, characterization of T-tubular protein in the membrane mixture is exceedingly difficult in the absence of any specific markers of the surface membrane, with the exception of ($Na^+ + K^+$)-dependent ATPase which, as shown here, is only a very minor constituent of muscle membranes.

Within this general context, the presence of basal Mg^{2+}-dependent ATPase activity in muscle microsomes is of considerable interest, since the present data, as well as previous work (Heilmann *et al.*, 1977; Wang *et al.*, 1979; Damiani *et al.*, 1981),

show that this activity is particularly high in the microsomes from rabbit slow muscle. Conflicting interpretations have been offered, however, to explain microsomal basal ATPase. According to one view (Inesi *et al.*, 1976), basal and Ca^{2+}-activated ATPase activities, as investigated in microsomes from rabbit fast muscle, are two different forms of the same enzyme. According to another view (Fernandez *et al.*, 1980), these activities are accounted for by three different enzymes, i.e. the sarcoplasmic-reticulum Ca^{2+}-dependent ATPase, mitochondrial ATPase and an ATPase enzyme of surface-membrane origin. On the other hand, we have found basal Mg^{2+}-dependent ATPase activity to be highest in light-microsomal fraction from rabbit slow muscle; this fraction has also been shown to be devoid of succinate:cytochrome *c* reductase activity and of the subunit polypeptides of F1-ATPase from mitochondrial contaminants. Furthermore, we found that basal ATPase activity was totally insensitive to either vanadate, oligomycin or azide and was largely insensitive to *N*-ethylmaleimide. According to some of these properties, the basal ATPase activity of our preparations appears to be similar to that investigated by Malouf & Meissner in a microsomal membrane fraction from chicken breast muscle (Malouf & Meissner, 1979) and from dog heart muscle (Malouf & Meissner, 1980), where it was shown to be localized in the plasma membrane and T-tubules, and not in sarcoplasmic-reticulum membranes. Therefore, these several findings seem to favour the interpretation that the basal ATPase activity of slow-muscle microsomes is associated with T-tubule fragments.

Little is known of the molecular-weight composition of basal ATPase that might help in identifying this enzyme in SDS/polyacrylamide-gel electrophoresis of slow-muscle microsomes. Interestingly, Fernandez *et al.* (1980) described a Triton X 100-insoluble membrane preparation from rabbit muscle microsomes having high basal ATPase activity and an electrophoretic pattern characterized by peptides of mol.wt. 55000, 50000 and 30000. We also have found peptides in this molecular-weight range in slow-muscle microsomes. In particular (see the Results section), our light fraction appeared to be enriched in a peptide of apparent mol.wt. 60000, as well as in peptides of mol.wt. 76000 and 28000. Brandt *et al.* (1980), on the other hand, reported the presence of peptides of mol.wt. 112000, 87000, 77000 and 68000 in purified T-tubules, and maintained that the component of mol.wt. 68000 was the most characteristic. A more important difference between our observations and those of both Brandt *et al.* (1980) and Fernandez *et al.* (1980), however, is that, whereas we found that the Ca^{2+}-dependent ATPase protein of mol.wt. 100000 was the main membrane constituent, this protein was virtually absent from their preparations.

Thus, because of the evidence supporting a polymorphism of the sarcoplasmic-reticulum Ca^{2+}-dependent ATPase related to fibre types (Damiani *et al.*, 1981) and of other evidence that basal ATPase activity is due to an alternative pathway for ATP hydrolysis of the Ca^{2+}-transport enzyme (Froelich & Taylor, 1976), it would be unwarranted, at the present time, to conclude that basal ATPase activity of slow-muscle microsomes is entirely due to an enzyme different from the Ca^{2+}-dependent ATPase enzyme.

Another category of proteins originally believed to be true constituents of the sarcoplasmic-reticulum membranes, such as the high-affinity Ca^{2+}-binding protein (see, e.g., Meissner & Fleischer, 1971), is now considered to be common to both sarcoplasmic-reticulum membrane and T-tubular membranes (Michalak *et al.*, 1980). Consequently, our evidence that this protein is present in similar amounts in the microsomes of fast and slow muscle does not provide information on the relative proportion of these membranes in the mixed microsomes from slow muscle.

Intrinsic enzyme proteins apparently specific to slow-muscle sarcoplasmic-reticulum membranes are the flavoprotein cytochrome b_5 reductase, cytochrome b_5 and stearoyl-CoA desaturase (Salviati *et al.*, 1979, 1981), which, on the basis of the present results, should be localized to membrane areas derived from the longitudinal sarcoplasmic reticulum. On the basis of previous observations, i.e. the scanty development of the sarcoplasmic-reticulum longitudinal tubules at the A-band level in slow-muscle fibres (Margreth *et al.*, 1974*b*; Eisemberg & Kuda, 1976), it is an interesting possibility that the above aspects of membrane specificity may relate to the existence of differences in macromolecular composition between sarcoplasmic-reticulum membranes at the A-band and I-band level. Further work along these lines with other membrane-separation methods will, however, be required in order to conclude with any certainty that these enzyme activities are truly characteristic of slow-twitch muscle longitudinal sarcoplasmic reticulum.

Freeze-fracture electron microscopy was carried out at the Institute of General Pathology, University of Modena. P. V. is a Postdoctoral Fellow of the Dino Ferrari Foundation. We thank Mr. R. Siligardi and Mr. G. A. Tobaldin for technical assistance. This work was supported by institutional funds from the Consiglio Nazionale delle Ricerche to the NRC Center for Muscle Biology and Physiopathology and, in part, by a grant from the Dino Ferrari Foundation to one of us (A. M.).

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