Characteristics of Sarcoplasmic Reticulum from Slowly Glycolysing and from Rapidly Glycolysing Pig Skeletal Muscle *post mortem*

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The composition and function of fragmented sarcoplasmic reticulum from pig skeletal muscle was examined in the period immediately *post mortem*. Muscle was defined as being either slowly glycolysing or rapidly glycolysing on the basis of colour, pH and concentrations of glycogen and lactate. The microsomal fraction was separated on a discontinuous gradient of 35, 40 and 45% (w/v) sucrose into heavy and intermediate fractions which sedimented to the interfaces, and a light fraction which remained on the surface of the 35%-sucrose layer. The sarcoplasmic reticulum from rapidly glycolysing muscle had a lower buoyant density than had that from slowly glycolysing muscle. This was reflected in the consistent lack of material in the heavy fraction and a greater proportion in the light fraction. The latter material had significantly lower ratios (w/w) of protein to phospholipid (2.3:1 versus 3.8:1) and of protein to cholesterol (10.4:1 versus 15.6:1). There were no gross differences in phospholipid content or in fatty acid composition of individual phospholipid classes in the membranes from the two types of muscle. Analysis of membrane proteins by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that ATPase (adenosine triphosphatase) was a major component of each fraction and that its contribution to the total protein content of the membrane was greater in rapidly glycolysing muscle, suggesting a loss of non-ATPase proteins. The two fractions of sarcoplasmic reticulum prepared from rapidly glycolysing muscle had approximately one-third the normal activities of Ca^{2+} binding and Ca^{2+} uptake in the presence of ATP and one-half the passive Ca^{2+} -binding capacity in the absence of ATP of the fractions from slowly glycolysing muscle. However, the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -stimulated ATPase activities were similar. Efflux from actively loaded vesicles, after the addition of EDTA, consisted of a rapid and a slow phase. Vesicles from rapidly glycolysing muscle lost 60% of associated Ca^{2+} (approx. $0.10 \mu\text{mol}$ of Ca^{2+} /mg of protein) during the rapid phase, compared with 30% (approx. $0.17 \mu\text{mol}$ of Ca^{2+} /mg of protein) in those from slowly glycolysing muscle. The efflux rate during the slower phase was comparable in both types of vesicles. Analysis of the temperature-dependence of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -stimulated ATPase activity revealed that a high-activation-energy process operating in the temperature range $31\text{--}45^\circ\text{C}$ in the intermediate and light fractions from slowly glycolysing muscle was not apparent in vesicles from rapidly glycolysing muscle. Conditions that result in the prolonged activation of glycogenolysis in pig muscle *post mortem* primarily affect the protein components of the sarcoplasmic-reticular membrane, giving rise to a loss of loosely associated proteins. The function of the membranes observed under these conditions does not appear to be due to enhanced permeability of the membrane to Ca^{2+} and may be the result of a defect in the transport of Ca^{2+} into the vesicles.

Glycogen degradation in skeletal muscle catalysed by glycogen phosphorylase (EC 2.4.1.1) is under complex control by hormones, metabolites and Ca^{2+} . During muscle contraction, enhanced glycolysis is accompanied by increased ATPase* activity. Both phosphorylase kinase (EC 2.7.1.38), a positive modifier of phosphorylase, and myofibrillar ATPase

(EC 3.6.1.3) are activated by Ca^{2+} in the concentration range $1\text{--}10 \mu\text{M}$ (Brostrom *et al.*, 1971; Caldwell, 1971). It has been suggested that Ca^{2+} may be the effector that synchronizes glycogen degradation and ATPase activity (Brostrom *et al.*, 1971).

The rate and extent of glycolysis under simulated conditions *post mortem* have been studied with a reconstituted muscle glycolytic system (Scopes, 1974).

* Abbreviation: ATPase, adenosine triphosphatase.

The rate of glycolysis depends on ATPase activity and the extent, represented by the ultimate pH, can be correlated with the amount of phosphorylase in the *a* form. Rates of glycolysis vary in the period *post mortem*. Rapid rates concomitant with high ATPase activity and accelerated pH decline, found particularly in pig muscles, are due to Ca^{2+} activation of myofibrillar ATPase, associated with accumulation of hexose monophosphates, indicating activation of phosphorylase kinase (Kastenschmidt, 1970).

The ability of the sarcoplasmic reticulum to regulate Ca^{2+} concentrations in the sarcoplasm is dependent on ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-stimulated ATPase (EC 3.6.1.3) activity, the permeability of the membrane and possibly the Ca^{2+} -binding proteins. We have studied the functional activity and lipid and protein composition of the sarcoplasmic reticulum isolated *post mortem* from skeletal muscle of pigs with widely differing rates of glycolysis. A preliminary report of this work has already appeared (Berman *et al.*, 1974).

Materials and Methods

Selection and sampling of muscle

Adult Landrace $\times$ Large White pigs were kept at the abattoirs for 12–24 h before slaughter, with free access to water. Animals were stunned by pressing electrodes carrying 80 V of alternating current (50 Hz) to the head for approx. 5 s. They were exsanguinated 15–30 s later by severing the right jugular vein. The carcasses were then subjected to the routine procedure of the abattoir, namely a momentary immersion in a water bath at 50°C and then firing in a butane-gas burner for 5–10 s to remove body hair. After this treatment, 10–15 min after stunning, samples (approx. 250 g) of longissimus dorsi muscle were removed. Muscle *in situ* was judged to be either slowly glycolysing or rapidly glycolysing on the basis of colour, consistency, amount of exudation and pH. The latter was measured by inserting a pH electrode (Radiometer, Copenhagen, Denmark) 2–3 cm into the muscle at four sites approx. 4 cm apart. Samples of slowly glycolysing muscle were selected from muscle that was red in colour, firm, had no obvious watery exudate and had a pH at 10–15 min *post mortem* of 6.50–6.80. Samples of rapidly glycolysing muscle were obviously pale and soft, exuded a watery fluid and had a pH of 5.40–5.70 at the time of excision. The muscle was cut into pieces (approx. 1 cm³) and placed on ice until it was minced and homogenized 45 min later. Selection was confirmed by determining the glycogen and lactate concentration of the muscle. Measurements of lactate (Hohorst, 1965) and of glycogen (Tarnocky & Nagy, 1963) were made on 0.5–1.5 g of muscle, which was cut from the bulk muscle sample immediately after excision from the carcass and

placed in ice-cold 50% (v/v) HClO_4 (5 ml) or 10% (w/v) trichloroacetic acid (5 ml) respectively. Determinations were performed within 2 h of excision.

Preparation of sarcoplasmic-reticulum vesicles

Muscle samples (usually 200 g) were minced and then homogenized for 45 s in a Waring Blendor with 4 vol. of 100 mM-KCl/5 mM-histidine buffer, pH 7.2. A crude microsomal fraction was isolated by differential centrifugation: 1000g for 20 min, followed by 8000g for 20 min and 30000g for 1 h (Greaser *et al.*, 1969c). After the first centrifugation, solid KCl was dissolved in the supernatant to give a final concentration of 0.6 M (Owens *et al.*, 1973). The final microsomal pellet was suspended in 6.0 ml of homogenization buffer. This was divided into three portions and these were layered on a discontinuous gradient of 35, 40 and 45% (w/v) sucrose in buffer (8 ml each) and centrifuged in a Beckman SW-25.1 rotor at 25000 rev./min for 2 h. The suspensions recovered from the two interfaces and from the top of the gradient were diluted with 100 mM-KCl/5 mM-histidine buffer, pH 7.2, and the suspended material was pelleted by centrifuging at 78000g for 1 h. Each pellet was suspended in the same buffer to a concentration of approx. 5 mg of protein/ml. In some instances the microsomal fraction was centrifuged through a continuous gradient of 15–45% (w/v) sucrose prepared by using the apparatus described by Stead *et al.* (1964). Centrifugation was then carried out in a Beckman SW-27.1 rotor at 25000 rev./min for 16 h. Sucrose density was measured with a refractometer (Bellingham and Stanley, London, U.K.) on fractions (0.5 ml) which were collected through a puncture in the bottom of the centrifuge tube. Homogeneity of the preparations was checked by electron microscopy (Greaser *et al.*, 1969c).

Lipid extraction and analysis

Suspensions of fragmented sarcoplasmic reticulum (2 mg of protein/ml) were homogenized with 17 vol. of chloroform/methanol (2:1, v/v). An additional 3 vol. of chloroform/methanol (2:1, v/v) was used to rinse the homogenizer blades and this was added to the bulk mixture. After filtration of the membrane extracts on a Millipore filter system by using Whatman no. 542 filter paper previously washed with chloroform/methanol (2:1, v/v), the filtrate was freed of non-lipid impurities by washing with 0.2 vol. of 0.9% (w/v) NaCl as described by Folch *et al.* (1957). The lower phase was separated and evaporated to dryness in a stream of nitrogen. The residue obtained was dissolved in chloroform which contained 0.005% (w/v) butylated hydroxytoluene as antioxidant.

Cholesterol and phosphorus in the lipid extracts

were determined colorimetrically by the methods of Courchaine *et al.* (1959) and of Bartlett (1959) respectively as described by Kates (1972). For the conversion of values for lipid phosphorus into phospholipid, the average molecular weight of phospholipids was taken as 750.

Polar lipids were separated on silica gel H (Merck) by t.l.c. with an eluent of chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) (Skipski & Barclay, 1969). The individual spots were detected under u.v. light after spraying lightly with aq. 0.05% (w/v) Rhodamine 6G. Each spot was excised and the phospholipids were extracted with successive solvents, namely 3 ml and then 2 ml of chloroform/methanol/acetic acid/water (25:15:4:2, by vol.), 2 ml of methanol and finally 2 ml of methanol/acetic acid/water (95:1:5, by vol.) (Skipski & Barclay, 1969), and the phosphorus content was then determined. Recoveries ranged from 75 to 95% of that applied to the plate. Individual classes of phospholipid were identified by chromatographing them alongside standards of phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine and sphingomyelin (Applied Science Laboratories, State College, PA, U.S.A.). Phosphatidylserine was detected by a positive reaction with 0.2% ninhydrin in butanol saturated with water. Phosphatidylinositol was presumed to migrate together with phosphatidylserine (Skipski & Barclay, 1969).

The fatty acids in isolated phospholipids were liberated and esterified with 14% (w/v) BF_3 in methanol (Merck) without eluting the phospholipids from the silical gel. The reaction was conducted at 100°C for 15 min in tubes flushed with nitrogen and sealed with Teflon-lined screw tops (Morrison & Smith, 1964). Water (2 ml) was then added after cooling and the methyl esters were extracted twice into 2 ml of *n*-heptane. Analyses of fatty acid methyl esters were carried out on a Pye gas chromatograph (104 series, Pye-Unicam, Cambridge, U.K.) equipped with dual flame-ionization detectors. Separation was achieved with 10% diethylene glycol adipate polyester on Chromosorb W, 80–100 mesh (Analab, North Haven, CT, U.S.A.) in a glass column [2.13 m $\times$ 0.46 cm (7 ft $\times$ 0.18 in)], operated isothermally at 188°C. An entire run lasted approx. 90 min. Identification was based on a comparison of the retention times of the samples peaks with those of a standard mixture examined under similar conditions. The standard mixture contained methyl esters of $\text{C}_{14:0}$, $\text{C}_{16:0}$, $\text{C}_{16:1}$, $\text{C}_{18:0}$, $\text{C}_{18:1\omega9}$, $\text{C}_{18:2\omega6}$, $\text{C}_{18:3\omega3}$, $\text{C}_{20:0}$, $\text{C}_{20:3\omega3}$, $\text{C}_{20:4\omega6}$, $\text{C}_{20:5\omega3}$, $\text{C}_{22:0}$, $\text{C}_{22:1\omega3}$ and $\text{C}_{24:1}$ fatty acids (Applied Science Laboratories). Peaks which did not correspond to any of the standards were tentatively identified from a graphic plotting of logarithms of retention times as a function of number of carbon atoms. Peak areas were evaluated by the triangulation method.

Protein analysis

Protein in fractions was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a reference standard.

Sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis was carried out as described by Weber & Osborn (1969). Albumin (mol.wt. 68000), pyruvate kinase (mol.wt. 57000), lactate dehydrogenase (mol.wt. 36000) and cytochrome *c* (mol.wt. 13000) were used as molecular-weight standards. Gels were scanned with a Varian Techtron spectrophotometer, model 635, at 600 nm, and peak areas were measured by the triangulation method.

Enzyme assays

($\text{Ca}^{2+} + \text{Mg}^{2+}$)-stimulated ATPase activity was measured either by a colorimetric (MacLennan, 1970) or by a pH-stat (Martonosi & Ferretos, 1964a) method. For the colorimetric procedure, the assay medium comprised 50 mM-Tris/HCl, pH 7.4, 100 mM-KCl, 5 mM-MgCl₂, 0.1 mM-EGTA and 0.1 mM-CaCl₂ (total volume 1 ml). The assay was conducted at 37°C with 50–200 μg of membrane protein. The reaction was initiated with ATP (final concn. 10 mM) and terminated after 4 min with a silicotungstic acid solution (4 ml) (Lindberg & Ernster, 1956). The concentration of P_i was determined by the Martin & Doty method (Lindberg & Ernster, 1956). The pH-titrimetric procedure was performed with an assay medium of 100 mM-KCl, 5 mM-MgCl₂, 0.1 mM-EGTA, 0.1 mM-CaCl₂ and 10 mM-ATP (total volume 3 ml). Addition of membrane suspension (25–50 μg of protein) initiated the reaction. The temperature of the reaction mixture was controlled by circulating water in a jacket surrounding the reaction vessel. The temperature was continuously monitored by a thermistor probe immersed in the reaction mixture. The rate of phosphate release was calculated on the basis that 1 mol of ATP on hydrolysis yields 0.7 mol of H^+ at pH 7.2 (Martonosi & Ferretos, 1964b).

Transport studies

Ca^{2+} transport by sarcoplasmic-reticulum vesicles was measured by the isotope-distribution method (Martonosi & Ferretos, 1964a). Ca^{2+} binding was carried out in a medium (1 ml) of 20 mM-histidine, pH 7.4, 50 mM-KCl, 5 mM-MgCl₂, 0.5 mM-EGTA and 0.5 mM-⁴⁵CaCl₂ (0.2 mCi/ml; The Radiochemical Centre, Amersham, Bucks., U.K.). The reaction was conducted at 25°C with 50–300 μg of membrane protein and initiated by the addition of ATP (final concn. 5 mM). Transport was terminated by filtering samples (200 μl) through Millipore filters (type HAWP 02500), which were then immediately washed with 50 mM-KCl/20 mM-histidine, pH 7.4. The quantity of Ca^{2+} accumulated was calculated from the radioactivity remaining on the filter. The

washed filters were transferred to liquid-scintillation-counting vials, 10ml of toluene/Bio-solv (BBS-3; Beckman Instruments, Palo Alto, CA, U.S.A.) scintillation 'cocktail' was added and the samples were assayed for radioactivity in a Beckman liquid-scintillation counter. Ca^{2+} uptake was determined in the same way, except that 5 mM-oxalate was included in the assay medium.

Ca^{2+} efflux was measured from vesicles that had been maximally loaded with Ca^{2+} (i.e. they had been incubated for 20 min at 25°C in Ca^{2+} -uptake medium). Efflux was initiated by the addition of EDTA (final concn. 6 mM) and was monitored by filtering samples with the Millipore system, as described above.

Passive Ca^{2+} binding

The method was adapted from that of Carvalho (1966). Samples of a suspension of sarcoplasmic reticulum (0.1 ml, containing 0.50 mg of protein) in 100 mM-KCl/5 mM-histidine, pH 7.2, were added to 10.0 ml of water and the pH was adjusted to 7.20 with 10 mM-KOH. CaCl_2 was added from a stock solution of either 0.01 or 0.1 M- CaCl_2 to give a range of nominal concentrations of 5–100 μM . The mixture was adjusted to pH 7.2 and equilibrated for 15 min at room temperature ($\sim 19^\circ\text{C}$) before centrifugation at 105 000g for 10 min. The Ca^{2+} concentration was determined in the supernatant and in the pellet (after it had been dispersed in 1 ml of water by sonication) with a Varian-Techtron atomic-absorption spectrophotometer, type AA-5, by using a nitrous oxide/acetylene flame.

Results

Selection of muscle type

On the basis of colour, texture, consistency, pH and lactate and glycogen concentrations, rapidly glycolysing muscle could clearly be distinguished from slowly glycolysing muscle (Table 1). The average pH of muscle selected as rapidly glycolysing was approx. 1.0 unit lower than that of slowly glycolysing muscle when measured at 10 min *post mortem*. Glycogen concentrations in rapidly glycolysing muscle were markedly diminished, whereas the lactate concentrations were more than double that of slowly glycolysing muscle.

Buoyant density of fragmented sarcoplasmic reticulum

The microsomal fraction from pig muscle that had undergone a slow decline in pH *post mortem* consisted of material ranging in buoyant density (ρ) from 1.10 to 1.20 g/ml (Plate 1a). An aggregated clump of material in the densest sucrose ($\rho > 1.20$ g/ml) was distinct from a broad disperse band in the central region of the gradient ($1.12 < \rho < 1.175$ g/ml). A third, narrow band was visible near the top of the gradient ($\rho = 1.10$ g/ml). The microsomal fraction from rapidly glycolysing muscle showed altered sedimentation behaviour, with a tendency towards lowered buoyant density. Aggregated material equilibrated at $\rho = 1.175$ g/ml and a sharply defined band was localized at $\rho = 1.07$ g/ml. Some disperse material also equilibrated in the latter region. Disperse material appeared at an intermediate zone in both preparations. In each of nine experiments in which the buoyant densities were compared there was a consistent pattern of lowered buoyant density of fractions from rapidly glycolysing muscle as outlined above, but the proportion of material present in the various density zones varied. In no instance was the buoyant density of the heavy aggregated material from rapidly glycolysing muscle greater than 1.18 g/ml.

For the purpose of further analysis, the microsomal fractions were separated on a preparative discontinuous gradient of 35, 40 and 45% (w/v) sucrose (Plate 1b). Three fractions from slowly glycolysing muscle were recovered, at the 40/45%-sucrose interface, at the 35/40%-sucrose interface and on the surface of the 35% sucrose layer, and were labelled heavy, intermediate and light fractions respectively. With this procedure, no heavy fraction was present in the microsomal preparation from rapidly glycolysing muscle. Sedimentation behaviour similar to that of rapidly glycolysing muscle has been observed in slowly glycolysing muscle 24 h *post mortem* (Greaser *et al.*, 1969d).

Protein and lipid content of sarcoplasmic-reticular membranes

Total protein in the microsomal fraction of rapidly glycolysing muscle was not significantly different from that of slowly glycolysing muscle (Table 2). The lack of material in the heavy fraction

Table 1. *Some of the parameters used to assess muscle type*

The overall pH of a muscle sample was a mean of three readings taken at sites in the muscle approx. 4 cm apart. Results are means $\pm$ S.D. for the numbers of observations given in parentheses.

Muscle type	pH	Glycogen (μmol of glucose equivalents/g of wet tissue)	Lactate (μmol /g of wet tissue)
Slowly glycolysing	6.73 ± 0.14 (10)	25.8 ± 9.9 (7)	48.6 ± 15.5 (10)
Rapidly glycolysing	5.69 ± 0.36 (10)	1.70 ± 1.22 (8)	107.5 ± 18.5 (10)

EXPLANATION OF PLATE I

Sedimentation of fragments of sarcoplasmic reticulum in continuous and in discontinuous sucrose gradients
 (a) Continuous sucrose gradient of 15–45% (w/v) sucrose. Each gradient contained approx. 25 mg of membrane suspension and was centrifuged for 16 h at 60000g_{av.}. (b) Discontinuous sucrose gradient of 35, 40, 45% (w/v) sucrose. Approx. 50 mg of membrane protein was layered on each gradient and centrifuged for 2 h at 64000g_{av.}. [Fragments of sarcoplasmic reticulum from slowly glycolysing (i) and from rapidly glycolysing (ii) muscle.]

EXPLANATION OF PLATE 2

Separation of the proteins of sarcoplasmic-reticular membranes by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The membrane suspensions were dissolved by heating at 50°C for 15 min in 10 mM-sodium phosphate buffer, pH 7.0, containing 1% (w/v) sodium dodecyl sulphate and 1% (v/v) β -mercaptoethanol. A portion of the solution (50 μ g of protein) was applied to the top of the gels and was electrophoresed at 8 mA/gel for 2–3 h. Staining was with Coomassie Blue. Proteins from vesicles of heavy (a), intermediate (b) and light (c) fractions from slowly glycolysing muscle and those from vesicles of intermediate (d) and light (e) fractions from rapidly glycolysing muscle are shown. The molecular-weight assignments were calculated for gel (a).

Table 2. *Protein and lipid content of sarcoplasmic-reticular membranes from slowly glycolysing and from rapidly glycolysing muscle*Results are means $\pm$ S.D. for the numbers of observations given in parentheses.

Fraction		Total protein (mg/g of wet muscle)	Protein/phospholipid (w/w)	Protein/cholesterol (w/w)
Light	Slow	0.16 $\pm$ 0.10 (14)	3.77 $\pm$ 0.72 (4)	15.58 $\pm$ 4.68 (5)
	Rapid	0.32 $\pm$ 0.15 (14)	2.31 $\pm$ 0.97 (5)	10.35 $\pm$ 3.59 (5)
Intermediate	Slow	0.26 $\pm$ 0.17 (14)	4.46 $\pm$ 0.99 (8)	23.28 $\pm$ 8.86 (7)
	Rapid	0.19 $\pm$ 0.16 (14)	4.20 $\pm$ 1.05 (7)	22.94 $\pm$ 8.86 (5)
Heavy	Slow	0.14 $\pm$ 0.09 (14)	4.78 $\pm$ 0.80 (2)	23.75 $\pm$ 8.05 (3)
	Rapid	<0.01	—	—
Total	Slow	0.56		
	Rapid	0.51		

* $P < 0.05$. ** $P < 0.0025$.Table 3. *Proportion of phospholipid classes in sarcoplasmic-reticular membranes from slowly glycolysing and from rapidly glycolysing muscle*The results are expressed as the percentage of total phosphorus present (means $\pm$ S.D.).

		Composition (% w/w)				
Phospholipid fraction ...		Light		Intermediate		Heavy
Nos. of preparations ...		Slow (2)	Rapid (2)	Slow (5)	Rapid (5)	Slow (1)
Solvent front		3.3 $\pm$ 1.7	1.4 $\pm$ 2.0	4.0 $\pm$ 3.2	4.5 $\pm$ 1.8	8.2
Phosphatidylethanolamine		18.6 $\pm$ 6.0	22.0 $\pm$ 0.95	19.2 $\pm$ 5.3	21.1 $\pm$ 1.9	17.5
Phosphatidylinositol + phosphatidylserine		15.5 $\pm$ 2.8	10.2 $\pm$ 2.2	11.0 $\pm$ 1.5	10.9 $\pm$ 1.0	10.9
Phosphatidylcholine		46.4 $\pm$ 1.1	56.7 $\pm$ 1.8	60.1 $\pm$ 6.2	55.9 $\pm$ 3.5	48.6
Sphingomyelin		11.9 $\pm$ 5.3	14.0 $\pm$ 2.3	5.0 $\pm$ 1.7	6.3 $\pm$ 1.4	11.6
Lysophosphatidylcholine		<1.0	<1.0	<1.0	<1.0	2.6
Origin		<1.0	<1.0	<1.0	<1.0	<1.0

from rapidly glycolysing muscle was balanced by a greater amount in the light fraction. The difference in buoyant density between the heavy, intermediate and light fractions of both muscle types could be correlated with protein/phospholipid ratios. The light fraction from rapidly glycolysing muscle had a significantly smaller ratio than the light fraction from slowly glycolysing muscle, in keeping with the lower buoyant density of this material. The protein/cholesterol ratio was also significantly lower in this fraction. The intermediate fractions were comparable with regard to both ratios. Taking into account the greater amount of material present in the light fraction and its low protein/lipid ratios, it is apparent that the vesicles derived from rapidly glycolysing muscle have lower protein/lipid ratios than those from slowly glycolysing muscle.

Each fraction was analysed by t.l.c. to determine the relative quantity of individual phospholipid classes (Table 3). The predominant phospholipid in each fraction was phosphatidylcholine, followed by phosphatidylethanolamine, phosphatidylinositol plus

phosphatidylserine and sphingomyelin. The relative proportions are similar to those found in purified fragmented sarcoplasmic reticulum of rabbit (Meissner & Fleischer, 1971; Waku *et al.*, 1971; Marai & Kuksis, 1973). No significant differences were noted between the lipids in the intermediate fractions from the two types of muscle. The light fraction from rapidly glycolysing muscle had a significantly greater quantity of phosphatidylcholine than the lightest fraction from slowly glycolysing muscle, and in this respect is more similar to the intermediate fractions, which is in keeping with a change to lower buoyant density of these vesicles.

The fatty acid composition of the various phospholipids was determined for the vesicles of intermediate buoyant density. Individual phospholipid classes in rabbit sarcoplasmic-reticular membranes have been shown to have characteristic fatty acid profiles (Marai & Kuksis, 1973). The values obtained for pig sarcoplasmic reticulum are similar (results not shown). The only statistically significant difference noted between fractions from slowly glycolysing and

from rapidly glycolysing muscle was a decrease in the latter of the proportion of arachidonic acid in the phosphatidylethanolamine fraction [14.5 ± 1.03 and $11.6 \pm 0.52\%$ of total area of all measurable peaks ($\pm$ s.d., $n = 8$ in both instances; $P < 0.001$).

The protein components of each fraction were examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Plate 2). ATPase (mol.wt. 105000) is a major component of all the membrane fractions. In the heavy fraction from slowly glycolysing muscle two other proteins are present in significant amounts, in positions in the gel corresponding to mol.wts. of over 150000 and approx. 45000. The intermediate and light fractions are similar in protein composition to each other and contain significant quantities of components of approx. mol.wt. 95000, 65000, 47000, 43000 and 32000. Some of these probably correspond to glycolytic enzymes, for example, phosphorylase (mol.wt. 94000) and lactate dehydrogenase (mol.wt. 36000), both of which have been shown to be loosely associated with membrane fragments from rabbit muscle (Margreth *et al.*, 1974). Owing to the large number of components it is not possible to identify unequivocally the high-affinity calcium-binding protein (mol.wt. 65000) and M_{55} protein or calsequestrin (mol.wt. 55000), which are known components of the sarcoplasmic reticulum of rabbit muscle (Meissner *et al.*, 1973; Ikemoto *et al.*, 1974; MacLennan & Wong, 1971; Ostwald & MacLennan, 1974). The relative proportions of the various protein components in the sarcoplasmic reticulum from slowly glycolysing and from rapidly glycolysing muscle are shown in Table 4. Both intermediate and low-buoyant-density fractions from rapidly glycolysing muscle contain relatively more ATPase protein and less of the

95000- (c), 65000- (d) and 42000- (i) dalton components. Rapidly glycolysing muscle exhibits a rapid decline in pH *post mortem*, which results in denaturation and aggregation of some soluble proteins (Scopes & Lawrie, 1963). Such denatured proteins would almost certainly sediment, at the low speed used during differential centrifugation, with the cell debris and mitochondria. Consequently, soluble proteins and possibly extrinsic membrane-bound proteins will not be expected to be found in association with the sarcoplasmic reticulum to the same extent as that from slowly glycolysing muscle. On the other hand, intrinsic membrane-bound proteins such as ATPase appear, from their concentration in the gels and from the lack of new lower-molecular-weight species, not to dissociate from the membrane or to be digested by proteolytic enzymes under the acidic conditions of rapidly glycolysing muscle.

Calcium transport

A typical time course of calcium accumulation by vesicles of intermediate buoyant density from both sorts of muscle is shown in Fig. 1. Accumulation in the absence of precipitable oxalate anions (Ca^{2+} -binding) is rapid, and meaningful rates are not obtainable by the Millipore method (Fig. 1a). However, the capacity to bind Ca^{2+} is diminished in vesicles from rapidly glycolysing muscle. In the presence of oxalate the rate of accumulation of Ca^{2+} and loading capacity for Ca^{2+} of these vesicles are diminished (Fig. 1b). The observations on seven preparations (Table 5) indicate that all fractions are able to accumulate Ca^{2+} and hydrolyse ATP under the conditions of assay. Ca^{2+} binding to vesicles from rapidly glycolysing muscle was one-half to one-third

Table 4. *Relative proportion of the main proteins of sarcoplasmic-reticular membranes from slowly glycolysing and from rapidly glycolysing muscle*

The gels shown in Plate 2 were scanned at 600 nm and peak areas were expressed as a percentage of the total area of all measurable peaks. Peaks with an area of less than 2% are excluded from the Table.

Protein band	Composition (%)					
	Slowly glycolysing			Rapidly glycolysing		
	Heavy	Intermediate	Light	Intermediate	Light	
a	21.9	<2	<2	<2	<2	
b(ATPase)	39.1	22.7	31.5	64.2	46.1	
c	<2	4.5	5.5	<2	5.4	
d	<2	29.1	19.1	2.1	5.2	
e	<2	<2	<2	2.0	<2	
f	2.7	3.0	5.3	4.0	3.5	
g	16.2	8.2	7.0	8.6	13.3	
h	<2	8.1	8.6	<2	<2	
i	2.2	7.1	4.8	2.5	2.9	
j	2.5	3.7	3.6	6.0	4.1	
k	<2	5.8	4.3	<2	4.4	
l	2.5	5.8	5.0	4.5	3.84

Fig. 1. Time course of Ca^{2+} binding and Ca^{2+} uptake by sarcoplasmic-reticulum vesicles of intermediate buoyant density

(a) Ca^{2+} binding; the complete reaction medium contained 20 mM-histidine, pH 7.4, 50 mM-KCl, 5 mM- MgCl_2 , 0.5 mM-EGTA, 0.5 mM- $^{45}\text{CaCl}_2$, 5 mM-ATP and 0.082 mg of membrane protein. (b) Ca^{2+} uptake; the same medium and protein concentration were used as for Ca^{2+} binding, except that the medium also contained 5 mM-oxalate. The reaction was stopped by Millipore filtration and washing. Ca^{2+} accumulation was estimated from the amount of radioactivity remaining on the filter. Results for vesicles from slowly glycolysing (●) and from rapidly glycolysing (○) muscle are shown.

and the rate of Ca^{2+} uptake was one-third to one-quarter of the values obtained for vesicles of slowly glycolysing muscle. Total ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase activity in the former vesicles was not decreased to the same extent as was their ability to accumulate Ca^{2+} . Activity in the intermediate fraction was diminished by one-third and that in the light fraction was unaffected.

Passive binding of Ca^{2+}

Fragmented sarcoplasmic reticulum of intermediate buoyant density from both types of muscle was capable of binding Ca^{2+} passively in the absence of ATP. The results, in the form of a Scatchard plot, are shown in Fig. 2. In the range of Ca^{2+} concentration studied (5–100 μM), vesicles derived from rapidly glycolysing muscle bound approximately one-half as much Ca^{2+} as did those from slowly glycolysing muscle. Some of the data obtained at low Ca^{2+} concentration (<7 μM), especially for binding to membranes from rapidly glycolysing muscle, show considerable scatter, which could be due to the endogenous Ca^{2+} , which has been recognized in membrane fragments from rabbit (Carvalho, 1968). Extrapolation to zero for added Ca^{2+} indicates that the reticulum from slowly glycolysing pig muscle has an endogenous Ca^{2+} content of 20–40 nmol of Ca^{2+} /mg of protein, whereas that from rapidly glycolysing muscle has 10–30 nmol of endogenous Ca^{2+} /mg of protein. The observations on binding of Ca^{2+} to fragments from slowly glycolysing muscle could be resolved into two linear portions in the Scatchard plot (Fig. 2). The higher-affinity binding site has a binding constant (K_D) of 7.6 μM and a capacity of 68 nmol of Ca^{2+} /mg of protein, and the lower-affinity site had values of 120 μM and 202 nmol of Ca^{2+} /mg of protein respectively. These two binding sites may be compared with the low-affinity ($K_D = 13$ –32 μM) and non-specific ($K_D = 320 \mu\text{M}$) binding sites respectively described by Chevalier & Butow (1971) for membrane fragments derived from rabbit muscle. These authors also found a third site with high affinity ($K_D = 0.4$ –1.3 μM) which could only be

Table 5. ATPase activity and Ca^{2+} -accumulating ability of sarcoplasmic-reticulum vesicles from slowly glycolysing and from rapidly glycolysing muscle

ATPase activity was measured in a medium containing 50 mM-Tris/HCl, pH 7.4, 100 mM-KCl, 5 mM- MgCl_2 , 0.1 mM-EGTA, 0.1 mM- CaCl_2 , 10 mM-ATP and 0.07–0.18 mg of membrane protein. The reaction medium for Ca^{2+} binding and Ca^{2+} uptake was as in Fig. 2. In both of the Ca^{2+} -transport assays, the reaction was stopped after 2 min by filtering through a Millipore filter. Results are means $\pm$ S.D. for seven preparations.

Fraction	ATPase activity (μmol of P_i /min per mg of protein)		Ca^{2+} binding (nmol of Ca^{2+} /mg of protein)		Ca^{2+} uptake (nmol of Ca^{2+} /min per mg of protein)	
	Slow	Rapid	Slow	Rapid	Slow	Rapid
Light	0.90 ± 0.22	0.97 ± 0.25	40.9 ± 7.1	17.7 ± 8.7	444 ± 73	136 ± 22
Intermediate	1.66 ± 0.18	1.13 ± 0.20	38.3 ± 12.8	13.0 ± 4.8	570 ± 94	121 ± 26
Heavy	1.39 ± 0.30	—	20.0 ± 15.2	—	256 ± 10	—

Fig. 2. *Passive Ca^{2+} binding to sarcoplasmic-reticulum membranes of intermediate buoyant density*
 Ca^{2+} binding to sarcoplasmic-reticulum membranes from slowly glycolysing muscle (●) and from rapidly glycolysing muscle (○) is shown in a Scatchard-type plot.

Fig. 3. *Passive efflux of Ca^{2+} from sarcoplasmic-reticulum vesicles preloaded with calcium oxalate*
 Vesicles of intermediate buoyant density from slowly glycolysing (●) and from rapidly glycolysing (○) muscle were incubated in Ca^{2+} -uptake medium at 25°C. Efflux was initiated after 20 min of loading (arrow) by the addition of EDTA (final concn. 6 mM). Loading and efflux was monitored by Millipore filtration of 0.2 ml samples and determining the radioactivity remaining on the filters.

detected when endogenous Ca^{2+} had been removed by a cation exchanger. Scatchard analysis of data on Ca^{2+} binding to vesicles from rapidly glycolysing muscle revealed only one type of binding site with an

affinity ($K_D = 31 \mu\text{M}$) intermediate between the two binding sites discernible in control reticulum. The total binding capacity (70 nmol of Ca^{2+} /mg of protein) of these vesicles was equal to that of the higher-affinity site in sarcoplasmic reticulum from slowly glycolysing muscle.

Ca^{2+} efflux

Passive efflux of Ca^{2+} from calcium oxalate-loaded vesicles was studied after inhibition of the transport enzyme by 6 mM-EDTA (Fig. 3). Addition of EDTA to control vesicles resulted in the rapid loss of approx. 25% of accumulated Ca^{2+} succeeded by slow efflux. Vesicles from rapidly glycolysing muscle lost a relatively greater proportion of Ca^{2+} initially (approx. 60%). The actual amount of Ca^{2+} that was rapidly released was, however, less (0.10 compared with $0.17 \mu\text{mol}$ of Ca^{2+} /mg of protein). Thereafter the first-order rate constant for efflux was $2.2 \pm 0.64 \text{ h}^{-1}$, compared with $2.8 \pm 1.21 \text{ h}^{-1}$ in control vesicles (mean $\pm$ S.E.M., $n = 3$).

Temperature-dependence of ATPase activity

The effect of temperature on the total ATPase activity of all the vesicular fractions was monitored continuously from 10 to 50°C by a pH-stat method. Arrhenius plots of the data are shown in Fig. 4. The most dense material in control preparations displayed a linear relationship over the temperature range 10–40°C with an apparent activation energy of 69.0 kJ/mol. At temperatures above 43°C, inhibition occurred, presumably owing to onset of denaturation

Fig. 4. Temperature-dependence of ATPase activity of sarcoplasmic-reticular membranes

(a) Light fraction; (b) intermediate fraction; (c) heavy fraction. ATPase activity was measured by a pH-stat method during a continuous temperature rise of approx. 2°C/min. Membrane fragments were obtained from slowly glycolysing muscle (●) and from rapidly glycolysing muscle (○).

of the enzyme. The ATPase activity of fragments of intermediate buoyant density from slowly glycolysing muscle exhibited two inflexion points, at approx. 15 and 33°C. The enzymic process between these two temperatures had a relatively low activation energy of 51.5 kJ/mol. A distinct high-activation-energy process (125.1 kJ/mol) became evident between 33 and 45°C. A similar temperature-dependence has been found for total ATPase activity of fragmented sarcoplasmic reticulum from rabbit skeletal muscle (Inesi *et al.*, 1973). Intermediate-density fragments from rapidly glycolysing muscle have an ATPase activity with a similar temperature-dependence in the range 20–35°C. However, in the higher range the fragments did not exhibit the high-activation-energy process, and in fact temperature did not appear to alter the reaction rate significantly between 38 and 46°C. The light vesicular fraction from slowly glycolysing muscle behaved in a manner similar to that of the intermediate fraction in having an essentially similar temperature-depen-

dent ATPase, except that the discontinuities were shifted to 20°C and to 38°C and the activation energies were somewhat smaller (36.8 kJ/mol for the range 21–38°C and 92.5 kJ/mol above 38°C). The light fraction of membrane fragments from rapidly glycolysing muscle also lacked a high activation energy of ATPase in the higher temperature range.

Discussion

Experimental conditions were chosen in the present study so that a comparison could be made between the sarcoplasmic reticulum of portions of muscle differing widely in sarcoplasmic pH and hence in rates of glycogenolysis. The samples of rapidly glycolysing muscle showed unequivocal and gross pale, soft and exudative features with depleted glycogen content and elevated lactate concentrations.

Sarcoplasmic-reticulum vesicles from rapidly glycolysing muscle had lower buoyant density than that from slowly glycolysing muscle. Not all the components of the fraction were equally affected, an indication of its heterogeneous composition. In this regard, Meissner (1975) distinguished two types of sarcoplasmic-reticulum vesicles in the microsomal fraction from rabbit skeletal muscle. He suggested that they may originate from different parts of the reticulum. In the present work it was observed that those fractions of vesicles with lower buoyant density also had diminished protein/phospholipid and protein/cholesterol ratios. It was inferred that lowered buoyant density could be the consequence of a loss of some of the protein constituents of the sarcoplasmic-reticular membrane. This conjecture was substantiated by analysis of the membrane proteins by polyacrylamide-gel electrophoresis. The pattern of proteins from rapidly glycolysing muscle differed from those of slowly glycolysing muscle in having a greater proportion of ATPase, the only protein considered to be intrinsic to the sarcoplasmic-reticular membrane in rabbit muscle (Stewart & MacLennan, 1974). ATPase is lipid-bound in membranes, but other proteins may be removed by relatively mild procedures such as incubation with 1 mM-EDTA (Duggan & Martonosi, 1970), with a solution of 1 M-KCl and 0.25% deoxycholate (Stewart & MacLennan, 1974), hypo-osmotic treatment or sonication (Margreth *et al.*, 1974). Thus it appears that by the time the muscle has become pale, soft and exudative, changes have taken place in the sarcoplasmic-reticular membranes which permit losses of protein from the vesicles during the course of fractionating procedures. Such proteins may have undergone denaturation owing to increased H⁺ concentration, or perhaps they may have undergone proteolysis by endogenous proteinases activated by the low sarcoplasmic pH. The former explanation is

favoured, since ATPase, which is known to be susceptible to tryptic digestion, was actually present in a greater proportion in the gels, whereas no lower-molecular-weight species were observed to accumulate. Sarcoplasmic reticulum from embryonic (Boland *et al.*, 1974) and from dystrophic muscle (Sabbadini *et al.*, 1975) has a lower buoyant density than that from mature and normal muscle respectively, but the reason in each of these cases is because of a smaller proportion of ATPase in the membrane rather than of non-ATPase protein.

The capacity and rate of accumulation of Ca^{2+} of both fractions of vesicles from rapidly glycolysing muscle was diminished. Greaser *et al.* (1969a,b) have documented the detrimental effects of conditions *post mortem* on the Ca^{2+} -accumulating ability of sarcoplasmic reticulum isolated from pig muscle. The activity declined 10–20-fold 24 h *post mortem* in slowly glycolysing muscle, although there appeared to be little morphological difference in the reticular membranes in the same period (Greaser *et al.*, 1969b). Sarcoplasmic reticulum from muscle undergoing rapid glycogenolysis showed an initial low activity, and thereafter it declined at the same rate as that from slowly glycolysing muscle, or faster (Greaser *et al.*, 1969a). They showed that the Ca^{2+} -accumulating ability could be correlated with the sarcoplasmic pH. Normally, hydrolysis of ATP and Ca^{2+} translocation are stoichiometrically related in the ratio 1:2. The results of Greaser *et al.* (1969a) and our own indicate that this stoichiometry does not exist in rapidly glycolysing muscle, since Ca^{2+} accumulation and ATPase activity are not altered equally.

Many agents and conditions are known to change the stoichiometry of Ca^{2+} accumulated and ATP hydrolysed, for example treatment with diethyl ether (Inesi *et al.*, 1967), phospholipase A (Mackinose, 1971) or with alkali and EGTA (de Meis & Carvalho, 1974). The mechanism in most of these instances is thought to be through increase of the passive permeability of the membrane to Ca^{2+} , rendering the vesicles 'leaky' and preventing inhibitory concentrations of Ca^{2+} from accumulating. The flux of Ca^{2+} into and out of the vesicles is increased, and such preparations usually display augmented ATPase activity. Passive efflux of Ca^{2+} from actively preloaded vesicles from both types of muscle consisted of a rapid and a slow phase. Two phases of release imply that either two types of vesicles are present or two pools of Ca^{2+} exist in the vesicles. We favour the latter explanation, since the vesicles used in the efflux experiments were all of intermediate buoyant density. Biphasic release of Ca^{2+} from vesicles of rabbit muscle origin was found by Meissner (1975), but the overall release was much faster (90% released within 4 min at 23°C) than that obtained here. Inesi & Scales (1974) and de Boland *et al.* (1975), using different methods, found an apparent monophasic

first-order release of Ca^{2+} with a half-time of 6–8 min and of about 20 min respectively at 25°C. We have previously (Berman *et al.*, 1977) obtained an efflux pattern from rabbit reticular vesicles similar to that found here. A rapid phase of release, complete within 2 min, was superseded by a slower phase with a half-time of 12 min (compared with 15 min in the present experiments). The slower phase of release corresponds approximately to that measured by Inesi & Scales (1974) and by de Boland *et al.* (1975), and the rate was taken by them as an indication of the permeability of the membranes to Ca^{2+} . It would follow that vesicles from slowly glycolysing and from rapidly glycolysing muscle are equally permeable to Ca^{2+} . The similarity in steady-state (Ca^{2+} + Mg^{2+})-stimulated ATPase activity would be in keeping with this conclusion. This interpretation implies that the rapid phase of release represents dissociation of Ca^{2+} from superficial binding sites and the slow phase the permeation of Ca^{2+} through the membrane from interiorly located Ca^{2+} stores. In this regard two types of ATP-dependent Ca^{2+} -binding sites have been identified in vesicles from rabbit muscle, which are considered to be superficially and lumenally located (Entman *et al.*, 1973). The smaller proportion of Ca^{2+} that effluxes slowly from vesicles of rapidly glycolysing muscle can be explained by the diminished Ca^{2+} loading exhibited by these vesicles. This is substantiated by the finding that the proportions of each phase are not significantly altered when rabbit sarcoplasmic-reticular vesicles, which have been acid-treated for sufficient time to eliminate Ca^{2+} -uptake activity, are loaded passively to the same extent as untreated vesicles and then allowed to release Ca^{2+} (Berman *et al.*, 1977). The diminished ability of vesicles from rapidly glycolysing muscle to accumulate Ca^{2+} may therefore be due to a defect in the mechanism for active loading of the slowly released pool of Ca^{2+} , leading to a disproportionate increase in the occupancy of the rapidly released pool.

Since transport of Ca^{2+} and ATPase activity are known to have specific lipid requirements (Martonosi *et al.*, 1968), the possible role of lipids in the altered functional state of the membrane was investigated. However, apart from a few minor differences, the membranes from the two types of muscle had very similar phospholipid and fatty acid compositions. Such differences as were observed appeared unlikely to alter membrane integrity in rapidly glycolysing muscle and hence account for the observed functional differences.

Passive binding of Ca^{2+} was diminished in membranes from rapidly glycolysing muscle when compared with slowly glycolysing muscle, a phenomenon attributable most likely to the loss of loosely associated proteins, some of which (in rabbit muscle) are known to bind large quantities of Ca^{2+} (Ostwald &

MacLennan, 1974). Their role in active Ca^{2+} transport, however, is still in doubt, since vesicles that partially (Meissner, 1975) or wholly (Racker *et al.*, 1975) lack them are still able to accumulate Ca^{2+} . Two types of binding sites were distinguished in membranes from slowly glycolysing muscle, whereas only one could be defined in those from rapidly glycolysing muscle. Loss from the membranes of some of the loosely associated proteins has given rise to a new type of binding site with intermediate affinity and diminished total capacity, e.g. by exposure of the polar head groups of phospholipids, some of which are known to be able to bind Ca^{2+} (Seimiya & Okhi, 1973). Poor data in the low- Ca^{2+} concentration range do not allow definition of the high-affinity site ($K_D \sim 1 \mu\text{M}$) involved in the hydrolysis of ATP and to which Ca^{2+} binds before being translocated.

ATPase in membranes from rapidly glycolysing muscle was observed to have altered temperature-dependence in the temperature range 33–45°C. Membranes from slowly glycolysing muscle have a relatively high-activation-energy process operating in this temperature range, whereas those from rapidly glycolysing muscle have a low one. Inesi *et al.* (1973) also observed a switch to a high activation energy in this temperature range with sarcoplasmic reticulum from rabbit muscle when the total ATPase activity was measured in the steady state. Although a change in membrane integrity occurred in this temperature range, allowing increased influx of Ca^{2+} , they concluded that the increase was not sufficient to affect Ca^{2+} accumulation and presumably ATPase activity. The use of spin labels attached to a thiol group in the ATPase protein also led them to conclude that the transition from a low- to a high-activation-energy process at approx. 35°C involved a conformational change in ATPase. If this is correct, then ATPase from rapidly glycolysing muscle may be unable to undergo such a transition. It is tempting to speculate that a conformational change of ATPase from normal may be related to diminished Ca^{2+} accumulation in these vesicles.

Conditions that develop in muscle *post mortem* and that lead to pale, soft and exudative features primarily affect the protein constituents of the sarcoplasmic-reticular membranes. Dissociation of loosely associated protein results in isolated vesicles of lowered buoyant density, and these vesicles display decreased efficiency in pumping Ca^{2+} . The susceptibility of a protein component, involved in Ca^{2+} transport through the sarcoplasmic-reticular membrane, to modification at low pH provides a mechanism whereby glycogenolysis in muscle *post mortem* once initiated could become self-perpetuating.

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