Biochimica et Biophysica Acta, 640 (1981) 285—297

BBA 79060

ISOPYCNIC-ZONAL CENTRIFUGATION OF PLASMA MEMBRANE, SARCOPLASMIC RETICULAR FRAGMENTS, LYSOSOMES, AND CYTOPLASMIC PROTEINS FROM PHASIC SKELETAL MUSCLE

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(Received June 2nd, 1980)
(Revised manuscript received August 26th, 1980)

Key words: Lysosome; Sarcoplasmic reticulum; Membrane fractionation; (Endoplasmic reticulum)

Summary

Homogenates of the posterior latissimus dorsi muscle, a phasic muscle, were fractionated by a one-step zonal centrifugation technique into four major organelle populations and cytoplasmic constituents. These were: (1) Plasma membrane fragments with a modal equilibrium density of 1.10 and containing 5′-nucleotidase, alkaline phosphodiesterase, *p*-nitrophenylphosphatase and acid phosphatase (β-glycerophosphate was used as the substrate). (2) Sarcoplasmic reticular fragments which could be further subdivided into calcium transport vesicles, with a modal equilibrium density of 1.16, that exhibited calcium uptake; K^+-ATPase; leucyl-β-naphthylamidase; acid phosphodiesterase; acid phosphatase (using cytidine monophosphate as the substrate); and sarcoplasmic reticular lysosomes, with a modal equilibrium density of 1.18, possessing dipeptidyl-aminopeptidase II, cathepsin D, α-glucosidase, *N*-acetyl-β-glucosaminidase, and NADH oxidase activity. (3) Mitochondria with a modal equilibrium density of 1.21. (4) Catalase-containing vesicles with a modal equilibrium density of 1.22; and cytoplasmic constituents (modal density of 1.25) with phosphorylase, pyruvate kinase, myosin-ATPase, aldolase, and protein and RNA content. The purity of these organelles was equal to or better than previous efforts, with a 30-fold purification achieved for 5′-nucleotidase and alkaline phosphodiesterase. These results lend support to the hypothesis that the sarcoplasmic reticulum of phasic muscle, in addition to its specialized role in excitation-contraction coupling, represents a multifunctional membrane system, and that, similar to the smooth endoplasmic reticulum of other

cells, it includes some membrane-bound lysosomal enzymes and NADH oxidase.

Introduction

There is considerable circumstantial evidence that skeletal muscle lysosomes are involved in the muscle atrophy accompanying a variety of diseases. Morphological studies have indicated that increased numbers of lysosomes occur in most muscle disease processes [1]. Similarly, assays of lysosomal enzymes have shown significantly increased activities in diseased as compared to normal tissue [2,3]. However, phasic muscles which characteristically contain few lysosomes and the least lysosomal enzyme activities in striated muscle [4], nevertheless, undergo selective atrophy. In contrast, the lysosome-enriched muscles do not. From these studies, it has been suggested that muscle lysosomes might play a very specialized catabolic role which does not include myofibrillar degradation [5].

The present studies were undertaken to evaluate quantitatively the components including lysosomes in homogenates from phasic muscles, by a one-step zonal centrifugation technique. 21 enzymes, protein, and RNA were measured in isolated fractions. Four distinct organelles were present in the separations: (1) plasma membrane, (2) sarcoplasmic reticulum, (3) mitochondria, and (4) catalase-containing organelles as well as cytoplasmic proteins. Lysosomes and NADH oxidase vesicles appeared as parts of the smooth endoplasmic reticulum in skeletal muscle.

Materials and Methods

6-week-old Cornish-cross chickens were obtained from the Poultry Science Department of Iowa State University. Posterior latissimus dorsi muscles were removed and pooled to yield approx. 10 g of tissue. The muscles were incubated for 1 h at 37°C in Hanks' solution containing 1 mg/ml each of collagenase (type I) and lysozyme (Sigma Chemical Co.) and bubbled continuously with O_2 (100%). Next, the solution of muscle fragments derived from enzymatic digestion was filtered through two layers of cheesecloth and collected by low-speed centrifugation (1500 × g · 10 min). This procedure allows: (1) the isolation of muscle material devoid of contaminating cells; and (2) easy homogenization with large yields of intact organelles [6]. The pelleted material was resuspended in 0.33 M sucrose (pH 7.2) and recentrifuged at the same speed. Subsequently, the muscle fragments were suspended in 55% sucrose (w/v) containing 50 units/ml of heparin and 20 mM imidazole buffer, pH 7.2, for homogenization which was effected by three strokes in an ice-slurry-cooled Potter-Elvehjem homogenizer (clearance 4—6 μm) rotating at 1000 rev./min. The homogenate volume was brough to 80 ml.

Subcellular fractionation of muscle homogenates

Gradients were formed from two (110 ml) solutions of sucrose (8.5 and 50%, w/v) containing 20 mM imidazole buffer (pH 7.2) and 50 units/ml of

heparin. The Beckman Z-60 zonal rotor was loaded while spinning at 1500 rev./min to two-thirds its volume with this linear gradient. 75 ml of homogenate (about 300 mg protein) were introduced into the rotor, followed by 65% (w/v) sucrose solution. The remaining 5 ml of homogenate were saved for other analysis. In order to minimize hydrostatic effects [7], pilot experiments were run at 15°C for a total of 1800 rad^2/ns. These studies were plagued by aggregation problems and were abandoned for the following protocol, at 4°C. In subsequent experiments at 4°C, the Z-60 rotor was loaded at 1500 rev./min, accelerated to 50 000 rev./min and spun for a total of 1800 rad^2/ns, decelerated to 1500 rev./min and unloaded while spinning. 22 15-ml fractions were obtained and their sucrose concentrations measured using an Abbe refractometer. These fractions then were diluted to a sucrose concentration of no more than 15% (w/v) for enzymatic and chemical analysis and calcium uptake studies.

Analytical techniques

RNA content was determined by using the orcinol method of Schneider [8], using yeast RNA as the standard. Protein content was approximated according to the method of Lowry et al. [9], using human serum albumin as the standard. Phosphate was determined by using the technique of Marinetti et al. [10].

Aldolase and alkaline phosphodiesterase were determined by using the method of Beaufay et al. [11] and pyruvate kinase by using that of Goldbarg et al. [12]. K^+-ATPase, which is specific for sarcoplasmic reticulum when corrections are made for basal Mg^{2+}-ATPase activity, was assayed according to the method of Duggan [13].

5′-Nucleotidase was determined by using the method of Headon et al. [14], using 5′-IMP at pH 6.5, a substrate and conditions which are specific for muscle. Acid phosphatase was monitored using 0.05 M β-glycerophosphate, cytidine monophosphate, or *p*-nitrophenylphosphate in 0.10 M acetate buffer, pH 5.0. Myosin-ATPase was determined by measuring the phosphate released from ATP in a solution containing 50 mM Tris, 5 mM EDTA, 400 mM KCl and 5 mM Tris-ATP, pH 7.5, after the membranes were disrupted using Triton X-100 (TVB solution). TVB solution (0.01% (w/v) Triton X-100, 1 mM EDTA, and 1 mM $NaHCO_3$) was used for dilutions of homogenates for lysosomal enzyme determinations as well as cytochrome oxidase and myosin-ATPase assays.

Assays for phosphorylase *a*, leucyl-β-naphthylamidase, catalase, *N*-acetyl-β-glucosaminidase, α-glucosidase, cathepsin D, cytochrome oxidase and calcium uptake for muscle fractions are described elsewhere [6]. β-Glucuronidase was detected by using the technique of Canonico and Bird [15] for muscle zonal fractions. Acid phosphodiesterase activity was determined according to the method of Kar and Pearson [16]. Pyruvate kinase was measured by using the method of Bucher and Pfliederer [17]. Assays for NADH oxidase and dipeptidyl aminopeptidase II were performed according to the methods of Avruck and Wallach [18] and McDonald et al. [19], respectively.

Data presentation

Fractionation data are plotted in histogram form as a function of the gradient volume. The ordinate is the normalized relative frequency of the constituent in the fractions relative to the concentration of the enzyme distributed homogeneously throughout the gradient [20], or it is the relative specific activity (% enzyme activity ÷% protein content for each fraction). Enzyme recoveries are calculated from the sum of the activities in each of the gradient fractions divided by the total activity in the starting material. Median density (g/cc) represents the calculated density at which 50% of each consitutent lies on either side, and modal density (g/cc) is the average density of the most frequently occurring fraction. All densities are true equilibrium densities.

Morphology of gradient fractions

Fractions containing the most activity (representing the modal density) for 5′-nucleotidase, calcium uptake, cytochrome oxidase and myosin-ATPase were selected for morphological evaluation using the technique of Baudhuin [21] for subcellular fractions in sucrose gradients. The thin sections derived from these fractions were prepared and electron photomicrographs were taken by conventional techniques.

Results

Distribution patterns for enzyme activities, protein, and RNA content generally were different for most constituents (Figs. 1 and 2) except acid phosphatase, acid *p*-nitrophenylphosphatase, and acid phosphodiesterase which overlapped the 5′-nucleotidase and calcium uptake profiles. 5′-Nucleotidase and alkaline phosphodiesterase (Fig. 1) showed single peaks of activity and represented material of lightest modal equilibrium density (1.10 g/cc). Cytochrome oxidase also showed a single peak of activity but with a modal equilibrium density of 1.21. Of the cytoplasmic proteins, myosin-ATPase, phosphorylase *a*, and pyruvate kinase were confined to a region of the gradient close to the starting sample zone. In contrast, some aldolase activity was found in other regions of the gradient and, along with protein content, was clearly bimodal. RNA distribution (Fig. 2) was similar to that of protein content with the majority of material associated with the sample zone and a small amount, representing about 12% of the total, distributing along with the membranous elements.

Fig. 2 shows the distribution of enzymes which might be associated with certain elements of the sarcoplasmic reticulum (smooth endoplasmic reticulum) of skeletal muscle. Markers for sarcoplasmic reticulum, calcium uptake [22], and K^+-ATPase [13] had narrow profiles with the same modal density of 1.16. Similarly, leucyl-β-naphtylamidase, acid phosphodiesterase, and acid phosphatase (using cytidine monophosphate as the substrate) were distributed around a modal density of 1.16, although the individual patterns were slightly different with different median densities (Table I). The lysosomal enzymes, α-glucosidase, dipeptidyl-aminopeptidase II, cathepsin D, β-glucuronidase, and *N*-acetyl-β-glucosaminidase were distributed around a modal density of 1.18 with a minor secondary peak for *N*-acetyl-β-glucosaminidase and cathepsin D at a

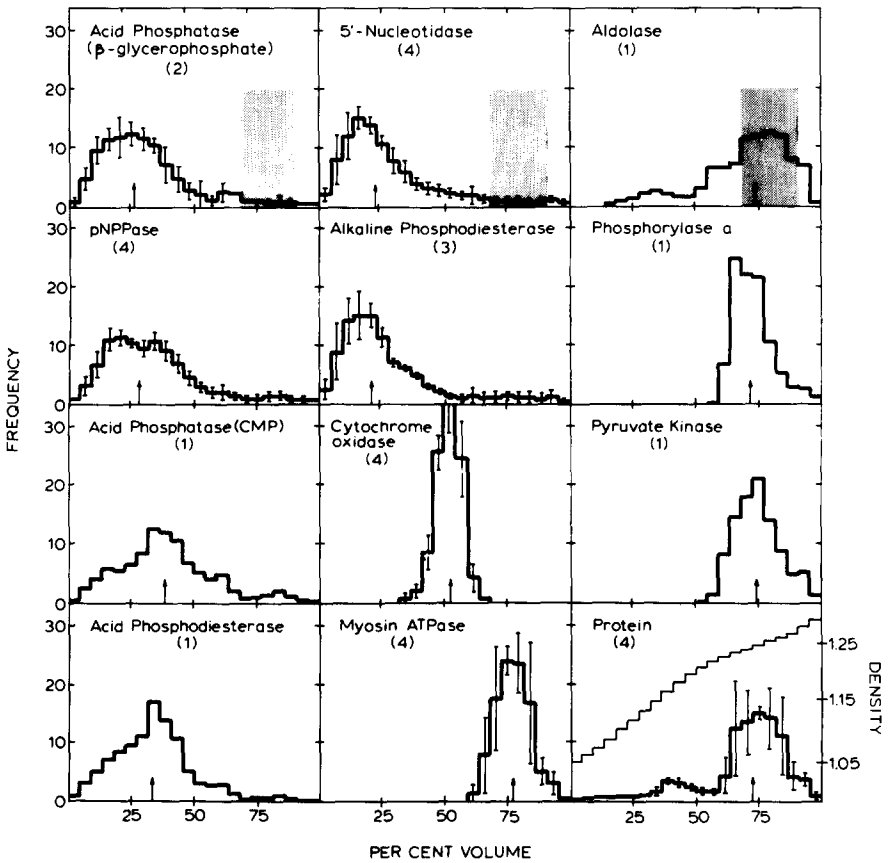

Fig. 1. Frequency distribution histograms as a function of rotor volume for enzyme activities and protein content. Density profiles are given along with the protein distribution. Values are means ±S.D. Numbers in parentheses represent the number of experiments. Arrow indicate median density. The shaded areas represent the starting sample zone. pNPPase, *p*-nitrophenylphosphatase.

modal density of about 1.22. The peak of activity at 1.22 corresponded to the major catalase peak. However, the catalase profile was distinctly different from the lysosomal enzyme and NADH oxidase distributions in both shape and primary modal density as well as median equilibrium density (Table I). The apparent recoveries of the constitutents are tabulated in Table I. Recoveries ranged from a low of 72.8% for alkaline phosphodiesterase to a high of 167.6% for calcium uptake but about two-thirds of the markers had recoveries from 90 to 100%.

Relative specific activity profiles (Fig. 3) for α-glucosidase, dipeptidyl-aminopeptidase II, and cathepsin D were quite similar to each other with two regions of enhanced purity, the greatest of which was about 8-times the activity of the total homogenate and located at the lighter equilibrium density peak. In contrast, NADH oxidase, which was also bimodal, had its greater relative specific activity at the heavier equilibrium density peak.

Although bimodal, both *N*-acetyl-β-glucosamindase and leucyl-β-naphthylamidase were different from the enzymes discussed above. The lighter equi-

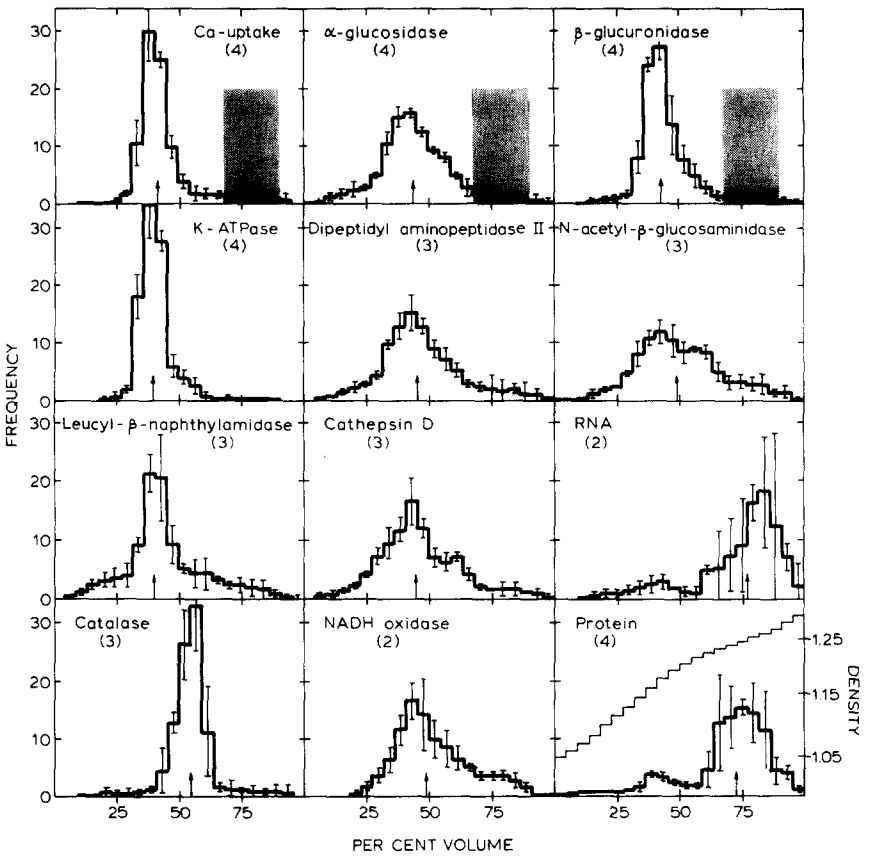

Fig. 2. Frequency distribution histograms as a function of rotor volume for calcium uptake, enzyme activities, and protein and RNA content. Values are means ±S.D. Numbers in parentheses represent the number of experiments. Arrows indicate median density. The shaded areas represent the starting sample zone.

librium density peaks corresponded to those of α-glucosidase, dipeptidyl-aminopeptidase II, and cathepsin D, but the right-hand part of the profile was skewed towards the heavier regions of the gradient. For leucyl-β-naphthylamidase, however, both modal profiles were skewed towards regions of lighter density. The real surprise was the distribution profile for the classical lysosomal marker enzyme, acid phosphatase, which most closely resembled that of the generally accepted plasma membrane marker enzymes (Fig. 4). In contrast, both acid phosphodiesterase and acid phosphatase (using cytidine monophosphate as substrate) had purification profiles that were between those of plasma membrane and lysosomal markers.

An approx. 13-fold increase in calcium uptake ability was recorded for the modal peak (1.16). These values (3.58 ± 0.3 μmol/mg protein) were greater than those found for any enriched fraction obtained from posterior latissimus dorsi muscle homogenates by differential centrifugation [5].

Relative specific activity profiles of the plasma membrane markers, 5′-nucleotidase and alkaline phosphodiesterase, indicate that an about 30-fold

TABLE I

% recovery given only for constituents with $n > 1$. For modal density groupings, see text.

Constituents	Median density	Modal density	% recovery
5′-Nucleotidase	1.100 ± 0.003	1.09	141.4 ± 14.3
Alkaline phosphodiesterase	1.117 ± 0.004	1.09	72.8 ± 2.4
Acid *p*-nitrophenylphosphatase	1.116 ± 0.003	1.101, 1.15 *	102.9 ± 10.4
Acid phosphatase (β-glycerophosphate)	1.131 ± 0.005	1.12	99.1 ± 14.7
Leucyl-β-naphthylamidase	1.175 ± 0.005	1.16	74.9 ± 10.8
K^+-ATPase	1.176 ± 0.004	1.16	91.0 ± 9.7
Ca^{2+} uptake	1.175 ± 0.004	1.16	167.6 ± 4.8
Acid phosphodiesterase	1.135	1.16	
Acid phosphatase (cytidine monophosphate)	1.141	1.16	
Dipeptidyl-aminopeptidase II	1.177 ± 0.003	1.18	109.9 ± 6.3
Cathepsin D	1.187 ± 0.004	1.18, 1.22 *	90.4 ± 9.6
α-Glucosidase	1.174 ± 0.003	1.18	95.2 ± 7.6
N-Acetyl-β-glucosaminidase	1.198 ± 0.005	1.18, 1.22 *	107.3 ± 2.2
β-Glucuronidase	1.173 ± 0.003	1.18	107.7 ± 3.4
NADH oxidase	1.185 ± 0.004	1.18	110.0 ± 6.7
Cytochrome oxidase	1.203 ± 0.002	1.21	82.8 ± 8.6
Catalase	1.205 ± 0.005	1.22	102.3 ± 4.7
Phosphorylase	1.236	1.24	
Pyruvate kinase	1.243	1.25	
Myosin-ATPase	1.242 ± 0.003	1.25	92.8 ± 12.4
Protein	1.235 ± 0.005	1.25	90.6 ± 8.5
Aldolase	1.236	1.26	
RNA	1.238 ± 0.009	1.27	81.6 ± 15.4

* Bimodal distributions.

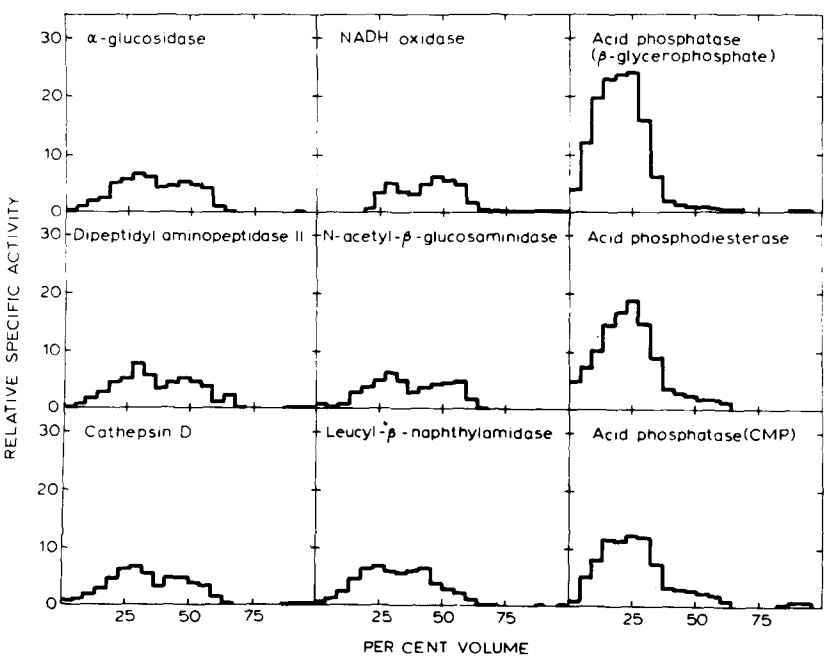

Fig. 3. Relative specific activity profiles of calcium uptake and enzyme activities as a function of rotor volume.

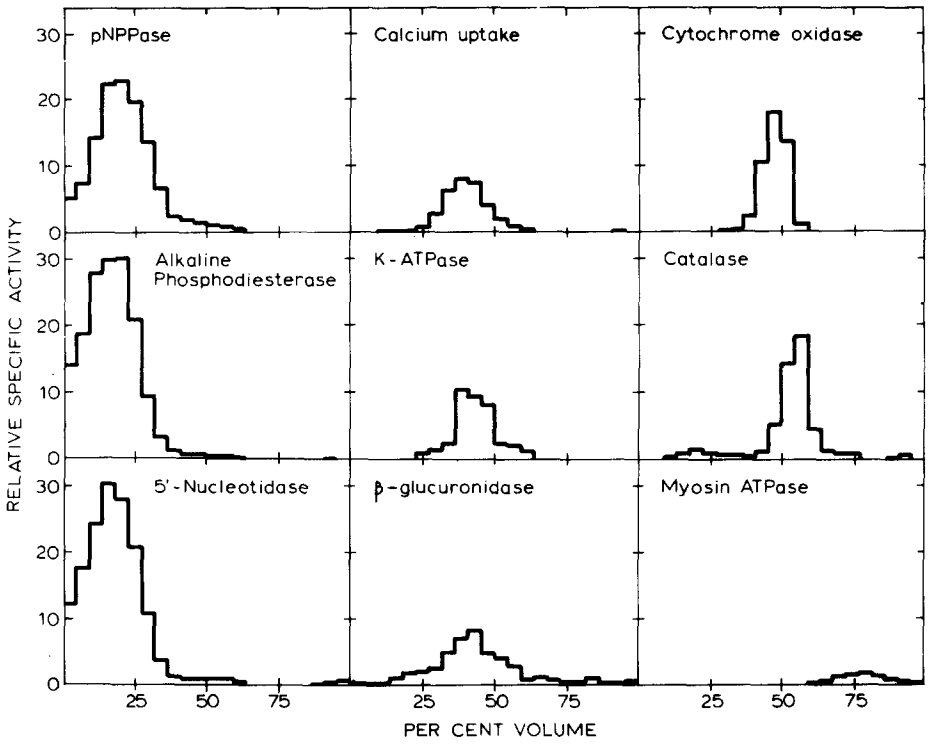

Fig. 4. Relative specific activity profiles of enzyme activities as a function of rotor volume.

purification was achieved. Acid *p*-nitrophenylphosphatase had a similar distribution profile, but with slightly less overall purification. For calcium uptake, K^+-ATPase, and β-glucuronidase, an 8–10-fold purification was recorded. Both cytochrome oxidase and catalase, although of distinctly different profiles, were purified about 20-fold. In contrast, myosin-ATPase, the major component of the cytoplasmic proteins, had a value slightly greater than 1.0.

Overlapping distributions exist for acid phosphatase and acid phosphodiesterase (i.e., some portion of activity was obtained from both plasma membrane and lysosomes) and if one assumes a bimodal distribution might prevail, then the percentage attributable to each of two distinct organelle profiles can be approximated. Thus, about 50% of acid phosphatase (cytidine monophosphate) and acid phosphodiesterase appears to be associated with plasma membranes with less than 50% being associated with lysosomes, whereas 70% of acid phosphatase (β-glycerophosphate) seemingly resides in the plasma membranes.

Micrographs of four fractions enriched in different cellular components are displayed in Fig. 5. Fraction A corresponds to the modal density (1.10) of plasma membrane markers. Fraction B corresponds to the fraction enriched in calcium transport capability (1.16) and was indistinguishable from that of modal density 1.18 (not shown) enriched in lysosomal hydrolases. Fraction C contains the mitochondrial enriched fraction (1.21). Fraction D contains the modal peak (1.25) for cytoplasmic proteins and RNA. Insert E shows

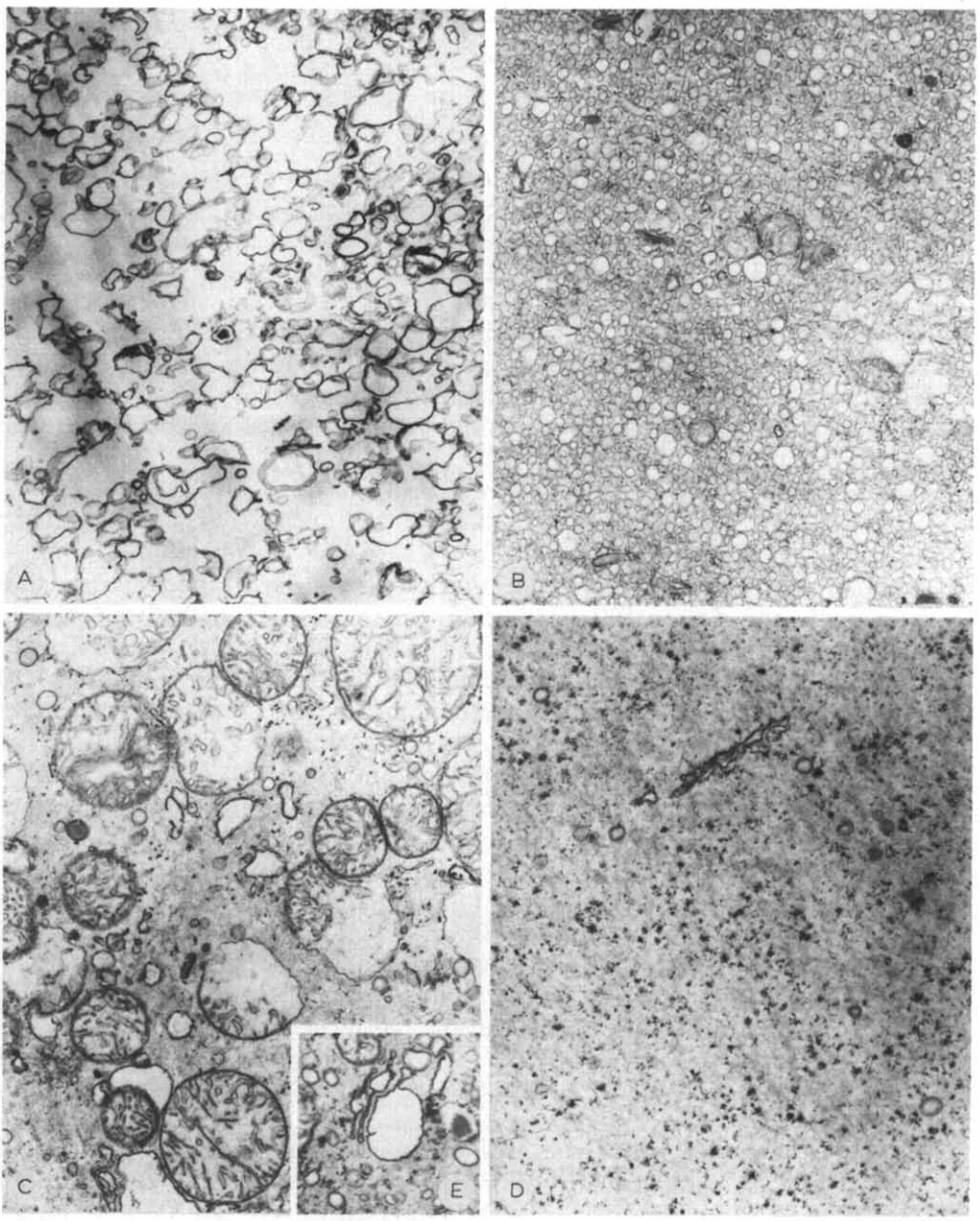

Fig. 5. Micrographs of selected gradient fractions representing the modal equilibrium densities of the major components. Fraction A, density 1.10 (plasma membrane); fraction B, density 1.16 (sarcoplasmic reticulum); fraction C, density 1.21 (mitochondria); fraction D, density 1.25 (sample zone). Insert E illustrates a t-tubule in the process of swelling into an electron-lucent vesicle. Magnification, ×13 230.

a t-tubule undergoing dilation to become an electron-lucent vacuole often seen in these fractions.

Discussion

Attempts to characterize subcellular organelles from skeletal muscle by zonal centrifugation techniques began with Schuel et al. [23] who, using zonal rotors for differential sedimentation, identified two distinct organelles. The larger (approx. $1 \cdot 10^4$ S) was associated with cytochrome oxidase activity (mitochondria) and the smaller (about $1 \cdot 10^3$ S) would cause suspended myofibrils to relax (sarcoplasmic reticular fragments). Later, other worker confirmed the existence of two organelle populations by similar rate-zonal centrifugation techniques, but they concluded that lysosomes and catalase-containing organelles could also be found in these samples, especially in the slower sedimenting one [24].

Since aggregation has been a recurrent problem during isolation of muscle membranes [25,26], the sedimentable acid hydrolase (lysosomal) activity might have resulted from adsorption to the sarcoplasmic reticulum and mitochondria. However, the selective alteration of the density of specific organelles by treatment with Triton WR-1339 [27], digitonin [14], as well as calcium uptake studies [24] would tend to disprove this criticism. Thus, the similarities in lysosomes and sarcoplasmic reticulum may indeed have resulted because some elements of the sarcoplasmic reticulum are also part of the vacuolar apparatus of skeletal muscle.

The results from the current experiments indicate that organelles can be separated with relatively high purity and yields from skeletal muscle. Furthermore, these organelles generally fall into four major categories: plasma membrane fragments, sarcoplasmic reticular fragments, mitochondria, and catalase-containing organelles. The category of sarcoplasmic reticular fragments can be further subdivided into calcium transport vesicles, acid hydrolase-containing vesicles, NADH oxidase-containing vesicles, and unassigned sarcoplasmic reticular vesicles. We feel that these results represent significant advances in state of the art of muscle cell fractionation and need to be discussed by category.

Plasma membrane

The marker enzymes for plasma membrane, alkaline phosphodiesterase and 5′-nucleotidase, had similar distribution patterns to those described for smooth muscle cells [28] and cardiac muscle [27]. However, the 30-fold purity achieved in the present quantitative fractionation studies was better than a 25-fold purity for 5′-nucleotidase obtained after a preparative approach consisting of four different centrifugation steps including one gradient separation [29]. The presence of acid *p*-nitrophenylphosphatase in these highly enriched plasma membrane fractions, the enzyme not being restricted solely to lysosomes, invalidates its use as a specific lysosomal marker enzyme. Similarly, the presence of leucyl-β-naphthylamidase in sarcoplasmic reticular fractions disqualifies its use as a plasma membrane marker enzyme in skeletal muscle tissue. On the other hand, acid phosphatase, measured by using β-gly-

cerophosphate as a substrate, recently has been shown to be localized in both sarcoplasmic reticulum and t-tubules which is in agreement with its two locales found in this study [30].

Although no specific enzymatic marker was used to localize t-tubules, morphological evidence (Fig. 5) suggests that the t-tubules swell into electron-lucent vesicles as a result of exposure to gradient materials and are, therefore, difficult to differentiate from other vesicles in gradient fractions. This swelling was similar to that seen in intact muscles exposed to hypertonic sucrose and may represent an interference in the relationship between opposing sides of the t-tubule. It is interesting that a similar finding of swollen t-tubules occurred in fatigued muscle and resulted in the cessation of the contractile response in vivo [31].

Sarcoplasmic reticulum

The occurrence of NADH oxidase and lysosomal hydrolases in vesicles that have sedimentation properties comparable to calcium transport vesicles supports an earlier hypothesis that the sarcoplasmic reticulum is a multifunctional tubular membrane system similar to that of smooth endoplasmic reticulum described for other tissues [32]. Moreover, an NADH-linked electron transport chain has been described [33] for slow and fast muscle sarcoplasmic reticular vesicles. The low activity of NADH oxidase is consistent with the small, but nevertheless measurable, activity associated with sarcoplasmic reticulum in this study.

Lysosomal enzymes have, for some years, been associated with sarcoplasmic reticular organelles in muscle, but because of their low activity relative to other tissues, they were assumed to be artifacts. However, Canonico and Bird [15] first established lysosomes as distinct organelles indigenous to skeletal muscle. Cytochemical studies in our laboratory recently resolved this problem by demonstrating that the sarcoplasmic reticular network lying in the region of the I band was reactive for lysosomal hydrolases [30]. In contrast, it has been documented that the longitudinal sarcoplasmic reticulum lying over the A band is principally involved in calcium sequestration [34]. Thus, we can account for the existence of two membrane vesicles which are morphologically indistinguishable, yet have similar sedimentation properties. Of the total population of sarcoplasmic reticular vesicles, only about one-third can be assigned to calcium transport [14,24,25]. Even if NADH oxidase vesicles and lysosomal vesicles represent entirely different populations, we are still left with approximately one-half of the total protein assigned to sarcoplasmic reticulum as yet functionally undefined.

Catalase-containing organelles

Additional oxidases have not been reported as being associated with catalase-containing vesicles in phasic muscle, and thus, peroxisomes cannot be considered to exist in this tissue. However, catalase was shown to be indigenous to skeletal muscle cells and it may be located in the cisternae of the sarcoplasmic reticulum. Christie and Stoward [35] have reported the terminal cisternae of the sarcoplasmic reticulum adjacent to t-tubules show peroxidative activity at pH 5.0. Furthermore, immunological localization of catalase [36] also con-

firmed a sarcoplasmic reticular locale. The role of catalase-containing organelles in non-oxidative muscles remains an unresolved question.

Mitochondria

Cytochrome oxidase showed the characteristic narrow band around a density of 1.21, which is slightly heavier than that reported in our earlier studies [24]. This may have resulted from the inclusion of larger or more dense mitochondria in our sample. In differential centrifugation procedures, many such mitochondria may be pelleted and lost in the nuclear fraction ($1000 \times g \cdot 10$ min) prior to gradient fractionation. In addition, less damage to mitochondria by homogenization would be expected due to the gentle techniques employed.

Cytosol

The cytoplasmic proteins such as phosphorylase *a*, pyruvate kinase, and myosin-ATPase were located together in the sample zone, as expected. The portion of aldolase activity that was not located in the sample zone may have been adsorbed to sarcoplasmic reticular membranes or, in fact, may have been an integral part of the sarcoplasmic reticulum [37]. Of the total RNA content, only 15% was associated with membranes, correspondingly, about 15% of the total RNA was reported to be membrane associated in rat muscle homogenates, but the RNA particles observed by morphological techniques did not seem to account for all the RNA found in the fractions [38]. We, too, failed to observe any rough microsomal membranes in any of our fractions, except occasionally in the sample zone.

In conclusion, more than 20 years of study have centerd around the physiological role of the sarcoplasmic reticulum in calcium transport, so specialized in phasic muscles. There has been almost total disregard of other possible functions of the reticulum, which might be similar to those of the endoplasmic reticulum found in other cells and equally important in the metabolism of normal and pathological muscles. Perhaps, the analytical fractionation techniques described in this study eventually will help to unravel the complexities of the endoplasmic reticulum of skeletal muscle tissue.

Acknowledgements

The authors express their appreciation to Anne-Marie Hedge for technical assistance, Francoise D. Stauber for the graphic presentations and Cynthia Martin for the manuscript preparation. Appreciation is also extended to Dr. Jerome J. Trout and Roger Ulrich for the micrographs of the fractions. This study was supported in part by a grant from the American Heart Association with local support from the Iowa Heart Association to B.A.S., by USPHS grant HL-25388 to W.T.S., and by a program project grant HL-14388 to the Cardiovascular Center, University of Iowa.

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