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An Isopycnic- Zonal Centrifugation Study of Smooth Muscle Organelles Isolated from Hog Carotid Artery (39071)

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Studies of calcium compartmentalization in smooth muscle organelles have been limited due to the operational methods of isolation of sarcoplasmic reticulum and mitochondria (1, 2). Although these methods give qualitatively excellent results their quantitation in respect to the whole cell is difficult. Since smooth muscle cells can now be isolated before fractionation (3), we employed these newer techniques to examine the distributions of sarcoplasmic reticulum fragments, lysosomes, catalase-containing organelles, mitochondria, and plasma membrane fragments in continuous sucrose gradients. This study confirms that smooth muscle organelles isolated from vascular tissue are heterogeneous and demonstrates that plasma membrane fragments and, to some extent, mitochondria can be separated from the other organelles.

Materials and methods. Hog carotid arteries (6–10 g) were obtained locally[3] from freshly slaughtered hogs. Fat and connective tissue were removed and the arteries were cut and placed in ice-cold complete Hank's solution for transport to the laboratory. There they were dissected and isolated cells were obtained by enzymatic digestion (3), omitting the hyaluronidase from the isolation mixture, since equally good results were obtained without its addition.

The washed cells isolated by this procecedure were disrupted in 0.25 *M* sucrose solution containing 0.02 *M* KCl with 25 strokes of a Type B pestle in a small Dounce homogenizer. This total homogenate was centrifuged at 600*g* for 10 min. The postnuclear supernatant (PNS) was removed and saved; the nuclear fraction (N) was resuspended in 0.25 *M* sucrose containing 0.02 *M* KCl.

The postnuclear supernatant (PNS) was adjusted to a sucrose concentration of 22% (w/v) and a volume of 160 ml and used as the light limiting solution in the construction of a 22–65% linear sucrose gradient that was pumped into a Z-60 zonal rotor (Beckman) spinning at 3000 rpm. After the loading had been completed, the rotor was accelerated to 50,000 rpm (Model L centrifuge, Beckman) for a total centrifugal effect of 33.0 rad^2/nsec. The rotor was kept at a constant 15° to avoid artifacts (4). Unloading of the rotor was at 3000 rpm and 22 15-ml fractions were collected. A 12 ml aliquant of each fraction was added to 10 ml of distilled water to reduce the viscosity and centrifuged in a Lourdes LRA centrifuge for 30 min at 30,000*g*. The supernatants were pipetted off and discarded; the pellets were resuspended in 0.25 *M* sucrose containing 0.02 *M* KCl and used for analysis.

Sucrose concentrations from the remaining 3 ml portion of the fractions were read on an Abbe Refractometer.

Cytochrome oxidase (EC 1.0.3.1.), cathepsin D (EC 3.4.4.23), and *p*-nitrophenyl phosphatase, *p*NPPase, (acid phosphatase EC 3.1.3.2.) were assayed by methods reported previously (5). *N*-acetyl-β-glucosaminidase (EC 3.2.2.30) was determined by enzymatic hydrolysis of *p*-nitrophenyl from (0.02 *M*) *p*-nitrophenyl-*N*-acetyl-β-glucosamide in 0.1 *M* acetate buffer at pH 5.0. The reaction was stopped with 1.25 *N* NaOH, filtered (Whatman #42), and the absorbance read at 440 nm on a Gilford (Model 300) spectrophotometer. Catalase (EC 1.11.1.6) was assayed by the method of Baudhuin *et al.* (6). The Ca-activated ATPase (EC 3.6.1.4.) was determined at 37° by the method

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of Greaser *et al.* (7). 5′-nucleotidase (EC 3.1.3.5.) was measured by the Amar-Costesec *et al.* method (8). Inorganic phosphate was determined at 27° by the method of Marinetti *et al.* (9). Protein equivalents were approximated as described by Lowry *et al.* (10) with human albumin (Sigma Chemical Co.) as the protein standard.

Ca-uptake was determined by the millipore filtration method of Martinosi and Feretos (11) using 0.45 μm Gellman filters. The incubation solution, pH 6.8, contained 0.020 m*M* $CaCl_2$ labelled with ^{45}Ca, 5 m*M* Mg-ATP, 0.1 *M* KCl, 5 m*M* potassium oxalate, 5 m*M* imidazole buffer. Incubation times were 10 min, which assured saturation.

Results from isopycnic-zonal centrifugation data are graphed by a modification of the Bowers and de Duve method (12). In this, the experimental results are presented as frequency histograms bounded on either side by arbitrary limits that refer to material accumulating above and below the desired limits of the gradient. The surface area of each block is the normalized percentage of enzymatic activity in each fraction, and the total surface area represents 100%. Median density represents the calculated density at which 50% of the enzyme activity, calcium uptake, or protein concentrations lie on either side. Modal density is the average density of the most frequently occurring fraction.

Enzyme recoveries are calculated from the sum of the activities in each of the gradient fractions divided by the sum of the activities in the nuclear fraction (N) plus the postnuclear supernatant (PNS).

Results. Distribution patterns for all enzymes, calcium uptake, and sedimentable proteins after isopycnic-zonal centrifugation are shown in Fig. 1. Cytochrome oxidase has the typical narrow distribution characteristic of mitochondria with a modal density of 1.172. The lysosomal enzymes, cathepsin D and pNPPase, have similar distributions with a modal density of 1.147 while *N*-acetyl-β-glucosaminidase distribution is close to that of the Ca-ATPase, having a modal density of 1.171. Catalase is distributed rather symmetrically around a modal density of 1.159, whereas 5′-nucleotidase is skewed towards the light end of the gradient with a modal density of 1.110. Ca-uptake activity occurred in particles that equilibrated about a modal density of 1.147, and protein concentration was distributed around a 1.171 modal density.

From the median densities (Table I) for these components, four groups can be characterized: (a) cytochrome oxidase with a median density of 1.175, (b) 5′-nucleotidase with a median density of 1.132, (c) proteins with a 1.169 median density and (d) the other components which equilibrated in the 1.15–1.16 region.

The apparent recovery of these components over the total homogenate is given in Table II. Approximately 20–30% of the cells' activity has been recovered in the twenty-two gradient fractions and about 7% of the protein. This represents a relative enrichment of organelles of about three- to fourfold as compared to the total unfractionated homogenate.

Calcium uptake values per mgm protein concentration are given in Table III. The peak calcium uptake value was 100.2 n*M* calcium/mg protein at an equilibrium density of 1.147. The values for the nuclear fraction (N) and post nuclear supernatant (PNS) were 17.8 and 23.1 n*M* calcium/mg protein, respectively. This indicates about a five-fold increase in activity at the modal density.

Discussion. The experiments described in this study demonstrate that smooth muscle from hog carotid artery can be fractionated and evaluated for sarcoplasmic reticular membranes, lysosomes, catalase-containing organelles, mitochondria, and plasma membrane fragments in continuous sucrose gradients. Peters and coworkers (3) comprehensively evaluated rabbit aortic smooth muscle organelles, but did not find a reliable marker enzyme unique to the sarcoplasmic reticulum. Our findings are generally consistent with theirs, except for some slight differences in the distributions of some organelles.

Mitochondria. Cytochrome oxidase shows the narrow equilibrium profile characteristic of mitochondria with a median density of 1.175. This density is greater than EDTA-

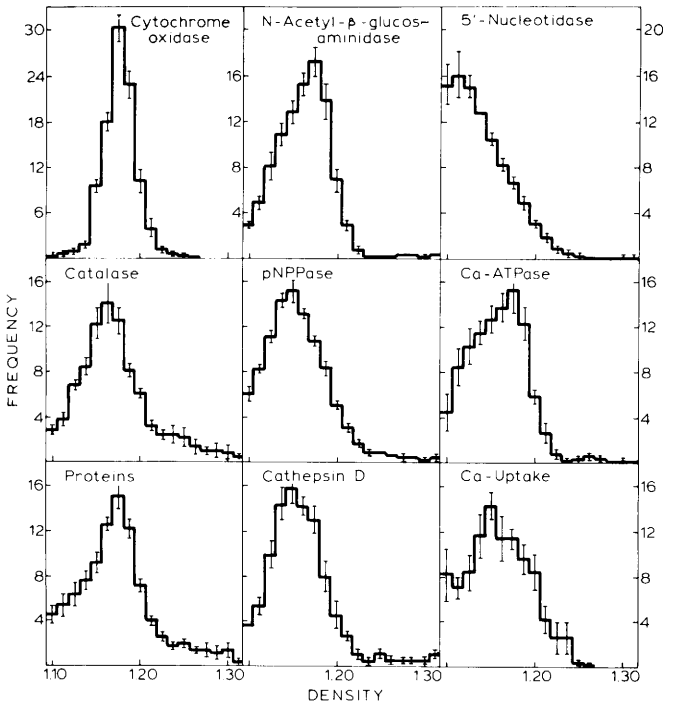

FIG. 1. Frequency distribution profiles for enzymes, calcium uptake and protein content. Data is plotted mean frequency ± standard error for each density interval (12). Number of experiments used to determine each point are given in Table II.

sucrose prepared mitochondria from rat skeletal muscle (1.172), but lighter than mitochondria prepared in sucrose alone (1.182) (13). However, aortic smooth muscle mitochondria prepared by the same technique, but in EDTA-sucrose, gave similar values (1.17) (3). The amount of calcium ions in the mitochondria may have been altered due to the isolation procedure for smooth muscle cells, either by depolarization or altered mitochondrial function.

Catalase-containing organelles. Catalase containing organelles had similar distributions to fraction A organelles isolated from rat skeletal muscle (13), but differed from catalase profiles from aortic smooth muscle (3). This probably reflects the differences in fractionation techniques and homogenization media, but confirms the presence of sedimentable particles with catalase activity.

Plasma membranes. The distribution of 5'-nucleotidase corresponds well to that described by Peters *et al.* (3) for plasma membranes from aortic smooth muscle and demonstrates that plasma membrane fragments can be partially separated from other organelles in sucrose gradients. Presence of some calcium uptake ability in this region

TABLE I. EQUILIBRIUM DENSITIES.[a]

		Modal ρ	Median ρ
Cytochrome oxidase	(7)	1.172	1.175 ± .001
Catalase	(5)	1.159	1.164 ± .003
5'-nucleotidase	(3)	1.110	1.132 ± .003
*p*NPPase	(4)	1.147	1.149 ± .002
Cathepsin D	(5)	1.147	1.154 ± .002
N-acetyl-β-glucos-aminidase	(3)	1.171	1.161 ± .004
Ca-ATPase	(4)	1.171	1.155 ± .005
Calcium uptake	(6)	1.147	1.154 ± .004
Protein	(6)	1.171	1.169 ± .005

[a] Values represent means ± standard error of the median density for each component. Modal density is the density of the most frequently occurring peak. Numbers in parentheses indicate the number of experiments.

TABLE II. PERCENT OF TOTAL ACTIVITY.[a]

		Recoverable PNS (%)	Recoverable gradient (%)
Cytochrome oxidase	(7)	64.6 ±7.1	24.3 ±2.1
Catalase	(5)	68.9 ±8.9	31.5 ±8.2
5′-nucleotidase	(3)	71.3 ±12.3	33.6 ±4.3
pNPPase	(4)	43.4 ±3.1	25.3 ±6.8
Cathepsin D	(5)	67.5 ±8.1	18.1 ±8.8
N-acetyl-β-glucosaminidase	(3)	79.0 ±15.7	25.7 ±5.1
Ca-ATPase	(4)	62.4 ±9.7	28.3 ±5.8
Calcium uptake	(6)	66.7 ±6.1	15.4 ±2.5
Protein	(6)	48.8 ±8.0	6.7 ±1.0

[a] Values represent means ± standard errors. Total activity = sum of activity in the Nuclear fraction (N) and postnuclear supernatant (PNS). Gradient values represent the sum of the activity in the 22-gradient fractions as a percent of the total activity. Numbers in parentheses indicate the number of experiments.

TABLE III. TOTAL CALCIUM UPTAKE.[a]

Fraction	n*M* Ca^{2+}/mg Pr/10 min
N	17.8 ± 4.7
PNS	23.1 ± 3.6
Modal peak	100.2 ± 8.3

[a] Values represent the means ± standard errors of six experiments. N = nuclear fraction, PNS = postnuclear supernatant, modal peak = gradient fraction with the greatest calcium accumulating content.

of the gradient agrees with the subfractionation studies of "microsomal" fractions from aorta where calcium uptake ability was present under the 5′-nucleotidase peak (2).

Lysosomes. The characterization of lysosomes from normal aortic smooth muscle cells have been extensively evaluated (3). Present results agree that all lysosomal enzymes studied equilibrate between a 1.15 and 1.16 median density. However, *N*-acetyl-β-glucosaminidase differed in its distribution from cathepsin D and *p*NPPase. This resembled the difference in acid phosphatase and *N*-acetyl-β-glucosaminidase observed in aortic smooth muscle. Two groups of lysosomes have been described for rat skeletal muscle. One group contained cathepsin D and *p*NPPase; the other contained acid ribonuclease, β-glucuronidase and arylsulfatase (5). It appears that lysosomes from hog carotid artery smooth muscle are similarly heteregeneous.

Sarcoplasmic reticulum. Morphologically, the sarcoplasmic reticulum has been shown to be organized in mammalian smooth muscle to function as a calcium store (14). Moreover, a calcium-activated pump which is capable of reducing calcium levels has been described for aortic smooth muscle (1, 2). The calcium uptake values measured for the modal peak of calcium uptake ability are consistent with those described for rat vascular smooth muscle (15). Nevertheless, the Ca-ATPase activity reflected mutiple sources of enzymatic activity such as mitochondrial, plasma, and sarcoplasmic reticular membranes and thus did not uniquely define the sarcoplasmic reticulum.

The presence of sarcoplasmic reticulum, lysosomes, and catalase-containing organelles in the same region of the gradient implied a heterogeneity of the sarcotubular system in muscle tissue. Recent histochemical findings have demonstrated a sarcoplasmic reticular location for lysosomal enzymes in cardiac tissue (16) and catalase in skeletal muscle tissue (17). Subfractionation studies of rat skeletal muscle organelles (13) have suggested that the reticulum in muscle tissues, besides its role in excitation-contraction coupling, may also be a multicompartmental system of enzymes.

1. Fitzpatrick, D. F., Landon, E. J., Debbas, G., and Hurwitz, L., Science **176,** 305 (1972).
2. Hurwitz, L., Fitzpatrick, D. F., Debbas, G., and Landon, E. J., Science **179,** 384 (1973).
3. Peters, T. J., Müller, M., and de Duve, C., J. Exp. Med. **136,** 1117 (1972).
4. Wattiaux-DeConninck, S., Ronveaux-Dupal, M., Dubois, F., and Wattiaux, R., Eur. J. Biochem. **39,** 93 (1973).

5. Canonico, P. G., and Bird, J. W. C., J. Cell Biol. **45,** 321 (1970).
6. Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O. Z., Wattiaux, R., Jacques, P., and de Duve, C., Biochem. J. **92,** 179 (1964).
7. Greaser, M. L., Cassens, R. G., Hoekstra, W. G., and Briskey, E. J., J. Food Sci. **34,** 633 (1969).
8. Amar-Costesec, A., Beaufay, H., Feytmans, E., Thines-Sempoux, D. and Berthet, J., *in* "Microsomes and Drug Oxidations" (J. R. Grillette, A. M. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts and G. J. Mannering, eds.), p. 41, Academic Press, New York (1960).
9. Marinetti, G. V., Albrecht, M., Ford, T., and Stotz, E., Biochim. Biophys. Acta **36,** 4 (1959).
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).
11. Martinosi, A., and Feretos, R., J. Biol. Chem. **239,** 648 (1964).
12. Bowers, W. E., and de Duve, C., J. Cell Biol. **32,** 349 (1967).
13. Stauber, W. T., and Bird, J. W. C., Biochim. Biophys. Acta **338,** 234 (1974).
14. Devine, C. E., Somlyo, A. V., and Somlyo, A. P., J. Cell Biol. **52,** 690 (1972).
15. Webb, R. C., and Bhalla, R. C., J. Cell. Mol. Card., **7,** (1975).
16. Hoffstein, S., Streuli, F., Hirsch, J., Fox, A. C., Gennaro, D. E., and Weissmann, G., J. Cell Biol. **63,** 142a (1974).
17. Hand, A. R., J. Histochem, Cytochem. **22,** 207 (1974).

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