LYSOSOMES IN SKELETAL MUSCLE TISSUE

Zonal Centrifugation Evidence
for Multiple Cellular Sources

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ABSTRACT

Postnuclear supernates from homogenates of skeletal muscle from rats subjected to starvation, injections of Triton WR-1339, dextran-500, and dextran + corticosterone were fractionated by means of rate and isopycnic zonal centrifugation in sucrose—0.02 M KCl gradients. Zonal fractions were analyzed for protein, RNA, cytochrome oxidase, and up to six acid hydrolases. The results indicate the presence of two groups of lysosome-like particles. One group contributes approximately 95% of the cathepsin D and acid phosphatase activity and 75% of the acid ribonuclease, β-glucuronidase, and arylsulfatase activity in muscle. It is characterized by a modal equilibrium density of 1.18 that is decreased by starvation, but is not shifted by dextran-500 or Triton WR-1339. The second group has a higher proportion of acid ribonuclease, β-glucuronidase, and arylsulftase; the equilibrium density can be shifted by dextran-500 and Triton WR-1339. It is suggested that this group of lysosomes is derived from macrophages and other connective tissue cells, whereas the former group represents lysosome-like particles from muscle cells.

INTRODUCTION

Tissue fractionation studies have established the presence of subcellular particles in normal skeletal muscle tissues having the biochemical and physical properties of lysosomes (3–5, 9, 23, 26, 37, 42). These studies, however, have been unable to establish the cellular origins of the particles. Morphological studies, on the other hand, have recognized lysosomes in myopathic cells, but consistently reported the conspicuous absence of lysosomes in normal skeletal muscle fibers (19, 25, 32, 38). Hence, a controversy has arisen concerning the presence of lysosomes in the normal fibers. The finding that skeletal muscle has a low specific activity of acid hydrolases has furthered the hypothesis that the activity in muscle tissues is derived from lysosomes of macrophages and connective tissue cells (34, 36).

This paper describes experiments designed to differentiate the contribution of lysosomes from various possible cellular sources in skeletal muscle tissues. By determining the distribution of acid ribonuclease, cathepsin D, β-glucuronidase, arylsulfatase, and acid phosphatase after rate and isopycnic zonal fractionation of a postnuclear supernate, two distinct sources of lysosomes have been resolved in normal skeletal muscle tissues.

MATERIAL AND METHODS

The following materials were obtained from Sigma Chemical Co. (St. Louis, Missouri): cytochrome c (type III), p-nitrophenylphosphate disodium tetrahydrate, yeast RNA (grade VI), bovine hemoglobin (type II), p-nitrocatechol sulfate dipotassium, phenolphthalein glucuronic acid, and dextran-500. *Micrococcus lysodeikticus* cells were obtained from the Worth-

ington Biochemical Corp. (Freehold, N. J.). Corticosterone was purchased from the Nutritional Biochemicals Corporation (Cleveland, Ohio) and Triton WR-1339 from Ruger Chemical Co. (Irvington on Hudson, N. Y.).

All experiments were performed on muscles from male Sprague Dawley rats, 200–250 g, maintained on Purina rat pellets. Animals were subjected to four different experimental treatments. One group was injected intraperitonealy with 20% dextran-500 in saline. The animals received at 5 ml injection for 4 consecutive days, and were sacrificed on the 5th day. A second group of dextran-500 treated animals received six 15 mg doses of corticosterone suspended in water and injected subcutaneously. Two injections of corticosterone were administered 12 hr apart on the first 2 days and single injection on each of the next 2 days. The animals were sacrificed on the 5th day. A third group of animals received a single intracardial injection of Triton WR-1339, diluted 1:4 (w/v) with saline, at a dosage of 850 mg/kg of body weight. These animals were sacrificed 4½ days later. The final group of experimental animals were fasted for 6 days and provided with tap water and normal saline for drinking.

All animals were sacrificed by decapitation. The lower leg and thigh muscles, except the soleus, were excised, chilled, and rinsed in 0.25 M sucrose containing 0.02 M KCl, and homogenized according to the method of Canonico and Bird (9). 0.02 M KCl was added to the homogenization media and sucrose gradients to minimize agglutination of particles. Preliminary experiments indicated that higher concentrations of KCl caused a congealing of muscle homogenates when used in association with 0.25 M sucrose.

Samples for isopycnic zonal centrifugation experiments were prepared by centrifuging a 20% (w/v) muscle homogenate, prepared from approximately 50 g of muscle, at 700 g for 10 min to remove the nuclear fraction and cellular debris usually associated with this fraction. This step was necessitated because the particulate matter constituting the nuclear fraction was found to occlude the entrance and exit channels of the zonal rotor. The nuclear pellet was washed by resuspension in 0.25 M sucrose-KCl to one-half the original volume and rehomogenization in a loose fitting, motor driven, Duall homogenizer (Kontes Glass Co., Vineland, N. J.) by three up and down strokes. This solution was readjusted to pH 7.2 by the addition of KOH, and centrifuged at 1000 g for 10 min. The postnuclear supernatants were pooled, and the washing procedure was repeated until a total volume of 450 ml of supernatant was obtained. This procedure yielded a postnuclear fraction containing nearly 80% of the total acid hydrolase activity of the muscles.

To the postnuclear supernatant sample sufficient 50% (w/v) sucrose was added to form a solution having a density of 1.1175 (28% sucrose). This solution was used in concert with an 80% (w/v) sucrose solution to construct a 30–50% linear sucrose gradient, using the gradient forming apparatus described by Anderson and Rutenberg (1). 600 ml of the gradient were pumped via the rim line at a rate of 30 ml/min into a Spinco B-XIV zonal rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) spinning at 3000 rpm. The remaining rotor volume was filled with a 50 ml cushion of 55% (w/v) sucrose. The incorporation of the muscle sample within the body of the gradient insured that a sufficient titer of sedimentable acid hydrolases would be available for reliable assays.

After loading, the rotor was accelerated to 32,000 rpm for approximately 4 hr. The integrated cen-
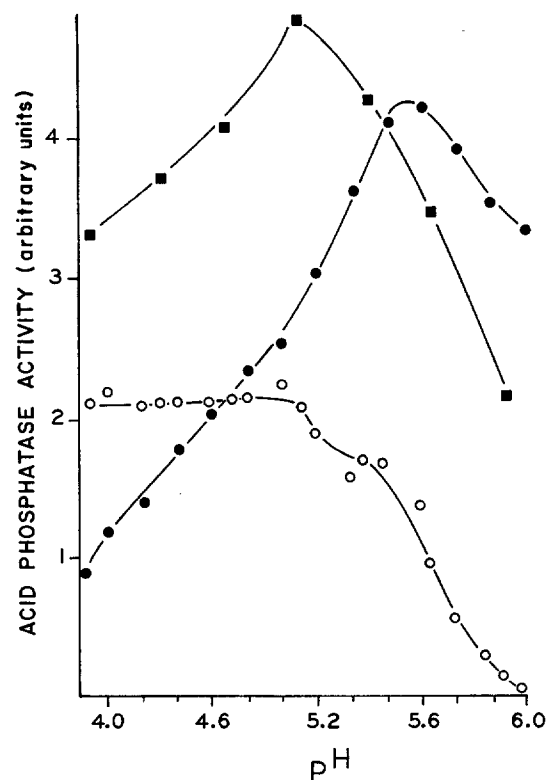

FIGURE 1 Acid phosphatase activity as a function of pH. Light mitochondrial fraction O——O; microsomal fraction ■——■; and soluble fraction O—O—O used in this study were isolated from a 10% muscle homogenate by the method of Canonico and Bird (9). Acid phosphatase activity was determined with p-nitrophenylphosphate as substrate and 0.12 M acetate buffer at designated pH values.

TABLE I

Incubation Conditions for Enzyme Assays

Enzyme (E.C.)	Final volume	Substrate final concentration	Buffer (pH)	Incubation at 37°C
	ml			*hr*
Acid phosphatase (3.1.3.2)	1.0	16 mM p-nitrophenylphosphate	0.12 M acetate (5.0)	1–2
Acid ribonuclease (2.7.7.16)	1.0	0.125% RNA*	0.16 M acetate (5.0)	8–10
Arylsulfatase (3.1.6.1)	1.0	10 mM nitrocatechol sulfate	0.08 M acetate (5.0)	8–12
β-glucuronidase (3.2.1.31)	1.0	1 mM phenolphthalein β-glucuronide	0.16 M acetate (5.0)	12–16
Cathepsin D (3.4.4.b)	2.0	2.0% hemoglobin‡	1.35 M acetate (3.8)	4–6
Cytochrome oxidase (1.9.3.1)	2.5	4.0 mg% reduced cytochrome c	0.03 M phosphate (7.4)	
Lysozyme (3.2.1.17)	3.0	8.0 mg% *Micrococcus lysodeikticus*	0.1 M phosphate (7.4)	8

* Dialyzed for 48 hr in the cold against 0.16 M acetate buffer, pH 5.0.
‡ Dialyzed for 48 hr in the cold against H_2O.

trifugal effect on the muscle sample was determined with a Beckman digital integrator, and the centrifuge was programmed to decelerate to 3000 rpm when the integral of $\omega^2 dt$ = 1.54 × 10^{11} rad²/sec. 26 25-ml fractions were collected at a rate of 25–30 ml/min by pumping 65% (w/v) sucrose through the rim line, and an aliquot of each fraction was measured for its refractive index (at 25°C) with an Abbe-3L-refractometer (Bausch & Lomb Inc., Rochester, N. Y.) for calculation of density. 20 ml of cold distilled water was added to a 10 ml aliquot from each fraction in order to lower the density of the sample and permit the sedimentation of particulate matter. The samples were centrifuged at 28,000 g for 20 min, the supernatant was discarded, and each pellet was resuspended in 4 ml of cold glass-distilled water containing 0.2% Triton X-100.

The reasons for isolating the particulate matter in each of the 26 fractions were the following: (a) to concentrate the particular acid hydrolase activity in each fraction; (b) to remove solubilized enzymes resulting from the labilization of lysosomes during the rigorous homogenization procedure, thus restricting enzymatic analyses to structure-linked sedimentable activity; (c) to remove the sucrose, present in varying concentrations in each fraction, which interferes with the analysis of β-glucuronidase and cathepsin D; and (d) to isolate the particulate matter in each fraction making it possible to analyze a structural-linked acid phosphatase with p-nitrophenylphosphate as the substrate. This is important in muscle because we find that the usual lysosomal

acid phosphatase, specific for β-glycerophosphate, has quite minimal activity in skeletal muscle. The atypical subcellular distribution of lysosomal acid phosphatase observed in tissue fractionation studies when the enzymatic activity is analyzed with p-nitrophenylphosphate as substrate (9) is attributed to the nonspecificity of the substrate and the presence of multiple nonlysosomal forms of acid phosphatase (20, 29). In the present study, however, the p-nitrophenylphosphatase assay is appropriate as a lysosomal acid phosphatase marker, since determinations were made on particulate mitochondrial fractions having little contamination from microsomal and cytoplasmic forms of the enzymes. The pH activity curves of p-nitrophenylphosphatase from three subcellular fractions (Fig. 1) support this conclusion by demonstrating that the multiple forms of the enzymes can be separated by simple differential centrifugation.

The techniques for rat zonal fractionation of our muscle tissues were exactly as reported previously (10).

Analyses of enzymatic activity were performed by modification of techniques described in previous papers (4, 26) and are summarized in Tables I and II. Lysozyme activity was analyzed by the method of Shibko and Tappel (30), modified by incubating the assay mixtures for 8–10 hr at 37°C. RNA determinations were made according to the method of Wannamacher et al. (39), and protein concentrations were measured according to Lowry et al. (18), using bovine serum albumin as the standard.

Results from rate zonal centrifugation experiments

TABLE II

Analytical Procedures for Enzyme Assays

Enzyme	Manipulations	Analysis
Acid phosphatase	Add 3 ml of 1.5 N NaOH, centrifuge	Colorimetric for nitrophenol (420 nm)
Acid ribonuclease	Add 1 ml of 10% PCA + 0.25% uranyl acetate, after 1 hr centrifuge, dilute aliquot 1:20 with glass-distilled H_2O	UV absorption at 260 nm
Arylsulfatase	Add 1 ml of 5% TCA, centrifuge, transfer 1 ml aliquot into 1.5 ml NaOH (2.5 N)	Colorimetric for nitrocatechol (515 nm)
β-glucuronidase	Add 3 ml of glycine-NaCl-Na_2CO_3 mixture, pH 10.7, centrifuge	Colorimetric for phenolphthalein (540 nm)
Cathepsin D	Add 4 ml of cold 5% TCA, filter in cold after 30 min, transfer 1 ml of filtrate into 2 ml NaOH (0.5 N)	Colorimetric at 660 nm 5 min after addition of 0.6 ml Folin-Ciocalteu reagent (0.67 N)
Cytochrome c		Rate of change in absorption of 550 nm for 4 min
Lysozyme		Rate of change in absorption at 450 nm, determined by measuring absorption at regular intervals during incubation

TCA, trichloroacetic acid; PCA, perchloric acid.

are expressed as the relative specific activity in each fraction versus the fraction number. The gradient cushion is represented by the last three fractions and its enzymatic activity is expressed as an average over fractions 24–26.

Isopycnic zonal centrifugation results are represented here by a modification of the method of Bowers and de Duve (6). The experimental results are presented in the form of density distribution histograms, divided into 18 equal sections of density span equal to 0.05, and flanked on either end by blocks of arbitrary density span ($\Delta\rho = 0.1$) referring to material accumulating below and above the limits of the gradient. The blocks on either end of the histograms were used to normalize slight variations in the absolute limits of the gradient and permit the comparison and averaging of results obtained from different experiments.

RESULTS

Rate Zonal Fractionation

Fig. 2 shows the distribution pattern observed for particulate enzymes and protein from control rat muscle after rate zonal fractionation of a post-nuclear supernate. The distribution pattern of the particulate lysosomal enzymes is bimodal with a major, slow sedimenting peak around

fraction 9, henceforth referred to as the A peak, and a smaller, faster sedimenting peak found against the cushion, fractions 24–26. The distribution of mitochondria, as indicated by cytochrome oxidase activity, was distinctly different from that of the acid hydrolases, with nearly 60% of the cytochrome oxidase activity found in the cushion. The observed acid hydrolase activity in the cushion might be due to the sedimentation of large, relatively dense lysosomes, agglutination of lysosomes, or absorption of lysosomes to mitochondria or residual material from the nuclear fraction. In any event, the results obtained from normal animals in the control experiments indicated a heterogeneity in the distribution of the acid hydrolases, since 30% of the total activity of RNase, β-glucuronidase, and arylsulfatase was found in the cushion as compared to 18% of the total cathepsin and acid phosphatase activity.

The influence of injected dextran-500 on the sedimentation characteristics of acid hydrolases is shown in Fig. 3. The results show an alteration in the distribution histograms of RNase, β-glucuronidase, and arylsulfatase as reflected by a significant increase in the activity of the enzymes in the cushion. The A peak for these enzymes,
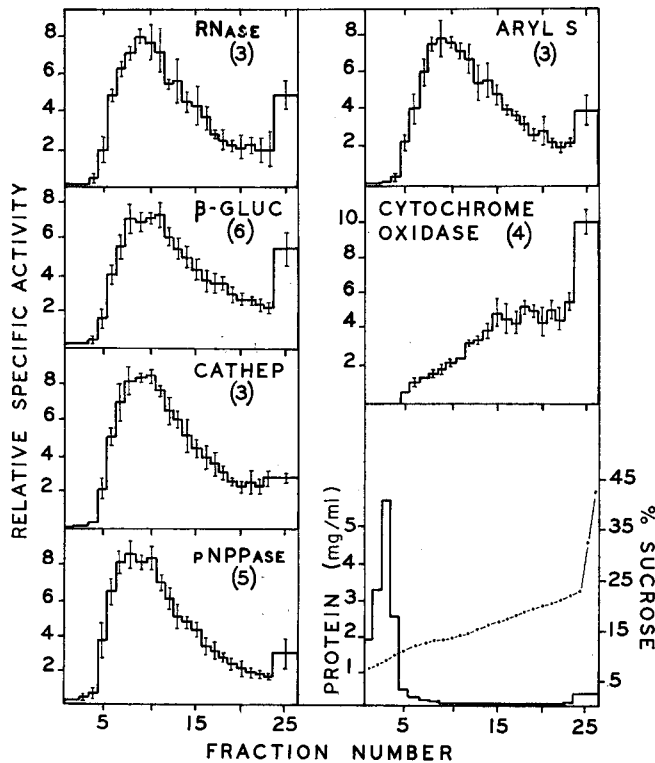
FIGURE 2   Distribution pattern of enzymes and protein after fractionation of postnuclear muscle homogenates by rate zonal centrifugation. Centrifugation conducted at 10,000 rpm for 1 hr $(\omega^2 t = 420 \times 10^7 \text{ rad}^2/\text{sec})$. KCl (0.02 M) was added to gradient and sample. Diagrams show average of results with standard error of the mean and number of experiments between parentheses. Ordinate is the relative specific activity in each fraction calculated by the method of de Duve et al. (1955). The activity in the cushion is represented as the average activity of fractions 24–26. Average per cent recovery of sedimentable activities was 67 for cathepsin D (cathep), 82 for acid phosphatase (p-NPPase), 69 for β-glucuronidase (β-gluc), 67 for acid ribonuclease (RNase), 76 for arylsulfatase (aryl s), and 71 for cytochrome oxidase.

while reduced in activity, remained stationed around fraction 9. On the other hand, the sedimentation characteristics of cathepsin and acid phosphatase were not affected by the administration of dextran-500.

When animals were treated with corticosterone in addition to dextran-500 the resulting distribution histograms (Fig. 4) for cathepsin and acid phosphatase showed a minor decrease in the relative specific activity of the A peak. There was however, a moderate increase in the relative specific activity of both enzymes in the latter half of the histograms. The distribution patterns of RNase, β-glucuronidase, and arylsulfatase were distinctly different from those of cathepsin and acid phosphatase. The A peaks for these enzymes were markedly reduced and a new peak around fraction 15–17 was observed. An increase in the total enzymatic activity of all five enzymes in the cushion was also found. The increase over controls was 38% for RNase, β-glucuronidase, and arylsulfatase and 30% and 25% for cathepsin and acid phosphatase, respectively.

Corticosterone was used in an attempt to modify the cellular composition of muscle tissues (16, 31).

Because little is known of the effects of high doses of glucocorticoids in skeletal muscle relative to the hydrolases in this tissue, the results of this experiment are difficult to interpret in terms of the cellular origins of the observed hydrolase peaks. Corticosterone administration produces a dramatic decrease in the total hydrolase activity of muscles (3). If this reduction is due to a loss of acid hydrolase from muscle cells, then the effect would be to minimize the contribution of hydrolases from muscle cells and emphasize possible alterations in the sedimentation rates of lysosomes from phagocytic cells due to their capability of storing dextran-500. This assumption seems to be supported by the experimental results. However, high doses of glucocorticoids have also been reported to inhibit endocytosis that may have prevented the accumulation of dextran-500 by phagocytic cells (12). Glucocorticoids while decreasing the number of circulating lymphocytes have also been shown to increase the macrophage population of muscle tissues (15). Consequently, the net effect of corticosterone on the acid hydrolase titers contributed by various possible cellular sources cannot be accurately estimated. These
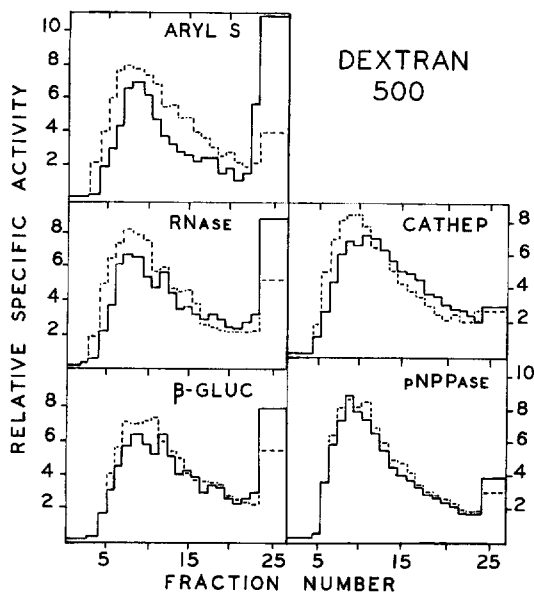
FIGURE 3 Influence of dextran-500 on the rate zonal sedimentation distribution pattern of muscle acid hydrolases. Animals were injected with dextran-500 (400 mg/100 g body weight) for 4 consecutive days and were sacrificed on the 5th day. Experimental distribution patterns represent the average of two experiments. Dotted lines show normal distributions.

factors make interpretation of the experimental results unclear with respect to the origin of the lysosomes responsible for the observed peaks.

The results of the above experiments, however, do confirm the heterogeneous distribution of muscle acid hydrolases and further suggest that the observed heterogeneity is due to the presence of two distinct groups of lysosomes. One group of lysosomes, whose distribution pattern seems affected by the administration of corticosterone, is rich in RNase, β-glucuronidase, and arylsulfatase, and is capable of increasing its sedimentation rate by the accumulation and storage of dextran-500. A second group proportionately richer in cathepsin and acid phosphatase does not accumulate dextran-500 and seems little affected by corticosterone administration.

The accumulation of Triton WR-1339 normally produces larger but less dense lysosomes (40). In experiments with Triton WR-1339-injected animals (Fig. 5) the distribution peak of acid hydrolase activity was not significantly different from the control values, probably because any increase in the size of the lysosomes was offset by a

decrease in density. In the cushion, however, there was a decrease in the activity of RNase, β-glucuronidase, and arylsulfatase.

Starvation, like Triton WR-1339, causes an increase in the size of liver lysosomes (35). In muscle, fluorescence microscopy of fresh frozen tissue sections vitally stained with acridine orange has demonstrated the presence of large orange fluorescent granules in muscle fibers of starved animals that were rarely seen within the muscle fibers of control animals (10). Thus, it seems that starvation, like vitamin E deficiency (38) or denervation (25), induces ultrastructural alterations resulting in the formation of large secondary lysosomes. These lysosomes would be expected to have faster rates of sedimentation; however, Fig. 6 demonstrates that the sedimentation pattern of acid hydrolases from starved animals did not differ greatly from control values. One can only conclude that an increase in the size of muscle fiber lysosomes occurring during starvation must be accompanied by a decrease in density, offsetting
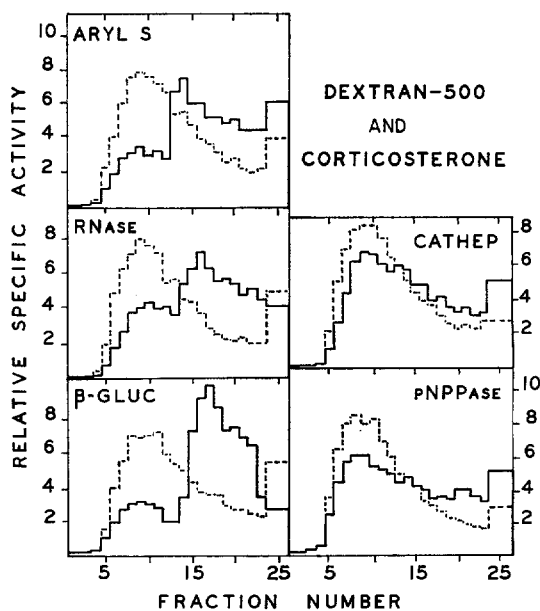

FIGURE 4 Influence of dextran-500 and corticosterone on the rate zonal sedimentation distribution pattern of muscle acid hydrolases. Animals were treated with dextran-500 (400 mg/100 g body weight) and corticosterone (15 mg) for 4 consecutive days as described in text. Experimental distribution patterns represent the average of two experiments. Dotted lines show normal distributions.

the effects of an increased particle size on the sedimentation rate.

Isopycnic Zonal Fractionation

The equilibrium density distribution histograms of five muscle acid hydrolases after isopycnic centrifugation demonstrate a broad distribution with a modal equilibrium density of 1.18 (Fig. 7). The heterogeneity in distribution of the acid hydrolases is demonstrated by the fact that while cathepsin and acid phosphatase histograms are skewed toward lighter densities, RNase, β-glucuronidase, and arylsulfatase are skewed toward the denser portions of the gradient. Cytochrome oxidase is concentrated within a narrow density span with a modal density slightly greater than 1.18. The broad distribution pattern of sedimentable protein reflected a pattern similar to that obtained from the lysosomal enzymes. The distribution of total protein, which included the sedimentable and unsedimentable protein in the gradient, is nearly uniform between densities 1.14 and 1.18, then declines steadily to a concentration of 4 mg/ml in the cushion. Lysozyme, a lysosomal

FIGURE 6 Effect of starvation on the sedimentation distribution pattern of rat muscle acid hydrolases. Animals were fasted for 6 days but provided with both tap water and normal saline. Experimental distribution patterns represent the average of two experiments. Dotted lines show normal distributions.
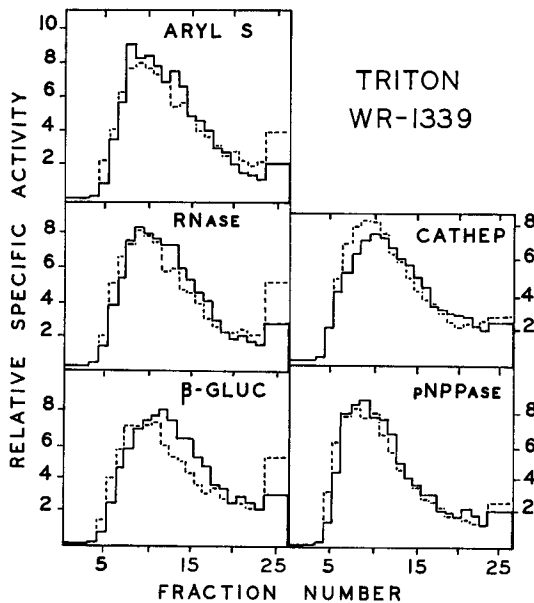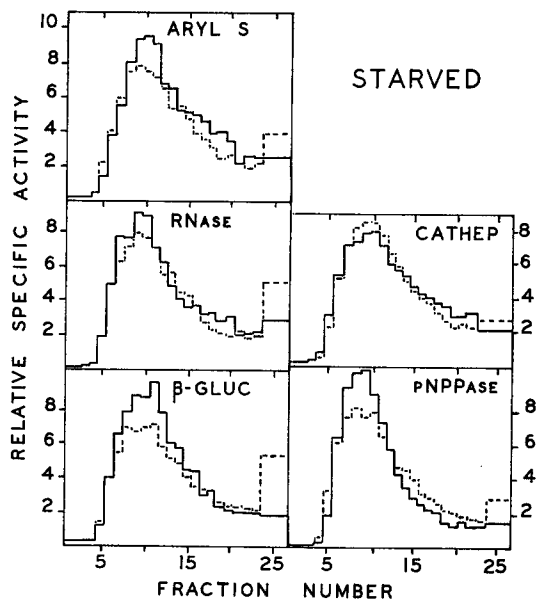

FIGURE 5 Influence of Triton WR-1339 on the rate zonal sedimentation distribution pattern of muscle acid hydrolases. Animals were given a single injection of Triton WR-1339 (85 mg/100 g body weight) 4½ days previously. Experimental distribution patterns represent the average of two experiments. Dotted lines show normal distributions.

hydrolase found in leukocytes (2) and macrophages (11), had a bimodal distribution with peaks at 1.15 and 1.20. Little lysozyme activity was found at 1.18, the corresponding modal equilibrium density peak for the other lysosomal enzymes.

The results represented in Fig. 8 show that starvation caused a decrease in the modal equilibrium density of all five hydrolases. The modal densities of cathepsin and acid phosphatase were decreased from 1.18 to 1.16; that of the remaining three enzymes to 1.165. These results are in agreement with the conclusion based on fluorescence microscopy and rate zonal experiments, which indicated that an increase in the size of muscle fiber lysosomes would be accompanied by a decrease in their density.

In experiments with dextran-500-injected animals (Fig. 9), the modal density of the majority of the five lysosomal enzymes was not shifted from the control value of 1.18. However, a portion of the activity was shifted to less bouyant parts of the gradient. This shift, which was greater for RNase, β-glucuronidase, and arylsulfatase than for cathepsin or acid phosphatase, clearly indicated that a small group of lysosomes rich in the former
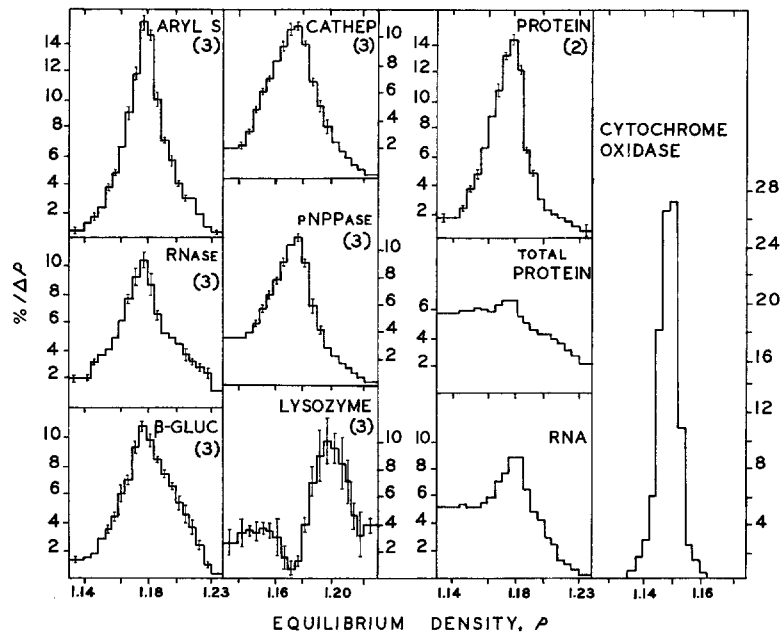
FIGURE 7   Distribution patterns of rat muscle acid hydrolases, cytochrome oxidase, protein, and RNA after isopycnic centrifugation in a sucrose-0.02 M KCl gradient. Centrifugation was conducted at 32,000 g for 4 hr ($\omega^2 t = 15,400 \times 10^7$ rad^2/sec). Patterns are normalized as explained under Materials and Methods. Diagram show averages of results with standard error of the mean and number of experiments between parentheses.

enzymes is present that is capable of altering its equilibrium density by the accumulation and storage of exogenous materials.

The influence of Triton WR-1339 on the equilibrium density distribution of the acid hydrolases is shown in Fig. 10. Triton WR-1339 did not alter the modal equilibrium densities of the hydrolases; however, the distribution of RNase, β-glucuronidase, and arylsulfatase was found to be skewed to more bouyant portions of the gradient, indicating that some particles did accumulate Triton WR-1339.

The interpretation of the isopycnic data is summarized in Fig. 11. Considering first the distribution histograms from the destran-500-treated animals, a best fit curve was drawn through the left face of the equilibrium density distribution with the apex coinciding with the modal density peak at 1.18. A mirror image of this curve was reproduced on the right side of the apex so as to construct a bell-shaped curve. The difference between this bell-shaped curve and the experimental data was used to construct a smaller bell-shaped curve (when extrapolated beyond the limits of the gradient).

The larger curves, defined above, were superimposed on the data from the animals injected with Triton WR-1339. A good fit was obtained on the right face of the distribution, verifying the validity of the curves derived from the dextran-500 data. The smaller bell-shaped curves were obtained as before. By this technique two groups of lysosomes with bell-shaped distributions were resolved. The smaller group contributes approximately 25% of the arylsulfatase, RNase, and β-glucuronidase and 5% of the cathepsin D and acid phosphatase activity in muscle, while the larger group contains approximately 75% of the former enzymes and 95% of cathepsin D and acid phosphatase. The contribution of hydrolases from each group was estimated from measurements of the relative area under the respective bell-shaped curves.

DISCUSSION

Tissue fractionation studies have shown the heterogeneous nature of lysosomes in such tissues as liver (27), spleen (7), and brain (28). The results of the present investigation have extended this current list by demonstrating the existence of two
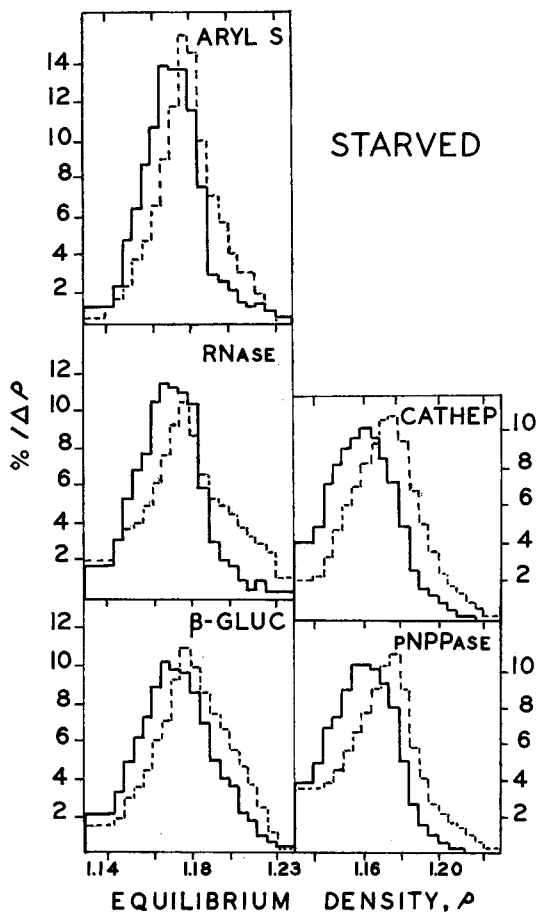
FIGURE 8 Effect of starvation on the density distribution patterns of rat muscle acid hydrolases. Animals were fasted for 6 days but provided with both tap water and normal saline. Experimental distribution patterns represent the average of three experiments. Dotted lines show normal distributions.

distinct groups of lysosomes in skeletal muscles, and suggest that the two groups have different cellular origins.

Lysosomes of Phagocytic Cells

The lysosomes of phagocytic cells are lysosomes having greater relative amounts of acid ribonuclease, β-glucuronidase, and arylsulfatase than of cathepsin D and acid phosphatase. They are largely characterized by their ability to accumulate the following: (a) dextran-500, as indicated by an increase in density and sedimentation rate; and (b) Triton WR-1339, resulting in a decrease in density. The cells comprising the connective

tissue components of muscle, including macrophages, fibroblasts, leukocytes, and epithelial cells, are the apparent source of this population. These cells are known to contain among the highest concentrations of lysosomes of any cell (34). Furthermore, they are noted for their ability to phagocytize exogenous materials and accumulate them in their lysosomes. In a recent study (10) we have observed lysosomes, as orange fluorescent granules, in fresh frozen sections of skeletal muscle vitally stained with acridine orange. The orange granules were abundant in macrophages, fibro-
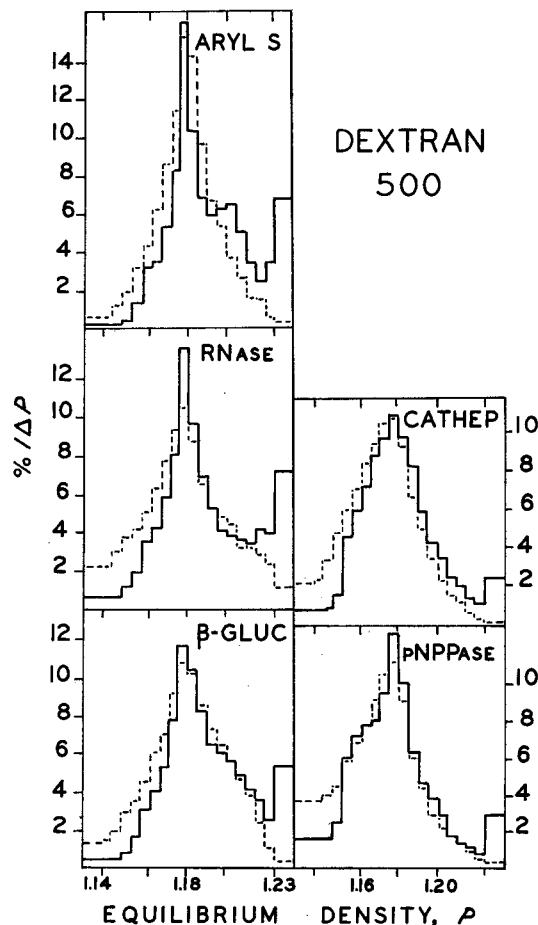

FIGURE 9 Influence of dextran-500 on the density distribution patterns of rat muscle acid hydrolases after isopycnic centrifugation. Animals were injected with dextran-500 (400 mg/100 g body weight) for 4 consecutive days and were sacrificed on the 5th day. Experimental distribution patterns represent the average of two experiments. Dotted lines show normal distributions.
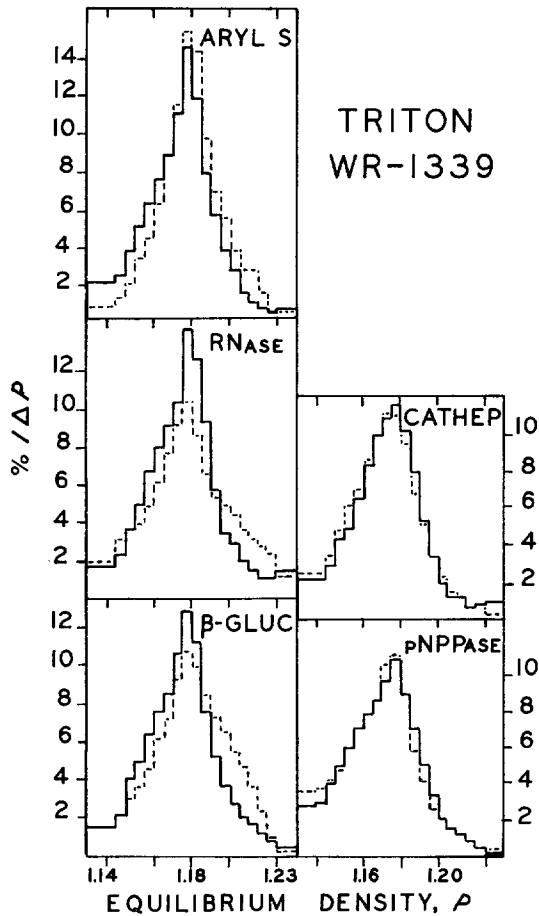
FIGURE 10 Influence of Triton WR-1339 on the density distribution patterns of rat muscle acid hydrolases after isopycnic centrifugation. Animals were given a single injection of Triton WR-1339 (85 mg/100 g body weight) 4½ days previously. Experimental distribution patterns represent the average of two experiments. Dotted lines show normal distributions.

lases in skeletal muscle tissues. A critical evaluation of the experimental design and the resolving capability of their apparatus indicates that their experiments, performed by layering small quantities of reconstituted mitochondrial pellets on sucrose gradients using Spinco SW-25.1 or SW-39 rotors, were subject to a number of limitations. Foremost among them were the following: (a) the insufficient resolution of the swinging bucket rotor technique, due to the limited number of fractions that can technically be obtained; (b) the use of reconstituted mitochondrial pellets as samples. We question this procedure because of our observations that agglutination and absorption of lysosomes to membranous elements cannot be completely eliminated in resuspending muscle mitochondrial pellets; and (c) the inability to assay for an adequate number of lysosomal enzymes, which would result in an incomplete picture of the distribution pattern of muscle hydrolases.

In the present study, the use of a Spinco B-XIV zonal-rotor in combination with a circuit for integrating the total centrifugal force on our particles made it possible to design experiments that circumvented a number of difficulties encountered by previous investigators. The B-XIV rotor provided the necessary resolution by permitting a greater number of fractions to be taken across the gradient and by allowing the total postnuclear supernate to be used as the sample rather than a resuspended mitochondrial pellet. It also permitted the use of larger amounts of starting material, resulting in fractions having sufficient activity and volume to accurately assay for at least five representative hydrolases.

Lysosomes of Muscle Cells

The group of lysosomes from muscle cells is the larger of the two groups and is characterized by relatively larger amounts of cathepsin D and acid phosphatase, and its apparent insensitivity to the effects of injected dextran-500 and Triton WR-1339. Muscle cells normally have no known phagocytic or secretory functions (14, 41) and were not expected to take up exogenous material such as dextran-500 or Triton WR-1339. Significantly, the inability of muscle cells to undergo endocytosis or exocytosis is dermatized by the progressive accumulation of lypofuscin granules. These residual granules, which may represent terminal forms of autophagio vacuoles, have cathepsin and acid phosphatase activity and are

blasts, and epithelial cells surrounding muscle fibers. These observations had emphasized the significant lysosomal contribution made by the nonmyogenic cells to the total acid hydrolase picture in muscle tissue.

Several investigators have recently published results of isopycnic gradient centrifugation of skeletal muscle tissue (16, 26, 33). Their findings failed to provide any evidence for the heterogeneity of muscle lysosomes. Some of these authors interpreted their results as suggestive evidence that phagocytic cells, specifically macrophages, do not make a significant contribution to the observed subcellular distributions of acid hydro-

ARYL S ACID RIBONUCLEASE
CATHEPSIN D PNPPASE
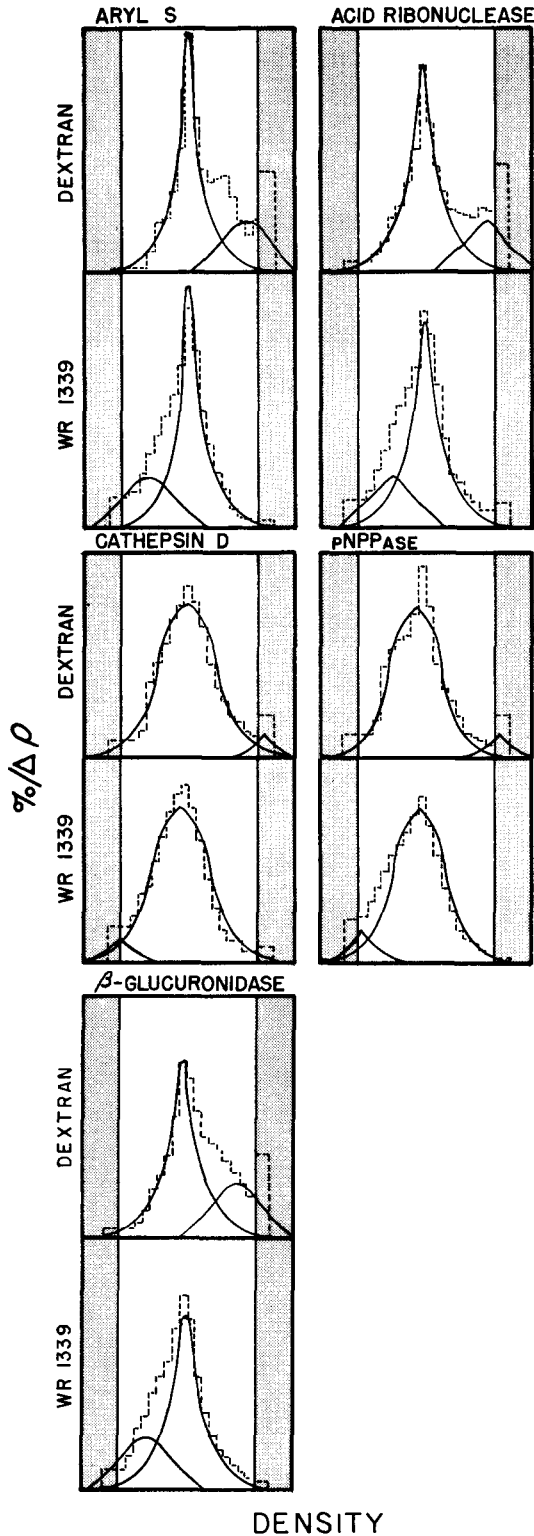β-GLUCURONIDASE

DEXTRAN
WR 1339

DENSITY

Figure 11

found in higher concentration in the muscle tissue of older animals (21).

Since the administration of exogenous materials did not affect the equilibrium density of the muscle lysosomes, starvation was used as an endogenous stimulus to promote the development of large secondary lysosomes (10), probably of the autophagic type (17). Swift and Hruban (35) have suggested that the formation of autophagic vacuoles is a response of the normal cell to starvation and other unfavorable conditions. Since muscle is a major storehouse of amino acids, it is not surprising that autophagic vacuoles would be formed during the muscle degeneration of starvation, and that cellular material sequestered within the vacuoles would, after digestion, enter the metabolic pool for reutilization by the animal. We feel that the decrease in equilibrium density of the muscle lysosomes during starvation, in fact the only method by which we could alter the density of this group of lysosomes, is further evidence in support of the muscle fiber origin of this group of organelles.

The observation that lysozyme was not associated with acid hydrolase activity at the modal density of 1.18 clearly indicates that little, if any, of the activity attributed to muscle fiber lysosomes was contributed to lysosomes of leukocytes and macrophages. The bimodal distribution of lysozyme in this study is in agreement with the findings reported for leukocytes by Baggiolini et al. (2). We are aware that our own investigation is bounded by a number of limitations. It must be emphasized that we are monitoring only a portion of the total acid hydrolase activity of muscle, specifically the activity of a particulate mitochondrial fraction. In the preparation of samples 20% of the activity was eliminated with the nuclear fraction and an additional 40% was later discarded as unsedimentable activity. A disproportionate number of dextran-500- H or Triton WR-1339-filled lysosomes may have been ruptured during the homogenization procedure, since such lysosomes are larger and more fragile (14). It must also be taken into consideration that the particles that remain intact after zonal centrifugation are said to have "survived" unfavorable

Figure 11 Bell-shaped curves of best-fit drawn through the equilibrium density distribution data of acid hydrolases in muscles of dextran-500- and Triton WR-1339-treated animals (Figs. 9 and 10). Theoretical curves are extrapolated beyond the limits of the gradient (shaded area) as explained in text.

osmotic conditions and may not be representative of the tissue or cell from which they are derived. On the basis of these various considerations, the relative contribution of lysosomes by the non-myogenic cells may be, in fact, significantly higher than our present estimates.

We wish to emphasize that, while providing evidence for the presence of lysosome-like particles in normal muscle fibers, no clues have been uncovered regarding the morphological nature of such particles. Thus, it remains to be explained why morphological studies have repeatedly failed to observe lysosomes in normal muscle cells. It is possible that primary muscle lysosomes may exist in a morphological configuration that is different from that of their counterpart in other tissues (34). Pearce has concluded from histochemical observations that muscle lysosomes may exist not as separate entities but as part of the longitudinal sarcotubular system (24). Hence, he coined the term sarcotubulo-lysosomal system. It is beyond the scope of this paper to present the biochemical

evidence that has led us to similar conclusions; however, an argument in favor of a sarcotubular-lysosomal system in skeletal muscle fibers has been recently presented (8). Since muscle fibers normally do not have absorptive, secretory, or excretory functions, we are presently persuaded they do not need to have a lysosomal vacuolar apparatus so highly developed as that of the reticulo-endothelial system. Instead, cellular economy in muscle cells may be maintained by the storage of minimal titers of hydrolases within portions of the sarcotubular elements, and used in the formation of autophagic vacuoles (22) during focal degradation of cell constituents or in response to pathological and physiological stresses.

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