

## THE USE OF ACRIDINE ORANGE AS A LYSOSOMAL MARKER IN RAT SKELETAL MUSCLE

PETER G. CANONICO and JOHN W. C. BIRD. From the Department of Physiology, Rutgers University, New Brunswick, New Jersey 08903

### INTRODUCTION

The uptake of vital dyes by lysosomes (1, 2) has permitted the microscopic observation of these subcellular organelles in living cells (3) and tissue homogenates (4). Acridine orange (3,6-bis-dimethylaminoacridine), a fluorescent metachromatic dye, has been shown to concentrate within lysosomes, causing them to appear as bright orange granules in fluorescence microscopy (5-7). We have observed these granules in fresh frozen sections of rat skeletal muscle and have determined their subcellular distribution by quantitating the dye with spectrophotofluorometry. Our technique demonstrates the use of acridine orange as a nonenzymatic marker for lysosomes in tissue fractionation studies.

### METHODS

Male Sprague-Dawley rats, 200-250 g, were injected intraperitoneally with acridine orange (50 mg/ml) in 0.9% saline, at a dosage of 500 mg/kg body weight. After 2 hr the animals were sacrificed

by decapitation. Lower leg and thigh muscles were rapidly excised, chilled in 0.25 M sucrose containing 0.02 M KCl, and homogenized according to the method of Canonico and Bird (8). Five subcellular fractions were isolated by differential centrifugation by the method of de Duve et al. (9), as modified for muscle tissue by Bird et al. (10). In rate-zonal centrifugation experiments a 10-30% linear sucrose gradient, resting on a 50 ml cushion of 50% sucrose-KCl, was gravity fed into a Spinco B XIV rotor spinning at 3000 rpm (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.). The samples for the zonal centrifugation experiments consisted of 50 ml of supernatant from a 20% muscle homogenate centrifuged at 700 *g* for 10 min to remove nuclei, contractile proteins, and cellular debris. The sample was injected into the rotor via the core line and followed by a 50 ml overlay of suspension media. The rotor was then accelerated to 10,000 rpm for approximately 1 hr. The total centrifugal effect on the sample was monitored on a Beckman  $\omega^2 dt$  integrator, and the centrifuge was programmed to decelerate to 3000 rpm when  $\omega^2 t = 412 \times 10^7$  (rad<sup>2</sup>/sec). Unloading began when  $\omega^2 t = 420 \times 10^7$ . 26 25-ml fractions were collected at a rate of 25-30 ml/min,

and an aliquot from each fraction was tested for its refractive index with an Abbe-3L refractometer (Bausch & Lomb, Inc., Rochester, N.Y.). A 10-ml aliquot from each fraction was centrifuged at 20,000 *g* for 60 min. The supernatants were discarded, and the pellets were resuspended in several milliliters of glass-distilled water containing 0.2% Triton X-100 (v/v). Enzyme activities of cathepsin D, arylsulfatase, acid phosphatase, acid ribonuclease,  $\beta$ -glucuronidase, and cytochrome oxidase were determined by methods reported in previous publications (10, 11).

Acridine orange was measured in the resuspended particulate matter of each zonal fraction as follows: 2 ml of 95% ethanol were added to a 1-ml aliquot of each fraction, the preparation was mixed and allowed to stand in the cold for 1 hr before the addition of 0.2 ml of 8% trichloroacetic acid. The preparations were then centrifuged at 32,000 *g* for 10 min. The concentration of acridine orange was then determined in the supernatants with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Inc., Silver Spring, Md.), using excitation and emission wavelengths of 485 and 530  $m\mu$ , respectively, and compared against reagent blanks. Preliminary experiments showed that tissue aliquots of nontreated animals did not fluoresce at wavelengths used for measuring acridine orange.

Fluorescence microscopy observations were made on medial gastrocnemius muscles from acridine orange-injected animals. Fresh frozen sections, 12  $\mu$  in thickness, were placed on a slide and covered with mineral oil or glycerol. The slides were illuminated by a Zeiss superpressure mercury lamp with a BG 12 exciter filter and a yellow gelatin (Wratten #9) barrier filter.

## RESULTS

Fluorescence microscopy observations of gastrocnemius muscles from the acridine orange-injected animals showed that nuclei and muscle fibers were pale green and green, respectively. Bright orange granules were seen in macrophages, mast cells, leucocytes, and endothelial cells surrounding muscle fibers. The orange granules were rarely seen within the muscle fibers of normal animals. However, after subjecting rats to 6 days of starvation before dye injection, large numbers of orange granules were also seen in the perinuclear region of the muscle fibers.

The subcellular distribution of acridine orange in muscle tissue (Fig. 1), as indicated by differential centrifugation, was similar to that of several representative acid hydrolases reported previously (8, 10). Acridine orange was concentrated in the lysosome-rich, light mitochondrial fraction. The

relative concentration of acridine orange in the nuclear fraction was higher than that of the acid hydrolases, which presumably reflects the binding of some acridine orange to nuclei and contractile proteins.

The distribution of acridine orange after rate-zonal fractionation of nuclei-free homogenates was similar to the distribution pattern of particle-bound acid hydrolases (Figs. 2 and 3). Sedimentation characteristics of lysosomal particles did not appear to be altered by acridine orange, since similar acid hydrolase distribution patterns were obtained from muscles of normal and acridine orange-injected animals. Distribution of relative specific activity of the lysosomal enzymes was bimodal, with peaks between fractions 7-12 and 24-26, and heterogeneous with respect to the individual enzymes.

Microscopic observation of tissue fractions in our laboratory and others (2) indicates that acridine orange does not bind to mitochondria. In our rate-zonal centrifugation experiments the mitochondria (as indicated by cytochrome oxidase activity) were found against the gradient cushion in fractions 24-26 (Fig. 2).

## DISCUSSION

Fluorescence microscopy of acridine orange-stained cytoplasmic granules has been limited to cells in tissue culture and cellular fractions in sucrose suspension (2-7). Observations on fresh frozen tissue sections have not been reported, possibly because of the known lability of lysosomes under conditions of freezing and thawing. Our observations of orange fluorescent granules in fresh frozen muscle sections demonstrate that the integrity of a large number of granules is preserved through the single freeze-thawing cycle necessary for the preparation of a slide.

Dingle and Barrett (12) have recently reported that actual counts of orange fluorescent granules in gradient centrifugation fractions of tissues are in good agreement with specific activities of lysosomal enzymes. We have extended these observations by the spectrophotofluorometric determination of acridine orange in cell fractions and have clearly shown that the subcellular distribution pattern of acridine orange corresponds to the distribution pattern of five representative lysosomal enzymes.

Koenig and Hurlebaus (13) studied the subcellular distribution of the basic dye neutral red, with acid phosphatase as their lysosomal reference

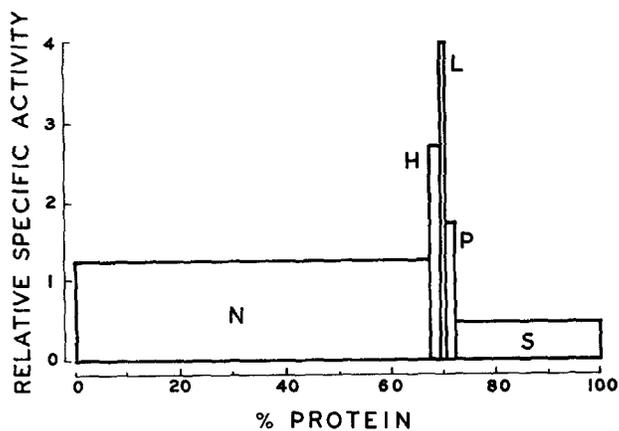


FIGURE 1 Distribution pattern of acridine orange. The ordinate shows the mean relative specific activity of fractions calculated by the method of de Duve et al. (9). The abscissa indicates the fractions, in the order of their isolation, represented by their relative protein content. From left to right: nuclear, *N*; heavy mitochondrial, *H*; light mitochondrial, *L*; microsomal, *P*; and unsedimentable, *S*.

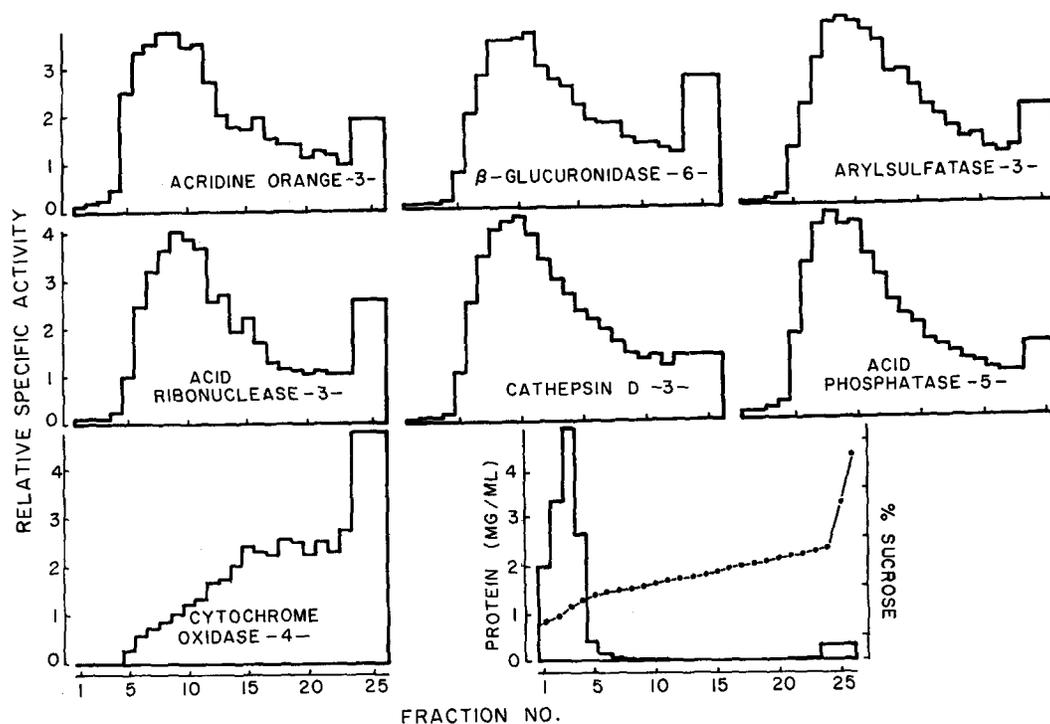


FIGURE 2 Distribution pattern of enzymes, acridine orange, and protein after fractionation of post-nuclear muscle homogenates by rate-zonal centrifugation ( $\omega^2 t = 420 \times 10^7$ ). Diagrams show averages of results; the number of experiments is shown between dashed lines. The ordinate measures the relative specific activity in each fraction calculated by the method of de Duve et al. (9). The activity in the cushion is represented as the average activity of fractions 24-26.

enzyme, in rat brain homogenates. They reported that neutral red reached a peak in concentration in the neural lysosomes at 1-2 hr after injection of the animals, and that the lysosomes underwent a decrease in density upon incorporation of the dye,

as shown by a shift in acid phosphatase activity and acid phosphatase-positive particles into lighter fractions of a sucrose density gradient. McDonald and Koenig (14) also reported that neuron lysosomes stained *in vivo* by neutral red were en-

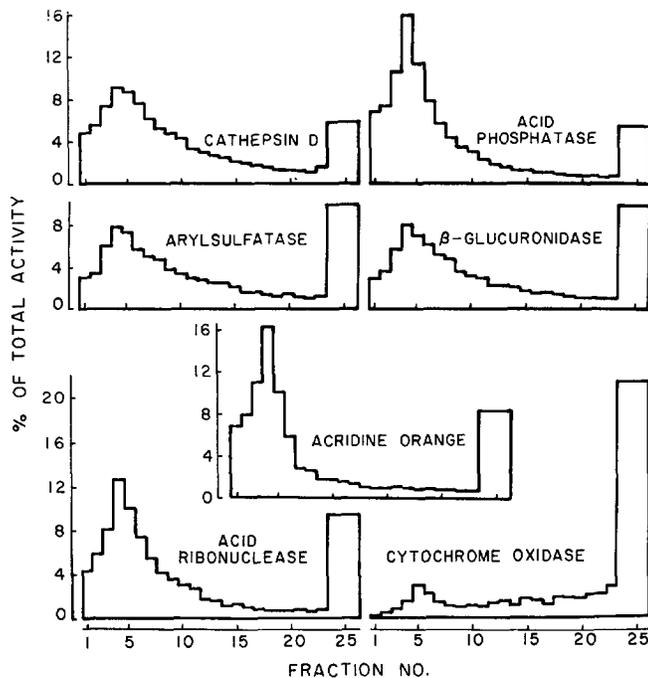


FIGURE 3 Same experiments as that of Fig. 2. The distribution patterns of lysosomal enzymes and acridine orange are represented as the percentage of the total activity in each fraction.

larged and showed electron-lucent areas as well as lamellae. We are not aware of any ultrastructure studies of muscle lysosomes from acridine orange-injected animals. However, isopycnic zonal centrifugation studies have shown that rat skeletal muscle lysosomes have a modal equilibrium density of 1.18 (15). We have found that the equilibrium density of muscle lysosomes does not change after injecting our animals with acridine orange. An analysis of muscle lysosome populations and the effects of various agents on these populations will be the subject of a later report.

Our study supports the interpretation that the orange fluorescent cytoplasmic granules observed

in acridine orange-stained tissues are lysosomes. Furthermore, under the conditions of our experiments, we propose that acridine orange may be useful as a nonenzymatic marker for lysosomes in tissue fractionation studies.

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