Isolation of Relaxing Particles from Rat Skeletal Muscles in Zonal Centrifuges

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ABSTRACT Supernatants of rat skeletal muscle homogenates were fractionated by differential centrifugation and by zonal centrifugation in sucrose density gradients. Cytochrome oxidase was employed as an enzymatic marker for locating mitochondria. The subcellular fractions were also assayed for their ability to prevent the ATP-induced contraction of myofibrils. Both the mitochondrial and microsomal fractions obtained by differential fractionation were found to be rich in such relaxing activity, and the microsomal fraction was appreciably contaminated by mitochondria. In contrast to this, when fractionation was carried out by means of zonal centrifugation (4200 RPM × 205 min. to 40,000 RPM × 60 min.), relaxing activity was found to be associated only with particles having the sedimentation characteristics of microsomes ($s_{20,w}$ estimated to be between 370 and 1880S). Relaxing activity was not detected in the regions of the gradient containing either the starting sample zone (soluble phase) or the mitochondrial peak. The microsomal relaxing particles showed negligible cytochrome oxidase activity.

Marsh (1952) demonstrated that muscle homogenates can prevent adenosine triphosphate–induced contraction of myofibrils. Many attempts have been made to isolate and define the components of this relaxing principle in muscle, and it is now generally agreed that vesicular fragments of the sarcoplasmic reticulum play a fundamental role in relaxation. Initially, it was proposed (Kumagai *et al.*, 1955) that relaxing activity was associated with the Kielley-Meyerhof granular adenosine triphosphatase. Then, by the method of differential centrifugation, the active principle was separated, and operationally defined, as a microsomal particle (Lorand *et al.*, 1958; Portzehl, 1957; Ebashi, 1961 *a*, *b*; Bendall, 1958). This assignment was also confirmed by electron microscopy (Nagai *et al.*, 1960; Muscatello *et al.*, 1961; Ebashi and Lipmann, 1962; Constantin *et al.*, 1965). Since myofibrils, actomyosin, and muscle systems in general require calcium ions for their contractile response (Heilbrunn and Wiercenski, 1947; Niedergerke, 1955; Heilbrunn, 1956; Ebashi, 1961 *a*, *b*; Weber and Winicur, 1961; Weber and Herz, 1963; Weber *et al.*, 1963; Lorand

et al., 1963; Seidel and Gergely, 1963; Podolsky and Constantin, 1964), and since the microsomal vesicles can effectively accumulate these ions in the presence of ATP (Ebashi and Lipmann, 1962), it is thought that the removal of calcium ions by the vesicles is the prime, if not the only, requisite for relaxation. Furthermore, substances which potentiate the uptake of calcium by the vesicles also enhance their relaxing activity, while treatments which destroy the calcium uptake ability also affect the vesicles relaxing activity (Lorand and Molnar, 1962; Hasselbach and Makinose, 1961; Molnar and Lorand, 1962; Martonosi and Feretos, 1964).

When muscle tissue is fractionated by procedures of differential centrifugation, relaxing activity is often (Baird and Perry, 1960; Weber *et al.*, 1964), but not always (Muscatello *et al.*, 1962), found also in the mitochondrial fraction. In fact, the mitochondrial fraction may display a greater activity than that of the microsomal fraction. Thus, there was an area of uncertainty as to whether there was a unique type of particle in muscle which was responsible for relaxation. Furthermore, it has also been claimed that the high-speed supernatant of muscle homogenates, believed to be free of microsomal particles, shows relaxing activity (Nagai *et al.*, 1962; Fuchs and Briggs, 1963; Baltscheffsky, 1964).

The zonal centrifuges recently developed by Anderson and collaborators have made it possible to achieve a fractionation of subcellular particles with very high resolution on a large scale (Anderson, 1962; Anderson and Burger, 1962; Anderson *et al.*, 1964; Schuel and Anderson, 1964; Schuel *et al.*, 1964) The present paper deals with the fractionation of rat skeletal muscle using zonal centrifugation. It will be demonstrated that relaxing activity is, indeed, associated with particles which have the sedimentation characteristics of microsomes. Furthermore, the active fraction is virtually free of contamination by mitochondria which by themselves, show no relaxing potency.

A preliminary account of part of this work was presented at a symposium sponsored by Federation American Societies Experimental Biology (Lorand, 1964).

MATERIALS AND METHODS

Preparation of Muscle Homogenates and Preliminary Fractionation Adult male Sprague-Dawley rats were allowed to eat and drink freely until killed. They were stunned by a blow on the head, decapitated, and allowed to bleed for several seconds while suspended by the tail. The skeletal muscles from the hind legs and back were quickly dissected out and placed in an ice cold solution of 250 mM sucrose, 130 mM potassium chloride, 20 mM potassium acetate, and 2 mM potassium oxalate (the homogenization medium). Twenty-five per cent (w/v) homogenates were prepared in this medium by treating the muscle preparation in a prechilled Waring blendor at full speed for 1 to 2 minutes. The resulting homogenate was then centrifuged for 30 minutes at 2000 RPM (approximately 900 × *g* max.) in the large swinging bucket

rotor (No. 284) of the International PR-I refrigerated centrifuge. The supernatant fluid was removed by decantation and filtered through several layers of cheese-cloth. This fluid was then subjected to fractionation by differential and by rate zonal centrifugation. The muscle material was maintained at 4–5°C at all steps of the isolation procedure.

Fractionation by Differential Centrifugation The 900 × *g*/30 min. supernatant of muscle homogenates was centrifuged for 15 minutes at 10,000 RPM (11,700 × *g* max.) in the No. 30 rotor of the Spinco model L preparative ultracentrifuge. The pellet was resuspended in the homogenization medium in one-tenth of the volume of the starting material, and was referred to as the "mitochondrial" fraction. The supernatant was recentrifuged in the No. 30 Spinco rotor for 60 minutes at 25,000 RPM (73,400 × *g* max.). This pellet was also resuspended in the homogenization medium in one-tenth of the volume of the starting material, and was referred to as the "microsomal" fraction. The supernatant of this high-speed centrifugation was referred to as the "soluble phase" fraction.

Fractionation by Zonal Centrifugation The 900 × *g*/30 min. supernatants also served as the starting material for fractionation with zonal centrifuge rotors A-IX and B-IV. The former is constructed of lucite with a capacity of 1300 ml and can operate at speeds up to 4200 RPM (3200 × *g* max.) on the International PR-II (Canning and Anderson, 1964; Anderson, Burger, Fisher, and Harris, unpublished data). The latter is constructed of aluminum with a capacity of 1710 ml and can operate at speeds up to 40,000 RPM (90,000 × *g* max.) on a Spinco model L drive (Anderson *et al.*, 1964). All operations, including the introduction of the sucrose gradient and sample into the rotors and the recovery of the gradient, with its concentric zones of separated particles, were accomplished while the rotors were rotating at low speed: 300 RPM for the A-IX rotor and 3000 RPM for the B-IV rotor. The gradient was collected in 40 ml fractions for subsequent analysis.

Visualization of Material in the Gradient The presence of subcellular components was determined by the continuous analysis of the gradient for ultraviolet absorbance at 260 mμ as it emerged from the rotor and passed through a quartz flow cell with a 0.2 cm light path. The data in this paper are presented in terms of the computed absorbance for a 1.0 cm light path.

Sucrose Gradients It has been our experience that microsomal granules isolated from skeletal muscle lose their capacity to function as relaxing agents immediately upon exposure to pure sucrose solutions. This effect appeared to be irreversible since the relaxing activity was not restored when the particles were centrifuged out of the sucrose and resuspended in a solution of 130 mM potassium chloride and 20 mM potassium acetate. Also, the appearance of the pellets suggested that exposure to 40 per cent (w/w) sucrose might have produced some structural changes in the particles. However, sucrose solutions containing oxalate have been successfully used to prepare relaxing granules (Briggs *et al.*, 1959). We confirmed their observation, and found that the relaxing activity of the granules was best preserved if the sucrose solutions contained 130 mM potassium chloride, 20 mM potassium acetate, and 2 mM potassium oxalate. Thus, microsomal particles as dilute as 2 to 4 mg protein/ml could be stored

at ice bath temperature in 40 per cent (w/w) sucrose made up with the above salt mixture for periods of more than 24 hours without loss in relaxing activity (Table I). Accordingly, sucrose gradients used in the zonal centrifuge runs included the salt mixture. The sucrose density gradients, linear with respect to rotor radius, were constructed and pumped into the zonal centrifuge rotors by means of a Beckman model 131 high capacity gradient pump.

TABLE I

STABILITY OF RELAXING ACTIVITY EXPOSED TO CONCENTRATED SUCROSE SOLUTIONS

	Percentage relaxation measured after incubation periods (hrs.) of									
	—	2	3	24		—	1	2½	5½	24
	Experiment 14					Experiment 15				
Incubation media					Incubation media					
A	23	31	36	23	D	63	63	71	71	71
B	19	14	8	10	E	90	88	100	100	99
C	3	2	1	2	F	93	84	86	85	90
Undiluted stock	33	34	—	34	Undiluted stock	51	—	—	—	35

Incubation media:

A. 130 mM potassium chloride and 20 mM potassium acetate;

B. 40 per cent (w/w) sucrose, 130 mM potassium chloride, and 20 mM potassium acetate;

C. 40 per cent (w/w) sucrose;

D. 130 mM potassium chloride, 20 mM potassium acetate, and 2 mM potassium oxalate;

E. 40 per cent (w/w) sucrose, 130 mM potassium chloride, 20 mM potassium acetate, and 2 mM potassium oxalate; and

F. 40 per cent (w/w) sucrose and 2 mM potassium oxalate.

Relaxing particles were isolated from rabbit skeletal muscle by differential centrifugation (Molnar and Lorand, 1962) in a solution of 130 mM potassium chloride and 20 mM potassium acetate. They contained 38 and 36 mg proteins/ml in experiments 14 and 15 respectively. These stocks were diluted tenfold into the media (A–F) specified and were incubated at 0° for the periods shown. When marked as "undiluted stock," the concentrated particles were incubated and dilution into medium A was carried out just prior to testing for relaxing activity. Aliquots of 0.5 ml were used in the relaxation assay with the stroboscopic centrifuge.

The concentration of sucrose within the isolated zonal centrifuge fractions was determined with a refractometer.

Assay for Relaxing Activity Myofibrils were obtained from fresh rabbit skeletal muscle by differential centrifugation. After decapitation, the hind leg and back muscles were quickly dissected out and chilled in crushed ice. The tissue was ground in a chilled meat grinder and then homogenized for 1 minute in a Waring blendor at 0°C in a solution of (200 ml/100 gm muscle) containing 130 mM of potassium chloride and 20 mM potassium acetate. The brei was stored in ice for 1 hour, and then another 100 ml/100 gm muscle of the above extracting solution was added. This preparation was

then centrifuged for 30 minutes at 2000 RPM (approximately 900 × *g* max.) at 0°C in rotor No. 284 in the International PR-I centrifuge. Pieces of connective tissue were removed from the pellet with a glass rod. The pellet was resuspended in approximately three volumes of ice cold extracting solution and centrifuged for 15 minutes at 2000 RPM. The white upper layer of the pellet which contained the myofibrils was removed with a glass rod. The myofibrils were washed four times in the extracting medium and then stored as a pellet in crushed ice until used. For the relaxing assays the myofibrils were suspended to a constant concentration of 20 mg protein/ml in a mixture of 250 mM sucrose, 130 mM potassium chloride, 20 mM potassium acetate, and 2 mM potassium oxalate.

The ATP-induced contraction of the myofibrils was followed by measuring the sedimentation rate of the myofibrils centrifuged at low speed (700 RPM) at room temperature in an International table model clinical centrifuge equipped with stroboscopic light (Marsh, 1952; Lorand and Molnar, 1962; Molnar and Lorand, 1959). The otherwise gradual settling of the myofibrils was observed to be drastically accelerated by the addition of ATP. The presence of relaxing agents, however, reduced this sedimentation rate to approximate that observed without ATP. Percentage reaxing activity (RA) could be defined by the following expression:

$$\text{Per cent } RA = \frac{T(\text{experimental}) - T(\text{contracted})}{T(\text{control}) - T(\text{contracted})} \times 100$$

where T represents the experimental time of centrifugation for the myofibrillar boundary to sediment a fixed distance down the tube in the presence of ATP and relaxing agent, T(contracted) stands for the centrifugation time for the myofibrils to sediment the same distance in the presence of ATP alone, and T(control) represents the centrifugation time required for the myofibrils to sediment the same distance in an identical milieu, but in the absence of ATP.

It is known that myofibrils contract on addition of ATP only in the presence of a small amount of ionic calcium, but many commercial ATP preparations are sufficiently contaminated with calcium ions so that these do not have to be added separately (Lorand *et al.*, 1963; Seidel and Gergely, 1963). All assays reported in the present study were performed with Sigma disodium ATP, lot 83B7280. With this preparation, it was always necessary to add calcium to the incubation medium in order to elicit a maximal contractile response.

The assay conditions were as follows: The subcellular fraction (0.5 ml) in sucrose, potassium chloride, potassium acetate, and potassium oxalate was added to a mixture consisting of 2 ml of 100 mM tris (hydroxymethyl)aminomethane adjusted to pH 7.0 with acetic acid and of 10 mM magnesium acetate; 0.4 ml of 50 mM ATP (pH 7.0) and 0.1 ml of 1 mM calcium chloride. This mixture was incubated for 3 minutes at room temperature. Then 2 ml of myofibrils (20 mg protein/ml, suspended in 250 mM sucrose, 130 mM potassium chloride, 20 mM potassium acetate, and 2 mM potassium oxalate) were quickly admixed and the sedimentation of the myofibrils was followed in the stroboscopic centrifuge. In order to determine the sedimentation rate of myofibrils alone under control conditions, 130 mM of potassium chloride and 20 mM potassium acetate were substituted for the ATP.

Cytochrome Oxidase Assay Cytochrome oxidase activity was determined spectrophotometrically with reduced cytochrome *c* (Sigma type II crystalline, from horse heart) as substrate (Cooperstein and Lazarow, 1951; Smith, 1955). The substrate concentration was 2×10^{-5} M, and sufficiently low levels of enzyme were employed so that the reaction appeared to follow zero-order kinetics (Hess and Pope, 1953; Schuel *et al.*, 1964).

Protein Assay The protein concentration of the myofibril suspensions was determined colorimetrically with the biuret reagent (Gornall *et al.*, 1949). Since sucrose

TABLE II

DISTRIBUTION OF RELAXING AND CYTOCHROME OXIDASE ACTIVITIES IN RAT MUSCLE FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION

Fraction	Cytochrome oxidase		Relaxing activity	
	Protein in test	($\Delta OD_{550m\mu}$/sec./mg of protein) $\times 10^5$	Protein in test	RA*
	mg		*mg*	*per cent*
Mitochondrial, (11,700 × *g*/15 min. sediment)	0.050	484	4.7	64
Microsomal (73,400 × *g*/60 min. sediment)	0.094	282	1.0	86
Soluble phase, D (73,400 × *g*/60 min. supernatant)	0.700	0	7.0	26
Soluble phase, A (73,400 × *g*/60 min. supernatant)	0.700	0	7.0	0

Soluble phase, D, supernatant obtained by decantation. Soluble phase, A, supernatant obtained from top of tube by aspiration.

* As defined in Materials and Methods section.

interferes with the biuret reaction (Anderson and Anderson, unpublished data, 1958), the protein contents of the fractions isolated in the zonal centrifuges were determined with the Folin-Ciocalteau reagent (Lowry *et al.*, 1951) in the absence of copper (Schuel and Anderson, in preparation) using the Technicon autoanalyzer (Skeggs, 1957).

RESULTS

Fractionation of Rat Skeletal Muscle by Differential Centrifugation. Table II shows the results of an experiment in which rat skeletal muscle was fractionated by differential centrifugation. The mitochondrial and microsomal fractions were found to be active in producing relaxation of rabbit myofibrils. A trace of activity was observed in the decanted high-speed supernatant fraction. Cytochrome oxidase activity was observed to be considerably higher in

the mitochondrial than in the microsomal fraction, and could not be detected in the high-speed supernatant.

Fractionation of Rat Skeletal Muscle by Zonal Centrifugation Results of an experiment in which a 30 ml sample of the 900 × *g*/30 min. supernatant of rat muscle homogenate was centrifuged at 4200 RPM for 205 minutes in rotor

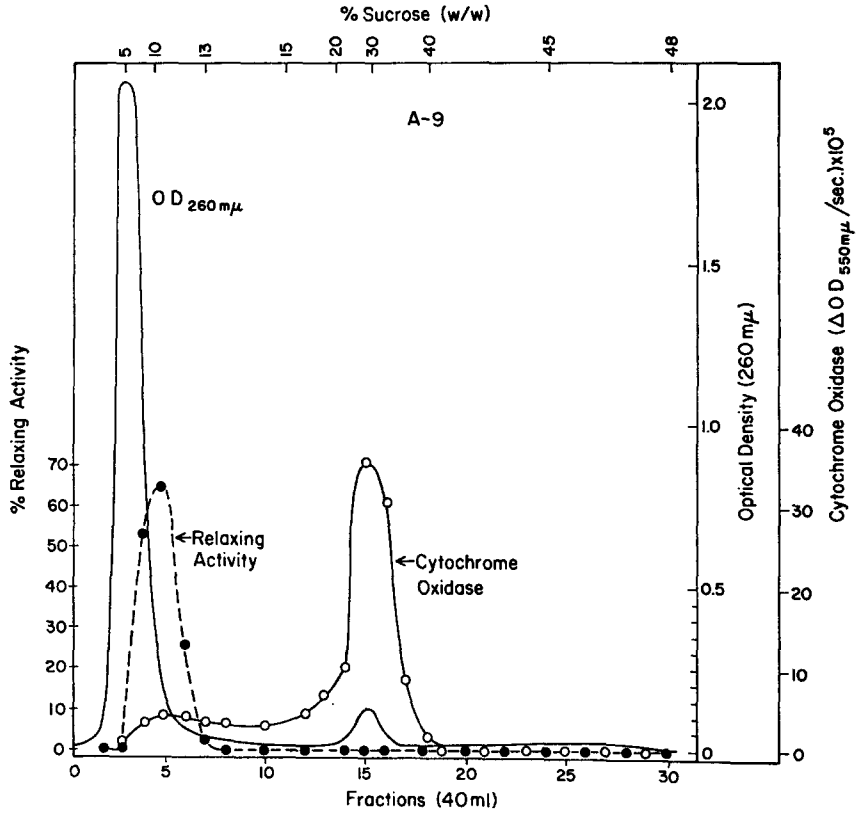

FIGURE 1. Distribution of relaxing and cytochrome oxidase activities in rat skeletal muscle fractions obtained by zonal centrifugagion in rotor A-IX at 4200 RPM for 205 minutes. Thirty ml of the 900 × *g*/30 min. supernatant of muscle homogenate served as starting material in the zonal centrifuge. The sucrose density gradient was made up in 130 mM of potassium chloride, 20 mM potassium acetate, and 2 mM of potassium oxalate.

A-IX, can be seen in Figs. 1 and 2. The distribution of relaxing activity WAS restricted to a narrow region of the gradient just beyond the sample zone (soluble phase fraction). The peak of the starting sample was in fraction 3, while the relaxing activity was found in fractions 4 through 8. The mitochondria, as indicated by the enzymatic marker of cytochrome oxidase, appeared to form a rather sharp peak around 31 per cent sucrose (fraction 15). No oxidase ac-

tivity could be detected in the gradient beyond this peak, but a very low level of activity appeared to be spread out in the gradient between the starting zone and the mitochondrial region.

In addition, the 900 × g/30 min. supernatant of rat skeletal muscle was also fractionated at high speed (20,000 RPM × 15 min. to 40,000 RPM × 60

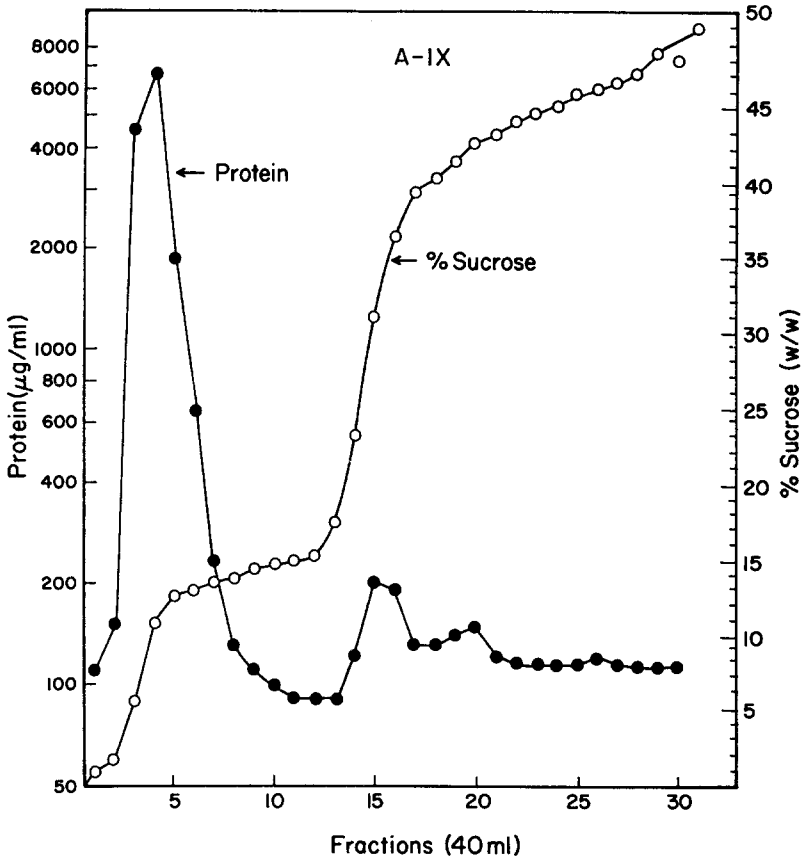

FIGURE 2. Protein contents of subcellular fractions from rat skeletal muscle subjected to 4200 RPM × 205 minutes of zonal centrifugation in rotor A-IX. Data pertain to run shown in Fig. 1.

min.) in the zonal centrifuge rotor B-IV. It was necessary to use a very large aliquot of the muscle preparation as the starting sample so that relaxing activity in the gradient fractions could be detected without further concentrating the subcellular particles by high-speed centrifugation. Figs. 3 and 4 illustrate the results of an experiment in which an 170 ml aliquot of the muscle brei supernatant (900 × g/30 min.) was fractionated at 20,000 RPM for 40 minutes. The total force generated in a centrifugal field is proportional to the integral of $\omega^2 t$, where ω is the angular velocity and t is the time of centrifugation in sec-

onds (Anderson, 1956). In this experiment, the total $\omega^2 t$, including acceleration and deceleration, was $11{,}878 \times 10^6$. The relaxing activity was restricted to a narrow region of the gradient (fractions 9 to 14) just beyond the sample zone (soluble phase fraction). The peak of the starting sample zone, in terms of protein concentration, was in fractions 4 and 5 (Fig. 4). The ultraviolet

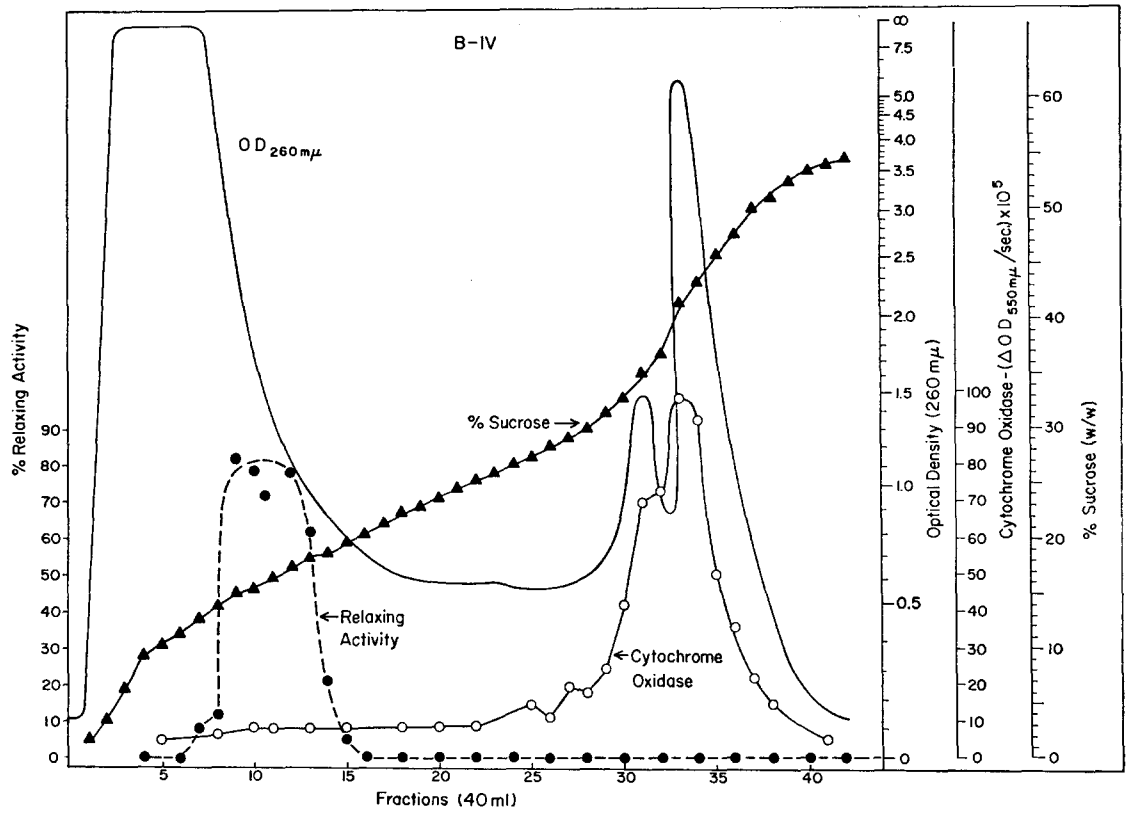

FIGURE 3. Distribution of relaxing and cytochrome oxidase activities in rat skeletal muscle fractions obtained in zonal centrifuge rotor B-IV at 20,000 RPM for 40 minutes. One hundred and seventy ml of the $900 \times g/30$ min. supernatant of muscle homogenate served as starting sample for zonal centrifugation. The sucrose density gradient was made up in 130 mM of potassium chloride, 20 mM potassium acetate, and 2 mM of potassium oxalate.

absorbance showed a distinct shoulder on the downward side of the sample zone. Unlike the soluble phase preceding it, the active fractions appeared to be free of pink color and were visibly turbid.

The mitochondria, as indicated by the cytochrome oxidase activity, formed a rather sharp peak much further out in the rotor at the level of 41 per cent (w/w) sucrose. A small amount of oxidase activity was again found to be present in the gradient between the starting sample and mitochondrial zones.

The identity of the two peaks detected by ultraviolet absorbance in the heavy end of the gradient has not been definitely established as yet, although the second peak contained large numbers of macroscopic clumps of material which could not be dissolved upon exposure to 0.2 to 1.0 per cent of the non-

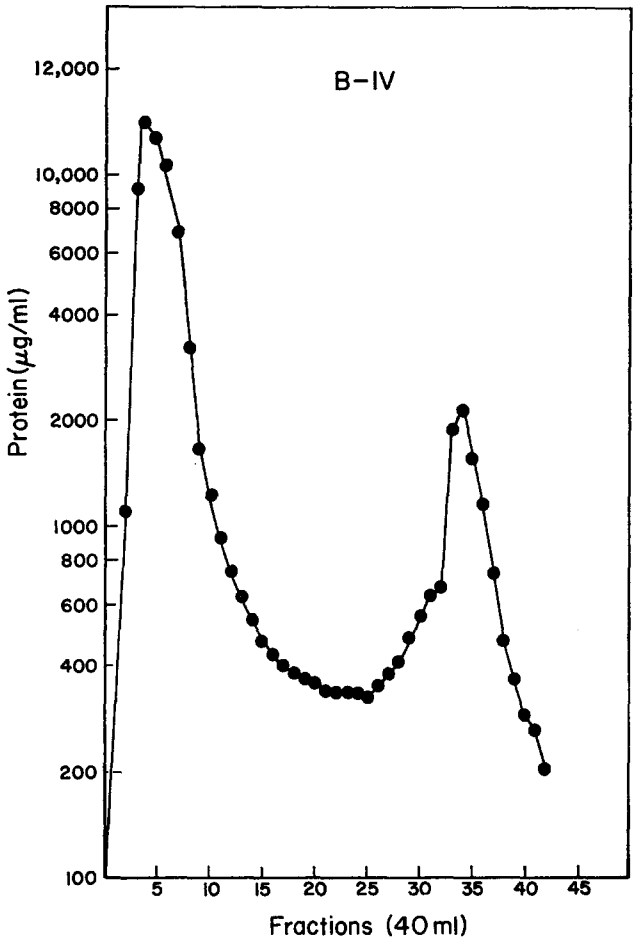

FIGURE 4. Protein contents of subcellular fractions from rat skeletal muscle subjected to 20,000 RPM × 40 minutes of zonal centrifugation in rotor B-IV. Data pertain to run shown in Fig. 3.

ionic detergent turgitol TMN (trimethylnonylether of polyethylene glycol, manufactured by Union Carbide Corp.—Chemicals Division). However, exposure to the detergent did result in an increase in cytochrome oxidase activity.

From the data obtained with zonal centrifuge rotor B-IV at 5°C using a sucrose gradient (Table III), the sedimentation coefficient was estimated with the aid of an electronic computer (Anderson *et al.*, 1964) for the relaxing par-

ticles in distilled water at 20°C. In order to calculate the sedimentation coefficient accurately it would be essential to know the density of the particles. Since this information was not available, sedimentation coefficients for particles ranging in density from 1.1 to 1.4 were calculated. At the assumed densities the range of sedimentation coefficients for relaxing particles was found to be 373 to 1880S.

The particles displaying relaxing activity could be sedimented completely free of the sample zone when subjected to the much higher centrifugal field of 40,000 RPM × 60 min. (90,000 × g max. and total $\omega^2 t$ = 62,500 × 10^6). Under these conditions, using the same gradient as in the 20,000 RPM run

TABLE III

SEDIMENTATION COEFFICIENTS (IN WATER AT 20°C) CALCULATED FOR PARTICLES WITH RELAXING ACTIVITY ISOLATED FROM RAT SKELETAL MUSCLE IN ZONAL ULTRACENTRIFUGE ROTOR B-IV

Fraction No.	$s_{20,w}$ (in Svedbergs) at assumed particle densities of			
	1.1	1.2	1.3	1.4
9	712	443	393	373
10	913	544	480	454
11	1123	643	565	532
12	1349	742	647	609
13	1603	841	730	685
14	1879	940	811	759

These data were computed from the experiment described in Fig. 3 (20,000 RPM × 40 minutes and $\omega^2 t$ = 11,878 × 10^6).

described above, the relaxing activity was restricted to a narrow region far out in the gradient (30 to 36 per cent sucrose w/w), while the mitochondria (cytochrome oxidase activity) formed a sharp peak slightly further out in the rotor (at a level ranging from 43 to 48° sucrose w/w).

DISCUSSION

The experiments described in this paper demonstrate the feasibility of using the zonal centrifuges, with suitably prepared sucrose density gradients, to isolate granules possessing relaxing activity from homogenates of mammalian skeletal muscle on a relatively large scale. Furthermore, the data show that the relaxing activity is indeed associated only with particles having sedimentation characteristics attributed to microsomes. These observations are consistent with the concept that the relaxing principle represents fragments of the sarcoplasmic reticulum. Furthermore, as a first approximation, the behavior of relaxing particles in the zonal centrifuge seems to parallel that already seen (Hasselbach and Makinose, 1963) in conventional small scale density gradient fractionation.

While the observation of Baird and Perry (1960) concerning the relaxing activity of mitochondrial preparations could be confirmed when rat skeletal muscle was fractionated by differential centrifugation, relaxing potency was found to be very definitely separated from mitochondria when fractionation was carried out by means of zonal centrifugation. This suggests that the mitochondrial fraction obtained by differential centrifugation must have been heavily contaminated by particles possessing relaxing activity. In fact, such an assumption has already been made (Martonosi and Feretos, 1964; Weber *et al.*, 1964) because the calcium binding of skeletal muscle mitochondrial fractions was not inhibited by compounds which interfere with calcium uptake by kidney and heart mitochondrial preparations.

The cytochrome oxidase activity was employed as an enzymatic marker in the present study to determine the distribution of the mitochondria. This appears to be a valid assumption in view of the observations that this enzyme is built into the structural elements of mitochondria (Gamble and Lehninger, 1956) and apparently is not associated with any other species of subcellular particles (de Duve *et al.*, 1962; Hogeboom *et al.*, 1953; Schuel *et al.*, 1964). A low level of oxidase activity appeared to be spread out in the gradient between the soluble phase and the mitochondrial regions. This may reflect the presence of various fragments of mitochondria produced during the homogenization of the tissue (Muscatello *et al.*, 1961).

The morphology of microsomal fractions obtained from skeletal (Nagai *et al.*, 1960; Muscatello *et al.*, 1961; Ebashi and Lipmann, 1962) and cardiac (Stram and Honig, 1962) muscle by differential centrifugation has been previously investigated with the electron microscope. These workers reported that the preparations rich in relaxing activity contained large numbers of membrane-limited vesicles and tubules which appeared to be fragments of the sarcoplasmic reticulum (Porter, 1961). Preliminary observations made by Dr. W. Harris in the course of the present investigation, using the negative staining technique, confirmed these earlier observations. Detailed results of an intensive morphological study on embedded sections of pellets obtained from active as well as inactive fractions will be published at a later date.

It has been reported that the high-speed supernatant fraction obtained from homogenates of mammalian skeletal muscle by differential centrifugation contained a potent relaxing substance (Baltscheffsky, 1964; Fuchs and Briggs, 1963). Our data do not confirm these observations, for no activity could be detected in the soluble phase. The trace of relaxing activity reported in the high-speed supernatant fraction obtained by differential centrifugation may result from the presence of a few residual microsomal particles.

Separations of subcellular particles in the zonal centrifuge systems can be achieved on the basis of differences in sedimentation rate and by sedimentation to the density-equilibrium (isopycnic) position in a density gradient. The

sedimentation characteristics of rat skeletal muscle mitochondria in the B-IV rotor appear to be similar to those already reported for rat liver mitochondria (Schuel *et al.*, 1964); *i.e.*, they are very quickly sedimented to their isopycnic position in sucrose at a density around 1.20 gm/cc. However, considerable additional data are necessary to establish more precisely the isopycnic point for skeletal muscle mitochondria. Furthermore, it is evident that the A-IX zonal centrifuge rotor is an ideal system for achieving separations of the larger subcellular particles of muscle based on differences in rates of sedimentation.

The isolation of particles with relaxing activity in the present study depended mainly upon their sedimenting velocity, but the results of the full speed run in the B-IV rotor (40,000 RPM × 60 min.) suggest that it may also be possible to band the relaxing activity granules isopycnically. In the latter system when used at maximum speed the relaxing particles are sedimented far out into the rotor away from the sample zone. Estimates for the equivalent $s_{20,w}$ values of relaxing particles were calculated to range between 373 and 1880S with assumed particle densities of 1.4 to 1.1 respectively. This estimate is expected to be useful for designing future centrifugation schedules and gradient compositions for isolating the relaxing particles on a large scale which is a prerequisite for further studies.

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