THE SARCOTUBULAR SYSTEM OF FROG SKELETAL MUSCLE

A Morphological and Biochemical Study

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ABSTRACT

In the frog skeletal muscle cell a well defined and highly organized system of tubular elements is located in the sarcoplasm between the myofibrils. The sarcoplasmic component is called the sarcotubular system. By means of differential centrifugation it has been possible to isolate from the frog muscle homogenate a fraction composed of small vesicles, tubules, and particles. This fraction is without cytochrome oxidase activity, which is localized in the mitochondrial membranes. This indicates that the structural components of this fraction do not derive from the mitochondrial fragmentation, but probably from the sarcotubular system. This fraction, called sarcotubular fraction, has a Mg^{++}-stimulated ATPase activity which differs from that of muscle mitochondria in that it is 3 to 4 times higher on the protein basis as compared with the mitochondrial ATPase, and is inhibited by Ca^{++} and by deoxycholate like the Kielley and Meyerhof ATPase. We therefore conclude that the "granules" of the Kielley and Meyerhof ATPase, which were shown to have a relaxing effect, are fragments of the sarcotubular system. The isolated sarcotubular fraction has a high RNA content and demonstrable activity in incorporating labeled amino acids, even in the absence of added supernatant.

INTRODUCTION

Three main types of structures are present in the cross-striated muscle fibers, namely, myofibrils, mitochondria, and the interfibrillar component called "sarcoplasmic reticulum" or "sarcotubular system." Myofibrils and mitochondria have been the object of intense morphological and biochemical studies in the past decade.

The interfibrillar component was described long ago by, among others, Retzius (1) and Veratti (2), and more recently by Bennett and Porter (3), Andersson (4, 5), and Porter and Palade (6). From these studies the suggestion was advanced that the interfibrillar component might be involved in the transmission of the excitatory impulse from the cell membrane to the contractile material. The possibilities were also considered that the sarcotubular system could be analogous to the endoplasmic reticulum of other cells and that it could be involved in the extramitochondrial metabolism of the sarcoplasm. However, no report has yet ap-

peared concerning the isolation of the sarcotubular system and the study of its biochemical properties.

On the other hand, important biochemical activities have been found associated with the "granules" prepared from muscle homogenate. A part of these "granules" consist of mitochondria (7), intact or fragmented, and has been used for studies on the mechanism of electron transport and oxidative phosphorylation. "Granular" preparations have also been used in the studies of muscle physiology and biochemistry, since it was found that muscle "granules" are needed for the relaxation of the muscle fibers and the inhibition of the myofibrillar ATPase (8–14).

Furthermore, muscle "granules" of different sizes have been found to exhibit ATPase[1] activity (15, 16). An ATPase preparation has been isolated by Kielley and Meyerhof (17, 18) and demonstrated to be lipoprotein in nature. It was suggested that this preparation was related to the "microsomal" component of the muscle. However, after the demonstration that muscle mitochondria also had an ATPase activity, it became difficult to decide whether the Kielley and Meyerhof ATPase preparation was the result of a fragmentation of mitochondria, or due to the presence among the myofibrils of "granular" structures endowed with a completely different physiological function (19).

The present investigation was undertaken to identify, by means of a correlated morphological and biochemical study, the muscle "granules" separated by differential centrifugation from a muscle homogenate, and to isolate a purified fraction from the sarcotubular system of frog skeletal muscle.

The term "sarcotubular fraction" will be used in the following presentation to denote a fraction obtained by means of differential centrifugation of muscle homogenate. As will be shown later, experimental support for the use of this term is given both by the morphological appearance of the structures present in the sediments, and by biochemical properties which exclude any possible derivation of these structures from fragmentation of mitochondria, the latter being the only components in the intact muscle cell expected to give rise to similar structures upon homogenization.

[1] The following abbreviations are used: RNA, ribonucleic acid; ATP, adenosine triphosphate; GTP, guanosine triphosphate; PEP, phosphoenolpyruvate; tris, tris(hydroxymethyl)aminomethane buffer; DOC, deoxycholate; TCA, trichloroacetic acid; DPNH, reduced diphosphopyridine nucleotide; Pi, inorganic phosphate; ATPase, adenosine triphosphatase activity.

METHODS

Frogs (*Rana temporaria*) weighing 20 to 30 g. were killed by decapitation and the hind legs were quickly skinned. One head of the semitendinosus muscle and sometimes also the sartorius muscle were immediately prepared for electron microscopy. The other muscles from the legs were excised, dropped into a chilled solution of 0.25 M sucrose, and weighed. The muscles were then blotted with filter paper, very finely cut with scissors, and transferred to chilled 0.88 M sucrose. An all glass Potter-Elvehjem homogenizer with a loosely fitting pestle was used for the homogenization of the minced muscle. The homogenization was not prolonged beyond 20 seconds, and the homogenized material was kept in an ice water bath. The homogenate was diluted with 0.88 M sucrose to a volume of 10 times the initial weight of the muscle, and centrifuged in a model L Spinco ultracentrifuge.

In the experiments where a fraction termed "sarcotubular fraction" was used, the muscle homogenate was first centrifuged at 75,000 *g* for 40 minutes, in order to sediment nuclei, myofibrils, mitochondria, connective tissue, and intact cells. Although a part of the sarcotubular system was lost in the sediment, this centrifugal force was selected in order to avoid contamination by mitochondrial fragments. The supernatant obtained from this centrifugation was centrifuged for 60 minutes at 105,000 *g*. The white, translucent pellet was resuspended in 0.25 M sucrose and used for biochemical analysis.

In the experiments where a fraction termed "mitochondrial fraction" was used, the muscle homogenate in 0.88 M sucrose was first centrifuged at 2,000 *g* for 7 minutes in order to sediment nuclei, myofibrils, and intact cells. The supernatant was centrifuged at 15,000 *g* for 10 minutes and the pellet obtained from this centrifugation was resuspended in 0.25 M sucrose. In some cases frog muscle mitochondria and sarcotubular fraction were prepared by using the Chappell and Perry tris-KCl medium (20).

Biochemical Analysis: The cytochrome oxidase activity was measured according to the procedure described by Potter (21). Concentration of the reagents in a final volume of 1.5 ml. was as follows: 33 mM phosphate buffer (pH. 7.4), 0.08 mM cytochrome C, 0.4 mM $AlCl_3$, 11.4 mM Na-ascorbate. The temperature was 30°C.

The RNA content was measured according to Schneider (22).

The ATPase activity was measured on the mitochondrial and sarcotubular fractions in a medium of the following composition in a final volume of 2 ml.:

50 mM KCl, 25 mM tris (pH 7.5), 5 mM ATP. The reaction was started by addition of 1 ml. of enzyme suspension (about 1 mg. protein per ml.) in 0.25 M sucrose and the tubes were incubated for 20 minutes at 30°C. with gentle shaking. The reaction was stopped by addition of 1 ml. 1 M perchloric acid, and the inorganic phosphate was analyzed according to the modified Martin and Doty method (23).

The protein concentration was measured by the biuret reaction.

Concentration of the reagents in the test system used for determination of the DPNH-cytochrome C reductase activity was as follows: 10 mM KCN, 2 mg. cytochrome C, 0.22 mg. DPNH, 20 mM phosphate buffer (pH 7.5), 0.1 to 1.0 mg. protein, and 200 mM sucrose. Final volume was 3 ml.

The amino acid incorporation was measured on the sarcotubular fraction in a medium of the following composition in a final volume of 1 ml: 10 mM $MgCl_2$, 25 mM KCl, 35 mM tris (pH 7.8), 250 mM sucrose, 1 mM ATP, 10 mM PEP, 0.2 mM GTP, 15 μg. pyruvate kinase, 1.6 mg. adenylate kinase, 0.08 mM ^{14}C-*l*-leucine, and resuspended sarcotubular fraction corresponding to 4 mg. of protein. After incubation at 20°C., 0.2 ml. of saturated solution of *dl*-leucine and TCA to a final concentration of 5 per cent was added to the tubes. The protein content was adjusted to the same amount and the protein was extracted as described by von der Decken and Hultin (24). The radioactivity was measured by a Tracerlab thin mica window counter. The activity values were recalculated for infinite sample thickness by means of an empirical saturation curve.

Electron Microscopy: The semitendinosus and sartorius muscles of the frog were fixed at rest length *in toto* in 1 per cent isotonic osmium tetroxide solution buffered with veronal acetate to pH 7.2. The preparation and immersion in the fixative were made at room temperature. The fixation for 4 hours, the rinsing in Ringer solution, and the dehydration in 70 per cent alcohol overnight were carried out at +2°C. The final dehydration in increasing concentrations of alcohol was carried out again at room temperature.

Two embedding media were employed, Araldite (25) and Epon (26). Before embedding, the muscle was cut into smaller pieces. The sectioning was performed with a Sjöstrand ultramicrotome and an LKB 4800 ultrotome. Both unstained sections and sections stained with saturated uranyl acetate (27, 28) were examined. The electron microscopes used were the RCA 3A at 100 kv. and the Akashi Transcope 50D. 50 μ molybdenum apertures were used in the objective pole pieces. The primary magnifications were 8,000 to 25,000.

Electron microscopical analysis of the pellets obtained by differential centrifugation was carried out as follows: The different layers of the fractions were most often separated by differential centrifugation, and each subfraction was prepared for electron microscopy. The pellets were fixed *in situ* for a few minutes with precooled 10 per cent formalin in veronal acetate buffer (pH. 7.2). Small pieces containing all layers were removed from the pellet and transferred to precooled, isotonic buffered 1 per cent osmium tetroxide solution. In the sectioning, care was taken to include all the different layers of the pellet.

Materials: ATP, GTP, cytochrome C, DPNH, and tris were obtained from Sigma Chemical Co., St. Louis, Missouri; PEP and pyruvate kinase from C. F. Boehringer und Söhne, Mannheim, Germany; ^{14}C-*l*-leucine (5.75 mc./mmole) from the Radiochemical Centre, Amersham, England; adenylate kinase was prepared from rabbit muscle and purified as described by Colowick and Kalckar (29).

RESULTS

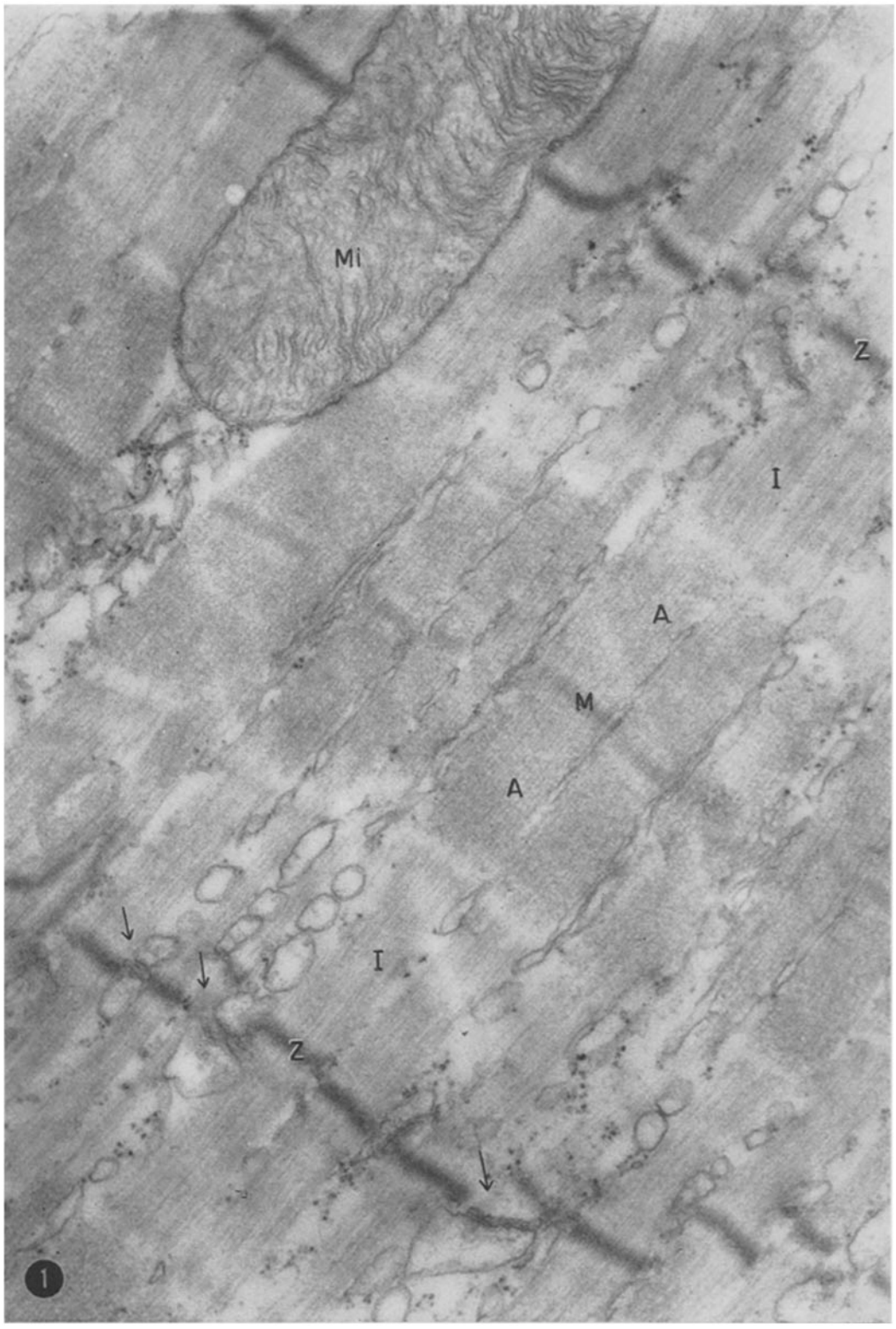
The Muscle Cell: Fig. 1 shows a longitudinal section through a frog semitendinosus muscle cell. Myofibrils, a mitochondrion, a well defined interfibrillar vesicular component, and small particles appear in the picture.

The *myofibrils* in the figure show the ordinary cross-striations (A, M, I, and Z bands) which are observed in a relaxed muscle cell. The *mitochondria* in the frog muscle are rather few in number in comparison with those in other types of muscle (for example, pigeon breast or mouse intercostal muscle). They are irregularly distributed between the myofibrils. In its detailed structure the mitochondrion shows the classical organization with an outer double membrane and a system of inner double membranes (30, 31).

Between the myofibrils there is a highly organized membranous system of tubules which is referred to as the *sarcotubular system* or sarcoplasmic reticulum.

As previously pointed out (5), the amount of this interfibrillar component varies in different skeletal muscle cells of the frog. The muscle cell illustrated in Fig. 1 represents the most common type appearing in the frog leg muscle.

The sarcotubular system seems to be composed of different parts. At the level of the A band of the myofibrils the sarcotubules, with a diameter of about 300 A, run parallel with the myofibrils. At level of the I band, the interfibrillar structures have a larger diameter (Fig. 1). At the Z band level a three-component structure, a "triad" (6), is located. In a longitudinal section the "triad"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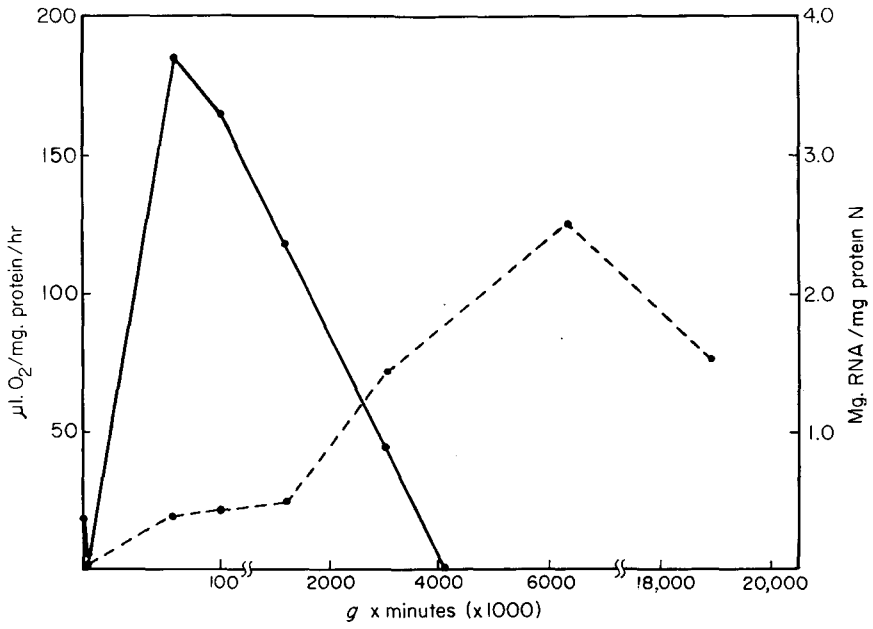


FIGURE 2

Distribution of cytochrome oxidase activity and RNA in the fractions obtained by differential centrifugation of frog skeletal muscle homogenate. After each centrifugation the supernatant was poured into a new tube for the next centrifugation and the pellets were resuspended in 0.25 M sucrose. Aliquots from each suspension were taken for determination of cytochrome oxidase and of RNA and protein content. For the cytochrome oxidase determinations each Warburg vessel contained 33 mM K-Na_2PO_4 buffer (pH 7.4), 0.08 mM cytochrome C, 0.4 mM $AlCl_3$, 11.4 mM Na-ascorbate; final volume, 1.5 ml.; gas phase, air; temperature, 30°C.; 0.2 ml. 2 M KOH in the center well. The solid line shows the cytochrome oxidase activity and the dashed one the RNA/protein N ratios.

appears as two larger vesicles located at each side of the Z band. The vesicles are elongated in a direction transverse to the myofibrils and have their surfaces flattened against each other. The space between these surfaces is about 500 A. In this interspace a small tubular or vesicular structure appears. Only one "triad" appears in each sarcomere.

The continuity of the different parts of the sarcotubular system of the frog muscle cell has not yet been analyzed in detail.

Small *particles* with a diameter of about 150 A are irregularly distributed in the sarcoplasm. The particles are not attached to the sarcotubular membranes.

Fractionation of the Muscle Homogenate: In Fig. 2 and in Table I data from two different experiments are reported concerning the cytochrome oxidase activity as well as the RNA and protein content of various fractions separated by differential centrifugation from a frog muscle homogenate. Frog skeletal muscle as the tissue and 0.88

FIGURE 1

Electron micrograph of a longitudinally sectioned semitendinosus muscle cell of the frog. Different bands of the myofibrils are indicated in one sarcomere (A, M, I, and Z bands). In the upper portion of the picture, part of a mitochondrion (*Mi*) is seen. Sarcotubules appear between the myofibrils. The interfibrillar components located at the I band level of the myofibrils appear as vesicular profiles. Arrows point to the three-component structure, the triad. Small dense particles not attached to the membranes are observed in the sarcoplasm. The specimens, fixed in osmium tetroxide, were embedded in Araldite. × 43,000.

TABLE I

Biochemical Properties of Various Fractions of Frog Skeletal Muscle Homogenate

Fraction	Centrifugal force	mg. protein per g. muscle	Cytochrome oxidase		mg. RNA per g. muscle	mg. RNA per mg. protein
			μl. O_2/g. muscle/hr.	μl. O_2/mg. protein/hr.		
Homogenate		166.0	1826	11.0	6.64	0.040
Myofibrils, nuclei	400 *g* × 7 min.	129.10	516.40	4.0	1.30	0.010
Mitochondria	6,500 *g* × 10 "	3.85	701.85	182.3	0.19	0.049
Mitochondria	10,000 *g* × 10 "	1.52	257.94	169.7	0.10	0.067
Mitochondria	40,000 *g* × 30 "	1.86	174.46	93.8	0.13	0.070
Sarcotubules	75,000 *g* × 30 "	1.40	49.0	35.0	0.26	0.185
Sarcotubules	105,000 *g* × 60 "	0.72	0	0	0.27	0.375
Sarcotubules	105,000 *g* × 180 "	0.92	0	0	0.21	0.228
Final supernatant		17.0	0	0	3.70	0.218

Experimental conditions as in Fig. 2. The gravity at which each fraction was obtained is indicated in the second column.

M sucrose as the homogenizing medium were selected after a number of preliminary experiments were carried out in which muscle of different animals (rat, mouse, and pigeon) and varying concentrations of sucrose in the presence or absence of ions ($MgCl_2$, KCl) were tested. When media containing ions or when muscles with a large number of mitochondria were used, the separation of the various components present in the muscle homogenate was less complete. For example, it was difficult to avoid the presence of mitochondrial fragments in the fractions sedimented at higher centrifugal force.

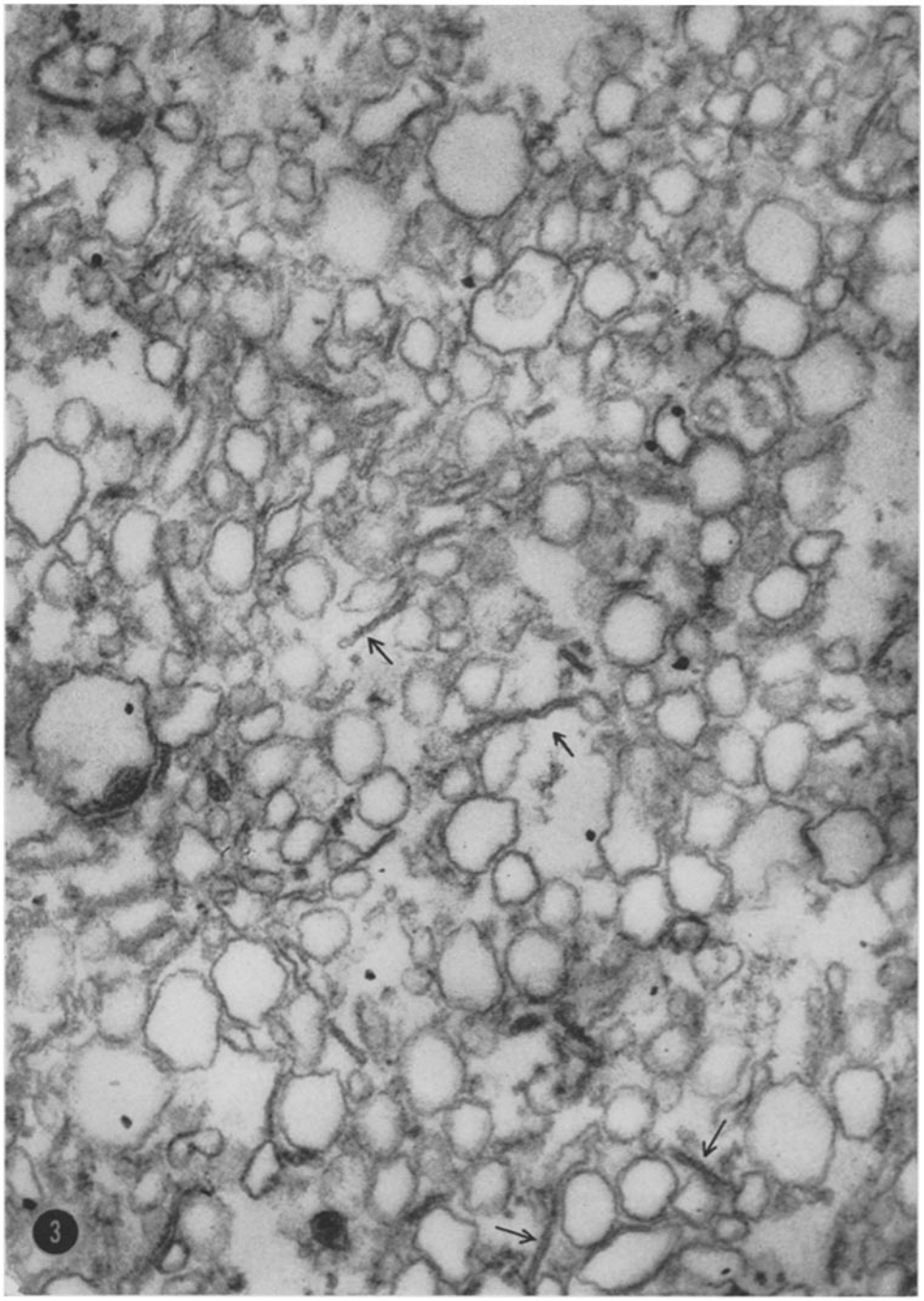
From the data of Fig. 2 and Table I it appears that the cytochrome oxidase activity was recovered in the fractions sedimenting at lower centrifugal force. Because of the presence of high cytochrome oxidase activity these fractions have been termed "mitochondrial fractions." About one-third of the cytochrome oxidase activity remained in the myofibrillar fraction, indicating that a large number of the mitochondria were not separated from the myofibrils during the muscle disintegration. The presence of mitochondria within intact parts of the muscle fibers was also confirmed by electron microscopical analysis performed on the myofibrillar fraction.

The fractions obtained at higher centrifugal force revealed different properties. The cytochrome oxidase activity disappeared and the RNA content increased considerably. This increase in RNA content together with a decreased amount of protein resulted in high RNA/protein ratios in these fractions.

Properties of the Mitochondrial Fragments: In order to exclude the possibility that the structures present in the fractions separated at higher centrifugal force could be derived from the fragmentation of the mitochondria, a detailed morphological and biochemical study was performed on the mitochondrial fragments obtained by mechanical treatment of the mitochondrial fraction, and the

FIGURE 3

Electron micrograph of mitochondrial fragments. The mitochondria were isolated by differential centrifugation between 15,000 *g* for 10 minutes and 40,000 *g* for 30 minutes, after treatment with the Ultra-Turrax blender. The homogenization medium used was 0.88 M sucrose. The rather tightly packed structures are mainly vesicles of different sizes, and tubular elements. Some of the vesicles contain smaller vesicles or a dark area. Tubular elements (arrows) with high density are seen. A few small particles are observed. A very high cytochrome oxidase activity is present in this fraction. The fraction, fixed in formalin and in osmium tetroxide, was embedded in Araldite. The section was stained in uranyl acetate. × 70,000.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properties of the mitochondria at different stages of fragmentation were compared with the morphological properties of the sarcotubules.

Figs. 3 and 4 show electron micrographs from sections of mitochondrial fragments. These fragments were isolated by centrifugation between 15,000 *g* for 10 minutes and 40,000 *g* for 30 minutes in 0.88 M sucrose (Fig. 3) or between 10,000 *g* for 10 minutes and 40,000 *g* for 25 minutes in Chappell and Perry tris-KCl saline solution (Fig. 4). In both micrographs a large number of rather tightly packed vesicles with a diameter up to 0.35 μ are seen. The vesicles were mostly bounded by a single membrane with a thickness of about 50 A. Most of the vesicles appeared empty, but in some cases they contained double-lined membranes, smaller vesicles, or dark areas, the latter being usually located at the periphery. Small particles, as well as single or diffuse aggregations, appeared in both preparations, but they seemed to be more abundant in the fragments obtained with Chappell and Perry medium (Fig. 4). In the preparations obtained with sucrose medium, several tubular elements (arrows, Fig. 3) with a high density are present between the vesicles. If the micrographs in Figs. 3 and 4 are compared with those obtained from the sarcotubular fractions (Figs. 5 and 6), the different average size of the vesicles present in the two preparations is particularly evident.

The biochemical analysis showed that the mitochondrial fragments illustrated in Figs. 3 and 4 had the following properties: (*a*) DPNH–cytochrome C reductase activity; and (*b*) high cytochrome oxidase activity. In additional experiments (Table II) it was demonstrated that extensive fragmentation of the muscle mitochondria, by treatment with an Ultra-Turrax blender (from Janke and Kunkel KG, Staufen), was not able to abolish the cytochrome oxidase activity. The mitochondrial fraction showed also a Mg^{++}-stimulated ATPase activity; but, as will be shown later, this ATPase activity was 3 to 4 times lower than that of the sarcotubular ATPase, and, in addition, it was not inhibited by Ca^{++}.

Properties of the Sarcotubular Fraction: In Figs. 5 and 6 are shown electron micrographs from sections of the sarcotubular fractions. The sarcotubular fraction illustrated in Fig. 5 was obtained by centrifugation between 75,000 *g* for 40 minutes and 105,000 *g* for 60 minutes, and the muscle was homogenized in 0.88 M sucrose. The structures present in this fraction are membrane-limited profiles and small granules or particles. The membranes appear either as single- or double-lined circular profiles of different size, or as small tubular elements. The vesicles have a diameter of 200 to 500 A and the tubules of about 150 to 200 A. The particles have a diameter of about 150 A and are not attached to the membranes. In the pictures with higher resolution the particles seem to be constructed of smaller, dark subunits in a less dense ground substance. The number of particles increases and the vesicles decrease in amount in the layers obtained at higher centrifugal force. In the latter layers there also appear thin filaments.

The fraction illustrated in Fig. 6 was obtained between 40,000 *g* for 30 minutes and 75,000 *g* for 30 minutes, and the muscle was homogenized in Chappell and Perry saline solution. The lower viscosity and the composition of this medium, containing $MgSO_4$ (0.005 M) and a high concentration of KCl (0.1 M), seemed to change the characteristics of the fractions. The 40,000 *g* to 75,000 *g* pellet in saline medium, for instance, contains mostly free particles, while in the fraction obtained at the same centrifugal force in 0.88 M sucrose practically no particles are visible in the electron micrographs. The thin filaments also are much more abundant in the fraction prepared in the Chappell and Perry medium (Fig. 6). It is very probable that these filaments are derived from

FIGURE 4

Electron micrograph of mitochondrial fragments. The mitochondria were isolated by differential centrifugation between 10,000 *g* for 10 minutes and 40,000 *g* for 25 minutes, after treatment with an all glass Potter-Elvehjem homogenizer. The homogenization medium used was the Chappell-Perry tris-KCl solution. Tightly packed vesicles of different sizes appear in the picture. Some of them contain smaller vesicles or dark spots mostly located at the periphery. Single small dark particles or aggregates are also observed. The fraction shows a very high cytochrome oxidase activity. The preparation for electron microscopy was as in Fig. 3. × 70,000.

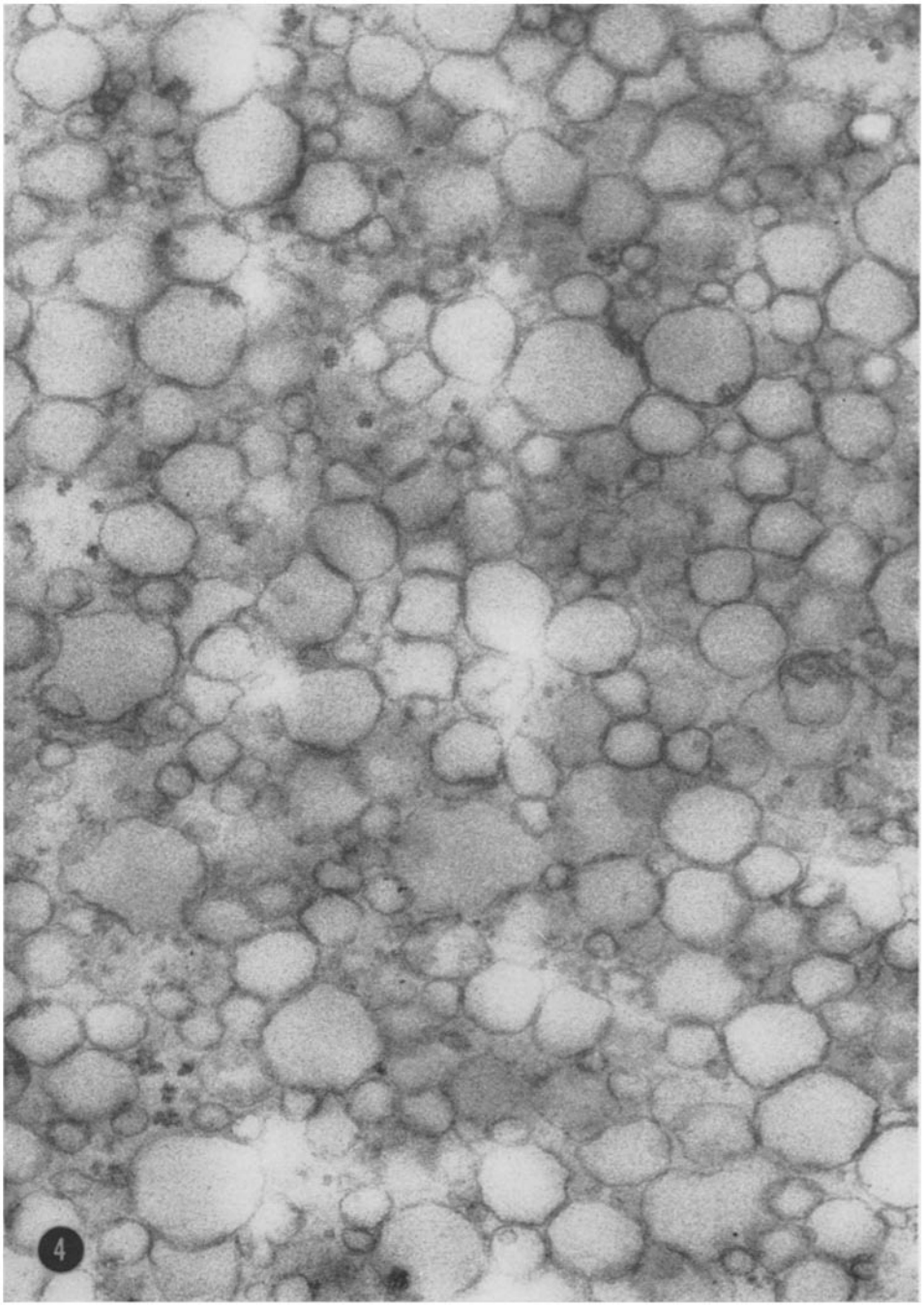
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TABLE II

Cytochrome Oxidase Activity in Intact and Fragmented Frog Skeletal Muscle Mitochondria

Material	Cytochrome oxidase activity	
	μl. O_2/mg. protein/hr.	μl. O_2/g. muscle/hr.
Intact mitochondria	105	388
Fragmented mitochondria		
Total fraction	102	377
Subfraction at 15,000 g × 10 min.	82	199
Subfraction at 105,000 g × 60 min.	285	177

Experimental conditions as in Fig. 2. The frog skeletal muscle mitochondria were isolated from a muscle homogenate in 0.88 M sucrose by differential centrifugation between 2,000 g for 7 minutes and 15,000 g for 10 minutes. One part of the mitochondrial suspension was tested for cytochrome oxidase activity, another part was treated for 60 minutes with an Ultra-Turrax blender. The fragmented mitochondria were either directly tested for cytochrome oxidase activity or divided into two subfractions, the first precipitated at 15,000 g for 10 minutes, and the second at 105,000 g for 60 minutes.

myofibrils. This suggestion is supported by the fact that myofibrillar proteins are more easily extracted from the myofibrils by saline solution, especially in the presence of ATP.

The fractions illustrated in Figs. 5 and 6 upon examination yielded the following biochemical observations: (*a*) no cytochrome component was found in the absorption spectra; (*b*) the cytochrome oxidase and the DPNH–cytochrome C reductase activities were absent; (*c*) a high Mg^{++}-stimulated, Ca^{++}-inhibited ATPase activity was present.

The ATPase Activity of the Sarcotubular Fraction: As mentioned in the introduction, a Mg^{++}-stimulated and Ca^{++}-inhibited ATPase activity has been reported by Kielley and Meyerhof (17, 18) in a lipoprotein fraction from muscle. A Mg^{++}-stimulated ATPase has recently been found in the microsomal fraction of liver cells (32). The following experiments were performed in order to test whether the sarcotubular fraction isolated from frog muscle contained a Mg^{++}-stimulated ATPase different in properties from that found to be associated with muscle mitochondria, and whether the sarcotubular ATPase corresponded to the "soluble muscle ATPase" described by Kielley and Meyerhof.

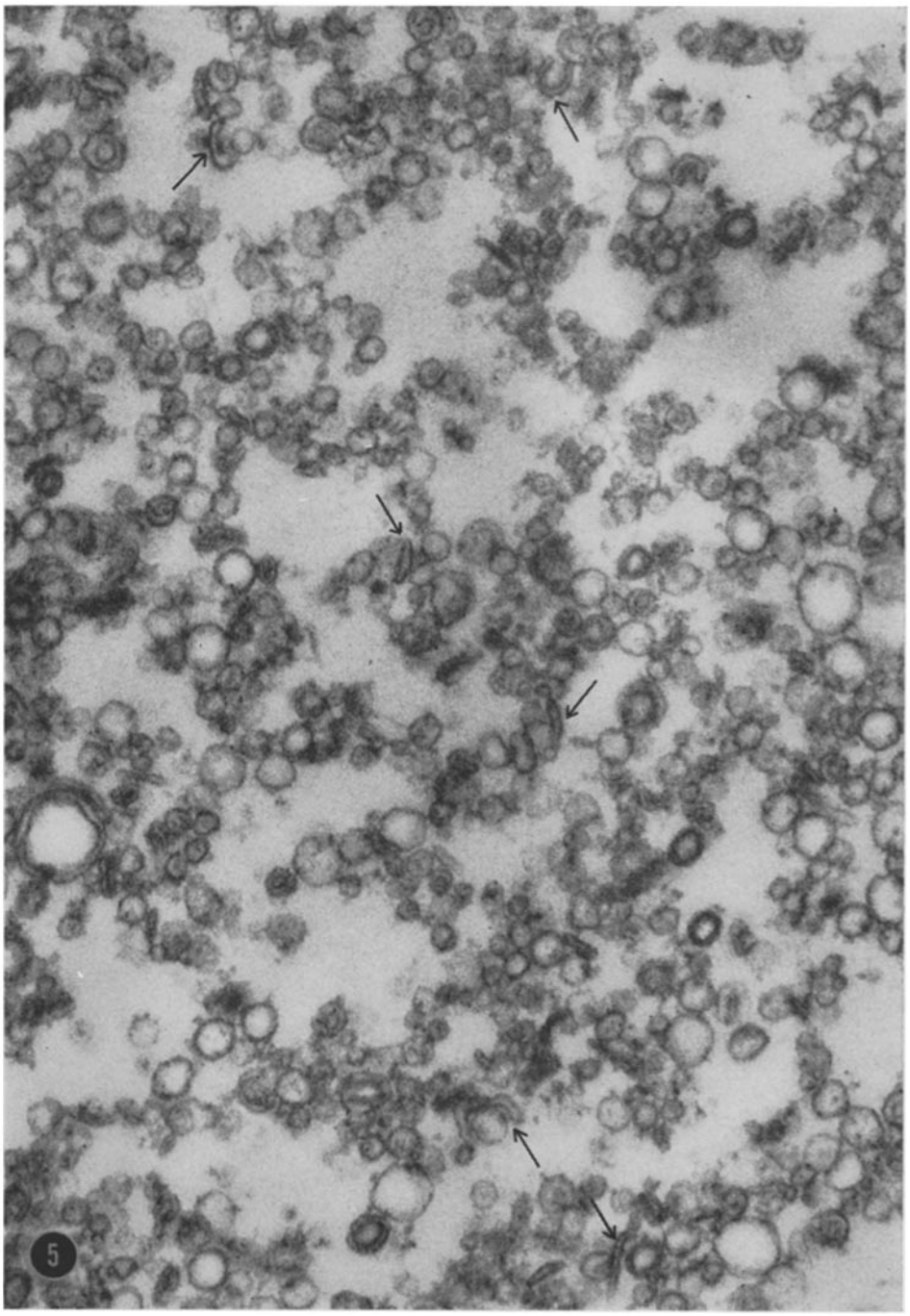
Fig. 7 shows the stimulation of the ATPase activity of the sarcotubular fraction isolated at 105,000 g in 0.88 M sucrose by various concentrations of Mg^{++}. Low concentrations of Mg^{++} stimulated 6 to 8 times the ATPase activity, whereas the stimulation was progressively lost at higher Mg^{++} concentrations. At a Mg^{++}/ATP ratio of 1.6 (Fig. 7) the stimulation was already reduced to about 50 per cent. At a Mg^{++}/ATP ratio of 10, used in the amino acid incorporation experiments, the Mg^{++}-stimulated ATPase activity was inhibited up to about 90 per cent.

An interesting characteristic of the Mg^{++}-stimulated ATPase of the sarcotubular fraction is its sensitivity to Ca^{++} (Fig. 8). Addition of 0.4 mM Ca^{++} inhibited about 50 per cent or more of the ATPase activity stimulated by 4 mM Mg^{++}. However, increasing concentrations of Ca^{++} were not able to abolish completely the ATPase activity.

The sarcotubular ATPase was found to be dependent, as in the case of the Kielley and Meyerhof ATPase, on the maintenance of the lipoprotein structure. As shown in Fig. 9, addition of low concentrations of deoxycholate, which brings about a solubilization of the lipoprotein structure, strongly inhibited the sarcotubular ATPase.

FIGURE 5

Electron micrograph of part of the sarcotubular fraction. This fraction was obtained by differential centrifugation between 75,000 g for 40 minutes and 105,000 g for 60 minutes. The medium used during the homogenization was 0.88 M sucrose. The main structures appearing are single- or double-lined vesicles, smaller in mean diameter than those appearing in Figs. 3 and 4. Tubular elements (at arrows) and a few small granules or particles are also seen. The biochemical analysis of the fraction revealed no cytochrome oxidase activity, a high Mg^{++}-stimulated Ca^{++}-inhibited ATPase activity, and a high RNA content. The preparation for electron microscopy was as in Fig. 3. × 70,000.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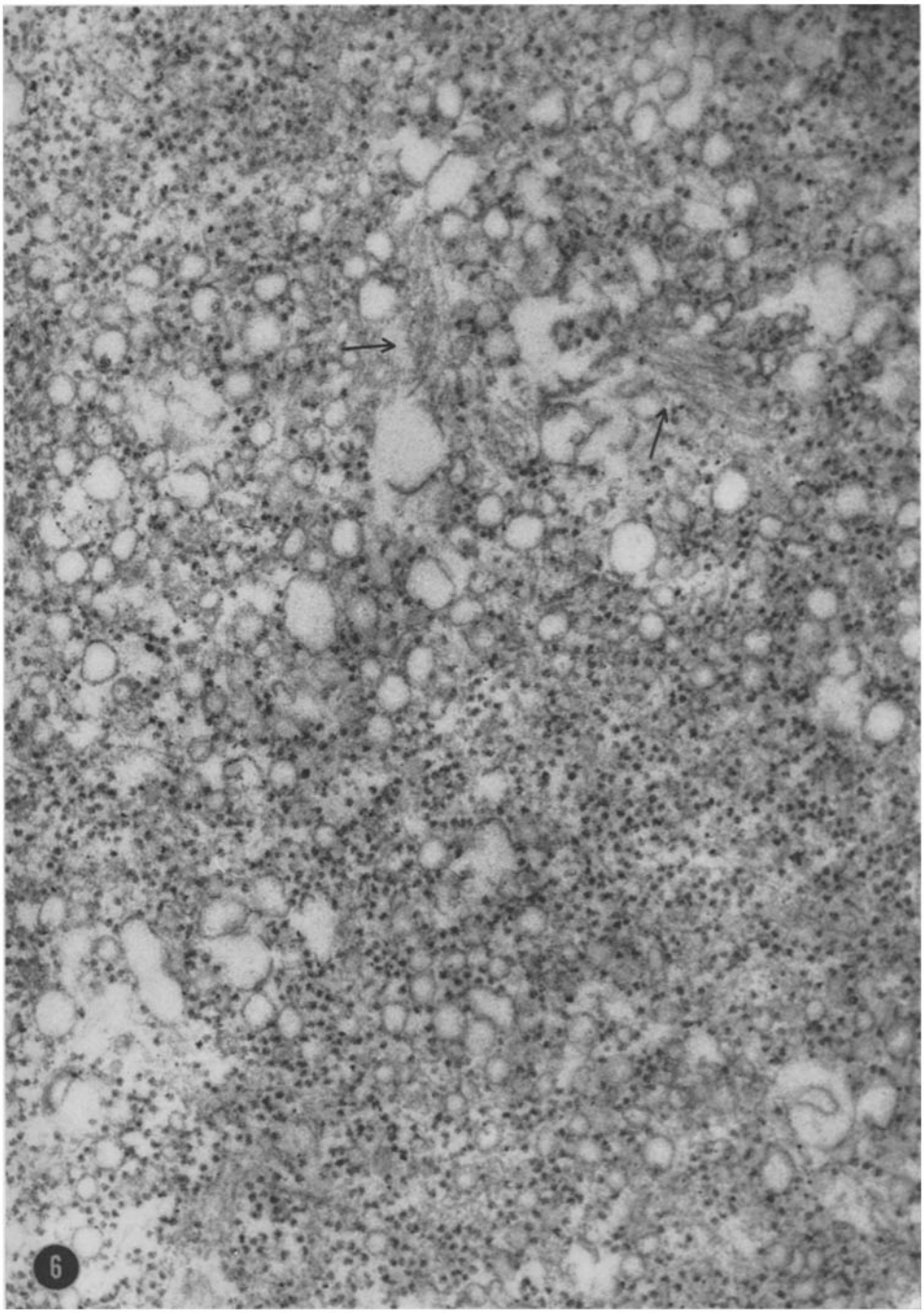
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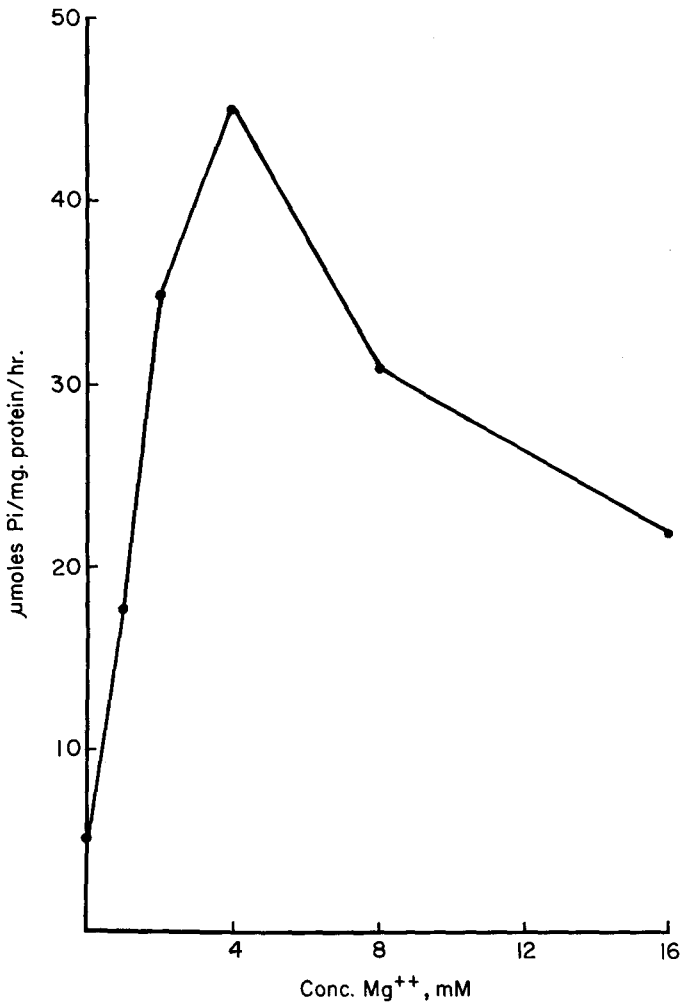

FIGURE 7

Effect of various Mg^{++} concentrations on the ATPase activity of the sarcotubular fraction. Each tube contained 50 mM KCl, 25 mM tris buffer (pH 7.5), 5 mM ATP, 125 mM sucrose; final volume, 2 ml; temperature, 30°C.; incubation time, 20 minutes. The ATPase activity was measured as μmoles of Pi liberated by 1 mg. of sarcotubular protein in 1 hour. The sarcotubular fraction was that shown in Fig. 5.

FIGURE 6

Electron micrograph of part of the sarcotubular fraction. The fraction was obtained by differential centrifugation between 40,000 *g* for 30 minutes and 75,000 *g* for 30 minutes. The medium used during the homogenization was the Chappell-Perry tris-KCl solution. The structures seen are vesicles of different sizes, a large number of small particles, and filaments. The small particles are composed of densely packed subunits in a less dense ground medium. Thin filaments appear in bundles (arrows) or are spread out between the other structures. The biochemical analysis revealed a very low cytochrome oxidase activity, a high RNA content, and a high ATPase activity. The preparation for electron microscopy was as in Fig. 3. × 70,000.

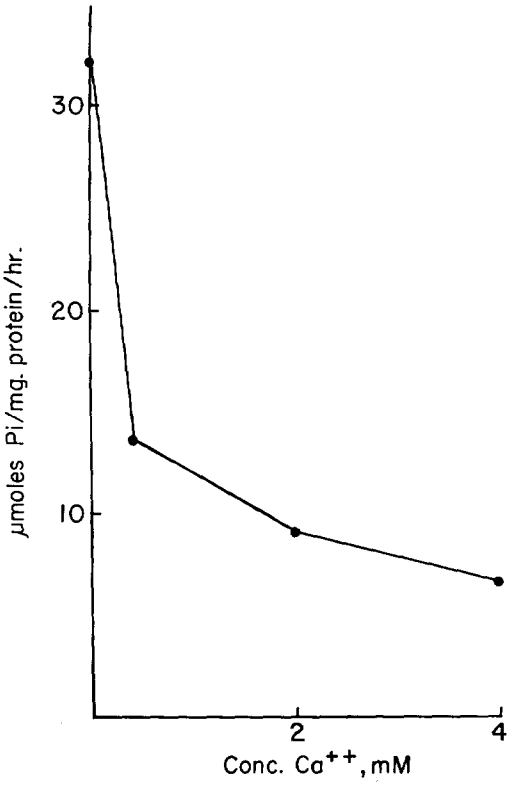

FIGURE 8

Inhibition by Ca^{++} of the Mg^{++}-stimulated ATPase activity of the sarcotubular fraction. Experimental conditions as in Fig. 7. 4 mM $MgCl_2$ was added to all tubes. In the absence of added $MgCl_2$, 2 μmoles Pi/mg. protein/hour were liberated by this preparation. The ATPase activity was measured as μmoles of Pi liberated by 1 mg. of sarcotubular protein in 1 hour. The sarcotubular fraction was obtained as in Fig. 5.

The different features of the Mg^{++}-stimulated ATPase found in the mitochondrial and sarcotubular fractions are illustrated in Table III. The Mg^{++}-stimulated ATPase activity was considerably higher in the sarcotubular fraction than in the mitochondria. About 8 to 10 μmoles Pi/mg. protein/hour at 30°C. were split by the mitochondrial ATPase, whereas 3 to 4 times more Pi was liberated by the sarcotubular ATPase. Furthermore, addition of Ca^{++}, in a molar ratio of 1:5 in relation to the Mg^{++} concentration, produced an inhibition by about 80 per cent of the Mg^{++}-stimulated ATPase of the sarcotubular fraction, but virtually did not interfere with the Mg^{++}-stimulated ATPase of the mitochondrial fraction. Fragmentation of the mitochondria with an Ultra-Turrax blender did not change this pattern. The Mg^{++}-stimulated ATPase of the fragmented mitochondria was increased by about 25 per cent, but no inhibition by Ca^{++} appeared.

Amino Acid Incorporation into the Sarcotubular Fraction: As shown in Table I, a relatively high RNA/protein ratio together with a lack of cytochrome oxidase activity was found in the fraction obtained by centrifugation between 75,000 *g* for 30 minutes and 105,000 *g* for 60 minutes in 0.88 M sucrose. To characterize this fraction as a fraction corresponding to the microsomes of other tissues, the ability to incorporate labeled amino acids into protein was investigated. As shown in Table IV, incorporation of amino acids was obtained also in the absence of soluble supernatant. Addition of 105,000 *g* supernatant from frog muscle even inhibited the incorporation slightly, whereas a small increase was observed in the presence of rat liver supernatant. The rate of amino acid incorporation was constant over a period of 2 hours; as shown in Fig. 10, the increase in isotope content was proportional to the time of incubation.

As shown in the electron micrographs (Figs. 5 and 6), the particles found in the sarcotubular fractions were not attached to the membranes. In spite of this lack of structural relation, the possibility of a functional relation between the particles and the sarcotubular membranes during the process of amino acid incorporation was investigated. The distribution of radioactivity after 2 hours of incubation is shown in Table V. Most of the radioactivity was found in the deoxycholate-insoluble fraction, which contained the ribonucleoprotein particles. The radioactivity of the deoxycholate-soluble fraction, which probably corresponds to the membrane part (33) of the sarcotubules, was almost negligible. About 16 per cent of the total activity was released from the ribonucleoprotein particles to the supernatant.

DISCUSSION

As described under Results, the components present in the fraction termed sarcotubular fraction were single- or double-lined small vesicles and tubules, together with a certain number of small particles not attached to the membranes. It seems that this fraction is not representative of the whole sarcotubular system present in the intact muscle cell. The necessity of avoiding mitochondrial con-

tamination has restricted the range of centrifugal force within which the sarcotubular membranes could be isolated. It is plausible to believe, and some experimental support has already been obtained for this conclusion, that some vesicles, originating from the larger sarcotubules located at the I band level, were sedimented at lower centrifugal force.

A large part of the present investigation was devoted to the question of the possible presence of mitochondrial fragments in the sarcotubular fraction. The conclusion that the fraction obtained at higher centrifugal forces contains little or no contamination from mitochondrial fragments was supported by the morphological and biochemical analysis.

The different morphological appearance of the vesicles in the electron micrographs, the presence and absence of cytochrome oxidase and DPNH-cytochrome C reductase activities and cytochrome absorption spectrum, together with the different features of the ATPase activities, were considered sufficient criteria to distinguish between mitochondrial fragments and sarcotubular structures.

The following evidence strongly supports the conclusion that the Kielley and Meyerhof ATPase preparation corresponds to the sarcotubular ATPase: (*a*) Both ATPases are strongly inhibited by Ca^{++}; (*b*) both ATPases are inhibited by a damage to the lipoprotein structure. On the contrary, the other two ATPases present in a muscle homogenate, the myofibrillar and the

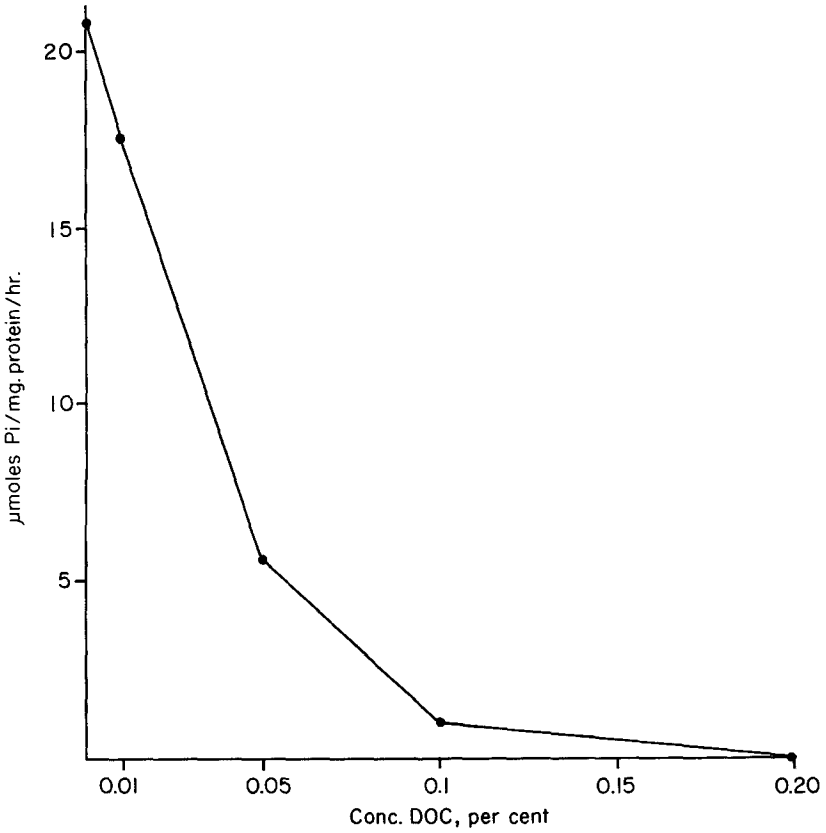

FIGURE 9

Inhibition by deoxycholate of the Mg^{++}-stimulated ATPase activity of the sarcotubular fraction. Experimental conditions as in Fig. 7. 4 mM $MgCl_2$ was added to all tubes. In the absence of added $MgCl_2$, 0.54 μmoles Pi/mg. protein/hour were liberated by this preparation. Resuspended sarcotubular fraction, corresponding to 0.50 mg. protein, was added to each tube. The ATPase activity was measured as μmoles of Pi liberated by 1 mg. of sarcotubular protein in 1 hour. The sarcotubular fraction was obtained as in Fig. 5.

TABLE III

ATPase Activity of Intact and Fragmented Mitochondria and of the Sarcotubular Fraction from Frog Skeletal Muscle

Material	ATPase activity (μmoles Pi/mg. protein/hr.)		
	Without Mg^{++}	With Mg^{++}	With Mg^{++} and Ca^{++}
Intact mitochondria	0.66	7.49	6.93
Mitochondrial fragments	0.76	9.49	8.77
Sarcotubular fraction	1.41	23.13	5.47

Experimental conditions as in Fig. 7. 4 mM $MgCl_2$ and 0.8 mM $CaCl_2$ were added when indicated. The mitochondrial fraction was isolated by centrifuging a frog muscle homogenate between 2,000 *g* for 7 minutes and 15,000 *g* for 10 minutes. The sarcotubular fraction was isolated by centrifuging the supernatant, after sedimentation of the mitochondria, between 75,000 *g* for 40 minutes and 105,000 *g* for 60 minutes. The mitochondria were tested for ATPase activity either directly or after being fragmented by treatment with an Ultra-Turrax blender for 60 seconds.

TABLE IV

Incorporation of ^{14}C-l-Leucine into Protein by the Sarcotubular Fraction of Frog Skeletal Muscle

Material	C.P.M./cm.^{2}
Complete system	34
Complete system plus 105,000 *g* supernatant from frog skeletal muscle	24
Complete system plus 105,000 *g* supernatant from rat liver	40

Quantities of the reagents in a final volume of 1 ml. were as follows: 10 mM $MgCl_2$, 25 mM KCl, 35 mM tris buffer (pH 7.8), 250 mM sucrose, 1 mM ATP, 10 mM PEP, 15 μg. pyruvate kinase, 0.08 mM ^{14}C-*l*-leucine, and resuspended sarcotubular fraction corresponding to 4 mg. of protein. The sarcotubular fraction was obtained as in Fig. 5. The added frog muscle supernatant and rat liver supernatant (34) contained 5 mg. and 1 mg. of protein, respectively. After incubation for 30 minutes at 20°C., 0.2 ml. of a saturated solution of *dl*-leucine and TCA to a final concentration of 5 per cent were added to the tubes. The protein content was adjusted to the same amount and the protein was extracted as described previously (24). The radioactivity was measured by a Tracerlab thin mica window counter. The activity values were recalculated for infinite sample thickness by means of an empirical saturation curve.

mitochondrial ATPases, are not inhibited by Ca^{++} or by lipoprotein-solubilizing agents.

It seems thus possible to distinguish among three types of muscle ATPase: (*a*) a water-insoluble, Mg^{++}- and Ca^{++}-stimulated ATPase, which belongs to the myofibrils; (*b*) a granular, Mg^{++}-stimulated ATPase, which belongs to the mitochondria; (*c*) a granular, Mg^{++}-stimulated and Ca^{++}-inhibited ATPase, which belongs to the sarcotubules. At present, however, the possibility cannot be excluded (35, 36) that a part of the mitochondrial Mg^{++}-stimulated ATPase is due to the presence in the mitochondrial fraction of part of the sarcotubular system (37).

The high RNA content and the amino acid incorporating capacity of the sarcotubular fraction would seem to indicate a certain analogy between the sarcotubules and the microsomes of other cells (38). However, the significance of this type of correlation is diminished by the following facts: (*a*) In both the intact muscle cell and the pellets, the ribonucleoprotein particles are free and not attached to the surface of the membranes; and no significant amino acid incorporation was found in the protein in the deoxycholate-soluble fraction. (*b*) When the amino acid incorporating capacity of the frog sarcotubular fraction was compared with that of rat liver microsomes on the basis of protein or RNA content, a lower activity was found in the muscle preparation. From a careful study of the electron micrographs obtained from different fractions and their relative RNA content, it seems that the RNA data are higher than might be accounted for by the number of particles present in the electron micrographs. The possibility cannot be excluded that at least part of the RNA may be present in the muscle in a physicochemical state different from that known to occur in the case of the "rough" membranes of the liver and pancreas cells, which might render it more difficult to observe in the electron micrographs. We would suggest, as an alternative explanation, that part of the RNA may not be in the form of compact ribonucleoprotein particles and that its presence in the sarcotubular fraction may be due to some structural relation of the RNA with the vesicular components of the sarcotubular fraction. A similar conslusion was recently reached for the liver microsomes by Moulé *et al.* (39).

TABLE V

Distribution of Radioactivity after Incorporation of ^{14}C-l-Leucine into Protein by Frog Skeletal Muscle Sarcotubular Fraction

Material	Total count	C.P.M./cm.^{2}
Unfractionated system	336	48
105,000 *g* supernatant	60	20
Sarcotubular fraction, DOC extract	9	4
Sarcotubular fraction, DOC residue	291	166

Experimental conditions as in Table IV, except that the final volume was 4 ml., pyruvate kinase was added to 4-fold amounts, and adenylate kinase was omitted. Rat liver supernatant (12 mg. protein) was also included. The sarcotubular fraction was obtained as in Fig. 5. The system was incubated for 120 minutes at 20°C. A sample of 0.7 ml. was taken for determination of radioactivity in the unfractionated system. The remaining incubation mixture was centrifuged at 105,000 *g* for 1 hour to isolate the supernatant. The pellet obtained was homogenized in 2.5 ml. of 0.25 per cent DOC and centrifuged for 1 hour at 105,000 *g* to give soluble fraction and residue. The three fractions obtained were precipitated with TCA and extracted as described in Table IV. The total number of counts was calculated from the activity values.

The isolation of the sarcotubular fraction without contamination by mitochondrial fragments raises the question of the composition of the muscle-relaxing factor system (RFS or Marsh factor) which is known to be associated with the muscle "granules." Since muscle "granules" apparently can be derived from the fragmentation of either the mitochondria or the sarcotubular system, one of these two components should contain the active principle of the relaxing factor system. The possibility cannot be excluded at present that the mitochondrial fragments, which are certainly contained in large amount in the "granules" so far used, might participate in inducing the relaxing effect. It is to be noticed, however, that the procedures used for the isolation or the storage of the relaxing factor system are not compatible with the maintenance of the most typical mitochondrial activities. Furthermore, the conclusion is reached in the present paper that the sarcotubular ATPase corresponds to the Kielley and Meyerhof ATPase preparation, which recently has been reported (40) to have a relaxing capacity. From the above guidances the suggestion is here advanced that the sarcotubular structures constitute the active part of the relaxing factor system. Of special significance therefore is the effect of Ca^{++}, which induces a strong inhibition of the Mg^{++}-stimulated ATPase of the sarcotubules, at the same time as it completely removes the relaxing effect of the "granules" (9, 41). The relaxing effect of the sarcotubular preparations and the relationship between the architecture of the sarcotubular system in the intact muscle and its possible physiological role in the process of muscle contraction and relaxation will be discussed in forthcoming papers.

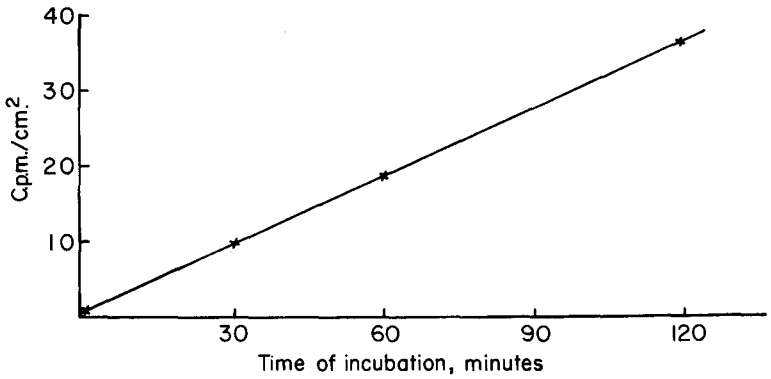

FIGURE 10

Time curve of incorporation of labeled amino acids into protein by the sarcotubular fraction. Incorporation system as in Table IV, but with 2 mg. sarcotubular protein and 2 mg. rat liver supernatant. Adenylate kinase was omitted. The sarcotubular fraction was obtained as in Fig. 5.

This work has been supported by grants No. 243 and No. 96 from the Swedish Medical Research Council and by a grant to the Institute of General Pathology, University of Padua, from the Muscular Dystrophy Associations of America, Inc.

The authors wish to thank Professor Ture Petrén, head of the Department of Anatomy, and Professor Olov Lindberg, Chairman of the Wenner-Gren Institute, for facilitating the work; Dr. Lars Ernster, of the Wenner-Gren Institute, for stimulating discussions; Dr. Marvin Karasek for correcting the English manuscript; and Mrs. Karin Persson, Miss Elisabeth Rosengren, and Miss Lillemor Lundberg, of the Department of Anatomy, for excellent technical assistance.

Received for publication, December 29, 1960.

REFERENCES

1. RETZIUS, G., *Biol. Untersuch.*, 1881, **1**, 1.
2. VERATTI, E., *Mem. r. Ist. lombardo sci. e lettere*, 1902, **19**, 87.
3. BENNETT, H. S., and PORTER, K. R., *Am. J. Anat.*, 1953, **93**, 61.
4. ANDERSSON, E., *Proc. Stockholm. Conf. Electron Microscopy, 1956*, New York, Academic Press, Inc., 1957, 208.
5. ANDERSSON-CEDERGREN, E., *J. Ultrastruct. Research*, 1959, suppl. 1.
6. PORTER, K. R., and PALADE, G. E., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 269.
7. CLELAND, K. W., and SLATER, E. C., *Biochem. J.*, 1953, **53**, 547.
8. MARSH, B. B., *Nature*, 1951, **167**, 1065.
9. PORTZEHL, H., *Biochim. et Biophysica Acta*, 1957, **24**, 474.
10. BRIGGS, F. N., and PORTZEHL, H., *Biochim. et Biophysica Acta*, 1957, **24**, 482.
11. PORTZEHL, H., *Biochim. et Biophysica Acta*, 1957, **26**, 373.
12. BRIGGS, F. N., KALDOR, G., and GERGELY, J., *Biochim. et Biophysica Acta*, 1959, **34**, 211.
13. MAKINOSE, M., and HASSELBACH, W., *Biochim. et Biophysica Acta*, 1960, **43**, 239.
14. NAGAI, T., MAKINOSE, M., and HASSELBACH, W., *Biochim. et Biophysica Acta*, 1960, **43**, 223.
15. SACKTOR, B., *J. Gen. Physiol.*, 1953, **36**, 371.
16. SACKTOR, B., *J. Gen. Physiol.*, 1954, **37**, 343.
17. KIELLEY, W. W., and MEYERHOF, O., *J. Biol. Chem.*, 1948, **176**, 591.
18. KIELLEY, W. W., and MEYERHOF, O., *J. Biol. Chem.*, 1950, **183**, 391.
19. PERRY, S. V., *in* Comparative Biochemistry, (N. Florkin and H. S. Mason, editors), New York, Academic Press, Inc., 1960, **2**, 245.
20. CHAPPELL, J. B., and PERRY, S. V., *Nature*, 1954, **173**, 1094.
21. POTTER, V. R., *in* Manometric Techniques, (W. W. Umbreit, R. H. Burris, and J. F. Stauffer, editors), Minneapolis, Burgess Pub., 1957, 170.
22. SCHNEIDER, W. C., *in* Methods in Enzymology, (S. P. Colowick and N. O. Kaplan, editors), New York, Academic Press, Inc., 1957, **3**, 680.
23. LINDBERG, O., and ERNSTER, L., *in* Methods of Biochemical Analysis, (D. Glick, editor), New York, Interscience Publishers, Inc., 1955, **3**, 1.
24. DECKEN, A. VON DER, and HULTIN, T., *Biochim. et Biophysica Acta*, 1960, **45**, 139.
25. GLAUERT, A. M., and GLAUERT, R. H., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 191.
26. LUFT, J., *in* Histological Techniques for Electron Microscopy, (D. C. Pease, editor), New York, Academic Press, Inc., 1960, 79.
27. WATSON, M. L., *J. Biophysic. and Bicchem. Cytol.*, 1958, **4**, 475.
28. BRODY, I., *J. Ultrastruct. Research*, 1959, **2**, 482.
29. COLOWICK, S. P., *in* Methods in Enzymology, (S. P. Colowick and N. O. Kaplan, editors), New York, Academic Press, Inc., 1955, **2**, 598.
30. PALADE, G. E., *Anat. Rec.*, 1954, **118**, 335.
31. SJÖSTRAND, F. S., *Nature*, 1953, **171**, 30.
32. JONES, L. C., and ERNSTER, L., *Acta Chem. Scand.*, 1960, **14**, 1839.
33. LITTLEFIELD, J. W., KELLER, E. B., GROSS, J., and ZAMECNIK, P. C., *J. Biol. Chem.*, 1955, **217**, 111.
34. DECKEN, A. VON DER, and HULTIN, T., *Exp. Cell Research*, 1958, **14**, 88.
35. AZZONE, G. F., CARAFOLI, E., and MUSCATELLO, U., *Exp. Cell Research*, 1960, **21**, 456.
36. AZZONE, G. F., EEG-OLOFSSON, O., ERNSTER, L., LUFT, R., and SZABOLCSI, G., *Exp. Cell Research*, 1961, **22**, 415.
37. AZZONE, G. F., Vth International Congress of Biochemistry, in press.
38. PALADE, G. E., and SIEKEVITZ, PH., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 171.
39. MOULÉ, Y., ROUILLER, C., and CHAUVEAU, J., *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 547.
40. EBASHI, S., *Arch. Biochem. and Biophysics*, 1958, **76**, 410.
41. BENDALL, J. R., *Nature*, 1958, **181**, 1188.