ON THE DIFFERENTIAL RESPONSE OF SARCOPLASM AND MYOPLASM TO DENERVATION IN FROG MUSCLE

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ABSTRACT

Electron microscopic evidence is presented that the early response to denervation ("simple atrophy") of the semitendinosus m. of the frog is characterized by a greater prominence of the sarcoplasmic reticulum and by the presence, in the interfibrillar spaces, of mitochondria which are more numerous and smaller than in normal muscle. In contrast with the dynamic changes of the sarcoplasmic structural components, the myofibrils showed a progressive decrease in diameter after denervation and throughout the period studied. By carrying out tissue fractionation experiments, the yield of microsome-protein was found significantly greater in the denervated muscles, as compared with the contralateral controls, in this initial stage. Under the conditions attending the overdevelopment of the sarcoplasmic reticulum (SR), denervated semitendinosus m. incorporated valine-C^{14} into proteins more actively than the control pairs. The denervated muscles also showed an increase in the number of freely scattered and membrane-bound ribosomes and of polyribosomes, suggesting a more active synthesis of the SR membranes. Pronounced atrophy of the myofibrils, disorganization of the SR, and an increased number of ribonucleoprotein particles lying in the enlarged interfibrillar spaces were the main ultrastructural features of "degenerative atrophy" in frog muscle in the late periods after denervation. The probably adaptive character of the early changes occurring on denervation of frog muscle is discussed.

INTRODUCTION

The amount of information concerning the ultrastructural correlates of skeletal muscle denervation atrophy has grown considerably in recent years, but only a slow progress has been made toward a substantial understanding of the problem.

The rapid decrease in the content of the structural proteins in skeletal muscle, upon sectioning of the motor nerve, has received considerable attention by biochemists (21, 56, 66, 60, 14), and later stimulated intensive efforts by electron microscopists to define the mode of atrophy of the myofibrillar component in denervation atrophy (65, 50), as well as in other types of muscle atrophy (64, 28).

The modifications occurring in the sarcoplasmic substructures after nerve sectioning have been comparatively less investigated, and still poorly understood are their correlations with the number of biochemical and functional abnormalities so far delineated in denervated muscle (see reference 4). Mitochondria share very rapidly in the retrogressive changes of denervated pigeon breast

muscle (1, 11, 12, 45), whereas they were reported practically unchanged, in both their structure and number, in rat skeletal muscles until relatively advanced periods after cutting the motor nerve (65, 50). Observations on the sarcotubular system have been briefly reported in the latter two studies of denervated rat muscles. The over-all architecture was found apparently well preserved (65, 50), except in the late stages of atrophy, when there was overdevelopment of tubules which were interpreted to be derivatives of the intermediate component of the triads (50).

Recent contributions by many workers with different approaches have provided a guide to an experimentally founded definition of the functions of the sarcoplasmic reticulum (SR) (6, 53) or sarcotubular system (2). The control exerted by the intermyofibrillar membrane-component over the contraction-relaxation cycle has been intensively investigated in several laboratories, in connection with studies on the "relaxing factor" (see reference 38). A metabolic function of the sarcotubular system, first suggested by Fawcett (18, 19), has recently received experimental support (3, 40, 41); in the light of the elegant cytochemical observations of Fahimi (17) it is probable that such metabolic function is related to the sarcoplasmic reticulum proper (see references 25, 26, and Terminology section). Novel interest has been aroused by the outstanding electron microscope observations of Franzini-Armstrong and Porter (25, 26), and of Huxley (37), concerning the previously suggested involvement of the T system (2) in the conduction of the nervous stimulus from the sarcolemma inward to the contractile material (36). Convincing evidence has also been presented that the T system is continuous with the sarcolemma (26).

It seemed, therefore, pertinent to conduct a study devoted to the early effects of nerve section on the ultrastructure of skeletal muscle, in order to investigate in some detail the changes that may be found in the several components of the sarcotubular system of the denervated skeletal muscle cell. Subordinately, this may provide an auxiliary approach to a better understanding of the relationship which the distinguishable parts of the sarcotubular system bear to the conduction of the excitation, the contraction-relaxation cycle, and muscle metabolism.

In the present investigation, the skeletal muscle of the frog (*Rana esculenta*) was studied since the literature offers a large amount of information regarding its ultrastructure, biochemistry, and physiology. In addition, the slow course of denervation atrophy in frog skeletal muscles (30, 8) and the greater development of the sarcotubular system in these muscles, as compared, for instance, with rat muscles (2, 41), provide more favorable conditions for studying the early substructural changes consequent upon nerve section.

Evidence will be presented here that, at variance with the progressive decrease of the myofibrillar component, the sarcoplasmic reticulum and the mitochondria of the frog muscle cell exhibit a transient increase in amount during the early stages after denervation.

MATERIALS AND METHODS

ANIMALS AND THEIR CARE: Frogs (*Rana esculenta*) weighing 20 to 60 gm were used. The animals were fed insect larvae and were kept, in groups of twenty to thirty, in large tanks containing shallow water. Fresh water was continuously circulated through the tanks which were fitted with a heater for ensuring constant temperature. This was maintained at 10°C in the experiments with frogs which were used for electron microscopy, or at either 10°C or 18°–20°C for other types of studies, as stated in the text. For complete denervation of the muscles of the left leg, the spinal roots of the sciatic and crural nerves were severed close to their emergence from the spinal canal and the nerves were removed distally as far as possible, in order to prevent re-innervation of the muscles from the regenerating nerve stumps.

MUSCLES: For the morphological studies, the semitendinosus muscle was used, whereas biochemical analyses were carried out either on the isolated semitendinosus m. or on the whole leg muscles. The symmetrical, normal muscles of the right leg were used as controls.

LIGHT AND ELECTRON MICROSCOPY: For routine histological examination, whole semitendinosus muscles were processed by the conventional procedures. Longitudinal and cross-sections of paraffin-embedded specimens were cut at 5 μ, and stained with hematoxylin and eosin. Measurements of muscle fiber areas were carried out on cross-sections which were obtained by the above procedure.

For phase microscopy of fresh fibers, the semitendinosus muscles were carefully dissected from the killed animals and placed in 0.44 M sucrose–2.5 mM EDTA solution, pH 7.0. Muscles were teased with a needle, and small bundles of fibers were examined in a phase-contrast Leitz apparatus, with an oil-immersion objective.

For electron microscopy, the semitendinosus

muscles were fixed at rest length *in situ* in a 1 per cent OsO_4 solution in isotonic saline, buffered with veronal-acetate, pH 7.4. Minor bundles of muscle fibers were isolated and stained, during the dehydration procedure, with 0.5 per cent uranyl acetate or 1 per cent phosphotungstic acid. Either Araldite or Epon was used as the embedding medium. Sections were cut on a Porter-Blum Ultramicrotome, or on an LKB-4800-Ultratome. The microscope used for examination was either the Akashi TRS 50 EI or the Siemens Elmiskop I. The measurements of myofibril areas were made, on the prints of cross-sections, with a planimeter.

INCORPORATION OF DL-4-C^{14}-VALINE INTO PROTEIN: Semitendinosus muscles were dissected from the denervated leg and the contralateral, normal leg of frogs and placed in cold frog Ringer's solution before transfer to the incubation flasks. The frog Ringer's solution had the same basic composition as Krebs-Ringer bicarbonate except that the concentration of NaCl was lowered from 0.118 to 0.083 M (46). For incubation, the muscles (25 to 50 mg fresh tissue weight) were put into 25-ml Erlenmeyer flasks containing: 3 ml of frog-Ringer bicarbonate solution that had been previously equilibrated with a gas mixture of 95 per cent O_2 and 5 per cent CO_2; 0.05 ml (1 unit) of insulin solution (prepared by dilution of U-40 Lilly insulin in Ringer bicarbonate); 0.05 ml of 0.6 M glucose and 0.05 ml (0.32 μmoles — 0.5 μc) of DL-4-C^{14}-valine (Radiochemical Centre, Amersham, England). Incubations were carried out at 30°C for 120 minutes, shaking at 100 oscillations per minute, with $O_2 + CO_2$ (95:5) as the gas phase. At the end of the incubation, the reaction flasks were chilled in an ice-cold bath and the medium was removed from the muscles and replaced with 10 ml of fresh, cold medium containing 0.05 M non-labeled valine. The muscles were allowed to soak for about 10 minutes, and were then homogenized in 3 ml of water again containing an excess of non-radioactive amino acid. Protein was isolated and purified for counting by a modified Schneider method (13).[1] The tissue homogenates were added to an equal volume of 20 per cent trichloroacetic acid and the precipitate obtained by centrifuging in the cold was then treated in this order: twice with 5 ml of 5 per cent trichloroacetic acid for 10 minutes at 90°, once with ethanol, ethanol–ethyl ether (3:1), ethanol–ethyl ether–chloroform (1:1:1), and ethyl ether. The defatted protein residue was dried *in vacuo* at room temperature, and dissolved in a small volume of 88 per cent formic acid.

In additional experiments, the matched (denervated and normal) frog muscles were used to determine the incorporation of radioactive amino acid into the crude "myofibril" and "myofibril supernatant" fractions obtained by low-speed centrifugation of homogenates prepared from the incubated tissues. The procedure was as follows. Incubation was terminated as above, by chilling the reaction flasks and by substituting the reaction medium with fresh medium containing an excess of non-radioactive valine. After brief soaking, the semitendinosus muscles were removed from the flasks, trimmed of any evident connective tissue and homogenized in 3 ml/muscle of 0.88 M sucrose by a small Potter-Elvehjem type homogenizer (A. H. Thomas, Philadelphia) provided with a teflon pestle. Myofibrils, nuclei, and unbroken tissue fragments were separated by centrifuging once for 10 minutes at 1000 *g*. After removing the supernatant fluid, the sediment was suspended in 3 ml of sucrose medium. Protein was isolated and purified for counting by the same procedure as described above, except that the extractions at 90° were carried out with 5 per cent trichloroacetic acid containing 0.05 M unlabeled valine.

PREPARATION OF CELL FRACTIONS: Symmetrical muscles were removed from the two legs of frogs and immediately placed in ice-cold 0.88 M sucrose-5 mM EDTA solution, pH 7.0. The muscles were freed from the main blood vessels, nerves, and any evident connective tissue, blotted on filter paper, weighed, and finely minced with scissors in a watchglass without delay. The muscle minces were homogenized in the sucrose-EDTA solution and were diluted to a final tissue concentration of 10 per cent (weight to volume). Homogenization was carried out in an all-plastic (Plexiglass) flat-bottomed homogenizer with a loosely fitting pestle driven by a stainless-steel shaft. The differential centrifugations were carried out in a PR-2 International Refrigerated Centrifuge and in an MSE automatic "Superspeed 50" Centrifuge. Equal volumes of the tissue homogenates (generally 30 ml) were centrifuged for 10 minutes at 1000 *g* to separate the nuclei and myofibrils. The sediment was resuspended by homogenizing in $\frac{2}{3}$ the original volume of sucrose-EDTA solution and recentrifuging. The combined washings and supernatant fluid were then centrifuged at 30.000 *g* for 30 minutes to sediment the mitochondria. Microsomes were isolated by centrifuging the mitochondrial supernatant at 100,000 *g* for 120 minutes. After removing the supernatant fluid, the microsomes were thoroughly dispersed by homogenization in a small volume of sucrose-EDTA solution, or of distilled water, and aliquots were taken for analysis of protein and RNA.

TREATMENT OF TISSUES FOR ANALYSIS OF NUCLEIC ACIDS: Aliquots of whole homogenates or of the microsome suspensions were subjected to the Schneider procedure (57). General conditions for removal of acid-soluble compounds and lipids were the same as described previously (42). Nucleic acids

[1] We are indebted to Dr. Alexandra von der Decken for giving us details of the procedure.

TABLE I

Changes in Wet Weight, Dry Weight, and Total Protein of Frog Semitendinosus Muscle at Early Periods after Denervation

Exp. No.	Wet weight			Dry weight or protein		
	Control	Denervated	Change	Control	Denervated	Change
	mg	*mg*	*per cent*	*mg*	*mg*	*per cent*
1	62	58	−6.4	9.6*	9.2*	−4.1
2	135	123	−8.8	21.0*	20.0*	−4.7
3	60	51	−15.0	8.8*	7.6*	−13.6
4	48.2	38.1	−20.9	9.6	8.6	−10.4
5	38.6	30.3	−21.5	7.7	6.5	−15.6
6	72.6	54.8	−24.5	13.9	11.4	−18.0
7	34.2	25.8	−24.6	7.0	5.5	−21.4
8	53.1	39.7	−25.2	10.7	8.6	−19.6

Experimental conditions: The left semitendinosus muscle of frogs was denervated by the procedures described in Methods and the animals were kept in shallow water at about 20°C. 27 to 74 days after denervation the frogs were killed by low spinal transection and the two semitendinosus muscles were quickly removed and weighed. The right semitendinosus muscle served as the control. Total tissue solids were determined on isolated semitendinosus muscle by drying to constant weight at 110°C. Protein was determined by the biuret method on trichloroacetic acid precipitates of the tissue homogenates in distilled water.

* Protein.

were extracted with 5 per cent trichloroacetic acid at 90°C, for 15 minutes.

Analytical Procedures

PROTEIN: This was determined by the biuret method or by the Lowry procedure (39).

RNA: This was estimated from ribose by the orcinol reaction (57).

DNA: This was estimated from deoxyribose measurements by the diphenylamine reaction, according to Burton (9).

RADIOACTIVITY OF PROTEIN: 1 to 3 mg of protein dissolved in formic acid was plated on aluminum planchettes (area 4.9 cm^2) and dried at 30°C. The radioactivity of the infinitely thin samples was determined in an end-window counter (Tracerlab, Boston).

Terminology

The highly differentiated intermyofibrillar system of tubules and cisternae of skeletal muscle cells has been named the "sarcoplasmic reticulum" (SR) (6, 53) or the "sarcotubular system" (2). In the present paper the latter term has been used in denoting the sarcotubular network in general, both the "T system" (2, 25, 37, 26) and the "sarcoplasmic reticulum" proper (26).

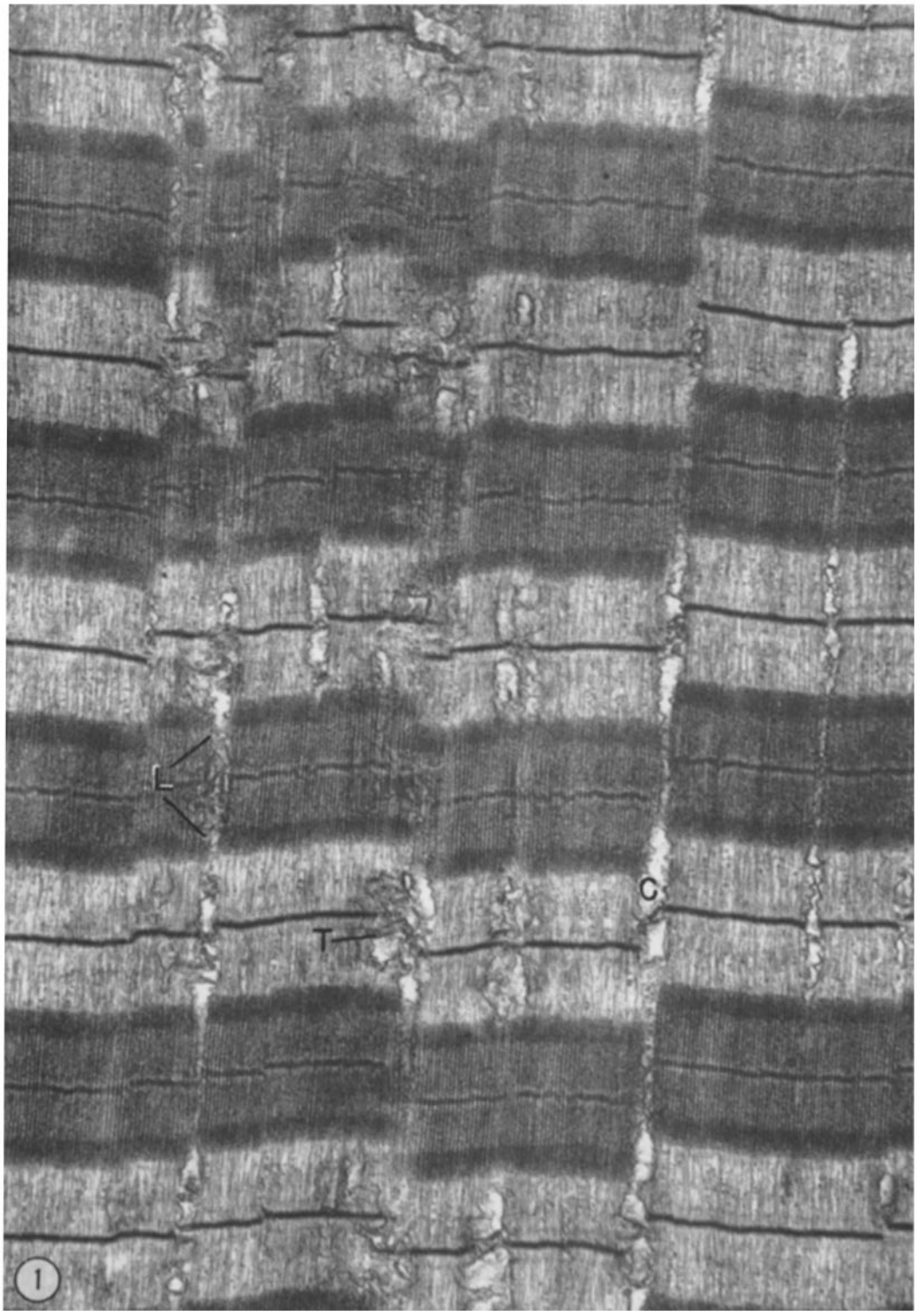
The high-speed preparations from frog muscle homogenate that, because of their structural origin from the sarcotubular system, were previously called by us "sarcotubules" or "sarcotubular fraction" (44, 43, 3, 40, 41, 42), will be referred to as "microsomes," in order to conform to current nomenclature (see references 48, 49, 58, 59).

RESULTS

General Remarks and Classification

It was found that the rate of atrophy of frog muscles was very sensitive to the environmental conditions to which the animals were exposed after denervation. Even under the same con-

FIGURE 1 Electron micrograph of a longitudinal section of frog semitendinosus muscle after denervation (Stage I). The contractile material does not show any appreciable change from normal. The terminal cisternae (*C*) of the SR appear somewhat enlarged. *T*, transverse system; *L*, predominantly longitudinally oriented tubules of the SR. Staining with uranyl acetate and PTA during dehydration. Embedding in Araldite. × 22,500.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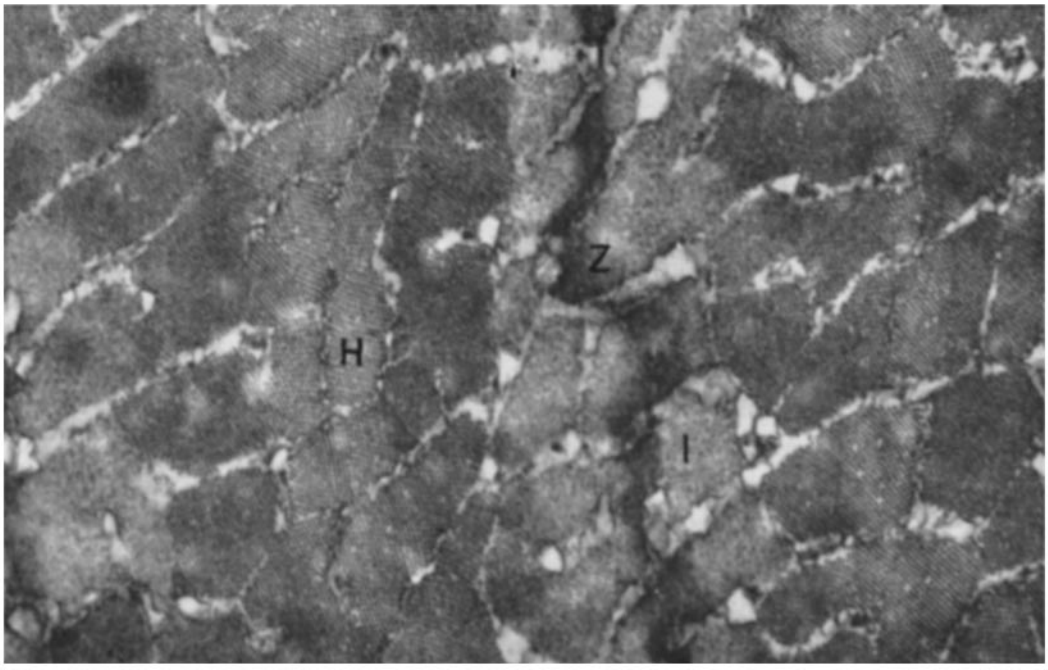

FIGURE 2 Electron micrograph of a cross-section of denervated semitendinosus muscle fiber of frog (Stage I). The section has passed through different layers of the myofibrils (*Z*, Z band level; *I*, I band level; *H*, H band level). A slight enlargement of the membrane-bounded spaces of the SR is particularly evident at the I band level (compare with Fig. 3). Conditions for staining and embedding as in Fig. 1. × 22,500.

ditions, there was a considerable variation from animal to animal; thus the course of atrophy was generally more rapid in the adult (50 to 60 gm) frogs as compared with the young and young adult animals. To relate the morphological and biochemical data gained in different experiments to the course of atrophy, the atrophy was traced by the changes in muscle wet weight. When the results were referred to the per cent loss in weight of the denervated muscles, rather than to the duration of atrophy (*i.e.* the time after denervation), a well defined course of changes could be recognized. For the sake of convenience, three main stages will be referred to, in presenting the morphological and biochemical correlates of muscle denervation atrophy in the frog.

(a) STAGE I (LAG PHASE): This corresponded to a loss of muscle wet weight of less than about 5 per cent. The intracellular spaces occupied by the myofibrils and intermyofibrillar components were essentially unchanged in their relative proportions.

(b) STAGE II (EARLY STAGE OF ATROPHY)[2]: This was characterized by decreases (5 to approximately 25 per cent) in the wet muscle weight, and by somewhat smaller decreases in dry weight (Table I). At this stage there was an appreciable reduction of the contractile material, whereas the sarcoplasmic structural components showed a relative and absolute increase in amount.

(c) STAGE III (ADVANCED ATROPHY OR DEGENERATIVE ATROPHY): The changes in the wet weight were greater than 25 to 30 per cent, and were associated with retrogressive changes affecting both the myoplasm and sarcoplasm.

Lag Phase

During this period the denervated muscle fibers

[2] In terms of duration of atrophy, Stage II corresponded to a period between 1 and 2 months after denervation in frogs kept in shallow water at 18–20°C, and between approximately 2 and 3 months in frogs kept at 10°C.

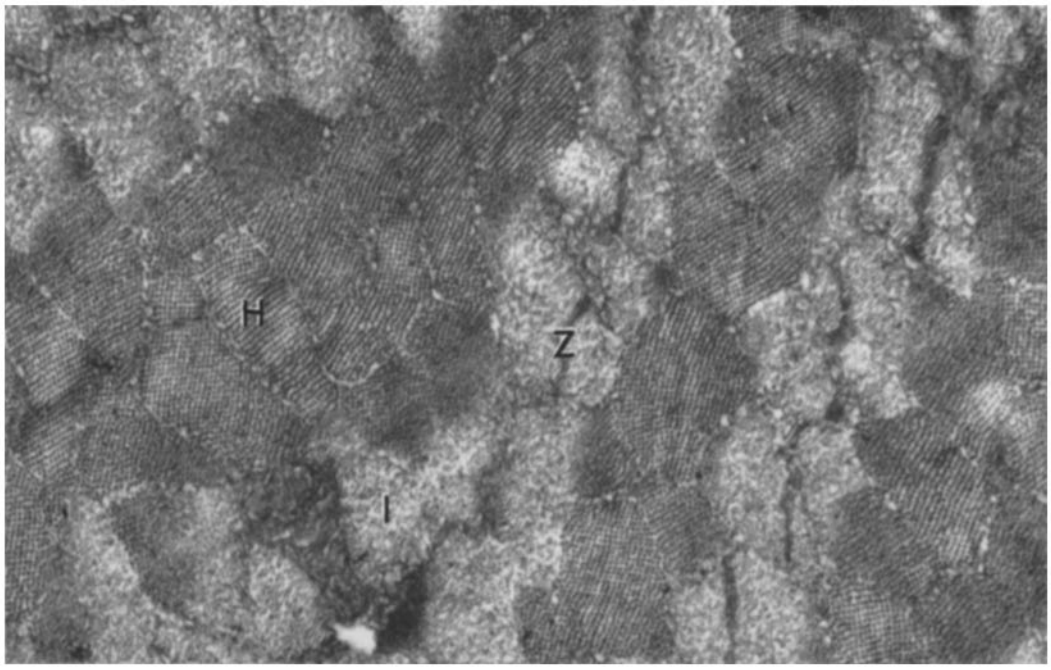

FIGURE 3 Electron micrograph of a cross-section of normal semitendinosus muscle fiber of frog. Sarcoplasmic components are seen between the myofibrils (*Z*, Z band level; *I*, I band level; *H*, H band level). Conditions for staining and embedding as in Fig. 1. × 22,500.

did not show appreciable modification from normal, when they were studied in the light and polarizing microscope. The electron microscopic appearance of the denervated semitendinosus m., at this early stage, is illustrated in Figs. 1 and 2. The banding of the myofibrils and the integrity of the myofilaments were completely preserved. The average myofibril-diameter was almost insignificantly reduced (Fig. 2, as compared with the control in Fig. 3). In contrast with that, the membrane-bounded spaces of the sarcoplasmic reticulum lying in between the myofibrils appeared slightly enlarged at the I band level, corresponding to the terminal cisternae of the longitudinal part of the system (Figs. 1 to 3).

Early Stage of Atrophy

The diameter of the denervated average muscle fiber was still not significantly changed from normal, when measurements were carried out of paraffin - embedded, hematoxylin - eosin–stained cross-sections of fibers, in muscles which had lost about 10 per cent of their weight. There was circumstantial evidence of progressive atrophy of the muscle fibers, from inspection of histological specimens which were obtained at slightly more advanced stages of muscle atrophy. More substantial losses of muscle weight (about 25 per cent, *i.e.* between Stage II and Stage III) entailed larger changes in fiber-diameter, which could be analyzed quantitatively with greater accuracy. In a light microscope comparison of transverse sections from the two semitendinosus muscles of one frog, the distribution of fiber areas was significantly different in the denervated muscle; the means of the values were $2.64 \times 10^3\ \mu^2$ and $3.61 \times 10^3\ \mu^2$, in the denervated and control fibers, respectively.

The myofibrillar component of the denervated muscle fibers showed a progressive decrease during the course of muscle atrophy. When measurements were carried out of myofibril-areas on electron micrographs of transverse sections from denervated frog muscles, an average 12 per cent decrease was found with respect to the control values, at some intermediate period of this stage. The frequency distribution of the myofibril-area values, in the

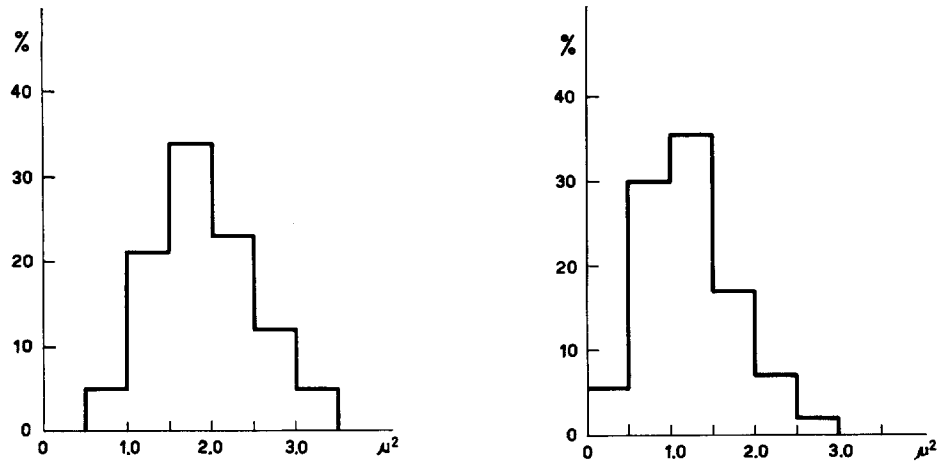

FIGURE 4 Distribution of myofibril areas in transverse sections from control (Fig. 4 *a*) and denervated (Fig. 4 *b*) semitendinosus muscle fibers of frog (Stage II). The mean values in the control and denervated muscle fibers were 1.93 and 1.69 μ^2, respectively. *Abscissa:* myofibril area in μ^2. *Ordinate:* number of myofibrils in individual groups as percentage.

denervated and control frog muscles, is shown in the histograms of Fig. 4 *a* and *b*.

The swelling of the terminal cisternae of the sarcoplasmic reticulum, which was observed in Stage I, appeared now associated with, or substituted by, a pronounced overdevelopment of the SR elements. When seen in longitudinal sections (Fig. 5), the spaces in between the myofibrils were occupied by tightly packed profiles of vesicles and tubules. Survey pictures of transverse sections showed that the aforementioned changes of the SR were generalized to wide areas of the denervated muscle fiber cytoplasm (Fig. 6). In contrast with the greater prominence of the SR, there was no evidence suggesting proliferation of the T system. When cut in the plane of the section, the small intermediate component of the triads appeared single and normally located with respect to the large cisternae at each side of the Z band.

To quantitatively determine the increase in amount of the sarcoplasmic reticulum components, homogenates of the denervated frog muscles were fractionated by centrifugation, and the yield of microsomes and of the soluble supernatant proteins were compared with those obtained from the paired control muscles. In line with previous biochemical observations (32), preliminary control experiments permitted exclusion of the existence of significant differences between the normal matched muscles of the two legs of frogs, concerning the content of microsome and soluble proteins (see Table II).

Greater amounts of soluble protein and microsome-protein were consistently extracted from the denervated muscles per unit of fresh tissue (Table III). The yield of microsome-protein was considerably larger than that of the soluble proteins, and it was still above normal values up to a loss of muscle weight of about 30 per cent.

The changes in the concentration and absolute amount of microsome-protein in the denervated muscles are shown in Fig. 7. It is apparent from this figure that the greater yield of microsomes from the denervated muscles was the result of both the absolute increase in amount of microsome-protein and the decrease in content of other subcellular components. Compared with the control muscles, the denervated muscles showed a pronounced rise in the concentration of microsome-protein, which reached a peak when the total muscle weight had decreased by 15 to 20 per cent. The absolute amounts of microsome-protein also increased in the denervated muscles, and only at the late stages of atrophy (degenerative atrophy) did they fall to normal. The increase in microsome concentration observed at the beginning of Stage II appeared to be almost completely accounted for by the absolute increases in microsome content in the denervated muscles. The subsequent rise in microsome-protein concentration during most of Stage II, while the absolute levels of microsomes remained constant and above normal, appeared then to be the reflection of the progressive decrease

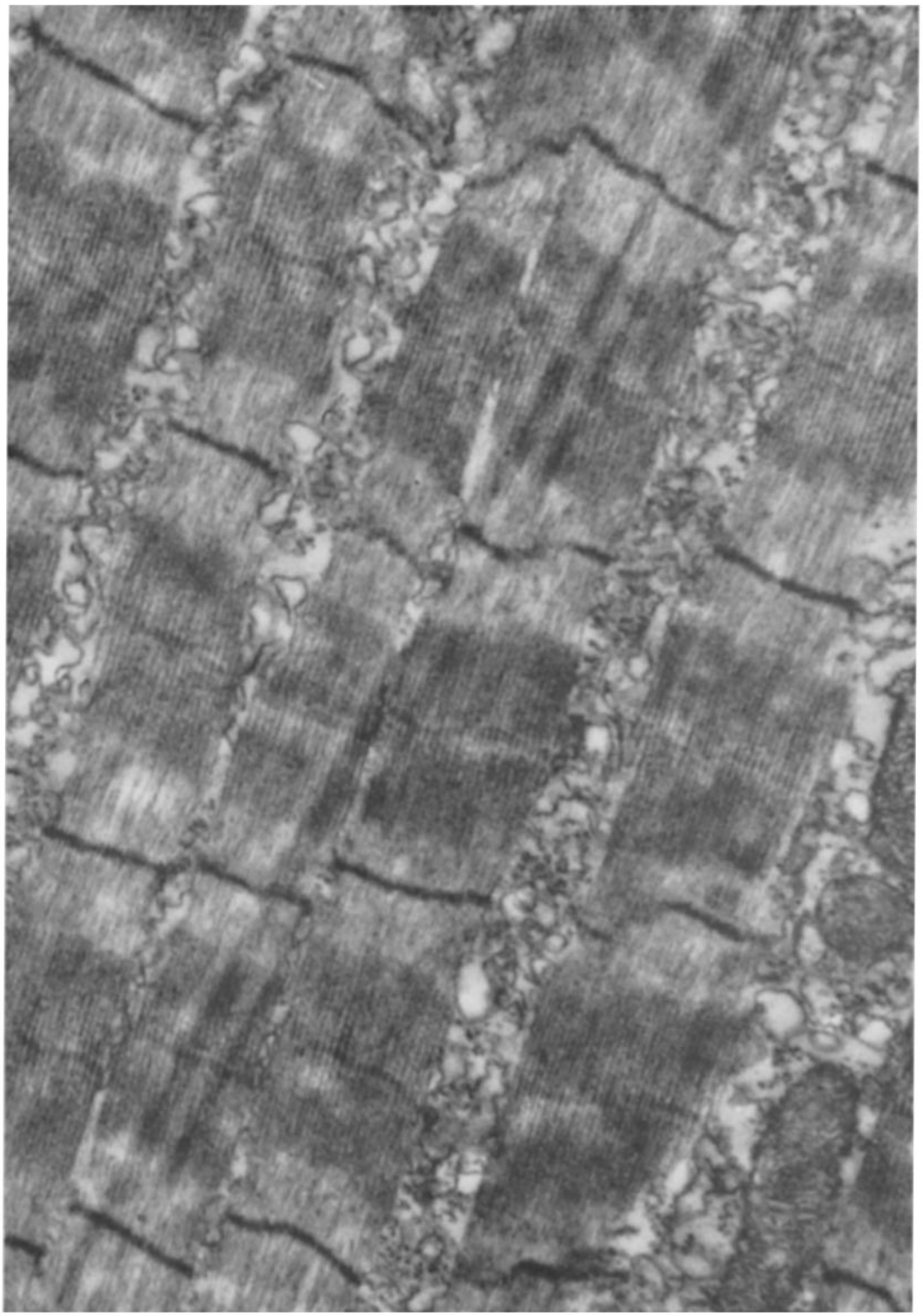

FIGURE 5 Electron micrograph of a longitudinal section of frog semitendinosus muscle (Stage II). The figure illustrates the initial reduction in diameter of the myofibrils which, however, preserve their normal banding. The intermyofibrillar spaces are slightly enlarged and are occupied by very numerous sarcotubular vesicles and mitochondria. Conditions for staining and embedding as in Fig. 1. × 38,000.

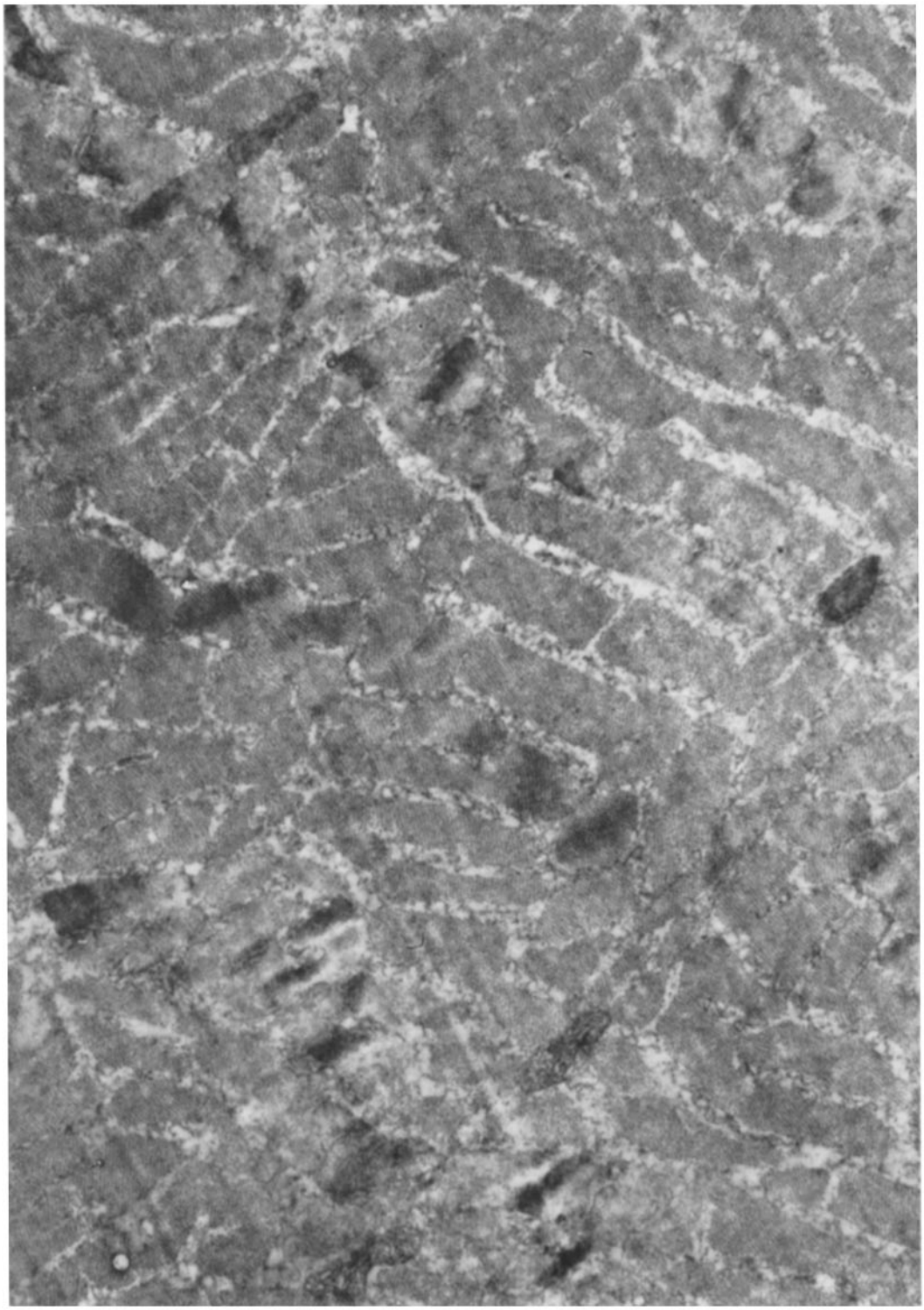

FIGURE 6 Electron micrograph of a cross-section of semitendinosus muscle fiber of frog at Stage II of atrophy. The survey picture illustrates a large part of a muscle fiber. The intermyofibrillar spaces appear occupied by numerous elements of the sarcoplasmic reticulum. Conditions for staining and embedding as in Fig. 1. × 20,000.

in content of contractile material in the denervated muscles.

In one experiment, the microsome-pellets obtained from the denervated (average loss of muscle weight: 19.5 per cent) and contralateral muscles of four frogs were examined under the electron beam. The survey showed profiles of tubules and vesicles of different size, as described by us in previous reports (44, 43, 41). A greater abundance of the smaller vesicular components was apparent in the pellet from denervated muscles, but the significance of these findings could not be assessed with certainty.

TABLE II

Yield of Microsome Protein and High-Speed Supernatant Protein from Symmetrical Leg Muscles of Frogs

	mg protein per gm muscle			
	Microsomes		Final supernatant	
Exp. No.	Right muscle	Left muscle	Right muscle	Left muscle
1	1.38	1.42	34.2	34.0
2	0.79	0.82	24.0	24.2
3	0.99	1.01	25.2	25.6

Experimental conditions: Symmetrical muscles from the two legs of frogs (2 frogs in each experiment) were homogenized in 0.88 M sucrose–5 mM EDTA solution, pH 7.0, and were fractionated by centrifugation according to the procedures described in Methods. Protein was determined by the Lowry procedure (39).

At the same stage when the hypertrophy of the sarcoplasmic reticulum was observed, the mitochondria of the denervated muscle fibers underwent appreciable changes in number, shape, and volume (Fig. 8).

Two different types of muscle cells, one endowed with few elongated mitochondria and the other with slightly more numerous, smaller mitochondria, can be recognized in fresh preparations of semitendinosus muscle of the frog when studied in the phase-microscope. The appearance of fibers belonging to the first type, which is the most common in frog leg muscles, is illustrated in Fig. 9 *a* and may be contrasted with that of the denervated muscle fibers (Fig. 9 *b*) in which more numerous, tightly packed mitochondria appear to form con-

TABLE III

Yield of Microsome Protein and Soluble Protein from Denervated and Control Leg Muscles of Frogs

Weight loss*				mg protein per gm muscle					
				Microsomes			Final supernatant		
Range	Average	No. of Exps.‡	No. of Frogs	Control	Denervated§	Change	Control	Denervated	Change
per cent	*per cent*					*per cent*			*per cent*
4–8	5.9	3	12	0.82	1.09	+32.9	36.1	40.8	+13.0
8–12	10.9	3	4	1.43	2.05	+43.4	43.6	50.1	+14.9
12–16	14.4	5	12	1.41 ± 0.31	2.12 ± 0.42	+50.4	35.8 ± 4.03	37.9 ± 4.12	+5.8
16–20	18.7	4	13	1.17 ± 0.06	1.95 ± 0.46	+66.7	38.9 ± 0.81	45.6 ± 2.94	+21.7
20–24	21.3	5	14	1.05 ± 0.19	1.71 ± 0.31	+62.9	30.7 ± 5.96	35.0 ± 6.50	+2.5
	29.8	1	3	0.56	0.78	+39.3	24.4	25.0	+2.5

Experimental conditions for tissue fractionation were the same as for Table I. Protein was determined by the Lowry procedure (39). Mean values, for each group, of weight loss are given ± S.E.M., when more than three determinations were carried out. The mean values for total number of determinations of microsome protein (21 determinations) and soluble protein (19 determinations), in denervated and control muscles, were, respectively: 1.77 ± 0.16 and 1.15 ± 0.11; 39.9 ± 6.43 and 35.6 ± 5.77. The difference between the two mean values of microsome-protein was significant at the $P < 0.01$ level in the t-test (23). The difference between the two mean values of soluble-protein was not statistically significant.

* Weight loss indicates the per cent change in fresh tissue weight of the denervated muscles with respect to the contralateral control muscles.

‡ Most of the experiments were carried out in December 1964 to February 1965, with frogs kept in shallow water at about 20°C.

§ Left side muscles were denervated as described in Methods. Frogs were sacrificed 18 days to 48 days after nerve sectioning.

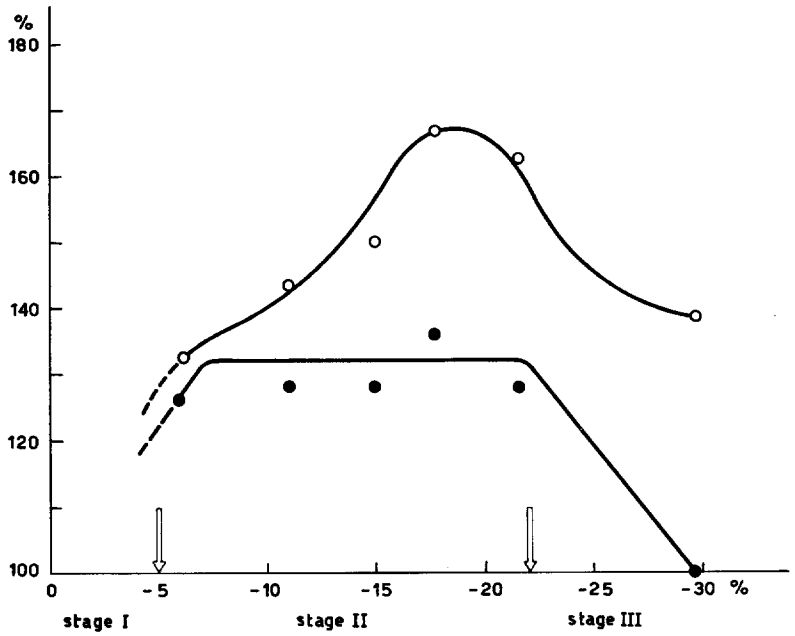

FIGURE 7 Changes in total amount and concentration of microsome-protein in denervated leg muscles of frog, as compared with the symmetrical control muscles. *Abscissa:* muscle weight change, per cent. *Ordinate:* microsome protein, per cent of control values. ○: microsome concentration (mg microsome-protein/gm muscle). ●: microsome content (total amount of microsome-protein/muscle).

The values reported in the chart were obtained from the same experiments as those of Table III.

tinuous rows which can be traced over large distances parallel to the direction of the fibers. In electron micrographs of normal muscle fibers belonging to the type just mentioned, the long axes of the elongated interfibrillar mitochondria appear to stretch along several sarcomeres. In addition, frog muscle mitochondria do not show the characteristic localization at the level of the I band, which has been described in rat (53, 41) and mouse (2) muscles, and are irregularly scattered here and there, along the side of the myofibrils (2, 44, 41). Most of the mitochondria which accumulated in rows between the myofibrils of denervated muscle fibers were round or irregularly shaped rather than elongated in form. They were also considerably smaller than the mitochondria of normal fibers, and their greatest diameter seldom exceeded the sarcomere length (Figs. 10 and 11). In agreement with the observations made on fresh fibers with the phase-contrast microscope, these small mitochondria could be traced over large distances in low-power views of longitudinal sections of the denervated muscle fibers. In no case, however, could significant differences be noticed between the mitochondria of denervated and control muscles regarding the organization of the outer-membrane and cristae.

In a limited number of experiments, attempts

FIGURE 8 Electron micrograph of a longitudinal section of frog semitendinosus muscle at Stage II of atrophy. The overdevelopment of the sarcoplasmic reticulum is, in places, associated with an increased number of mitochondria which are accumulated in rows between the myofibrils. Arrows show profiles of vesicles and tubules (T system) which appear to join with the terminal cisternae of the sarcoplasmic reticulum at the Z band level. There are no signs of proliferations of the T system elements. Conditions for staining and embedding as in Fig. 1. × 26,000.


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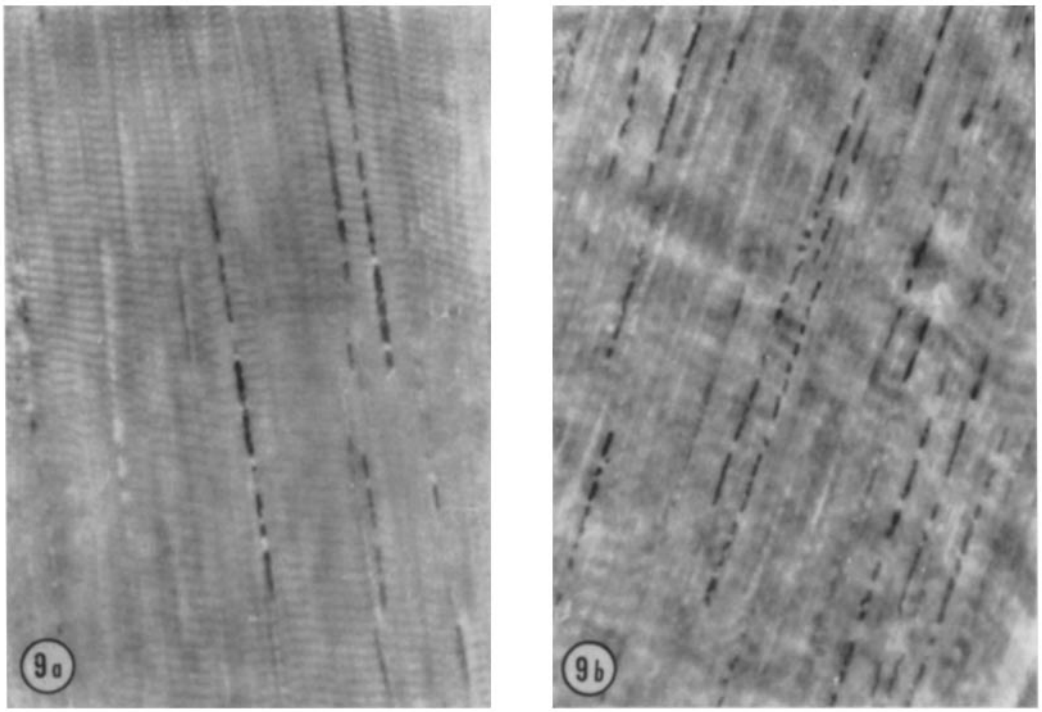

FIGURE 9 Phase contrast photomicrograph of fresh frog semitendinosus muscle fibers. Fig. 9 *a*, normal muscle fibers; Fig. 9 *b*, muscle fibers at Stage II of atrophy. The pictures illustrate the number, shape, and distribution of mitochondria in the normal and denervated muscle fibers, respectively. Oil immersion. × 900.

were made to determine the average content of mitochondria in the denervated and control leg muscles of frogs, by using the general fractionation procedures described in Methods. The experimental conditions were so designed as to obtain complete sedimentation of the mitochondria, by centrifuging the myofibril-supernatant (1000 *g* for 10 minutes) at high gravities (40,000 *g* for 30 minutes). These mitochondrial preparations appeared to contain an appreciable quantity of non-mitochondrial material, mostly myofibrils. With these reservations in mind concerning the interpretation of the results, it is of interest that at a degree of atrophy corresponding to an average 17.6 per cent loss of muscle weight (7 experiments), there was a 29.5 per cent increase in the yield of "mitochondrial" protein. The average values ± average deviation, for the denervated and control muscles, were, respectively, 1.36 ± 0.62 and 1.05 ± 0.49 mg protein/gm muscle.

To learn whether the hypertrophy of the sarcoplasmic reticulum and the apparent increase in the number of mitochondria might correspond to their more active formation in the denervated muscle cell, experiments were carried out in which the incorporation of radioactive DL-valine into the protein of frog semitendinosus muscle was studied *in vitro*. It may be seen from Table IV that the incorporation of labeled amino acid into protein, under conditions where the rate of incorporation was approximately linear with time (A. Margreth and F. Novello, unpublished experiments), was consistently higher in the denervated muscles, 15 days after nerve section. In three additional experiments (not shown in the Table) with denervated frog muscles (39 days after nerve section), the concentration of valine was increased threefold without altering the total amount of radioactivity in the medium. Under these conditions, the labeling of denervated muscle protein was found to be 67 per cent higher than the control values.

The average values of the results obtained from five additional experiments are also included in Table IV, to show the greater increase of radioactivity in the "myofibril-supernatant," as compared with the "myofibrillar fraction," of de-

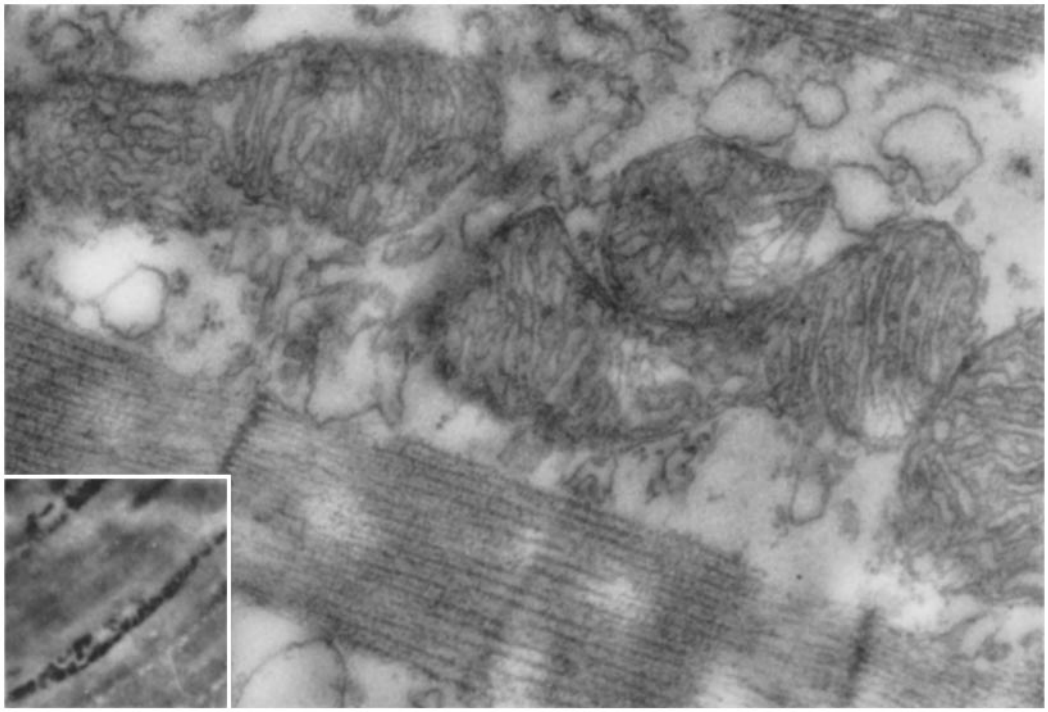

FIGURE 10 Electron micrograph of longitudinal section of frog semitendinosus muscle at Stage II of atrophy. A cluster of small, but otherwise normal mitochondria is seen in a considerably enlarged intermyofibrillar space. At the lower left, a detail illustrates a low-power view of similar clusters of mitochondria as seen in the phase-contrast microscope. Conditions for staining and embedding as in Fig. 1. Magnifications: electron micrograph, × 45,000; phase-contrast micrograph, × 900.

nervated frog muscles. Because of some difficulty in separating accurately the sarcoplasmic proteins from the myofibrils with the low amount of material (single muscles were used in each experiment —see Methods), these figures should be regarded as approximate. It is likely that a fairly large proportion of the radioactivity in the "myofibrillar fraction" was accounted for by the presence of non-myofibrillar material in the low-speed sediment.[3] Likewise, some contamination of the supernatant fluid by unsedimented myofibril fragments could not be completely avoided. With these reservations in mind, it may be sufficient to note, from the data presented in Table IV, that the average per cent change in the uptake of radioactivity into protein after denervation was about threefold as high in the "myofibrillar supernatant," as compared to the "myofibrillar fraction."

In view of the role played by RNA in protein synthesis, analyses were also carried out of the total RNA and the microsomal RNA in denervated muscles at this early stage of atrophy. It is apparent from the data shown in Table V that,

[3] Congruous with this interpretation and lending further support to the suggestion made in the present paper, that whereas the synthesis of myofibril-protein is impaired, that of sarcoplasmic proteins is increased in denervated muscles, are preliminary results obtained from radioactivity measurements of purified myofibril preparations, microsomes, and high-speed soluble proteins (Margreth and Novello, this Laboratory). In one comparison, the denervated (Stage I) and the contralateral semitendinosus and semimembranosus muscle of 14 frogs were used. After incubation with radioactive valine, the muscles were pooled, homogenized, and fractionated by centrifugation in sucrose-medium. The radioactivity of total muscle protein was only slightly increased in the denervated muscles. Specific activities of purified myofibril, microsome, and high-speed supernatant fractions of homogenates from denervated muscles were 75, 137, and 119 per cent, respectively, of the values found in the same fractions from the contralateral muscles.

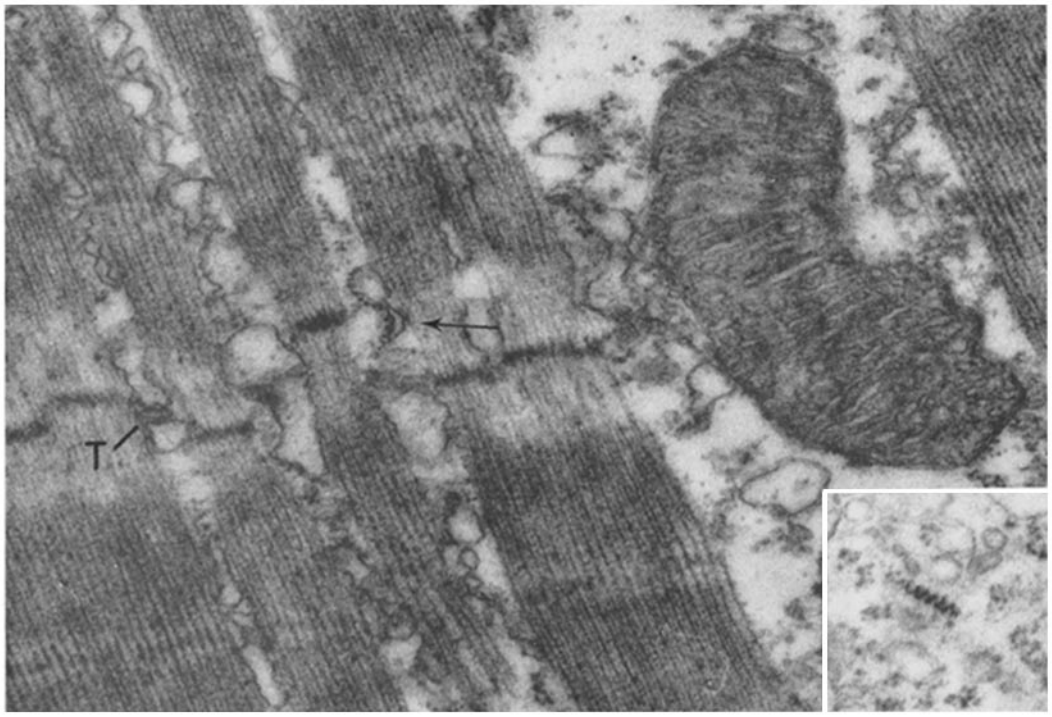

FIGURE 11 Electron micrograph of longitudinal section of frog semitendinosus muscle at Stage II of atrophy. The two large vesicles and intermediate component of a typical triad are shown at *T*. The arrow indicates numerous ribosomes which are in close relation to the SR membranes. At the lower right, a helical arrangement of ribosomes (polyribosomes) is shown. Conditions for staining and embedding as in Fig. 1. × 45,000. Detail, × 45,000.

TABLE IV

Rates of Incorporation of DL-4-C^{14}-*Valine into Muscle Protein in the Paired, Denervated, and Normal, Semitendinosus Muscles in Vitro*

Time after denervation	Tissue fraction	Incorporation		
		Control	Denervated	Change
days		CPM/*mg protein*	CPM/*mg protein*	*per cent*
15	Whole tissue	18.6	25.0	+34
120	Whole tissue	15.5	28.6	+85
120	Whole tissue	31.9	50.6	+59
22 to 30	Unpurified myofibril	31.3 ± 7.7*	36.7 ± 14.6*	+17
	Myofibril supernatant	43.7 ± 13.5*	63.7 ± 9.7*	+46

Experimental conditions: Left (denervated) and right (normal control) semitendinosus muscles (50 to 100 mg, fresh tissue weight) of the same frog were quickly removed and after brief soaking in cold Ringer's solution were transferred to flasks each containing 3 ml of frog Ringer-bicarbonate (46) to which were added: glucose, 50 μmoles; insulin, 1 u; and DL-4-C^{14}-valine, 0.32 μmoles (0.5 μc), dissolved in a total volume of 0.15 ml distilled water. Incubation was at 30°C, for 120 minutes, with shaking. The gas phase was O_2 + CO_2 (95:5). At the end of the incubation the isolated semitendinosus muscles were treated as described in Methods.

* Average values of 5 Exps. ± average deviation.

TABLE V

Changes in the Concentration of Total RNA and Microsomal RNA after Denervation

Exp. No.	Muscle	Muscle wt. change	RNA fraction	μg RNA/gm muscle (fresh tissue weight)		
				Control	Denervated	Change
		per cent				*per cent*
1	Semitendinosus muscle	−2.6	Total	613	761	+24.1
2	Semitendinosus muscle	−8.3	Total	719	802	+11.5
3	Gastrocnemius muscle	Not determined	Total	487	793	+62.8
4	Gastrocnemius muscle	Not determined	Total	501	756	+50.8
5	Whole leg muscles*	−6.4	Microsomal	7.45	8.30	+11.4
6	Whole leg muscles	−14.1	Microsomal	13.86	23.50	+69.6
7	Whole leg muscles	−15.4	Microsomal	10.12	20.77	+105.2
8	Whole leg muscles	−23.5	Microsomal	7.27	19.32	+165.7

Experimental conditions: Denervation was obtained by cutting the sciatic nerve of one side. It was carried out in May and in August, in Exps. 1 to 4 and 5 to 6, respectively. Frogs were kept in shallow water at about 10°C and were sacrificed at 74 days after nerve section. The contralateral symmetrical muscles served as the controls. 10 per cent homogenates in distilled water (Exps. 1 to 4), or in 0.88 M sucrose—1 mM EDTA solution (Exps. 5 to 8) were prepared from the two muscles of single frogs (Exps. 1 to 4), or from the pooled muscles of 5 to 6 frogs (Exps. 5 to 8). Microsomes were isolated from the sucrose homogenates by differential centrifugation under the conditions described in Methods. The pellet obtained by centrifuging for 120 min. at 100,000 *g* was suspended in a small volume of water. Aliquots of whole homogenates and of the microsome-suspensions were subjected to the Schneider procedure (57) and RNA was estimated by the orcinol reaction (57). Protein was determined by the biuret method (whole homogenates) or by the Lowry procedure (39) (microsomes).

* Dorsal thigh muscles plus gastrocnemius muscle.

with no exception, there was an increase in the concentration of total and microsomal RNA with respect to the control values. There resulted also an absolute increase in the content of RNA per muscle when account was taken of the loss of muscle weight (see reference 66).

At the stage when the aforementioned changes of RNA were recorded, freely scattered ribosomes and ribosomes bound to the SR membranes were frequently observed in the intermyofibrillar spaces of the denervated muscle fiber (Fig. 11). The ribosomal nature of these granules could be assessed on the basis of their dimensions (140 to 160 A in diameter) and strong staining with uranyl acetate and phosphotungstic acid (7). The appearance of ribosome-encrusted membranes of the sarcoplasmic reticulum, which are not usually observed in normal skeletal muscle, was in good agreement with the results obtained in 4 experiments which showed a 27.7 per cent increase of RNA per mg microsomal protein (control: 13.61 ± 2.39; denervated: 17.38 ± 3.57). Helical arrangements of ribosomes (polyribosomes) were also observed (see detail to Fig. 11).

Degenerative Atrophy

Histological examination of the denervated muscles showed pronounced atrophy of the fibers. The atrophy of the denervated muscle fibers was accounted for, to a considerable extent, by the decrease in diameter of the myofibrils as shown by the histogram in Fig. 12 (to be compared with Fig. 4 *a*). The thinning of the myofibrils could be traced to the decrease in the number of myofilaments per myofibril. The mutual arrangement of the residual myofilaments within the different zones of the sarcomere was generally well preserved. However, irreversible contracture and subsequent degeneration of the myofibrils occurred, occasionally, at this late stage of muscle atrophy (Fig. 13). This mode of degeneration was presumably the outcome of fibrillation (see reference 35).

The intermyofibrillar spaces were considerably enlarged and were occupied by still numerous sarcotubular vesicles and mitochondria. The sarcoplasmic reticulum exhibited, however, a marked degree of disorganization which involved mostly the longitudinal part of the system. It is of interest

that the mutual relationship of the "triad" (53) components at the Z band level was essentially unaltered (Fig. 14).

The increase in number of ribosomes noted in the previous stage was still observed at late periods after nerve section. It was particularly evident in the perinuclear sarcoplasm (Fig. 15). Measurements of RNA in the denervated muscles expressed in relative units (μg RNA per mg protein) revealed a 75 to 79 per cent increase over the control values (see also reference 42), at 6½ months after denervation. The values for semitendinosus and gastrocnemius muscles were: 4.54 (control) and 8.13 (denervated); 4.98 (control) and 8.65 (denervated), respectively.

DISCUSSION

The general proposition was put forward, first by Orbeli (47), that skeletal muscle cells regress toward a fetal or embryonic type when the regulating influence of the nerve is removed by denervation. This hypothesis, according to which the different denervated muscles show a tendency to converge to a common type through a loss of the specific properties associated with red and white muscles, has received considerable attention by many investigators (see reference 34) and was recently restated in physiological and biochemical terms by Eccles (15) and by Perry (51). A critical appraisal of the present status of the "dedifferentiation" hypothesis may require thoughtful consideration of the elements of uniformity and heterogeneity in the ultrastructural and biochemical features of different skeletal muscle cells upon sectioning of the motor nerve. An over-all assessment of this matter cannot, as yet, be made, but what is most clearly beginning to emerge is the remarkable uniformity, among different types of denervated muscle cells, in the mode of atrophy of the contractile material, and, just opposite to that, the large variability of changes in the sarcoplasm according to the type of muscle.

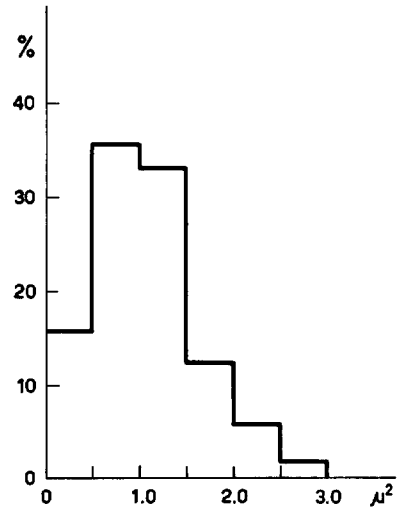

FIGURE 12 Changes in the distribution of myofibril areas in transverse sections of denervated frog semitendinosus muscle fibers at Stage III of atrophy. (Compare with normal muscle in Fig. 4 *a*). Mean value 1.07 μ^2.

The earlier observations by Fischer and Ramsey (22) and Fischer (20), regarding the decrease in content of contractile proteins in denervated muscles, have been recently correlated with the decrease in diameter of the myofibrils. There is conclusive evidence from electron microscope studies of the denervated gastrocnemius (65, 50) and soleus muscle (50) of the rat, and of several skeletal muscles from this rodent species after a chronic deficient diet (64), that the reduction of contractile material takes place mainly through a loss of myofilaments at the outer periphery of the myofibrils.

On the basis of the experimental evidence presented in this paper, it is apparent that the contractile material of the frog semitendinosus muscle is affected by nerve section in a manner similar to that described for the mammalian muscles just mentioned, although the rate of atrophy of the frog myofibrils is considerably smaller.

As far as the sarcoplasm is concerned, it was mentioned in the Introduction that the attention of several workers is centered mainly on mitochondrial changes occurring on denervation. A striking decrease in the mitochondrial complement of the highly aerobic pigeon breast muscle has been shown by us to occur at very early stages after denervation (3 to 5 days) (1, 11, 12, 45). Conflicting results have appeared with regard to the mitochondria of denervated rat muscles. Modifications in the number of mitochondria could not be ascertained in the gastrocnemius muscle of the rat 4 to 8 weeks after nerve section (65, 50). It was recently observed, however, in an electron microscope study of denervated rat muscles (16) that the number of mitochondria actually increases in the very early stages after denervation.

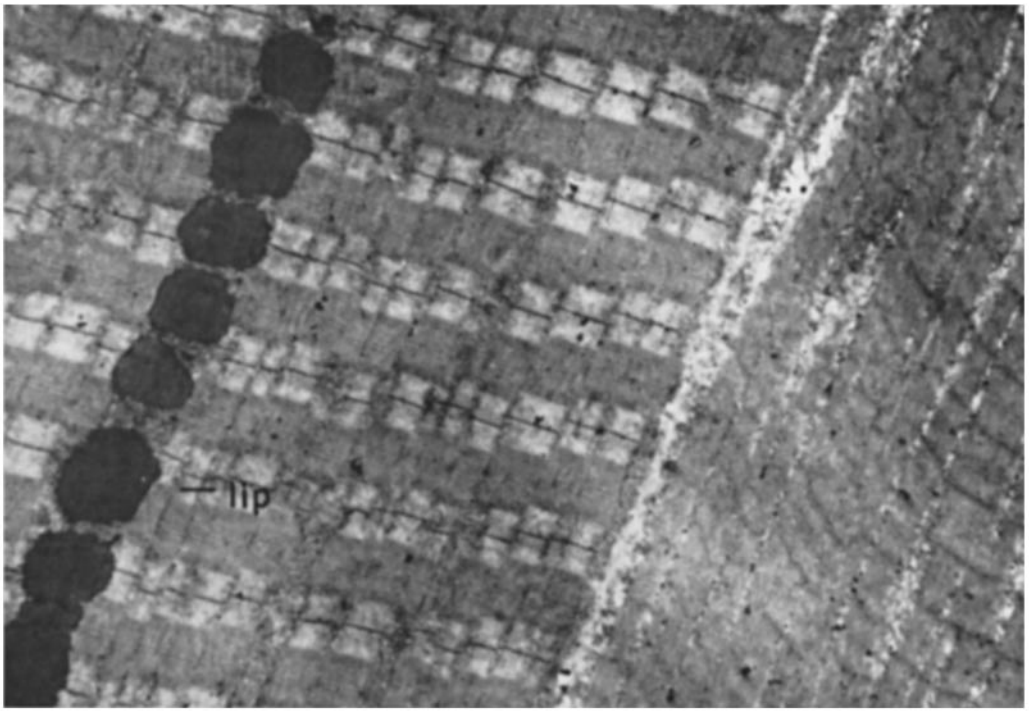

FIGURE 13 Electron micrograph of longitudinal section of frog semitendinosus muscle at Stage III of atrophy. The figure illustrates two adjacent muscle cells at low magnification. The cell shown in the right part of the figure appears under high contracture, as demonstrated by the extreme reduction in length of the sarcomeres. The adjacent fiber shows a row of lipid bodies (*lip*) in close relation to small mitochondria. The specimen, embedded in Araldite, was stained with a saturated solution of uranyl acetate after sectioning. × 6,000.

Wechsler and Hager (65) and Pellegrino and Franzini (50) were unable to find significant changes in shape, volume, and internal structure of the mitochondria, in their studies on denervated gastrocnemius and soleus muscles. Accompanying the linear course of atrophy of the myofibrils, considerable changes were found to occur in both the sarcoplasmic reticulum and the mitochondria on denervation in the present investigation with frog skeletal muscles. Three different stages, corresponding to increasing degrees of atrophy of the myofibrils, have been described in the Results. For the purposes of this Discussion, only the early period of atrophy (Stage I and Stage II; see Results) will be considered in detail here, since the available evidence indicates that "simple atrophy" (61) was later superseded by "degenerative atrophy." It is worthy of mention, for comparing the present findings with those gained with other muscle types, that the decrease in weight of the denervated frog muscles, at those "late" stages, was of the same order of magnitude as that found after just 1 week in denervated rat muscles (about one-third of the original weight) (50). Thus, the early stages of atrophy of frog skeletal muscle may probably correspond to a period of a few hours to a few days in denervated mammalian (rat) muscles, and to even less time in the avian (pigeon) red muscles. A greater prominence of the sarcoplasmic reticulum and an increase in the number of mitochondria were the main ultrastructural changes seen in the sarcoplasm of the denervated frog muscle cell during these transient stages.

Several lines of evidence, both morphological and biochemical, support the view, held in the present paper, that the frog muscle cell responds to denervation by making more of these subcellular components. The possibility was entertained that the greater prominence of the sarcoplasmic reticulum might be the result of the displacement of membranes from adjacent sarcomeres through interruptions in the transverse continuity

of the T system across the denervated muscle fibers. In contrast with this simple interpretation is the finding, however, that the three-component structure, the triad (53), appeared normally located at the Z band level in denervated muscles. The elaborate proliferations of the SR membranes repeated in regular pattern with the sarcomeres, suggesting that only the longitudinal part of the SR reacted to the early effects of denervation in frog muscle. At variance with the results obtained by Pellegrino and Franzini with denervated rat muscles (50) is the observation in the present study that there were no signs indicative of the overdevelopment of the T system, even at late stages of atrophy.

The additional evidence obtained from tissue fractionation studies of the denervated and control muscles support completely the interpretation we have just offered of our morphological observations on the SR. As reported in the Results, there was a statistically significant increase in the yield of microsomes isolated by centrifugation from the denervated muscles. Since the amount of microsomes per unit (fresh tissue weight) increased more than could be accounted for by the initial atrophy of the myofibrils, we could conclude that there was an absolute increase in content of microsomes per muscle.

The hypertrophy of the sarcoplasmic reticulum was associated with changes of the mitochondria in the denervated muscles. Convincing evidence from morphological observations in the phase-contrast and electron microscope and additional suggestions from tissue fractionation experiments indicate that the mitochondria were considerably smaller and more numerous in the denervated than in the control muscles.

Experimental evidence has been presented, in connection with studies on the denervated hemidiaphragm (10), and on the anterior tibialis muscle of the rat (29), that muscle atrophy is preceded by a period of increased protein synthesis. However, attempts were not made by the above authors to identify the proteins whose synthesis was increased following denervation.

Consistent with, though not providing compelling evidence for, the increased formation of SR membranes and mitochondria is the finding that the incorporation *in vitro* of C^{14}-labeled valine into muscle protein was enhanced following denervation, and that the increase in the rate of incorporation of radioactivity was apparently greater in the sarcoplasmic fraction, as compared with the myofibrils. On the basis of these incorporation experiments, it could not be established which of the series of reactions leading to protein synthesis was accelerated in the atrophic muscles. It would seem likely, however, that the increased incorporation *in vitro* of amino acid into the sarcoplasmic proteins, in part at least, reflected the increased number of amino acid incorporating sites, since the available evidence indicated that in the denervated muscles there was increase of total RNA and, to an even larger extent, of microsomal RNA. In these muscles the existence of granular membranes and of clusters of ribosomes in the areas of overdeveloped sarcoplasmic reticulum deserves a special comment because of the very infrequent occurrence of rough-surfaced membranes and of polyribosomes in differentiated skeletal muscles. The polyribosomes seen in denervated frog muscles were similar to those previously described in differentiating cells of the rat embryonic small intestine (5) and in the differentiating muscle cells of *Rana pipiens* embryos (62). Since "messenger" RNA has been proposed as the limiting factor in muscle protein synthesis (24), and since polyribosomes are regarded as ribosomes linked by "messenger" RNA (55, 63, 27), the above morphological observations raise the possibility that in denervated muscles there may be acceleration of the DNA-directed synthesis of RNA molecules acting as templates for the synthesis of sarcoplasmic proteins.

The correlations, observed in the course of the present study of denervated frog skeletal muscle, between proliferation of the SR membranes and of the mitochondria, increase in the rate of incorporation of amino acid into the sarcoplasmic proteins, and the formation of ribosome-aggregates, do not support the hypothesis of Zack and Gutmann (67) that in denervated muscle there is dissociation between RNA synthesis and protein synthesis. A different interpretation which fits better the experimental findings presented in this paper is that the more immediate effect of the removal of the regulating influence of the nerve is the uncoupling of the synthesis of the contractile proteins from that of the sarcoplasmic proteins, the former process being inhibited and the latter being stimulated (or de-repressed), respectively. Thus, the changes in the proportions of cell constituents occurring on denervation of frog muscle would conform to the concept, put forward by

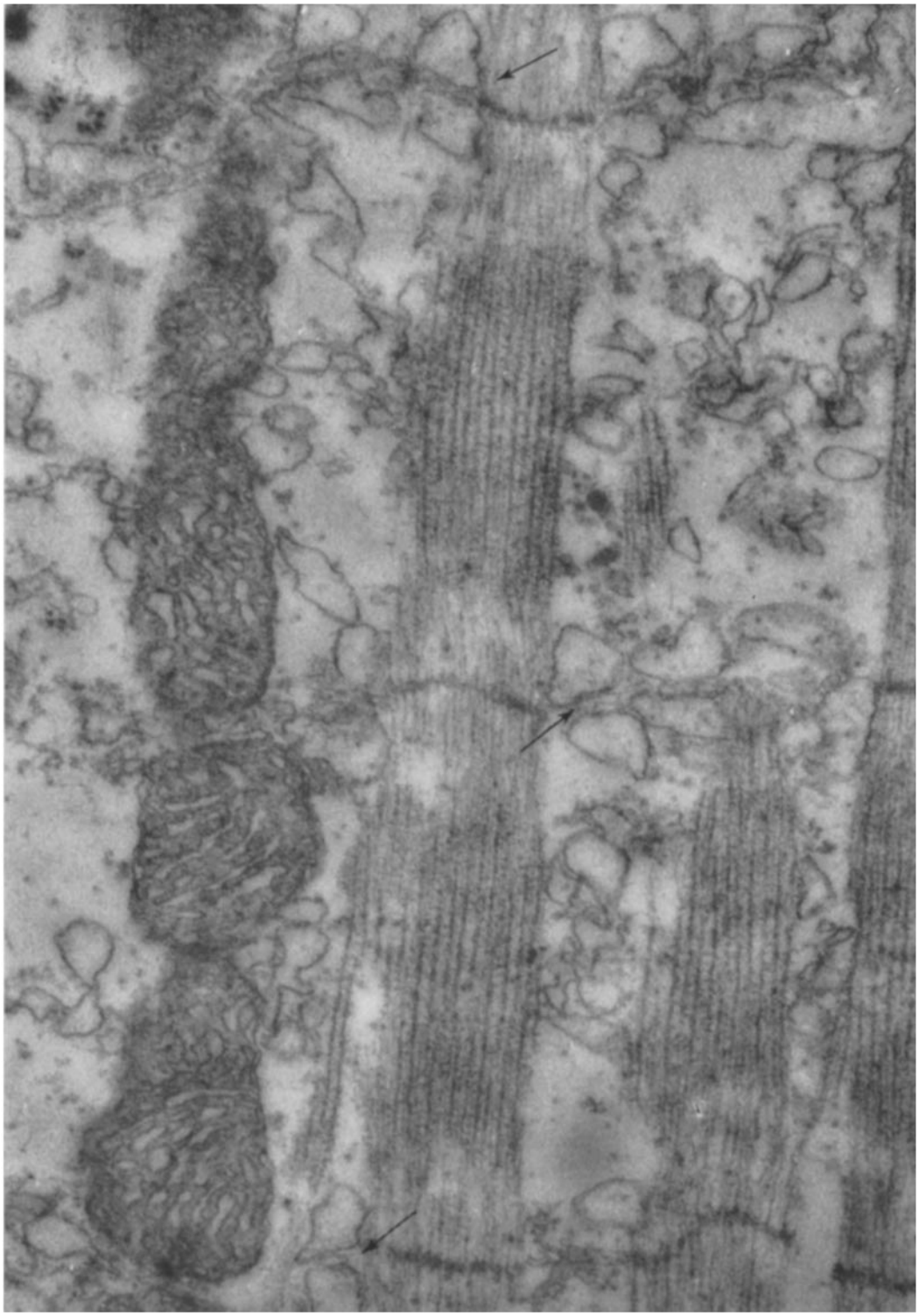

FIGURE 14 Electron micrograph of longitudinal section of frog semitendinosus muscle. Stage III of atrophy. This illustrates the considerable atrophy of the myofibrils and the disorganization of the sarcoplasmic reticulum. The mutual arrangement of the triad components at the Z band level is well preserved (arrows). Conditions for staining and embedding as in Fig. 1. × 60,000.

Helander (33) and further developed by Perry in his recent studies of developing muscle (31, 52), of "adaptive muscular allomorphism." Since there are probably different sites in the muscle cell for the synthesis of specific protein molecules, it is of interest, in the present context, that accompanying the elaborate proliferation of the sarcoplasmic reticulum there is a tendency in the denervated frog muscles to find RNA particles becoming attached to the surfaces of the intracellular membranes. These findings are in agreement with the suggestion made previously, on the basis of other lines of evidence (42), that microsomal RNA may be specifically involved in the synthesis of the SR membranes.

In order to make a functional evaluation of the modifications of the sarcotubular system described in this paper, it is noteworthy that the T system and the sarcoplasmic reticulum responded to the early effects of denervation in a different fashion. These findings bear some general resemblance to the observations made by Price *et al.* (54) in the diaphragm muscle of rats given the myotoxic agent Plasmocid. They also lend further support to the idea that there exist different functions associated with the two morphologically distinguishable parts of the sarcotubular system. The overdevelopment of the sarcoplasmic reticulum, but not of the T system is, presumably, the morphological expression of metabolic changes occurring on denervation of frog muscle, since the sarcoplasmic reticulum has been implicated in glycogen metabolism and glycolysis (3, 40, 41, 17). The hypertrophy of the sarcoplasmic reticulum appears thus to characterize a phase of "simple atrophy" during which, conceivably, new metabolic patterns become delineated which provide fitness to the denervated condition with regard to cell survival and, ultimately, muscle repair. Congruous with this interpretation of the dynamic changes of the SR is additional information gained from study of carbohydrate metabolism in denervated frog muscles which will be the subject of forthcoming papers.

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BIBLIOGRAPHY

1. Aloisi, M., Azzone, G. F., and Carafoli, E., *Arch. De Vecchi*, 1960, **32**, 33.
2. Andersson-Cedergren, E., *J. Ultrastruct. Research*, 1959, suppl. 1.
3. Andersson-Cedergren, E., and Muscatello, U., *J. Ultrastruct. Research*, 1963, **8**, 391.
4. Bass, A., *in* The Denervated Muscle, (E. Gutmann, editor), Prague, Publishing House of the Czechoslovak Academy of Science, 1962, 203.
5. Behnke, O., *Exp. Cell Research*, 1963, **30**, 597.
6. Bennet, H. S., and Porter, K. R., *Am. J. Anat.*, 1953, **93**, 61.
7. Biava, C., *J. Lab. Invest.*, 1963, **12**, 1179.
8. Birks, R., Katz, B., and Miledi, R., *Nature*, 1959, **184**, 1507.
9. Burton, K., *Biochem. J.*, 1956, **62**, 315.
10. Buse, M. G., Newton, N., Jones, D. J., and Worthington, W. C., *Clin. Research*, 1963, **11**, 40.
11. Carafoli, E., Margreth, A., and Buffa, P., *Nature*, 1962, **196**, 1101.
12. Carafoli, E., Margreth, A., and Buffa, P., *J. Exp. and Molec. Path.*, 1964, **3**, 171.
13. Decken, A. von der, and Hultin, T., *Exp. Cell Research*, 1958, **14**, 88.
14. Dreyfus, J. C., Kruh, J., and Schapira, G., *in* Protein Metabolism, (F. Gross, editor), Berlin, Springer Verlag, 1962, 326.
15. Eccles, J. C., *in* The Effect of Use and Disuse on Neuromuscular Functions, (E. Gutmann and P. Hnik, editors), Amsterdam, Elsevier Publishing Company, 1963, 549.
16. Eliakova, G. V., Laboratory of Electron Microscopy, SSSR Academy of Science, Moscow, personal communication.
17. Fahimi, H. D., and Amarasingham, C. R., *J. Cell Biol.*, 1964, **22**, 29.
18. Fawcett, D. W., *Circulation*, 1961, **24**, 336.
19. Fawcett, D. W., and Revel, J. P., *J. Biophysic. and Biochem. Cytol.*, 1961, **10**, No. 4, suppl., 89.
20. Fischer, E., *Arch. Phys. Med.*, 1948, **29**, 291.
21. Fischer, E., Proceedings of the Third Medical Conference of the Muscular Dystrophy Associations of America, New York, Published by the M.D.A.A. Inc.,1954, 212.

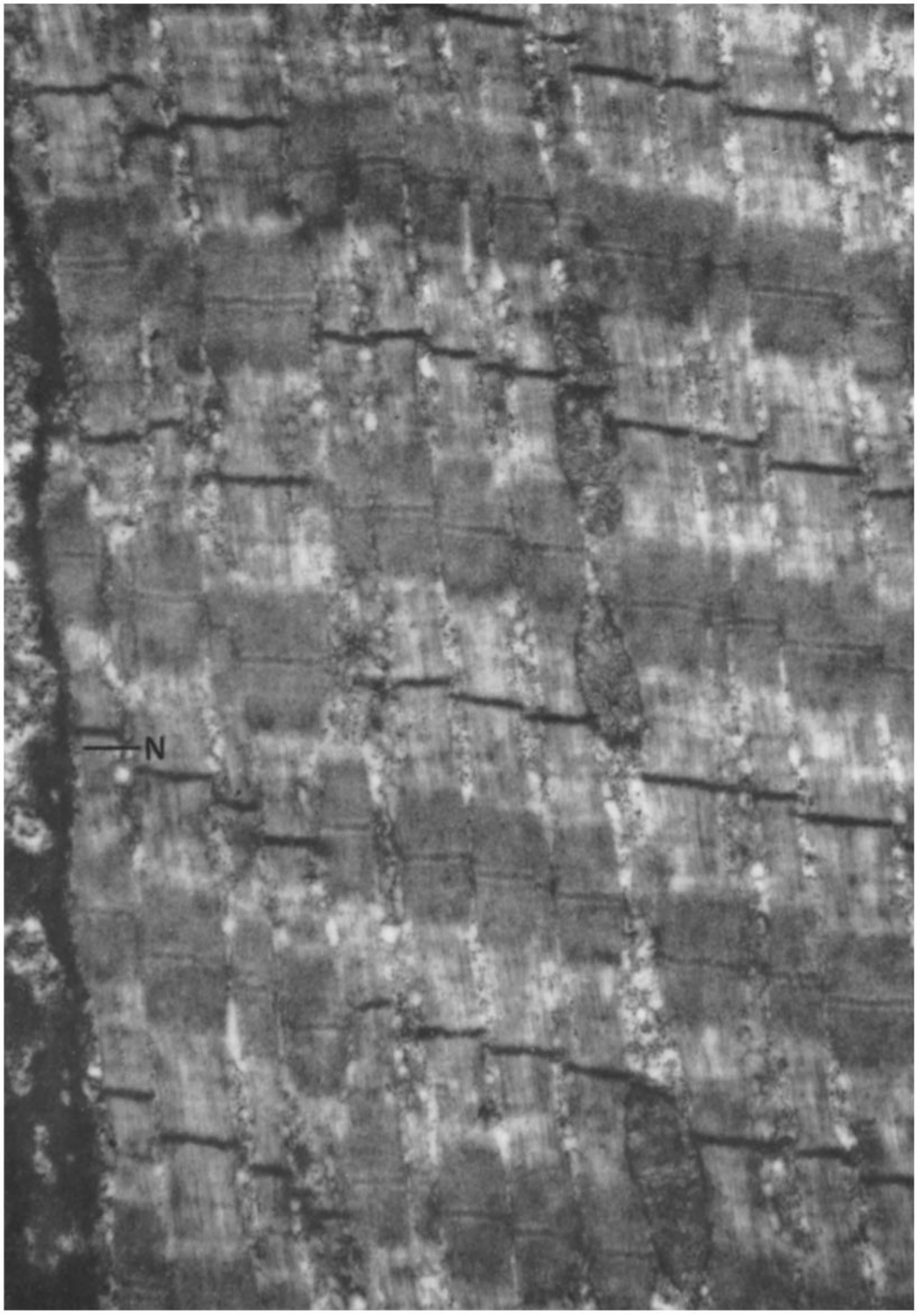

FIGURE 15 Electron micrograph of longitudinal section of frog semitendinosus muscle. Stage III of atrophy. The profiles of the SR membranes appear poorly resolved. Very numerous ribosomes are seen in the intermyofibrillar spaces. *N*, nucleus. Embedding in Araldite. Stained with a saturated solution of uranyl acetate. × 18,000.

22. FISCHER, E., and RAMSEY, V. W., *Am. J. Physiol.*, 1946, **145**, 571.
23. FISHER, R. A., and YATES, F., Statistical Tables, Edinburgh, Oliver and Boyd, 1963.
24. FLORINI, J. R., and BREUER, C. B., *Biochem.*, 1965, **4**, 253.
25. FRANZINI-ARMSTRONG, C., and PORTER, K. R., *Nature*, 1964, **202**, 355.
26. FRANZINI-ARMSTRONG, C., and PORTER, K. R., *J. Cell Biol.*, 1964, **22**, 675.
27. GIERER, A., *J. Molec. Biol.*, 1963, **6**, 148.
28. GIESEKING, R., *Frankfurter Z. Pathol.*, 1962, **71**, 584.
29. GUTMANN, E., *Physiol. Bohemoslov*, 1960, **9**, 382.
30. HARRIS, E. J., and NICHOLLS, J. G., *J. Physiol.*, 1956, **131**, 473.
31. HARTSHORNE, D. J., and PERRY, S. V., *Biochem. J.*, 1962, **85**, 171.
32. HEGNAUER, A. H., and CORI, G. T., *J. Biol. Chem.*, 1934, **105**, 691.
33. HELANDER, E., *Nature*, 1958, **182**, 1035.
34. HNIK, P., *in* The Denervated Muscle, (E. Gutmann, editor), Prague, Publishing House of the Czechoslovak Academy of Science, 1962, 341.
35. HNIK, P., and SKORPIL, V., *in* The Denervated Muscle, (E. Gutmann, editor), Prague, Publishing House of the Czechoslovak Academy of Science, 1962, 135.
36. HUXLEY, A. F., and STRAUB, R. W., *J. Physiol.*, 1958, **143**, 40.
37. HUXLEY, H. E., *Nature*, 1964, **202**, 1067.
38. KIELLEY, W. W., *Ann. Rev. Biochem.*, 1964, **33**, 403.
39. LOWRY, H. O., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J., *J. Biol. Chem.*, 1951, **193**, 265.
40. MARGRETH, A., *Biochim. et Biophysica Acta*, 1963, **77**, 337.
41. MARGRETH, A., MUSCATELLO, U., and ANDERSSON-CEDERGREN, E., *Exp. Cell Research*, 1963, **32**, 484.
42. MARGRETH, A., and NOVELLO, F., *Exp. Cell Research*, 1964, **35**, 38.
43. MUSCATELLO, U., ANDERSSON-CEDERGREN, E., and AZZONE, G. F., *Biochim. et Biophysica Acta*, 1962, **63**, 55.
44. MUSCATELLO, U., ANDERSSON-CEDERGREN, E., AZZONE, G. F., and DECKEN, A. VON DER, *J. Biophysic. and Biochem. Cytol.*, 1961, **10**, No. 4, suppl., 201.
45. MUSCATELLO, U., CARAFOLI, E., and BUFFA, P., Proceedings 3rd European Regional Conference on Electron Microscopy, Prague, Publishing House of the Czechoslovak Academy of Science, 1964, 85.
46. NARAHARA, H. T., ÖZAND, P., and CORI, C. F., *J. Biol. Chem.*, 1960, **235**, 3370.
47. ORBELI, L. A., *Trudy Fiziol. inst. im. I.P. Pavlova Akad. Nauk SSSR*, 1945, **1**, 1.
48. PALADE, G. E., and SIEKEWITZ, P., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 171.
49. PALADE, G. E., and SIEKEWITZ, P., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 671.
50. PELLEGRINO, C., and FRANZINI, C., *J. Cell Biol.*, 1963, **17**, 327.
51. PERRY, S. V., *in* The Effect of Use and Disuse on Neuromuscular Functions, (E. Gutmann and P. Hnik, editors), Amsterdam, Elsevier Publishing Company, 1963, 551.
52. PERRY, S. V., HARTSHORNE, D. J., *in* The Effect of Use and Disuse on Neuromuscular Functions, (E. Gutmann and P. Hnik, editors), Amsterdam, Elsevier Publishing Company, 1963, 491.
53. PORTER, K. R., and PALADE, G. E., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 269.
54. PRICE, H. M., PEASE, D. C., and PEARSON, C. M., *Lab. Invest.*, 1962, **11**, 549.
55. RISEBROUGH, R. W., TISSIERES, A., and WATSON, J. D., *Proc. Nat. Acad. Sc.*, 1962, **48**, 430.
56. SCHAPIRA, G., and DREYFUS, J. C., *Am. J. Phys. Med.*, 1959, **38**, 207.
57. SCHNEIDER, W. C., *J. Biol. Chem.*, 1945, **161**, 293.
58. SIEKEWITZ, P., *in* Methods in Enzymology, (S. P. Colowick and N. O. Kaplan, editors), New York, Academic Press, Inc., 1962, **5**, 61.
59. SIEKEWITZ, P., *Ann. Rev. Physiol.*, 1963, **15**, 25.
60. STEWART, D. M., *Am. J. Physiol.*, 1962, **202**, 281.
61. TOWER, S. S., *Physiol. Revs.*, 1939, **19**, 1.
62. WADDINGTON, C. H., and PERRY, M. M., *Exp. Cell Research*, 1963, **30**, 599.
63. WARNER, J. R., KNOPF, P. M., and RICH, A., *Proc. Nat. Acad. Sc.*, 1963, **49**, 122.
64. WECHSLER, W., *Naturwissensch.*, 1964, **51**, 91.
65. WECHSLER, W., and HAGER, H., *Naturwissensch.*, 1960, **47**, 185.
66. ZAK, R., *in* The Denervated Muscle, (E. Gutmann, editor), Prague, Publishing House of the Czechoslovak Academy of Science, 1962, 273.
67. ZAK, R., and GUTMANN, E., *Nature*, 1960, **185**, 766.