Journal of Clinical Investigation
Vol. 44, No. 8, 1965

Ca^{++} Uptake and ATPase of Human Sarcoplasmic Reticulum *

F. J. SAMAHA † AND J. GERGELY

(From the Kennedy Memorial Laboratories, Massachusetts General Hospital; the Departments of Neurology and Biological Chemistry, Harvard Medical School; and the Department of Muscle Research, Institute of Biological and Medical Sciences, Retina Foundation, Boston, Mass.)

In 1951 Marsh showed that an aqueous KCl extract of skeletal muscle reversed syneresis and inhibited ATPase activity of myofibrils (1). The same extract inhibited the ATP-induced contraction of glycerol-extracted muscle fibers (2) and became known as the Marsh-Bendall relaxing factor (3). It is generally believed that the active component of the relaxing factor preparation consists of fragments of sarcoplasmic reticulum present as small vesicles (microsomal fractions or grana) that act by removing calcium from the actomyosin system (4–10). Recently it has been shown that grana prepared from fast (white) muscle of the rabbit have a greater calcium uptake activity than grana prepared from slow (red) muscle (11). The ATPase activity of these two types of grana reacts differently to aging and to azide (11).

In animal studies the relaxing microsomal fraction has usually been obtained from muscle homogenates by centrifugation between 10,000 and 30,000 × *g*. This paper is a report on similar microsomal preparations from normal human skeletal muscle showing Ca^{++}-Mg^{++} stimulated ATPase and calcium uptake activity. Studies on grana prepared from human muscles anatomically analogous to the fast (white) and slow (red) muscles of the rabbit are also presented.

* Submitted for publication December 21, 1964; accepted May 10, 1965.

This investigation was supported by grants from the Muscular Dystrophy Associations of America, Inc., and the National Heart Institute, U. S. Public Health Service (HE-05949-02-03).

† Special fellow of the National Institute of Neurological Diseases and Blindness, U. S. Public Health Service (2F11 NB 1133 02). Address requests for reprints to Dr. F. J. Samaha, Department of Neurology, Massachusetts General Hospital, Boston, Mass. 02114.

Methods

Gastrocnemius, vastus lateralis, and soleus muscles were obtained from cadavers. The muscles were taken within 5 hours after death from refrigerated cadavers in which no neuromuscular disease was present. The muscles were removed 4 to 6 cm short of each tendinous insertion. Vessels, fat, and connective tissue were removed, and each muscle was minced separately with scissors. The mince was homogenized for 90 seconds in a Waring blendor with 3 vol of ice-cold 0.25 M sucrose and 0.005 M histidine, pH 7.0. The myofibrils were sedimented by centrifugation for 20 minutes at 600 × *g*, and the supernatant was fractionated by centrifugation in a Spinco model L preparative ultracentrifuge. The fractions sedimented during 20 minutes at 8,000 × *g*, 1 hour at 30,000 × *g*, and 1 hour at 80,000 × *g* were resuspended in the above sucrose-histidine medium; the 30,000 × *g* fraction (crude grana) was spun again for 20 minutes at 8,000 × *g*. The supernatant will be referred to as "purified" grana. The above procedures were all carried out at 2 to 5° C.

Calcium uptake was measured by incubating 0.02 to 0.3 mg of protein of the appropriate fraction in a medium containing 0.1 M KCl, 20 mM histidine, pH 7.35, 5 mM $MgCl_2$, 5 mM ATP, and 0.10 to 0.15 mM $Ca^{45}Cl_2$, to which potassium oxalate, sodium azide, and dinitrophenol were added as indicated in the text and legends. The reaction was carried out at 33° C and terminated by passing 1 to 2 ml of the mixture through a type HA Millipore filter (0.45 to 0.3 μ average pore diameter) (10). The amount of bound calcium was calculated from the radioactivity of the filtrate measured in a Packard liquid scintillation counter according to Loftfield and Eigner (12).

Adenosine triphosphatase activity was measured in 2.0-ml samples by determining, after precipitation of the protein by the addition of 0.5 ml ice cold 10% trichloroacetic acid and centrifugation, the amount of inorganic phosphorus liberated from ATP in the supernatant by the Fiske-SubbaRow method (13). Protein was measured by the biuret method.

Results

As shown in Figure 1, the calcium uptake by grana in the presence of oxalate is complete in 10

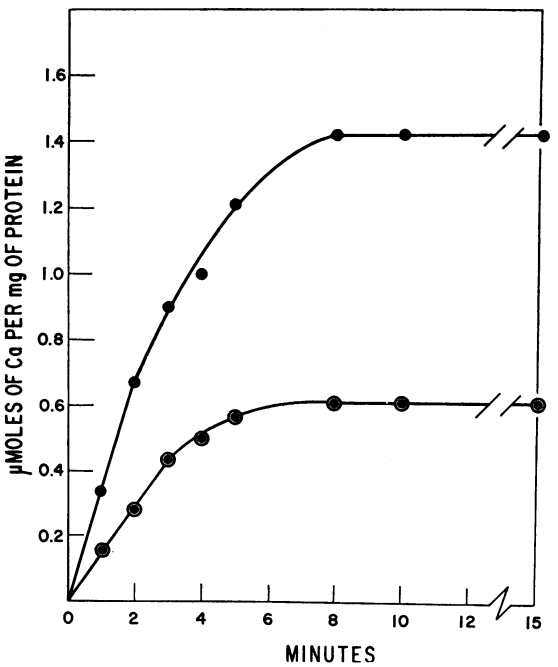

FIG. 1. CALCIUM UPTAKE OF HEAVY AND LIGHT MICROSOMES. ●, 8,000 to 30,000 × *g*; ◉, 30,000 to 80,000 × *g*. For details see Methods.

minutes. Calcium uptake by the heavier microsomal fraction exceeded that of the lighter microsomal fraction both in initial rate (over first 2.5 to 3.0 minutes) and in total uptake. Total uptake was routinely estimated after 15 minutes incubation. The dependence of calcium uptake activity on the presence of ATP and magnesium is shown in Table I. The grana fail to take up calcium when these constituents are absent. The enhancement by oxalate of calcium uptake found with animal muscle grana (5–7, 10) can also be observed with human muscle grana (Table I), and dependence of calcium uptake on oxalate concentration is similar

TABLE I

Requirements for calcium uptake by grana

System	µmoles of Ca^{++} per mg protein per 15 minutes
No oxalate	0.17
1.0 mM oxalate	0.37
3.0 mM oxalate	2.32
5.0 mM oxalate	3.64
7.0 mM oxalate	4.30
5.0 mM oxalate, no ATP	0
5.0 mM oxalate, no Mg^{++}	0

to that described by Martonosi and Feretos (10). For the studies reported in this paper 5 mM oxalate was used, since this concentration produces good calcium uptake and has been used in studies on animal grana.

It should be pointed out that according to the data of Hasselbach and Makinose (14) the solubility product of Ca-oxalate is exceeded with 1.5×10^{-4} M Ca, 5×10^{-3} M oxalate present under the conditions of our experiments; in fact, no formation of Ca-oxalate precipitate—as would be indicated by loss of radioactivity on filtration in the absence of grana—was observed.

Twenty-three muscle preparations were studied. Data on ATPase activity, calcium uptake, and the ratio of initial calcium uptake to ATP hydrolyzed are presented in Table II. Values shown are means with their standard deviation.

The data on gastrocnemius and vastus lateralis (white) were grouped together, as distinct from those on the soleus (red) in view of the recently found differences between white and red muscles in rabbit (11). The initial rate and total uptake of calcium are higher in the fast or white muscles, gastrocnemius, and vastus lateralis, but the differ-

TABLE II

Mean ATPase and calcium uptake activities of grana prepared from gastrocnemius plus vastus lateralis and soleus muscles

Muscle	No. preparations	ATPase	Initial rate* of Ca^{++} uptake	$\Delta Ca^{++}/\Delta P_i$†	Total Ca^{++} uptake
		µmoles P_i/mg protein/min	*µmoles/mg protein/min*		*µmoles/mg protein*
Gastrocnemius + vastus lateralis	15	0.84 ± 0.06	0.38 ± 0.04	0.43 ± 0.04	2.04 ± 0.22
Soleus	8	0.66 ± 0.09	0.17 ± 0.04	0.25 ± 0.04	1.01 ± 0.19

* Measured over first 2.5 minutes.
† P_i = inorganic phosphorus.

TABLE III

Effect of 0.5 mM sodium azide on ATPase activity of grana and mitochondrial preparation

	No. preparations	% of normal ATPase (mean ± SE)
White grana	12	76.3 ± 3.4
White grana, purified	9	91.0 ± 2.1
Red grana	9	55.7 ± 6.7
Red grana, purified	7	80.5 ± 4.4
Mitochrondria	16	24.4 ± 2.4

TABLE IV

Effect of 0.1 mM EGTA on ATPase activity of grana and mitochondrial preparations*

	No. preparations	% of normal ATPase (mean ± SE)
White grana	10	41.2 ± 3.8
White grana, purified	6	28.2 ± 2.8
Red grana	8	65.3 ± 4.0
Red grana, purified	6	47.6 ± 3.6
Mitochondria	10	88.1 ± 3.2

* EGTA = ethylene glycol bis tetraacetic acid.

ences are not so striking as those found for rabbit muscles (11). There is no significant difference between ATPase activities of the two. No correlation between calcium uptake and ATPase activity, on the one hand, and age, sex, or any non-neuromuscular disease with which the patient may have been afflicted, on the other hand, has been found.

The question of contamination with mitochondrial fragments of muscle microsomal fractions has been raised in connection with studies on dog cardiac muscle (15) and on red muscles of the rabbit (11), and it seemed advisable to investigate this aspect of human muscle grana. Siekevitz, Löw, Ernster, and Lindberg (16) showed that the ATPase activity of liver mitochondria was considerably inhibited by sodium azide. We found that ATPase activity of human skeletal muscle mitochondria (600 to 8,000 × *g* fraction) was similarly inhibited by azide (Table III). ATPase activity of grana obtained from human white muscle was only slightly inhibited by sodium azide; purified grana activity showed little or no inhibition by azide (Table III). The ATPase activity of grana prepared from red muscle is inhibited almost 50% by azide, and the purified grana activity was inhibited by 20%.

Siekevitz and his co-workers (16) also showed that mitochondrial ATPase activity was only slightly inhibited by EDTA, and other investigators have shown that animal grana ATPase ac-

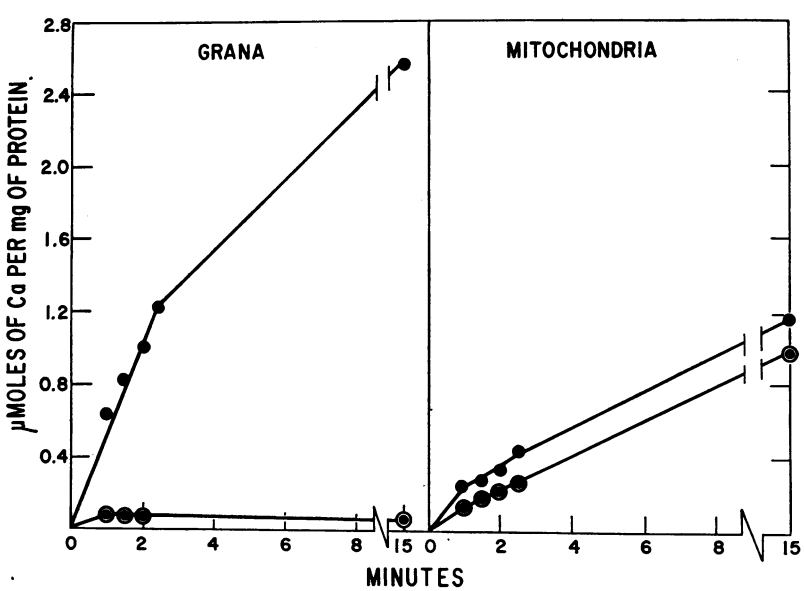

FIG. 2. EFFECT OF 5 mM POTASSIUM OXALATE ON CALCIUM UPTAKE ACTIVITY OF GRANA AND MITOCHONDRIAL PREPARATIONS. ◉, no oxalate; ●, 5 mM potassium oxalate. For details see Methods.

TABLE V

Effect of 0.5 mM azide and 0.1 mM DNP on total calcium uptake of grana and mitochondrial preparations*

	No. preparations	No. normal Ca^{++} uptake (mean ± SE)
Grana		
+ 0.5 mM azide	18	100.8± 4.0
+ 0.1 mM DNP	8	101.9± 5.8
Mitochondria		
+ 0.5 mM azide	12	21.1±10.8
+ 0.1 mM DNP	6	29.0± 7.0

* DNP = dinitrophenol.

tivity was considerably inhibited if calcium was removed from the incubation medium with EGTA [1] (17–18).

Table IV shows that human mitochondrial ATPase activity is only slightly inhibited by EGTA, whereas that of grana is more markedly inhibited. Mitochondrial ATPase activity inhibited by azide is not present in purified grana prepared from white muscle; hence the EGTA effect is more striking. This does not hold true for the activity of purified grana prepared from red muscle, as significant azide inhibition is still present.

Calcium uptake in grana and mitochondria. Since it is well known that oxalate potentiates the calcium uptake of animal grana (5–7, 10), its effect on human grana and mitochondria was studied. Figure 2 shows that 5 mM oxalate enhanced both the initial rate and total uptake of calcium by grana, whereas the mitochondrial uptake was only slightly increased. The calcium uptake by mitochondria from liver, kidney, brain (19), and beef heart (20, 21) is inhibited by sodium azide and dinitrophenol. Grana from human muscle are unaffected by these agents, but the calcium uptake of the mitochondrial fraction is depressed (Table V).

Effects of aging. The ATPase activity of grana from white rabbit muscle increases on aging, whereas the activity of grana from red muscle decreases with time (11). The ATPase activity of human grana from either red or white muscle decreases with time at 0° C (Figure 3A). This is accompanied by a decrease of the calcium uptake activity of both types of human grana (Figure 3B).

[1] Ethylene glycol bis tetraacetic acid = EGTA.

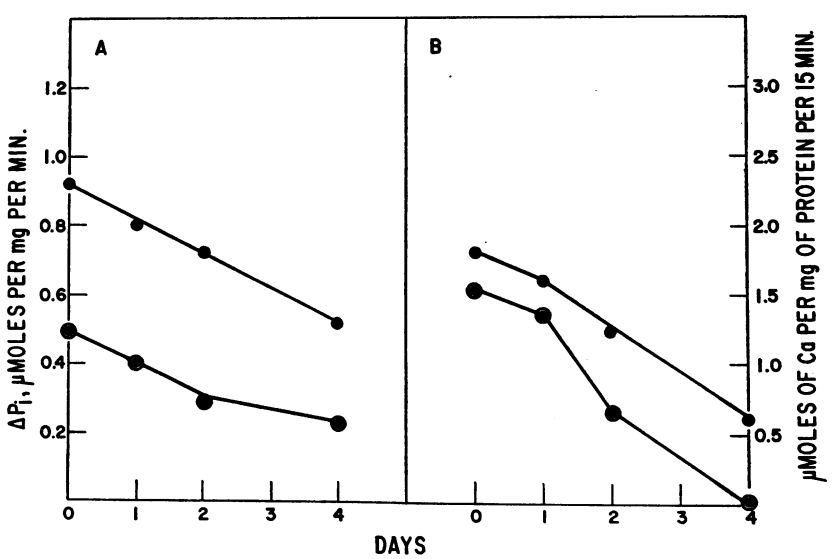

FIG. 3. A. EFFECTS OF AGING AT 0° C ON ATPASE ACTIVITY OF GRANA. B. EFFECT OF AGING AT 0° C ON TOTAL CALCIUM UPTAKE ACTIVITY OF GRANA. ●, grana from white muscle; ◉, grana from red muscle. P_i = inorganic phosphorus. For details see Methods.

Discussion

A considerable amount of evidence has accumulated implicating calcium as a link between the excitation-contraction and relaxation of striated muscle. It has been shown that calcium entry into muscle increases with contraction (22) and that skeletal muscle fails to undergo contracture in a calcium free solution (23, 24). Contraction of skeletal myofibrils, actomyosin, and glycerinated muscle fibers requires the presence of calcium (25–28). The relaxing effect of chelating agents (28–31) and skeletal muscle grana (4–10) is explained by their ability to lower the Ca^{++} of the test system to below a 10^{-6} M concentration. The grana probably do this by active uphill transport of calcium with the hydrolysis of ATP as the source of energy. Most investigators agree that these grana or heavy microsomal fractions represent vesicles of fragmented sarcoplasmic reticulum.

Whether or not human myofibrils or glycerinated fibers respond to removal of calcium by relaxation, and—if they do—whether grana preparations from human muscle can produce relaxation will require further study.

All the work on ATPase and calcium uptake activity on grana has been done on animal skeletal (4–10) and cardiac muscle (18). The data presented in this paper show that a grana fraction similar to animal preparations can be prepared from human striated muscle homogenates that have a Ca^{++}-Mg^{++} stimulated ATPase activity and ability to take up calcium. During the early phase of this study we found that muscle from cadavers properly refrigerated, obtained less than 5 hours after death, yielded preparations that were comparable to those obtained from fresh biopsied material.

The grana obtained from human striated muscle are relatively free of mitochondrial contamination, as measured by small or no inhibition of ATPase activity by azide, marked inhibition of ATPase activity by EGTA, no inhibition of calcium uptake by azide or dinitrophenol, and a marked stimulation of calcium uptake by oxalate. The slight inhibition of grana ATPase activity by azide is probably due to contamination with some fragmented mitochondria. In contrast, the mitochondrial fraction showed a marked inhibition of ATPase activity by azide but little effect by EGTA. The calcium uptake activity was inhibited by azide and dinitrophenol, whereas oxalate showed only slight enhancement of Ca-uptake. The small inhibition of ATPase activity by EGTA and the slight stimulation of calcium uptake by oxalate probably indicated contamination of the mitochondria by intact sarcoplasmic reticulum vesicles. Studies at present being carried out in our laboratory show that succinic dehydrogenase and cytochrome oxidase activities, which are high in the mitochondrial fraction, are low in grana and virtually absent in purified grana (32).

The molar ratio of calcium taken up to ATP hydrolyzed is lower in human preparations than in those obtained from rabbit muscle (17). However, a direct comparison of ATP hydrolyzed with the calcium taken up by the grana may be difficult because of the heterogeneity of the ATPase in the same fraction. In addition to the microsomal Ca^{++}-Mg^{++} stimulated ATPase, there is ATPase activity, but no calcium uptake in some fragmented mitochondria and also the Mg-Na-K stimulated ATPase recently described in a heavy microsomal fraction of human striated muscle (33).

The difference between grana preparations from human red and white muscle in their ability to take up calcium is not so striking as that found in the case of the rabbit (11) where grana from white muscle take up almost 20 times as much calcium as grana from red muscle. This is in accord with other work, showing that the preponderance of a fiber of one type over that of the other found in some animal muscles (34–36) is not so pronounced in human muscle (37). The ATPase activity of grana prepared from both types of human muscle decreased when aged at 0° C. In the case of the rabbit the ATPase activity of grana prepared from white muscle increased with aging, whereas that prepared from red muscle decreased (11).

These studies probably represent the first work on the preparation, ATPase activity, and calcium uptake activity of grana from human striated muscle. Work is now in progress on grana prepared from muscle biopsy material from patients with various myopathies. We hope that these studies will yield information on metabolic defects in certain muscle diseases and lead to a better understanding of the excitation-contraction coupling of human striated muscle.

Summary

1. The preparation of grana (fragments of sarcoplasmic reticulum) from human striated muscle is described.

2. The Ca^{++}-Mg^{++} moderated ATPase and calcium uptake activities of human grana have been studied and compared with those of human muscle mitochondria.

3. The difference in calcium uptake activity in grana prepared from human fast (white) and slow (red) muscles is not so pronounced as that found in studies on rabbit muscle.

Acknowledgments

The authors are grateful to Mrs. Ingeborg Mansell for skillful technical assistance, and to the members of the staff of the Pathology Department, Massachusetts General Hospital, for their cooperation in securing material for these studies.

References

1. Marsh, B. B. A factor modifying muscle fibre synaeresis. Nature (Lond.) 1952, **167**, 1065.
2. Bendall, J. R. Effect of the 'Marsh factor' on the shortening of muscle fibre models in the presence of adenosine triphosphate. Nature (Lond.) 1952, **170**, 1058.
3. Gergely, J. Muscle proteins and energy utilization. Ann. N. Y. Acad. Sci. 1959, **72**, 538.
4. Ebashi, S. Calcium binding and relaxation in the actomyosin system. J. Biochem. (Tokyo) 1960, **48**, 150.
5. Hasselbach, W., and M. Makinose. Die Calciumpumpe der "Erschlaffungsgranna" des Muskels und ihre Abhängigkeit von der ATP-Spaltung. Biochem. Z. 1961, **333**, 518.
6. Hasselbach, W., and M. Makinose. ATP and active transport. Biochem. biophys. Res. Commun. 1962, **7**, 132.
7. Hasselbach, W., and M. Makinose. The calcium pump of the relaxing vesicles and the production of a relaxing substance *in* Biochemistry of Muscle Contraction, J. Gergely, Ed. Boston, Little Brown, 1964, p. 247.
8. Ebashi, S., and F. Lipmann. Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. J. Cell Biol. 1962, **14**, 389.
9. Weber, A., R. Herz, and I. Reiss. On the mechanism of the relaxing effect of fragmented sarcoplasmic reticulum. J. gen. Physiol. 1963, **46**, 679.
10. Martonosi, A., and R. Feretos. Sarcoplasmic reticulum I. The uptake of Ca^{++} by sarcoplasmic reticulum fragments. J. biol. Chem. 1964, **239**, 648.
11. Sréter, F. A., and J. Gergely. Comparative studies of the Mg activated ATPase activity and Ca uptake of fractions of white and red muscle homogenates. Biochem. biophys. Res. Commun. 1964, **16**, 438.
12. Loftfield, R. B., and E. A. Eigner. Scintillation counting of paper chromatograms. Biochem. biophys. Res. Commun. 1960, **3**, 72.
13. Fiske, C. H., and Y. SubbaRow. The colorometric determination of phosphorus. J. biol. Chem. 1925, **66**, 375.
14. Hasselbach, W., and M. Makinose. Uber der Mechanismus des Calciumtransportes durch die Membranen des sarkoplasmatischen Reticulum. Biochem. Z. 1963, **339**, 94.
15. Honig, C. R., and A. C. Stam, Jr. Relaxing systems of cardiac muscle. Fed. Proc. 1964, **23**, 926.
16. Siekevitz, P., H. Löw, L. Ernster, and O. Lindberg. On a possible mechanism of the adenosinetriphosphatase of liver mitochondria. Biochim. biophys. Acta (Amst.) 1958, **29**, 378.
17. Martonosi, A., and R. Feretos. Sarcoplasmic reticulum II. Correlation between adenosine triphosphatase activity and Ca^{++} uptake. J. biol. Chem. 1964, **239**, 659.
18. Fanburg, B., R. M. Finkel, and A. Martonosi. The role of calcium in the mechanism of relaxation of cardiac muscle. J. biol. Chem. 1964, **239**, 2298.
19. Ross, C. S., and A. L. Lehninger. Stoichiometric relationships between accumulation of ions by mitochondria and the energy-coupling sites in the respiratory chain. Biochem. Z. 1963, **338**, 698.
20. Fanburg, B. Calcium in the regulation of heart muscle contraction and relaxation. Fed. Proc. 1964, **23**, 923.
21. Brierley, G. P., E. Murer, and E. Bachmann. Studies on ion transport III. The accumulation of calcium and inorganic phosphate by heart mitochondria. Arch. Biochem. 1964, **105**, 89.
22. Bianchi, P., and A. M. Shanes. Calcium influx in skeletal muscle at rest, during activity and during potassium contracture. J. gen. Physiol. 1959, **42**, 803.
23. Frank, G. B. Effects of changes in extracellular calcium concentration on the potassium-induced contracture of frog's skeletal muscle. J. Physiol. (Lond.) 1960, **151**, 518.
24. Frank, G. B. Utilization of bound calcium in the acetylcholine contracture of frog skeletal muscle. J. Pharmacol. exp. Ther. 1963, **139**, 261.
25. Weber, A. On the role of calcium in the activity of adenosine 5'-triphosphate hydrolysis by actomyosin. J. biol. Chem. 1959, **234**, 2764.
26. Weber, A., and S. Winicur. The role of calcium in the superprecipitation of actomyosin. J. biol. Chem. 1961, **236**, 3198.
27. Weber, A., and R. Herz. Requirement for calcium in the synaeresis of myofibrils. Biochem. biophys. Res. Commun. 1961, **6**, 364.

28. Ebashi, S. Calcium binding activity of vesicular relaxing factor. J. Biochem. (Toyko) 1961, **50**, 236.
29. Bozler, E. Relaxation in extracted muscle fibers. J. gen. Physiol. 1954, **38**, 149.
30. Watanabe, S. Relaxing effects of EDTA on glycerol-treated muscle fibers. Arch. Biochem. 1955, **54**, 599.
31. Watanabe, S., and W. Sleator, Jr. EDTA relaxation of glycerol-treated muscle fibers and the effects of magnesium, calcium and manganese ions. Arch. Biochem. 1957, **81**, 68.
32. Pragay, D., F. J. Samaha, and J. Gergely. Unpublished work.
33. Samaha, F. J., and J. Gergely. Na^+- and K^+-stimulated ATPase in human striated muscle. Arch. Biochem. 1965, **109**, 76.
34. Dubowitz, V., and A. G. E. Pearse. A comparative histochemical study of oxidative enzyme and phosphorylase activity in skeletal muscle. Histochemie 1960, **2**, 105.
35. Pellegrino, C., and C. Franzini. An electron microscope study of denervation atrophy in red and white skeletal muscle fibers. J. Cell Biol. 1963, **17**, 327.
36. Slater, E. C. Biochemistry of sarcosomes *in* Structure and Function of Muscle, G. Bourne, Ed. New York and London, Academic Press, vol. 2, p. 105.
37. Engel, W. K. The essentiality of histo- and cytochemical studies of skeletal muscle in the investigation of neuromuscular disease. Neurology (Minneap.) 1962, **12**, 778.