The extraction of cytochrome *c* from the fragments by 0.1 M phosphate buffer also removes uncoupling agents. During the further treatment the accessibility of electrons to PMS from succinate dehydrogenase is steadily increased, while the level of respiration with β-hydroxybutyrate remained constant or diminished. TSOU[1] has shown a difference in the two forms of cytochrome *c* based on their complexing capacity with cyanide. He has pointed out that exogenous cytochrome *c* combines with cyanide to form a stable complex, while the endogenous cytochrome *c* is not affected by cyanide even in a prolonged incubation. Behavior of the fragments in the presence of trapped cytochrome *c* indicated that the exogenous cytochrome *c* was in good equilibrium with the endogenous cytochrome *c*, and that the bypass of cytochrome oxidase by denatured cytochrome *c* was not increased over the control.

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Department of Biochemistry,
State University of New York at Buffalo,
Buffalo, N.Y. (U.S.A.)

A. N. MALVIYA
W. B. ELLIOTT

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The effect of soluble proteins on the fragmented sarcoplasmic reticulum

In an investigation into the nature of the dialyzable cofactor of the fragmented sarcoplasmic reticulum (FSR) (refs. 1–3) it was found that the soluble fraction of a muscle homogenate extended the delay in syneresis of myofibrils produced by FSR (ref. 4). One of the effective substances appeared to be inorganic phosphate and the

Abbreviation: FSR, fragmented sarcoplasmic reticulum.

other was not dialyzable and could be replaced by a commercial preparation of myokinase[4]. It had been reported earlier that myokinase could potentiate the inhibition by FSR of myofibrillar ATPase activity[5]. The present results indicate that the effect of the non-dialyzable fraction of the supernatant is the result of a non-specific effect of protein and is not related to the enzymatic activity of myokinase.

FSR was prepared as described by MARTONOSI AND FERETOS[6]. Actin was extracted at 0° as described by FEUER *et al.*[7] and purified according to MOMMAERTS[8]. Tropomyosin B was extracted with 1 M KCl from the residue left after extraction of actin and purified by $(NH_4)_2SO_4$ fractionation as described by BAILEY[9]. Bovine serum albumin (Fraction V) was obtained from Calbiochem. In measuring the effect of FSR on myofibrillar ATPase activity, FSR was added to the complete medium (except for myofibrils, see Table I). After 5 min myofibrils were added and the reaction was terminated 5 min later with an equal vol. of 10% trichloroacetic acid. P_i was determined by the method of FISKE AND SUBBAROW[10]. A correction was made for the ATPase activity of the FSR preparation separately determined. Myokinase activity was determined by measuring the release of P_i in the ATPase assay system in which ATP was replaced by 2 mM ADP and 1.0 mg of myofibrillar protein per ml was included. One unit of activity was defined as the amount of enzyme liberating 1 μmole of P_i per min under the conditions described.

TABLE I

EFFECT OF SERUM ALBUMIN, TROPOMYOSIN B AND F-ACTIN ON RELAXING FACTOR SYSTEM

ATPase activity was assayed in a medium containing 0.05 M Tris, 4 mM $MgCl_2$, 4 mM ATP, 4 mM potassium oxalate, 0.05 M KCl, $2 \cdot 10^{-5}$ M $CaCl_2$, pH 7.5, 25°. FSR 1 was assayed 14 days after preparation, final FSR concentration in assay, 0.04 mg of protein per ml. FSR 2 was purified before using by centrifugation at 8000 × *g* for 3 min and the residue discarded and was assayed 1 day after preparation. FSR concentration in assay, 0.006 mg of protein per ml. Note the higher protein concentration required to reduced myofibrillar ATPase activity in the case of FSR 1.

Additions to assay system	*Myofibrillar ATPase activity (μmoles P_i/mg of protein per min)*		
	FSR 1	*FSR 2*	*FSR omitted*
None	0.42	0.37	0.44
FSR	0.19	0.23	—
FSR + 0.72 mg bovine serum albumin/ml	0.04	0.19	0.52
FSR + 0.42 mg tropomyosin B/ml	0.11	0.20	0.44
FSR + 0.38 mg F-actin/ml	0.14	0.20	0.47

Bovine serum albumin, tropomyosin B and, to a small extent, F-actin potentiated the relaxing activity of FSR (Table I). This potentiation was observed with preparations of FSR that had been aged 14 days but was markedly reduced with FSR only 1 day old. The potentiation by the dialyzed supernatant of the relaxing activity of FSR was not observed with freshly prepared FSR but was observed only after FSR preparations were aged (Fig. 1). The same pattern was observed with serum albumin as with the supernatant with respect to the effect of age of FSR preparation. In the absence of FSR these proteins did not inhibit myofibrillar ATPase activity, indicating that the effect is exerted on FSR and is not a direct inhibition of myofibrillar ATPase activity (Table I).

With a highly purified preparation of myokinase having 4- to 5-fold higher enzymatic activity than the original preparation, we were unable to observe any potentiation of relaxing activity by myokinase when assayed at the same level of myokinase activity which was effective in earlier experiments[4]. In view of these

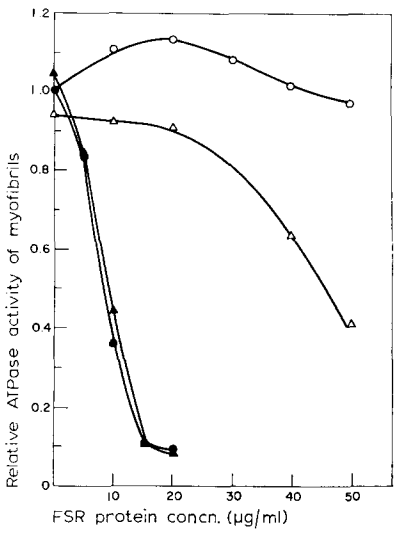

Fig. 1. Effect of dialyzed supernatant on relaxing activity of fresh and aged preparations of FSR. ATPase activity was assayed under conditions described in Table I. The assay medium contained (when added) 1.2 mg of supernatant protein per ml. The supernatant was prepared by centrifugation at 100000 × *g* for 3 h and was dialyzed against 500 vol. of 10 mM Tris (pH 7.5) for 48 h. ●, fresh FSR (2 h after completing preparation); ▲, fresh FSR + dialyzed supernatant; ○, aged FSR (11 days after completing preparation); △, aged FSR + dialyzed supernatant.

results and in view of the effectiveness of serum albumin and tropomyosin and the dependence of this potentiation on the age of FSR preparations, it is apparent that the effect of the non-dialyzable fraction of the supernatant cannot be attributed to the myokinase activity present in this fraction. The effectiveness of myokinase which we found previously[4] may have been due to a contaminant in the myokinase preparation or to an effect of protein in view of the higher protein concentration used with the less active preparation. However, it should not be inferred that myokinase cannot under any conditions affect the relaxing activity of FSR, particularly since it has been reported that ADP strongly inhibits the calcium accumulation of FSR (ref. 11). The present experiments were designed to study the effect of the supernatant and were not carried out under conditions most favorable for detecting an effect of myokinase. In our experiments the accumulation of calcium by FSR was largely completed during the 5-min preincubation in which time very little ADP was formed. It is, therefore, not too surprising that myokinase was not effective in our experiments. It appears that LORAND AND MOLNAR did not use preincubation in their studies with myokinase[5] and thus may have had conditions more favorable for detecting an effect of myokinase mediated through an effect of ADP on calcium accumulation of FSR.

During attempts to measure the effect of soluble proteins on the accumulation

of calcium by FSR (refs. 12, 13) discrepancies were observed between results obtained by the centrifugation method[12] and the Millipore filtration method[6] of separating FSR from the incubation medium. In the presence of 1.2 mg of serum albumin per ml, 0.05 mg of FSR protein per ml and 10^{-4} M $CaCl_2$, 75 % of the calcium was removed by centrifugation (100000 × *g*, 30 min) while only 50 % was removed by filtration (pore size of filter, 0.45 μ). This discrepancy was not observed in the absence of serum albumin. The cause of this discrepancy appears to be leakage of particles of FSR through the Millipore filters when serum albumin is included in the medium, as judged by the presence of significant ATPase activity in the filtrate (Table II). With serum albumin present in the assay medium, the amount of P_i liberated in 2 h after removal of FSR by centrifugation was less than 3 % of that released after filtration. If bovine serum albumin was omitted essentially no P_i was liberated in 20 h after filtration. Clearly, the use of the filtration method for measuring calcium accumulation by FSR must be limited to conditions where appreciable amounts of soluble proteins that may affect the removal of FSR by Millipore filtration are not present.

TABLE II

HYDROLYSIS OF ATP IN ASSAY MEDIUM AFTER FILTRATION OR CENTRIFUGATION TO REMOVE FSR

Incubation of FSR was carried out under conditions described in Table I except that 0.2 mg of FSR protein/ml, 1.2 mg bovine serum albumin/ml and 10^{-4} M $CaCl_2$ were used, and no myofibrils were present. After a 10-min incubation samples were filtered or placed in the centrifuge. The assay was carried out 13 days after the preparation of FSR. Total ATP present was 4.0 μmoles/ml. F indicates filtration of 2-ml samples through Millipore filters of 0.45 μ pore size and C indicates centrifugation at 100000 × *g* for 30 min.

Addition to assay system	*Method of separation of FSR*	*P_i released after filtration or centrifugation (μmoles/ml)*	
		After 2 h	*After 20 h*
None	F	0.00	0.00
FSR	F	0.00	0.02
FSR + bovine serum albumin	F	2.6	6.9
FSR + bovine serum albumin	C	0.06	0.32
Bovine serum albumin	F	0.00	0.00

It appears that serum albumin and the other proteins do not solubilize the calcium-binding factor or the ATPase of FSR, since the ATPase activity and radioactive calcium are readily removed by centrifugation, but these proteins in some way alter the properties of FSR so that it is not removed by Millipore filtration.

In conclusion, FSR becomes less effective in inhibiting myofibrillar ATPase activity on aging and its activity can be partly restored by several soluble proteins. The potentiation of the relaxing effect of FSR by a soluble, non-dialyzable fraction of a muscle homogenate appears to be a similar non-specific effect of protein.

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Department of Muscle Research,
Retina Foundation,
Institute of Biological and Medical Sciences,
Boston, Mass. (U.S.A.)

J. C. SEIDEL

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